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Alpinia officinarum mediated copper oxide nanoparticles: synthesis and its antimicrobial anticancer and antioxidant activity

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ABSTRACT

Objective

Copper nanoparticles (CuNPs) were synthesized from root extracts of Alpinia officinarum and tested for antibacterial as well as cytotoxic effects against HT-29 and MCF-7 cancer cells in this work.

Methods

UV-visible, FT-IR, FESEM, EDX, and DLS spectroscopy were used to analyze the CuNPs that were synthesized from Alpinia officinarum extracts. Several microorganisms, including the bacterial Staphylococcus aureus, the protozoan Pseudomonas aeruginosa, the cyanobacterium Escherichia coli, and the yeast Candida albicans, were tested for their antibiotic properties. We used the MTT test to measure cytotoxicity and DCFH-DA staining to quantify intracellular reactive oxygen molecules (ROS) generation. Also examined were alterations in apoptotic morphological characteristics and mitochondrial membrane potential (MMP).

Results

CuNPs displayed 550- and 400-nm surface plasmon resonance peaks, confirming their synthesis. There was a noticeable antimicrobial effect of the ethanolic root extract, with inhibition zones for bacteria measuring between 1.1 ± 1.3 mm and 2.4 ± 0.7 mm, and for fungi reaching up to 2.4 ± 1.3 mm. At IC50 of 30 µg/ml was observed in the HT-29 cell line, while the cell line MCF-7 exhibited an IC50 in 45 µg/ml. Elevated ROS levels and altered MMP in the treated cells demonstrated improved apoptotic activity. Further evidence of oxidative stress as its destructive mechanism was the marked effect on scavenging enzyme production and lipid peroxidation markers.

Novelty:

The study's originality comes from its eco-friendly and sustainable green production of nanoparticles of copper oxide utilizing Alpinia officinarum is a natural mediator. This approach can improve the therapeutic efficacy of nanoparticles by enhancing their antibacterial, anticancer, and antioxidant capabilities. They show promise as a medicinal agent due to the synergistic effects that may result from combining plant-derived bioactive chemicals.

Keywords: *Alpinia officinarum, antioxidant, antimicrobial, cytotoxic, copper oxide*

1.INTRODUCTION

Plants have been widely accepted as a rich source of bioactive substances capable of treating a wide range of human ailments, including cancer, diabetes, inflammation, and microbial infections. More than 8000 bioactive chemicals, including phenolics and flavonoids, have been found in various plant components such as leaves, roots, stems, flowers, seeds, and fruits. These bioactive chemicals are well-known for their varied biological functions, which benefit human health by providing macro and micronutrients [1].

Plants with high therapeutic characteristics are frequently resistant to microbial infections and demonstrate their power through brilliant colors, tempting scents, and rich flavors. Furthermore, they feature systems for expelling harmful chemicals, emphasizing their therapeutic potential. Combating microbial infections, however, is a considerable issue, particularly given the increasing possibility of multidrug resistance [2].

Plant phytochemicals have an important function in supplying antioxidants. Flavonoids, phenolic acids, saponins, tannins, catechins, and phenolic triterpenes all contribute to antioxidant activity, which is critical for countering the reactive oxygen species (ROS) pathways that cause a variety of diseases. These ROS mechanisms are involved in cell proliferation, death, inflammation, and differentiation, demonstrating the varied roles of phytonutrients in biological processes[3].

Tumor-induced inflammation is an important step in the development of cancer. Phytochemicals have been proven to successfully reduce tumor formation as well as balance tumor tissues by inhibiting cell proliferation, causing apoptosis, and improving anticancer immunity. Breast cancer, among other types of cancer, is a serious threat to the well-being of women, demanding the development of new and successful therapies [4].

In this study, we looked at the antibacterial activities of the ethanol extract of the root of *Alpinia officinarum*. In addition to analyzing its cancer-fighting potential, we investigated its antibacterial and antioxidant properties. Preliminary phytochemical analysis was performed using three different solvents, demonstrating a wide range of phytochemicals found in the ethanolic root extract, such catechins, polysaccharides, saponins and tannins.By investigating the varied qualities of *Alpinia officinarum*, we hope to add to the expanding body of information about medicinal plants' therapeutic potential. Our research aims to find new insights on the mechanisms underlying plant extracts' bioactivity, paving the path for the development of novel therapeutics for a variety of human ailments, including cancer as well as microbial infections.**2. MATERIALS AND METHODS**

2.1. Chemicals and Reagents

Dulbecco's Modified Eagle Medium (DMEM), phosphate-buffered saline (PBS), fetal bovine serum (FBS), antibiotics (penicillin, streptomycin), dimethyl sulfoxide (DMSO), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), 2,7-diacetyl dichlorofluorescein (DCFH-DA), ethidium bromide (EtBr), Rhodamine 123, Acridine orange (AO) stain and other biochemical reagents such as Heparin, reduced nicotinamide adenine dinucleotide (NADPH), Thiobarbituric acid (TBA), 1-chloro-2,4-dinitrobenzene (CDNB), Nitro blue tetrazolium (NBT), reduced glutathione (GSH), Trichloroacetic acid (TCA), tris buffered saline tween-20 (TPST) phenazine methosulphate (PMS), Low melting agarose (LMPA), normal melting agarose (NMPA), Protease inhibitor, Acrylamide, Bisacrylamide, Sodium dodecyl sulfate (SDS), N,N,N',N'-tetramethylethylenediamine (TEMED), Glycine, β-Mercaptoethanol, Ascorbic acid, Potassium ferricyanide, Ferric chloride, Glacial acetic acid, Hydrogen peroxide, $(CuSO4)_2 \cdot 3H_2O$, Whatman filter paper NO:1S) ethanol, were purchased from Himedia laboratories, Mumbai, India.

2.2. Plant material collection

The rhizome of *Alpinia officinarum* (L.) Wild was obtained in the Trichy district.

2.3. Preparing *Alpinia officinarum* Rhizome

Alpinia officinarum rhizomes were properly cleaned and rinsed with tap and distilled water to remove dust particles. They were then dried in the shade for 15 days to minimize the moisture content. The dried rhizomes were pulverized with a grinding machine and placed into brown bottles for storage[5].

2.4. Extraction Process:

Extraction was carried out by placing 20 g of powdered *Alpinia officinarum* rhizomes in a 500 ml beaker with 400 ml of deionized water. To protect the beaker from light, it was covered in aluminium foil. The mixture was then agitated for 90 minutes with a mechanical shaker before being warmed on a magnetic stirrer at 50°C for an hour. After cooling to ambient temperature overnight, the solution was filtered using Whatman No.1 filter paper, yielding a clear solution that was kept at 4°C for future studies[5].

2.4.1. Green Copper Nanoparticle Synthesis

A 1 mM aqueous solution of copper sulfate ($CuSO_4 \cdot 2H_2O$) was stored in brown bottles. Then, drop wise, 100 ml of plant leaf extract was combined with 400 ml of 1 mM copper sulfate solution (1:4 ratio) while stirring continuously. The combination was incubated at the ambient temperature for 24 hours, with the color change measured every 30 and 60 minutes. The change in color from blue to dark green showed the creation of copper nanoparticles. The solution was then centrifuged for 15 minutes in 10,000 rpm, and the Cu NPs supernatant was filtered through What man filter paper No.1 to eliminate contaminants. The nanoparticles were then dried, crushed, and ready for further investigation. The synthesis of CuNPs was confirmed by observing the spectra in UV-Vis spectroscopy using a PerkinElmer (Lambda 750) UV-Vis-NIR spectrophotometer, which range from 300 nm to 800 nm[6].

2.4.2. Characterization of the synthesized sample

The Synthesis of CuNPs was validated by UV-visible spectroscopy, and FT-IR, FE-SEM, XRD, DLS, and zeta potential were used for characterization.

