Biosynthesis of colloidal Copper Oxide Nanoparticles using *Manilkara hexandra* (Roxb.) Dubard leaf extract and its Physicochemical Characterization and Pharmaceutical Evaluation

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Available online at: www.isroset.org

Received: 04/Dec/2018, Accepted: 18/Dec/2018, Online: 31/Dec/2018

Abstract- In the present investigation, plant mediated bio-synthesis of copper oxide nanoparticles is synthesized using *Manilkara hexandra* leaf extract and were further characterized using Fourier transform infrared spectroscopy (Presence of biomolecules), UV-vis spectroscopy (250-300 nm), Field electron scanning electron microscope (30 to 60 nm), Energy dispersive x-ray analysis (Cu, O elements), X-ray diffraction analysis (face-centered cubic structure), Particle size analyser (343 nm), Zeta potential (-9.50mV). The Thermogravimetry/Differential Thermal Analysis and Differential scanning calorimetry is also examined to find the stability of materials. This confirms that copper oxide nanoparticles are well formed and synthesized. They are further tested with anti-microbial assays and antioxidant evaluation (DPPH method in-vitro) these reports show high estimation. This confirms that the copper oxide nanoparticles can be produced in large scale and can be implied for prevention of food, crops and drug delivery system.

Keywords- *Manilkara hexandra*, Copper oxide nanoparticles, Anti-microbial assays, DPPH method.

I. INTRODUCTION

Traditionally, plants play a major role in the drug in the human. The presence of phytochemicals present in a medicinal plant is useful for curing and healing human diseases [1]. Nanomaterials have a wide view of the application in human life and its environment. Some knowledge of nanoscale fabrication was about 5000 years old Indian system of medicine, *Ayurveda* for medicinal purposes [2]. The ancient book Ras-Ratnakar by Nagarjuna (50 B.C.) clearly describes the process of formation of *Bhasma* which is a special kind of Ayurvedic medicine. A combination of traditional method and new scientific method formulate a new approach to metal nanoparticles. The physical and chemical method can produce pure and well-defined nanoparticles, it is quite expensive and dangerous to the environment and not eco-friendly [3]. Among various methods, plant-mediated nanoparticles have a high impact because of its cost-effective, environmentally friendly and safe for human therapeautic use [4]. It is a bottom-up approach. The novel nanoparticles were synthesized by researchers with continuous efforts for industrial and technological advancements. The metal nanoparticles (Cu, Ag, Au & Pt) have the high impact of microorganisms and is been reported [5-7]. Among various metal CuNPs has versatile applications such as optical, catalytic, antibacterial and antifungal [8-9]. It is also used in gas sensors, dye-sensitized solar cells (DSSCS), paints, filters, plastics and textiles [10-12]. Etc.

The aim of the present work is to synthesize copper oxide nanoparticles using *Manilkara hexandra* leaf extract. The extract act as reducing and stabilizing agent. This green synthesized copper oxide nanoparticles are characterized by various methods such as UV, FT-IR, FE-SEM, EDAX, XRD, DLS, Zeta Potential, TG/DTA and DSC. Silver nanoparticles was tested against antimicrobial and antioxidant assay. This shows a high zone of inhibition and good antioxidant property.

II. MATERIALS AND METHODS

2.1 Chemicals and scientific name of plants

All chemicals and solvent of AR grade were purchased from Sigma Aldrich, India. The *Manilkara hexandra* (Roxb.) Dubard leaves were collected from Jayakondam district, Tamil Nadu. Identified in Rapinet herbarium, St. Joseph College, Trichy, India. (Voucher No: AAL 001 and Figure 1).
2.2. Scientific name and medicinal properties of plant

The botanical name of the plant is *Manilkara hexandra* (Roxb.) Dubard belong to Sapotaceae family, Genus - Manilkara, Species - Manilkara hexandra, in Tamil it is called as Ulakaiippaalai or Kanuppaalai. The *Manilkara hexandra* grow in wild conditions and has various medicinal values such as anorexia, loss of consciousness, dyspepsia, astringent, odontopathy, burning sensation, leprosy etc [13].

