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Eco-friendly synthesis of CuO-NPs from *Momordica charantia* agro-waste: characterization and *in vitro* biological evaluation of anti-bacterial, anti-oxidant and anti-cancer properties

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An eco-friendly green strategy was developed to synthesis stable nanomaterials to be applied in the pharmaceutical area, which can be turned into innovative drugs with minimal side effects and much more cost effective. The present work is novel in that it uses an aqueous extract from mature and complete *Momordica charantia* L. (MC) plant, an agro-waste taken from the natural farmed field to synthesize copper oxide (CuO) nanoparticles (NPs). Eco-friendly MC-CuO-NPs were assessed using UV-Visible, FTIR Spectroscopy, XRD, and SEM. Furthermore, we entrenched the *in vitro* biological activities of these NPs in anti-bacterial (multidrug-resistant bacteria), anti-oxidant (DPPH), and anti-cancer (MCF-7) applications using standard methodologies. The generated MC-CuO-NPs have UV-Vis absorption spectra with bands at 485 nm. SEM micrographs showed spherical-like structure, whereas XRD spectra identified the crystalline phase and revealed four diffraction peaks of metallic copper. Biosynthesized MC-CuO-NPs can effectively suppress both *Staphylococcus aureus* (gram-positive) and *Escherichia coli* (gram-negative) bacteria in a dose-dependent manner. *E. coli* was the most inhibited, with a zone of inhibition measuring 24 mm. In addition, MC-CuO-NPs effectively inhibited radical DPPH with an IC_{50} of 63.15 μg/mL. MTT was used to assess the cytotoxicity of MC-CuO-NPs on MCF-7 cells, and the results revealed that NP cytotoxicity increased linearly with NP concentration. At 24 hrs, the median inhibitory concentration (IC_{50}) for MCF-7 cells was 100 μg/mL. The LDH release assays further validated the cytotoxic effects on the cells. As a result, they may be a feasible source of therapeutic molecules in the treatment of oxidative stress-induced diseases.

Keywords : Agro-waste, copper oxide nanoparticles, cytotoxicity, green nanotechnology, MCF-7 cell line, LDH release, valorization,

INTRODUCTION

Agricultural production has increased more than thrice over the previous 50 years as population growth has increased demand for food (Flores-Contreras *et al*. 2024). Presently, about 30% (2 billion tons) of global agricultural production is classed as agro-waste (including leaves, stems, seeds, pulp, stubble, bagasse etc.). Inadequate agro-waste disposal produces greenhouse gases, which contribute to climate change and ecosystem changes (Duque-Acevedo *et al*. 2020).

To limit its impacts, some techniques have been proposed to alleviate its consequences, such as "3R approaches" like reuse, recycling, and remanufacturing (Carmona-Cabello *et al*. 2018; Singh *et al*. 2021). These measures may lead to cleaner and ecological waste management (Duan *et al*. 2022; Barragán-Ocaña *et al*. 2023) and converts biowaste into value-added products (VAP) like biofertilizers, biogas, bioplastics, biofuels, therapeutic phytochemicals, and coatings etc. (Senthilkumar *et al*. 2024). This vast spectrum of VAP entails lower manufacturing costs and more accessibility (Nath *et al*. 2023). In addition, extracting bioactive chemicals are defined as food products or supplements that benefit the hosts health beyond addressing basic nutritional needs (Sulaiman *et al*. 2011). These substances have antioxidant, anti-microbial, and anti-inflammatory properties, although their effects depend on bioactivity, chemical structure, dosage, and other factors (Nath *et al*. 2023). Wastes are rich in bioactive components that can

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be used to make functional materials. Furthermore, these wastes can serve as a starting point for developing valuable nanomaterials (Carmona-Cabello *et al*. 2018; Aswathi *et al*. 2023).

