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# Green synthesis of copper oxide nanoparticles from leaf extract of *Glycosmis pentaphylla*: its efficiency as antifungal, photocatalytic and cytotoxic agent

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**Abstract**
The present study demonstrates a simple, beneficial and eco-friendly method for the facile synthesis of copper oxide nanoparticles (CuONPs) using leaf extracts of *Glycosmis pentaphylla*. The formation of the biosynthesized nanoparticle was identified from the surface plasmon resonance at 262 nm using UV-Visible spectrophotometer. The functional group of the plant extract responsible for capping on CuONPs was confirmed by FTIR analysis. The surface morphology of biosynthesized CuONPs were predominantly spherical, as per TEM studies. The CuONPs, relatively of size 10 nm, did not have any physical contact with each other. The biosynthesized CuONPs exhibited strong photocatalytic activity in the degradation of Coomassie Brilliant Blue dye. Antifungal activity of CuONPs when screened against two plant pathogenic fungi, *Rhizoctonia solani and Sclerotium rolfsi* by poisoned food technique revealed the high efficacy of copper oxide nanoparticles as a strong antifungal agent. Apart from the beneficial aspects of biosynthesized CuONPs, the cytotoxicity analysis on *Allium cepa* root tips displayed anomalies in cell division and chromosome behaviour at a higher concentration of 6 mg/L.

**Keywords:** *Glycosmis pentaphylla*; copper oxide nanoparticles; photocatalytic degradation; antifungal activity; cytotoxicity

## Introduction

Nanotechnology is an expeditiously developing multidisciplinary field with wide scope of applications in cancer therapeutics, targeted drug delivery, electronics, cosmetic industry and biosensors [1]. Nanoparticles are atomic or molecular aggregate with at least one dimension between 1 and 100 nm [2, 3]. They arise as valuable and interesting green impetuses whose proficiency is ascribed to their characteristic high surface area to volume ratio. However, there are a lot of environmental concerns while using traditional nanoparticles. This can be rectified to some extent by using biomolecules present in plant concentrates. Improvement of green nanotechnology is producing enthusiasm of analysts towards eco-friendly biosynthesis of nanoparticles. The biosynthesized nanoparticles may be effectively applied in all fields, and also have much more potential target application than conventionally produced nanoparticles.

Recently copper nanoparticles have gained the interest among research community due to its unique physio-chemical properties and economical production. Copper nanoparticles have great importance in applications like heat transfer systems [4], antimicrobial materials [5], super strong materials [6] and catalysts [7]. Although copper is one of the most widely utilized materials in various fields, its synthesis in nano sizes is challenging due to its high tendency of oxidation. Unlike gold and silver, copper is extremely sensitive to air, and the oxide phases are thermodynamically more stable [8]. Therefore, the formation of a surface oxide layer on copper nanoparticles is inevitable.

Among the various methods involved in the synthesis of CuONPs, the physical and chemical methods are traditionally used. However, these approaches entail certain drawbacks which cannot be neglected. In physical approach, expensive instruments, high temperature and pressure, and high energy consumption are some of the hurdles that are often faced. In chemical approach, the toxicity of chemicals causing environmental hazards is of major concern [9–11]. The biological approach is gaining acceptance due to its cost-effective and eco-friendly way of fabricating nano materials.

Various biological agents such as bacteria [12, 13], fungi [14, 15], actinomycetes [16–19], yeast [20] and plants [21–24] are used in the green route for the synthesis of metallic nanoparticles. *Glycosmis pentaphylla* is one of the medicinally important plants belonging to the family Rutaceae. Traditionally, leaves of *Glycosmis pentaphylla* are used in the treatment of fever, hepatopathy, eczema, skin disease, helminthiasis and wounds [25]. *G. pentaphylla* is reported to contain the alkaloid arborinine as the active ingredient [26]. Leaf extract and crude alkaloid possesses antibacterial and antifungal properties [27].

Copper based antifungal chemicals has revolutionised plant disease management since the use of Bordeaux mixture in 1885. Overruling the beneficial aspects of copper fungicide, the disadvantages include phytotoxicity, soil accumulation and adverse effect on soil biota, crop yield and quality. At high concentrations, copper is toxic to plant roots as it interferes with nutrient uptake [28, 29]. This led to the search for alternatives to copper based plant therapeutics for sustainable agriculture. A significant reduction in the use of copper based fungicide in plant disease management is the major challenge. CuONPs can be suggested as an alternative to copper fungicide as it significantly reduces the dose.

