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RESEARCH ARTICLE OPEN ACCESS

Preparation of Zinc Oxide Nanoparticles using *Aspergillus niger* as Antimicrobial and Anticancer Agents

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Abstract

In the current study, zinc oxide nanoparticles (ZnO-NP) were prepared using extracellular extracts of *Aspergillus niger*. Hence, the morphological structure, optical, and surface features of the synthesized nanoparticles were studied by X-ray diffraction, transmission electron microscopy, ultraviolet-visible and infrared absorption by Fourier transform. Use dynamic light scattering and zeta potential measurements to assess colloidal stability. The mean size of the synthetic particles is approximately 20 ± 5 nm and they have a hexagonal crystal structure. In addition, the prepared nanoparticles have strong light absorption in the ultraviolet region of λ = 265 and 370 nm. To achieve the goal of this study, the efficiency of ZnO-NP was determined as an antibacterial and antifungal against different bacterial and fungal strains. It was found that ZnO-NP showed significant antibacterial activity, where the inhibition zones were varied from 21 to 35mm in diameter against six bacterial species (i.e. *K. pneumoniae, E. coli, A. baumannii, P. aeruginosa, S. aureus,* and *S. haemolyticus*). In such a case, the minimal inhibitory concentration of zinc oxide nanoparticles against bacterial strains were 50, 12.5, 12.5, 50, 12.5, and 12.5µg/ml for *K. pneumoniae, E. coli, A. baumannii, P. aeruginosa, S. aureus,* and *S. haemolyticus,* respectively. Furthermore, ZnO-NP exhibits an antifungal behaviour against four fungal species (i.e., *A. niger, P. marneffei, C. glabrata,* and *C. parapsilosis*) with inhibition zone from 18 to 35mm in diameter. Whereas, the MICs for fungal isolates were 12.5µg/ml except *A. niger* was at 25µg/ml. Wi-38 cells were treated with ZnO-NPs exhibited different levels of cytotoxicity dependent upon the concentration of ZnO NPs using the MTT assay with IC_{50}~800.42. Therefore, the present study introduces a facile and cost-effective extracellular green-synthesis of ZnO-NP to be used as antimicrobial and anticancer agents.

Keywords: Zinc oxide nanoparticles, extracellular green synthesis, nanoparticles characters, anti-bacterial and anti-fungal

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INTRODUCTION

Recently, in the presence of extracellular or intracellular extracts of microorganisms such as bacteria, fungi, and yeast strains, the biosynthesis of nanostructures has attracted attention due to its ease of preparation, photoelectric and physicochemical properties; in addition, it has excellent antimicrobial activity[1-4]. Although the reduction rate of metal ions in nanoparticles is faster, the existence of extracellular extracts (i.e. plants and microorganisms) is much faster under environmental conditions of temperature and pressure[5-7]. Several studies have been devoted to improving the synthetic efficiency such as control of particle size, shape, and mono-dispersity[8-10]. Zinc oxide (ZnO) is an inorganic metal oxide that has a wide range of applications, such as medicines and cosmetics, and is mainly used to fight infections in the form of ointments and creams. Similarly, ZnONPs combined with other metal oxides (such as SiO_2 NPs) is used as UV blockers in textiles and induce superhydrophobicity and bacterial growth inhibitory properties[11-13]. In addition, ZnO NPs are widely used as an additive in ceramics, glass, cement, rubber, lubricants, paints, ointments, adhesives, pigments, foods, batteries, first-aid tapes, etc.[14,15]. Moreover, ZnO NPs have higher catalytic efficiency, and chemical stability, the antimicrobial mechanism of ZnO NPs is still open and not well known. Some authors suggest that ZnO NPs didn't exhibit any antimicrobial activity by themselves, but enhances the antimicrobial activity of certain antibiotics[16]. The antimicrobial effects of ZnO NPs against many foodborne pathogens have been reported, such as *E. coli* O157:H7[17], *C. jejuni*[18], *P. aeruginosa*[19], *Salmonella* spp.[20] and *Staphylococcus aureus*[21]. In addition, ZnO-NPs have antibacterial efficacy against both the Gram-negative and Gram-positive bacteria and many viruses such as *Escherichia coli, Streptococcus pyogenes, Staphylococcus aureus, Enterococcus faecalis, Bacillus subtillis, B. atrophaenus, Salmonella typhimurium, Klebisella pnumoniae, Alfalfa mosaic virus,* etc.[22-26]. Furthermore, ZnO-NPs have anti-fungal activity against several types of molds such as *Aspergillus flavus* and *A. fumigatus* and many other microscopic molds, such as *Botrytis cinerea, Penicillium expansum, Phanerochaete salmonicolor, Candida albicans*, and *Fusarium oxysporum*[27,28]. Up-to-date, some studies have been devoted to reporting that fungal-mediated synthesis is more advantageous because fungi are super accumulators, showing economic viability and easy expansion of synthesis[29,30]. Furthermore, a large number of extracellular proteins and enzymes have the dual function of producing and coating mono-disperse nanoparticles[31]. In addition, fungi are very tolerant of higher concentrations of metals and, due to the presence of a large number of extracellular proteins and redox enzymes; they have a large number of functional groups that can reduce metal ions to zero-valence metal nanoparticles[32]. The cytotoxic mechanism of ZnO-NPs is not yet fully understood, but hydroxyl radicals ($OH^{\bullet}$), superoxide anions (O_2^-), and per-hydroxyl radicals ($HO_2^{\bullet}$) generated from the surface of ZnO are believed to be the main components. When Nanoparticles interact with cells, cellular protection mechanisms are activated to minimize damage; however, if the production of highly active free radicals more exceeds the cell's antioxidant defence capabilities, biomolecules will undergo oxidative damage, leading to cell death[33,34].

In this study, green ZnO nanoparticles have been synthesized by using extracellular proteins and enzymes present in *Aspergillus niger* extract to reduce Zn^{2+} present in zinc acetate as a metal precursor. In addition, antimicrobial agents will be studied, including antibacterial and fungal activity against bacterial and fungal species.

Experimental Section

Materials

Zinc acetate salt ($Zn(CH_3COO)_2$) was pursued from Sigma-Aldrich, Whatman filter paper. Glassware was washed with sterile distilled water more than one time then dried in an electric oven before using to remove any contaminations. Muller-Hinton agar[35], Nutrient Agar medium[36]. Nutrient Broth medium[37], Potato Dextrose Agar (PDA)[38] and Potato Dextrose Broth (PDB)[39], from Sigma Aldrich- Germany.

Methods

Isolation and purification of fungal isolates

Soil samples were taken from the garden of the Botany and Microbiology Dep., Faculty of Science, Al-Azhar University, Cairo, Egypt, at approximately 10 cm depth. One gram of soil sample collected was suspended

in 100 ml of distilled and sterilized water. One milliliter (1 ml) from 10^{-1} to10^{-6} dilutions of soil suspension of the sample was placed on the plate[40], with media Czapex-Dox Agar (CDA) media g/L: Sucrose: 30g. $NaNO_3$: 2g. , KH_2HPO_4: 1g. KCl: 0.5g. $MgSO_4.7H_2O$: 0.5g, $FeSO_4.7H_2O$: 0.01g, Agar: 20g, and distilled water: 1000 ml, pH7, supplemented with chloramphenicol 0.5% to suppress bacterial growth[41]. A triplicate of plates was used for each particular dilution. Then, the plates were incubated for seven days at 27±2°C until colonies appeared. The isolation of fungi was carried out by using a CDA medium according to the method described by[42].

Identification of most potent fungal isolate

Morphological, cultural and microscopic examination for fungal isolate

A Purified fungal isolate was identified based on routine cultural and morphological characteristics. Fungal genera and species were identified according to standard manuals[43,44]. Macroscopic examinations were carried out to study the culture characteristics, including colony surface color, reverse color, colony growth rate, and pigmentation.