Researchers worldwide are becoming interested in using nanoparticles (NPs) in biomedical fields due to their unique ability to interact with and within cells at the molecular level, resulting in increased specificity and efficacy in fighting infectious diseases (Nair *et al*. 2022; Sathiyaseelan *et al*. 2023). These nano-materials are widely used for special charcteristics – such as size, flexibility in design, and high surface-to-volume ratio (Dahoumane *et al*. 2017). Multiple studies have been undertaken utilizing metals and metal oxides for synthesizing NPs and uncovering their potential effects in diverse domains (Grigore *et al*. 2016). Among these, copper (Cu) NPs are one. Cu-NPs are gaining popularity for their distinct optical, thermal, electrical, chemical, and biological capabilities (Grigore *et al*. 2016). These features have numerous uses, including sensors, storage devices, infrared filters, super-capacitors and health and environment sectors (Bhattacharjee and Ahmaruzzaman, 2016; Nasrollahzadeh *et al*. 2016; Gawande *et al*. 2016). Cu-NPs exhibit antibacterial action, making them promising therapeutic agents (Palaniappan *et al*. 2015; Joshi *et al*. 2017; Alamri *et al*. 2023; Palanisamy *et al*. 2024). Various enhanced synthesis methodologies are being developed on a daily basis, including physical, chemical, and biological processes. While CuO-NPs are less expensive to synthesize than gold (Au), platinum (Pt) and silver (Ag) nanoparticles, maintaining their stability remains a challenge (Nair *et al*. 2022; Sathiyaseelan *et al*. 2023). Kim *et al*. (2012) and Deepa *et al*. (2022, 2023) describe a unique combination of generating Ag-NPs from vegetable waste. Senthilkumar *et al*. (2024) recorded the green synthesis of CuO-NPs from Agricultural Waste Garlic Husk. Aswathi *et al*. (2023) enlisted the NPS synthesis from biodegradable waste extracts. Generally, the outer peels of fruits and vegetables, which are typically discarded as waste, contain a range of beneficial substances. These ingredients could be utilized to make NPs, which could be a useful food waste management strategy (Patra and Baek, 2016). Furthermore, biologically synthesized NPs have been shown to increase efficiency, productivity, and sustainability (Chung *et al*. 2016).

Momordica charantia L. (family Cucurbitaceae), is grown extensively in Asia and Africa for its exceedingly bitter edible fruit (Jia *et al*. 2017; Gayathry and John, 2022). *M. charantia*, sometimes known as bitter gourd, possesses excellent antifungal, antiviral, anti-HIV, and antitumor activities (Fang and Ng, 2011). *M. charantia* fruit appears to be a viable alternative to current antidiabetic medicines including fenofibrate, rosiglitazone, thiazolidinediones, and GW50156 (Saeed *et al*. 2021). Agrawal and Beohar (2010) discovered C57 Bl mice with melanoma tumor models treated with *Momordica* extracts had a longer life span than mice in the control group, demonstrating its anticarcinogenic effects. Thus, *M. charantia* phytochemicals has been shown to efficiently portray antibacterial properties (Kubola and Siriamornpun, 2008; Krithiga and Briget, 2015; Ekezie *et al*. 2017; Alamri *et al*. 2023; Palanisamy *et al*. 2024). Furthermore, Joshi *et al*. (2017) and Palanisamy *et al*. (2024) found that Ag-NPs derived from *M. charantia* fruit and leaf extract were effective against human multidrug-resistant bacteria. In addition, Ekezie *et al*. (2017) and Alamri *et al*. (2023) also recorded *M. charantia* fruit extracts produced CuO-NPs and Cu-Fe-NPs greatly inhibited bacterial growth and cytotoxic effects on human liver and breast cancer cells. As a result, despite its abundance of metabolites, *M. charantia* has received little attention. Specifically, the mature complete plant (agro-waste) are concerned. The current study intends to synthesis CuO-NPs by a green biological approach, using an aquous extract produced from whole mature plant waste (*Momordica charantia*), and characterization of the created nanoparticles employing UV–vis spectroscopy, X- ray diffraction (XRD) analysis, Scanning Electron Microscopy (SEM) and Fourier- Transform Infrared (FTIR) Spectroscopy, and to evaluate their antibacterial efficacy against *Escherichia coli* and *Staphylococcus aureus* bacterial strains, anti-oxidant activity (DPPH) and anti-cancer activity in MCF-7 cells by MTT and LDH assay.

