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Green synthesis of *Tecoma stans* leaves-mediated copper oxide nanoparticles: Preparation, antioxidant, antimicrobial activities and *in vitro* MTT assay against MG-63 cell line

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Abstract
The present study investigates the *in vitro* antimicrobial and anticancer efficacy of *Tecoma stans* leaves-mediated copper oxide nanoparticles synthesized by a biological method. The antimicrobial and anticancer activity of *Tecoma stans* leaves-mediated copper oxide nanoparticles was assessed using the well diffusion method and MTT Assay against the (cell line name). The nanoparticles showed antibacterial and antifungal effects against (specify the organisms). The cytotoxic activity of *Tecoma stans* leaves -mediated copper nanoparticles was evaluated using the MTT assay against MG-63 (Human osteosarcoma cell line), confirming that copper oxide nanoparticles possess cytotoxic activity.

Keywords: *Tecoma stans* leaves, copper oxide nanoparticles, antimicrobial assay, MTT assay

Introduction
Nanoparticles and Nanomaterials have a wide range of multidisciplinary applications, including targeted drug delivery, imaging, diagnosis, catalysis, electronics, cosmetics, surfaces-enhanced Raman scattering, and biosensing [1]. Due to their high surface area-to-volume ratio, nanoparticles exhibit properties influenced by classical and quantum effects. Hybrid nanoscale structures, consisting of metal and metal oxide nanoparticles, often incorporate functionalized organic ligands that form protective organic coronas. This allows for direct immobilization of bioactive substances or their incorporation into the nanoparticle surface [2]. In recent years, metallic nanoparticles or metal nanoparticles have gained significant importance in the field of nanotechnology [3]. These nanoparticles can be synthesized using physical, chemical, and biological methods [4]. Among these methods, biological synthesis is preferred due to its ability to produce benign nanostructured materials and reduce the utilization and generation of toxic substances [5]. However, it should be noted that these nanostructures may release harmful by products into the environment, which can lead to toxicological issues [6].
Green synthesis is a promising approach that addresses this problem by producing clean, non-toxic, biodegradable, and environmentally friendly materials [7]. Medicinal plants have been found to be excellent sources of metallic nanoparticles with anticancer activity. This is attributed to the presence of various phytochemicals such as carbohydrates, flavonoids, saponins, proteins, amino acids, and terpenoids, which play a crucial role in the synthesis of nanoparticles [8].
Copper oxide nanoparticles (CuONPs) have numerous applications, including superconductors, solar energy transformation, synthesis of nanostructure composites, as well as antifungal, antimicrobial, and antibiotic agents [9]. CuO NPs can be synthesized using different physical and chemical methods, such as microwave irradiation, sol-gel, electrochemical techniques, thermal decomposition, and alkoxide-supported methods [10]. Researchers have found that the green synthesis of copper oxide nanoparticles from plant extracts, such as *Acalypa indica* [11], *Ficus religiosa* [1], *Syzygium alternifolium* [12], *Azadirachta indica* [13], *Hibiscus rosa-sinensis* [14], *Murraya koenigii* [15], *Moringa oleifera* [11], and *Tamarindus indica* [11] exhibit potent anticancer activity.
Tecoma stans, commonly known as yellow bell and belonging to the Bignoniaceae family, is widely distributed in tropical and subtropical regions worldwide. Extensive phytochemical studies have revealed the presence of various compounds in this plant, including alkaloids,

Iridoid glycosides, lapachol, sugars, triterpenoids, sterols, and phenolics. Research has demonstrated the diverse therapeutic properties of different parts of the *Tecoma stans* plant [16].

For example, the leaves possess anthelmintic [17], antispasmodic [18], antibacterial [19], anticancer [20], and wound-healing properties [21], while the flowers exhibit antidiabetic [22] and anticancer activity [23]. Notably, *T. Stans* has shown significant anticancer activity against Ehrlich ascites carcinoma (EAC) tumors, human breast cancer (MCF-7) [24], and lung cancer (A-549) [25] cell lines.