Molecular identification for fungal isolate

Use the Easy Pure Genomic DNA Extraction Kit to isolate DNA samples from the tested fungal strains, and use Nano-Drop to measure the purity and appropriate concentration of the DNA samples. Four samples of *Aspergillus niger* DNA for PCR reactions. Two fungal universal primers, FW 5-ATGGGCAAGGCACCAAATAA-3 and RW 5-TGGAAATGGATC CAAGAATG-3 were used to carry out the PCR reaction of 4 fungal 18s rDNA gene amplification. The PCR reaction conditions were adjusted to 40 ng (6 µl) DNA template (fungi) and 8.5 µl master mix, which includes a mixture of dNTP, $MgCl_2$, Taq polymerase, and PCR buffer. Add primers separately after preparation from the lyophilized stock solution (each primer is 1 µmol/l). The PCR conditions were adjusted for the denaturation step of 92.3°C, the hybridization step of 55.6°C and the extension step of 71.9°C. The number of PCR cycles was 36 cycles, and the El-Dokki PCR thermal cycler from the National Research Center (NRC) of Giza, Egypt was used. Use a 1% agarose gel to separate the amplified DNA product and, DNA markers [Gene Ruler 100 bp DNA Ladder (SM0241)] by electrophoresis. The gel was stained with ethidium bromide and the band profile was recorded using the UV gel documentation system. Purify, and sequence the amplified DNA products. The 4 sequences were analyzed and tested against the most closed sequence in Gen Bank NCBI through BLST, and the phylogenetic tree was designed using MEGA 7 software.

Preparation of fungal biomass extract

A Purified fungal isolate was tested for ZnO NP biosynthesis by growing in Czapex Dox broth (CBD)[45]. Two discs (0.7 mm) of purified fungal isolates were freshly incubated in 50 ml of CBD in a 250 ml Erlenmeyer flask as a fermentation medium. Incubate at rpm for 6 days. The fungal biomass is collected by passing through two layers of medical gauze and washed with distilled and sterile water to remove any adhering medium components. Then, ten (10) g of fungal biomass were suspended in 100 ml of distilled water in a 250 ml Erlenmeyer flask and stirred at 150 rpm at 28 ± 2°C for 72 hours. Then, the cells were discarded by Whatman No. 1 filter paper to obtain a biomass filtrate for nanoparticle synthesis.

Extracellular green synthesis of Zinc oxide nanoparticles using fungal isolates

Typically, in a 250 ml reaction vessel, an equi-volume ratio (1:1) aqueous solution of zinc acetate (1 mM/100 ml, Zn $(CH_3COO)_2$) was added to 100 ml of fungal extract filtrate under orbital shaking at 150 rpm. The reaction was incubated at pH ~ 6.5, and a temperature of 32°C for 72 hr. in dark conditions. A white precipitate is formed indicating to the formation of ZnONPs. The as-prepared ZnONPs were washed several times with sterile distilled water then centrifuged at 10,000 rpm for 10 min[46].

Characterization

Use the JASCO 730 Double Beam Spectrophotometer to obtain the optical UV-Vis absorption characteristics. The absorption spectrum was recorded in the range of 200 to 900 nm and the increase in wavelength was approximately 0.2 nm. The JEOL Transmission Electron Microscope (TEM), model 1200EX, is used to study micrographs of samples obtained at an operating voltage of 120 kV. In addition, the Ultima IV powder diffractometer (Rigaku) has been used for X-ray diffraction (XRD) measurements in the 2 rango range of 20-70 degrees using a Cu

objective with Kα1 = 1.54060 Å. X-ray scanning is performed in a 2θ / θ continuous mode at a speed of 2 degrees per minute with a step size of 0.02. The tube voltage and tube current are maintained at 40 kV and 40 mA. It Uses the Malvern Zetasizer Nano (ZS) instrument and He/Ne laser (633 nm) to collect backscattering optics at an angle of 173° to measure the size distribution and zeta potential of the sterilized ZnONP. In addition, according to Vivek et al.[47], the JASCO 6700 Fourier Transform Infrared Spectrometer (FT-IR) was used to obtain the FT-IR of green synthetic ZnONP in the range of 400 to 4000 cm^{-1}.

Antimicrobial property

Tested microorganisms

Bacteria

Six isolates, *Klebsiella pneumoniae, Escherichia coli, Acinitobacter baumannii, Pseudomonas aeruginosa, Staphylococcus aureus,* and *Staphylococcus haemolyticus* were isolated from clinical samples and identified based on culture, morphology, and biochemical analysis according to Bergey's manual[48]. In addition, a Vitek2 system was carried out to confirm the identity.

Fungi/Yeast

Four isolates were *Aspergilus niger, Penicillium marneffei, Candida glabrata,* and *Candida parapsilosis* isolated from clinical samples and identified in the Mycology Lab. of the Botany and Microbiology Dep. Faculty of Science, Al-Azhar University, Cairo, Egypt.

Antimicrobial Experiments

The zinc oxide nanoparticles (ZnO-NPs) synthesized from *Aspergillus niger* biomass were tested for antimicrobial activity via two methods; the agar-well diffusion method and the broth micro-dilution assay.

Agar well diffusion method

Apure cultures of the tested bacteria were sub-cultured in nutrient broth and each strain was uniformly spread on sterilized petri plates with Muller-Hinton agar. A circular well of 6 mm in diameter was made in plates using a sterile cork-borer. Each well was loaded with (50 µl) ZnO-NPs to check the antibacterial activity and the plates were incubated at 37°C overnight and the zones of inhibition were measured. Additionally, the antifungal activity of zinc oxide nanoparticles was examined against tested fungal species. The strains were maintained on CDA at 28°C and 5-d old cultures were used for antifungal analysis. Pour three to four milliliters of sterile normal saline onto the fungal growth; gently scrape to collect the conidia. Spread 100 µl of this liquid spore suspension evenly on a fresh potato dextrose agar (PDA) plate. Use a sterile cork-borer to make a 6 mm in diameter circular hole in the plate. Each well was filled with (50 µl) zinc oxide nanoparticles to check antifungal activity, then incubated the plate at 25°C for 2-3 days and measured the zone of inhibition[49].

Broth microdilution assay

The suspension was turbid to 0.5 McFarland standards (10^8 cfu / ml) produced by fresh subcultures of bacterial and fungal/yeast Mueller Hinton Broth (MHB) and Potato Dextrose Broth (PDB), respectively. The corresponding suspension was diluted to 10^6 cfu / ml. Prepared microbial inoculation (100 µl) was added to each well of a sterile flat bottom 96-well microtiter plate containing the tested concentrations of ZnO-NP (100 µl/well). As a result, a final inoculation concentration of 5x105cfu/ml was obtained from each well. The tested ZnO-NP used in the growth control of the tested microorganisms was contained in wells containing microbial suspensions and other well plates containing double background control. The optical density was measured at 620 nm after 24 hours at 37°C for bacteria, 48 hours at 28°C for mold / yeast, using an ELISA microplate reader (Sunrise ™ -TECAN, Switzerland) at the Faculty of Science Al-Azhar University in Cairo, Egypt. Finally, the cell concentration was converted to an average growth inhibition percentage (%). The rate of decrease in microbial growth (GR %) was estimated as follows, based on the treatment of the control group (excluding the ZnONPs). GR% = CT / C x100 where C is the treated cell concentration of the control group and T is the ZnONPs process. Three replicas were considered. The Results were reported as the mean ± SE of the experiments[50].

Preparation of Resazurin Solution

The concentration of resazurin stain solution is 0.02% (w/v)[51]. Dissolve 0.002 g of resazurin stain salt fine powder in 10 ml of sterile distilled water and vortex. The complete mixture is filtered through a Millipore membrane filter (0.2 µm). This solution can be stored at 4°C for 2 weeks.

Determination of Minimum Inhibitory Concentration (MIC)

The minimum inhibitory concentration of biosynthesized ZnO NPs was evaluated against bacterial and fungal strains via a method described in the guide[50]. The MIC test is evaluated on a 96-well microtiter plate with a round bottom by a standard broth microdilution assay.

For bacterial strains; the concentration of the bacterial inoculum was adjusted to 106 cfu /ml. Add 100 µl of biosynthesized ZnO-NPs stock solution (400µg/mL) to the first well, dilute 2 times with the bacterial inoculum, and use 100 µL media MHB from column 4 to column 12. The fourth column of the plate contains the highest concentration of ZnO NP, while the 12th column has the lowest concentration. Column 1 serves as a positive control (medium and bacterial inoculum), and column 2 serves as a negative control (medium only). Add 30 µl resazurin solutions to each well of the microtiter plate and incubate for 24 h at 37°C. A color change is observed. Blue/purple means no bacterial growth, while pink/colorless means bacterial growth. The MIC value is obtained at the lowest concentration of the antibacterial agent that inhibits the growth of bacteria, the color remains blue.

