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Pterocarpus marsupium Derived Phyto-Synthesis of Copper Oxide Nanoparticles and their Antimicrobial Activities

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Abstract

In present study, copper oxide nanoparticles (CuONPs) synthesized by quick and eco-friendly phytogenic-reduction of copper salt (copper sulphate $CuSO_4.H_2O$) solution with *Pterocarpus marsupium* extract. UV-VIS spectrometry indicated formation of nanoparticles via absorption spectra of copper colloidal solution at 442 nm. Phytosynthesis of CuONPs were further characterized by Transmission electron microscopy; scanning electron microscopy and Fourier transform infrared spectroscopy. The experimental results showed that diameter of CuONPs in colloidal solution were < 40 nm. Further, antibacterial activities of CuONPs were determined against Gram negative *Escherichia coli*- MTCC-9721, *Proteus vulgaris*- MTCC-7299, *Klebsiella pneumonia*- MTCC-9751 and Gram positive i.e. *Staphylococcus aureus*- MTCC-9442, *Staphylococcus. epidermidis*- MTCC- 2639, *Bacillus cereus*- MTCC-9017 bacteria by well agar diffusion and microdilution method. Notably, The CuONPs showed an effective antibacterial activity against all test microorganisms where K. pneumonia and *E.coli* showed maximum ZOI and MIC respectively i.e. 24 mm and 6 µg/ml.

Keywords: Phyto-reduction; Copper oxide nanoparticles; *Pterocarpus marsupium*; Antibacterial activity; SEM; TEM

Introduction

Conventionally, copper and its complexes have been used as water purifiers, algaecide, fungicides, and as antibacterial and anti-fouling agents [1,2]. In last decade metal is being tried set to minimal size of particles as nanoparticles due to idiosyncratic properties. CuONPs exhibited huge potential as catalysis, optics, electronics, batteries, gas sensors, solar energy conversion tools, high temperature superconductors [3-7] and found very persuasive in past studies, evaluating antibacterial activity and roles in other ailment [8,9]. Notably, CuONPs explored their potential in antimicrobial activities against many infectious organisms such as *Vibrio cholera, Syphillis typhus, Staphylococcus aureus, E. coli, Pseudomonas aeruginosa* and *Bacillus subtilis*. [10-12]. These particles showed great possibilities in malignant condition as mediator in chemotherapeutic doses delivery and targeting tumor cell. Synthesis of metallic nanoparticles is carried out by several physical and chemical methods that include laser ablation [13], ion sputtering [14], solvo thermal synthesis [15], chemical reduction [16], and sol-gel [17,18] method, involve toxic solvents, high temperature, energy and high pressure conversion. Few studies held, were focused on Microbe mediated synthesis as of not practical for industries due to laboratory maintenance. The growing need of environmental supporting phenomenon for synthesis nanoparticles, phytogenic reduction methods (phytosynthesis) are more acceptable, easy, efficient, and eco-friendly. *Pterocarpus marsupium* belongs family *Fabales*, also known as Vijayasar or the Indian Kino Tree, is a medium to large, deciduous tree that can grow up to 30 meters tall. It is native to India, Nepal, and Sri Lanka. Notably, parts of the Indian Kino (heartwood, leaves and flowers) have long been used for their medicinal properties in Ayurveda.

Reportedly, Deepa et al. assessed antimicrobial activity of ethanolic extract of Pterocarpus marsupium Roxb Bark [19].

In present study *Pterocarpus marsupium* plant extract is used as reducing and capping agent [20] and synthesized CuONPs were further, characterized by UV-visible spectroscopy, TEM, SEM and FTIR followed by anti-bacterial activities. Umesh et al. reported green synthesis of silver nanoparticles using Artocarpus heterophyllus Lam. seed extract [21]. Das et al. described green synthesis of silver nanoparticles using Sesbania grandiflora leaf extract [22]. Abuelmagd et al. studied green synthesis of gold nanoparticles using *Punica granatum* L. extract [23]. Some of plant materials such as *Aegle marmelos* leaf extract [24] and *Syzygium aromaticum* aqueous extract [25] have been used for the synthesis of CuONPs.

