Biogenesis of copper nanoparticles using peel extract of *Punica granatum* and their antimicrobial activity against opportunistic pathogens

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**ABSTRACT**

Copper nanoparticles (CuNPs) were biologically synthesized using peel extract of *Punica granatum* as reducing agent as well as capping agent. On treatment of aqueous solutions of CuSO₄·5H₂O with peel extract of *P. granatum*, stable CuNPs were formed. UV-Visible spectrophotometer analysis confirmed the formation of CuNPs. The synthesized nanoparticles were characterized with Fourier transform infrared spectroscopy, particles size analyzer and transmission electron microscopy (TEM). The electron microscopy analysis of CuNPs indicated that they ranged in size from 15 to 20 nm. The biologically synthesized CuNPs demonstrated high antibacterial activity against opportunistic pathogens, that is, *Micrococcus luteus* MTCC 1809, *Pseudomonas aeruginosa* MTCC 424, *Salmonella enterica* MTCC 1253 and *Enterobactor aerogenes* MTCC 2823 in vitro. Nanoparticles synthesized biologically using plant extracts have the potential to serve as possible ecofriendly alternatives to chemical and physical methods for biomedical applications and research.

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**Introduction**

Environment-friendly procedures for the synthesis of metal nanoparticles using biological means are evolving rapidly as an important branch of nanobiotechnology (1, 2). Among green synthesis methods, the use of plants for synthesis of nanoparticles could be advantageous over other environmentally benign biological processes, as this eliminates the elaborate process of

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maintaining cell cultures \cite{3, 4}. The biosynthesis of nanoparticles by using plant materials involves fairly rapid reduction of metallic materials. Shankar et al. \cite{5} reported the extracellular synthesis of silver nanoparticles by Pelargonium graveolens (the extract of geranium leaves) for reduction of Ag\(^{+}\) ions to Ag nanoparticles. Some reports were showed the synthesis of gold nanoparticles by Avena sativa (Oat) biomass \cite{6} and Syzygium cumini leaf extract and seed extract \cite{7}, in a mechanistic way. The extracellular synthesis of highly stable silver and gold nanoparticles has been reported with the use of Emblica officinalis fruit extract acting as a reducing agent \cite{8}.

**Figure 1.** UV-Visible absorption spectra of synthesized CuNPs.

**Figure 2.** FTIR spectrum of CuNPs synthesized using the peel extract of P. granatum.
Copper nanoparticles (CuNPs) have vast number of applications, but there are only a few studies on their biosynthesis. CuNPs have been biologically synthesized using plant leaf extract (Magnolia), as a reducing agent (9) and peptide-capped CuNPs using stem latex of a medicinally important plant, Euphorbia nivulia and their biological effect on tumor cells studied (10). There are some reports on antimicrobial activity of cuprous oxide (Cu$_2$O) nanoparticles on Gram-positive and Gram-negative bacteria (11–14). A low melting point soda-lime glass powder containing CuNPs with high antibacterial (against Gram-positive and Gram-negative bacteria) and antifungal activity has been reported by Esteban-Tejeda et al. (15).

In this paper, the synthesis of CuNPs from copper sulfate solution was studied using the peel extract of Punica granatum as a natural reducing agent. The aim of this work is to study the antibacterial activity of the CuNPs by using the antibacterial peel extract against opportunistic pathogenic microflora.

**Results and discussion**

The resultant CuNPs were characterized with a UV-Visible Spectrophotometer, particle size analyzer, zeta potential and transmission electron microscopy (TEM).

The characteristic absorption peak at around 585 nm is due to the surface plasmon band of Cu colloids (Figure 1). The strong surface plasmon absorption band observed at 585 nm may be due to the formation of non-oxidized CuNPs (16, 17). Hashemipour et al. (18) also reported that CuNPs synthesized by the chemical reduction method showed absorption peaks at 580 nm. So an absorption peak around 585 nm confirms the formation of CuNPs by the peel extract. The broadness of the absorption band probably arises from the wide size distribution of CuNPs (19). More absorbance peak around 300 nm may be due to the presence of proteins, enzymes and flavanoid-like biomolecules in the extract that might be responsible for the reduction in CuSO$_4$ (20).