MATERIALS AND METHODS

Plant and Chemical

Chemicals and reagents were obtained from Sisco Research Laboratories Pvt. Ltd (India) and Sigma Aldrich. *Momordica charantia* L. (MC) mature complete plant were recovered before eradication from farm filed as waste in Pudukkottai (10º 24' 29" N Latitude; 78º 47' 20" E Longitude), Tamil Nadu, India, during March 2024. The Department of Botany assisted in identifying the plant species, and the microbial cultures used in the antimicrobial research were performed at the Department of Microbiology, JJ College of Arts and Science (A), Pudukkottai, Tamil Nadu. The *M. charantia* plant was washed using sterilized water to eliminate dust particles and dried for 15 days.

Preparation of Momordica charantia aqueous extract for CuO-NPs synthesis

The entire shade-dried *M. charantia* plant (MC) was pulverized into fine powder using an electrical blender. The powdered MC (10 g) and 100 mL of sterilized water (1:1) were cooked at 70 ºC for 90 mins and filtered through Whatman No.1 paper. The resulting extracts were refrigerated at 4 °C for future investigations (Palaniappan *et al*. 2015). To screen the nanoparticles synthesis, the *M. charantia* (CM) extract (100 mL) was introduced into a 1 mM concentration of aqueous copper nitrate ($CuNo_3$) solution (100 mL) and placed in a shaker incubator (110 rpm) at 28 ^{0}C for 24 hrs. The reduction of metal ions was regularly monitored by visual inspection of color changes that ranged from blue to green to dark brown, indicating the production of CuO nanoparticles. The precipitates were collected and dried at 150 °C before further characterization.

MC-CuO-NPs characterization
UV-vis spectra

The production of CuO-NPs by *M. charantia* extract was investigated using a range of spectroscopic and microscopic methods. The UV-Vis spectra of MC-CuO-NPs was investigated at 300-800 nm using a Shimadzu UV-2450 spectrophotometer to evaluate their optical characteristics (Deepa *et al*. 2022).

XRD

The crystalline nature of *M. charantia* extract generated CuO-NPs was evaluated using XRD (Shimazdu, Model LabX-XRD-6000), operated at a voltage of 40 kV and an output current of 30 mA on Cu-Ká radiation in the range of 2θ (20º-80º) (Palaniappan *et al*. 2015).

SEM

SEM used to study the size and exterior surface morphology of MC-CuO-NPs (Jeol JSM-6480 LV-SEM).

FTIR

FTIR spectroscopy experiments were performed to identify potential bio-reducing agents in the *M. charantia* extract-CuO-NPs. The spectra were acquired on a Perkin Elmer-spectrum RX1 instrument with a resolution of 400–4000 cm^{-1} (Senthilkumar *et al*. 2024).

Biological applications of M. charantia extract assisted CuO-NPs

In vitro antibacterial activity

The well diffusion method (Rashid *et al*. 2017) was utilized to assess the antibacterial activity of biosynthesized CuO-NPs derived from crude aqueous *M. charantia* extract against human pathogenic bacteria such as *Escherichia coli* (gram-negative) and *Staphylococcus aureus* (gram-positive). Bacterial strains isolated clinically were subcultured in nutrition broth (NB) for 24 hrs at 28 ± 2 °C. To begin, sterile petri plates (90 mm diam.) were filled with 15 mL Mueller Hinton Agar (MHA) and inoculated with each bacterial strain (10^6 CFU cells/mL). The plates were then swabbed uniformly with sterile cotton swabs, and various concentrations (50, 100, and 150 µg/mL) of MC-CuO-NPs were poured into each well. As a positive control, 1 mg/mL of streptomycin was used. Later, inoculated plates were kept at 37 °C for 24 hrs to measure the zone of inhibition (mm). This experiment was repeated three times.