2.5. Antimicrobial Assay

The antimicrobial activity of green generated Cu NPs was assessed against six distinct microorganisms utilizing the agar well diffusion method. Mueller Hinton Broth is used as a nutrient agar media to promote bacterial growth. This agar medium comprises beef extract, peptone, sodium chloride, and yeast. The prepared medium was then autoclaved at 120°C for 20 minutes, put into a sterile petri dish, and allowed to harden in a laminar airflow chamber. After solidification, a sterile cotton swab was used to disseminate fresh bacterial culture throughout the plate using the spread plate technique. Three wells were drilled into the plates using a sterile cork borer of 5 mm in diameter, one for the control and one for the standard antibiotic, Ampicillin (10 µg/mL). The wells were loaded with bacterial cultures, and then the plates had been incubated at 37°C for 24 hours. The plates were then examined to ensure that there was a distinct inhibition zone around each well. The inhibitory zone's diameter was measured in millimetres. Three gram-positive bacterial cultures, *Escherichia coli* (MTCC584), *Pseudomonas aeruginosa* (MTCC1034), and *Staphylococcus aureus* (MTCC9542), as well as fungal cultures *Candida albicans* (ATCC20), Mucor (ITCC20), and *Aspergillus niger* (ATCC902), were used as test microbes in this study[7].

2.6. Cell Culture and Drug Treatment

HT-29 and MCF-7 cell lines were obtained from the National Centre for Cell Science (NCCS) in Pune, India. Cells were cultivated in 75 cm^2 tissue culture flasks at 37ºC in a humidified, 5% CO_2 atmosphere using Minimum essential medium (Eagle) supplemented with 10% Fetal bovine serum (FBS), 1% glutamine, and 100 u/ml penicillin-streptomycin[8].

2.6.1 Cell Proliferation Assay

Following the protocol outlined by Rama doss H et al; 2021, the effect of Alpinia officinarum CuNPs on the

proliferation of HT-29 and MCF-7 cells was evaluated using an MTT test, which measures the activity of mitochondrial dehydrogenase in healthy cells. A total of 100 ml of DMEM was added to 96-well plates containing HT-29 and MCF-7 cells, and the cells were cultured for up to 24 hours. The cell density was 5X103 cells per well. The cells were treated with different amounts of *Alpinia officinarum* CuNPs. After 24 hours, the cells had been treated with 100 ml of MTT solution (1 mg/ml) at 37°C for 2 hours. Read well touch, ELISA plate reader (Robotic, India) was used to remove the MTT solution and add 100 ml of DMSO to dissolve the crystals of form azan. The plate was then read at 570 nm[8].

2.6.2. Evaluation of intracellular reactive oxygen species production

To measure ROS within cells, researchers utilized DCFH-DA, a non-fluorescent assay that can easily cross cell membranes and convert to the fluorescent dye dichlorofluorescein when exposed to reactive oxygen species (ROS). According to Oves M*et al*, the amount of ROS created is directly correlated with the intensity of fluorescence. The cells were seeded into a 6-well plate with $1X10^6$ cells/well and then treated with different concentrations of *Alpinia officinarum* CuNPs. After 24 hours, they were placed in a CO_2 incubator. Following a 24-hour incubation period, 100 ml DCFH-DA was added to 1 ml of cells and incubated for 10 minutes at 37°C. The Suzuki RF-5301 PC spectrofluorometer was used to measure the intensity of the fluorescent signals with an excitation filter set at 485 + 10 nm and an emission filter set at 530 + 12.5 nm. The researchers measured the effects by looking at the percentage raise in fluorescence intensity[8].

2.6.3. Determination of the mitochondrial membrane potential

Johnson employed the lipophilic cationic dye Rhodamine-123 (Rh-123) to measure mitochondrial membrane potential (MMP)[8]. In a 6-well plate with 1X106 cells/well, cells were treated with Alpinia officinarum CuNPs. Rh-123 dye was added to the cells for 30 minutes following the 24-hour treatment. For the qualitative evaluation of the MMP, an in vitro cell imaging station (Invitrogen, USA) was utilized. Cells had been trypsinized and their fluorescence intensity was evaluated at 485/530 nm with a Spectrofluorometer (Schimadzu, USA). The graphical findings were compared using a positive control.

2.6.4. Assessing apoptotic morphological alterations

The presence of apoptotic cells was identified by staining with ethidium bromide (EtBr) and acridine orange (AO)[9]. For 24 hours, cells were cultured on a 6-well plate with $3X10^4$ cells/well after being exposed to different doses of CuNPs from *Alpinia officinarum*. The cells were then fixed for 30 minutes at 4°C in a 3:1 combination of methanol plus glacial acetic acid. The cells were washed with PBS before being stained with AO/EtBr in a 1:1 ratio for thirty minutes at 37°C. After rinsing with PBS, the stained cells were examined with a fluid cell imaging apparatus (Invitrogen, USA). The percentage of cells in the field displaying apoptotic features was determined by multiplying the entire number of cells by this percentage.

2.6.5. Biochemical assays

***Alpinia officinarum* CuNPs** treated cells were harvested, and the cell suspension was used for biochemical estimations. LPO (lipid peroxide) byproduct of TBARS reactive substance (Thiobarbituric acid reactive substance), superoxide dismutase (SOD), catalase (CAT), and measurement of the intracellular enzyme of reduced glutathione (GSH).glutathione peroxidase (GPx), Glutathione reductase (GR), and Glutathione-S-Transferase (GST) levels were determined[10,11].

2.13. Data analysis with statistics

Statistical analysis was performed on the collected data and the results were presented as means, standard deviations, and correlation coefficients. This analysis was carried out using IBM® SPSS® Statistics, 2017. We used GraphPad Prism 8.4.1 (GraphPad Software, San Diego, CA, www.graphpad.com) to conduct a two-way ANOVA on the IC50 data, which is presented as the mean ± SD (n = 3). The interaction was deemed significant with a p-value of less than 0.05.

3. RESULT AND DISCUSSION

The production of CuNPs is accomplished by a variety of biological processes. Since bacterial and fungal-mediated synthesis cannot be commercialized and necessitates sterile conditions, plant extracts were preferable for synthesis[12]. As part of this investigation, CuNPs were produced from *Alpinia officinarium*root extracts, and their efficacy against microbes was evaluated. FT-IR, FESEM, EDX, DLS, UV-visible spectra, and CuNps were used for characterization as well as zeta potential. Surface plasmon resonance peak at 550 nm and 400 nm were observed during the UV-visible spectroscopic characterization of CuNPs produced from Alpinia officinarum. (Figure 1A), proving the production of CuNPs. According to earlier research, CuNPs exhibit surface resonate with plasmons in the 350–800 nm spectrum. However, it has been noted that Cu nanoparticles exhibit absorption bands between 550 and 650 nm [13]. The presence of copper nanoparticles was confirmed by the observation of a single surface plasmon resonance at 580 nm after reduction in the colloidal suspension [13]. The distinctive characteristics of metal nanoparticles are due to surface plasmon resonance and are affected by variables such nanoparticle size, shape, and interparticle distance [14,15].Electron resonance bands on surfaces are impacted by various attributes, such as the synthesis solvent and particle size and geometry, but the bandwidth grows as the nanoparticle size decreases[16].

Figure 1 displays the CuNPs' FTIR spectra. Peaks in the 3700-3100 cm^{-1} wavenumber range indicated O-H stretching modes in both water and CuNPs.The C-H stretching vibrations were responsible for the peaks observed between 3000 and 2800 cm-1 was reported [17].In addition, the carbonyl group (C½O), stretching C½C, C-OH stretching vibrations, and C-O stretching were demonstrated by peak values at 1643, 1561, 1277, and 1103 cm1, respectively[15,18,19]. These peaks indicated that a sample of *E. caucasicumTrautv* extract contained phenolic acids and flavonoids. It is possible that the presence of flavonoids as well as polyphenol functional groups in the extract is responsible for the reduction of metal ions and the creation of nanoparticles[20]. Nanoparticles have the potential to adsorb functional groups such as flavonoids and phenolics. According to Nasrollahzadeh et al. (2015), this interaction is likely accomplished when no other significant ligating agents are present[15].

