

Green-fabrication of Copper Nanoparticles Using *Pistia Stratiotes* for Sustainable Synthesis and Assessment of *in vitro* Apoptosis Cell Death Mechanism and other Biomedical Potential

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Abstract

Objective: Cancer remains a major global health challenge, ranking among the leading causes of death due to increasing incidence and mortality rates, which hinder efforts to achieve longer life expectancy worldwide. Since its conceptual introduction, nanomedicine explored for potential applications in a wide variety of illnesses. The plant-mediated synthesis of nanoparticles offers cost-effective and environment-friendly alternative to conventional methods. **Methods:** The current research designed to focus on the effective utilization of aquatic weed for biomaterials synthesis and the investigation of its biomedical potential *in vitro*. In this study, copper nanoparticles (CuNPs) was synthesized using *Pistia stratiotes* leaves extract, reflecting novelty of this work in the field of nanosciences. The synthesized CuNPs were characterized using UV-Vis absorption spectra, Fourier Transform Infrared Spectroscopy, Transmission Electron Microscope, and X-ray diffraction. The cytotoxicity, apoptotic mechanism, morphological changes revealed by AO/EtBr and DAPI staining, and cell migration of CuNPs were evaluated using MCF-7 human breast cancer cells. The present study further investigated the antimicrobial and antioxidant activity of the CuNPs. **Results:** UV-Vis analysis revealed the surface plasmon resonance property of CuNPs, showing a characteristic absorption peak at 320 nm, while HRTEM indicated the nanoparticles were mono-dispersed, spherical in shape, and ranged in size from 20 to 30 nm. The anticancer study clearly demonstrated that CuNPs induced apoptosis as the cancer cell death pathway and inhibited the proliferation and migration of MCF-7 human breast cancer cells. The antimicrobial effects of the green-synthesized CuNPs were examined against human pathogenic and non-pathogenic microorganisms. This research further highlighted the possible applications of CuNPs for *in vitro* targeting of free radical scavenging. **Conclusion:** In conclusion, this commencement research clearly demonstrated the effective utilization of aquatic weed for the production of biomaterials and highlighted novel strategies for biomedical potential.

Keywords: Copper Nanoparticles, *Pistia stratiotes*, MCF-7 cells, Apoptosis, Antioxidant and Antimicrobial activity

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Introduction

Cancer is a complicated disease that arises through a multistep progression involving resistance to programmed cell death (apoptosis), uncontrolled cell growth, variations in cellular signalling, and invasion of tissues, metastasis, and the formation of new blood vessels i.e. angiogenesis [1]. Globally, it's documented that cancer leads to 19.3

million new diagnoses and approximately 10.0 million deaths annually, with this figure projected to reach 30 million by 2030 [2-4]. Early detection and intervention are crucial in combating cancer; however, current standard diagnostic and treatment techniques, including surgery, radiotherapy, and chemotherapy, often lack efficiency

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[5, 6]. The dosage of chemotherapy is frequently limited due to the adverse effects on organs and toxicity to normal cells, which lowers the effectiveness of anti-cancer therapy [7]. Therefore, developing novel, enhanced, harmless, and more targeted treatments is essential for cancer patients. In recent years, nanomedicine has shown significant promise in cancer diagnosis and treatment through drug delivery using nanomaterials, minimizing side effects [8].

Recently, nanotechnology has emerged as a fundamental field focused on the development and application of nano-sized materials that possess structural characteristics between atoms and bulk materials, showing novel or improved properties based on their size, morphology and single dimension [9]. Nanomaterials are garnering significant interest from various scientific fields owing to their vast applications in electronics, optoelectronics, drug delivery, and biosensors [10]. The synthesis of nanomaterials using plant extracts and microorganisms has received considerable attention as a green, sustainable, and environmentally friendly method [11]. Many researchers are now focusing on the biological synthesis of metal and metal oxide nanoparticles through plant extracts for biomedical purposes [12-17]. Given the involvement of nanomaterials in various domains, they are perceived as a groundbreaking innovation that could significantly enhance human health and serve as a valuable resource for improving human well-being [18, 19].

Pistia stratiotes (water lettuce) is one of the most productive freshwater aquatic plants in the world. This invasive, stoloniferous, floating plant grows in lime-rich waters of lakes, rivers, and still water ponds throughout India and is distributed across tropical and subtropical regions of Asia, Africa, and America [20]. The dense canopy of *P. stratiotes* reduces light penetration to submerged macrophytes and planktonic algae. It can also lower water temperature, reduce pH, promote stratification, and prevent water mixing by wind [21]. Mats of *Pistia stratiotes* provide breeding grounds for disease-carrying mosquitoes, including malaria vectors such as *Anopheles* and *Mansonia* [22, 23]. While herbicides like diquat, glyphosate, terbutryn, and 2,4-D have been used successfully to manage *Pistia stratiotes* [24], their use requires caution due to potential harm to the environment, marine life and human health [25].

Aquatic weeds thrive in abundant water bodies such as rivers, streams, canals, lakes, and wetlands. Most of these exotic species alter the water's nutritional balance, causing eutrophication, serve as breeding grounds for disease vectors like mosquitoes, and disrupt local navigation. The present study focused on the efficient use of aquatic weeds for the synthesis of CuNPs and investigated their biomedical properties. The biologically synthesized CuNPs were characterized by UV-visible spectroscopy, X-ray diffraction (XRD), Fourier transform infrared spectroscopy (FT-IR), field emission scanning electron microscopy (FE-SEM), and high-resolution transmission electron microscopy (HR-TEM) analysis. The study also aimed to explore the anticancer activity of green-synthesized CuNPs by examining their role in inducing apoptosis and inhibiting

cell migration. Furthermore, the antimicrobial and antioxidant effects were studied *in vitro*.

Materials and Methods

2.1. Preparation of plant extract

All glassware were properly cleaned before used. The leaves of *Pistia stratiotes* were collected from water bodies in Kottayam District, Kerala, India. The leaves were thoroughly rinsed with deionized water and sterilized using 0.02% mercuric chloride. After sterilization, the leaves were dried and finely powdered. For extract preparation, 10 g of leaf powder was dissolved in 100 mL of deionized water. The mixture was boiled at 80 °C for 5 minutes, and the resulting solution was filtered. The filtrate was stored for further analysis.