For fungal strains, a suspension of the original inoculum in sterile saline containing 1% Tween 80 (supplied by Trek Diagnostic Systems) was prepared from 7-day old cultures grown on a PDA slope. For an inoculation density of 95%, the actual stock inoculation suspension calculated by quantifying the inoculation varies between 0.9 × 10^6 and 4.5 × 10^6 cfu / ml. One hundred (100 µl) biosynthesized ZnO NPs stock solution (400µg/mL) was added to the first well of another new plate (96-well microtiter plate with a round bottom). Followed by a 100 µl of fungal conidia inoculum suspension in liquid potato dextrose agar (PDA) was added. The microdilution tray is incubated at 30°C and checked after 4 days of incubation. The endpoint of MIC is the lowest concentration of ZnNP showing no growth or complete inhibition of growth (100% inhibition)[52]. On the other hand, as described above, unicellular fungi are evaluated as bacteria.

Cytotoxicity assay to evaluate nanoparticles toxicity by using tissue culture

According to Riss and Moravec[53] (MTT protocol), the culture plate with (96-well tissue) was cultivated with 1×10^5 cells/ml (100 µl/well) and incubated for 24 hours at 37°C to form an integrated monolayer. After forming a collected cell sheet, pour out the growth medium from the 96-well microtiter plate, and the cell monolayer was washed twice with a washing medium (duple dilution) in a medium containing 2% serum (maintenance medium). 0.1 ml of each dilution test was prepared in different wells, 3 wells were left as controls, and only contained medium. Incubate the plate at 37°C. Check the cells for any changes that indicate physical toxicity, such as partial or complete damage of monolayer, shrinkage, rounding, or granulation of the cells. 20ul of MTT solution was added to each well, then shacked for 5 minutes at 150 rpm to optimum mix the MTT in the medium completely, then incubated for 1-5 hours at 37°C and 5% CO_2 to let the MTT metabolize. Then remove the medium, dry the towel if necessary to remove debris, resuspend the formazan (metabolic product MTT) in 200 ul DMSO, and shake it for 5 minutes at 150 rpm to remove the formazan mixed thoroughly into the medium. Finally, record the optical density at a wavelength of 560 nm and subtract the background at 620 nanometers. The optical density must be directly related to the number of cells.

Statistical analysis

All the experiments were performed in triplicate and data were analysed. Analyses were performed as prescribed by Kareem et al.[54].

RESULTS AND DISCUSSION

Identification of Fungal Isolates

Cultural and morphological characterization

The obtained results showed that the isolate (MEKKY A1) belonged to *Aspergillus niger*. Cultural and morphological characterization data are depicted in Table 1 and Fig. 1. The colonies' diameter was in the range of 35-40 mm after incubation for 4 days at 30°C. Rapid rate growth with black or brownish black appearance in observed colour and Pale begin reverse colour. Conidia are spherical, rough walled, 2-5 µm in size. The sterigmata were Biseriate, (Metulae and Phialides), vesicle globose in shape 40.0-60.0 µm, conidial heads with a radial shape, and the conidiophore was 11.9-19.9 µm in diameter, up to

Table 1. Culture and microscopic characteristics of fungal isolate (MEKKY A1) growing on CYA

Character	Examination
Culture Examination	
Growth characteristics	Colonies grow moderately with 35-40 mm in diameter in 4 days at 30°C on CYA, with black or brownish black. Reverse was pale beige.
Microscopic Examination	
Conidiophore	11.9-19.9 µm in diameter, up to 4.0 mm extent
Vesicle	Globose shape 40.0-60.0 µm.
Sterigmata	Biseriate, (Metulae and Phialides)
Conidia	Spherical, rough walled 2-5 µm.
Conidial heads	Radiate shape.

4.0 mm in extent. From previous data, the fungal isolate (MEKKY A1) followed the *Aspergillus* sp.

Molecular identification of the most potent fungal isolate

Phylogenetic analysis of isolate (MEKKY A1) suggested that this isolate have high similarity (99%) with strain *Aspergillus niger*. The sequence obtained from the current study was deposited under accession number MT645619.1 in Gene Bank for *Aspergillus* Fig. 2.

Synthesis and characterization of extracellular synthesized zinc oxide nanoparticles

Using fungal biomass from *A. niger*, ZnO-NPs were completely synthesized outside the fungal cell. In this process, the formulation of ZnO-NP is determined through the formation of a white precipitate at the end of the reaction. This is due to the reduction of Zn^{2+} ions in zinc acetate in ZnO-NP in the presence of an aqueous medium, (Fig. 3). The fungal filtrate from the fungal isolate acts as both a reducing and a protective agent.

This is due to the presence of a set of extracellular proteins and enzymes. Jane et al.[55] *A. aeneus*, as a stabilizer, was found to synthesize spherical ZnO nanoparticles coated with protein molecules. Furthermore, they showed that the fungal extracellular proteins' role in the synthesis of nanoparticles suggests that the biosynthesis process is not enzymatic, but involves amino acids found in the protein chain.

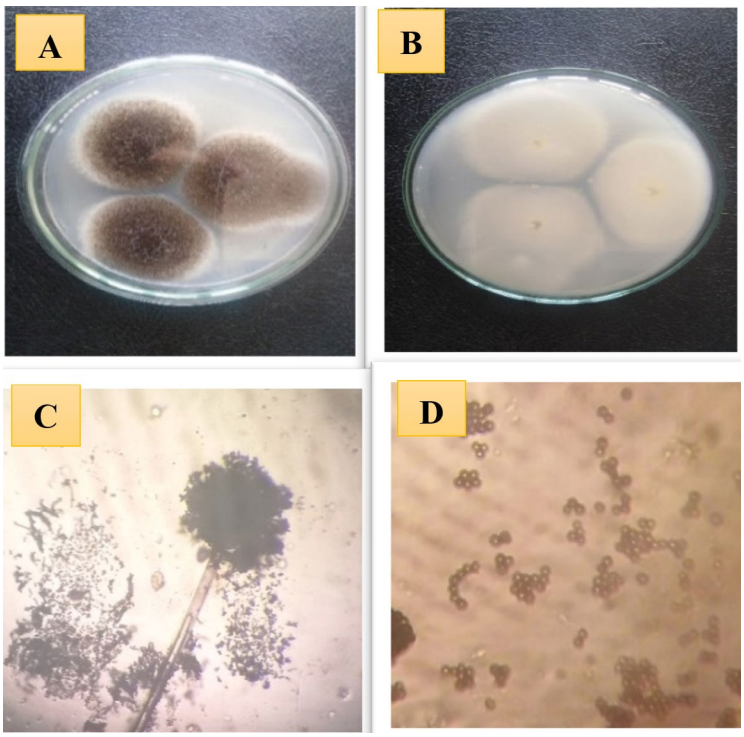

Fig. 1. Morphological and microscopic characteristic of fungal isolate (MEKKY A1) *Aspergillus* sp. (A) colony of fungal isolate *Aspergillus* sp. on CYA, (B) reverse colony of fungal isolate *Aspergillus* sp., (C and D), bright field microscope (X= 20×40).

Several authors showed that *A. fumigatus*[56], *A. niger*[57], *F. oxysporum*[58] and *P. citrinum*[59], have the ability to synthesize nanoparticles from metal salts. Moreover, the formation of the biosynthesized zinc oxide nanoparticles was confirmed using UV-Vis spectroscopy measurements shown in Fig. 4.

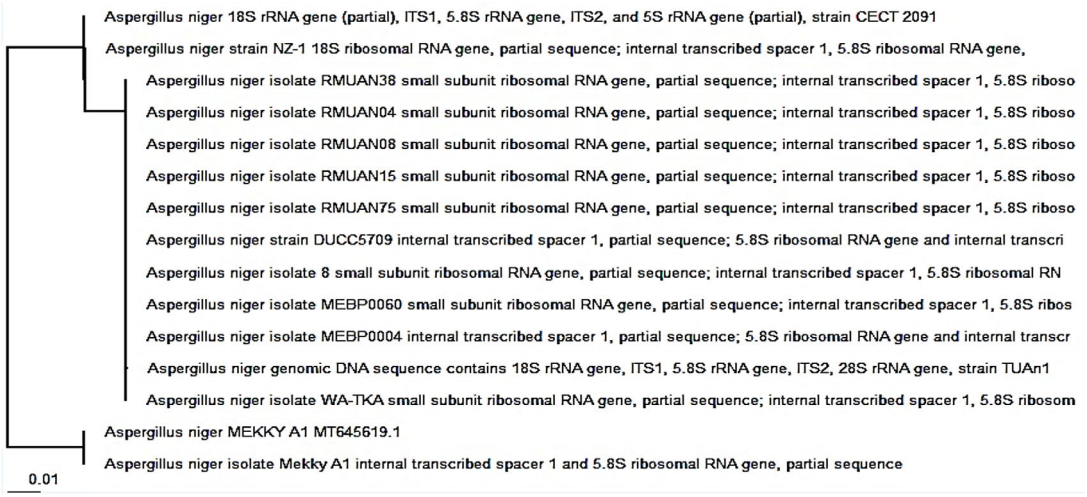

Fig. 2. Phylogenetic tree of gene sequences of the *Aspergillus niger* isolate with the sequences retrieved from NCB Gene Bank site.