Figure 2 represents Fourier transform infrared (FTIR) spectrum of Cu nanoparticles synthesized using the P. granatum peel extract. The absorption peaks located mainly at 3244 cm$^{-1}$ are generally attributed to aromatic or aliphatic C–H stretching. 2935 cm$^{-1}$ are generally assigned to the alkyl C–H stretching, whereas peaks at 1452, 1375 and 1332 cm$^{-1}$ are due to C–O–O stretching bands, 1152 and 1050 cm$^{-1}$ are due to C–C stretching vibrations, 713 and 624 cm$^{-1}$ are due to acetylenic C–H bending vibrations (21) in the region of 40–4000 cm$^{-1}$. Huang et al. (22) reported that peaks of BSA-Cu$^{2+}$ at 1021 and 824 cm$^{-1}$ might be contributed to the interaction of Cu$^{2+}$ and BSA. So Peaks at 1050 and 713 cm$^{-1}$ corroborate with the work reported by Haung et al. and showed the interaction between CuNPs and proteins present in the peel extract of P. granatum.

The morphology of CuNPs synthesized with the peel extract of P. granatum was found by dispersing them on a copper grid coated with carbon and observing in TEM (Figure 3). It can be seen from the TEM images that nanoparticles of size 15–20 nm with spherical shape were formed.

Figure 4 shows the size distribution of the nanoparticles given by zeta sizer for CuNPs. This analysis presents that the average size of nanoparticles is about 21 nm which is in agreement with the TEM image analysis. Hashemipour et al. (18) also represented a similar size distribution of the CuNPs by the particle size analyzer and TEM.

In this study, the zeta potential was negative (−56 mV) and remained constant over a period confirming the stability of CuNPs (Figure 5). This observation can be attributed to the presence of biomolecules as stabilizing agents in the leaf extract. The zeta potential measures the surface charge of particles. As the zeta potential increases, the surface charge of the particles will be also increase. The zeta potential greatly influences particle stability in suspension through the electrostatic
repulsion between particles (23, 24). These results are consistent with TEM images.

In Figure 6 it can be seen that the colloidal CuNPs synthesized by the plant extract show high antimicrobial activity against all bacterial strains, *Pseudomonas*, *Salmonella*, *Micrococcus* and *Enterobacter* which show high susceptibility in terms of zones of inhibition, 18.67 ± 1.53, 19.67 ± 1.53, 20.33 ± 1.53 and 19 ± 1 mm, respectively. So the agar well diffusion test suggests that for all cultures of bacteria, the antimicrobial action of the CuNPs was superior. Control (peel extract) also shows a clear zone against bacteria, that is, 7.66 ± 0.57, 7.33 ± 1.5, 8.67 ± 0.58 and 7.17 ± 0.29 mm for *Pseudomonas*, *Salmonella*, *Micrococcus* and *Enterobacter*, respectively. Standard antibiotic, that is, streptomycin was also used to compare the antimicrobial efficacy of CuNPs (not shown in Figure 6). It is clear from Table 1 that biosynthesized CuNPs showed higher antimicrobial activity than standard antibiotic. It has been reported that *P. granatum* peel extracts have shown antibacterial activity against various bacteria (25–27). So the biosynthesis of CuNPs by using the antimicrobial peel extract of *P. granatum* is very promising alternate of a novel antimicrobial agent, that is, antibiotics.

A few studies have been performed to elucidate the mechanism of bactericidal action of nanoparticles. It is difficult to distinguish between the bactericidal activities of nanoparticles from that of the ions released by the nanoparticles (28). Ruparelia et al. (14) reported that in case of CuNPs, a good negative correlation was observed between the inhibition zone observed in disc diffusion test, and minimum inhibitory concentration/minimum bactericidal concentration determined based on liquid cultures against *Escherichia coli* (four strains), *Bacillus subtilis* and *Staphylococcus aureus* (three strains).

We assume that CuNPs have greater affinity to surface active groups of bacterial strain which may have led to its greater bactericidal effect. The mechanism of action of the CuNPs as antimicrobials is not yet fully established.

Figure 4. Size distribution of CuNPs biosynthesized by the peel extract of *P. granatum*.

