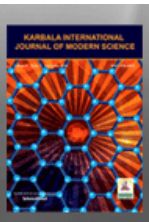

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Elucidation of antibacterial, synergistic, antioxidant, and anticancer activities of green synthesized copper oxide nanoparticles against human breast cancer cells

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Abstract

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Keywords

Aspergillus niger, Copper oxide nanoparticles, Antibacterial activity, Cytotoxic activity.

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RESEARCH PAPER

Elucidation of Antibacterial, Synergistic, Antioxidant, and Anticancer Activities of Green Synthesized Copper Oxide Nanoparticles Against Human Breast Cancer Cells

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Abstract

The study conducted biosynthesis of copper oxide nanoparticles using an aqueous extract of *Aspergillus niger* and investigated their potential biomedical applications. The nanoparticles were characterized using various techniques including UV-Vis, SEM, TEM, and EDX, revealing their spherical to crystalline shape with sizes ranging from 21 nm to 48 nm. The nanoparticles demonstrated notable antimicrobial activity against *Staphylococcus aureus* with a zone of 8 mm and *Escherichia coli* recorded high synergistic activity with 20.93%, as well as antioxidant properties. Furthermore, selective cytotoxicity towards breast cancer cells was observed with an IC_{50} of 107.81 ug/ml and an IC_{50} of 250.93 ug/ml on normal cells, suggesting their potential use as an anticancer agent and for targeted drug delivery.

Keywords: *Aspergillus niger*, Copper oxide nanoparticles, Antibacterial activity, Cytotoxic activity

1. Introduction

Cancer is a leading cause of death and a major impediment to increasing life expectancy. According to GLOBOCAN 2020, lung, liver, and female breast cancers remain significant causes of cancer death, accounting for 18%, 8.3%, and 6.9% respectively [1]. In 2020, it was reported that around 2.3 million women were diagnosed with breast cancer and a mortality of 0.685 million cases worldwide [2]. However, the statement from WHO has brought to light that female breast cancer has already surpassed lung cancer in cases of morbidity, in 2020 it was reported that breast cancer recorded 2.26 million cases, followed by 2.21 million lung cancers [3]. Chemotherapy is typically the first line of cancer treatment. However, the efficacy of the current chemotherapeutic drugs is limited, particularly in late-stage tumours. Hence, a new treatment profile should be identified to counter the breast cancer cells at an early stage of infection.

In recent years, the remarkable success of nanotechnology has been well demonstrated through various ongoing nanoparticle research projects. The innovation shown in the field of nanotechnology has influenced the day-to-day activities of humans. Thus, the importance of nanosized particles was well accepted in various fields, including diagnostics, targeted medication delivery, cosmetics, health care, fuel cell, food industries, energy storage, tunable resonant devices, computing, biosensors, and many more [4–6]. Furthermore, novel

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research on nanoparticles (NPs) has led to various milestones in the field of biological applications through nanomedicine formulations which perhaps widens the ground for studying more about this technology [7]. NPs are the product of specialized dispersions or solid particles with sizes varying from 1 to 100 nm, where the medication can be dissolved, entrapped, encapsulated, or linked to its matrix [8]. Since the NPs are of ultra-small size, this in turn creates a large surface to volume ratio thus showing a tremendous improvement in their melting point, optical absorption, catalytic activity, biological properties, electrical and thermal conductivity, which was not the same in their bulk form [9,10].

In common practice, metallic nanoparticles were synthesised and stabilised using chemical and physical techniques, either bottom-up or top-down approach [11]. Chemical methods like soft assembly, plasma processing, soft lithography, chemical vapour deposition, chemical etching, etc … were used. However, physical methods employed in the destruction of samples were high energy ball milling, lithography, and gas condensation [12]. The usage of conventional methods in the synthesis of NPs either chemical or physical suffers a few disadvantages, such as that it causes toxic effects to humans, animals, and the environment while using hazardous chemicals, there is difficulty in filling the gap with its mechanism and modelling factors, expensive analysis is required, reuse/recycling of materials used, etc … [13–16].

The above-listed limitations have opened the door for synthesizing metal NPs which should be eco-friendly to nature, and commercially viable, thus approaches like biosynthesis or green synthesis of metal nanoparticles from plant-based extracts, microorganisms like fungi, bacteria, yeast, and algae are in practice [17]. Microorganisms like fungi act as reducing enzymes both intracellularly and extracellularly [7]. Unlike conventional methods, NPs produced by biosynthesis showed good yield, a wide range of shapes, sizes, and stable physiochemical properties. Due to the enormous benefits of biosynthesis method, the research community considered this a promising way to produce metallic NPs using inorganic metals like copper, tin, iron, zinc, and nickel which are used in medicine, energy, and environmental research in very interesting ways [9]. In recent years metal oxides in general have gained more attention due to their variety of applications in different fields compared to their metal NPs [18]. In addition, recent studies have revealed that metal oxides have higher acceptance in biomedical research. One such metal oxide of interest is copper oxide. Copper is among the chemical elements with 29 nucleons that are generally soft, easily shaped, and possess great flexibility as well as thermal and electrical conductivities [19]. This element also possesses key physical and chemical properties, including optical, catalytic, heat transfer, magnetic, and biocidal properties, as well as a high proportion of surface area to volume [20]. Once the particle size and size-dependent characteristics can be manipulated, NPs of copper and its oxides should be applied to a wider variety of applications such as anticancer, antimicrobial, and antioxidant [21]. Studies have shown that copper oxide nanoparticles (CuONPs) inhibit cell viability through various mechanisms like necrosis and apoptosis [22].

Fungal mediated biosynthesis of copper (Cu) or CuONPs, was achieved through the production of the extracellular protein that helps in reducing metal ions and acts as a capping agent in the synthesis of metallic NPs [23]. Due to various reasons, fungi are one of the important microorganisms which are widely accepted in the field of pharmaceutical research. It was estimated that planet earth is filled with about one and a half million fungi out of which only 60% of species were identified, leaving plenty of room to discover [24]. Fungi, in comparison to other types of organisms, can produce NPs with a greater rate of consistency since they can resist high flow pressure as well as agitation properties [25]. Green synthesizes of NPs from fungi strategies themselves explain that in cases where both an internal and an external pathway will be used to manufacture NPs of multiple sizes, those formed from the external pathway can be regarded as relatively small, but with a better diversity profile and proportions [26]. Furthermore, fungi sources were established for their medicinal and nutritional benefits, which made the pharmaceutical industries shift their investigations to a wide range of discoveries [27]. For the current study, *Aspergillus niger* was selected as a reducing agent for the biosynthesis of CuO NPs.