The non-degradable and carcinogenic dye effluents released into the water bodies by the textile and paper industries without any treatment leads to contamination of resources [30]. Among the various techniques, heterogeneous

photocatalysis is a popularly employed process to eliminate hazardous waste materials especially organic compounds which are degraded to less toxic materials [31]. The classic works [32] have demonstrated that the *Allium cepa* test is reproducible and successful model for assessing and checking cytotoxicity and genotoxicity of chemicals. The present work details a green chemistry approach to the synthesis of Copper oxide nanoparticle using *Glycosmis pentaphylla* leaf extract. The obtained product was characterised using UV- Visible spectrophotometer; Fourier transformed infrared spectroscopy and Transmission Electron Microscope. Further, the synthesized nanoparticles were evaluated for its antifungal activity, cytotoxicity and photocatalytic efficiency.

## Materials and methods

### Preparation of plant extract

The leaves of *Glycosmis pentaphylla* were collected from a local medicinal plant garden at Thrissur, Kerala, India. Subsequently, ground dried leaf powder (20 g) was boiled with 200 mL of deionized water to obtain the aqueous extract. Thereafter, the extract was filtered and filtrate was centrifuged at 2000 rpm for 10 minutes. The supernatant thus obtained was stored in amber coloured reagent bottles at $4^0$C for further experiments.

### Synthesis of CuO nanoparticles

The aqueous solution of *Glycosmis pentaphylla* leaf extract was taken in Erlenmeyer flask and 3 mM $CuSO_4$ solution was added to it in the ratio 1:10 by volume. The reaction was allowed to proceed for 8 hours at 120 $^O$C with constant stirring in a sealed glass container. The nanoparticles thus formed were separated by repeated centrifugation at 1200 rpm. Finally they were washed with deionized water to remove water soluble molecules and the nanoparticle suspension was lyophilized. The lyophilized CuONPs was used for all the subsequent experiments.

### Characterization of CuONPs

The formation of CuONPs using the aqueous leaf extract of *Glycosmis pentaphylla* was monitored by recording the UV-Visible spectra of the solution using UV-Visible spectrophotometer (Shimadzu UV Probe 1800) in the scanning range of 300-700 nm. The Fourier Transform Infrared (FTIR) spectra were recorded for the CuONPs through potassium bromide (KBr) pellet (FTIR grade) method in the ratio 1:100 using Thermo Nicolet Avatar 370 FTIR spectrophotometer in the spectral range of 4000-400 cm $^{-1}$. FTIR analysis was done to identify the bioactive molecules of the plant extract responsible for the reduction of nanoparticles. Transmission Electron Microscopic analysis (TEM) was carried out to study the size distribution and shape of the biosynthesized CuONPs. The 200 kV ultra-high resolution transmission electron beam (JEOL, Model No. JEM 2100 HR with EELS) was used in TEM analysis.

### Photocatalytic degradation of toxic dye

Coomassie Brilliant Blue (CBB), a triphenyl methane dye used in the textile industry, is now commonly used for staining proteins in analytical biochemistry. To 1% CBB solution, CuONP was added in 10:1 ratio (v/v) with continuous stirring to establish adsorption equilibrium. Thereafter, the resulting mixture was irradiated under sunlight. After specific time intervals (10, 20, 30 min), 2 mL mixture was taken and absorbance spectrum (480-680 nm) was recorded to assess the photocatalytic activity of biosynthesized CuONPs.

### Anti-fungal activity assay

The pure cultures of the test fungi *Rhizoctonia solani* and *Sclerotium rolfsi* were obtained from Plant Pathology Department, Kerala Agricultural University, Kerala, India. The assessment of fungi toxicity was done by poisoned food technique [33, 34]. The inoculum disc of 4 mm in diameter was prepared with the seven days old pure culture of the test fungus. A volume of 0.5 ml each of CuO nanoparticle suspension in different concentrations (25 mg/L, 50 mg/L, 75 mg/L, 100 mg/L) and 3mM $CuSO_4$ was aseptically included into the petriplate. This was followed by the addition of 9.5 ml of molten Potato Dextrose Agar (Himedia, Mumbai) and swirled gently to accomplish thorough mixing of the contents. Deionized water served as control. The prepared inoculum disc of the test fungus was inoculated at the centre of the petriplate upside down asceptically, followed by incubation at 25 $\pm$ $2^0$C. The mycelial growth inhibition percentage was calculated on the 7$^{th}$ day of incubation using the formula:

$$I = C\text{-}T/C \times 100$$

Where, I is inhibition percent, C is colony diameter in control (mm) and T is colony diameter in treatment (mm) [35].

