Biogenic synthesis of Copper nanoparticles using aquatic pteridophyte Marsilea quadrifolia Linn. rhizome and its antibacterial activity

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Abstract

The spread of contagious diseases and the increase in the drug resistance amongst pathogens has enforced the researchers to synthesize biologically active nanoparticles. Development of eco-friendly practice for the synthesis of nanoparticles is growing bit by bit in the field of nano-biotechnology. The present investigation outlines the development of a method to biosynthesize copper nanoparticles (CuNPs) by mixing copper chloride solution with aqueous rhizome extract of Marsilea quadrifolia. The synthesized nanoparticles were characterized by using the UV-vis spectroscopy, Fourier Transform Infrared Spectroscopy (FTIR), Scanning Electron Microscopy (SEM), X-ray Diffraction (XRD) and Atomic Force Microscope (AFM). The UV-vis spectra showed an absorption band at 324 nm. The FTIR measurement revealed the presence of all functional groups having control over reduction and stabilization of the nanoparticles. The SEM micrograph depicts the morphology of biogenically synthesized CuNPs with leaf like structure. The X-ray diffraction pattern confirmed the formation of crystalline nature of CuNPs with an average size of 25.20 nm. Regular gravel like structure of CuNPs was displayed in the AFM image. Additionally, the biosynthesized CuNPs were found to be extremely toxic against two gram positive bacterial strains Bacillus thuringiensis and Streptococcus faecalis.

Keywords: Antibacterial; Biogenic Synthesis; Copper Nanoparticles; Marsilea quadrifolia; Scanning Electron Microscopy (SEM).

How to cite this article


INTRODUCTION

Today, there is an ever-increasing attention of the biosynthesis of nanoparticles as an emerging highlight of the intersection of nanotechnology and biotechnology. It is due to a rising need to expand environment-friendly technologies in material synthesis. Biomolecules as reductants are found to have a significant advantage over their counterparts as protecting agents [1]. The need for biosynthesis of nanoparticles rises for the reason that the physical and chemical processes are expensive, unsafe, take a longer time, and they are tedious process to isolate nanoparticles. The application of plant extracts to the biosynthesis reaction is one of the vital branches of biosynthesis
of nanoparticles. Based on this method, the plant extract is used as a capping and reducing agent for the synthesis of nanoparticles [2-4].

Among different metal nanoparticles, copper nanoparticles (CuNPs) are used in a wide array of applications such as in electronics, air and liquid filtration, ceramics, wool products, films, lubricants oil and in inks [5-7]. Copper (Cu) is an active transition metal involved in many redox process in animal and plant cells. In plants, copper is a component of regulatory proteins, a cofactor of phenol oxidases, ascorbate oxidase, superoxide dismutase (SOD) and participates in electron transport in the respiratory and photosynthetic chains [8].

*Marsilea quadrifolia* Linn, a member of Marsileaceae is a creeping herbaceous perennial plant widely distributed in tropical and temperate regions of world and originated in marshy places and along the banks of canals and rivers [9]. As per the traditional claims the plant has been employed as astringent, hypnotic, diuretic, psychopath, leprosy, hemorrhoids, skin diseases, expectorant, aphrodisiac, anodyne, ophthalmic, constipating, fever, insomnia, febrifuge, reduces mental tension inducing sleep, reduces anxiety and stress in emotional conditions [10]. The phytoconstituents like marsilin (1-triacontanol-cerotate), 3-hydroxytriacontan-11-one, hentriacontan-6-ol, methylamine, betasitosterol, marsileagenin A, flavonol-O-monoo-and-diglycoside, C-glucoylflavones and C-glucosylxanthones have been isolated from the plant [11]. Due to the presence of above active constituent, plant has been reported to possess various pharmacological activities. Hence, the present study was undertaken with an aim to synthesize the CuNPs via novel biological method using aquatic pteridophytic plant extract of *Marsilea quadrifolia* rhizome, in addition, the antibacterial activity of the CuNPs and the *M. quadrifolia* rhizome extract against pathogenic bacteria is assessed.

**MATERIALS AND METHODS**

*Collection of Marsilea quadrifolia Rhizome*

*Marsilea quadrifolia* Linn. rhizome was collected from Puthallam, Kanya Kumari District. The collected samples were cut into minute fragments and shade dried till the fracture is uniform and smooth. The dried material was granulated or powdered by using a blender and sieved using sieve No. 60 to get uniform particles. The powder finally obtained was employed for the extraction of active ingredients of the plant material. The plant samples were identified with the aid of local flora and validated by Botanical Survey of India, Southern Circle, Coimbatore, Tamil Nadu, India.