The resistance of bacteria to existing antibiotics and the emergence of multidrug-resistant bacteria are increasing at an alarming pace, providing one of the biggest challenges in the fight against bacterial infection and related disorders [28]. According to the World Health Organization (WHO), it was estimated that every year, almost 700,000 people die from antibiotic-resistant illnesses. In addition, the WHO also forecasted that by 2050, antibiotic-resistant illnesses might be responsible for 10 million deaths annually. *Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumonia, Acinetobacter baumannii, Pseudomonas aeruginosa*, and *Enterobacter* species

comprise the bulk of nosocomial infections in hospital settings [29].

The main objective of this novel study is to green synthesis the CuONPs from the *A. niger* and to study their anticancer effect on Human M.D. Anderson - Metastatic Breast 231 cancer cells (Human MDA -MB 231).

2. Materials and methods

2.1. Materials

DMEM-high Glucose - (AL111, Himedia), Fetal Bovine Serum (RM10432, Himedia), MTT Reagent (5 mg/ml) (4060 Himedia), DMSO (#PHR1309, Sigma), D-PBS (TL1006, Himedia), Camptothecin (Cat No: C9911, Sigma), MDA MB 231−Human breast adenocarcinoma cell line (NCCS, Pune), MCF-10A −Human Non-malignant breast cell line (ATCC, USA).

2.2. Methods

2.2.1. Cultivation of fungal spore

Cultivation of fungal spore was processed from the forest soil collected from Bukit Kledang (coordinates are 4°34′60″ N and 101°1′1″ E, Ipoh, Malaysia) at a depth of 15−30 cm using a sterile shovel and transferred to a sterile zipper bag and taken to the laboratory. Collected soil was dried at room temperature, and about 1 g of soil was weighed and transferred to a sterile test tube, solubilised using distilled water, and vortexed to obtain homogenously. The homogenous suspension of the soil sample was serially diluted in a concentration ranging from 10 to 1 to 10−5. About 10 μl of extract from serial dilution was inoculated aseptically onto the respective Sabouraud Dextrose Agar (SDA) plates in the middle and dispersed in a circular motion using an L-shaped rod. The inoculated SDA plates were kept at room temperature for 5−7 days for the growth of fungal spores.

2.2.2. Identification of A. niger

Upon the growth of fungal spores, selected spores were subjected to the microscopical identification for *Aspergillus* species, a minute portion of the fungal spores were collected using sterile needle, transferred to a glass slide, covered with lacto phenol cotton blue solution, and covered with a cover slip. The slide was examined under 10x and 40x for the microscopical structure identification and recorded. To confirm the species, a small portion of the spore was cut and transferred to a sterile sample tube and examined for 18s RNA sequencing for gene identification and recorded. Subculture was done on the pure species for further study.

2.2.3. Synthesis of A. niger biomass

Using a sterile loop, portion of spore from the subcultured species of *A. niger* (*A.niger*) was inoculated into a 250 ml Erlenmeyer flask containing 200 mL of freshly prepared and sterilised Potato dextrose agar broth (PDA), a few sets of broth was prepared for sub cultivation of biomass for synthesising nanoparticles. The inoculated broth was kept in an orbital shaker at 200 rpm for about 96 h, at a temperature of 25 ± 2 °C to produce the mycelium biomass. Upon incubation period, the biomass was filtered through Whatman filter paper No. 1 and subjected to washing with distilled water to remove excess PDA broth. The filtrate was collected and used further in the biosynthesis of CuONPs extracellularly.

2.2.4. Fungal biosynthesis of CuONPs

Using a 250 mL Erlenmeyer flask, 200 mL of fungal biomass filtrate was added and treated with 5.0 mM of copper sulfate ($CuSo_4$) with a weight of 0.159g crystals as a metal precursor. The required quantity of $CuSo_4$ crystals was calculated based on the available filtrate volume. The solution was kept on the orbital shaker at room temperature (25 ± 2 °C) for 96 h at a speed of 150 rpm. During the mentioned period, bioconversion of metal precursors to CuONPs was recorded for the change in biomass colour. In addition, a 1:1 diluted biomass solution was taken and tested for UV spectroscopy (scan range 800 nm−200 nm) to determine the lambda maximum against blank biomass without metal precursor, to evaluate the formation of CuONPs. Furthermore, in extracting CuONPs microbial pellets the solution was centrifuged at 11,000×*g* for 15 min, washed a few times with double distilled water, and finally treated with 70% ethanol to remove any media residuals. Dried pellets were weighed to calculate the percentage yield and stored in airtight containers for further analysis. Simultaneously, two control experiments were carried out one with metal precursor and media without fungal filtrate, and the other with fungal inoculum without metal precursor.

2.3. Characterisation techniques for the biosynthesised CuONPs

2.3.1. UV-visible spectrophotometer analysis

UV visible characterisation on the biosynthesized CuONPs solution was performed in a UV-visible spectrometer (Shimadzu UV 1900) at a scan range between 200 and 800 nm. The analysis solution was diluted with distilled water in a 1:1 ratio and a

negative control consisting of filtrate without the addition of CuSO4 was used as a blank. The absorbance maximum was measured and recorded [30].

2.3.2. Fourier transform infrared spectroscopy (FTIR) analysis

The CuONPs were identified for their functional group by using FTIR spectrometer (Shimadzu, Japan), in which the dried NPs were mixed with potassium bromide (KBr) at a ratio of 1/10 and carried out for the FTIR spectra from 4000 to 40 cm^{-1} with 32 scans and a resolution of 4.0 The emitted energy was measured and used to generate the spectra for further detection on functional groups confirmation of the CuONPs [31].

2.3.3. Scanning electron microscopy (SEM) & energy -dispersive X-ray spectroscopy (EDS)

The surface morphology of the CuONPs was studied using SEM analysis. The liquid sample was sonicated at 10,000 rpm for 30 min, the supernatant was removed, and the pellet was collected carefully, washed, and lyophilized. Powdered CuONPs were coated with conductive layers, such as gold, using a sputter-coater, and then they were positioned in the middle of the device. Upon being coated, the sample was subjected to SEM analysis (Hitachi SEM S-4700) under vacuum conditions. EDS is used to detect the composition of the NPs. EDS systems are typically attached to the SEM. The presence of elemental Copper and Oxygen was confirmed using EDX detector [32].