2.3 Preparation of leaf extract

The leaves were collected and washed in flowing water and then again washed with de-ionized water and dried in the room for two weeks. The dried leaves were grained using pestle mortar into a fine powder. Ten grams of leaf powder were weighted using an electronic balancing scale and soaked in 100 ml of de-ionized water of 30mins, then heated at 60˚C for 10 minutes in a heating mantle. The extracts were then filtered using What man No.1 filter paper and stored at 5˚C for future use.

2.4. Phytochemical screening

The stored extract was analyzed qualitatively to identify phytochemicals such as alkaloids, flavonoids, terpenoid, saponin, tannin, amino acid, protein, carbohydrates, emodins, quinone, resin, phlobatannin, coumargins, anthraquinone, oils and fat, gum, anthocyanin, glycosides, steroids, and xanthoprotein [14].

2.5. Preparation of copper sulfate solution

1.248 grams of copper sulfate pentahydrate (CuSO₄·5H₂O) were weighted and mixed with 1000 ml of de-ionized water and made for 5mM copper sulfate solution shown in Figure 2.

![Figure 2. Preparation of Copper Sulfate solution (5mM).](image)

2.6. Biosynthesis of copper oxide nanoparticles

The copper oxide nanoparticles were synthesized using 90 ml of copper sulfate solution mixed with 10 ml of *Manilkara hexandra* leaf extracts. The change of color is observed, which indicate the presence of copper oxide nanoparticles. MHL-CuO
NPs were collected using centrifuged techniques (Remi 12C) at 12,000 rpm. Then the particles are collected, dried and purified using alcohol and it was powdered.

2.7. Characterization
These copper oxide nanoparticles were characterized by using various instruments such as the UV-Visible spectrometer (U-2910 Hitachi) in the wavelength range 200-1100 nm. The functional group was identified by FTIR (IRAffinity-1S Shimadzu) wavelength range from 400 to 4000 cm⁻¹. The morphological studies and elemental composition of MHL-CuO NPs were studied using FE-SEM and EDX (FEI QUANTA-250 FEG). The crystallinity of MHL-CuO NPs was analyzed by X-ray diffraction (X’ Pert Pro-P Analytic). The size distribution and Zeta Potential of MHL-CuO NPs was also analyzed using the particle size analyzer (Zetasizer 7.11, Malvern, Instruments Ltd). The MHL-CuO NPs thermally examined by Thermogravimetry/Differential Thermal Analysis (TG/DTA) model number ( SDT Q600 V20.9 Build 20) and Differential scanning calorimetry (DSC) model number ( NETZSCH DSC 214 Polyma).

2.8. Antimicrobial assays
2.8.1. Collection of bacterial strains and antibacterial assays
Four diverse human pathogenic microbes are inspected in the trial. Two-gram +ve bacterial strains Staphylococcus aureus (MTCC 25923) and Bacillus subtilis (MTCC 2451). Two gram –ve bacterial strains Escherichia coli (MTCC 25922) and Pseudomonas aeruginosa (MTCC 27853). These strainssecured from microbial sort culture and gathered (MTCC) at Chandigarh, India. Every one of these strains was developed at 37°C and kept up on supplement agar (Difco, USA) incline at 4°C. The antibacterial potential for MHL-CuO NPs, Manilkara hexandra leaf extract, Copper sulfate solution (5mM), the standard was analyzed utilizing disc diffusion method. The muller hintor agar was set up in Petri dishes (60mm) and immunized with testing organisms [15]. Sterile disc (6mm width) was impregnated with 10µl of MHL-CuONPs, Manilkara hexandra leaf extract, Copper sulfate solution, Amoxicillin. The sterile circle put on the top layer of the agar plates. The dishes are then brooded for 24h at 37°C and the zone of inhibition is recorded. Amoxicillin is utilized as the standard for an antibiotic.