**Preparation of Extracts for Phytochemical Screening**

*Hot Maceration Method Using Soxhlet Apparatus*

Freshly collected plant materials were dried in shade, and after that coarsely powdered in a blender. The coarse powder (100 g) was extracted successively with water, 250 mL in a Soxhlet apparatus for 24 hrs. The extract was filtered using Whatman No. 41 filter paper. The aqueous extract was subjected to qualitative tests for the identification of various phytochemical constituents as per standard procedures [12].

**Green Synthesis of Nanoparticles**

*Preparation of Rhizome Extract (Reducing Agent)*

The rhizome extracts were prepared by taking 20 gm of rhizome. Rhizome was washed thoroughly with double distilled water and cut into fine pieces. Then, the pieces are boiled in 100 ml double distilled water for 20 minutes at 60 °C in a glass beaker. After boiling the extract was filtered using Whatman No. 1.

**Preparation of Precursors**

Precursors for copper nanoparticles (CuCl₂ respectively) were purchased from Hi-media chemicals, India and prepared freshly. Precursor for preparing copper nanoparticles was 2 mM of copper chloride using double distilled water.

**Synthesis of Copper Nanoparticles**

In a typical reaction mixture, 10 mL of aqueous 2 mM copper chloride dehydrate CuCl₂·2H₂O was treated with 10 mL of aqueous rhizome extract of *M. quadrifolia* and stirred magnetically at room temperature, until the light blue colour changes to light green colour. Then, the mixture is heated at 80 °C for 2 minutes. Afterwards, the mixture was treated with 1 M sodium hydroxide drop by drop. As soon as, the sodium hydroxide comes in contact with copper ions that spontaneously change the green mixture into brown precipitate, indicating the formation of water soluble mono dispersed copper oxide nanoparticles.
Characterization of the Synthesized Copper Nanoparticles

UV – Vis Spectroscopy

Ultraviolet-visible spectroscopy (UV-Vis) submits to absorption spectroscopy in the UV-visible spectral area. The copper nanoparticles were characterized in a Shimadzu V 650 UV-vis spectrophotometer. Being the scanning range for the samples at 300-700 nm, the double distilled water is made use as a blank reference.

Fourier Transform Infra-red Spectroscopy (FTIR)

The nanoparticles were characterized using a Fourier Transform Infra red Spectrophotometer (FTIR Thermoscientific iS5). Two milligrams of the sample were mixed with 100 mg Potassium bromide (KBr). Compressed to prepare a salt disc roughly 3 mm in diameter, the discs were at once kept in the sample holder. The FTIR spectra were recorded in the absorption range between 400 and 4000 cm⁻¹.

Scanning Electron Microscope (SEM) Analysis

The SEM is a kind of electron microscope (Carl Zeiss EVO 18) that images a sample by scanning it with an elevated energy beam of electrons in a raster scan models. This film of the sample was prepared on a carbon coated copper grid by just dropping a very small amount of the sample on the grid. The extra solution was removed by a blotting paper and then the films on the SEM grid were permitted to dry by putting it below a mercury lamp for 5 min.

X-Ray Diffraction (XRD) Analysis

The particle size and nature of the copper nanoparticles were determined by using the XRD. This was done using Shimadzu XRD–6000/6100 model with 30 kv, 30 mA with Cukα radians at 2θ angle. X-ray powder diffraction is a fast analytical technique. This is chiefly used for phase identification of a crystalline material and can supply information on unit cell dimensions. The analyzed material is thinly ground, and the normal bulk composition is established. The particle or grain size of the copper nanoparticles was determined using Debye Sherrer’s equation.

\[ D = \frac{0.94 \lambda}{B \cos \theta} \]

AFM Analysis

Surface topology of the synthesized copper nanoparticles were studied by 1µm x 1 µm Atomic Force Microscopy (AFM Nanosurf 2) analysis. 0.01 g synthesized nanoparticles were mixed with 20 ml of acetone and sonicated for 5-10 minutes using ultra sonicator. The solution was poured in a clean glass slide and was allowed to dry until all the acetone gets evaporated. Now, this glass slide is studied by using the Atomic Force Microscopy (AFM) in a non contact mode and the captured image was processed using the XEI software.

Antibacterial Activity

Antibacterial activity of synthesized nanoparticles was carried out by disc diffusion method [13]. The test bacteria Bacillus thuringiensis, Streptococcus faecalis, Salmonella paratyphi and Escherichia coli was obtained from the Research Laboratory, Department of Microbiology, Bharathidasan University, Tiruchirapalli, Tamil Nadu. The overnight incubated bacterial culture was spread over the freshly prepared nutrient agar plates. The 6 mm sterile disc (Hi media) was kept at the centre and different concentrations of synthesized nanoparticles (40 µg/mL, 80 µg/mL and 100 µg/mL) were poured on disc and then placed on the plate. The tetracycline disc (reference or positive control), copper solution without extracts and plant aqueous extract were also kept and then incubated at 37 °C for 24 h and after incubation the zone of inhibition was measured.