2.2. Synthesis of CuNPs

In the phyto-synthesis protocol, for copper ion reduction, 10 mL of the filtrate was mixed with 90 mL of 1 mM of copper sulphate ($\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$) solution (Riedel-de-Haen Ag Seelze Havvonee™ Seelze, Germany). The mixture was placed in a water bath at 70 °C for 30 minutes. The preliminary formation of CuNPs was detected by visual observation of the colour change. The resulting samples were centrifuged at 6000 rpm for 20 minutes. The pellet was lyophilized, and the nanoparticles were stored at 4 °C.

2.3. Characterization of CuNPs

UV-Visible spectroscopy: The colour changes from pale yellow to colloidal brown indicated the formation of copper nanoparticles. UV-Visible spectroscopy was used for the characterization of synthesized CuNPs. The reduction of CuNPs was monitored by recording the absorbance of the reaction mixture in the range of 200–700 nm using a Shimadzu UV-1800 Spectrophotometer.

FTIR analysis: The chemical compositions of both the plant extract and synthesized copper nanoparticles were analysed using an FTIR spectrometer (SHIMADZU IR Prestige-21). The samples were characterized in the range of 4000–400 cm^{-1} using KBr pellet.

XRD analysis: The synthesized CuNPs were subjected to XRD analysis using a multipurpose X-ray diffractometer equipped with a D/teX Ultra 250 1D detector. The target was Cu $K\alpha$ radiation with a wavelength of 1.54 Å. The generator was operated at 40 kV and with a 30-mA current. The average grain size of CuNPs was calculated using Scherrer's formula: $D = 0.94\lambda / (\beta \cos \theta)$, where D is the average crystal size, k is the Scherrer coefficient (0.94), λ is the X-ray wavelength, θ is Bragg's angle and β is the full width at half maximum in radians.

FESEM, EDX and HRTEM imaging: The morphology and elemental composition of the synthesized CuNPs were studied using FESEM equipped with EDX spectroscopy (TESCAN MAIA 3 XMH). The size and morphology of the CuNPs were further characterized using HRTEM (JEOL-JEM-2100 with LaB6 electron source). For HRTEM imaging, the CuNPs solution was drop-coated onto a carbon-coated copper grid.

2.4. Cell Culture

MCF-7 human breast cancer cells (passage number: 23) and HEK293 human embryonic kidney cells (passage number: 12) were obtained from National Centre for Cell Sciences (NCCS), Pune, India. Both cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% antibiotics (penicillin-streptomycin) in a humidified incubator maintained at 37°C with 95% O₂ and 5% CO₂. Once the cells reached the logarithmic growth phase, they were detached from culture flasks using 0.25% trypsin containing 0.02% EDTA, followed by gentle tapping of the flask bottom. The cell count was determined using trypan blue dye exclusion assay. The cells were seeded into 6-well and 96-well plates as required for specific experiments. Stock solutions of the synthesized CuNPs derived from *Pistia stratiotes* (1 mg/ml) were prepared using water followed by sonication. These stock solutions were subsequently diluted to the desired working concentrations (µg/ml) for use in respective assays.

The treatment groups were: a) Group I: Control - untreated MCF-7 cells; b) Group II: MCF-7 cells treated with CuNPs; c) Group III: MCF-7 cells treated with the standard drug Doxorubicin.

2.5. Cell Viability Assessment

The cytotoxic effect of CuNPs on both MCF-7 and HEK293 cells were evaluated using the MTT assay [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide]. Briefly, MCF-7 cells and HEK293 cells were seeded at a density of 8,000 cells per well in 96-well plates and incubated for 24 hours at 37°C in a humidified atmosphere containing 5% CO₂ and 95% O₂ for adherence and confluency. The cells were then treated with CuNPs at concentrations of 5, 10, 15, 20, 25, and 30 µg/ml and with the standard drug, Doxorubicin at concentrations of 1, 3, 5, 7 and 10 µg/ml, followed by incubation for another 24 hours. After treatment, the culture medium was completely removed, and 10 µl of MTT solution (5 mg/ml) along with 90 µl of fresh medium was added in the dark. The plates were incubated for 4 hours to allow metabolically active cells to form water-insoluble formazan crystals. Subsequently, 100 µl of DMSO was added to solubilize the formazan, producing a purple colour measurable at 570 nm. After 15 minutes in the dark, absorbance was read at 570 nm using a microplate reader. The absorbance of control (untreated) cells was considered 100% viability. The experiment was done in triplicate, and cell viability (%) was calculated:

$$\text{Cell viability (\%)} = \left(\frac{\text{Absorbance of treated cells}}{\text{Absorbance of control cells}} \right) \times 100$$

2.6. Calculation of IC₅₀ value

The IC₅₀ (Inhibitory Concentration 50) value, representing the concentration of CuNPs required to inhibit 50% of cell viability, was calculated by generating a scatter plot and applying the linear regression equation $y = mx + c$, where m is the slope, x and y represent drug concentration and % cell viability, and c is y-intercept. The

IC₅₀ was determined using AAT Bioquest's IC₅₀ calculator, providing a reliable estimate of CuNPs cytotoxic potency.

2.7. Acridine orange/Ethidium bromide (AO/EtBr) dual staining

Acridine Orange/Ethidium Bromide (AO/EtBr) dual staining was performed to assess morphological apoptotic responses. MCF-7 cells were seeded in 6-well plates at a density of 300,000 cells/ml and incubated for 24 hours at 37 °C for cell adherence. The cells were then treated with a working concentration of 15 µg/ml of CuNPs (prepared from 1 mg/ml stock) and the standard drug Doxorubicin at a concentration of 5.7 µg/mL concentration [26], followed by incubation for 24 hours. After treatment, the cells were washed with 1X PBS and stained with 5 µg/ml AO/EtBr (from a 100 µg/ml stock). A total of 2 ml of staining solution was added per well, and plates were incubated for 30 minutes at 37°C. Excess dye was removed by washing with PBS, fresh medium was added, and the cells were observed under a fluorescence microscope (Olympus IX51, Japan) at an excitation wavelength of 300–360 nm using a 40X objective lens (filter channel 2).