2.8.2. Collection of fungal strains and antifungal assays
Two distinctive parasitic strains Aspergillus flavus (MTCC-3396) and Aspergillus Niger (MTCC-227) were gained from National substance laboratory (NCL), Pune, Maharashtra, India. It is vaccinated independently in Sabouraud's dextrose stock for 6h and is checked to give roughly 10⁵ CFU/ml. The Antifungal movement is dissected for MHL-CuO NPs, Manilkara hexandra leaf extract, Copper sulfate solution (5mM), standard. The Sabouraud's dextrose agar is set up in Petri dishes (60mm) and immunized with strains. The sterile disc (6mm) was impregnated with 10µl of MHL-CuO NPs, Manilkara hexandra leaf extract, Copper sulfate, and fluconazole. The dishes were brooded for 24h at 37°C and the zone of restraint is recorded. Fluconazole is utilized as the standard for anti-infective.

2.9. Antioxidant assays (in-vitro DPPH method)
The 2, 2-diphenyl-2- picrylhydrazyl (DPPH) is used for examining free radical scavenging activity. DPPH radicals (0.2mM) are prepared in methanol solution. MHL-CuO NPs (20-100µg/ml) in water mixed with one ml of prepared DPPH solution in a test tube. It is shaken vigorously and kept in a dark room for 30 min after absorbance is measured. Similarly, ascorbic acid is used as standard. After measuring the IC₅₀ value is calculated [16].

The scavenging ability is calculated using formula.

\[ \text{% of inhibition} = 100 \times \left( \frac{A-B}{A} \right) \]

Where, I (%) is inhibition percentage
A- Absorbance of control reaction
B- Sample absorbance of test compound.

III. RESULT AND DISCUSSION
The phytochemical screening of Manilkara hexandra aqueous leaf extract revealed the presence of alkaloid, flavonoids, terpenoids, amino acid, protein, quinone, anthraquinone, saponin, resin, carbohydrates, steroids, tannin, phlobatannin, gum & mucilage, glycosides, and xanthoprotein. However, Manilkara hexandra aqueous leaf extract shows the negative answer for phytochemicals such as anthocyanin, emodins, fixed oils & fats and coumarins. The medicinal behaviors depend on the
phytochemicals present in the plant. In recent centuries, green synthesis of metal nanoparticles plays a major role in research due to its boundless application [17].

### 3.1. Characterization of Copper oxide nanoparticles

Formation of copper oxide nanoparticles is easily recognized by the change of color of the solution shown in Figure 3.

![Figure 3. CuSO₄·5H₂O (5mM) mixed with leaf extract gives Copper oxide nanoparticles (CuO NPs).](image)

The *Manilkara hexandra* leaf extract added with 5mM of cupric sulfate solution start the change in color reaches to dark brown after incubation for 48 hours after the color of the solution is been unchanged. The UV-vis spectra clearly project a strong absorbance between 250-300 nm. This suggests the formation of copper oxide nanoparticles as in Figure 4. This SPR absorption initiates the reflection of the size and shape of nanoparticles [18].

![Figure 4. UV-vis spectroscopy indicating the synthesis of MHL-CuO NPs](image)

Figure 5a shows the FTIR spectrum was observed for *Manilkara hexandra* aqueous leaf extract 3437cm⁻¹ (−OH group), 1638 cm⁻¹ (C=C of aromatic ring overlapped with the carbonyl group), 1384 cm⁻¹ (NO₃ group), 1069 cm⁻¹ (C-N stretching of amines) and 660cm⁻¹ (C-H stretching). The disappearance of 1452 cm⁻¹ peak and the relative shift of copper oxide nanoparticles are observed at 3437 cm⁻¹ to 3447 cm⁻¹, 2075 cm⁻¹ to 2076 cm⁻¹, 1638 cm⁻¹ to 1637 cm⁻¹, 1069 cm⁻¹ to 1116 cm⁻¹ and 660 cm⁻¹ to 675 cm⁻¹. This clearly projects that oxidized polyphenols capped the surface of copper oxide nanoparticles. The mode of vibration for CuO nanoparticles range from 500 cm⁻¹ to 700 cm⁻¹. The major peak 675 cm⁻¹ were observed to be CuO stretching as in Figure 5b.
Figure 5. Fourier Transform Infrared Spectroscopy of a) Leaf Extract and b) MHL-CuO NPs.