In this study, CuO nanoparticles were synthesized using Tecoma stans leaves extract through the green synthesis method. The primary objective of the study was to investigate the antibacterial activity of the synthesized CuO nanoparticles against both Gram-positive and Gram-negative bacteria. Additionally, the synthesized nanoparticles were evaluated for their potential anticancer activity against MG-63 (Human osteosarcoma cell line).

Methods

Plant collection and Authentication

The leaves of *T. stans* (commonly called yellow bells) were collected from the trees growing around the local areas of Salem, Tamil Nadu, India. The plant was identified and authorized by the Professor Dr. K Kannan, Taxonomist, Department of Botany, Vivekanandha College of Arts and Sciences for Women, Namakkal, Tamil Nadu, India. The collected plant material was washed with double distilled water to eliminate any surface impurities, and subsequently minced into small fragments. The leaves were then subjected to shade drying for a period of 7-10 days.

Preparation of Plant extract

The leaves were ground into a fine powder using a mixer grinder. A total of 100 grams of the powder was then dispersed in 100 mL of distilled water and boiled at 60ºC for 20 minutes. After cooling, the extract was filtered using a Whatman No.1 filter paper and stored in a refrigerator for further investigation [18].

Green Synthesis of Copper Oxide Nanoparticles.

The synthesis of copper oxide nanoparticles was carried out using copper sulphate and plant extract. A solution of 0.1 M copper sulphate in double distilled water was prepared. Copper sulphate and plant extract were mixed together in ratios of 5:5, 6:4, 7:3, 8:2, and 9:1. The reaction mixture was heated below the boiling point and continuously stirred at 800 rpm using a magnetic stirrer. Within 1 hour, the mixture turned green in colour. The entire reaction process was conducted in the dark. The resulting suspension was then centrifuged at 15,000 rpm for 15 minutes, and the pellet containing copper oxide nanoparticles was washed 3–4 times with deionized water to remove impurities. The precipitated nanoparticles were subsequently lyophilized and stored in a cool, dry, and dark place for further characterization.

Antioxidant Activity

DPPH Assay

The DPPH radical scavenging assay was performed in a 96-well microtiter plate. To each well, 100 µl of DPPH solution was added to 300 µl of *Tecoma stans* leaves-mediated CuONPs sample at different concentrations (500, 250, 100, 50, and 10 µg/mL). The mixture was vigorously shaken and allowed to stand at room temperature for 30 minutes. The absorbance was measured at 517 nm using a UV-VIS spectrophotometer, with ascorbic acid serving as a reference. A lower absorbance value of the reaction mixture indicates higher free radical scavenging activity [26]. The capability of scavenging the DPPH radical can be calculated using the following formula.

DPPH scavenging effect (% inhibition) = [(absorbance of control- absorbance of reaction mixture)/absorbance of control] X 100.

Nitric Oxide Radical Scavenging Assay

The extracts were prepared from a 50 mg/mL crude extract. These were then serially diluted with DMSO to make concentrations from 500-10 µg/mL of the *Tecoma stans* leaves-mediated CuONPs. These were stored at 4°C for later use. The150 µl extract was mixed with an equal volume of freshly prepared Griess reagent (150µl). Control samples without the extracts but with an equal volume of buffer were prepared in a similar manner as was done for the test samples. After 30 minutes of incubation period, 100 µL of the reaction mixture was transferred to a 96-well plate [27]. The absorbance was measured at 570 nm using a UV-Vis microplate reader (Molecular Devices, GA, USA).

Antibacterial activity

Petri plates were prepared by adding 20 mL of nutrient agar medium and then inoculated with a 24-hour bacterial culture. Bacterial Strains *Aeromonas hydrophila* and *Streptococcus pyogenes* were procured from Microbial Type Culture Collection and Gene Bank, Chandigarh, India. The bacterial culture was adjusted to an optical density (OD) value of 0.5 according to the McFarland standard, using bacterial strains from the year 1928. Wells were then cut into the agar, and *Tecoma stans* leaves-mediated CuONPs samples were added at concentrations of 500, 250, 100, and 50 µg/mL. The plates were incubated at 37 °C for 24 hours. The antibacterial activity of *Tecoma stans* leaves-mediated CuONPs was evaluated by measuring the diameter of the inhibition zone formed around the wells [28, 29, 30]. Gentamicin antibiotic was used as a positive control. The data were analyzed using Graph Pad Prism 6.0 software.