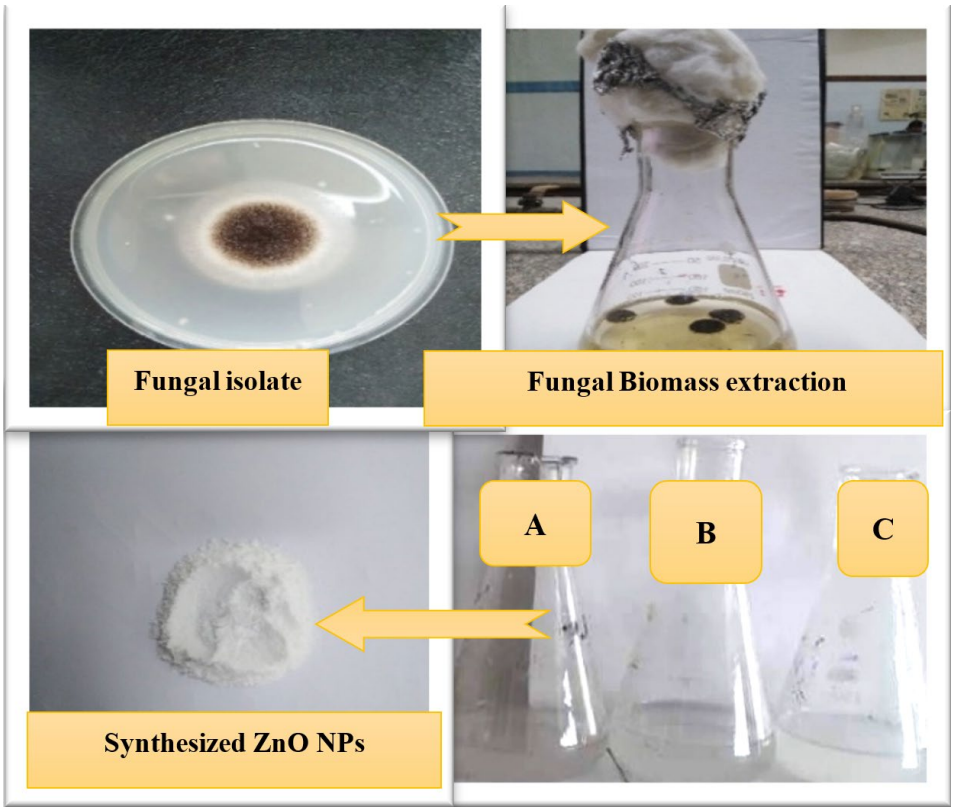

Fig. 3. Scheme for extracellular synthesis of ZnO NPs, (A) fungal biomass extract, (B) Zinc acetate solution and (C) synthesized ZnOPs solution.

ZnO-NPs showed strong absorption bands at 265 and 370 nm in the ultraviolet region, indicating the formation of ZnO-NPs. The morphology and structural characteristics of the prepared ZnO-NPs were confirmed by TEM and XRD, as shown in Figure 5 and 6, respectively. TEM micrographs show that ZnO NPs are quasi-spherical without any agglomeration and have a polydispersity distribution. The average particle size is about 20 ± 5 nm Fig. 5. The XRD pattern shown in Fig. 6 shows three stronger and narrower reflections near 2θ 31.7°, 34.5°, and 36.2°, indicating that the lattice spacing (dhkl) is 2.81, 2.6, and 2.5 Å, which respectively revealed the (100), (002), (101) crystal reflections of the hexagonal structure (hcp) of zinc oxide atoms (reference code 010890510)[60].

Furthermore, the colloidal stability of ZnO NP has been studied using dynamic light scattering technology (DLS) and zeta potential measurement, as shown in Table 2. The hydrodynamic diameter (HD) of the ZnO NPs prepared in the solution carrier is approximately 680.7 ± 84.79 nm, and the polydispersity index (PDI) is 0.908, indicating that the polydisperse particles aggregate because of the high hydrophilicity of the ZnO-NPs prepared[61], as shown in Fig. (7a) and Table (2) However, the zeta potential (η) of ZnO NPs prepared from *Aspergillus niger* extracellular extracts is approximately -14.4

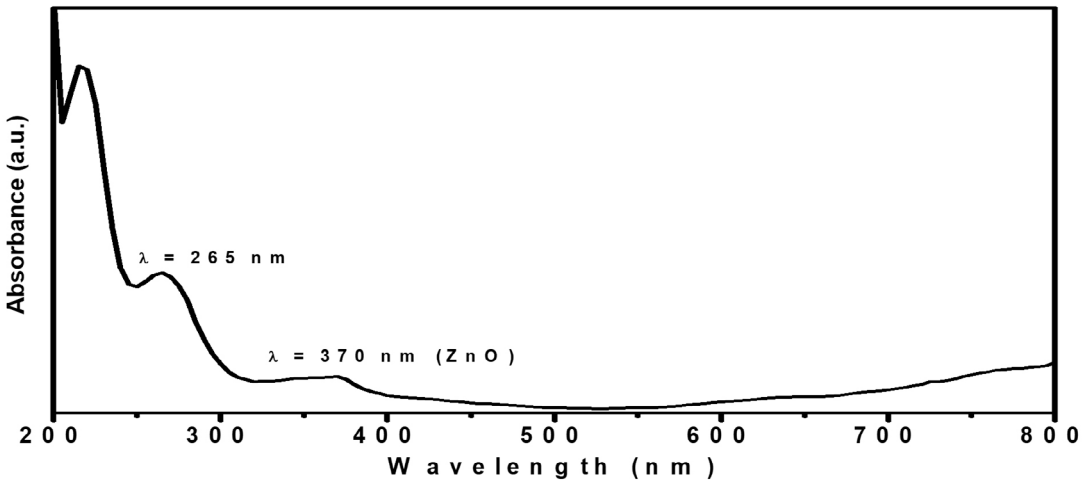

Fig. 4. The spectra of UV-Vis absorption of green synthesized ZnO NPs using (A. niger) extracellular extract.

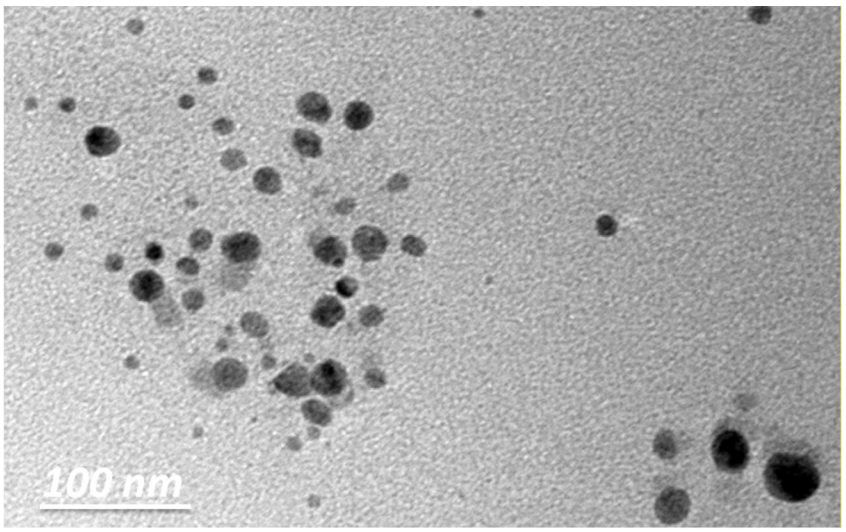

Fig. 5. TEM micrographs at scale bar 100 nm of green synthesized ZnO-NPs using (*A. niger*) extracellular extract.