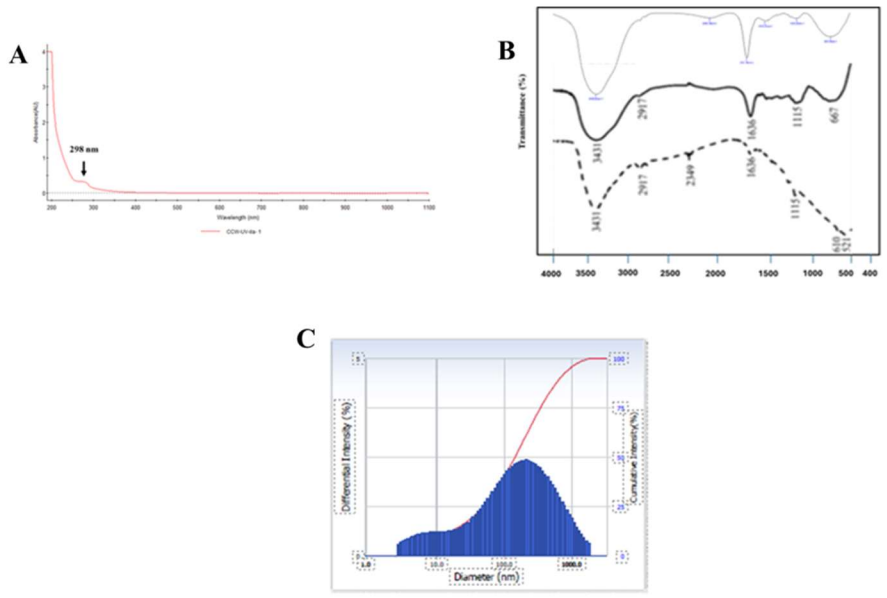


Figure 1. (A) UV wavelength spectroscopy of CuNPs and *Alpinia officinarum*root extracts; (B) Fourier transform infrared spectroscopy of *Alpinia officinarum* extracts; (C) Dynamic light scattering analysis with particle size distribution

The prepared various *Alpinia officinarum* CuNPs possess Figure2 shows the morphological structure of Rhizome*Alpinia officinarum extract* and *Alpinia officinarum*-CuNPs by SEM elucidated *Alpinia officinarum*-CuNPs had smooth surface and the solid inclusion complex with the nano hydrophobic cavity of CuNPs. Copper nanoparticles exhibiting almost spherical forms and average dimensions below 40 nm were visible in the scanning electron micrograph. Nanoparticles' size determines their characteristics and bioactivity[21]. The size of the nanoparticles that are formed can be influenced by process parameters, variations in plant species, and the concentration and fluctuation of the reducing substances in the extracts[22].

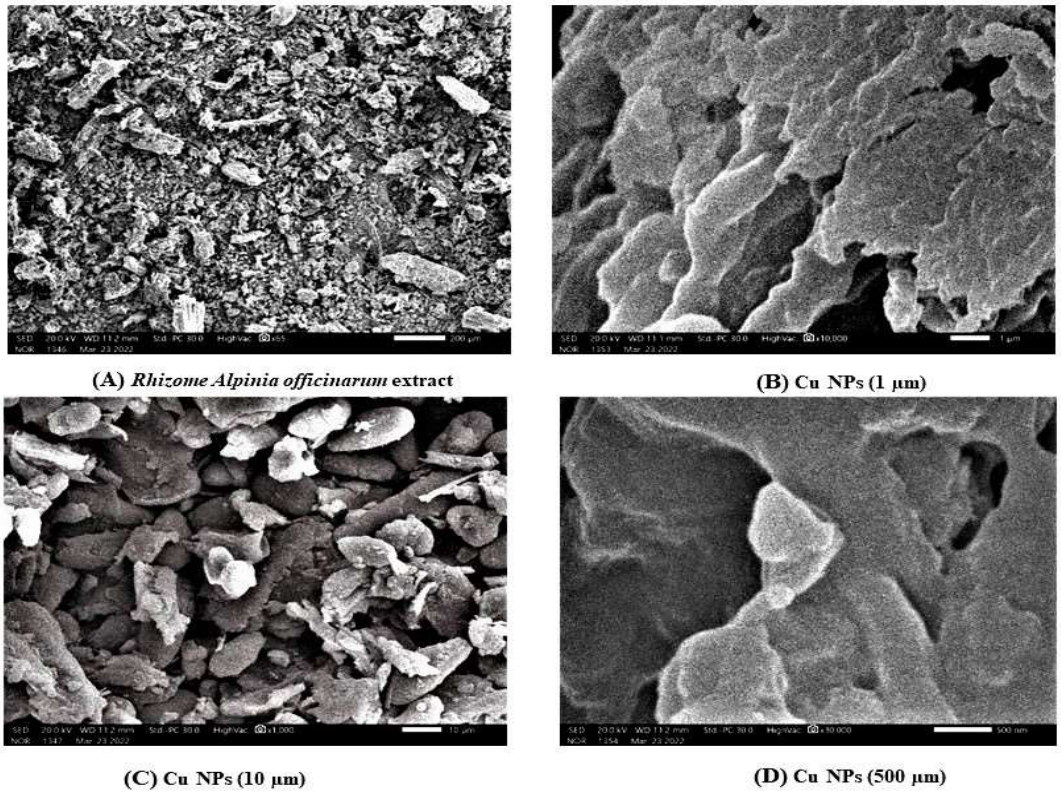


Figure 2. Morphological structure of (A) ***Rhizome*** from ***Alpinia officinarum extract*** and(B), (C),(D), ***Alpinia officinarum*** **Cu** nanoparticles

3.1. Antimicrobial activity of *A. officinarum* ethanolic root extract

Herbal ailments consider one of the crucial fields of traditional medicine in India especially in rural areas. Thus, chemotherapy is used by a large scale of Indian population for the curing of human disease. To support the appropriate use of plant medicine and to validate their effective sources of new finding, as well as it is necessary to study medicinal plants, which have ancient character in a more strengthen way[23]. Antimicrobials of plant origin have enormous therapeutic potential. Herbal medicines are contributing effective in the curing of infections and disease, but the synthetic drugs may cause side effect and responsible for the microbial resistant[24]. The *A. officinarum* ethanolic root extract have significant antimicrobial activity against *Escherichia coli*, *Pseudomonas aeruginosa* and *Staphylococcus aureus* shows 2.1 ± 1.2 (mm), 1.1 ± 1.3 (mm) and 2.4 ± 0.7 (mm) at 5mg/mL concentration range were shown in figure 3 and table 1. Fungal strains Mucor and *Candida albicans* possess 2.4 ± 1.3 (mm) and 1.6 ± 1.1 (mm) zone of inhibition in the 5mg/mL concentration, respectively. But commercial antibiotic disc ampicillin did not affect against both bacterial and fungal cultures. Crude extract contains effective antimicrobial activity because of its synergetic activity of mixed compounds.

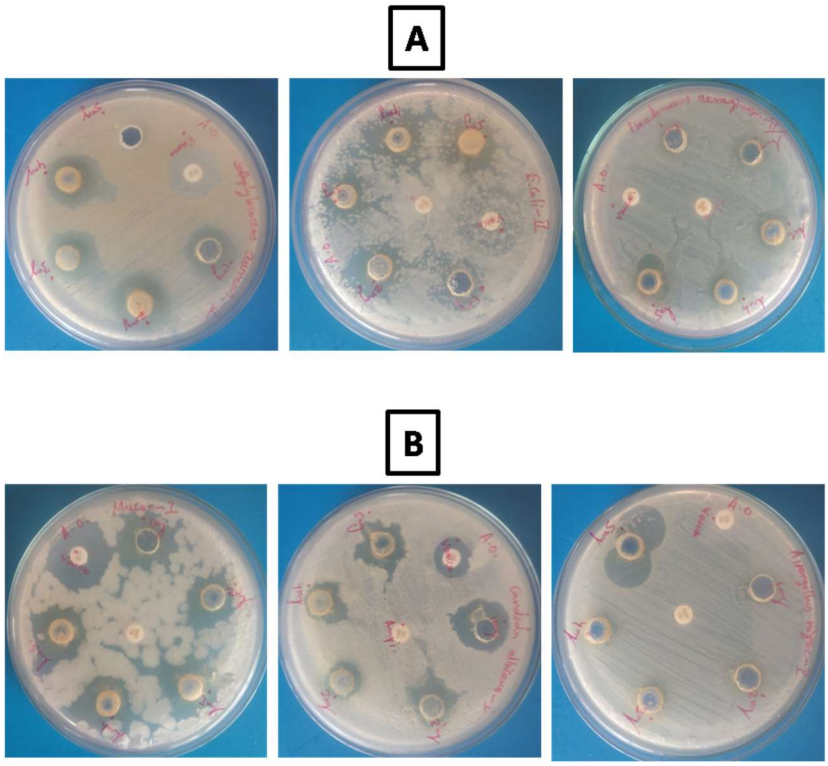


Figure 3. Zone of inhibition was obtained both bacterial and fungal strains comparison commercial of antibiotic disc.