The morphology and size of synthesized CuO NPs are analyzed by using FE-SEM. The shape of the particle is spherical. The size of the copper nanoparticle was found to be about 30-60 nm as shown in Figure 6a, and Figure 6b. The elemental composition of copper (Cu) and oxide (O) are highly present in EDAX analysis and a strong signal for Si which is due to glass wafer used as the substrate to prepare the thin film. The minor peaks elements correspond to the protein, which act as a capping over the copper oxide nanoparticles [19].
Figure 6.a) Field Emission Scanning Electron Microscopy (FE-SEM) image of MHL-CuO NPs and b) Energy Dispersive Spectroscopy (EDAX) spectrum of MHL-CuO NPs.

XRD analytic view a small distinct peak at 32.523, 38.873, 46.323, 48.773, 51.323, 58.373 and 75.223, which indexed the planes 110, 200, 112, 202, 112, 202 and 222 of the face-centered-cubic (FCC) structure of copper oxide nanoparticles (JCPDS-05-0661) as in Figure 7. The XRD pattern project that green synthesized CuO NPs are crystalline in nature [20].

Figure 7. X-ray powder diffraction (XRD) of MHL-CuO NPs
The particle size is analyzed by using DLS. The average particle size distribution was 343.1 nm shown in Figure 8a. Particle size is small in FE-SEM compared to DLS, since DLS is measured based on the hydrodynamic diameter of particles. The zeta potential value was found to be -9.50 mV shown in Figure 8b. The zeta potential value is high, which denote a strong repellent force within the particles and prevent aggregation [21, 22].

![Size Distribution by Intensity](image1)

| Size (d.nm) | Intensity (%) |
|-------------|---------------|
| 0.1         | 0             |
| 1           | 2             |
| 2           | 4             |
| 3           | 6             |
| 4           | 8             |
| 5           | 10            |
| 6           | 12            |

![Zeta Potential Distribution](image2)

| Apparent Zeta Potential (mV) | Total Counts |
|------------------------------|--------------|
| -100                         | 50000        |
| -90                          | 150000       |
| -80                          | 250000       |
| -70                          | 350000       |
| -60                          | 450000       |

Figure 8. a)The hydrodynamic size of MHL-CuO NPs and b) Zeta potential of MHL-CuO NPs.

The MHL-CuO NPs was examined by TG/DTA to find the thermal stability. In TG curve, we observe weight lost from 3.8290 mg to 0.5565 mg from room temperature to 900°C and the residue is 14.53% after 900°C it is constant there is no weight loss. The weight is been lost at six stages from room temperature to 104.27°C, 104.27°C to 201.08°C, 201.08 to 387.64°C to 627.08°C, 627.08° to 773.82°C, 773.82°C to 900°C. The primary weight loss is due to the moisture content present in the nanoparticles. The major reduction, weight loss occurs at 201.08°C to 387.64°C and 387.64°C to 627.08°C this is due to decomposition of materials shown in Figure 9.
Figure 9. Thermogravimetry/Differential Thermal Analysis (TG/DTA) of MHL-CuO NPs.

The DTA thermograms reveals an endothermic peak at 65°C. The exothermic peak at 240°C shows the decomposition of copper nanoparticles and another exothermic peak shown at 345°C depict the stability of the material. The nanoparticles can be examined by DTA for thermal decomposition and crystallization of the material [23]. In the DSC curve, we observe four endothermic peak at 61.7°C, 84.9°C, 125°C, 220°C and small other bumps. There is also an exothermic peak at 76°C, 120°C, 170°C, 210°C and small other bumps. They show clearly that the particles are stable up to 250°C. The peaks are due to degradation of molecules present in the materials and is shown in Figure 10.