In vitro antioxidant activity by DPPH assay

To test the ability to scavenge 2,2-diphenyl-1-picrylhydrazyl (DPPH), different quantities (20, 40, 60, 80, 100 and 120 µg/mL) of *M. charantia* extract produced NPs i.e. CM-CuO-NPs was mixed with 1 mL of DPPH (0.1 mM), then incubated for 30 mins in the dark. A UV-visible spectrophotometer (Shimadzu UV-2450) was used to detect the absorbance at 517 nm. Ascorbic acid was utilized as the standard (Rehana *et al*. 2017). The proportion of radical inhibition or scavenging was calculated using the equation, where $A_{control}$ stays for the absorbance of the control and A_{sample} for the absorbance of the sample

Antioxidant activity (%) = ($A_{Control}$ - A_{Sample} / $A_{Control}$) × 100

In vitro cytotoxic activity against MCF-7 cell line by MTT assay

The cytotoxic effect of CM-CuO-NPs was investigated against MCF-7 breast cancer cells using a slightly modified MTT test (Palanisamy *et al*. 2024). MCF-7 cells were grown in DMEM (Dulbecco's Modified Eagle Medium) with 10% fetal bovine serum and incubated at 37 ^{0}C in a 5% humidified CO_2 environment. A concentration of 1×10^5 cells/well was grown on sterile 96-well microtitre plates at 37 ^{0}C for 24 hrs in 5% CO_2. After incubation, cells were treated with varied quantities of *M. charantia* produced CuO-NPs. Plates were then incubated for 24 hrs to assess their cytotoxic efficacy. Next, add 20 ìL of MTT (5 mg/mL) solution to the wells and incubate for another 3 hrs. Following incubation, the medium was carefully removed without damaging the produced formazan crystals. The crystals were solubilized in 100 µL of DMSO and the absorbance was measured at 570 nm using an ELISA microplate reader (Epoch BioTek). MCF-7 cells that had not been treated with CuO-NPs served as controls. The equation for calculating the percentage of cell viability was as follows:

Cell viability (%) = (Abs of cells with CuO-NPs / Abs of cells without CuO-NPs) × 100

Assessment of Lactate dehydrogenase release assay

MCF-7 cells were planted into 96 well microtitre plates at a density of 1×10^5 cells/well, with 100 µL DMEM medium added. The plate was then incubated at 37 ^{0}C with 5% CO_2 for 24 hrs to allow cells to adhere (Kumar *et al*. 2018). After 24 hrs, the spent media was withdrawn and 100 ìL fresh media was given to the seeded cells in the microtitre plates. The cells were treated with *M. charantia* generated CuO-NPs at varied concentrations (1-10 µg/0.1 mL in DMSO) and incubated for another 24 hrs. Cells are lysed with 50 mM 0.1 mL Tris-HCL buffer, pH 7.4, 0.1 mL 20 mM EDTA, and 0.1 mL 0.5% Sodium Dodecyl Sulfate (SDS) and centrifuged at 10000 rpm for 15 mins. The resulting precipitate was mixed with 0.2 mL of 1 mM pyruvate and 0.2 mL of 0.2 mM NADH. After 15 mins incubation decrease in NADH was seen at 340 nm in Shimadzu UV-2450 spectrophotometer.

% of Growth Inhibition = (Control OD- Treated OD/ Control OD) × 100

Statistical Analysis

Each experiment's data is reported as the mean ± standard deviation from three replicates.

RESULTS AND DISCUSSION

Green synthesis of CuO-NPs

CuO-NPs nanoparticles were successfully formed using aqueous extracts of *M. charantia*, as evidenced by the initial change in color from blue-green to brown agglomerated precipitate in the reaction medium after 24 hours of incubation with the precursor (copper nitrate) solution. In this case, the investigated plant extract aids in the reduction of copper nitrate to nanoscale. The UV-Vis spectra of the reaction supernatant was analyzed, and an absorption peak in the range of 485 nm (Fig. 1) was identified, indicating the creation of nano-copper oxide particles in agreement with previous observations (Qamar *et al*. 2020).

Characterization of CuO-NPs

The structural identification of the *M. charantia* biosynthesized CuO-NPs was established using XRD patterns (Fig. 2A). The typical peaks were seen at 2θ angles ranging from 20º to 90º (JCPDS-45-0937). The XRD planes (021), (131),

(110), (-202), (020), (112), (202), and (-113) are confirmed to have corresponding values of 27.8, 32.3, 38.2, 46.7, 54.5, 57.3, 64.8, and 76.4, respectively, which may be indexed as the band for the centered cubic structure of copper ions. Broad peaks represent nanoparticle particle size, while pointed peaks represent nanoparticle crystallinity (Alamri *et al*. 2023). The mean size of CuO-NP particles was estimated using the Scherrer equation, and it ranged from 100 nm to 150 nm. The XRD pattern clearly shows that the CuO-NPs produced using the present green approach are crystalline.