### Cytotoxicity on onion root tip

### Determination of Mitotic and Active Mitotic Index

Bulbs of *Allium cepa*, a standard material for chromosome studies, were used for cytotoxicity analysis. Mitotic squash preparation of the onion root tips was done with improved techniques [36].

The germination of onion bulbs was done using different concentrations of CuONPs (0, 2, 4, 6 mg/L) suspension and 3mM $CuSO_4$ for 48 hours, thereafter transferred to distilled water for the removal of test solutions. Each treatment comprised of 5 replicas, and distilled water served as negative control.

After treatment, the root tips were fixed in Carnoy's fluid, hydrolysed with 1N HCl, stained and squashed with 2% acetocarmine solution followed by microscopic examination. The mitotic index and cytological abnormalities were scored in mitotic cells [37], and mitotic index was calculated as follows:

$$\text{Mitotic index (\%)} = \frac{\textit{Number of dividing cells}}{\textit{Total number of cells observed}} \times 100$$

$$\text{Active mitotic index (\%)} = \frac{\textit{Number of metaphase+Number of anaphase}}{\textit{Total number of cells observed}} \times 100$$

### Determination of chromosomal aberrations

The slides were screened for both the numerical and structural aberrations. A minimum of 100 dividing cells per sample was assessed to score for any chromosomal aberrations that may be induced due to the uptake of CuONPs.

This permits evaluation of chromosome damage and cell division aggravations, giving additional data with respect to the severity of potential cytotoxicity.

**Digital imaging**
The slides were screened under oil immersion objective (100×) of Labomed E200 light microscope and the chromosomes were photographed using Coolpix 995 digital imaging camera.

## Results and Discussion

**Synthesis of copper oxide nanoparticles**
Upon addition of aqueous leaf extract of *Glycosmis pentaphylla* to 3 mM $CuSO_4$, an immediate change in colour from yellowish brown to green was observed (Figure 1) indicating the formation of CuONPs.

**Characterization of CuONPs**

**U V - Visible spectroscopy**
The formation of CuONPs was analysed by UV-Visible spectroscopy. A broad absorption peak was observed at 262 nm (Figure 2) indicating the formation of CuONPs. Absorption peak at 262 nm is typical of CuONPs [38]. The broadness of the obtained absorption peak attributes to the wide size distribution of NPs.

**FTIR analysis**
The FTIR spectra (Figure 3) revealed prominent absorption bands at 3366.89 $cm^{-1}$, 2361.94 $cm^{-1}$, 1632.81 $cm^{-1}$, 1522.87 $cm^{-1}$, 1383.98 $cm^{-1}$, 1197.85 $cm^{-1}$, 1151.55 $cm^{-1}$, 1099.47 $cm^{-1}$, 1002.06 $cm^{-1}$, 861.25 $cm^{-1}$, 799.53 $cm^{-1}$, 662.58 $cm^{-1}$, 593.14 $cm^{-1}$ and 470.65 $cm^{-1}$. Earlier, Shobha *et al.*, [39] had confirmed that the FTIR analysis could be used to identify the capping, reducing and stabilising capacity of the leaf extract in the synthesis of copper nanoparticles. The broad peak is observed at 3366.89 $cm^{-1}$ in the spectra of CuONPs and it corresponds to free OH in molecule and OH group forming hydrogen bonds of stretching groups of macromolecular association. The bands observed at 1522.87 $cm^{-1}$, 1383.98 $cm^{-1}$, 1197.85 $cm^{-1}$ and 1151.55 $cm^{-1}$ is be assigned to the C=C aromatic ring and C-H or C-O stretching vibrations of methyl, methylene and methoxy groups [40]. Thus, the FTIR analysis confirms the capping of plant derived secondary metabolites onto the biosynthesized CuONPs and could serve as a better candidate for the drug delivery systems.

**TEM analysis**
Figure 4 shows the TEM image of biosynthesized CuO NPs. The image reveals that the biosynthesized CuONPs were predominantly spherical and were not in physical contact with each other. The size of the CuONPs was determined to be about 10 nm. Bright spots on image (Figure 4, b) represent particularly well represented orientations of microcrystals, revealing the polycrystalline nature of the biosynthesized CuONPs.