Figure 10. Differential scanning calorimetry (DSC) of MHL-CuO NPs.
3.2. Antimicrobial evaluation

The antibacterial activity of various samples such as MHL-CuO NPs, Manilkara hexandra leaf extract, copper sulfate (5mM) and Standard was tested against pathogens by disk diffusion method. The CuO NPs showed growth inhibitory action against *Escherichia coli* (8 mm) and *Staphylococcus aureus* (7 mm). The Manilkara hexandra leaf extract exhibited the antibacterial activity all the four bacteria, but was more susceptible against *Escherichia coli* (10 mm), *Pseudomonas aeruginosa* (7 mm). However, the leaf extract and copper oxide nanoparticles showed better inhibitory actions against bacteria. Copper sulfate (5mM) shows no zone of inhibition. *Amoxicillin* is used as the standard to find the range of activity. The antifungal susceptibility test of the different samples such as MHL-CuO NPs, Manilkara hexandra leaf extract, copper sulfate (5mM) and the standard was tested against the test organisms. The MHL-CuO NPs were the most effective and the highest activity was seen against *Aspergillus niger* (9 mm zone of inhibition), followed by the highest activity against *Aspergillus flavus* (6 mm zone of inhibition). The Manilkara hexandra leaf extract showed the activity against *Aspergillus niger* (7 mm zone of inhibition) shown in Table 1. and Figure 11. *Fluconazole* was used as the standard to analyze testing organisms. Antimicrobial assays are represented in the bar chart, it is shown in Figure 12.

Figure 11. Various Antibacterial and Antifungal strains. Zone of Inhibition of MHL-CuO NPs, Leaf Extract, CuSO₄ and Standard.
Figure 12. Zone of inhibition of CuO NPs, Leaf extract, CuSO₄, Standard with different microbes is shown in bar chart.

Table 1. Zone of Inhibition of CuO NPs, Leaf Extract, CuSO₄, and standard against various microbes.

| S.No | Name of the organism                  | Zone of inhibition in mm |
|------|---------------------------------------|--------------------------|
|      |                                       | CuO NPs (D) | Leaf Extract (C) | CuSO₄ (A) | Standard (B) Amoxicillin / Fluconazole |
| 1.   | *Staphylococcus aureus* (MTCC 25923)  | 7            | 6              | 0         | 9                                   |
| 2.   | *Bacillus subtilis* (MTCC 2451)       | 0            | 6              | 0         | 9                                   |
| 3.   | *Escherichia coli* (MTCC 25922)       | 8            | 7              | 0         | 9                                   |
| 4.   | *Pseudomonas aeruginosa* (MTCC 27853) | 4            | 7              | 0         | 9                                   |
| 5.   | *Aspergillus flavus* (MTCC-3396)      | 6            | 0              | 0         | 10                                  |
| 6.   | *Aspergillus niger* (MTCC-227)        | 9            | 7              | 0         | 10                                  |

3.3. Antioxidant (DPPH method in-vitro)
The antioxidant activity is examined using the DPPH method for various concentrations of copper oxide nanoparticles from 20 µg/ml to 100µg/ml. They are alternatively compared with standard (ascorbic acid). Here MHL-CuO NPs show a good antioxidant property but low compared with the standard. But, MHL-CuO NPs are non toxic and has no side effect. The ascorbic acid (standard) causes side effect. They are compared and the IC50 value is calculated as in Figure 13 and Table 2.
IV. CONCLUSION

The present bio-synthetic method is an easy, eco-friendly, economical and rapid form of synthesis at room temperature. To synthesize CuO NPs, there is no need of an external capping agent. This method produced spherical, a polydispersed form of nanoparticles. It is well separated from each other and no aggregation was observed. Based on the above experiment we observe that this method can also be indulged in a large industrial scale of fabrication of nanomaterials. The antimicrobial properties of synthesized CuO NPs having the size range from 30 nm to 60 nm were effective against pathogens. It also show good antioxidant potential. This CuO NPs can be used for the improvement of agriculture, food safety product and for drug delivery system.

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