Materials and Methods

Materials

Pterocarpus marsupium (Fabales) a deciduous tree, belonging to the group called rasayana in ayurvedic system of medicine. Rasayana drugs are immunomodulators and relieve stress of the body. In ayurveda, aqueous extract of heart-wood of *P. marsupium* is used in treatment of diabetes. Although there are several reports on *P. marsupium* as an anti-diabetic drug.

The heart-wood of *P. marsupium* were collected and identified from Department of Botany, University of Rajasthan, Jaipur. Further, wood were thoroughly washed, dried and finally powdered to store in air-tight container at cool and dry place for next preparation as phytogenic reduction of copper sulphate salt.

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Synthesis of copper oxide nanoparticles

The broth of *P. marsupium* wood was prepared by boiling 5 gm plant material (heart-wood) with 100 ml of distilled water for 15 min before final decantation. The broth was filtered through pal funnel using Whatman filter paper no 1 and stored at -18°C. Next, as filtrate was introduced in 1 mM $CuSO_4.H_2O$ aqueous solution at 50°C on temperature controlled magnetic stirrer, a greenish solution was formed, indicating the formation of copper oxide nanoparticles. The resultant solution was centrifuged at 15000 rpm for 15 min, followed by several time washing of finally collected pellet. Sequentially, the pellet was vacuum dried and stored for further use.

Characterization of the synthesized copper oxide nanoparticles

The optical absorbance of CuONPs solution was recorded on UV–visible spectroscopy 1800 of Shimadzu, Kyoto, Japan. The fractional amount of CuONPs powder was coated with a thin layer of gold by sputter coating and examined in a scanning electron microscope, carl zeiss EVO-18, 30KV. FTIR spectra were recorded on IR-Affinity 1, Shimadzu Fourier transform infrared spectrometer at room temperature by implanting < 0.01 g of copper nanopowder with KBr salt. Further high resultant images were obtained by HRTEM using Techni G2, S-twin 200KV.

Determination of anti-bacterial activities of CuO nanoparticles

The CuONPs and aqueous extract prepared by *P. marsupium* were used to evaluate antimicrobial activity against Gram (-) and Gram (+) Bacteria (*E. coli*- MTCC-9721, *P. vulgaris*- MTCC-7299, *K. pneumonia*- MTCC-9751, *S. aureus*- MTCC-9442, *S. epidermidis*- MTCC- 2639 and *B. cereus*- MTCC-9017) on MHA plates by agar well diffusion method [26,27]. Bacterial strain were kept up on nutrient agar slants that included peptone (5.0 g), meat extract (1.0 g), yeast extract (2.0 g), sodium chloride (5.0 g), and agar (15.0 g) per liter of distilled water.

The Minimum Inhibitory Concentration (MIC) method for all test bacterial strains were also determined [28,29]. MIC of the CuONPs and Gentamicin performed by the broth micro dilution method. The 50 µL of nutrient broth were added in 96-well plate followed by 50 µL of stock solution of CuONPs by Two-fold serial dilutions method producing 100 µg/mL to 0.78 µg/mL concentration. The each will inoculated with 5 µL bacterial culture incubated overnight at 37°C in nutrient broth and adjusted to a final density of 10 CFU/mL by 0.5 McFarland standards. Briefly, different concentrations of Gentamicin and CuONPs prepared by using regular ascending interval i.e. 1 µg/mL to 55 µg/mL were used for MIC. Sterilized nutrient broth was used as the (-) control and inoculated broth was used as the (+) control. After incubation for 24 hours at 37°C, MIC were determined with an ELISA reader (Infinite 200) as the lowest concentration of compound whose absorbency was comparable with the negative control wells. Results are expressed as the mean values of three independent determinations.