**Photocatalytic degradation of toxic dye**
The Coomassie brilliant blue R-250 (Hi media, India) dye is used as a model system for the photocatalytic degradation experiment [41]. The UV- Visible spectra of CBB alone has a very prominent absorption peak at 580 nm. There was a gradual decolourization and decrease in this peak intensity when nanoparticle suspension was added to CBB solution. This indicates the efficiency of copper oxide nanoparticles in the removal of toxic dyes from environmental system. Photocatalytic degradation of CBB by CuONPs is depicted in the Figure 5. The colloidal copper oxide nanoparticles effectively degrade the Coomassie brilliant blue R-250 dye beneath the sunlight [42]. Our results comply very well with the previous studies and thereby find application in bioremediation.

**Antifungal assay**
The presented study indicates that CuONPs synthesized *via* green route were promising antifungal agents against the plant pathogens *Sclerotium* and *Rhizoctonia*. It was found that CuONP solution inhibited the development of *Sclerotium rolfsii* in all the concentrations tried, while only 75 % and 100 % concentration of CuONP solution inhibited *Rhizoctonia solani* (Table1, Figure 6, 7). It was observed that with the increase in concentration of CuONPs, mycelial growth in the treatment plates decreased. Both $CuSO_4$ and CuONP solution exhibited more antifungal property for *Sclerotium* than *Rhizoctonia*. One of the suitable alternatives to copper fungicide is to use CuONPs which requires low dosages of copper, but effectively checks fungal growth. The CuONPs used here being synthesised from *Glycosmis pentaphylla* has the added advantage of antimicrobial property [25] because the secondary metabolites caps over the NPs.

**Cytotoxicity assay**
The cytotoxicity assay was conducted to find out the impact of biosynthesized CuONPs on the cell division of onion root tip, thereby ascertaining the degree of toxicity to plant cells. The potential cytotoxicity and genotoxicity of biosynthesized CuONPs was analysed by observing cytological parameters such as mitotic index and number of chromosomal abnormalities, including sticky chromosomes (metaphase) and cells with damaged nucleus.

**Determination of Mitotic and active mitotic index**
Mitotic index measures the proportion of cells in the M – phase of the cell cycle and inhibition could be interpreted as cellular death or delay in the cell proliferation kinetics [43]. Mitotic index frequencies of onion root tips treated with various concentrations of biosynthesized CuONPs (2 mg/L, 4 mg/L and 6 mg/L) and 3 mM $CuSO_4$ were recorded as 9.41 %, 8.5 %, 4.97 % and 5.98 % respectively. The results indicated low mitotic index frequency for treatment groups when compared to control with a mitotic index of 13.93 %. This suggests that CuONP solution might interfere with cell division. The biosynthesized CuONPs at lower concentration exhibited higher mitotic index when compared to $CuSO_4$ solution (3 mM). This indicates that at low concentrations, the biosynthesized CuONPs exhibits lesser toxicity. The highest mitotic index was expressed in onion root tip cells treated in deionized water, whereas the treatments with various concentrations of biosynthesized CuONPs exhibited dose dependent decrease in mitotic index. Among the various concentrations of biosynthesized CuONPs, higher mitotic index (9.41 %) was observed at 2 mg/L. Reduction in the mitotic activity could be due to inhibition of DNA synthesis or a blocking in the G2 phases of the cell cycle, preventing the cell from entering mitosis [44]. Therefore, the exposure of onion root tips to higher concentration of biosynthesized CuONPs revealed significant inhibition of mitotic index (Table 2).
A decrease in active mitotic index with increase in concentration of biosynthesized CuONPs was observed. Maximum active mitotic index was at 2 mg/L (3.36 %) and

gradually decreased with increase in concentration of biosynthesized CuONPs (Table 2). When compared to $CuSO_4$ solution (3 mM), the biosynthesized CuONPs exhibited higher active mitotic index. These results indicated that low concentrations of the biosynthesized CuONPs possessed low inhibitory and mitodepressive effects on the cell division and chromosome behaviour of *Allium cepa* when compared to the $CuSO_4$ solution.