Antifungal Activity

Petri plates were prepared by adding 20 mL of potato dextrose agar medium and then inoculated with a 72-hour fungal culture of *Aspergillus niger*. Wells were cut into the agar, and different concentrations of *Tecoma stans* leaves-mediated CuONPs samples (500, 250, 100, and 50 µg/mL) were added. The plates were incubated at 28°C for 72 hours [31]. The anti-fungal activity of *Tecoma stans* leaves-mediated CuONPs was evaluated by measuring the diameter of the inhibition zone formed around the wells [32]. Amphotericin B was used as a positive control. The data were analyzed using Graph Pad Prism 6.0 software.

Cell culture

MG-63 (Human osteosarcoma cell line)was purchased from NCCS, Pune and were cultured in liquid medium (DMEM) supplemented 10% Fetal Bovine Serum (FBS), 100 ug/ml penicillin and 100 µg/ml streptomycin, and maintained under an atmosphere of 5% CO_2 at 37 ºC.

MTT assay

The *Tecoma stans* leaves-mediated CuONPs Test sample was tested for *in vitro* cytotoxicity, using MG-63cells by MTT assay. Briefly, the cultured MG-63cells were harvested by trypsinization and pooled in a 15 ml tube. Then, the cells were plated at a density of 1×10^5 cells/ml cells/well (200 µL) into the 96-well tissue culture plate in DMEM medium containing 10 % FBS and 1% antibiotic solution for 24-48 hour at 37°C.

The wells were washed with sterile PBS and treated with various concentrations of the *Tecoma stans* leaves-mediated CuONPs sample in a serum-free DMEM medium. Each sample was replicated three times and the cells were incubated at 37°C in a humidified 5% CO2 incubator for 24 h. After incubation, MTT (20 µL of 5 mg/ml) was added to each well and the cells were incubated for another 2-4 h until purple precipitates were clearly visible under an inverted microscope. Finally, the medium together with MTT (220 µL) was aspirated off the wells and washed with 1X PBS (200 µl). Furthermore, to dissolve formazan crystals, DMSO (100 µL) was added and the plate was shaken for 5 min. The absorbance for each well was measured at 570 nm using a microplate reader (Thermo Fisher Scientific, USA) and the percentage cell viability and IC50 value were calculated using Graph Pad Prism 6.0 software (USA) [33].

Formula Cell viability % = Test OD/Control OD X 100

Results

Antioxidant Activity

DPPH radical scavenging

The DPPH scavenging assay is a commonly used method for assessing the antioxidant potential of a material. DPPH is a stable nitrogen-centered free radical that changes color from violet to yellow when reduced through hydrogen reduction or electron donation. The scavenging activity of the prepared Copper oxide nanoparticles is presented in Table 1. The results indicate that the scavenging percentage increases with an increase in the concentration of *Tecoma stans* leaves-mediated CuONPs. At a concentration of 10 µg/mL, the solution exhibits a scavenging percentage of approximately 24.19%. Furthermore, the concentrations of 50 µg, 100 µg, 250 µg, and 500 µg demonstrate scavenging percentages of 35.30%, 44.80%, 50.35%, and 55.10%, respectively.