Statistical Analysis

The grouped data were statistically evaluated using ANOVA with SPSS16 software. Values are presented as the mean ± SD of the three replicates of each experiment.

Results and Discussion

The copper sulphate solution and fresh broth of *P. marsupium* were sky-blue and Yellowish-brown respectively. The addition of *P. marsupium* extracts to copper sulphate solution turned the solution to green color at 50°C confirmed formation of CuONPs (Figure 1).

UV-visible spectroscopy

Synthesis of CuONPs using extract of heart-wood of *P. marsupium* observed by UV-visible spectroscopy. Reduction of copper sulphate solution to CuoNPs was confirmed by measuring the UV-Vis spectrum at the range of 200–800 nm. The UV-Vis absorption spectrum of sample is recorded and shown in Figure 2. As expected, CuO nanoparticles show an absorption peak between 400 and 500 nm i.e. 442 nm that can be contributed to the characteristic absorption of CuONPs.

Scanning Electron Microscope (SEM) and Transmission Electron Microscopy (TEM)

SEM is source for information about the size and morphology details of the CuONPs. As understanding the image of CuONPs, The fractional amount of CuONPs powder was coated with a thin layer of gold by sputter coating and examined in a scanning electron microscope. Figure 3b shows the scanning electron micrograph of the copper oxide nanoparticles obtained from the phytogenic-reduction method.

TEM proficiency was used to project the sound structure of the CuONPs (Figure 3a). TEM grids were fitted out by addition of 5 µL of the CuONPs solution on carbon-coated copper grids and drying under

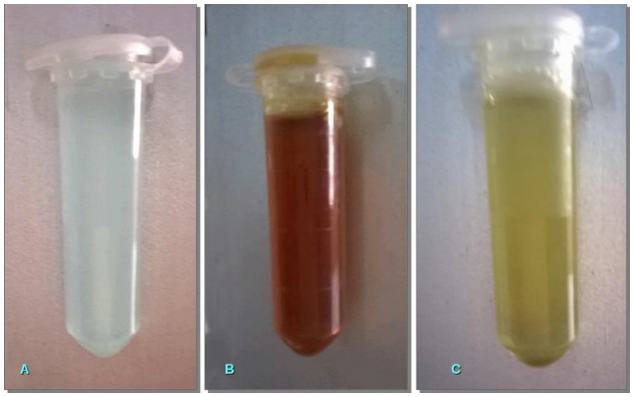

Figure 1: A visual image of (a) copper sulphate solution (b) broth of *P. marsupium* and (c) CuONPs colloid.

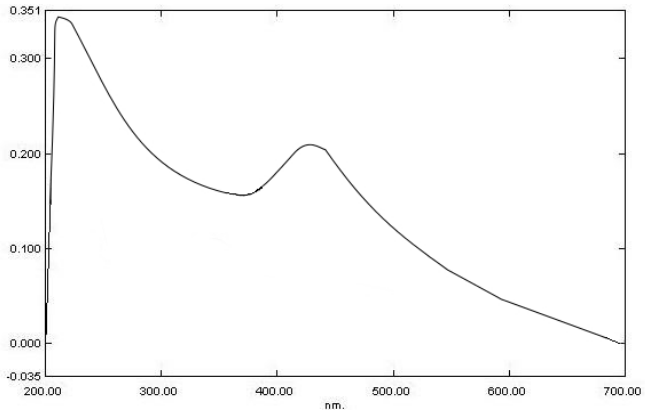

Figure 2: UV-Vis spectrum of CuO nanoparticles phyto-synthesized by *P. marsupium*.

UV lamp. The experimental results showed that the shape of prepared CuONPs was spherical with diameters that ranged from 20 nm to 50 nm (Figure 3a) and found in form of nanocluster. The larger copper particles may be due to the aggregation of the smaller ones, during the TEM analysis. Facsimile etiology was accessed when employing $Cu(NO_3)2.3H_2O$ as demonstrated by Anandan et al. [30].