**Determination of chromosomal aberrations.**

The frequency of mitotic aberration was nil in the control (Figure 8). But the root tip exposed to either $CuSO_4$ solution or higher concentration of biosynthesized CuONPs had different kinds of chromosome anomalies. It was supported by the high occurrence of sticky metaphase and anaphase (Figure 9b and 9e), diagonal metaphase (Figure 9c), Vagrant chromosome (Figure 9d), damaged chromosomes and Dislocation of chromosome (Figure 9f). Greater number of aberrations was observed at both 3 mM $CuSO_4$ solution and higher concentration (6 mg/L) of biosynthesized CuONPs (Figure 9). The observation of sticky metaphase reinforces the hypothesis of the toxic effect of copper in plant cells.

When $CuSO_4$ solution and various concentrations of biosynthesized CuONPs suspensions were tested on *Allium cepa* cells to evaluate their action on the kinetics of the cell cycle, a decrease in the mitotic index was observed. This was prominent for 3mM $CuSO_4$ solution and 6 mg/L of biosynthesized CuONPs. The $CuSO_4$ solution and higher concentrations the biosynthesized CuONPs suspension proved to be extremely cytotoxic and inhibited *Allium cepa* root growth. The decline of mitotic index below 22 % in comparison to control could contribute to lethality on the organism [45], while a decrease below 50% usually has sub-lethal effects called cytotoxic limit value [46]. Previous studies have reported the mitodepressive effects on the cell division of some plant extracts, including their ability to block of DNA synthesis and nucleus proteins [47]. Therefore, the obtained results indicate that lower concentrations of biosynthesized CuONPs are beneficial, but its higher concentrations might possess inhibitory and mitodepressive effects on cell division and chromosome behaviour of *Allium cepa*. Hence, before its application to the biological systems, biocompatibility of CuONPs has to be further analysed.

**Conclusion**

The present study was carried out to synthesize CuONPs using the leaves of *Glycosmis pentaphylla.* The biosynthesized CuONPs were characterized using UV-Visible Spectroscopy, Fourier Transform Infrared Spectroscopy and Transmission Electron Microscopy. The surface plasmon resonance at 262 nm in UV-Visible spectra indicated the formation of CuONPs, the FTIR spectra confirmed capping of plant derived secondary metabolites on CuONPs. TEM analysis revealed the nanosized spherical nature of CuONPs. The results indicate that biosynthesized CuONPs can be used as antifungal agents against two plant pathogenic fungi, *Rhizoctonia solani* and *Sclerotium rolfsii.* The presented study also reveals the photocatalytic activity of biosynthesized CuONPs in the efficient degradation of toxic textile dye – CBB. The comparative cytotoxicity analysis of biosynthesized CuONPs and $CuSO_4$ which is a common ingredient of copper based fungicide in *Allium cepa* root tip revealed that CuONPs at a lower dose is safer while $CuSO_4$ treatment results in cellular anomalies. These results are promising as they can contribute to the utilization of biosynthesized CuONPs as an alternative to copper fungicide and also find its application in bioremediation.

**Conflicts of Interest**

The authors declare no conflicts of interest.