Table 1: DPPH scavenging power of the *Tecoma stans* leaves-mediated CuONPs and of Ascorbic acid as standard

S. No	Tested sample concentration (µg/ml)	Percentage of inhibition (in triplicates)			Mean value (%)
1.	Ascorbic acid	78.49462	70.43011	67.74194	72.22222
2.	500 µg/ml	56.72043	54.30108	54.30108	55.10753
3.	250 µg/ml	49.19355	48.92473	52.95699	50.35842
4.	100 µg/ml	47.84946	47.31183	39.24731	44.80287
5.	50 µg/ml	37.90323	36.02151	31.98925	35.30466
6.	10 µg/ml	25.53763	22.58065	24.46237	24.19355

Nitric Oxide Free Radical Scavenging Activity

The results of the NO radical scavenging assay for the synthesized *Tecoma stans* leaves-mediated CuONPs are presented in Table 2. The findings indicate that the inhibition percentage increases with an increase in the concentration of CuO NPs. At a concentration of 10 µg/mL, the inhibition percentage was found to be 44.75%. Furthermore, at concentrations of 50 µg/mL, 100 µg/mL, 250 µg/mL, and 500 µg/mL, the inhibition percentages were 51.94%, 63.83%, 67.60%, and 73.61%, respectively. These results suggest that the synthesized *Tecoma stans* leaves-mediated CuONPs exhibit moderate antioxidant activity in NO radical scavenging assays.

Table 2: Nitric oxide scavenging power of *Tecoma stans* leaves-mediated CuONPs and of Ascorbic acid as standard

S. No	Tested sample concentration (µg/ml)	Percentage of inhibition (in triplicates)			Mean value (%)
1.	Control	100	100	100	100
2.	500 µg/ml	75.61837	72.79152	72.43816	73.61602
3.	250 µg/ml	65.01767	71.37809	66.4311	67.60895
4.	100 µg/ml	63.60424	65.01767	62.89753	63.83981
5.	50 µg/ml	55.47703	50.53004	49.82332	51.94346
6.	10 µg/ml	47.70318	45.22968	41.34276	44.75854

Antibacterial Activity

The antimicrobial potential of CuO nanoparticles synthesized via a green method using *Tecoma Stans* was investigated. The effectiveness of these nanoparticles was evaluated against two human pathogenic bacteria, namely *Streptococcus pyogenes* (Gram +ve) and *Aeromonas hydrophila* (Gram-ve), as shown in Table 3 and Fig 1. Initially, at lower concentrations, the nanoparticles exhibited a modest antibacterial activity with a zone of inhibition of around 7.5 mm. However, as the concentration of the CuO nanoparticles increased, the antibacterial activity against both Gram +ve and Gram -ve bacteria significantly improved. The control drug Gentamicin displayed the highest zone of inhibition, with 25.5 mm and 13.5 mm against the two organisms, respectively.

Interestingly, the green-synthesized *Tecoma stans* leaves-mediated CuONPs nanoparticles demonstrated greater antibacterial activity against *Aeromonas hydrophila* compared to *Streptococcus pyogenes*, indicating their stronger potential against Gram -ve bacteria. The nanoparticles' spherical shape and size, ranging from 2 to 20 nm, are likely contributing factors to their enhanced biological activity. Overall, the results clearly suggest that *Tecoma stans* leaves-mediated CuONPs nanoparticles synthesized via a green method possess potent antibacterial properties against both Gram +ve and Gram -ve bacteria, with a zone of inhibition greater than 7.5 mm.

Table 3: Antibacterial activity (Zone of inhibition) of *Tecoma stans* leaves-mediated CuONPs

S. No	Name of the test organism	Zone of inhibition (mm) SD ± Mean				
		500 µg/ml	250 µg/ml	100 µg/ml	50 µg/ml	Gentamicin
1.	*Aeromonas hydrophila*	25.5±0.7	21.5±0.7	11.5±0.7	10.5±0.7	12.5±0.7
2.	*Streptococcus pyogenes*	13.5±0.7	11.5±0.7	10.5±0.7	7.5±0.7	13.5±0.7