2.8. DAPI (4',6-diamino-2-phenylindole) staining

DAPI (4',6-diamidino-2-phenylindole) staining was used to assess DNA damage and apoptotic morphology. Briefly, MCF-7 cells were seeded in 6-well plates at a density of 300,000 cells/ml and incubated for 24 hours at 37 °C. The cells were then treated with 15 µg/ml CuNPs (from a 1 mg/ml stock) and the standard drug Doxorubicin at a concentration of 5.7 µg/mL, followed by incubation for 24 hours at 37 °C. After treatment, the cells were fixed with 4% paraformaldehyde for 10 minutes to preserve cellular architecture and permeabilized with 0.1% Triton X-100 for 5 minutes to allow dye penetration. The cells were then washed three times with 1X PBS. Subsequently, the cells were then stained with 1 µg/ml DAPI (from a 1 mg/ml stock) for 10 minutes at 37 °C. Excess dye was removed by washing with PBS, fresh medium was added, and the cells were observed under a fluorescence microscope (Olympus IX51, Japan) at 359 nm excitation (channel 4) using a 20X objective lens.

2.9. Wound healing Assay

For the wound healing assay, cells were seeded in 6-well plates at a density of 50,000 cells per well and allowed to grow to 90–100% confluency. After 12 hours of serum starvation, a uniform scratch (wound) was made across the cell layer using a sterile 200 µL pipette tip. The wells were then washed with 1X PBS to remove any dislodged cells. The treatment wells were then supplied with fresh DMEM-HG containing 15 µg/mL, while the control wells received only the culture medium. Images were captured immediately after scratch formation (0 hours) and after 12 hours using a phase contrast microscope. The wound areas at 0 and 12 hours were measured using ImageJ software by manually tracing the wound margins. The percentage of wound closure was calculated using the formula:

$$\text{Wound Closure (\%)} = \frac{\text{Area at 0 h} - \text{Area at 12 h}}{\text{Area at 0 h}} \times 100$$

Area at 0 h × 100

2.10. Microbial cultures

Pathogenic bacterial strains like *Escherichia coli* (ATCC 35218), *Pseudomonas* sp. (ATCC 27853), and *Staphylococcus aureus* (ATCC 25923), as well as non-pathogenic strains such as *Bacillus* sp., *Escherichia coli* and *Staphylococcus Aureus*, were maintained on nutrient agar slants at 4 °C.

Agar diffusion assay

Human pathogenic and non-pathogenic microbial cultures were grown overnight in nutrient broth, then centrifuged at 5000 rpm for 2 minutes. The pellets were washed with 1X PBS, resuspended in fresh nutrient broth, and allowed to grow for 6-7 hours. Nutrient agar plates were swabbed on three axes with sterile cotton swabs dipped in the freshly prepared diluted cultures. Wells of 6 mm diameter were bored aseptically using a sterile cork borer. Each well was loaded with CuNPs at concentrations of 50 and 100 µg/mL and left for 1 hour to allow the perfusion of nanoparticles. The plates were kept for further incubation at 30 °C for 24 hours. The antimicrobial activity of CuNPs was determined by measuring the diameter of the zone of inhibition (mm) around each well.

2.11. Free radical scavenging activity

1,1-Diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging potential of green- synthesized CuNPs was determined using a modified method by Hanato et al. [27]. CuNPs at various concentrations (50, 100, 250, 500 and 1000 mg/mL) and standard vitamin C were mixed with 1 mL of freshly prepared DPPH (0.15 % in methanol). The mixtures were vortexed thoroughly and incubated in the dark for 30 minutes. Absorbance was measured at 515 nm. A control (DPPH + Methanol) was processed identically. The percentage of radical scavenging was calculated as:

$$\text{Radical scavenging activity (\%)} = (A_0 - A_1) / A_0 \times 100$$

Where, A₀ is the absorbance of control, and A₁ is the absorbance of sample (CuNPs or vitamin C).

2.12. Hydroxyl radical (HO) scavenging activity

Hydroxyl radical scavenging activity was determined as per the modified method of Chung et al. [28]. The reaction mixture contained 200 µL of 10 mM FeSO₄·7H₂O, 200 µL of 10 mM EDTA, 200 µL of 10 mM 2-deoxyribose, and 1.2 mL of 0.1 M phosphate buffer (pH 7.4) with different CuNPs concentrations (50, 100, 250, 500 and 1000 mg/mL). To this, 200 µL of freshly prepared 10 mM H₂O₂ was added and incubated for 4 hours at 37 °C. Subsequently, 1 mL of 2.8 % TCA and 1 mL of 1 % TBA were added, and the mixture was boiled for 10 minutes. After cooling, samples were centrifuged at 2000 rpm for 5 minutes, and absorbance was recorded at 532 nm. Radical scavenging activity was calculated using the same formula as above.

2.14. Statistical Analysis

To ensure the reliability and reproducibility of the findings, all experiments were conducted in triplicates. Statistical analysis was performed using one-way Analysis of Variance (ANOVA). Tukey's post-hoc tests were applied as appropriate to compare experimental groups with controls. Results with *p* < 0.05 were considered statistically significant.

3. Results

3.1. Green Synthesis, Characterization, and Morphological Analysis of Copper Nanoparticles

In the present study, CuNPs were synthesized by a biological approach using *Pistia stratiotes* leaf extract. The formation of CuNPs was initially confirmed by a visible colour change of the reaction mixture from pale yellow to colloidal brown, indicating nanoparticle synthesis. To further validate the formation of CuNPs and study their optical properties, UV–Visible spectroscopy was employed. The UV–Visible absorption spectrum of the synthesized CuNPs recorded a characteristic surface plasmon resonance band at 320 nm (Figure 1).

Following optical characterization, FT-IR spectroscopy was conducted to identify functional groups involved in CuNP stabilization. FT-IR spectrum measurements of CuNPs exhibited (Figure 1) seven prominent bands, which were compared with the standard values to identify the functional groups. FT-IR showed the following bands at approximately 3278, 2916, 1624.06, 1552, 1398, 977, and 686 cm⁻¹. The intense peaks detected at ~3278 cm⁻¹ and 2916.37 cm⁻¹ attributed to ≡C-H (alkyne stretch) and N-H stretches, respectively. The solid intense peak at ~1624 cm⁻¹ showed the C=C and C-N stretching, which confirmed the presence of proteins. The infrared spectra at 1095–1075 / 1055–1020 cm⁻¹ are characteristic for functional groups like organic siloxane or silicone (Si-O-Si) in the synthesized CuNPs.