**Figures**

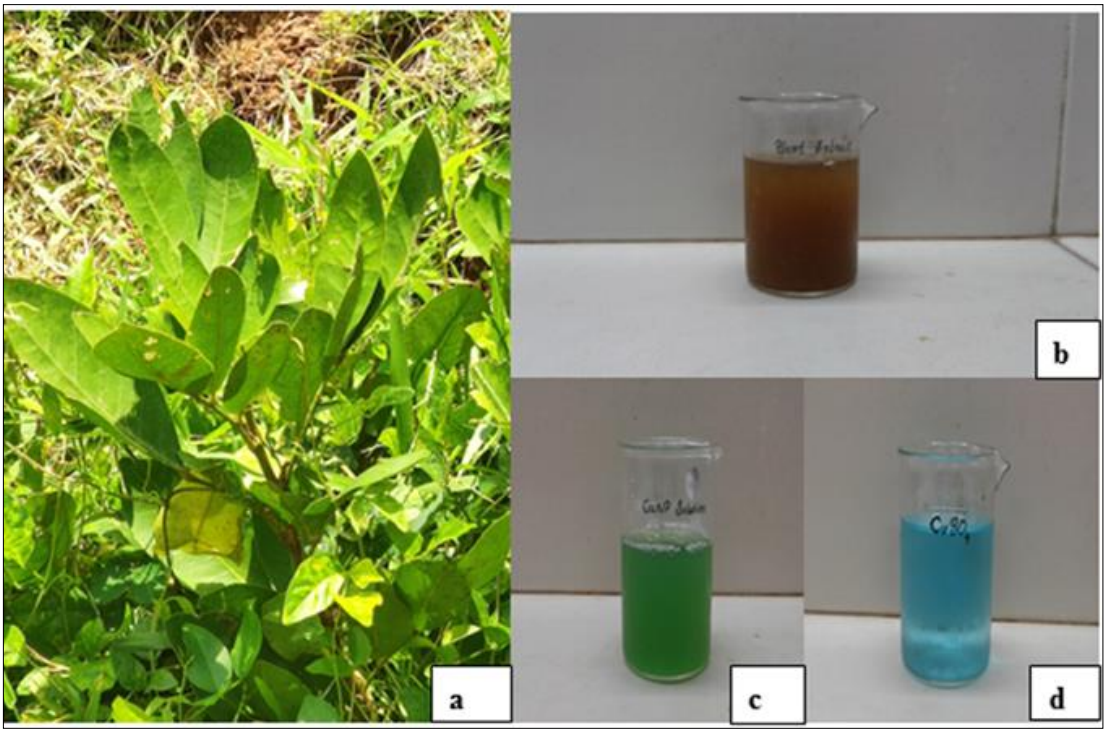


**Fig 1:** a. Glycosmis pentaphylla b. Plant extract c. Copperoxide nanoparticle solution d. CuSO4

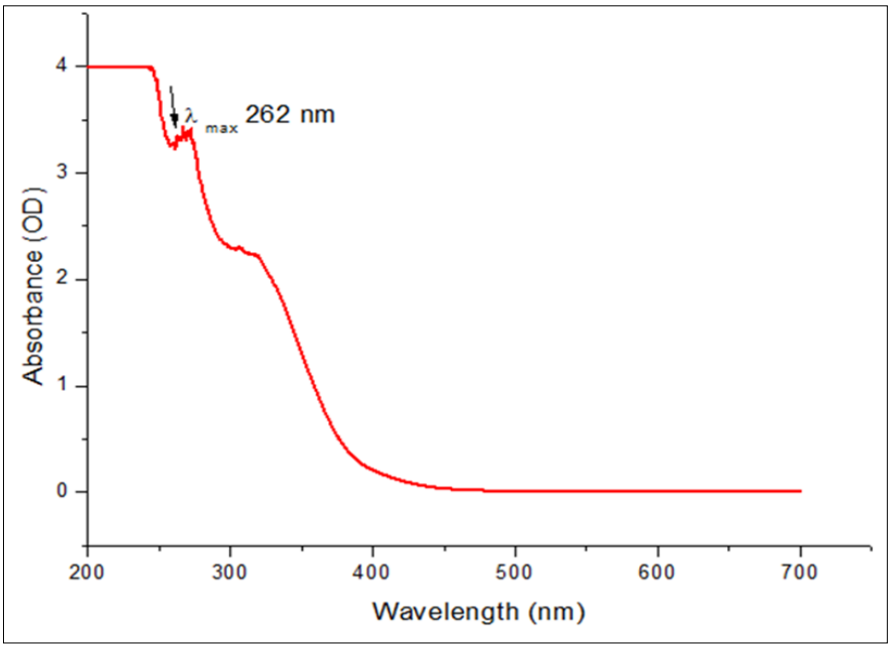


**Fig 2:** UV – Visible absorption spectra of copper oxide nanoparticles synthesized using aqueous leaf extracts of *G. pentaphylla* with $\lambda_{max}$ = 380 nm