Table 1. Antimicrobial activity of *A. officinarum* ethanolic root extract

Antibiotic	zones are represented as radius (mm)						
	Conc. of the root extract	***Escherichia coli***	***Pseudomonas aeruginosa***	***Staphylococcus aureus***	***Mucor***	***Aspergillus niger***	***Candida albicans***

(µg/disc)	(mg/mL)						
-	1	1.2 ± 2.1	0.3 ± 1.1	1.5 ± 1.2	1.8 ± 1.0	-	1.1 ± 1.8
-	2	1.5 ± 0.5	0.5 ± 1.6	1.8 ± 0.9	1.9 ± 1.4	-	1.3 ± 0.8
-	3	1.6 ± 0.4	0.8 ± 0.8	1.9 ± 2.0	2.1 ± 2.4	-	1.2 ± 0.6
-	4	1.8 ± 1.1	0.9 ± 0.6	2.2 ± 1.5	2.2 ± 0.2	-	1.4 ± 1.0
-	5	2.1 ± 1.2	1.1 ± 1.3	2.4 ± 0.7	2.4 ± 1.3	-	1.6 ± 1.1
Ampicillin (10 µg)	-	-	-	-	-	-	-

According to Khan, Shahid, et al. 2018, nanoparticles' antibacterial characteristics are influenced by their size. Smaller particles have a larger surface area, which means they interact with bacteria more effectively. As a result, their antibacterial properties are enhanced. Nanoparticles with a small size have more antibacterial activity because their surface ions release more metal ions[25]. The antibacterial characteristics of the synthesized CuNPs, which in this study might be because of their tiny size. While the exact processes by which metallic nanoparticles exert their antimicrobial effects remain a mystery, three distinct pathways have been identified in the literature: 1) When metal nanoparticles build up and then dissolve in bacterial membranes, it alters permeability, which in turn releases lipopolysaccharides, proteins, along with intracellular biomolecules, and it weakens the plasma membrane's proton motive force. oxidative stress on cell structures due to the generation of reactive oxygen compounds (2). (3) The process whereby nanoparticle-derived metallic ions are absorbed by cells, resulting in decreased intracellular ATP levels and an impairment in the replication of DNA [13,15,18].

Tables 1 further demonstrated that CuNPs had a stronger antibacterial impact on gram-negative bacteria than gram-positive bacteria. Hasheminya et al. 2018 found results that agreed with these findings[26]. Because of structural differences in their cells, gram-positive as well as gram-negative bacteria do not respond similarly to nanoparticles (Khan, Kanwal, et al. 2018). The intricate cell wall structure and thick coating of peptidoglycan make it very difficult for nanoparticles to penetrate gram-positive bacteria. In contrast, the outer membrane of gram-negative bacteria is negatively charged and covers a thin layer of peptidoglycan. According to Hasheminya et al. 2018, gram-negative bacteria exhibit increased antibiotic activity due to this structure's tendency to draw in more Cu^{+2} ions[26]. Beyond the mentioned factors, the antibacterial activities of the copper nanoparticles may be influenced by flavonoids and phenolic chemicals that have been adsorbed onto their surface. However, according to Tables 1, the ethanolic extract exhibited greater antibacterial effects against gram-positive bacteria than gram-negative bacteria using both techniques. The decrease in sensitivity in gram-negative microorganisms is caused by the existence of the outer membrane, which encases the cell wall and prevents hydrophobic substances from diffusing into the lipid layer of the cell membrane[17].

3.2. Effect of Copper nanoparticles (CuNPs) from *Alpinia officinarum*on cell cytotoxicity in HT-29 and MCF-7 cells

Figure 4. Shows the cytotoxic effect of *Alpinia officinarum* CuNPs on HT-29 and MCF-7 cells by MTT assay. Cells were treated with different concentrations of *Alpinia officinarum* CuNPs (10-100 µg/ml) for 24 h

incubation, which revealed a time-dependent inhibition of cell proliferation.

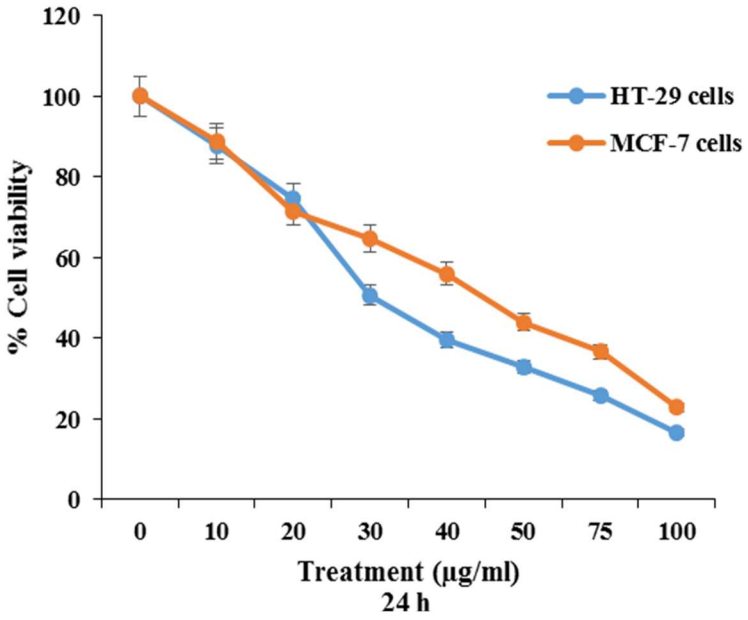
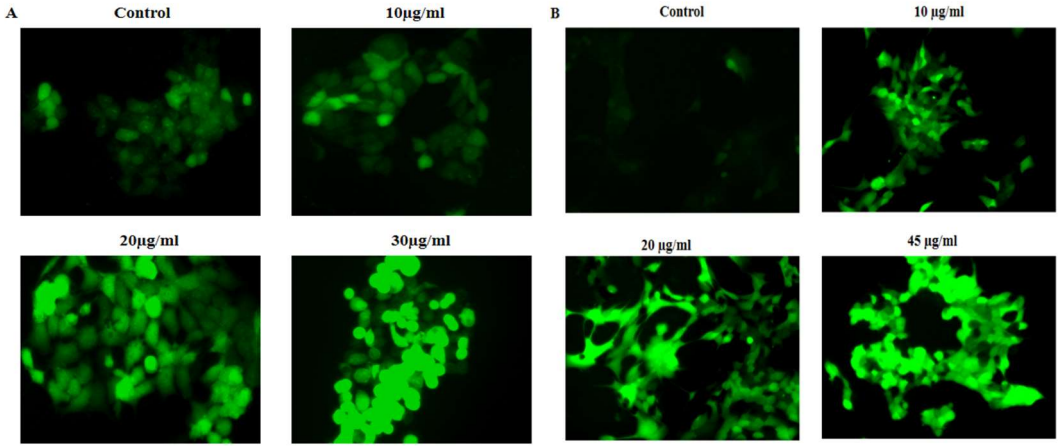


Figure 4. Represents the results of the cytotoxic effect of *Alpinia officinarum* CuNPs in HT-29 and MCF-7 cells.

The inhibitory concentration (IC_{50}) of HT-29cells observed was 30 µg/ml, and 45 µg/ml for MCF-7 cells (24 h). Maximum cell death was observed at 100 µM concentrations.The statistical analysis was carried out by using one-way ANOVA. Values are represented as the Mean ± SD of three experiments. $p < 0.05$ was significantly different from the control sample.

