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Original Article

Novel and Facile Green Synthesis of Copper Nanoparticles Using Aqueous Extract of *Phragmites australis* Leaves and Evaluation of their Antioxidant, Antihemolytic, Anti-inflammatory, and Anticancer Effects

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Abstract

Introduction: There is a growing interest in producing green nanoparticles with a variety of biological activities. For that, the leaf extract of *Phragmites australis* was used to mediate copper nanoparticle (CuNPs) synthesis, and its antioxidant, antihemolytic, anti-inflammatory, and anticancer effects were evaluated.

Materials and Methods: The characterization of CuNPs was done using different techniques: UV-Vis spectroscopy, FT-IR spectroscopy, scanning and transmission electron microscopy (SEM and TEM), and X-ray diffraction. The antioxidant and anti-inflammatory activities were assessed using two methods for each. Anti-cancer potential was evaluated by means of the MTT assay.

Results: Phytochemical screening demonstrated the richness of the plant in different biomolecules. UV-Vis spectra showed a peak at 312 nm. The crystalline structure of the phyto-synthesized CuNPs was confirmed by XRD, and the grain size was estimated to be 18.06 nm. Furthermore, the obtained nanoparticles exhibited remarkable antioxidant activity, with IC_{50} values of 4.62 µg/ml and 78.99 µg/ml for DPPH and FRAP tests, respectively. The IC_{50} values for the anti-inflammatory activity in both tests were conspicuous and revealed a substantial capability of green CuNPs. The cytotoxicity of Phragmites australis-mediated synthesized CuNPs against MCF-7 cell lines disclosed a dose-dependent efficacy.

Conclusions: In conclusion, our research suggests that *Phragmites australis* leaves' aqueous extract can be used as reducing and stabilizing agents for the green synthesis of CuNPs, which provided antioxidant, anti-inflammatory, and anticancer effects.

Keywords: *Phragmites australis*, Green Copper Nanoparticle, Antioxidant, Grain Size, Cytotoxic Activity

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Introduction

By utilizing green technologies, researchers can create nanoparticles using environmentally friendly methods such as plant extracts, microorganisms, and biodegradable materials. These approaches not only minimize the use of hazardous chemicals but also offer a more sustainable alternative to traditional synthesis methods. Additionally, green synthesis techniques have shown promising results in terms of controlling the size, shape, and properties of nanoparticles, making them suitable for a wide range of applications. Overall, the integration of green technologies in nanoparticle (NP) synthesis represents a significant step towards achieving a more sustainable and environmentally conscious approach to nanotechnology. As research in this field continues to advance, we can expect to see further innovations that harness the potential of nanoparticles while minimizing their environmental footprint.[1-3] Phytonano technology is the biogenic manufacture of nanomaterials using bioactive chemicals from living entities such as plants, bacteria, fungi, and algae. The phytochemicals such as phenols, flavonoids, and alkaloids serve as bioreducing, stabilizing, and capping agents.[4] The green synthesis of NPs is ecologically harmless, cost-effective, simple and scalable.[5] Furthermore, green synthesis methods often result in nano particles with enhanced biocompatibility and applicability in biomedical and environmental applications.[6] The potential usage of metal and metal oxide NPs is determined by the metal utilized in the biogenic production of NPs. Several metal and metal oxide nanoparticles were synthesized using green technologies, including Au, ZnO, Se, Cu, CuO, Fe_3O_4, Ag, and many more, for diverse biological purposes.[4,7]

Copper nanoparticles (CuNPs) are gaining popularity due to their inexpensive cost and unique optical, mechanical, catalytic, electrical, and thermal characteristics that differ from those of bulk metals.[8,9] Many studies have focused on the green method of producing copper NPs using plant leaf extract, rhizomes, flowers, barks and juice.[8,10]

Phragmites australis, or common reed, is a wetland species known for its abundance of bioactive components in aqueous plant extracts such as tannins, phenolic compounds, flavonoids, terpenoids, and glycosides.[11] The genus Phragmites contains more than ten species in the world.[12] It is used in traditional medicine to cure a variety of human and livestock ailments.[13]

This study presents the green synthesis of copper NPs via *P. australis* leaf aqueous extract, highlighting its antioxidant activity, anti-inflammatory activity and cytotoxic potential, while underscoring its physicochemical characteristics.

Materials and Methods

Plant Collection

The plant was collected from Touggourt state in Algeria. Leaves were detached from specimens, washed with water to remove dust and debris, and allowed to dry in the shade before being crushed into a fine powder.