Figure 5. Zeta potential of CuNPs using the *P. granatum* peel extract was negative (−56.9 mV).
Experiments

Copper sulfate was purchased from Sisco Research Laboratories, India. The peel extract was prepared from pomegranate fruit purchased from local vegetable shop (Hisar, Haryana, India). The test strains Micrococcus luteus MTCC 1809, Pseudomonas aeruginosa MTCC 424, Salmonella enterica MTCC 1253, and Enterobactor aerogenes MTCC 2823 were procured from the Institute of Microbial Technology, Chandigarh, India.

Synthesis of Cu nanoparticles

CuNPs were synthesized using a biological agent, that is, the peel extract of pomegranate as a catalyst. The pomegranate peel extract was prepared in methanol using Soxhlet apparatus according to the method described previously (29). For this, peels were air-dried in a vacuum oven at 40°C for 48 h and ground to fine powder; 100 g powdered sample was extracted with 1000 ml methanol at room temperature by the Soxhlet extraction method for 8 h. 0.05M CuSO4 (50 ml) aqueous solution was added to 50 ml peel extract dropwise with continuous stirring. After heating at 80°C for 10 min it was continuously stirred for 4 h at 40°C. The solution was centrifuged at 10,000 rpm, at 4°C for 30 min and pellet was dissolved in distilled water for periodic probe sonication for 5 s for 5 min at 30 ± 0.5°C. Nano suspension thus obtained was dried in oven at 70°C for 24 h to obtain nanoparticles in powder form for further experiments.

Characterization of CuNPs

Product samples were subjected to UV–Visible spectroscopic (UV-2450, Shimadzu, Japan) studies, the most confirmatory tool for the detection of surface plasmon resonance (SPR) property (of CuNPs.

The interactions of peel extract and CuNPs were analyzed with FTIR spectroscopy. For FTIR measurements, sample ground with KBr and pellet was analyzed using an FTIR spectrophotometer (Affinity-FTIR, Shimadzu, Japan) in the range of 400–4000 cm⁻¹. Morphological details of the synthesized CuNPs were revealed under a transmission electron microscope (Hitachi FEI Philips Morgagni 268D TEM, Japan) at an accelerating voltage of 100 kV. To prepare for TEM, the specimen was suspended in distilled water, dispersed ultrasonically to separate individual particles, and one or two drops of the suspension was deposited onto carbon-coated copper grids and air-dried well, before mounting and observed under TEM.

The size and zeta potential of CuNPs was measured by using a particle size analyzer (Zeta sizer ZS-90, Malvern, UK). All measurements were performed at 25°C. Each measurement was obtained as an average of 20 runs.

Antimicrobial activity of CuNPs

The antimicrobial activity of CuNPs was tested by the agar well diffusion method against M. luteus MTCC 1809, P. aeruginosa MTCC 424, Salmonella enterica MTCC 1253, and Enterobactor aerogenes MTCC 2823 in vitro. Nutrient agar media plates were prepared and solidified in laminar air flow, after solidification bacterial cultures were swabbed on repeats. Wells were prepared by cutting agar with 1 ml micro tips and filled with the solution containing nanoparticles at a concentration of 100 µg ml⁻¹. The plates were incubated at 37°C for 24 h, after which, the zone of inhibition was measured. The experiments were performed in triplicates.

Conclusion

This work clearly demonstrates the application in green chemistry of a method for synthesizing CuNPs with desirable characteristics using the leaf extract from P. granatum. Synthesized CuNPs were well capped and stable. This is a very important aspect for their various biomedical applications, such as contrasting agents in bioimaging in future. Furthermore, the nanoparticles so synthesized were found to possess superior antibacterial property against the test bacteria. The use of environmentally benign materials, such as plant extract, bacteria, fungi and enzymes, for the synthesis of nanoparticles.
offers numerous benefits of eco-friendliness and compatibility for pharmaceutical and other biomedical applications, as they do not use toxic chemicals for the synthesis protocol. The green synthesis also helps create more awareness about the importance of plants. A combination of antibacterial peel extract and CuNPs may give rise to more comprehensive bactericidal effect against mixed bacterial population. Before commercialization, detailed research and comparative study of strain-specific variability are required to determine the bactericidal efficiency of metal nanoparticles.

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Disclosure statement

The authors have no financial interests or benefits from this work.

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