To corroborate these findings and gain insight into

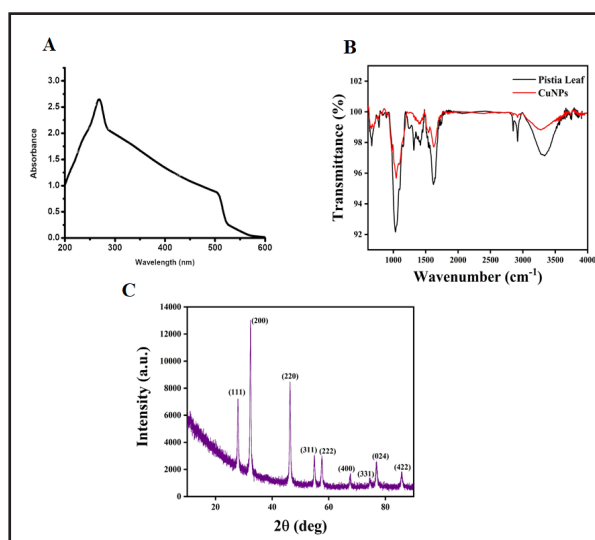


Figure 1. UV-visible Absorption Spectrum of the BioLogically Synthesized CuNPs (A), FT-IR Spectra of Green Synthesized Copper Nanoparticles (B), XRD Spectrum of the CuNPs from *Pistia stratiotes*.

the crystalline nature of the CuNPs, XRD analysis was carried out. The XRD pattern of the green-synthesized CuNPs from *Pistia stratiotes* is shown in Figure 1. In XRD analysis, four peaks were identified at 2θ values of 27.88° , 32.30° , 46.28° , 54.88° , and 57.52° . Sets of lattice planes were observed which may be indexed to the (111), (200), (220), (311) and (222) reflections of face-centered cubic structures of copper (JCPDS File No: 01-073-1667). XRD pattern clearly demonstrated that the CuNPs were crystalline in nature and the average crystal size was found to be 20 nm calculated by Scherrer's equation is $d = 0.9\lambda/\beta \cos \theta$ where, d is the average crystallite size of the nanoparticles, λ is the wavelength of X-ray radiation source, β is the angular FWHM of the XRD peak at the diffraction angle θ .

Finally, to investigate the morphology and elemental composition of the CuNPs, FE-SEM, EDX, and HR-TEM analyses were conducted. The morphology of the bio-synthesized CuNPs was determined by FE-SEM analysis. The FE-SEM images confirmed the spherical-shaped exterior morphology of CuNPs (Figure. 2A-C). The FE-SEM and EDX clearly revealed that the nanoparticles were mono-dispersed and spherical in shape with a size range of 20 to 30 nm. The EDS analyses confirmed the elemental composition and stability of green synthesized CuNPs (Figure. 2D). The purity levels of the particles were examined, and the EDX spectrum illustrated the presence of strong metallic Cu signals, which indicated that *Pistia stratiotes*-mediated CuNPs had 92.7 % of Cu, and it also exhibited some weak signals of Cl (4.7 %) and Ag (2.5 %). The spherical nature of the copper nanoparticles was also confirmed by the HR-TEM analysis

as shown in Figure. 3A-C. The average diameter of the copper nanoparticles varies between 20 and 30 nm. The selected area electron diffraction (SAED) pattern of the CuNPs witnessed the polycrystalline nature of the green synthesized CuNPs as shown in Figure. 3D.

3.2. Anti-Proliferative and Cytotoxic Effects of CuNPs on MCF-7 Cells

Following the successful synthesis and characterization of the copper nanoparticles, it was essential to evaluate their biological efficacy, particularly their potential anti-proliferative activity against cancer cells. This investigation assessed the cytotoxic effects of CuNPs' on MCF-7 breast cancer cells using the MTT assay, where a significant reduction in cell viability was observed after 24 hours of treatment. This effect was dose-dependent across concentrations ranging from 10 to 30 $\mu\text{g/ml}$ when compared to untreated control cells. The morphological changes and cytotoxic trend are visually represented in Figure. 4A and B.

The IC_{50} value was determined as 10.119 $\mu\text{g/ml}$ using AAT Bioquest's IC_{50} calculator, which highlights the cytotoxic efficacy of CuNPs's against breast cancer cells (Figure. 4 C). In addition, comparatively lower cytotoxicity was observed in normal HEK 293 cells treated with CuNPs (Figure. 4 D). The anticipated anticancer mechanism of action of metal nanoparticles such as copper, gold, and silver involves the generation of ROS due to oxidative stress. This process induces physiological alterations by disrupting the membrane permeability, ultimately leading to cancer cell death while exerting minimal effects on normal cells.

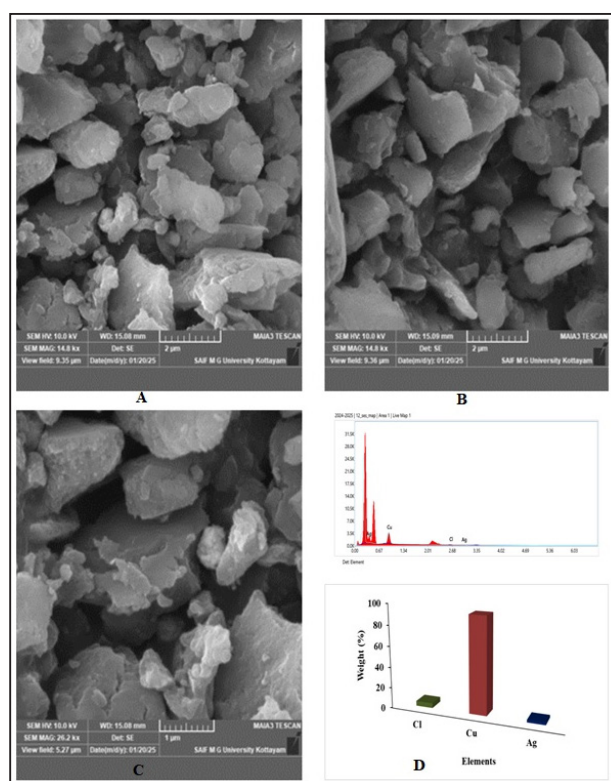


Figure 2. Morphological Characterizations Using FESEM (A-C). Elemental composition analysis of synthesized CuNPs using EDX (D).