Extract Preparation

50 ml of distilled water was poured into a beaker containing 50 g of the plant's powder. It was then heated to 50 °C while being stirred for 60 minutes. The preparation was allowed to macerate for 24 hours at ambient temperature, then it was filtered using both muslin cloth and filter paper. The resulting filtrate was then dried in a laboratory oven and stored in the freezer for future applications.[14]

Phytochemical Screening

The presence of compounds such as tannins, alkaloids, phenols, flavonoids, steroids, terpenoids, and saponins, in the plant extract was analyzed using standard protocols for phytochemical screening.[15-17]

Phyto Synthesis of CuNPs

CuNPs were prepared by adding 4g of copper sulfate to a volume of 400 ml of distilled water. Then, 20 ml of leaf extract and 10 ml of ascorbic acid were added to the mixture. The mixture was placed on a heater-stirrer set at 70 °C/1500 rpm. The color changed from blue to dark green within the first 15 minutes, indicating the formation of CuNPs. The pH of the solution was determined beforehand and maintained at a basic level.[18,19] After four hours, the samples were centrifuged and rinsed twice with distilled water and once with ethanol before being dried in an electric furnace set at 70 °C. Figure 1 provides a schematic representation of the biological reaction between biomolecules present in the plant extract and the copper metal ions to obtain CuNPs.

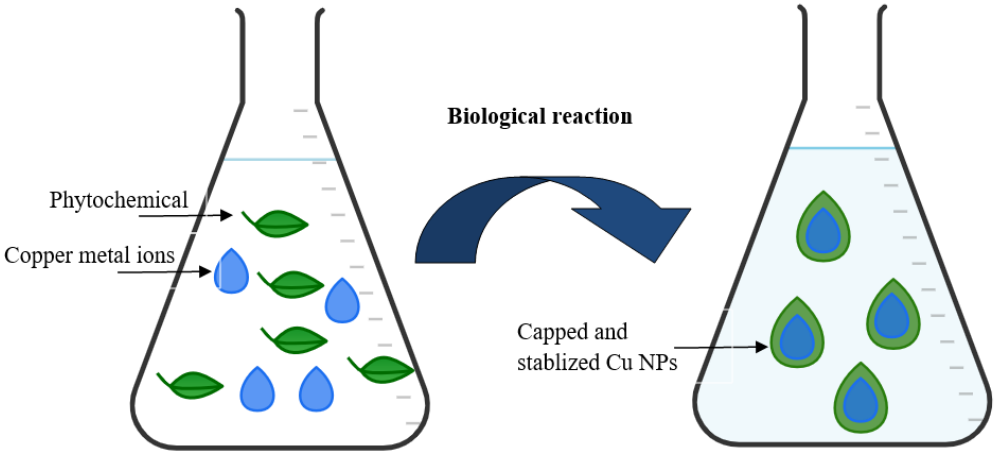

Figure 1. Schematic Representation of the Formation of CuNPs via *P. australis* Leaf Extract.

Characterization of Synthesized Copper NPs

Different techniques were used to characterize our green synthesized copper NPs. UV-Vis spectroscopy was used to confirm the synthesis of NPs, using a PG Instruments T80 Spectrometer, in the range between 200 and 400 nm. Distilled water was used as a blank. FT-IR spectroscopy was employed to demonstrate the involvement of bioactive phytochemicals of the plant extract in reducing and capping NPs (Agilent Technologies). Morphology and size were estimated using SEM (Zeiss) and TEM (Tecnai). Crystal structure and grain size were determined using an X-ray diffractometer (PROTO® AXRD Benchtop), scaling angle (2θ) range of 10-80° with a Cu Kα radiation (λ=1.540593Å).

Evaluation of Antioxidant and Anti-inflammatory Activities of NPs

The antioxidant activity was measured using two assays including DPPH (2,2-diphenyl-1-picrylhydrazyl as a stable

free radical molecule) free-radical scavenging activity as described by Nwidu *et al.*[20] and the ferric reducing anti-oxidant power assay (FRAP) as described by Oyaizu.[21] The ascorbic acid used as a standard agent. In addition, the anti-inflammatory effect was investigated *in vitro* using two methods. The first measures protein denaturation inhibition as described by Vennila *et al*.[22] and the second measures red blood cells protection against haemolysis as stated by Vinjamuri.[23] For the first method, BSA protein was used and for both methods, diclofenac was used as a positive control.