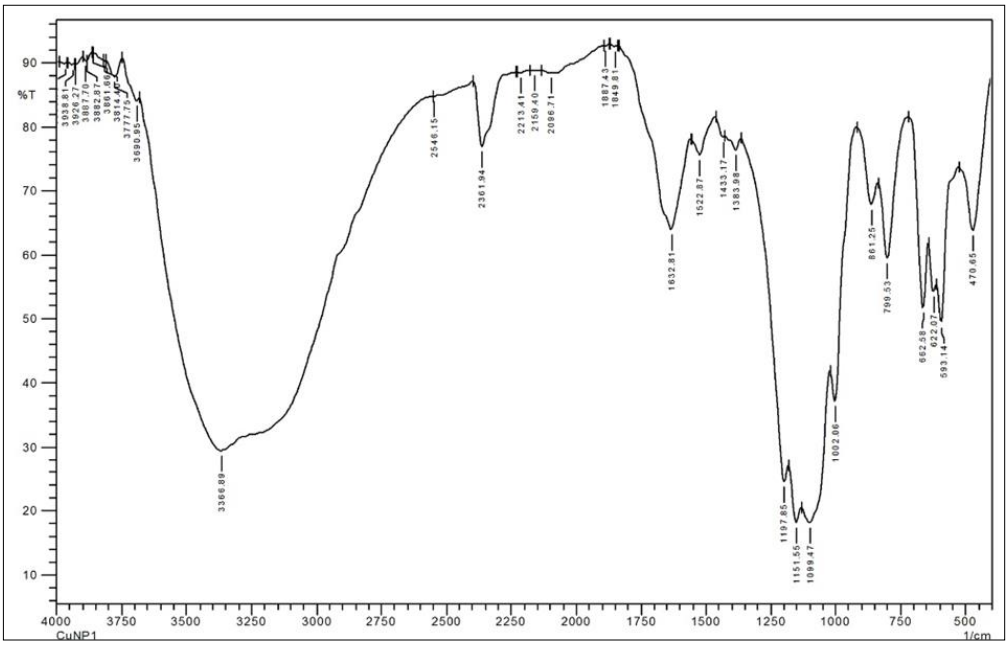


**Fig 3:** FTIR analysis of CuO nanoparticles indicating the involvement of various functional groups in the formation of metal oxide nanoparticles in the range 4000-400 $cm^{-1}$.