RESULT AND DISCUSSION

Phytochemical Screening

The resulting preliminary phytochemical screening of aqueous extract of M.quadrifolia rhizome reveals the presence of various phytochemicals like, alkaloids, coumarin, flavonoid, phenol, saponin, tannin, terpenoid, sugar, glycoside and xanthoprotein (Table 1).

Synthesis of Copper Nanoparticles (CuNPs)

The first step in nanoparticles characterization is the transformation of solution colour (Fig. 1a, b, c). The reduction of copper ions into CuNPs using aqueous extract of M.quadrifolia rhizome is indicated in Fig. 1c. It has occurred so because of visual colour change of solution from light blue to green colour and then brownish (brown precipitate), indicating the formation of water soluble nanodispersed CuNPs. Colour change of reacting solution from green to reddish brown using fruit extract of Zizyphus spina-christi and
leaf extract of *Ageratum houstonianum* was also confirmed by Khani *et al.* and Chandrakar *et al.* [14, 7].

**Characterization Study of CuNPs**

*UV-Vis Spectroscopy*

The formation of the CuNPs was confirmed mainly based on the colour change of reaction mixture and also by UV-Visible spectroscopy. The change of colour in aqueous solution is due to Surface Plasmon Resonance (SPR) Phenomenon. The position of the peak and shape of the spectra are using sensitive to metal size, dielectric constant of metal, nature of the reducing agent and stabilizer(s). In the present investigation, the UV-Vis spectrum of CuNPs confirmed absorption band at 324 nm (Fig. 2). This absorption band can be attributed to the plasma resonance absorption of the copper particles [15]. Similar observation has been reported in previous studies showing the absorption maxima of CuNPs at 384, 354, 350 and 326 nm using the flower extract of *Millettia pinnata* [16], leaf extracts of *Nerium oleander* and *Ageratum houstonianum* [17], *Cissus arnotiana* [18] respectively.

*Fourier Transform Infrared Spectroscopy (FTIR)*

The FTIR analysis is used to identify the possible bioactive molecule, which are accountable for the reduction of the copper ions, and the capping ability of the bioreduced CuNPs using *M.quadrifolia* rhizome extract. The FTIR absorption spectra have been shown in Fig. 3 and 4 to confirm the occurrence of different functional groups (Table 2)

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Table 1. Preliminary Phytochemical Screening of Aqueous Extract of *M. quadrifolia* Rhizome.

<table>
<thead>
<tr>
<th>Phytochemicals</th>
<th>MR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alkaloid</td>
<td>+</td>
</tr>
<tr>
<td>Anthraquinone</td>
<td>-</td>
</tr>
<tr>
<td>Catechin</td>
<td>-</td>
</tr>
<tr>
<td>Coumarin</td>
<td>+</td>
</tr>
<tr>
<td>Flavonoid</td>
<td>+</td>
</tr>
<tr>
<td>Phenol</td>
<td>+</td>
</tr>
<tr>
<td>Quinone</td>
<td>-</td>
</tr>
<tr>
<td>Saponin</td>
<td>+</td>
</tr>
<tr>
<td>Steroids</td>
<td>-</td>
</tr>
<tr>
<td>Tannin</td>
<td>+</td>
</tr>
<tr>
<td>Terpenoids</td>
<td>+</td>
</tr>
<tr>
<td>Sugar</td>
<td>+</td>
</tr>
<tr>
<td>Glycoside</td>
<td>+</td>
</tr>
<tr>
<td>Xanthoprotein</td>
<td>+</td>
</tr>
<tr>
<td>Fixed oil</td>
<td>-</td>
</tr>
</tbody>
</table>


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Fig.1. Synthesis of CuNPs from *M. quadrifolia* rhizome a) Plant extract b) Copper Chloride c) Copper nanoparticles.
Fig. 2. UV-visible spectrum of CuNPs of M. quadrifolia rhizome.

Fig. 3. FT-IR Spectrum of M. quadrifolia rhizome powder.