Evaluation of Cytotoxicity Effect of NPs Using MTT Assay

MTT (3-[4,5-dimethylthiazole-2-yl]-2, 5-diphenyl tetrazol iumbromide) assay was used to evaluate the cytotoxic effect of phytosynthesized copper NPs on MCF7 cells as breast cancer cell line. Briefly, the cells were treated with various concentrations of CuNPs (0.15, 0.31, 0.62, 1.25, 2.5 mg/ml) for 72 hours after being seeded in a 96-well plate and incubated for 24 hours. Dimethyl sulfoxide was used to solubilize the produced formazan and the absorbance was measured at 520 nm using a microtiter plate reader. Finally, the cytotoxicity percentage was calculated using the following equation:

$$\text{Cytotoxicity percentage } \% = \left(\frac{(\text{A c} - \text{A bg}) - (\text{A s} - \text{A bg})}{\text{A c} - \text{A bg}}\right) \text{x } 100$$

Where Ac is the control's absorbance, Abg is the background's absorbance, and As is the sample's absorbance.

Results

Phytochemical Screening

Numerous bioactive components, including phenolics, terpenoids, tannins, alkaloids, carbohydrates, and flavonoids, were identified in *P.australis* leaves aqueous extract after phytochemical screening as shown in Table 1.

Table 1. Phytochemical Screening of *P. australis* Leaf Aqueous Extract

Phytochemical compound	*P. australis* leaf aqueous extract
Polyphenols	+
Flavonoids	+
Alkaloids	+
Tannins	+
Saponins	+
Terpenoids	+
Reducing compounds	+

Characterization

The findings show that the addition of the aqueous leaf extract of *P. australis* to the solution of copper sulfate induces a colour change from blue to greenish-dark, indicating the formation of copper nanoparticles.

UV-Vis Spectroscopy

Surface Plasmon Resonance (SPR) of the phytosynthesized CuNPs was evaluated using a UV–Vis spectrophotometer. A peak at 312 nm was detected (Figure 2), which is characteristic of CuNPs.

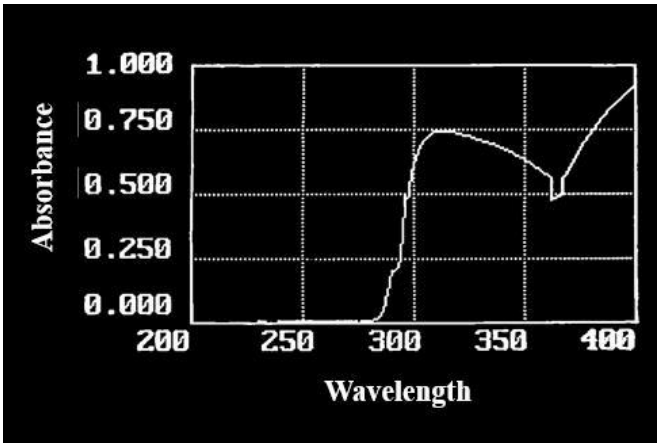

Figure 2. UV-Vis Spectrum of Green-synthesized CuNPs Using *P. australis* Leaf Extract.

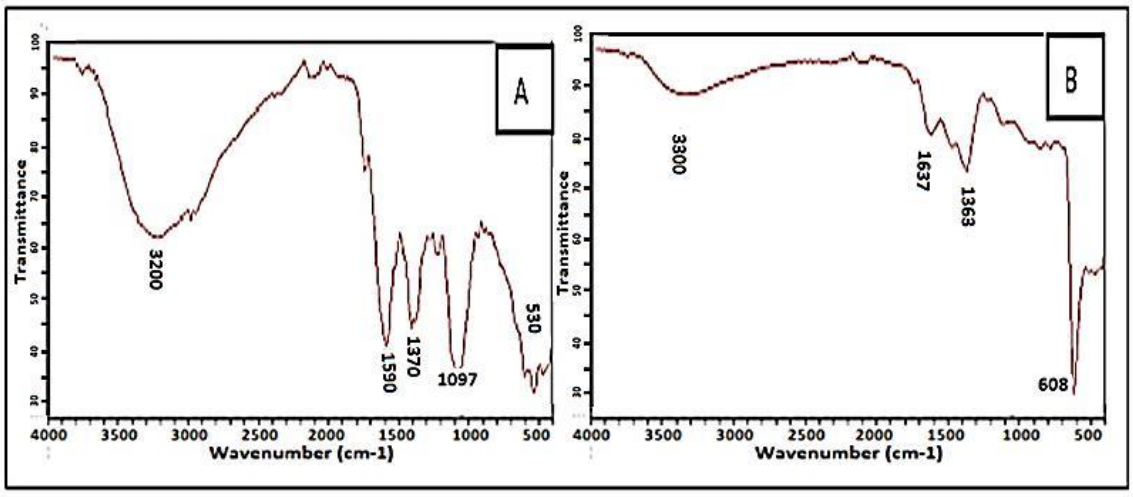

Figure 3. FT-IR Spectra of *P. australis* Leaf Extract (A) and Phytosynthsized CuNPs (B).