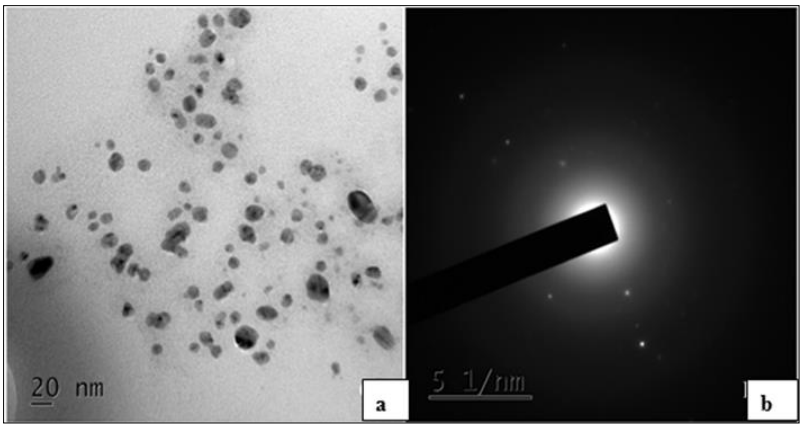


**Fig 4:** a - b TEM images of CuONPs around 10 nm size; b. SAED pattern-Crystal lattice structure of CuONP.

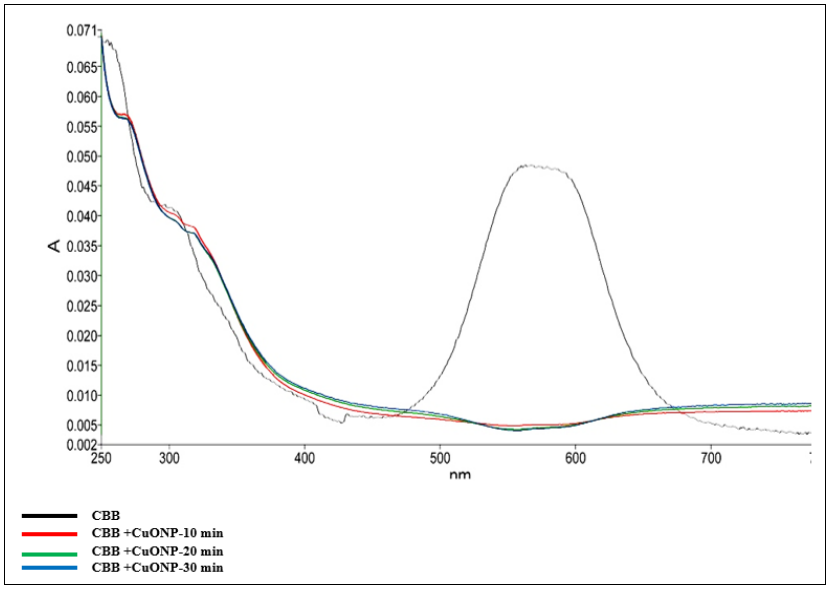


**Fig 5:** Photocatalytic degradation kinetics of CBB by CuONPs.

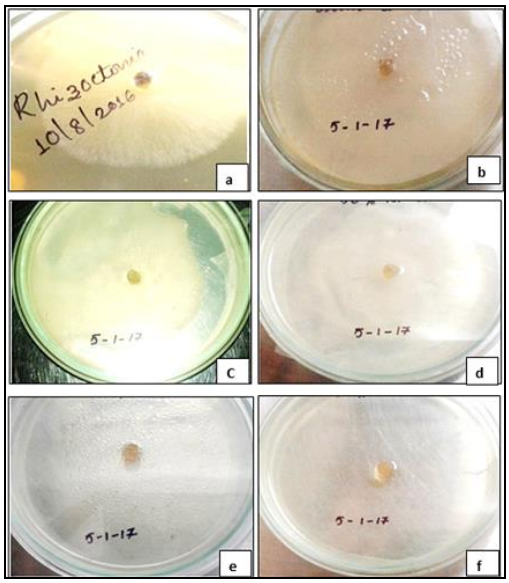


**Fig 6:** Evaluation of the antifungal activity against *Rhizoctonia solani*; a. Control (Deionized water); b-e *Rhizoctonia* cultured in different concentration of CuONPs; b. 25 mg/L; c. 50 mg/L; d. 75 mg/L; e. 100 mg/L; f. 3mM $CuSO_4$

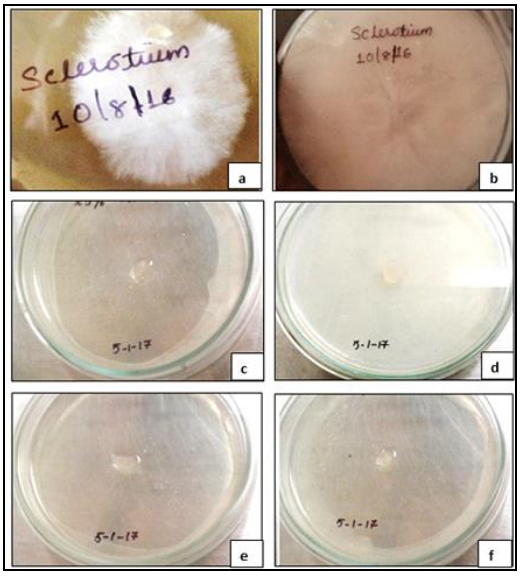


**Fig 7:** Evaluation of the antifungal activity against *Sclerotium rolfsii*; a. Control (Deionized water); b-e *Sclerotium* cultured in different concentration of CuONPs; b. 25 mg/L; c. 50 mg/L; d. 75 mg/L; e. 100 mg/L; f. 3mM $CuSO_4$

## Tables

**Table 1:** Effect of copper oxide nanoparticles on fungal pathogens

| SI. No | Treatments (Concentratıon) | Mycelıal Growth ın treatment plate (mm) | | Percentage of ınhıbıtıon = C-T/C × 100 | |
|---|---|---|---|---|---|
| | | *Rhizoctonia solani* | *Sclerotium rolfsii* | *Rhizoctonia solani* | *Sclerotium rolfsii* |
| 1 | Control | 60 | 60 | 0 | 0 |
| 2 | 25 mg/L | 33 | 0 | 45 | 100 |
| 3 | 50 mg/L | 29 | 0 | 51.6 | 100 |
| 4 | 75 mg/L | 0 | 0 | 100 | 100 |
| 5 | 100 mg/L | 0 | 0 | 100 | 100 |

Where, C = Average increase in mycelial growth in control plate.
T = Average increase in the mycelial growth in the treatment plate.

**Table 2:** Effect of copper oxide nanoparticles on mitosis in A. Cepa

| Treatment | No. of cells observed | No. of dividing cells | | | | Total no. of dividing cells | Mitotic index frequency (%) | Active mitotic index frequency (%) |
|---|---|---|---|---|---|---|---|---|
| | | P | M | A | T | | | |
| Control | 1500 | 134 | 38 | 19 | 18 | 209 | 13.93 | 3.8 |
| 2mg/L | 1823 | 79 | 37 | 17 | 22 | 155 | 8.50 | 2.96 |
| 4mg/L | 1956 | 74 | 19 | 13 | 11 | 117 | 5.98 | 1.63 |
| 6mg/L | 2110 | 71 | 14 | 9 | 11 | 105 | 4.97 | 1.09 |

P – Prophase, M – Metaphase, A – Anaphase, T – Telophase

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