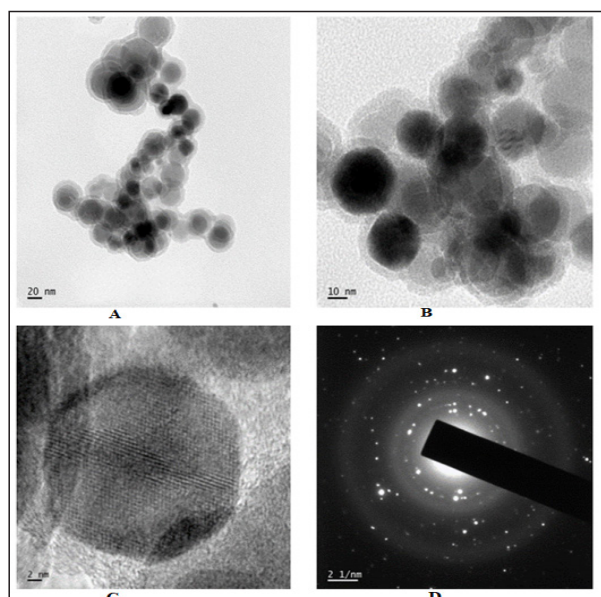


Figure 3. HR-TEM Image of Copper Nanoparticles Synthesized with *Pistia stratiotes* Leaf Extract. The CuNPs exhibited a spherical shape (A-C), SAED pattern of copper nanoparticles (D)

3.3. CuNPs Induced Apoptosis in MCF-7 Cells as Evidenced by AO/EtBr Dual Staining

To determine whether the inhibition of MCF-7 cell growth was due to apoptosis, AO/EtBr dual staining was performed using fluorescence microscopy after 24 hours of treatment with CuNPs and Doxorubicin (Standard drug). Fluorescence microscopy revealed the characteristic morphological changes of apoptosis, wherein acridine orange stains both live and early apoptotic cells green, while ethidium bromide stains late apoptotic cells with red-orange fluorescence, indicating compromised membrane integrity. Nuclear shrinkage or blebbing leads to cells stained with EtBr (cells lacking cytoplasm) appearing orange or red, whereas cells stained with AO appear green as their membranes remain intact.

Interestingly, cells treated with doxorubicin (standard drug) and CuNPs exhibited a notable reduction of viable (green-stained) cells along with an increase in orange-red fluorescence, signifying apoptotic cell death (Figure. 5B & C). However, untreated control cells showed minimal apoptosis (largely viable with intact membranes), as evidenced by the predominance of green fluorescence (Figure. 5A). Compared to the control untreated group, CuNPs-treated cells exhibited a significant increase in apoptotic cells ($p < 0.05$) (Figure. 5D).

3.4. CuNPs Induce Dose-Dependent Reduction and Morphological Alterations in MCF-7 Cells Revealed by DAPI Staining

To further investigate whether the induction of apoptosis in MCF-7 cells was due to CuNPs treatment, DAPI labelling was performed 24 hours post-treatment. Nuclear fragmentation is one of the hallmarks of apoptotic cell death.

In the control untreated MCF-7 cells, a greater number of cells with intact nuclei were observed

(Figure. 6A). Treatment with standard drug showed a significant decrease in the number of cells along with the appearance of apoptotic features (Figure. 6B). However, following treatment with CuNPs, a noticeable decrease in the number of cells was observed (Figure. 6C). Markedly, the CuNPs-treated group displayed a significant decrease in cell number relative to the control (Figure. 6D). Morphological analysis by fluorescence microscopy revealed distinct changes in cell morphology following treatment, with CuNPs-treated cells showing more pronounced apoptotic features compared to control cells.

3.5. CuNPs Inhibit Migration of MCF-7 Cells as Evidenced by Wound Healing Assay

Consistent with the reduced viability and increased apoptotic features observed in AO/EtBr and DAPI staining, the wound healing assay further demonstrated the anti-migratory and anti-proliferative effects of CuNPs on MCF-7 cells. The assay revealed a significant reduction in the wound gap closure in CuNPs-treated cells compared to untreated controls at both 0 hour (Figure. 7 A and C) and after 12 hours in MCF-7 cells.

While control cells exhibited approximately 75.77% wound closure, indicating normal cell migration and proliferation, CuNPs-treated cells showed a 36.23% increase in wound area with no significant reduction in the gap. This clearly indicates that CuNPs impair both cell migration and proliferation of MCF-7 cells. Representative images further illustrate these findings, as CuNPs-treated cells displayed a markedly wider wound gap at the 12-hour time point (Figure. 7D) compared to control cells (Figure. 7B).

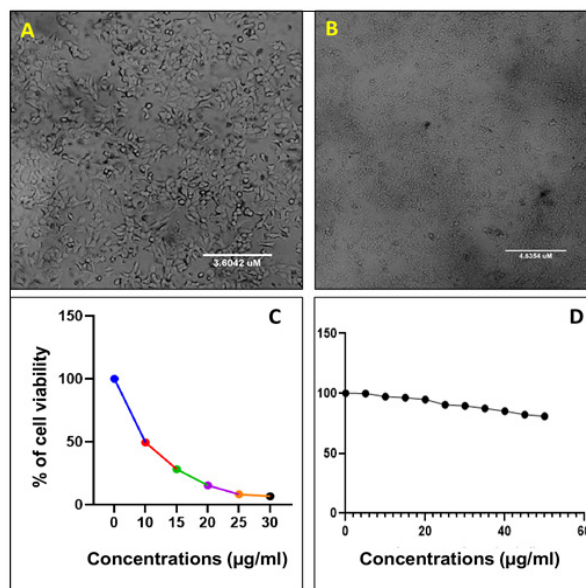


Figure 4. Morphological Appearance of MCF-7 Cells in Phase-contrast Microscopy: (A) on Day 3 of the twenty forth subculture and (B) on Day 1 after treatment with CuNPs and (C) Dose-dependent cytotoxic effect of CuNPs on MCF-7 cells and IC_{50} was determined to be 10.119 µg/ml (D) Dose-dependent cytotoxic effect of CuNPs on HEK293 cells after 24 hours, assessed by MTT assay. Data are presented as mean $\pm$ SD ($n=3$).