± 4.75 mV (Fig. 7a. 7b, Table 2). Negative values indicate the stabilization of nanoparticles and prevent the clustering of nanoparticles[62]. The negative potential value may be due to the capping

Table 2. DLS and zeta-potential measurements of green synthesized ZnO NPs

Sample Name	DLS Data Hydrodynamic Diameter (HD, nm)	Polydispersity Index (PDI)	Zeta-potential (η, mV)
Zinc oxide (ZnO)	680.7 ± 84.79 nm	0.908	-14.4 ±4.75

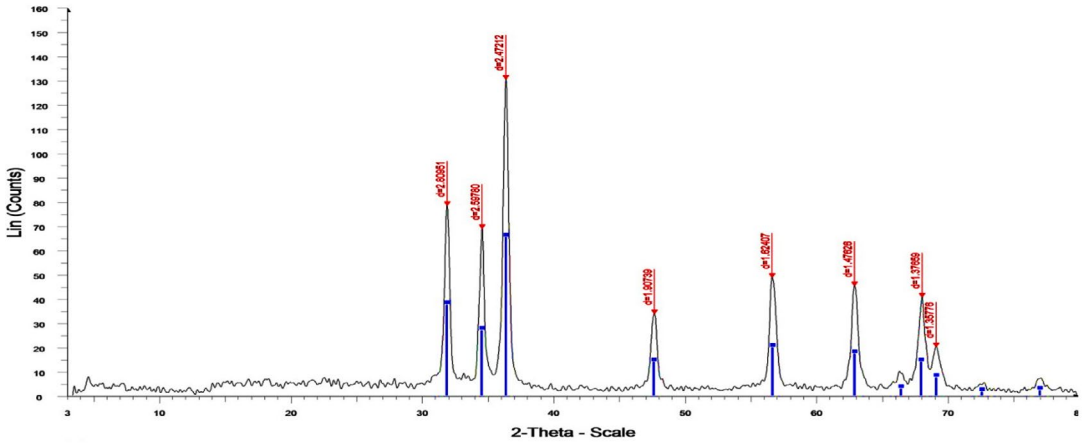

Fig. 6. XRD patterns of green synthesized ZnO-NPs using (*A. niger*) extracellular extract.

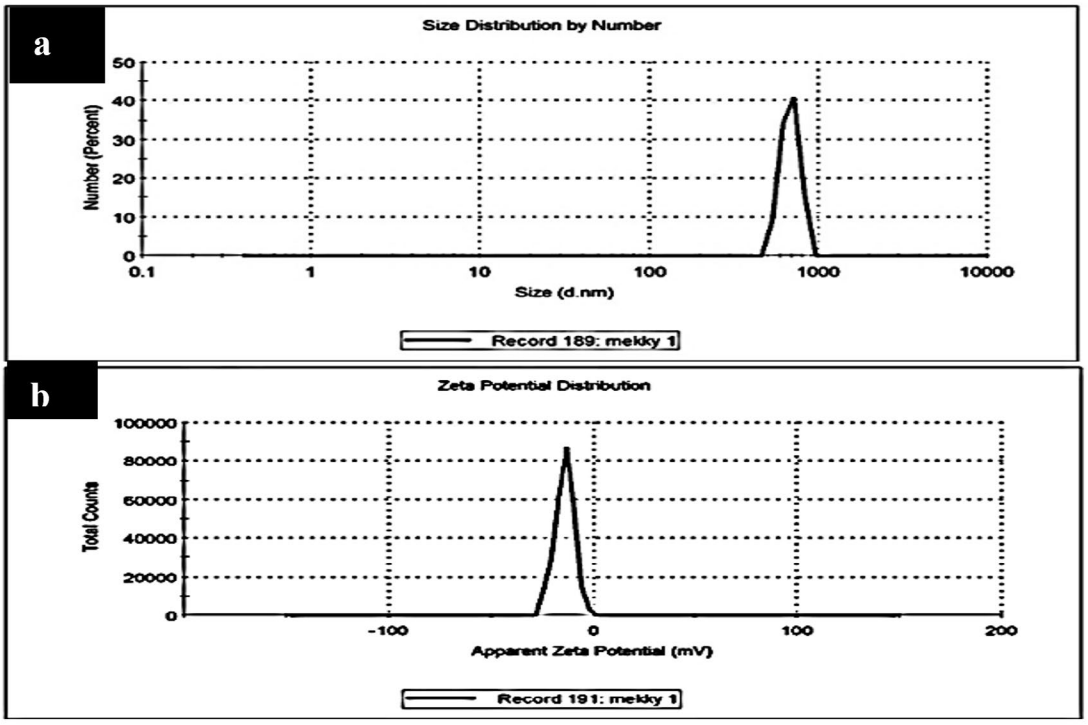

Fig. 7. (a) DLS and (b) Zeta-potential data for green synthesized ZnO NPs using (*A. niger*) extracellular extract.

effect of biomolecules present in the water extract of *A. niger*.

Finally, FT-IR analysis was performed to determine the probable interactions between ZnO and biologically active molecules, which might be the reason for the composition and stabilization (end-capping) of ZnO NPs (Fig. 8). Peaks Strong 3463, 2343, 1637, 1384, 1255 and 526 cm^{-1}, respectively, indicate the presence of hydroxyl groups (OH), benzene rings, carboxyl groups (C = O), and halogenated alkyl groups. FT-IR results are used to identify potential biomolecules for ZnO-NPs. The significant peaks of the FT-IR results exhibit the conformable values of the amide group (NH stretched 3428 cm^{-1}), the alkene (CC-1637, 1384, 1255, and 526 cm^{-1}), and the ether group (COC-1043, 3 cm^{-1}). Similar observations have been found in flavonoids, triterpenes, and polyphenols[63].

Therefore, terpenoids have been shown to have perfect potency to transform aldehyde groups on metal ions into carboxylic acids.

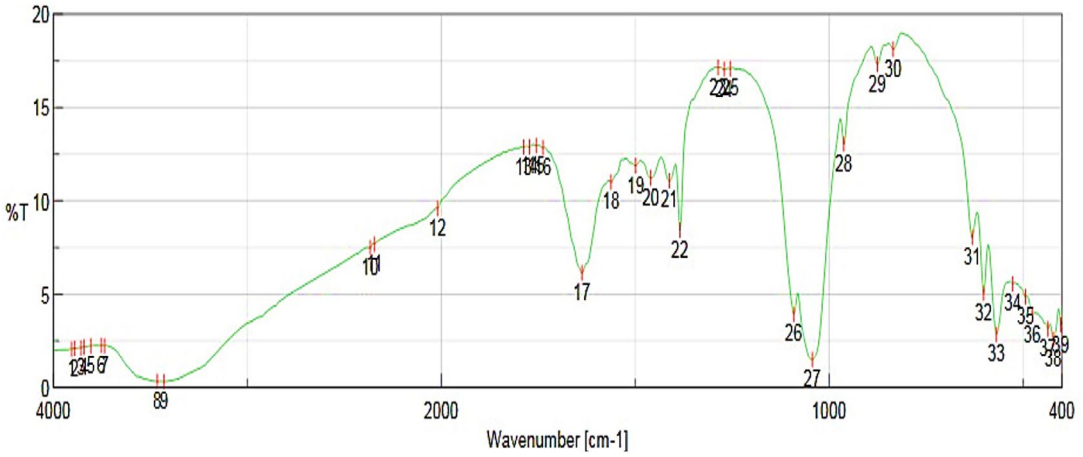

Fig. 8. FT-IR spectra for green synthesized ZnO NPs using *A. niger* extracellular extract.

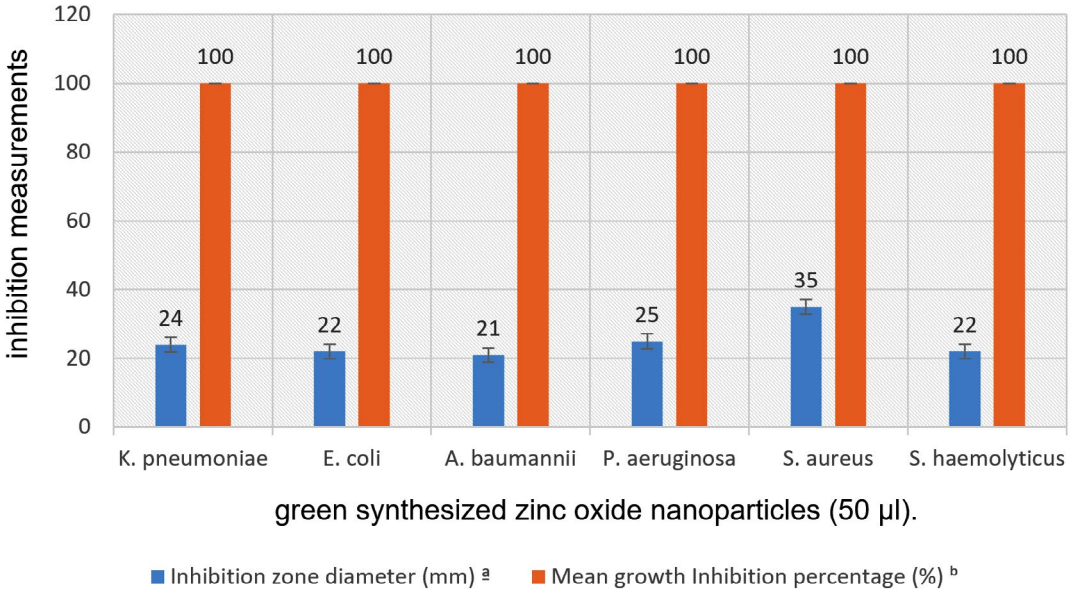

Fig. 9. Antibacterial activity of ZnONPs against the tested bacterial strains diameter of inhibition zone and mean growth inhibition percentage.