Fourier transform infrared spectroscopy (FTIR)

CuONPs solution was centrifuged at 15,000 rpm for 20 minute and obtained solid residue subjected to several time washing with distilled water followed by drying for next 24 hours. The dusty amount of powder were used for FTIR analysis, which were performed on a IR-Affinity-1-Shimadzu. The FTIR peaks were identified and expressed in wave numbers (cm^{-1}).

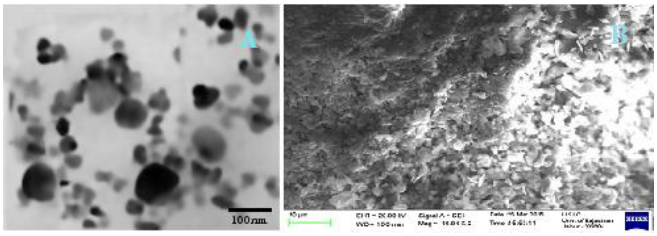

Figure 3: (a) Bright field TEM image of CuONPs.(b) A typical of SEM image of phyto-reduced copper nanoparticles.

The refined CuONPs possessed absorption peaks at 1149, 1616, 1645, and 3333 cm^{-1} due to cyclic C–O–C, C=O and OH functional groups, respectively (Figure 4). It may be inferred that the bioactive ingredients of *P. marsupium* was the presumptive reducing agent which was concerned in the Phyto-synthesis of CuONPs and might have organized a layer on the CuONPs (i.e., Phyto-capping) that may have hindered the agglomeration of the Nano-sized particles would have stabilized them.

Determination of anti-bacterial activities of CuO nanoparticles

Six bacterial strains were used for antimicrobial activity where three Gram (-) bacteria *E. coli*- MTCC-9721, *P. vulgaris*- MTCC-7299, *K. pneumonia*- MTCC-9751 and three Gram (+) bacteria *S. aureus*- MTCC-9442, *S. epidermidis*- MTCC- 2639, *B. cereus*- MTCC-9017. Bacterial response to CuONps and antibiotics is evaluated using well diffusion assay. Prepared CuONPs suspension was added into the wells. The prepared samples were incubated for 15 minute at 4°C followed by 37°C for 24 h. Zone of inhibition (ZOI) were measured on scale in mm around the well after the incubation period. The mean values of each triplicate were reported.

The outcomes pointed that CuONPs have bear down dose-dependent state against both gram negative and gram positive microorganisms. All these bacteria were found susceptible against the CuONPs as growth of these microbes reduced with the increase in concentration of CuONPs (Figure 5).