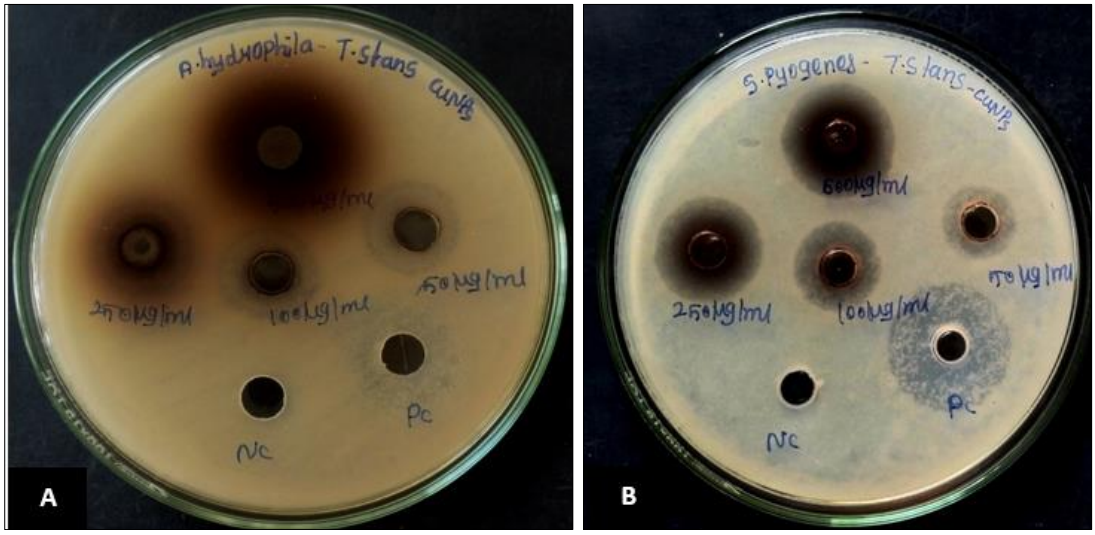

Fig 1: Antibacterial activity of T*ecoma stans* leaves-mediated CuONPs against *Aeromonas hydrophila* (A) & *Streptococcus pyogenes* (B)

Antifungal Activity

The antifungal activity of CuO nanoparticles against *Aspergillus niger* was evaluated in vitro at various concentrations, such as 500, 400, 300, 200, 100, 80, 60, 40, 20, and 10 µg/ml (Fig 2; Table 4). Amphotericin B was used as a positive control, while DMSO served as the negative control. The results demonstrated that the copper oxide nanoparticles exhibited a maximum zone of inhibition of 19.5±0.7 mm against *Aspergillus niger*.

Table 4: Antifungal activity (Zone of inhibition) of *Tecoma stans* leaves-mediated CuONPs

S.NO	Name of the test organism	Zone of inhibition (mm) SD ± Mean				
		500 µg/ml	250 µg/ml	100 µg/ml	50 µg/ml	Amphotericin B
1.	*Aspergillus niger*	19.5±0.7	15.5±0.7	11.5±0.7	10.5±0.7	21.5±0.7

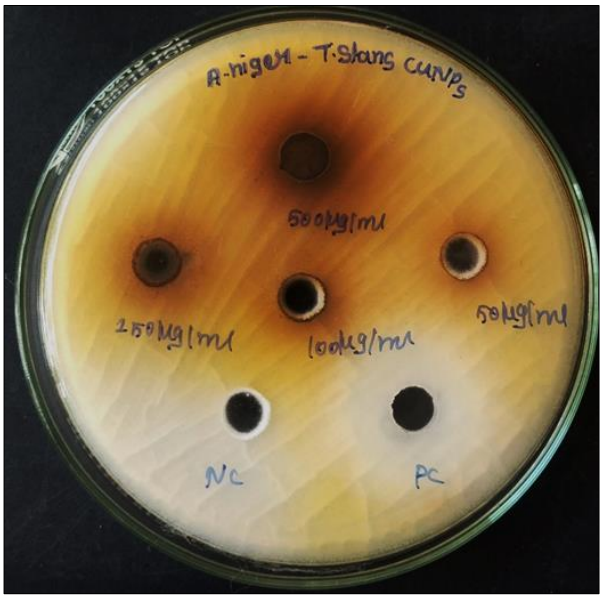

Fig 2: Antifungal activity of T*ecoma stans* leaves-mediated CuONPs against *Aspergillus niger* (A)