FT-IR Spectroscopy Analysis

The FT-IR analysis was performed on both *P. australis* leaf extract and the green-synthesized CuNPs to discover probable biomolecules involved in the bioreduction of the latter. The scan range was from 500 to 4000 cm^{-1}. Figure 3A shows the FT-IR spectrum of the plant extract where different peaks are visible. A broad peak at 3208 cm^{-1} correlates with the H-bonded (-OH) group, which slightly shifted to a higher wavenumber of 3300 cm^{-1} but with lower intensity in the CuNPs solution (Figure 3B). Peaks detected at 1596 cm^{-1} and 1407 cm^{-1} correlate with stretching vibrations of C-C, C=C, or C-O of the aromatic cycles, with mild changes observed in the values of the CuNPs spectrum, yet their intensity was much lower. The peak detected at 1097 cm^{-1} indicates the existence of C-H and C-O stretching of carboxylic acids, alcohols, esters, and ether groups.

SEM and TEM Analysis

As shown in the SEM images (Figure 4A & B), the particles have irregular shapes, although they are approximately spherical. TEM images consolidated our findings about the spherical-like shape and provided more insight into the size (Figure 4C). The TEM scan showed very fine nanoparticles with sizes ranging between 7.59 nm and 16.61 nm. The average size was found to be around 13.22 ± 3.53 nm (Figure 4D).