2.3.4. Transmission electron microscopy (TEM)

The particle size, shape, and size distribution of the produced CuONPs were characterised using TEM analysis (Zeiss Libra 120). About 1 ml of the NPs liquid sample was sonicated for 10 min. One drop of the sonicated solution was placed in a carbon grid on filter paper and allowed to be dried at room temperature for several minutes. The dried sample was loaded into a specimen holder and analysed for the TEM characterisation [33].

2.3.5. Thermogravimetric analysis (TGA)

Thermogravimetric analysis was used to study the change in mass of CuONPs subjected to a controlled temperature program in a controlled atmosphere. The analysis was performed using the PerkinElmer Thermal Analysis TGA 8000, which measures the initial weight of the sample using a hang down pan balance and then places it in a standard furnace at temperatures ranging from 30.00 °C to 900 °C at a rate of 20 °C per minute [34].

2.4. Evaluation of antibacterial efficacy

The antibacterial activity of CuO NPs was evaluated against both gram-positive and negative bacteria like *S. aureus* (ATCC 33862), *Bacillus aureus, P. aeruginosa* (ATCC 15442), *Escherichia coli* (ATCC 8739) *Salmonella sp,* respectively using disc diffusion method with 20 μg/mL, 40 μg/mL, 60 μg/mL, and 80 μg/mL of CuONPs solution against standard antibiotics under a controlled incubation environment. Results were compared with control levofloxacin. Furthermore, synergistic antibacterial efficacy was determined with the selected antibiotics like tetracycline, meropenem, gentamicin, erythromycin, and cefuroxime added with 80 μg/mL of biosynthesised CuO NPs, studied for its zone of inhibition and the data were tabulated and analysed.

2.5. Determination of antioxidant activity using DPPH assay

The antioxidant evaluation was conducted using the DPPH free radical scavenging assay. Stock solutions were made from the dried sample of CuONPs by diluting it with 25 mg per 5 mL of distilled water and then sonicated for about 10 min to ensure complete solubility. The stock solution was diluted to a concentration of 2000 μg/mL, 4000 μg/mL, 6000 μg/mL, and 8000 μg/mL. The diluted solutions in the test tubes were allowed to stand for 30 min in a dark place. The absorbance of coloured changed sample was measured with a spectrophotometer at a wavelength of 517 nm. Ascorbic acid was used as standard and DPPH solution was used as blank.

DPPH free radical scavenging activity is calculated using the following equation:

$$\text{DPPH scavenging activity}\ (\%) = (1 - A_S / A_c) \times 100 \quad (1)$$

where A_s is the absorbance of the sample and A_c is the absorbance of control at 517 nm for control.

2.6. Cell culture maintenance

The MDA MB 231 (Human breast adenocarcinoma cell line) was purchased from NCCS, Pune, India, and MCF-10A (Human Normal breast cell line) were procured from ATCC, USA. The cells were maintained in DMEM high glucose media supplemented with 10% FBS along with the 1% antibiotic-antimycotic solution in the atmosphere of 5% CO2, 18–20% O2 at 370C temperature in the CO2 incubator and sub-cultured every 2 days.

2.7. Determination of cytotoxicity activity through MTT assay

Cytotoxicity activity of the biosynthesised CuONPs was studied using MTT (3- [4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) assay. 200 µl of cell suspension was seeded in a 96-well plate at the required cell density (20,000 cells per well), without the test agent. Cells were allowed to grow for about 24 h. Added 12.5, 25, 50, 100, and 200ug/ml of CuONPs solution and incubated for 24hrs at 37 °C in a 5% CO2 atmosphere. Upon incubation period, plates were taken out from the incubator, and spent media was added with MTT reagent to a final concentration of 0.5 mg/mL of total volume. Plates were wrapped with aluminium foil to avoid light exposure and incubated. MTT reagent was removed and added with 100 µl of solubilisation solution (DMSO). The absorbance was measured on an ELISA reader at 570 nm [35].

% Cell viability was calculated using below formula:

% Cell viability = [Mean absorbance of treated cells / Mean absorbance of Untreated cells] x 100

The linear regression equation i.e., $Y = Mx + C$ (2) was used to calculate the IC_{50} value. Where $Y = 50$, M and C values were derived from the viability graph.

3. Result and discussion

3.1. Isolation and identification of A. niger sp from soil sample

The inoculated SDA plates were observed for the growth of *A.niger* sp. Plates with 10^{-4} & 10^{-5} dilutions showed well separated growth of *A. niger* sp colonies. A study report from Aryal et al. on the SDA plates was used to analyse the morphological growth of this species, it states colony formation starts with a white-cotton-textured colony before rapidly transforming into a black colour. According to the statement, colonies of the species selected appeared to be black and at the same time reverse from white to yellow. To confirm the selected species of *A.niger* it was further analysed using lacto phenol staining method [36]. Microscopical examination revealed the hyphal structure, conidia, and spore arrangement as shown in Fig. 1.

3.2. 18s RNA gene sequencing identification

The *A.niger* was confirmed with the 18s RNA sequencing method and results showed the following arrangement. Fig. 2 shows the graph on species confirmation. The percentage similarity was found to be 96%.