Table 3. The antibacterial activity of prepared ZnO-NPs using agar well diffusion method and broth micro dilution assay.

Bacterial Isolates	Antibacterial activity	
	Synthesized ZnONPs nanoparticles (50 µ L)	
	Diameter of Inhibition zone (mm) [a]	Mean growth Inhibition percentage (%) [b]
K. pneumoniae	24 ± 0.75	100 ±0.00
E. coli	22±0.55	100 ±0.22
A. baumannii	21±0.64	100 ±0.44
P. aeruginosa	25±0.82	100 ±0.42
S. aureus	35±0.42	100 ±0.26
S. haemolyticus	22±0.61	100 ±0.22

a) Diameter of Inhibition zone including the well diameter of 6 mm was determined by the agar well diffusion method.
b) Mean growth inhibition percentage (%) was determined by the broth micro dilution method.

Furthermore, the amide group is also responsible for the presence of enzymes, which are responsible for the reductive synthesis and establishment of metal ions. Furthermore, polyphenols have also been shown to be potential reducing agents in the synthesis of NP from ZnO[64,65].

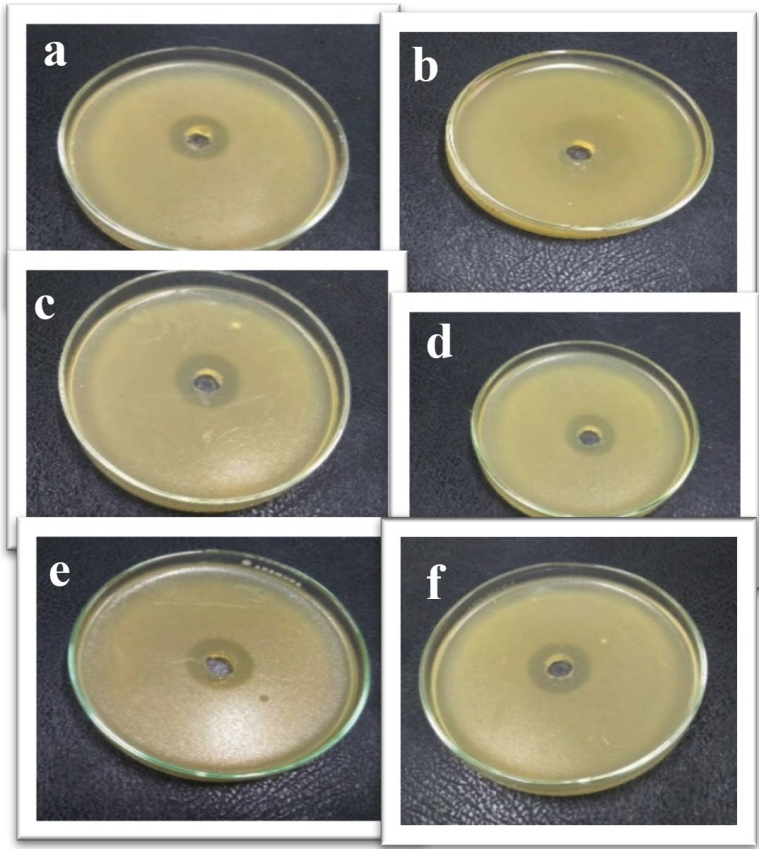

Fig. 10. Inhibition zones produced against tested bacterial isolates (a) *K. pneumoniae,* (b) *E. coli,* (c) *A. baumannii,* (d) *P. aeruginosa,* (e) *S. aureus* and (f) *S. haemolyticus* using green synthesized zinc oxide nanoparticles.

Table 4. The antifungal activity of prepared ZnO-NPs using agar well diffusion method and broth micro dilution assay

Fungal Isolates	Antifungal activity	
	Synthesized ZnO-NPs nanoparticles (50 µ L)	
	Diameter of Inhibition zone (mm) [a]	Mean growth Inhibition percentage (%) [b]
A. niger	35±0.55	100 ±0.22
P. marneffie	31±0.24	100 ±0.31
C. glabrata	20±0.43	100 ±0.22
C. parapsilosis	18±0.41	100 ±0.25

a) Diameter of Inhibition zone including the well diameter of 6 mm was determined by the agar well diffusion method.
b) Mean growth inhibition percentage (%) was determined by the broth micro dilution assay.

Table 5. Minimum inhibitory concentration (MIC) against bacterial strains

Bacterial Strains	MICs of ZnO nanoparticles (µg /ml)
K. pneumoniae	50
E. coli	12.5
A. baumannii	12.5
P. aeruginosa	50
S. aureus	12.5
S. haemolyticus	12.5

Antimicrobial property (antibacterial and antifungal activity)

The biggest healthy risk in every place in the world nowadays is antimicrobial impedance, which damages the health of humans and raises the risk of diseases and death-rate linked to serious life- menacing diseases. Therefore, scientists from many different domains are examining the antibacterial effects of plants on multi-drug resistant bacteria in a new way[66]. The antimicrobial property, including the antibacterial action of green synthesized ZnO- NPs against bacterial strains, has been determined for *K. pneumoniae, E. coli, A. baumannii, P. aeruginosa, S. aureus,* and *S. haemolyticus* as bacterial isolates. In addition, the antifungal activity of *A. niger, P. marneffei, C. glabrata,* and *Candida parapsilosis* as fungal isolates using well diffusion agar prescribed by other authors[67]. The experiment was completed

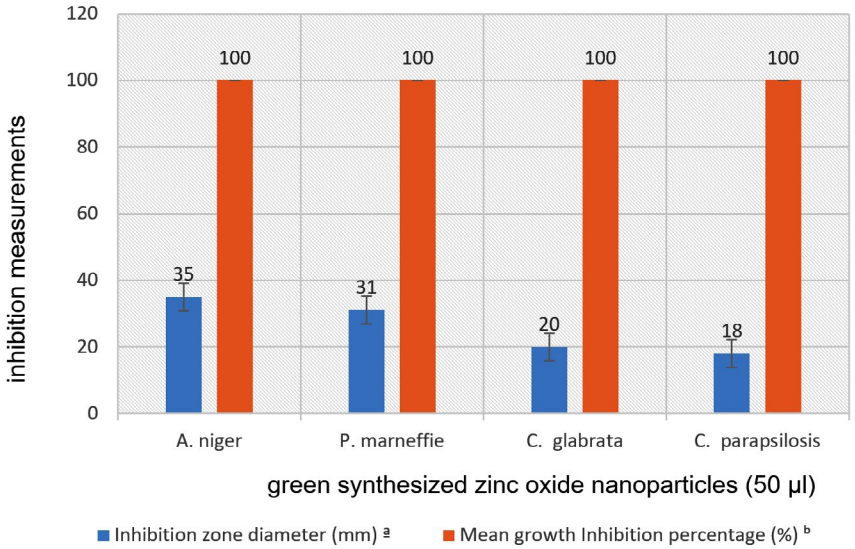

Fig. 11. Anti-fungal activity of ZnONPs against the tested fungal strains diameter of inhibition zone and mean growth inhibition percentage

in triplicate and the data is exhibited in the form of mean ± SE.