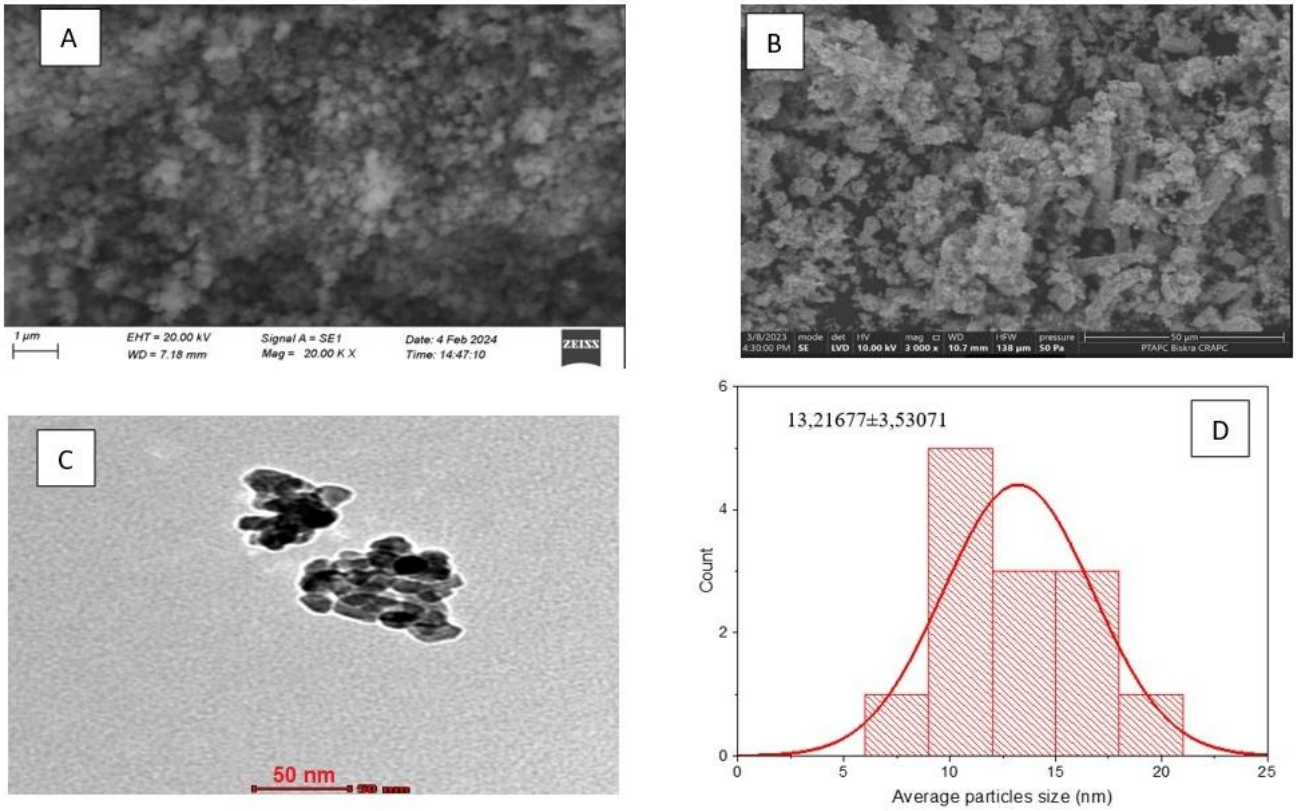

Figure 4. Characterization of Green-synthesized CuNPs Using *P. australis* Leaf Extract by SEM (A and B), TEM (C), and Size Distribution Analysis (D).

X- ray Diffraction Analysis

In Figure 5, major diffraction peaks were observed at 29.71°, 36.49°, 42.44°, 61.48°, and 77.53° corresponding to the following Miller indices 110, 111, 200, 220, 222 (JCPDS 01-077-0199) assigned to Cu_2O. Additionally, another diffraction peak at 50.46° assigned to Miller indices 200, corresponding to Cu (JCPDS 01-070-3038). These peaks align with the cubic crystal system. The sharp peaks of the XRD patterns indicate the crystalline nature of copper NPs. The grain size of the crystallite was calculated using the Scherrer equation, which can be written as: $D = 0.9\lambda/\beta\cos\theta$, where λ is the X-ray wavelength, β is the line broadening at half the maximum intensity (FWHM), and θ is the Bragg angle. The result demonstrates an average size of 18.06 nm.

Antioxidant Activity

In this study, the antioxidant activity of our green CuNPs was estimated using the DPPH and FRAP methods. The results, expressed as IC_{50} values, demonstrate significant scavenging power. However, the IC_{50} level of ascorbic acid, used as a standard, is lower than that of our green synthesized CuNPs (Figure 6). The antioxidant activity assessed using the FRAP assay shows similar results to the DPPH assay, with the IC50 level of vitamin C being lower than that of our green CuNPs.

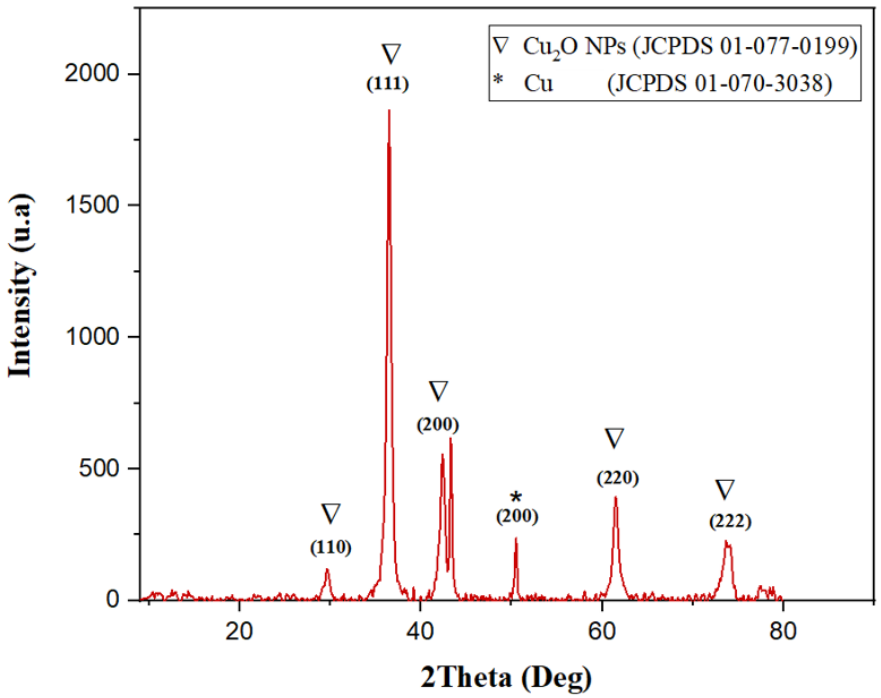

Figure 5. XRD Patterns of Synthesized CuNPs.