Anticancer activity

The *in vitro* anticancer potential of CuO nanoparticles synthesized using *Tecoma stans* leaves was assessed against the human osteosarcoma MG-63 cell line. The MTT assay was employed to determine cell viability and proliferation based on the reduction of Tetrazolium salt. Various concentrations of the synthesized CuO nanoparticles, ranging from 10 to 500 µg/mL, were evaluated (Fig. 3 & Fig. 4), and the percentage of cell viability was calculated from the OD value of the MTT assay (Table 5).

The results indicated that the CuO nanoparticles exhibited weak cytotoxicity against the human osteosarcoma cell line, with 92% cell viability observed at an initial concentration of 10 µg/mL. However, the viability of the cancer cells decreased with an increase in the concentration of the green-synthesized CuO nanoparticles. At a higher concentration of 500 µg/mL, the nanoparticles demonstrated potent anticancer activity against human osteosarcoma, with a cell viability of 36%.

The IC50 value of *Tecoma stans* CuONP against human osteosarcoma MG-63 was found to be 106.3 µg/mL. The reduced cell viability observed in this study may be attributed to the small size of the synthesized CuO nanoparticles, which allows for easier penetration inside the cells, resulting in a

higher rate of cell death. Increased penetration of nanoparticles inside the cells can cause damage to the cell membrane, leading to the leakage of enzymes and subsequent cell death. Furthermore, treatment with high levels of CuO nanoparticles can elevate oxidative stress by increasing the production of reactive oxygen species (ROS). This oxidative stress mainly affects the mitochondria and initiates apoptosis within the cells.

Table 5: Percentage of cell viability of *Tecoma stans* leaves mediated CuONP against Human osteosarcoma MG-63 cell line

S. No	Tested sample concentration (µg/ml)	Cell Viability (%)
1.	Control	100
2.	500 µg/ml	36.013387
3.	400 µg/ml	46.620802
4.	300 µg/ml	53.89046
5.	200 µg/ml	56.149103
6.	100 µg/ml	63.461616
7.	80 µg/ml	67.0922
8.	60 µg/ml	71.817559
9.	40 µg/ml	77.866059
10.	20 µg/ml	86.672656
11.	10 µg/ml	91.911842