Based on the observed results, ZnO-NP synthesized via *A. niger* watery extract was an active antibacterial substance for Gram negative and Gram-positive bacteria. In such cases, the diameter of the inhibitory area (left) is about 24, 22, 21, 25, 35 and 22 mm. In addition Mean growth inhibition percentage was about 100 for all bacterial strains (Table 3 and Fig. 9, 10), for *K. pneumoniae, E. coli, A. baumannii, P. aeruginosa, S. aureus,* and *S. haemolyticus*. These results

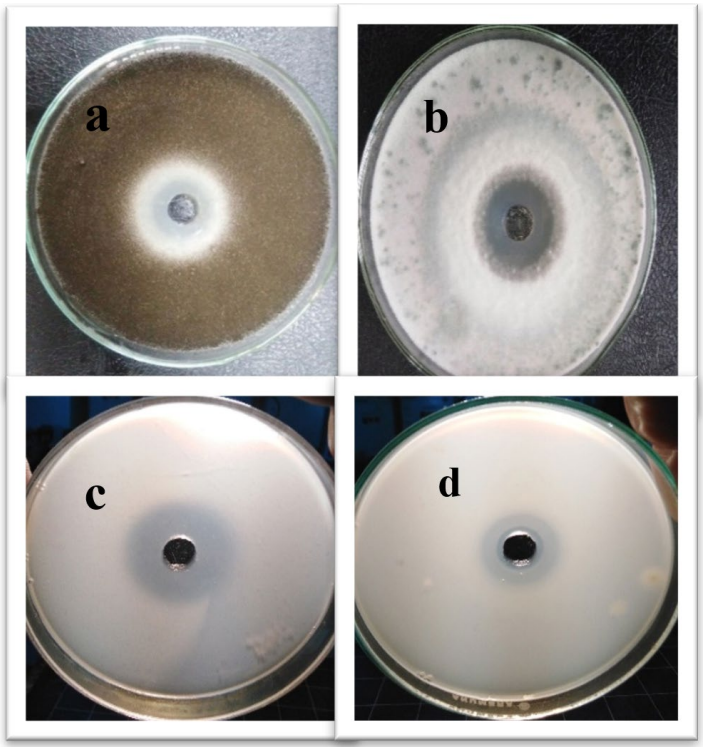

Fig. 12. Inhibition zones produced against (a) *A. niger,* (b) *P. marneffie,* (c) *C.glabrata* and (d) *C. parapsilosis* using as-prepared zinc oxide nanoparticles.

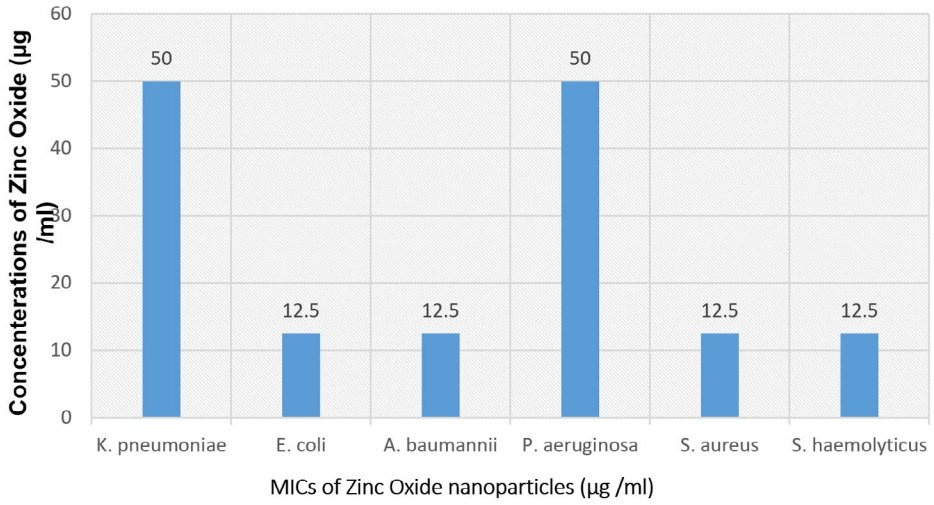

Fig. 13. Minimum inhibitory concentrations (MICs) histogram against the pathogenic bacterial strains.

were consistent with the results informed by Jan et al.[68]. In addition, *Klebsiella Pneumoniae* is a dangerous opportunistic pathogen that is involved in many serious human diseases and is considered to be an important dietary source that is found in many types of food[69]. Jan and colleagues reported that the antimicrobial effect of ZNO-NPS is more effective against the yellow conveyor than against *pseudomonas aeruginosa.*

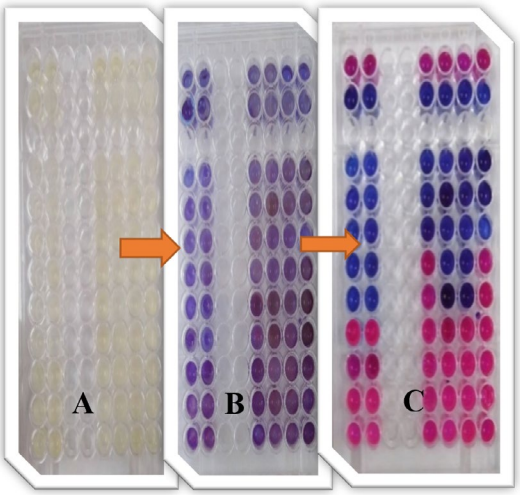

Fig. 14. Ninety-six well Microtiter plates of the colorimetric-XTT assay for determination of MICs values of ZnO NPs against bacterial strains using resazurin salt, (A) cart preparation, (B) after addition resazurin dye and (C) the results after incubation.

Table 6. Minimum inhibitory concentration (MIC) against fungal isolates

Fungal Isolates	MICs of Silver nanoparticles (µg /ml)
A. niger	25
P. marneffie	12.5
C. glabrata	12.5
C. parapsilosis	12.5

Other studies developed by ZnO Spherical nanoparticles by biosynthesis of ZnO-NPs via *Catharanthus roseus* were in the range of 23-57 nm, and we revealed excellent antibacterial activity against *S. aureus, B. thuringiensis,* and *E. coli* Green injury[70]. Not only for bacteria; Sofy et al. reported that spraying Tomato plants with ZnO-NPs Tomv (100 mg / l ZnO-NPs) and to overcome the HVV infection by inducing antioxidant defence systems, it was reported that it is the desired strategy[71]. Other researchers have successfully prepared new salicylidene-iminothiazole and benzylidene-bis-iminothiazole bases to explore their antibacterial and antifungal performance[72]. Because manufactured antibacterial have a negative effect on food quality, and human health, so natural antibacterial are urgently required[73].

Regarding the antifungal activity of as-prepared ZnO NPs against *A. niger, P. marneffie, C. glabrata,* and *C. parapsilosis* as tested fungal isolates have been investigated using the method

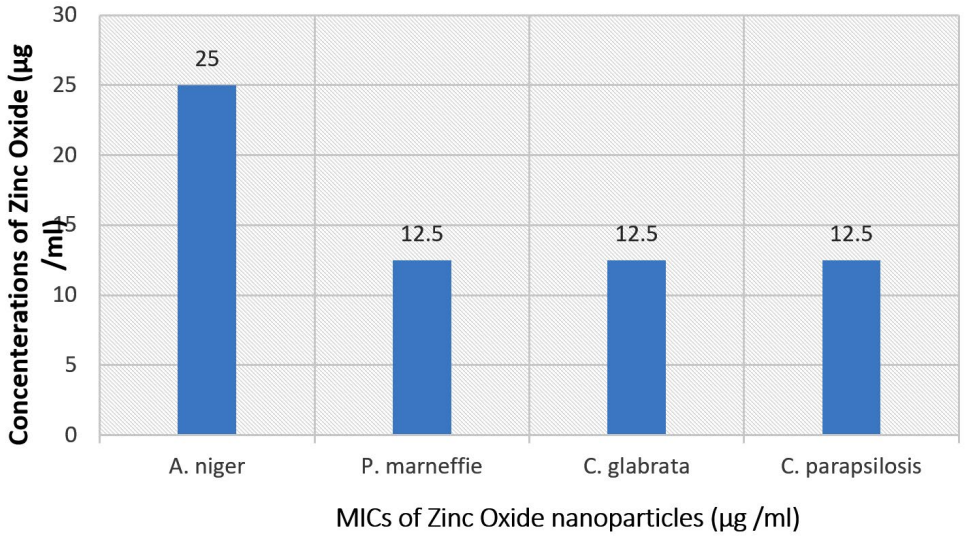

Fig. 15. Minimum inhibitory concentration (MIC) histogram against the pathogenic fungal strains.

of agar well diffusion. The results in Table 4 and Fig. 11, 12 showed that *A. niger* is more susceptible to ZnO NPs than other fungal isolates with an inhibition zone of about 35 mm at an appropriate volume of 50 µl of as-prepared ZnO NPs solution.