SEM was utilized to investigate the morphological shape and size of *M. charantia*-produced CuO-NPs (Fig. 2B). The SEM investigation of CuO-NPs revealed spherical particles with aggregation, which is consistent with X-ray diffraction (XRD). Furthermore, we concur that larger concentrations of bioactive chemicals in colloidal solution may result in the development of nanoclusters (Qamar *et al*. 2020). Furthermore, the results indicated that CuO-NPs are formed as a result of the action of *M. charantia* extract, which serves as an effective bioreductant for biosynthesis.

FTIR has emerged as a key method for understanding the functional groups involved in the interaction of metal particles and biomolecules (Kubola and Siriamornpun, 2008; Jia *et al*. 2017). FTIR assessment of biosynthesized *M. charantia*-CuO-NPs was carried out to discover the possible interaction between proteins and copper ions at room temperature. The FTIR spectrum study of CuO-NPs by *M. charantia* revealed several peaks at 3574 cm^{-1}, 3226 cm^{-1}, 2078 cm^{-1}, 1637 cm^{-1}, 1385 cm^{-1}, 1154 cm^{-1}, and 657 cm^{-1} (Table 1). The broad peak at 3574 cm^{-1} and 3226 cm^{-1} corresponds to O-H and N-H bonds, which could be caused by the presence of phenolic chemicals in the solution (Rashid *et al*. 2017). The band at 2078 cm^{-1} represents Ca≡N bonds. The observed band at 1637 cm^{-1} is most likely caused by C=N or C=O stretching. As a result, the signal at 1385 cm^{-1} indicates C-H bending or acyl C-O (or phenol C-O) stretching, whereas the peak at 1154 cm^{-1} represents alkoxy C-O. Additionally, the presence of bioactive phytochemicals (triterpenes, proteins, steroids, carbohydrates, alkaloids) in the solution may cause unsaturated C-H bending below 1000 cm"1. These compounds may impart capping, which aids in the stability of CuO-NPs. Furthermore, a peak in the IR spectra at low frequencies (590-680 cm"1) corresponds to CuO vibrations, as previously reported (Kumari *et al*. 2017; Qamar *et al*. 2020).

Table 1: FTIR spectra analysis of biosynthesized CM-CuO-NPs

FTIR peaks (cm^{-1})	Functional groups
3574	O-H bonds
3226	N-H bonds
2078	C≡N bonds
1637	C=N or C=O stretching
1385	C-H bending or acyl C-O
1154	D-phenol C-O- stretching alkoxy C-O
657	C-H bending

In vitro antibacterial activity

The well-diffusion technique was used to assess antibacterial activity of *M. charantia* produced CuO-NPs. *E. coli* and *S. aureus* showed the highest zone of inhibition at 150 µg/mL (Fig. 3; Table 2). CuO-NPs showed considerable efficacy against both test strains compared to standard drugs (+ve). The aqueous extract of *M. charantia* (-ve) did not exhibit any action. *E. coli* showed the best efficacy, with a zone of inhibition measuring 24 mm (Table 2). Krithiga and Briget (2015), Ekezie *et al*. (2017), and Qamar *et al*. (2020) have all demonstrated antibacterial activity against multidrug-resistant bacteria using *M. charantia* leaf and fruit extract-mediated silver and copper NPs. Previous research suggests that NPs can enter bacterial cells through changes in membrane shape, leading to increased permeability and decreased transport over the plasma membrane, ultimately leading to cell death (Brayner *et al*. 2006; Raju *et al*. 2022). CuO-NPs antibacterial activity has been attributed to a variety of processes (Chatterjee *et al*. 2014). In bacterial cells, this includes the production of reactive oxygen species, protein oxidation, lipid peroxidation, membrane disintegration, and DNA degradation. Nanoparticles antibacterial properties vary depending on their form, size, and oxidation number (Chatterjee *et al*. 2014; Qamar *et al*. 2020; Sathiyaseelan *et al*. 2023).