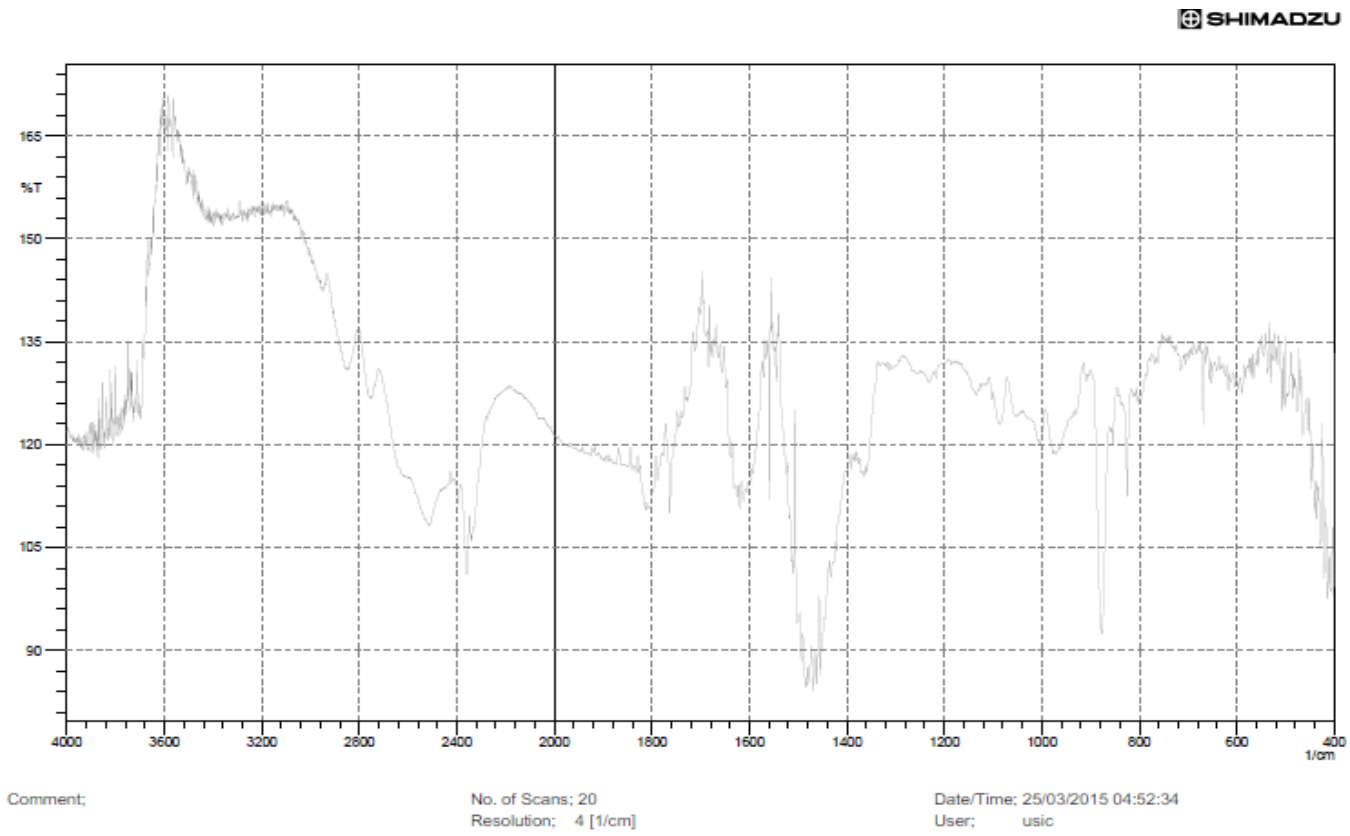

Figure 4: FTIR spectrum of *P. marsupium* capped CuONPs.

The effectiveness of CuONPs against all six microbes compared with the control, the diameters of ZOI (mm) vary for all the test bacteria at contrastive level of CuONPs concentration. It was revealed that 0.08 mmol/mL colloid solution of CuONPs displayed 25 mm clear inhibitory zone against *K. pneumonia* after incubation for 24 h followed by *E. coli* (24 mm), then 23 mm for *B. cereus* and 22 mm for *P. vulgaris,* minimum for *S. epidermidis* and *S. aureus* (20 mm) proposing that phytosynthesized CuONPs have valuable antibacterial effect against Gram (-) than Gram (+) Bacteria (Table 1).

The upshot of the present study are compatible with other findings that point greater action of CuONPs against Gram (-) bacteria [31]. Notably, results signal that the CuONPs are more active against *K. pneumoniae* a Gram (-) bacteria that is may be due to the expedited influx of Nano-sized CuONPs into the cell wall of Gram (-) microorganism which made-up of a specific outer membrane with a one-on-one layer of peptidoglycan alike to cell wall of Gram (+) microorganism with many layer of peptidoglycan [32,33]. The idiosyncratic high surface/volume ratios of CuONPs ease to interact with the cell membrane bacteria [34]. Which prompt the necrosis of the microorganism cell [35].