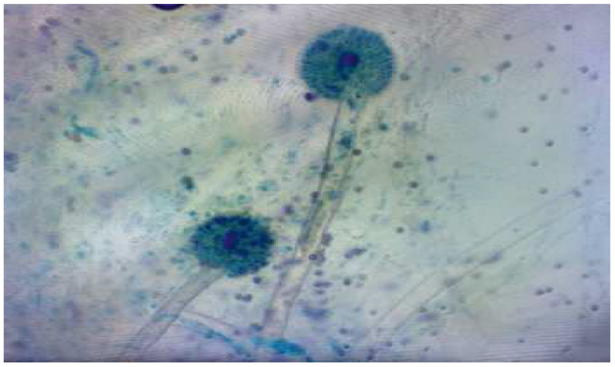

Fig. 1. Microscopical image of lacto phenol blue stained A.niger *spore.*

TTGCGGAAGGAACATTACCGAGTGCGGGTC
CTTTGGGCCCAACCTCCCATCCGTGTCTATTAT
ACCCTGTTGCTTCGGCGGGCCCGCCGCTTGTC
GGCCGCCGGGGGGGCGCCTTTGCCCCCCGG
GCCCGTGCCCGCCGGAGACCCCAACACGAAC
ACTGTCTGAAAGCGTGCAGTCTGAGTTGATTG
AATGCAATCAGTTAAAACTTTCAACAATGGAT
CTCTTGGTTCCGGCATCGATGAAGAACGCAG
CGAAATGCGATAACTAATGTGAATTGCAGAAT
TCAGTGAATCATCGAGTCTTTGAACGCACATT
GCGCCCCCTGGTATTCCGGGGGGCATGCCTG
TCCGAGCGTCATTGCTGCCCTCAAGCCCGGC
TTGTGTGTTGGGTCGCCGTCCCCCTCTCCGGG
GGGACGGGCCCGAAAGGCAGCGGCGGCACC
GCGTCCGATCCTCGAGCGTATGGGGCTTTGTC
ACATGCTCTGTAGGATTGGCCGGCGCCTGCC
GACGTTTTCCAACCATTTTTTCCAGGTTGACCT
CGGATCAGGTAGGGATACCCGCTGAACTTAA
GCATATCAATAAGCGGAGGAGTCATAGCTGTT
TCCATGA.

3.3. Biosynthesis of CuONPs

The biosynthesis of CuONPs was prepared using 5g biomass of the isolated strain of *A. niger sp*. as shown in Fig. 3 a [37]. The mycelium free filtrate was used as a reducing agent for the biosynthesis of CuONPs from 5 mM Copper sulfate (CuSO4) crystals as its metal precursor as shown in Fig. 3 b [38]. Furthermore, upon the completion of the incubation period, initially, the change in colour from light blue to pale green was observed on the solution, as an indication of a reduction in the metal precursor to CuONPs as shown in Fig. 3 c. The extracted CuONPs were shown in Fig. 3 d. Percentage recovery of CuONPs was calculated to be 6.3 mg/100 ml of the culture. There were no obvious colour changes

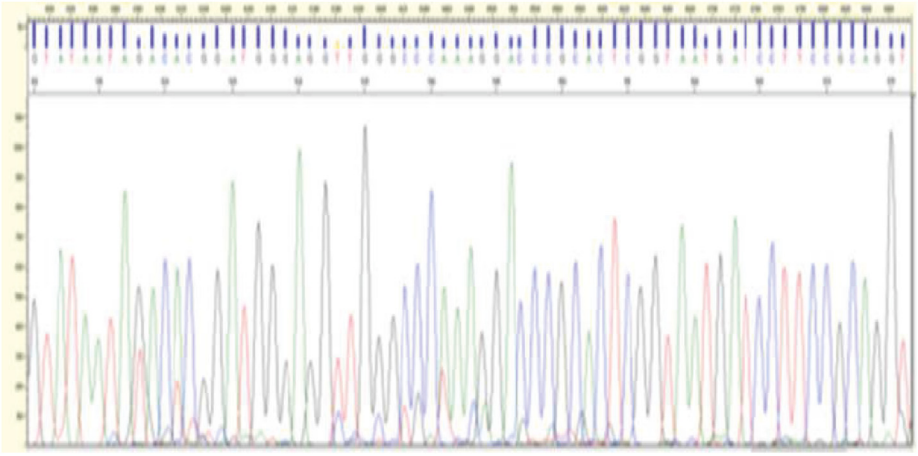

Fig. 2. 18s RNA gene sequencing identification of Aspergillus niger.

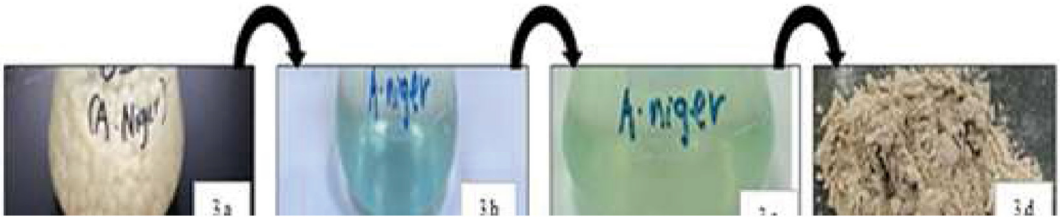

Fig. 3. a; Biomass of A.niger *b; $CuSo_4$ + biomass filtrate solution c; CuO formed solution d; Cuo NPs.*

observed in control cultures which confirms that the metal precursor was successfully reduced to its NPs.

3.4. Characterisation of biosynthesised CuONPs

3.4.1. UV-spectroscopy analysis

UV-Visible spectroscopy is one of the most pertinent ways to find out about the morphology and stability of nanoparticles. The UV-Vis spectra of the synthesized CuONPs were recorded at different wavelength ranges, ranging from 800 nm to 200 nm as shown in Fig. 4 a. The peak that was found at the wavelength provided evidence that the CuONPs that are synthesized are in their active state. This absorption band is related to the interbond transition of core electrons of CuO [39], and its absorption maximum was found to be at 265 nm. The findings of this result were coherent with the investigations that were conducted by Mani et al. [40], stating that any absorption that occurred in the range of 260–270 nm is an indication of the presence of CuONPs.

3.4.2. FTIR spectroscopy analysis

FTIR is considered one of the most used techniques in analysing any IR spectra formed by chemical bonds present in a molecule. IR spectra were obtained for the biosynthesised CuONPs that range from 400 cm^{-1} to 4000 cm^{-1} were shown in Fig. 4 b. At a wavenumber of 3373.50 cm^{-1}, a broad intense absorption band was detected, and this observation points to the presence of a hydroxyl functional group (OH) [41]. It is either an alcoholic group or a phenolic group that corresponds to the binding of copper ions that resulted in the description of O–H stretching [35]. Additionally, the stimulated absorption band at 1629.85 cm^{-1}, was recorded as the bending vibrations of amide I group (C=O) [42]. Meanwhile, the bands found at 1195.87 cm^{-1} and 1157.29 cm^{-1} are considered to also possess sulphur-containing amino acids, which originate from proteins produced by Aspergillus [43].