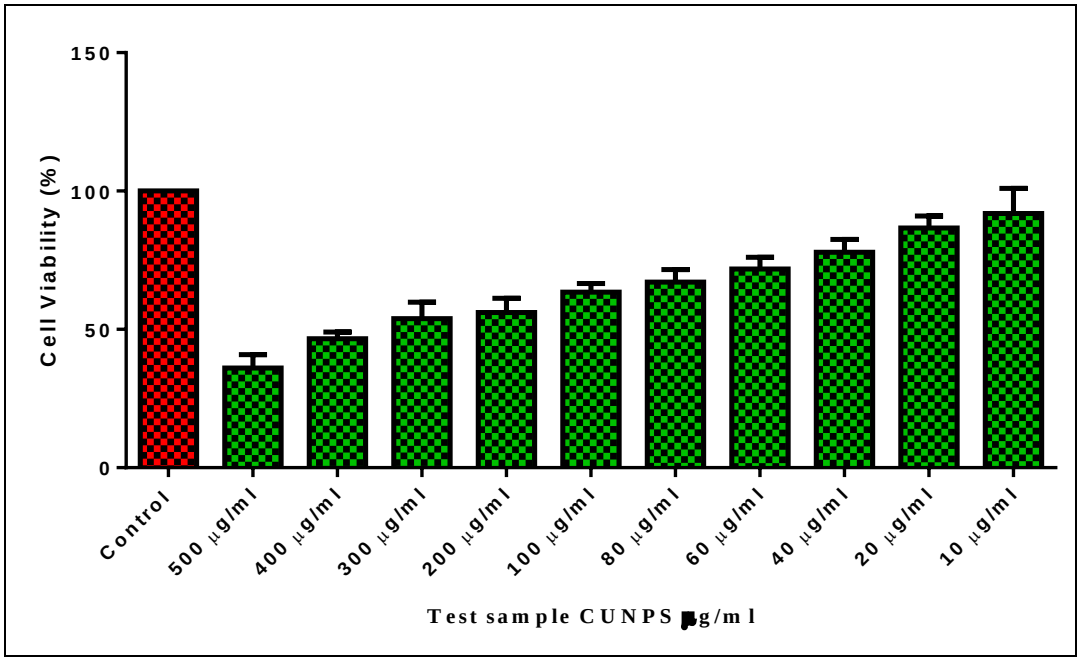

Fig 3: Cytotoxicity assessment in Human osteosarcoma MG-63 cell line following the exposure of various concentrations of *Tecoma stans* leaves mediated CuONP for 24 h:(A) MTT assay; (B) Morphological changes

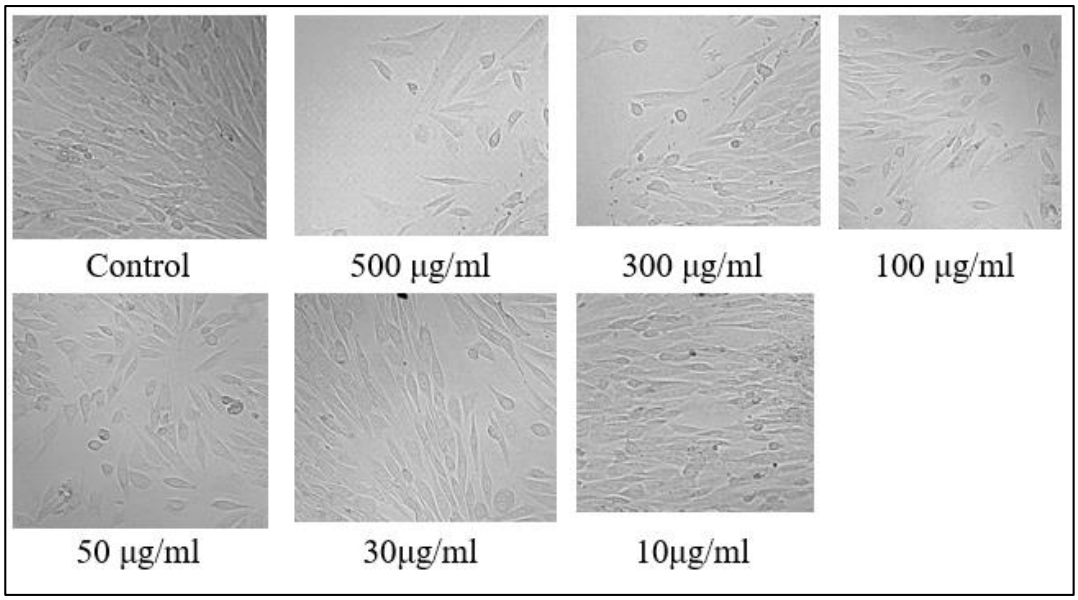

Fig 4: Cytotoxicity assessment in Human osteosarcoma MG-63 cell line following the exposure of various concentrations of *Tecoma stans* leaves mediated CuONP for 24 h: (B) Morphological changes

Conclusion

In this study, we present an eco-friendly and simple method for synthesizing copper oxide nanoparticles (CuONPs) using *Tecoma stans* leaf extract. The synthesis process involved the use of copper sulfate and *Tecoma stans* leaf extract at 80 °C.

The antioxidant studies conducted revealed that CuONPs exhibited significant antioxidant activity, surpassing the performance of the standard antioxidant. In the antimicrobial studies, CuONPs demonstrated enhanced antibacterial activity, particularly against Gram-positive bacteria such as *Aeromonas hydrophila*.

Furthermore, in the cytotoxicity studies, CuONPs exhibited potent cytotoxicity against the human osteosarcoma MG-63 cell line, suggesting their potential as an effective anticancer agent.

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