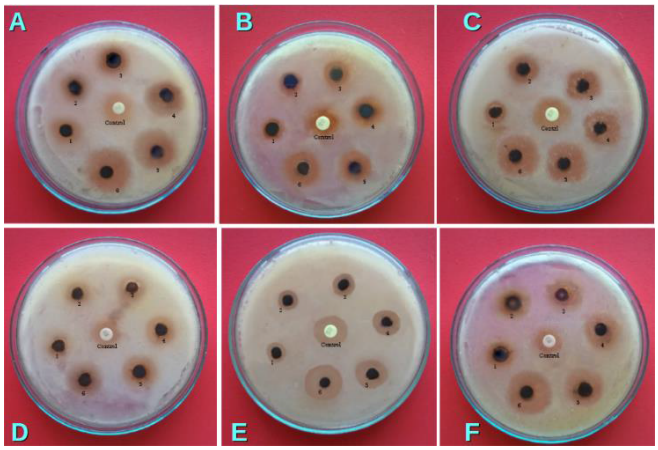

Figure 5: A visual image of antibacterial activity of CuONPs against (a) *K. pneumoniae,* (b) *S. epidermidis,* (c) *B. cereus,* (d) *S. aureus,* (e) *P. vulgaris and* (f) *E. coli.*

Minimum inhibitory concentration of CuONPs (Table 2) was assessed against all test bacteria. The copper nanoparticles exposed muffled MIC against *E. coli* and *K. pneumoniae* at 6 µg/mL autem blaring MIC was found against *S. aureus* i.e. 28 µg/mL. Effectiveness of CuONPs against both Gram negative and Gram positive bacteria proposing as broad spectrum potential of nanoparticle. Bacterial colony stamp down by cell filaments formation influenced by CuONPs subjected to bacterial cell membrane destruction [36] and CuONPs may encounter multiple toxic effects such as genesis of free radicals assaulting membrane lipids and sequential free-radical induced impairment of membranes [37-39], formation of irregularly shaped pits in outer bacterial membrane and changes in permeability were linked up with metal exhaustion and liberal spillage of membrane proteins and lipopolysaccharides [40], Metallic nanoparticles undergoes slow oxidation with vent of their ions which lead to lose of replicative ability by DNA [41,42] and to the inhibition of expression of ribosomal subunits and to the inactivation of some cellular proteins and enzymes, essential to ATP production

Conclusion

In this study CuONPs were prepared using extracts of *P. marsupium*, a plant abundantly found throughout in India, as reducing agent. The outcomes were very gleaming since the extract supports the fabrication of CuONPs at 50°C with an accelerated kinetics, without noxious chemicals. The method was easy to execute in one-on-one step. The copper nanoparticles synthesized by this method were bear-down nominees for its use in biological instrumentation and antibacterial activity. The diameter of the copper nanoparticles is < 40 nm, as shown by TEM analysis. Surely, the CuONPs produced by this method, initiative to a new itinerary to study conductive activity and antimicrobial properties.

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copper nanoparticle Concentration (mmol/mL)	Bacterial Sp (zone of inhibition-mm)					
	K. pneumoniae (A)	*S. epidermidis* (B)	*B. cereus* (C)	*S. aureus* (D)	*P. vulgaris* (E)	*E coli* (F)
.0025	12	11	10	11	12	10
.005	13	12	13	13	15	11
.01	15	14	15	14	16	14
.02	18	16	17	16	17	17
.04	21	17	20	18	18	19
.08	25	20	23	20	22	24
control	13	12	14	13	14	12

Table 1: Antibacterial activity (ZOI in mm) of CuONPs against test bacterial strains.

Bacterial Sp	MIC (µg/mL)	
	CuONPs	Gentamicin
K. pneumoniae (A)	6 µg/mL	6 *µg/mL*
S. epidermidis (B)	21 µg/mL	15 *µg/mL*
B. cereus (C)	10 µg/mL	6 *µg/mL*
S. aureus (D)	28 µg/mL	15 *µg/mL*
P. vulgaris (E)	15 µg/mL	10 *µg/mL*
E Coli (F)	6 µg/mL	6 µg/mL

Table 2: Minimal Inhibitory Concentration (MIC), in µg/ml, of CuONPs against test bacteria.

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