3.3. Effect of Copper nanoparticles (CuNPs) from *Alpinia officinarum*on generation of intracellular ROS in HT-29 and MCF-7 cells.

The intracellular ROS generation was measured by DCFH-DA staining. Fig.1.6. indicates the levels of ROS generation in control and *Alpinia officinarum* CuNPstreated (10, 20, 30 µg/ml- HT-29, and 10, 30, 45 µg/ml- MCF-7) cells. HT-29 and MCF-7 cells were treated with *Alpinia officinarum* CuNPs (IC_{50}) shows significantly increased levels of ROS generation at a different time point which was evidenced by extreme green fluorescence intensity as compared to untreated control cells.



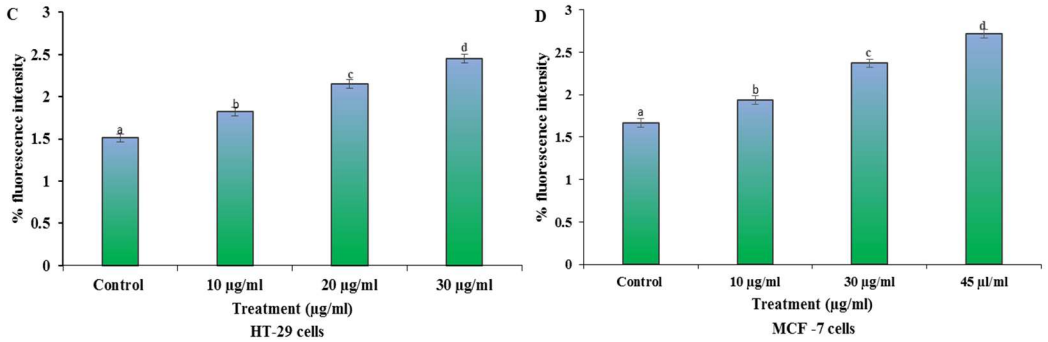
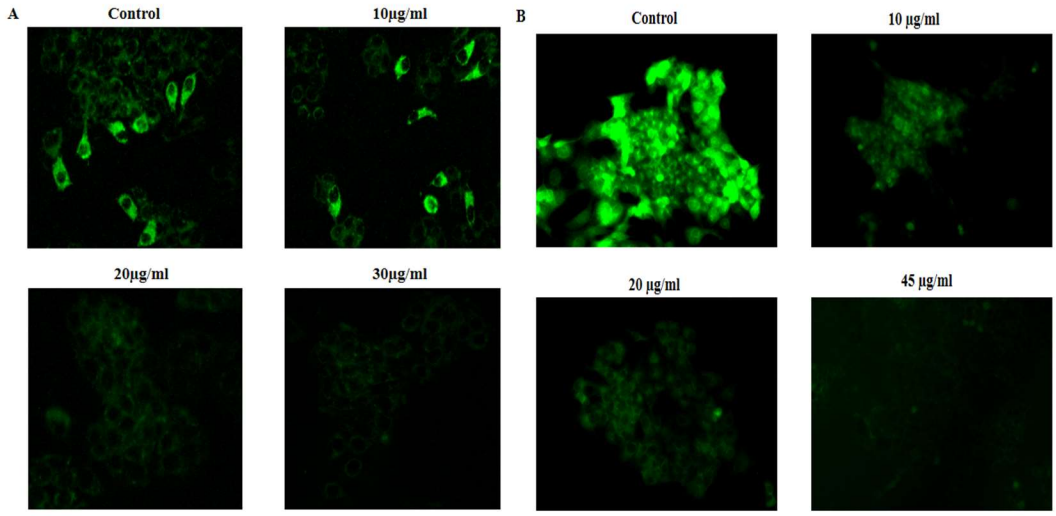


Figure 5. The effect of ***Alpinia officinarum*** **CuNPs** on intracellular ROS generation was evaluated with HT-29 (A) and MCF-7(B) cells by using DCFH-DA staining (40X). The photo micrographic image shows control and treated cells. Untreated control of HT-29 and MCF-7 cells show weak fluorescence with DCF. ***Alpinia officinarum*** **CuNPs** (24 h) treated cell shows increased ROS generation, indicating deep DCF fluorescence intensity. The diagrammatic graph (C&D) shows the percentage of ROS that was detected by the spectrofluorometer. All experiments were performed in triplicate and all the values were expressed as Mean ± SD. Statistical significance was determined by a one-way ANOVA followed by DMRT. Asterisks indicate significant different from control ($p < 0.05$).

3.4. Effect of Copper nanoparticles (CuNPs) from *Alpinia officinarum*on Mitochondrial membrane potential (MMP) in HT-29 and MCF-7 cells.

Apoptosis was initiated in mitochondria by the altered mitochondrial membrane potential, which was assessed by lipophilic cationic dye - Rhodamine-123. The control cells emit high intensity of green fluorescence indicating a polarized mitochondrial membrane. Conversely, HT-29 and MCF-7 cells were treated with *Alpinia officinarum* CuNPs for 24 hr. (10, 20, 30 µg/ml- HT-29, and 10, 30, 45 µg/ml- MCF-7), showed significant alteration in mitochondrial membrane potential causing diminished green fluorescence in Figure 6.



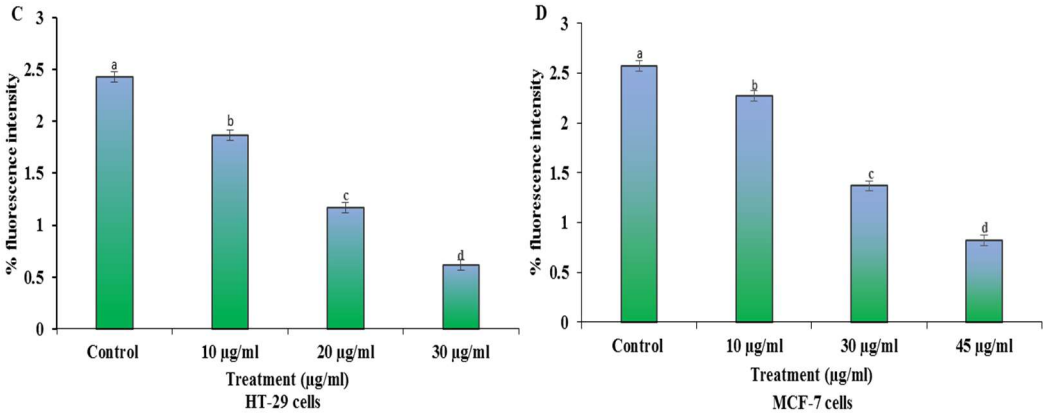


Figure 6. The effect of *Alpinia officinarum* CuNPs on mitochondrial membrane potential *(ΔΨM)* was evaluated with HT-29 (A) and MCF-7(B) cells by Rh-123 staining.

Untreated cells show high fluorescence indicating a polarized mitochondrial membrane. *Alpinia officinarum* CuNPs for 24 hr treated (10, 20, 30 µg/ml- HT-29, and 10, 30, 45 µg/ml- MCF-7) cell shows decreased fluorescence intensity indicating depolarized mitochondrial matrix (A&B). The diagrammatic graph shows fluorescence intensity detected by a spectrofluorometer (C&D). All experiments were performed in triplicate and the values were expressed as Mean ± SD. Statistical significance was determined by a one-way ANOVA followed by DMRT. Asterisks indicate significant different from control ($p < 0.05$).Mitochondria, the cell's powerhouse, is involved in energy production, apoptosis regulation, reactive oxygen species production from oxygen, and calcium balance[27]. Because the roles of mitochondria in normal and malignant cells can be distinguished, mitochondria have become a target for anticancer medicines [28]. The treatment of HT-29 and MCF-7 cancer cells with *Alpinia officinarum* CuNPs resulted in a decrease mitochondrial membrane potential in our study. The dye (Rhodamine 123) accumulated and aggregated within the mitochondria of non-apoptotic live cancer cells, resulting in intense green fluorescence, whereas apoptotic cells showed faint staining, highlighting *Alpinia officinarum* CuNPs anticancer properties. When HT-29 and MCF-7 cancer cells were treated with *Alpinia officinarum* CuNPs, the concentration of Rhodamine 123 decreased, indicating a breakdown in the mitochondrial membrane potential, indicating enhanced ROS generation. ROS was found to be involved in apoptotic signaling by causing mitochondrial depolarization and an increase in the amounts of intracellular pro-apoptotic molecules[29,30].