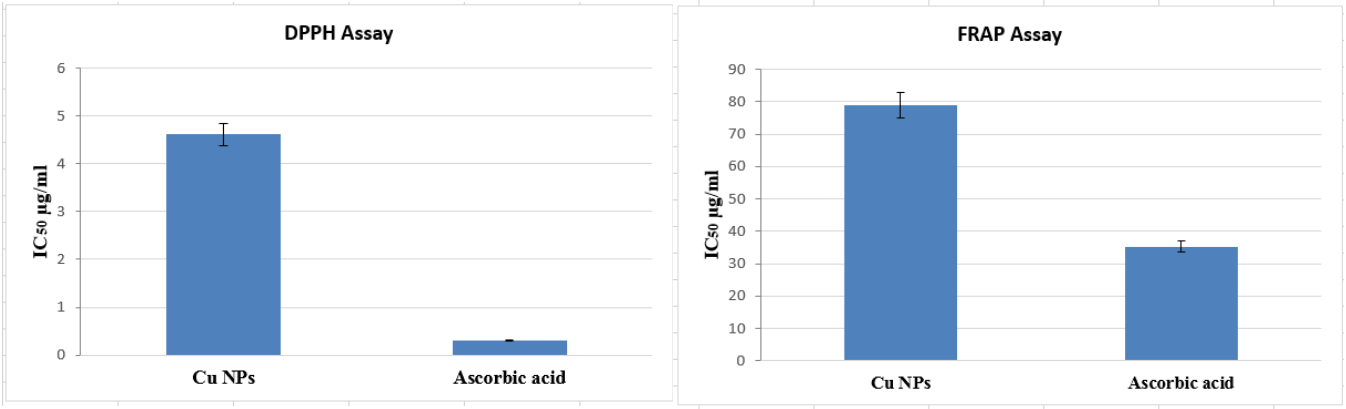

Figure 6. Antioxidant Activity of the Green-synthesized CuNPs as IC_{50} Values Compared to Ascorbic Acid Evaluated by DPPH and FRAP Assays.

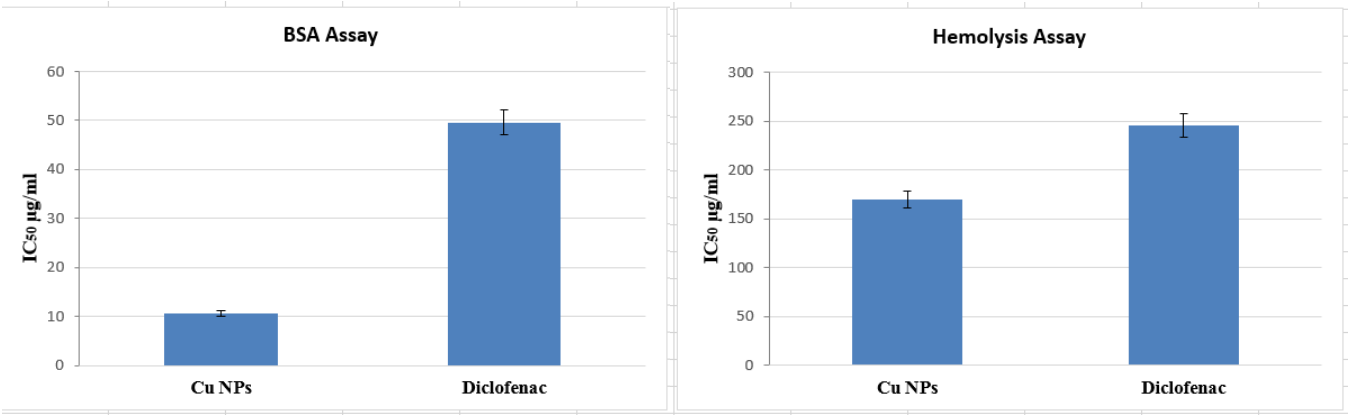

Figure 7. Anti-inflammatory Activity of Green CuNPs as IC_{50} Values Compared to Diclofenac Evaluated by BSA and Hemolysis Assays.

Anti-inflammatory Activity

Anti-inflammatory results, obtained from bovine serum assay and red blood cell protection against hemolysis, are presented as IC_{50} values for synthesized CuNPs and the anti-inflammatory drug "diclofenac", which was used as a positive control. The albumin denaturation inhibition test revealed the significant ability of the green CuNPs to protect proteins from denaturation, which is linked to inflammation. On the other hand, human erythrocytes were used to analyze the anti-hemolytic activity of our green CuNPs, showing their potential to protect red blood cells against hemolysis (Figure 7).