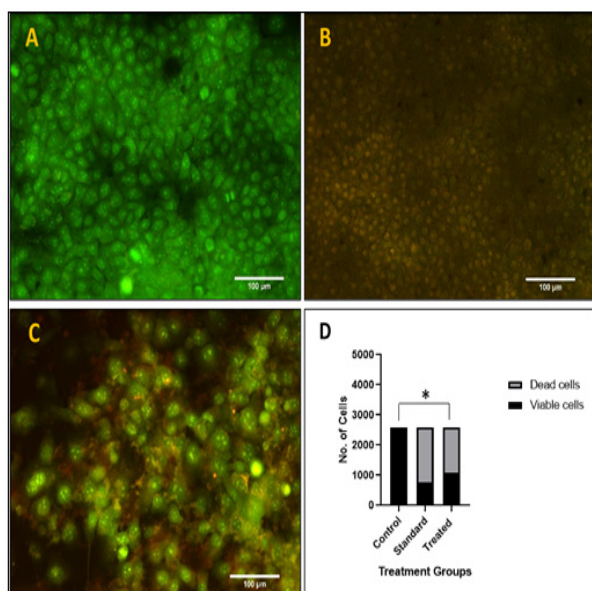


Figure 5. Assessment of Apoptosis by AO/EtBr Staining in the experimental groups on MCF-7 breast cancer cells (A) Control, (B) Standard drug treated, (C) CuNPs treated, and (D) Percentage of apoptotic cells calculated by distinguishing viable and dead cells using image J software. * Indicates $p < 0.05$ compared to untreated control.

3.6. CuNPs Exhibit Broad-Spectrum Antimicrobial Activity against Human Pathogens

Building on the demonstrated anti-proliferative, anti-migratory, and pro-apoptotic effects of CuNPs on MCF-7 cells, the present study further evaluated their anti-microbial potential against human pathogens. The CuNPs synthesized from *Pistia stratiotes* leaf extract demonstrated promising antimicrobial activity against all the human pathogens used in this study. After 24 hours of incubation at 37 °C, a zone of inhibition was observed on the plates loaded with copper nanoparticles. All plates exhibited clear zones around the wells, as illustrated in Figure. 8A, and the measured diameters of these zones are shown in Figure. 8B.

3.7. Antioxidant Activity of Green Synthesized Copper Nanoparticles

In continuation of the observed anticancer, anti-migratory, and antimicrobial properties of CuNPs, the effectiveness of spherical-shaped nano-copper as an antioxidant was examined through *in vitro* assays. The findings regarding the impact of varying concentrations of CuNPs on DPPH and hydroxyl radical scavenging activity are presented in Figure. 9A & B. It was observed that increasing the concentration of CuNPs tends to boost DPPH radical scavenging activity, with copper nanoparticles exhibiting a scavenging rate ranging from 37% to 86% at concentrations of 50 to 1000 mg/mL. The aforementioned activity was greater than that of the standard vitamin C (74%). Interestingly, the DPPH scavenging effect of CuNPs was visually countersigned by the colour change from violet to yellow. Moreover, the study was extended to determine the antioxidant properties of CuNPs using the hydroxyl radical scavenging

technique. At a CuNP concentration of 1000 $\mu\text{g/mL}$, the highest rate of inhibition was 79%, although this was lower than the standard ascorbic acid, which showed a 92% scavenging capability. Both DPPH and hydroxyl scavenging assays clearly revealed that the antioxidant activity of copper nanoparticles was dose dependent, as increased concentrations led to enhanced antioxidant activity.

4. Discussion

In the current study, CuNPs were rapidly formed after the addition of *Pistia stratiotes* extract, the reaction mixtures turned dark green to bluish-green upon after 3 min of incubation. The immediate and spontaneous colour transformation upon interaction of *P. stratiotes* phytochemicals with Cu^{2+} ions further supported the formation of CuNPs. This colour change is attributed to the interaction between the conduction electrons of metal NPs and incident photons, a phenomenon known as surface plasmon resonance [29]. The UV-Visible absorption spectrum detected at 320 nm. This absorbance corresponds to the excitation of surface plasmons, which is size-dependent. This result aligns with earlier findings where CuNPs synthesized from *Withania somnifera* exhibited maximum absorbance at 330 nm [30], further confirming nanoparticle formation.

FT-IR study confirmed the following bands at approximately 3278, 2916, 1624.06, 1552, 1398, 977, and 686 cm^{-1} . The intense peaks noticed at $\sim 3278 \text{ cm}^{-1}$ and 2916.37 cm^{-1} attributed to $\equiv\text{C-H}$ (alkyne stretch) and N-H stretches, respectively. The strong intense peak at $\sim 1624 \text{ cm}^{-1}$ indicated the C=C and C-N stretching, commonly associated with proteins [31, 32]. Proteins are known to play important roles in the biosynthesis of metallic

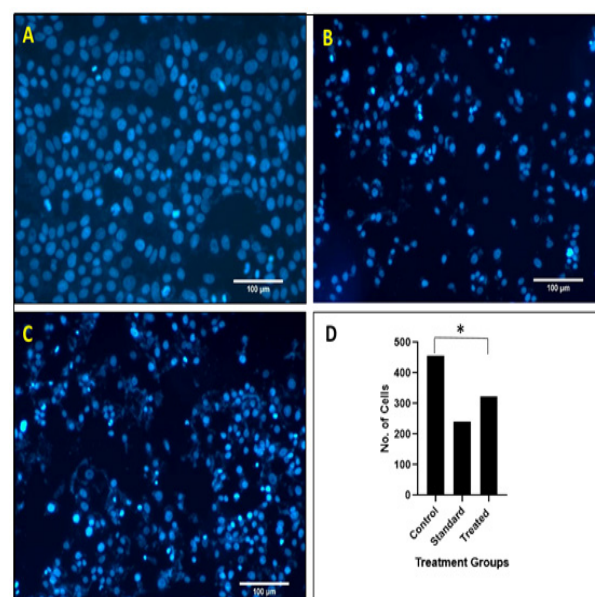


Figure 6. Assessment of Apoptosis by DAPI Staining in the experimental groups MCF-7 (A) Control, (B) Standard drug treated, (C) CuNPs treated, and (D) Number of cells calculated by using imageJ software. * indicates $p < 0.05$ compared to untreated control.