CuNPs from *Alpinia officinarum* further induced cytotoxicity in cancer cells at a specific IC50. According to several studies, mitochondria are a popular target for anticancer drugs since they are involved in cellular metabolism and function, as well as inducing cytotoxic effects. Throughout a 24-hour incubation period, CuNPs from Alpinia officinarum generated a substantial number of apoptotic bodies in HT-29 and MCF-7 cells. In HT-29 and MCF-7 cancer cells, changes in mitochondrial membrane potential indicate *Alpinia officinarum*CuNP's role in mitochondrial-mediated apoptosis. In MCF-7 cells, Alpinia officinarum reduced mitochondrial membrane potential (MMP) and increased apoptotic capacity, both of which were attributable to the formation of reactive oxygen species (ROS)[32].

3.5. Effect of Copper nanoparticles (CuNPs) from *Alpinia officinarum*on apoptotic morphological changes in HT-29 and MCF-7 cells.

A microscopic image shows the characteristic features of apoptotic morphological changes on Alpinia *officinarum* CuNPs for 24 hr treated cells (10, 20, 30 µg/ml- HT-29, and 10, 30, 45 µg/ml- MCF-7) stained with EtBr/AO. Ethidium bromide a red fluorescence dye has selectively penetrated condensed nuclei of apoptotic cells, while the acridine orange (green) had only taken up healthy cells. In our results, untreated control cells had a highly green fluorescence nucleus which indicates the presence of live cells. *Alpinia officinarum* CuNPs

(IC_{50}) treated cells showed early apoptotic cells-stained orange in color and red-stained fragmented nuclei indicating late apoptosis at different time points i.e 24 h was represented in Figure 7.

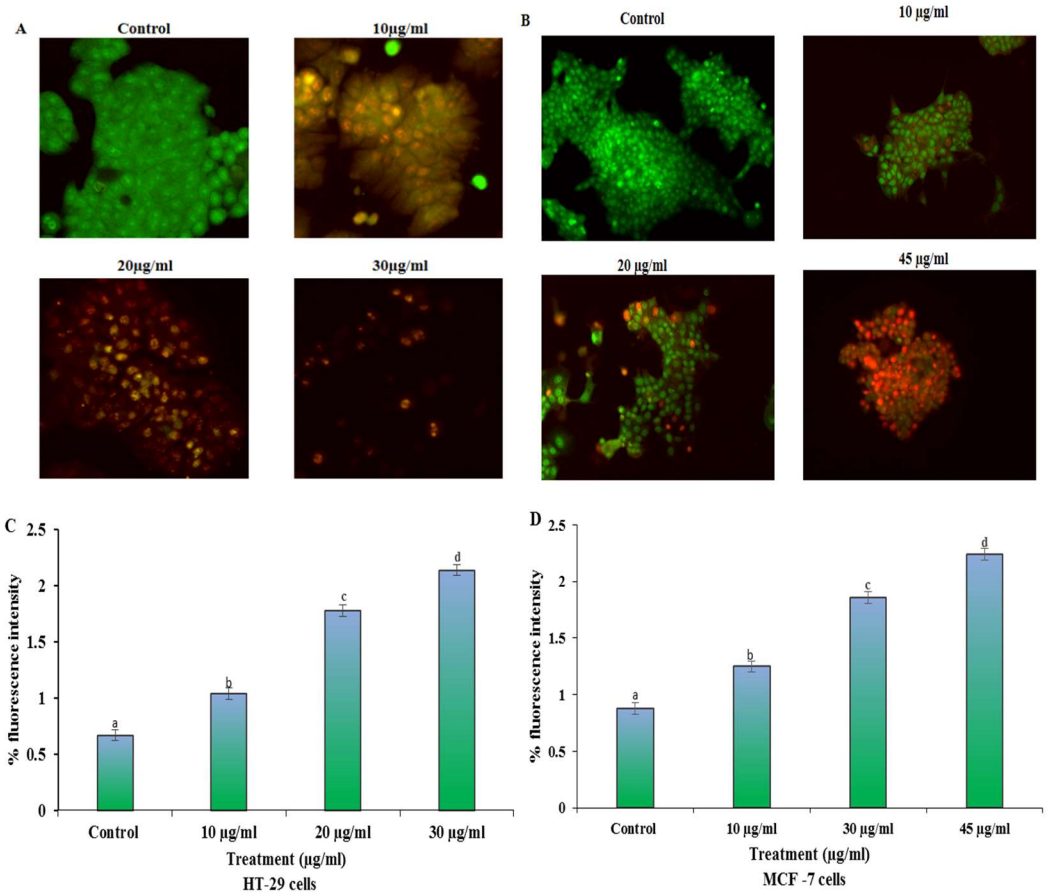


Figure 7. The effects of *Alpinia officinarum* CuNPs on morphological changes in the HT-29 (A) and MCF-7(B) cells were observed on dual staining with AO/EtBr in 24 treatments.

The percentage of apoptotic cells was significantly increased in comparison with the control (A&B). The values are expressed as Mean ± SD from the three independent experiments. p <0.05. The diagrammatic graph shows fluorescence intensity detected by a spectrofluorometer (C&D). All experiments were performed in triplicate and the values were expressed as Mean ± SD. Statistical significance was determined by a one-way ANOVA followed by DMRT. Asterisks indicate significant different from control ($p < 0.05$).

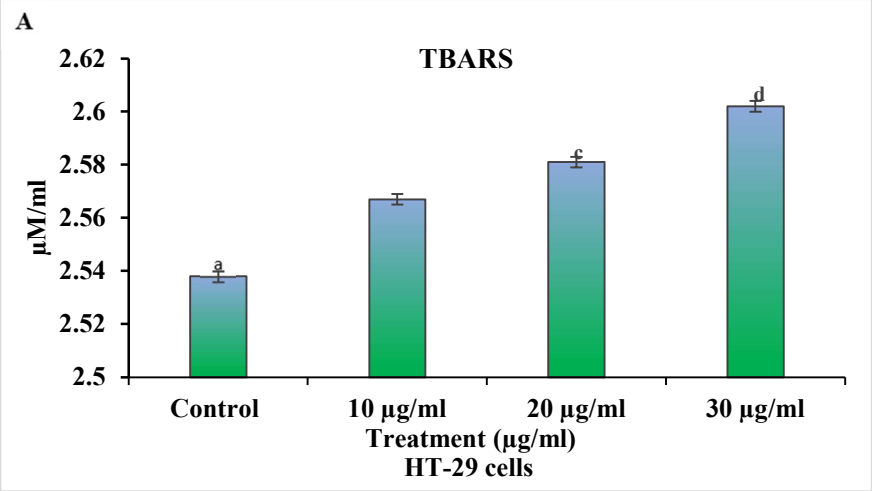
The cell's inherent process for programmed cell death is apoptosis. It is especially important in long-lived mammals [37] because it is involved in both development and homeostasis (Hassan M; 2014). It is a carefully regulated process that seeks to eradicate any excess or undesirable cells. The apoptotic pathway can be triggered by a variety of factors, including DNA damage or excessive growth (Lopez J; 2015). Intracellular and extracellular signals both trigger the apoptotic process. Apoptosis dysregulation is a sign of a wide range of disorders. Infertility, immunodeficiency, and acute and chronic degenerative illnesses all have accelerated apoptosis, while cancer and autoimmunity have delayed or inhibited apoptosis (Hassan M; 2014). Apoptosis screening of medicinal plants and their derivatives has become a popular method in anticancer medication development. (Pfeffer CM and Singh AT; 2018).