Cytotoxic Activity

The MTT assay is based on the ability of succinate dehydrogenase, a mitochondrial enzyme, to convert tetra zolium yellow dye into formazan crystals, which are purple in color. The rate of formazan crystal formation is directly proportional to cell viability, measured in terms of optical density. As shown in Figure 8A, the cytotoxic effect of green CuNPs on the MCF-7 cell line is dose-dependent. The IC_{50} level, the concentration value that decreases the viability of 50% of cancer cells, is found to be approximately 3 µg/ml. Microscopic observations also showed the anti-proliferative effects of the nanoparticles on MCF-7 cells (Figure 8B).

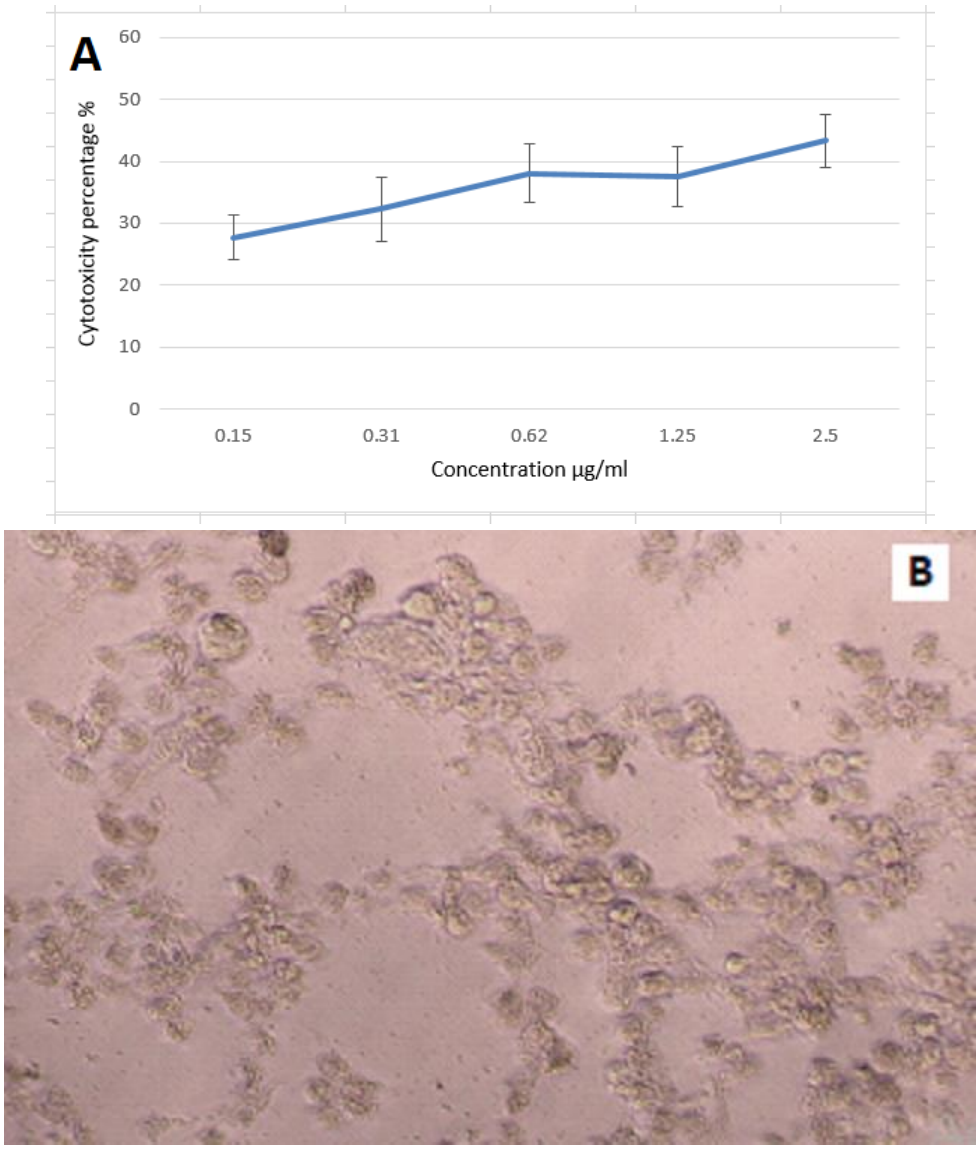

Figure 8. Cytotoxic (A) and Anti-proliferative Effects (B) of CuNPs on Breast Cancer MCF-7 Cells Line.