Table 2 : Antibacterial activity of green synthesized CM-CuO-NPs

Test organisms	Zone of inhibition (mm in diameter) / Concentrations of CM-CuO-NPs (µg/mL)				
	CM extract (-ve)	Std. Abs (+ve)	50	100	150
Escherichia coli	-	23	22	23	24
Staphylococcus aureus	-	20	20	21	23

Standard antibiotic- streptomycin; Values are presented the mean (n=3)

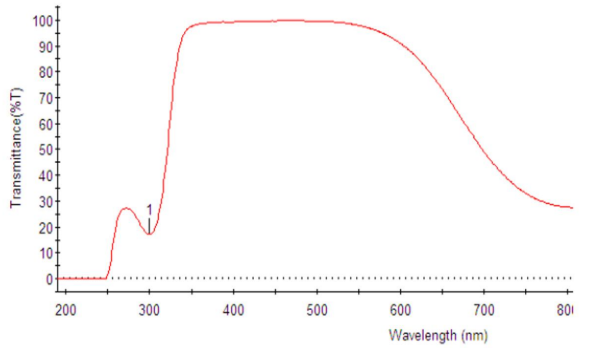

Fig. 1 : UV-vis spectrum of synthesized copper nanoparticle using *Momordica charantia* (MC) agro-waste extract

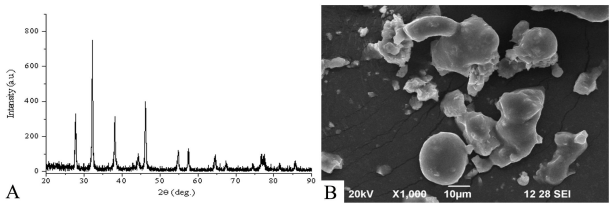

Fig. 2 : Powder XRD spectrum (A), scanning electron microscopy (SEM) images (B) of MC mediated CuO-NPs

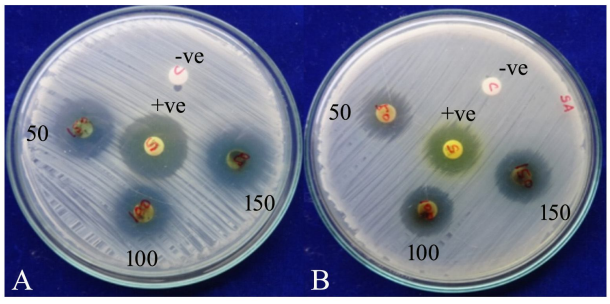

Fig. 3 : Assessment of antibacterial potential of CM-CuO-NPs against human infectious pathogens using well diffusion assay. A. *Escherichia coli*; B. *Staphylococcus aureus*. The CM-CuO-NPs concentration was 50, 100, 150 µg/mL, respectively. +ve-standard antibiotic streptomycin (10 µg/well). -ve - CM extract

Antioxidant activity

The *M. charantia* produced CuO-NPs ability to scavenge radicals *in vitro* was assessed using the DPPH test. The free radical scavenging activity of NPs at various concentrations was