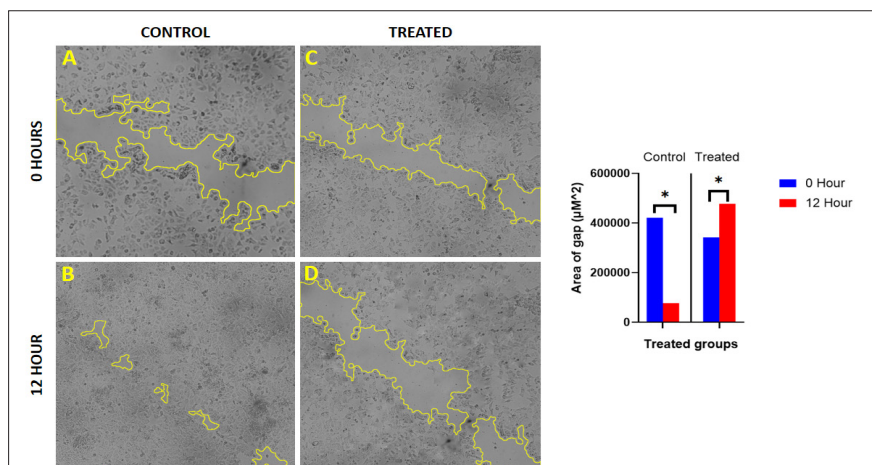


Figure 7. Evaluation of CuNPs's anti-proliferative effects through the wound healing assay. (A) Control cells at 0 hours, (B) Control cells at 12 hours, showing 75.77% wound closure, (C) CuNPs treated cells at 0 hours, (D) CuNPs treated cells at 12 hours, showing a 36.23% increase in wound area. *indicates significant difference $P < 0.05$ between control and treated groups.

nanoparticles (NPs), which are biological substitutes for conventionally used chemical capping and stabilizing agents [33]. Proteins contribute to stabilization through capping mechanisms of AgNPs and may enhance the stability of biosynthesized nanoparticles [34]. They can adsorb onto the surface of nanoparticles, forming a protective layer that prevents agglomeration. The unique structure of proteins allows them to stabilize nanoparticles by providing steric hindrance and electrostatic repulsion [35]. The IR spectra at 1095–1075 / 1055–1020 cm^{-1} representing the organic siloxane or silicone (Si-O-Si), and this finding was also in agreement with earlier report of John [36]. The present FT-IR investigation was in concurrence with previous report of Shohei et al. [37], which revealed that the Cu nanoparticle is one of the most difficult nanoparticles to be handled due to its absence of oxidation stability. The silica may be involved in the coating of Cu nanoparticles and enhanced its stability to oxidation; therefore, CuNPs coated by silica shells had remarkable stability even in the presence of a strong oxidizing agent. The XRD analysis confirmed the face-centered cubic structures of copper nanoparticles and Scherrer's equation indicated the average size of NP were 20 nm. In the XRD spectra, some additional peaks were also observed which are due to the presence of bioorganic matters and capping agents for silver nanoparticles formation [38–40]. The EDX spectrum revealed the presence of Cu (92.7%) and additionally a few weak signals, these weak signals may be due to the X-ray emission from the macromolecules like flavonoids, phenolic compounds, carbohydrates, glycosides, steroids and tannins present in the extracts [38]. It is quite normal in the green as well as bio-synthesized nanoparticles [41]. A report of Ryntathieng et al. [42] suggested that the presence of chlorine and oxygen further supports their stability, making them suitable for biological applications.

Following the successful synthesis and physicochemical characterization of CuNPs, it was very much important to investigate their biological efficacy, mostly their anticancer potential against breast cancer cells. Therefore, the cytotoxic and apoptotic effects of the synthesized CuNPs

were investigated using MCF-7 human breast cancer cells. Inverted brightfield microscopy analysis evidently exposed the characteristic morphological changes of apoptosis in CuNPs-treated MCF-7 cells. The current study exhibited enhanced anticancer effects at a lower concentration (10.119 $\mu\text{g}/\text{ml}$) on MCF-7 cells. This finding aligns with earlier reports where CuNPs synthesized from *Hippophae rhamnoides* exhibited cytotoxicity at 48 $\mu\text{g}/\text{mL}$, with cancer cell viability reduced to 28.18% in HeLa cervical cancer cells [43]. Similarly, Hasanin et al. [44] reported potential anticancer properties of CuNPs synthesized from *Talaromyces pinophilus*, with

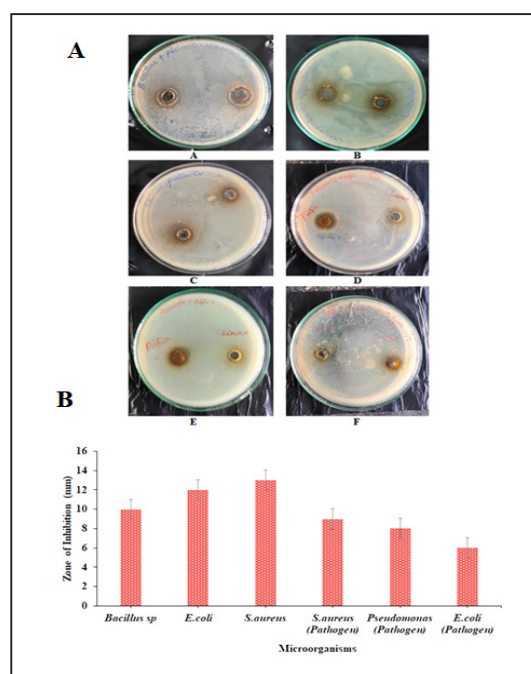


Figure 8. Depicts the antimicrobial activity of *Pistia stratiotes* mediated CuNPs against various human pathogenic and non-pathogenic microorganisms using agar well diffusion method (A). The diameter of zone of inhibition which indicates the effectiveness of green synthesized copper nanoparticles on antimicrobial activity (B).

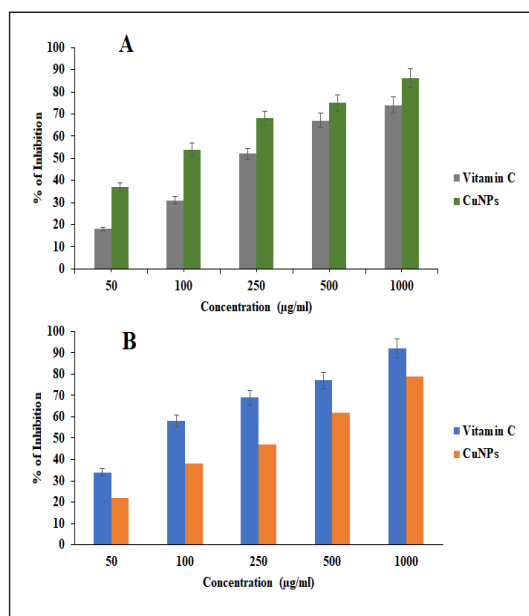


Figure 9. Demonstrates the Antioxidant properties of CuNPs using DPPH (A) and hydroxyl radical (B) assays

cytotoxic effects noted at higher concentrations of 210.9 µg/mL. Interestingly, the present investigation also observed similar dose- and time- dependent anticancer activity of CuNPs when compared with the standard drug, doxorubicin [45-47]. This observation was corroborated by earlier findings in which MCF-7 cells treated with CuNPs synthesized from *Prunus nepalensis* displayed concentration- dependent cytotoxicity [46].