Discussion

In current study, chemical screening results demonstrate a variety of secondary metabolites, including polyphenols, alkaloids, tannins, terpenoids and carbohydrates. The phenolic compounds are known to be antioxidants that neutralize free radicals, which are triggers of oxidative damage.[14,24,25] Secondary metabolites have played a significant role in preventing and treating a range of diseases, including cancer,

inflammation-related disorders, diabetes, osteoporosis, cardiovascular disease, and neurodegenerative diseases.[26]

The formulation of the plant-based CuNPs was first confirmed by the colour change from blue to greenish-dark, suggesting the biological reaction between the plant's secondary metabolites and copper metal ions. The result is consistent with previous investigations on green synthesis of CuNPs.[18] CuNPs were then characterized using different techniques. UV-Vis spectroscopy enabled the detection of a peak at 312 nm, which is characteristic of CuNPs. Other studies showed peaks at 300 nm[27] and 327 nm.[28] According to Mie's theory, nanoparticle form determines the number of SPR bands, with spherical nanoparticles typically having a single band.[29] FT-IR spectroscopy performed on both plant extract and plant-based formulated CuNPs shows different peaks with different intensities. The previously stated functional groups do exist in the structure of polyphenols. Their lower intensity in green-synthesized CuNPs is proof of their role in reducing copper ions and stabilizing the produced CuNPs. The sharp and intense peak detected at 608 cm^{-1}, as presented in Figure 3B, is attributed to vibrations of CuO validating the formation of copper nanoparticles.[30] XRD analysis results demonstrate an average size of 18.06 nm which is in line with the TEM size finding (Figure 4D). Other studies have found similar results to ours.[30,31]

Regarding the antioxidant activity, both tests revealed significant scavenging power of the green CuNPs. The IC_{50} values of CuNPs and vitamin C as determined by the DPPH test are 4.62 µg/ml and 0.31 µg/ml, respectively. Similarly, the IC_{50} values of CuNPs and vitamin C as determined by the FRAP test are 78.99 µg/ml and 35.24 µg/ml, respectively. It is known that the lower the IC_{50} level, the higher the antioxidant activity. Our findings are consistent with the majority of existing literature.[27,32]

Results of the anti-inflammatory tests displayed the captivating potential of the green CuNPs to protect both red blood cells and protein from haemolysis and denaturation, which was even higher than the standard. The mechanism of protecting erythrocytes from haemolysis may be due to the ability of anti-inflammatory compounds to stabilize the red blood cell membrane and prevent them from releasing their intra-cellular content at the inflammation site. Al-Jubouri et *al.* found similar results; however, the percentage of inhibition Aspirin, used by them as a positive control, showed the highest level compared to CuNPs synthesized using *Myrtus communis* leaf extract.[33]

Regarding the anti-cancer activity of the plant-based formulated CuNPs, Figure 8 shows that the cytotoxic effect on the MCF-7 cell line is dose-dependent. Many other studies have reported the anticancer potential of plant-based synthesized CuNPs against different cancer cell lines. In a study conducted by Shobha et al. the IC_{50} was about 133 µg/ml.[34] In addition, Ramaswamy et *al.* found that brown algae-mediated CuNPs had a significant anticancer effect on the MCF-7 cell line, with cells being inhibited by 93% at a dosage of 100 µg/ml.[35] Studies indicate that nanoparticles should be smaller than 100 nm to pass through endothelial cells. Spherical nanoparticles ranging from 20 to 200 nm are more effective for drug delivery and *in vivo* applications.[36] Antitumor effect of CuNPs against cancer cell lines is likely due to oxidative stress, lipid peroxidation, cellular breakage, membrane functions loss, involvement in some interactions with intracellular macromolecules, DNA fragmentation, and chromosomal aberrations.[37]

Conclusion

The green synthesis of CuNPs using *Phragmites australis* aqueous leaf extract was successful. XRD and TEM results revealed fine-sized copper nanoparticles (13.22 ± 3.53 nm). Green-synthesized CuNPs showed promising anti-inflammatory activity and a prominent anticancer effect at very small doses.

Authors' Contributions

AF design of activity tests and experiments; AF and SD analysed and interpreted the data; JN and SD Supervising and edited the manuscript. The manuscript has been reviewed and approved by all authors.

Conflict of Interest Disclosures

The authors declare that they have no conflicts of interest.

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