Fig. 4. FT-IR spectrum of CuNPs of M. quadrifolia rhizome.
in adsorbent. The FTIR spectra of M.quadrifolia rhizome powder (Fig. 3), which clearly shows the peak 3420 cm\(^{-1}\) corresponds to the O-H stretching hydroxyl group, peak at 2853 cm\(^{-1}\) assigned as C-H stretching of alkanes, peak at 1628 cm\(^{-1}\) represent > N-H band of secondary amine, peak at 1441 cm\(^{-1}\) corresponding to the C-C stretching of aromatics, peak at 1383 cm\(^{-1}\) represent C-N stretching of aliphatic amines, peak at 767 cm\(^{-1}\) represents C-H 'oop' of aromatics and peak at 568 cm\(^{-1}\) corresponds to the C-Br stretching of alkyl halides. Fig. 4 shows the FTIR spectrum of the biosynthesized CuNPs, peak at 3838 and 3384 cm\(^{-1}\) corresponds to the O-H stretching hydroxyl group/alcoholics or phenolics, peak at 2921 cm\(^{-1}\) represent O-H stretching of carboxylic acid, peak at 1599 and 1541 cm\(^{-1}\) represents C-C stretching (in ring) of aromatics, peak at 1376 cm\(^{-1}\) assigned as C-H rock of alkanes, peak at 1049 cm\(^{-1}\) represents C-N stretching of aliphatic amines, peak at 849 cm\(^{-1}\) represents C-H 'oop' of aromatics peak at 568 cm\(^{-1}\) assigned as C-Br stretching of alkyl halides. The small shift is observed in the absorbance peak in loaded nanoparticles on to M. quadrifolia rhizome powder in comparison with M.quadrifolia rhizome extracts (Fig. 3 and 4). The broad band 3420 cm\(^{-1}\) is changed to 3838 and 3384 cm\(^{-1}\). In addition, the peak 1628 cm\(^{-1}\), 1441 cm\(^{-1}\), 1383 cm\(^{-1}\), 1028 cm\(^{-1}\), 568 cm\(^{-1}\) are shifted to 1559 cm\(^{-1}\), 1541 cm\(^{-1}\), 1376 cm\(^{-1}\), 1049 cm\(^{-1}\) and 849 cm\(^{-1}\) respectively. The results obtained from FTIR studies indicate that the phenolic compounds and flavonoids act as capping agents, thus presenting agglomeration and stabilize the formation of nanoparticles [19].

**SEM Analysis of Copper Nanoparticles**

The morphological analysis of Cu based nanoparticles, driven from M.quadrifolia rhizome by SEM, was observed to be leaf like structure (Fig. 5). According to the previous reports nanoparticles synthesized by plant extracts show different shapes which depend on the extract chemical compositions, concentrations and pH of media [20-22].

**XRD Analysis of Copper Nanoparticles**

Fig. 6 represents the XRD pattern of the synthesized CuNPs using the aqueous extract of M. quadrifolia rhizome. The size, phase identification and crystalline nature of the CuNPs are determined by the XRD analysis. One intense and sharp peak at 2q=35.52°can then indexed to the 111 plane of Bragg’s reflection of copper. Therefore, the XRD pattern indicates that the CuNPs are organized by the reduction of Cu\(^{2+}\) and Cu\(^{+}\) ions. This reduction is by the aqueous extract of M.quadrifolia rhizome which is crystalline in nature. The size range of the synthesized CuNPs is 25.20 nm. This is consistent with findings published by Rama Devi et al. [23].