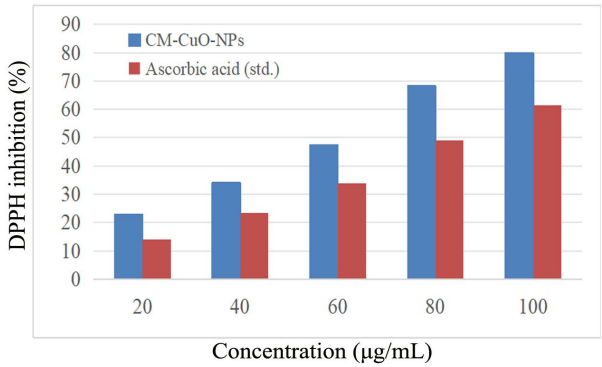

Fig. 4 : *In vitro* antioxidant activity of CM-CuO-NPs by DPPH assay

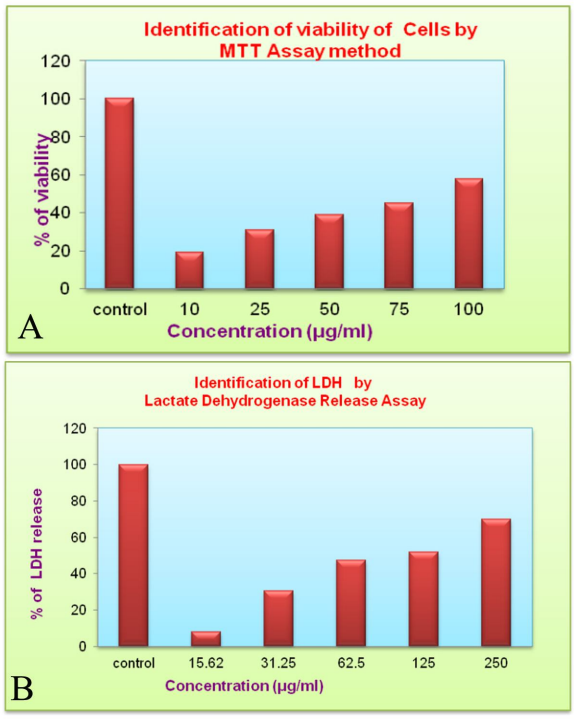

Fig. 5 : Cytotoxic effect of CM mediated CuO-NPs against MCF-7 cells using MTT assay (A), Lactate dehydrogenase release assay (B)

compared to the IC_{50} values of ascorbic acid, a typical antioxidant (Fig. 4). The results revealed significant differences between ascorbic acid and CuO-NPs, with enhanced scavenging activity as

sample concentration increased. The IC_{50} values for CuO-NPs and ascorbic acid were 63.15 and 81.53 μg/mL, respectively. That means CuO-NPs are less active than ascorbic acid, as the lower the IC_{50} value, the greater the activity. *M. charantia* produced CuO-NPs exhibits increased activity owing to the inclusion of different biochemicals (Rehana *et al*. 2017).

Cytotoxicity

Total cell viability is determined by the number of living cells that transform the tetrazolium salt (MTT) to a purple formazan crystal through metabolic activity. MCF-7 cells were treated to various concentrations of *M. charantia* synthesized CuO-NPs (control, 10, 25, 50, 75, and 100 μg/mL) for 24 hrs (Fig. 5A). Cell viability in the MCF-7 cell line decreased significantly (100%, 19%, 31%, 40%, 45%, and 57%). Furthermore, the reduction was seen in a dose-dependent way. Similarly, Alamri *et al*. (2023) found that the cytotoxicity of g-Cu-FeNPs derived from *M. charantia* fruit extract increased in a concentration-dependent way on HuH-7 and MCF-7 cells.

LDH release assay

LDH release was used to evaluate cell death in culture. Fig. 5B shows that *M. charantia* produced CuO-NPs had a concentration-dependent lethal effect on MCF-7 cell lines, as indicated by the LDH release curves. After 72 hrs of exposure to CuO-NPs doses of 15.62, 31.25, 62.5, 125, and 250 μg/mL, MCF-7 cell lines released 8.29, 33.33, 42.60, 56.52, and 73.60% LDH, respectively. Higher doses (250-1000 μg/mL) of the NPs led to increased LDH release (Abdullah *et al*. 2014).

CONCLUSION

This study describes a safe and effective approach for synthesizing CuO-NPs from a whole plant aqueous extract of *M. charantia*. These green-synthesised CM-CuO-NPs were thoroughly examined by analytical techniques. FTIR data indicated that *M. charantia* phytoconstituents were liable for reducing copper ions and capping the CuO-NPs. CM-CuO-NPs showed substantial antibacterial and antioxidant activities, with the maximum efficacy at 150 μg/mL. Notably, these CM-CuO-NPs had a strong suppressive impact on the growth of breast cancer MCF-7 cells, indicating that they could be a good candidate for future antibacterial, antioxidant, and *in vitro* cytotoxic studies. This study emphasizes the environmental friendliness of producing CuO-NPs from an aqueous extract of *M. charantia*, as well as its potential as a life-saving drug in anticancer therapy. Further research is needed to determine the possible applications of CuO-NPs in the treatment of metastatic breast cancer.

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DECLARATION

Conflict of Interest: Authors declare no conflict of interest.

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