3.4.3. SEM analysis

The surface morphology of the biosynthesised CuONPs was analysed using SEM and the results were shown in Fig. 4 d. The particle sizes of the NPs range from 15.51 nm to 25.05 nm respectively, with its mean particle size of 20.72 ± 3.747156 nm. The 3D obtained images were found to be spherical to crystalline shape and have a high level of homogeneity. The results observed are substantiated by the study conducted by Gunalan et al., where the

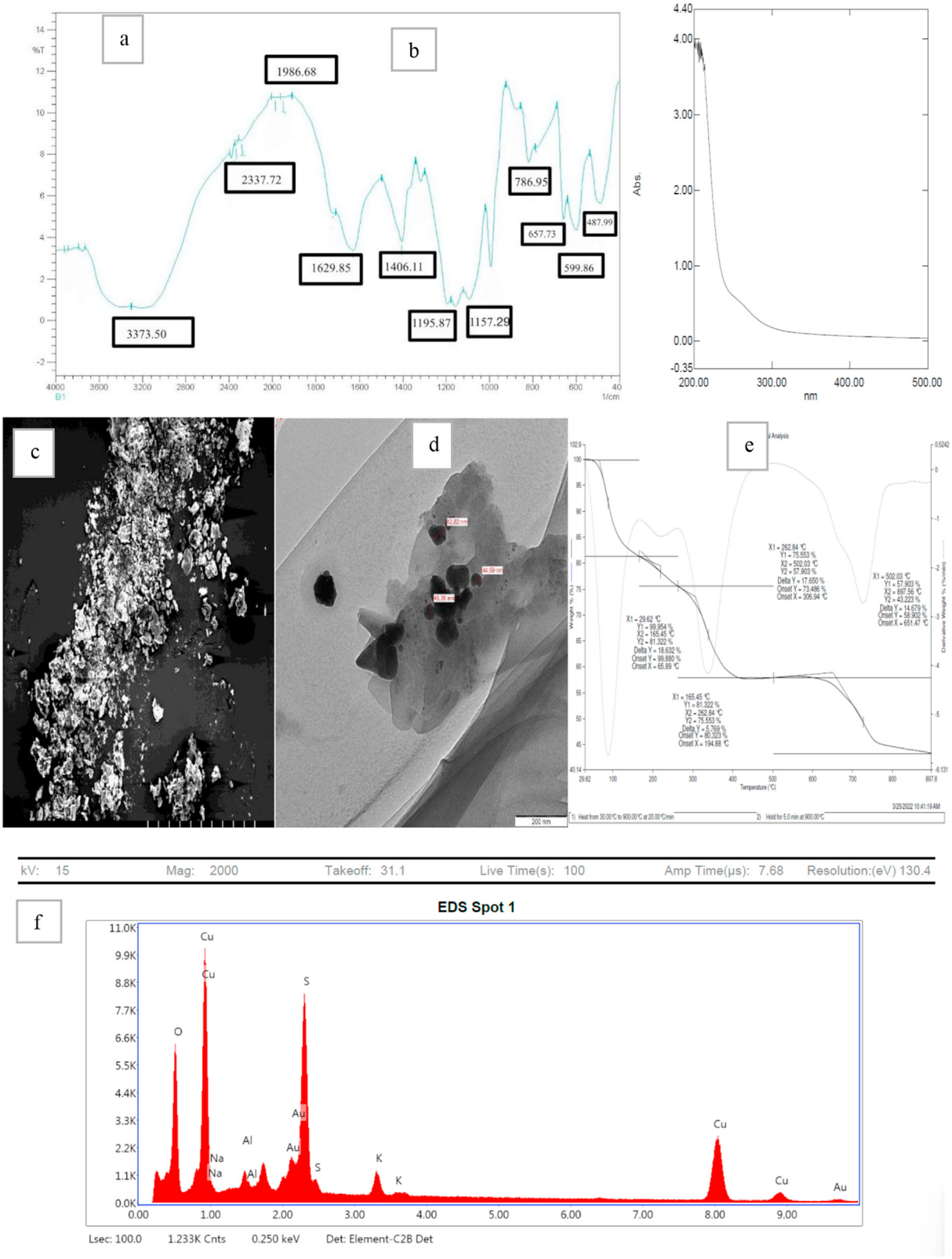

*Fig. 4. Characterisation of biosynthesised CuONPs. **a:** UV-Visble absorption spectra **b:** FTIR spectrum of CuONPs **c:** SEM image of CuONPs **d:** TEM image of CuONPs **e:** TGA analysis of CuONPs **f:** EDS analysis of CuONPs.*

green synthesized CuONPs from Aloe extract showed spherical NPs ranging from 15 to 30 nm [44].

3.4.4. TEM analysis

The TEM analysis confirms the morphology of the biosynthesized CuONPs as shown in Fig. 4 e. The 2D images on the NPs showed spherical shaped particles with a size ranging from 21 nm to 48.36 nm accordingly and the average particle size was found to be 37.39 ± 11.2 nm. Furthermore, the studied results proved their high efficiency compared to the study conducted by Moon et al., on the plant synthesized spherical shaped CuONPs with particles ranging from 25 to 67 nm [45].

3.4.5. Thermogravimetric analysis

The thermal stability, moisture, oxidation content, and volatility were confirmed using TGA. The thermogram was used to study the temperature dried CuONPs ranging from 30 °C to 900 °C at a ramp rate of 20.00 °C per min. At 100 °C no loss of protein was observed, however most protein loss resulted at 262.84 °C as can see in Fig. 4 c. According to the earlier study the protein thermal breakdown was observed from 166° to 306.94° based on the origin and purity of the NPs [46]. At its final temperature still, the sample remaining in the pan indicates that biosynthesised CuONPs are stable [47].

Table 1. Antibacterial efficacy of biosynthesised CuONPs on selected gram positive and negative bacteria.

	Zone of Inhibition (mm)				
Concentration (µg/mL)	Escherichia coli	Salmonella	Pseudomonas aeruginosa	Bacillus aureus	Staphylococcus aureus
Control	34.0	40	30	33	32
20 µg/mL	-	-	-	9	8
40 µg/mL	7	8	7	10	9
60 µg/mL	8	10	8	12	10
80 µg/mL	9	13	9	19	21

3.4.6. Energy-dispersive X-ray spectroscopy

Elemental analysis of the biosynthesised CuONPs was determined using EDX spectra as shown in Fig. 4 f. The findings proved the presence of signals corresponding to the elements copper (Cu) and oxygen (O), and the percentage weight was found to be 61.51% and 12.08%, respectively. Furthermore, the peak that was established around 5.8 keV corresponds to oxygen, while the peak that was established around 8.2,9.9,2.2, and 0.01 keV corresponds to copper.