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Frequency (cm(^{-1}))</th>
<th>Chemical Bond</th>
<th>Phytoconstituents present</th>
<th>Peak Observed (Rhizome Powder)</th>
<th>Peak Observed (CuNPs)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3850-3500</td>
<td>O-H Stretch</td>
<td>Hydroxyl group</td>
<td>-</td>
<td>3838</td>
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<tr>
<td>2</td>
<td>3500-3200</td>
<td>O-H Stretch</td>
<td>Alcohols or Phenols</td>
<td>3420</td>
<td>3384</td>
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<tr>
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<td>3300-2500</td>
<td>O-H Stretch</td>
<td>Carboxylic acid</td>
<td>2924</td>
<td>2921</td>
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<tr>
<td>4</td>
<td>3000-2850</td>
<td>C-H Stretch</td>
<td>Alkanes</td>
<td>2853</td>
<td>2852</td>
</tr>
<tr>
<td>5</td>
<td>2700-2250</td>
<td>NH(^{+}) Stretch</td>
<td>Tertiary amine salt</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>6</td>
<td>1650-1550</td>
<td>&gt;N-H bend</td>
<td>Secondary amine</td>
<td>1628</td>
<td>-</td>
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<tr>
<td>7</td>
<td>1600-1585</td>
<td>C-C Stretch (in ring)</td>
<td>Aromatics</td>
<td>-</td>
<td>1559,1541</td>
</tr>
<tr>
<td>8</td>
<td>1500-1400</td>
<td>C-C Stretch</td>
<td>Aromatics</td>
<td>1441</td>
<td>-</td>
</tr>
<tr>
<td>9</td>
<td>1390-1350</td>
<td>C-H rock</td>
<td>Alkanes</td>
<td>1383</td>
<td>1376</td>
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<tr>
<td>10</td>
<td>1360-1290</td>
<td>N-O Symmetric Stretch</td>
<td>Nitro Compound</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>11</td>
<td>1320-1000</td>
<td>C-O Stretch</td>
<td>Esters, Ethers</td>
<td>1155</td>
<td>-</td>
</tr>
<tr>
<td>12</td>
<td>1250-1020</td>
<td>C-N Stretch</td>
<td>Aliphatic amines</td>
<td>1028</td>
<td>1049</td>
</tr>
<tr>
<td>13</td>
<td>910-665</td>
<td>N-H Wag</td>
<td>1(^{st}),2(^{nd}) amines</td>
<td>-</td>
<td>-</td>
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<tr>
<td>14</td>
<td>900-675</td>
<td>C-H 'oop'</td>
<td>Aromatics</td>
<td>767</td>
<td>849</td>
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<tr>
<td>15</td>
<td>690-400</td>
<td>C-Br Stretch</td>
<td>Alkyl halides</td>
<td>617</td>
<td>568</td>
</tr>
</tbody>
</table>
AFM Analysis of Copper Nanoparticles

An Atomic Force Microscopy (AFM) technique was employed to characterize the copper nanoparticles for its detail size and morphology of copper. The topographical image of the CuNPs synthesized by plant extract *M. quadrifolia* rhizome, are shown in Fig. 7 (a, b). The AFM image of CuNPs exhibited regular gravel like structure.

Antibacterial Activity

The antimicrobial activity of the CuNPs synthesized by *M. quadrifolia* rhizome extract was investigated against various pathogenic organisms such as *Bacillus thuringiensis* (+), *Streptococcus faecalis* (+), *Salmonella paratyphi* (-) and *Escherchia coli* (-). The diameter of inhibition zones (nm) around each disc with the CuNPs solution is represented in Table 3. Copper nanoparticles synthesized from *M. quadrifolia* rhizome showed greater inhibition zone 14mm and 15mm against *Bacillus thuringiensis* and *Streptococcus faecalis* at 100µg/ml concentration respectively. The antimicrobial activity was due to the fact that the copper ions released from the CuNPs permeated the bacterial cell membrane and damaged the structure of the cell membrane by disturbing the negatively charged cell wall [24, 25]. Copper ions are involved in cross linkage of nucleic acid strands by binding them with the DNA molecule of bacteria. These outcomes in a disordered helical
structure of DNA molecule, roots denaturation of proteins and some other biochemical procedures in the cell lead to total destruction of the bacterial cell [26].

In general, CuNPs synthesis has attracted particular interest, compared with other NPs, as their useful properties are achievable at cost lower than silver and gold [27]. Research into CuNPs has made significant progress in the areas of nanotechnology and nanomedicine with in the last decade due to their excellent antifungal/antibacterial applications. In recent years, plant mediated biological synthesis of nanoparticles has gained interest due to its simplicity and eco-friendliness.

CONCLUSION

Biological synthesis of nanoparticles has increased in the field of nanobiotechnology. It creates novel materials that are eco-friendly cost effective and with a great significance to apply in the different areas. In this research, M. quadrifolia rhizome extract is used in green chemistry approach towards the synthesis of CuNPs. Phytochemicals studies confirm the presence of flavonoids and phenolics compounds, which are responsible for the formation of CuNPs. The structure of the synthesized nanoparticles has been appropriately characterized using UV-Vis spectroscopy, FT-IR, SEM, XRD, and AFM analysis. UV-vis spectra of CuNPs synthesized by M. quadrifolia rhizome extract revealed characteristic absorption peak at 324 nm. FT-IR analysis confirmed that phenolics compounds and protein molecules in the extracts are responsible for capping of the Cu nanoparticles. XRD pattern revealed the crystalline nature of the CuNPs. SEM studies confirmed the structure of the synthesized CuNPs. The AFM image had also confirmed the morphology of the CuNPs. Moreover, the synthesized CuNPs showed considerable antibacterial activity against the treated gram positive and gram negative bacterial strains. This is the first on the synthesis of copper nanoparticles using M. quadrifolia rhizome extract and their effect as antibacterial agents.

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DISCLOSURE STATEMENT

No potential conflict of interest was reported by the authors.
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