Alpinia officinarum CuNPson apoptosis induction in HT-29 and MCF-7 cells were analyzed and it was

able to induce apoptosis in both the cell line studied. Apoptosis removes DNA damage caused by stress stimuli, which helps to prevent carcinogenesis (Halazonetis et al; 2008; Negrini et al; 2010) and maintain genomic integrity (Fulda; 2010). Apoptosis dysregulation promotes tumour growth and therapeutic resistance due to its altered function. As a result, apoptosis is being investigated as a potential therapeutic target in cancer research, and drugs that specifically induce apoptosis in tumour cells could be an ideal anticancer medication. Apoptotic cells' nuclei undergo morphological changes such as chromatin condensation, nuclear fragmentation, plasma membrane bulging, and the formation of many vesicles known as apoptotic bodies (Martins et al; 2008). Many plant-derived chemotherapeutic drugs can cause cell death and generate apoptotic bodies (Wink; 2007). *Alpinia officinarum* CuNPs treatment on HT-29 and MCF-7 cells revealed chromatin condensation and apoptotic bodies at two separate time points using AO/EtBr staining (24h).

3.6. Copper nanoparticles (CuNPs) from Alpinia officinarum and antioxidant activity

The results of lipid-peroxidation status in control and ***Alpinia officinarum* CuNPs** treated HT-29 and MCF-7 cells were shown in Figure 8 (A&B). The activity of TBARS conjugated dienes, and lipid hydroperoxide levels were found to be increased in ***Alpinia officinarum* CuNPs** treated HT-29 and MCF-7 cells when compared to untreated cells. Likewise, the results of antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), and reduced glutathione (GSH) in ***Alpinia officinarum* CuNPs** treated and untreated control HT-29 and MCF-7 cells showed significant changes. There was a reduction in the level of antioxidants in both the cells when studied with IC_{50} concentration of ***Alpinia officinarum* CuNPs** for 24 hr (Figure 4 and 5)



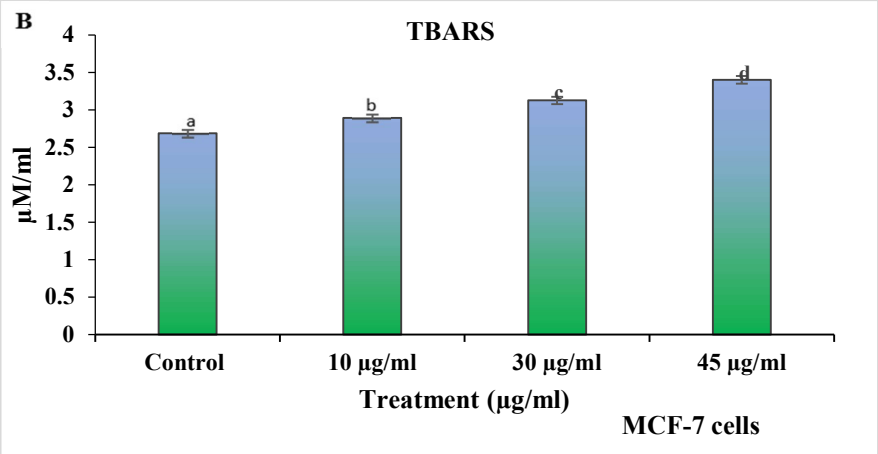
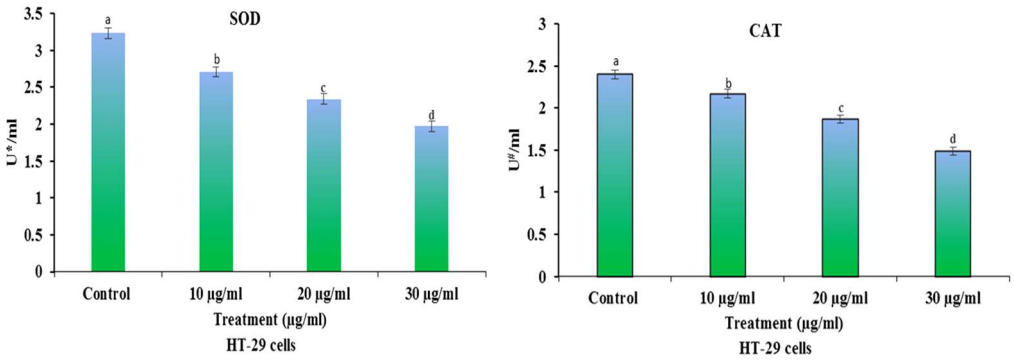


Figure 8A. Effect of Copper nanoparticles (CuNPs) from *Alpinia officinarum* induced lipid peroxidation in HT-29 and MCF-7 cells. Bars represent the changes in the levels of lipid per oxidative markers (TBARS) in HT-29 and MCF-7 cells. Values are given as means S.D. of six experiments in each group. Values not sharing a common marking (a, b, c, . . .) differ significantly at P <0.05 Vs control (DMRT).

Increased lipid peroxidation (TBARS), and decreased antioxidant enzyme activities are all caused by excessive ROS (Pajovic and Saicic; 2008). Alkaloids reduce antioxidant enzymes, resulting in a buildup of superoxide radicals and H_2O_2 in the mitochondria of apoptotic cells (Erguder IB et al; 2005). In this study, Alpinia officinarum CuNPs therapy on HT-29 and MCF-7 cancer cells resulted in decreased antioxidant enzymes and elevated TBARS when compared to untreated control cells. The findings support the hypothesis that *Alpinia officinarum* CuNPs can operate as a potent activator of ROS generation. HT-29 cells treated with *Ficushispida Linn* yielded similar results (Sathiyamoorthy J et al; 2018). Thus, ***Alpinia officinarum*** **CuNPs** exert a beneficial action in cancer cells and its effects could be associated with the inhibition of cell proliferation, induction of tumor cell death, and alterations in the levels of oxidative stress markers.



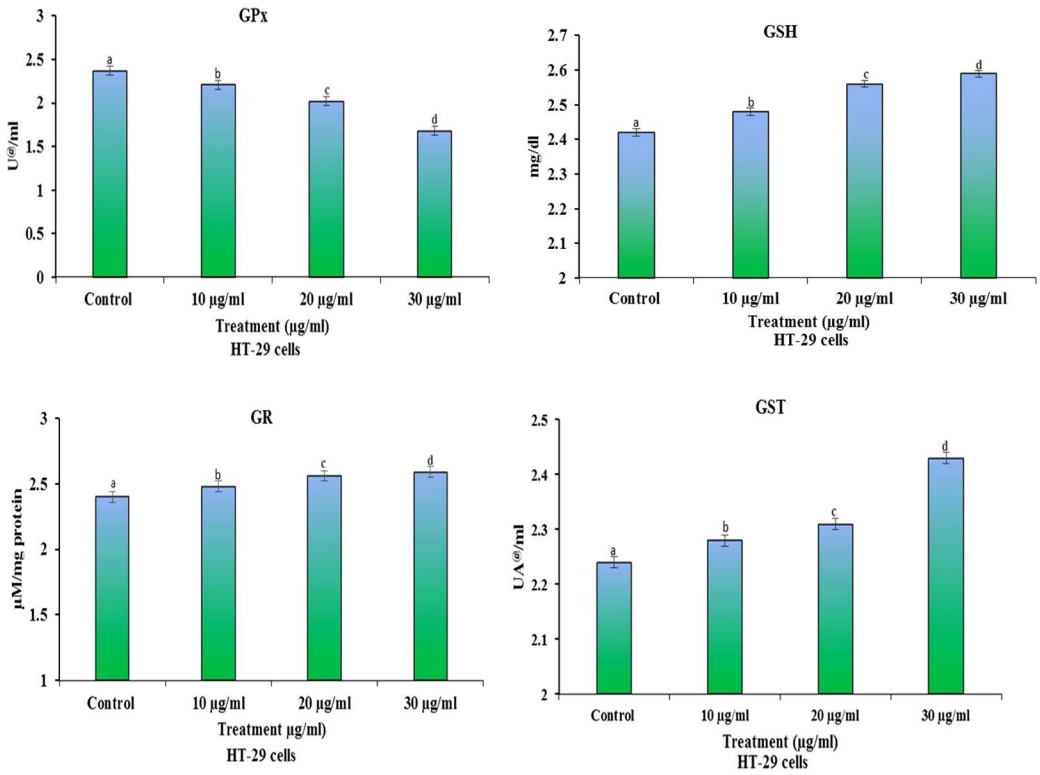
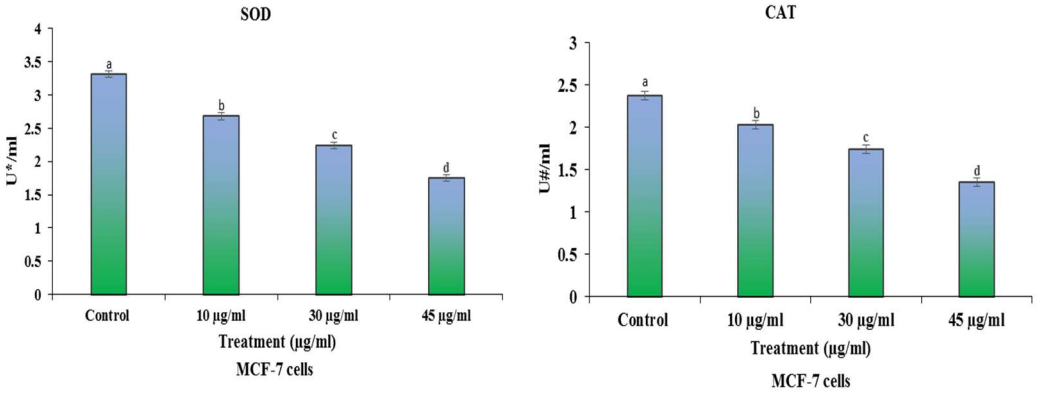