The induction of apoptosis in cancer cells plays a pivotal role of the activity of anticancer substance [48]. In the present study, apoptosis in MCF-7 cells was confirmed by AO/EtBr dual staining and DAPI staining following treatment with CuNPs. These fluorescence-based techniques are widely used for examining membrane integrity and nuclear morphology. In AO/EtBr staining, acridine orange permeates both live and dead cells and stains the nuclei green, whereas ethidium bromide typically enters the cells with compromised membrane integrity and exhibits yellow-orange-red nuclei with green cytoplasm depending on the stage of apoptosis [45]. Activation of tumour suppressor genes and mitochondrial pathways is regulated by CuONPs to mediate their anticancer effect against a myelogenous leukemia (K562) cell line [49]. Apoptotic cell death was further confirmed by AO/EtBr dual staining in MCF-7 cells treated with chitin incorporated silver and copper nanocomposite [50]. The staining results indicated that CuNPs induced apoptosis rather than necrosis in treated cells, as evidenced by increased orange-red fluorescence. This observation was further corroborated by DAPI staining, a fluorescent DNA-binding agent that confirms apoptotic nuclear alterations [51]. Interestingly, examination revealed distinct changes in nuclear morphology in the cells treated with CuNPs. The consistency between the AO/EtBr results, which reflects membrane integrity (Figure 5), and DAPI staining, which highlights nuclear condensation and fragmentation (Figure 6), strongly indicates that

the cytotoxic effects observed in CuNPs-treated cells predominantly through apoptotic mechanism.

In addition to inducing apoptosis, the synthesized CuNPs also exhibited significant anti-migratory effects on MCF-7 cells, as demonstrated by the wound healing assay. Cell migration is a critical process involved in cancer progression and metastasis. In the present study, CuNPs-treated cells showed a marked reduction in wound closure compared to untreated control cells, indicating inhibition of cell migration and proliferation. While control cells exhibited rapid wound closure due to normal migratory activity, CuNPs-treated cells maintained a wider wound gap even after 12 hours of incubation (Figure 7). These findings suggest that CuNPs not only induce apoptotic cell death but also suppress the migratory potential of MCF-7 breast cancer cells. The observed inhibition of cell migration further supports the anticancer potential of CuNPs and highlights their possible role in preventing cancer cell proliferation and metastasis. The same effects were observed in copper nanoparticles from *Petalium murex*-treated cells included cell contraction, nucleus disintegration, and cell budding, resulting in cells of varying sizes in the human lung cancer A549 cell line [52].

The present study on antimicrobial activity of CuNPs synthesized from *Pistia stratiotes* confirmed the zone of inhibition around the wells. The zone of inhibition evidenced the antibacterial activity of AgNPs against selected Gram-negative and Gram-positive bacterial pathogens [17, 53]. Research into the antibacterial mechanisms of CuNPs is increasing, and the green- synthesized CuNPs from *Panchagavya* revealed strong antibacterial activity against *Bacillus cereus* and *Pseudomonas aeruginosa*; the mechanism behind this was that CuNPs disrupted the bacterial cell membrane, causing leakage of cellular contents as confirmed by SEM examination [54]. Previous studies have indicated that the antimicrobial potential of green-synthesized copper nanoparticles is influenced by types of bacteria studied [55, 56]. Copper nanoparticles exhibited strong bactericidal properties, with robust adsorption and antimicrobial activity, and possess a broad spectrum of long-lasting antibacterial, antifungal, and anti-mildew effects [57]. The copper nanoparticles seem to release reactive hydroxyl radicals that can cause irreversible harm, such as the oxidation of proteins, the breakdown of RNA and DNA molecules, and the mutilation of cell membranes via lipid peroxidation, which ultimately led to cell death in the strains of *E. coli* and *Enterococcus* sp. [58]. The both DPPH and hydroxyl scavenging assays revealed the potential antioxidant properties of green synthesized CuNPs. The colour change from violet to yellow in the DPPH reaction mixture indicates the effective scavenging of CuNPs. The reduction of DPPH by hydrogen atom donation, which exhibited significant scavenging activity, was visibly seen through this colour change [59]. The phytochemicals from the plant, consisting of abundant polyphenols and flavonoids, are linked to nanoparticles and might be boosting the antioxidant effects of copper nanoparticles, potentially revealing a novel approach to

improving traditional medicine and addressing various incurable illnesses [60].

In conclusion, the current study focused on the synthesis of sustainable, economical, and relatively non-toxic copper nanoparticles (CuNPs) using the leaves of the aquatic weed *Pistia stratiotes*. Notably, the biological process proposed here represents a commencement report for the production of CuNPs from *P. stratiotes*. The synthesized CuNPs were well-characterized and predominantly spherical and monodispersed in nature, confirming the successful formation and stability of the nanoparticles.

The well-characterized CuNPs demonstrated promising potential as a nanodrug by effectively triggering the apoptotic pathway and inducing cell death in MCF-7 breast cancer cells. The outcome of the present study in human breast cancer cells provided clear evidence of molecular events such as chromatin condensation, nuclear fragmentation, membrane blebbing, and DNA fragmentation, which ultimately led to apoptosis induced by CuNPs. Importantly, the study also demonstrated the efficacy of CuNPs in inhibiting MCF-7 cell migration and proliferation, further supporting their potential role in suppressing cancer progression. In addition, the green synthesized copper nanoparticles exhibited significant antibacterial activity against selected bacterial pathogens and also demonstrated notable antioxidant potential. Overall, the biomedical efficacy of CuNPs was successfully highlighted in the present investigation. These findings may ultimately pave the way for further exploration into the development of copper nano-based drugs for the treatment of various cancers and infectious diseases.

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Author contributions

N. K.: Conceptualization, N. K., A. M., and M. P.: Experiments performed, N. K. and A. T. S.: Writing original draft, N. K., A. M., and M. P.: Editing and preparation of figures, N. K., A. T. S., M. M.: Review, editing and formal analysis, M. M.: Supervised, directed, and managed the study. All authors approved the final version of the manuscript.

Declaration

All authors have read, understood, and have complied as applicable with the statement on "Ethical responsibilities of Authors" as found in the Instructions for Authors.

Availability of data and material

Not applicable.

Competing interests

The authors declare that they have no competing

interests regarding the publication of this research manuscript.

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