The overall characterization reports confirm the biosynthesized CuONPs have shown good stability, are highly durable, and could be used for various biomedical applications.

3.5. Antibacterial activity of copper oxide nanoparticles

Antibacterial efficacy of the biosynthesised CuONPs was evaluated against *E. coli, Salmonella sp, P. aeruginosa, B. aureus, and S. aureus*. The CuONPs have resulted in a maximum zone of inhibition of 80 µg/mL and a minimum zone of inhibition of 20 µg/mL. At a concentration of 80 µg/mL maximum inhibition was observed in *S. aureus* with 21 mm and minimum inhibition was also observed in *S. aureus* with 8 mm. However, there was no zone observed with *E. coli, Salmonella sp, P. aeruginosa* at 20 µg/mL. From the results, it was proven that CuONPs have

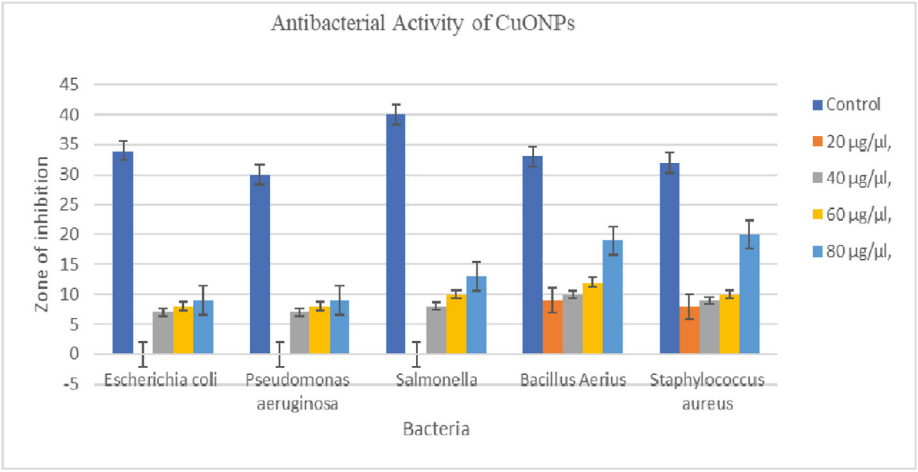

Fig. 5. Antibacterial activity of CuoNPs against selected gram positive and negative bacteria.

Table 2. Synergistic activity of CuONPs with various antibiotics. Mer:Meropenen; Mer + NPs: Meropenen + CuONPs; Tet: Tetracycline; Tet + NPs: Teracycline + CuONPs; Ery: Erythromycin: Ery + NPs: Erythromycin + CuONPs; Gen: Gentamycin; Gen + NPs: Gentamycin + CuONPs; Cef: Cefuroxime; Cef + NPs; CuONPs.

	Zone of Inhibition (mm)				
Concentration (µg/mL)	Eschenichia coli	Salmonella	Pseudomonas aeruginosa	Bacillus aureus	Staphylococcus aureus
Control	34.0	40	30	33	32
20 µg/mL	-	-	-	9	8
40 µg/mL	7	8	7	10	9
60 µg/mL	8	10	8	12	10
80 µg/mL	9	13	9	19	21

shown commendable antibacterial activity against gram positive compared to the tested gram-negative organisms as shown in Table 1 and Fig. 5. A previous study on the antibacterial activity against *S. aureus* using CuONPs showed the highest increments of zone inhibition [48]. The catalysed interaction of copper metallic ions with amines and carboxyl groups is likely to be the fundamental reason for CuONPs' high antibacterial activity against Gram-positive, *S. aureus* rather than Gram-negative pathogens [49]. In addition, synergistic activity was tested against the same set of organisms using 20 µg/mL of CuONPs impregnated on the following antibiotic discs like tetracycline, meropenem, gentamicin, erythromycin, and cefuroxime. The measured zone of inhibition on the synergistic bactericidal effect is shown in Table 2. Among the tested bacteria *E. coli* (20.93%) and Salmonella (15.58%) showed the greatest zone of inhibition for the antibiotics combined with CuONPs. This is since the cell wall composition of this gram-negative bacterium differs compared to gram positive ones [50]. It was found that gram-negative bacteria contain a layer of lipopolysaccharides on their outer surfaces, which is then followed by a layer of peptidoglycan that is very thin (7–8 mm). The peptidoglycan, also known as polymer, composed of amino acids and sugars, which makes it less hard and resistant, allowing CuONPs and antibiotics to easily enter the bacterial cell and disrupt the usually controlled system [51]. On the other hand, gram-positive bacteria's cell wall was primarily composed of a dense layer of peptidoglycan that forms a three-dimensional structure generated by cross-linking linear polysaccharides with short peptides [52]. As a result, this supports the findings that *S. aureus* (5.28%) recorded the lowest percentage of synergistic bactericidal effects among the other bacteria in the study. Based on the findings, it was evident that synergistic effects between CuONPs and antibiotics are feasible owing to the development of a complex that aids in the efficient suppression of a certain bacterial strain, either by deferring cell wall synthesize or causing bacterial lysis [53]. A study report from Balasubramanian et al., revealed that green synthesised CuONPs showed significant antibacterial activity against *S.aureus* [54]. Abdolrazagh Marzban et al., previous study report proved that polysaccharides-copper nanoparticles exhibited greater antibacterial effects and biofilm inhibition against *S.aureus* [55]. The Study report from Mohamad S. Hasanin on the biosynthesis of CuoNPs based biopolymers shown good antibacterial activity on both gram positive and negative organisms [15]. Raman Krishnamoorthi et al., study on silver nanoparticles synthesised by edible mushrooms showed good antibacterial activity against the gram-positive microorganism [56]. Furthermore, a study report from Muhamad Ikram et al. on the synthesis of calcium Oxide nanoparticles exhibited good antibacterial activity against *S.aureus* [57]. Study on starch grafted polyacrylicacid doped barium oxide nanostructures recorded good antibacterial activity against *S.aureus* [58].Our study reports are in line with the previous research findings and showed excellent antibacterial activity. The percentage synergistic activity of the tested bacteria against CuoNPs and antibiotics were shown in Fig. 6.