Figure 8B. Effect of Copper nanoparticles (CuNPs) from *Alpinia officinarum* on the cellular antioxidant status on HT-29 cells. Bars represent the changes in the activities of SOD, CAT, GPxGSH, GR, and GST in Alpinia ***officinarum*** **CuNPs**treated HT-29 cells compared to control cells. Values are given as the mean S.D. of six experiments in each group. Bars not sharing the common superscripts differ significantly at $P < 0.05$ vs. control (DMRT).



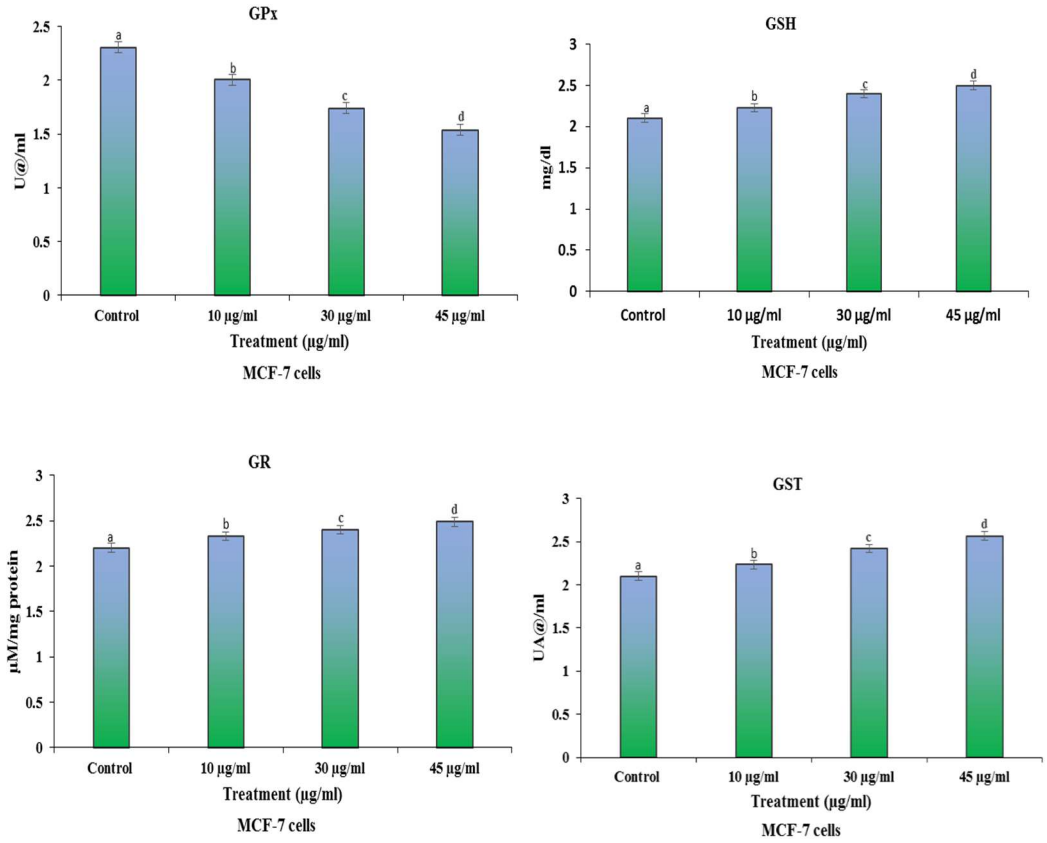


Figure 9. Effect of Copper nanoparticles (CuNPs) from *Alpinia officinarum* on the cellular antioxidant status on MCF-7 cells. Bars represent the changes in the activities of SOD, CAT, GPx, GSH, GR, and GST in Alpinia *officinarum* CuNPs treated MCF-7 cells compared to control cells. Values are given as the mean S.D. of six experiments in each group. Bars not sharing the common superscripts differ significantly at $P < 0.05$ vs. control (DMRT).

Endogenous production of reactive oxygen species agents increases in mitochondria, peroxisomes, the endoplasmic reticulum, and the plasma membrane. Radicals such as hydroxyl and hydroperoxyl destroy lipids in cell membranes and have been related to diseases like cancer (Dizdaroglu and Jaruga; 2012).

Antioxidant enzymes (SOD, GPx, and CAT) may be increased or decreased in these cells to defend the situation (Kumar RR et al; 2014). In this work, the contents of SOD, GPx, CAT, and GSH were evaluated in the HT-29 and MCF-7 cell lines. Excessive accumulation of reactive oxygen species had a crucial role in producing oxidative damage when *Alpinia officinarum* CuNPs were applied to apoptosis-inducing factors. Due to lipid peroxidation, there is a reduction in SOD, CAT, and GSH to counteract the damaging effects of reactive oxygen species (Liao JC; 2015). ***Alpinia officinarum* CuNPs** also reduced the activities of antioxidant enzymes, SOD, CAT, and GPX in HT-29 and MCF-7 cells, in a time-dependent manner, but the same was higher in untreated HT-29 and MCF-7 cells(Figure 8 and Figure 9). The results obtained from *in vitro* studies conducted against HT-29 and MCF-7 cell lines depicted that ***Alpinia officinarum* CuNPs** have significant antioxidant activity against tested cell lines in a time-dependent manner.

CuNPs from *Alpinia officinarum* further induced cytotoxicity in cancer cells at a specific IC50. According to several studies, mitochondria are a popular target for anticancer drugs since they are involved in

cellular metabolism and function, as well as inducing cytotoxic effects. Throughout a 24-hour incubation period, CuNPs from Alpinia officinarum generated a substantial number of apoptotic bodies in HT-29 and MCF-7 cells. In HT-29 and MCF-7 cancer cells, changes in mitochondrial membrane potential indicate *Alpinia officinarum* CuNP's role in mitochondrial-mediated apoptosis. In MCF-7 cells, Alpinia officinarum reduced mitochondrial membrane potential (MMP) and increased apoptotic capacity, both of which were attributable to the formation of reactive oxygen species (ROS)[31].

CONCLUSION

The green synthesis of copper oxide nanoparticles using Alpinia officinarum offers a promising avenue for developing environmentally friendly and biocompatible materials with significant antimicrobial and anticancer properties. The phytochemicals present in the plant facilitate the effective reduction and stabilization of CuOnanoparticles, which demonstrate potent activity against various pathogens and cancer cells. These findings highlight the potential of CuOnanoparticlesin medical applications, particularly in the fields of antimicrobial agents and cancer therapeutics. However, further research, including *in vivo* studies and safety assessments, is essential to fully explore their therapeutic potential and to optimize their application in clinical settings.**REFERENCES**

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