3.6. Antioxidant evaluation of biosynthesised CuONPs

The percentage of free radical scavenging activity of CuONPs and standard ascorbic acid is shown in Fig. 7. CuONPs record IC_{50} values between 6000 and 8000 µg/ml. However, standard ascorbic acid recorded great free radical activity at its lower concentration. It has been demonstrated that the antioxidant activity of both the samples and the standards increased in a concentration-dependent manner, following a uniform linearity pattern as the concentration increased, as evidenced by the R2 values of 0.9837 and 0.9792 for the samples of CuONPs and the standard ascorbic acids, respectively. This concentration-dependent result was also observed in a study conducted by Brajesh et al., in which the antioxidant activity of CuONPs synthesized from Andean blackberry (*Rubus glaucus* Benth) was evaluated at various concentrations (0.5–2.5 mM) the whereby highest percentage of inhibitions is being observed at 89.02% which was increased linearly from the lower to higher concentration [59]. Electrostatic attraction between

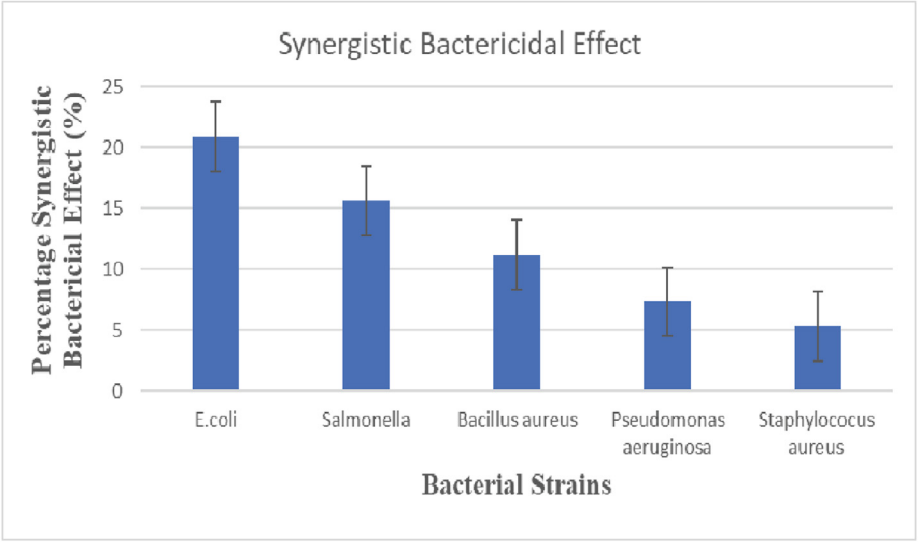

Fig. 6. Percentage synergistic inhibition of CuONPs and antibiotics against tested bacteria.

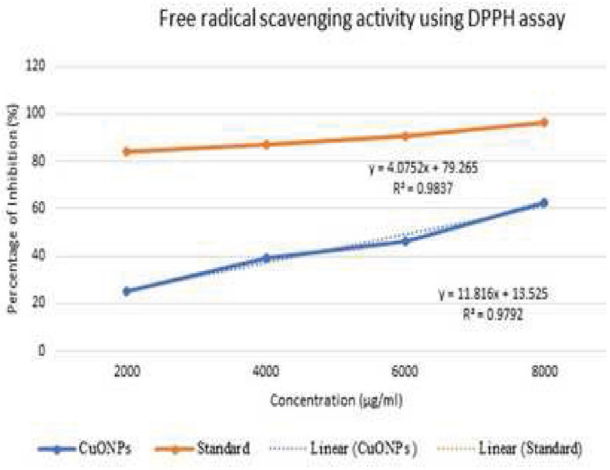

Fig. 7. Percentage synergistic inhibition of CuONPs and antibiotics against tested bacteria.

negatively charged bioactive compounds like phenolate ions and positively charged NPs is thought to be the mechanism by which CuONPs function as antioxidant agents. This explains why binding between two different charged molecules increases their bioactivity and phytochemical synergy [60]. This assertion is then further confirmed by the fact that the effect of antioxidant activity will be influenced by the site of its attachments to the metal and that its resultant impact will be on the activity of antioxidant agents themselves [61].

3.7. Toxicity evaluation of CuONPs

The toxicity of CuONPs has been evaluated against MDA MB 231 breast cancer cells in a dose dependent manner and was compared with a

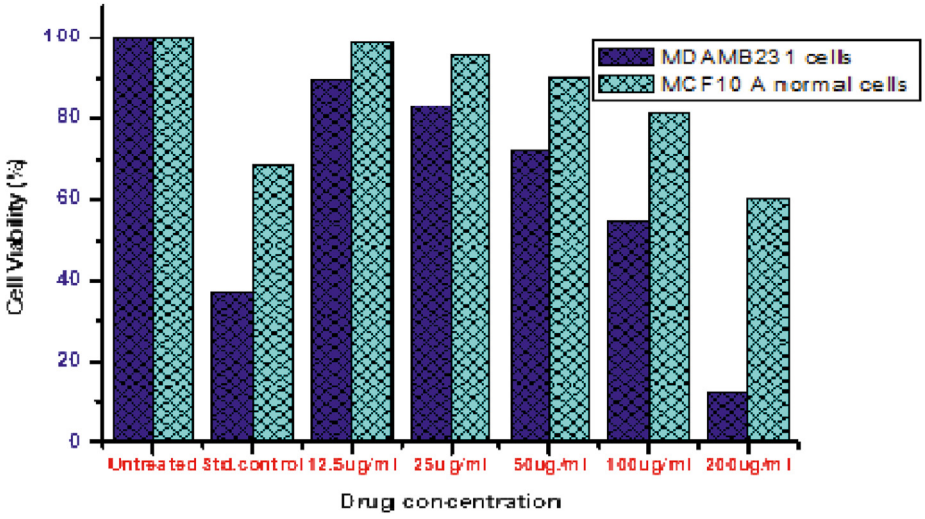

Fig. 8. Percentage cell viability on MDA MB-231cells and MCF-10A normal cells at various drug concentrations.

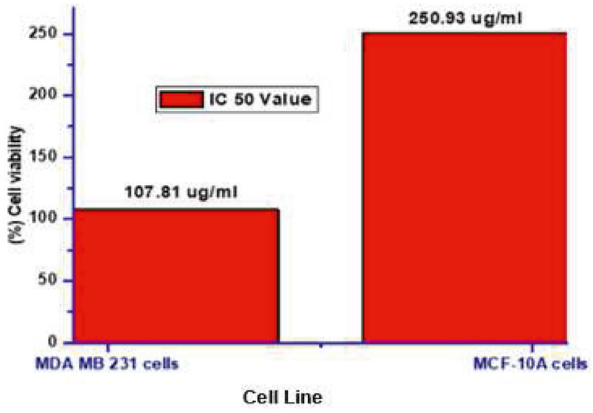

Fig. 9. IC_{50} Concentration comparison of CuO NPs on MDA -MB 231 cells and MCF-10A cells.

positive control camptothecin and results are shown in Fig. 8. The CuONPs showed a very commendable IC_{50} value of 107.81 ug/ml in selected cancer cells compared with positive control which showed an IC_{50} value of 250.93 ug/ml. Graphical representation of IC_{50} value of CuO NPs compared with positive control as shown in Fig. 9. Fig. 10 (A-D) showed morphological changes in the cells after being treated with nanoparticles at their minimum (12.5 ug) and maximum concentration (200 ug). Percentage cell viability on MDA MB-231 and MCF-10 A normal cells at various drug concentrations shows the CuONPs have good activity on cancer cells and minimal action on normal cells, as shown in Fig. 10. The CuO NPs treated with A549 and human cervical carcinoma showed excellent toxicity [62,63]. Thus, our study is in line with previous studies.

Alongside these CuONPs were compared with normal MCF-10A human breast cells. Our results showed that these nanoparticles are less toxic compared to cancer cells. as shown in Fig. 10 (E-H). Henceforth, these nanoparticles are less toxic to normal cells but highly toxic to cancer cells. The higher toxicity is due to cancer cells containing leaky vasculature compared to normal cells and nanoparticles can easily enter cancer cells [64]. Rajamma et al., 2020 reported that copper oxide nanoparticles showed higher toxicity towards cancer cells as compared with normal cells [39]. The toxicity is due to the production of reactive oxygen species (ROS) which cause significant cell death in HeLa cells [65]. Previous studies also showed that biologically synthesized copper oxide nanoparticles release ROS that induce cell death in A549 cells due to mitochondrial membrane damage [66]. A study report from Vijayakumar et al., reported that the

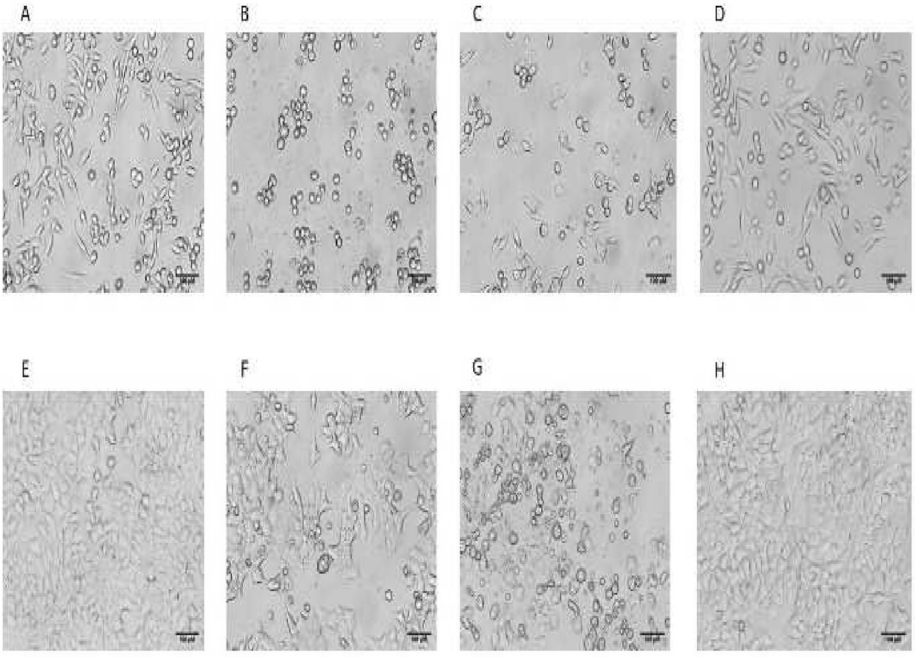

Fig. 10. Morphological changes occurred on MDB MA 231 cells and MCF 10 A upon using biosynthesised CuONPs; A: MDA- MB 231 Breast cancer cells treated with CuONPs at 12.5 uG; B: MDA- MB 231 Breast cancer cells treated with CuONPs at 200 uG; C: MDA- MB 231 Breast cancer cells treated with standard; D: Untreated MDA- MB 231 Breast cancer cells; E: MCF- 10 A normal human breast cells treated with CuONPs at 12.5 uG; F: MCF- 10 A normal human breast cells treated with CuONPs at 200 uG; G: MCF- 10 A normal human breast cells treated with standard; H: Untreated MCF- 10 A normal human breast cells.

green synthesis of gold nanoparticles shows effectiveness in inhibiting the viability of human A549 lung cancer cells [67]. Thus, as per the previous reports, the toxicity of CuONPs towards breast cancer cells is due to the release of ROS which causes cell damage and cell death in the breast cancer cells. Therefore, these nanoparticles could be used to treat breast cancer as well as antibacterial agents, and further studies on their toxicity are recommended.

4. Conclusion

In conclusion, this *A.niger* is a good candidate for the reduction of copper metal into copper oxide nanoparticles. The synthesised NPs are well stable and within the nanorange in size. These NPs exhibit excellent antibacterial activity against both gram positive and negative pathogens, and showed excellent antioxidant activity. Furthermore, these NPs proved good toxicity towards breast cancer cells and least toxic to the normal cells. Therefore, these CuONPs could be used as a potential antibacterial and anticancer agent which require in-depth toxicity analysis before using them as a potential antibacterial and anti-cancer agent.

Conflicts of interest

We wish to confirm that there are no conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

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