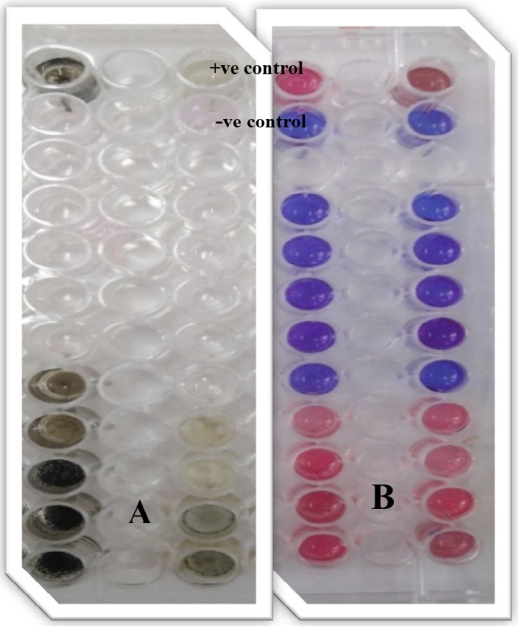

Fig. 16. Ninety-six well Microtiter plates of the colorimetric-XTT assay for determination of MIC values of ZnO NPs against fungal isolates. A: multi cellular fungi (1- *A. niger* and 2- *P. marneffie*), B: unicellular fungi (3- *C. glabrata* and 4- *C. parapsilosis* using resazurin salt).

Whereas, the inhibition zone diameters (IZD) for other fungal isolates were about 31, 20 and 18mm for *P. marneffie, C. glabrata,* and *C. parapsilosis* respectively. In addition, the mean growth inhibition percentage was about 100 for all fungal isolates. Also, ZnO-NPs may have anti-viral activity against several types of viruses such as *Cucumovirus* and *tobamovirus*, and treat many of viral diseases such as Fruit Tree Viroid Diseases, Citrus Gummy Bark Disease and Potato Spindle Tuber Viroid[74-78]. Other researchers successfully prepared a new compound called polyquaternary phosphonium oligochitosans (PQPOCs) to be used as a natural synergistically bio reductant compound to reduce silver (Ag^+) ions into silver nanoparticles and as a stabilizing factor for these silver nanoparticles to manufacture PQPOCs-AgNPs Nano-biocompunds for use as an antiviral[79]. The experiment was completed in triplicate and the data is exhibited in the form of mean ± SE.

Determination of minimum inhibitory concentrations (MICs)

The minimum inhibitory concentrations (MICs) values of ZnO NPs against the bacterial strains ranged from 12.5 µg/ml to 50 µg/ml (Table 5). *E. coli, A. baumannii, S. aureus* and *S. haemolyticus* showed an MIC of 12.5 µg/mL. While *K. pneumoiae* and *P. aeruginosa* showed an MIC of 50 µg/ml.

Resazurin tincture was used in this study as an indicator to determine microbial cell

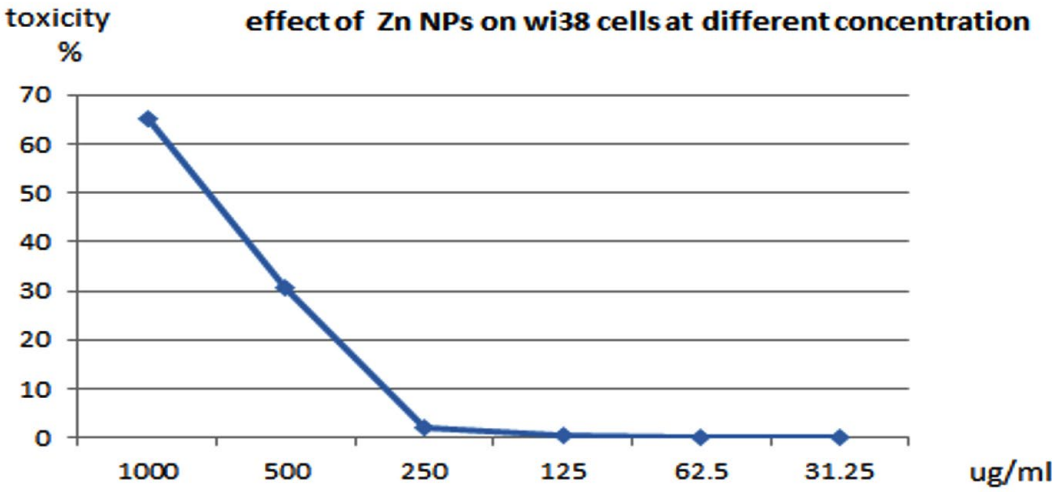

Fig. 17. Effect of ZnONP on wi38 cells at different concentrations.

Table 7. Effect of ZnONPs on wi38 cells at different concentrations

ID	Conc. ug/ml	O.D			Mean O.D	ST.E	Viability %	Toxicity %	IC_{50}
Wi38	1:2	0.362	0.358	0.378	0.366	0.00611	100	0	------
	1000	0.14	0.125	0.116	0.127	0.007	34.69945355	65.30054645	
	500	0.268	0.251	0.243	0.254	0.007371	69.3989071	30.6010929	
	250	0.348	0.375	0.352	0.358333	0.008413	97.90528233	2.094717668	800.42
ZnONPs	125	0.368	0.36	0.364	0.364	0.002309	99.45355191	0.546448087	
	62.5	0.373	0.365	0.362	0.366667	0.003283	100.1821494	0	
	31.25	0.377	0.359	0.363	0.366333	0.005457	100.0910747	0	

Wi-38: human cell strain used. O.D: mean optical density. ST.E: Standard Divisions Error. IC50: half maximal inhibitory concentration.

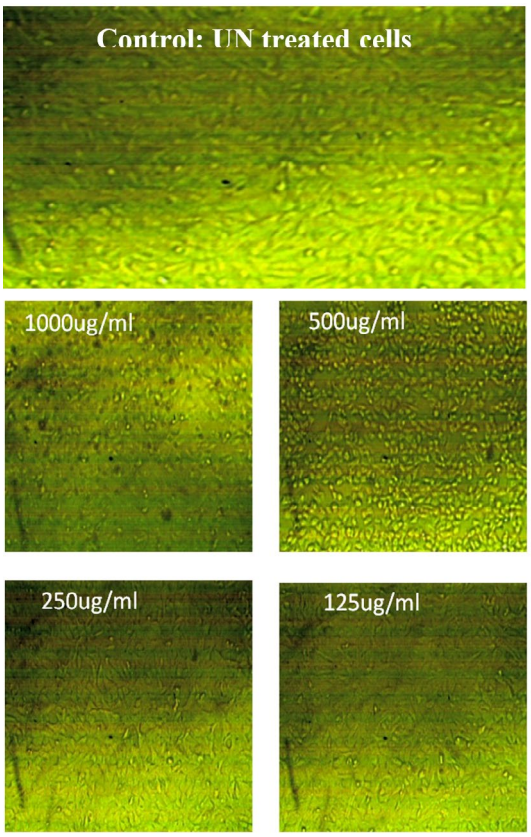

Fig. 18. Effect of ZnONPS on wi38 cells (Homo sapiens, lung, fibroblast, adherent and normal) at different concentrations.

growth[81]. The oxidoreductases enzyme inside living microbial cells reduces the resazurin salt to resorufin and changes the blue non-fluorescent colour of resazurin salt to pink and the fluorescent colour of resorufin (Fig. 13, 14).

While the determination of MIC for ZnO NPs against fungal isolates have been depicted in Table 6 and Fig. 15, 16. The potential antifungal activity of ZnO NPs on *A. niger, P. marneffie, C. parapsilosis,* and *C. glabrata* was assured by MICs experimentation. The minimum inhibitory concentrations of zinc oxide nanoparticles on *P. marneffie, C. parapsilosis, C. glabrata* tested were 12.5 µg/ml. The MIC on *A. niger* was higher than other tested species, about 25 µg/ml.

Cytotoxicity assay

Viability screening is fundamental for evaluation of the cellular response to toxic materials. To determine total cell viability after nanoparticles exposure, we used the MTT method described by Riss and Moravec[53]. Zinc oxide nanoparticles, because of their physical and chemical characteristics, are considered an effective material in cancer therapy[81].

New biosynthetic methods of ZnO-NP use natural environmental sources such as water extracts of plants and fungi that reduce metal ions compared with physical methods and chemicals, they are easy to apply and non-toxic[82].

The cytotoxic mechanism of ZnO-NP is not fully understood, but it is believed that the major components of hydroxyl radicals (OH*), superoxide anions (O_2^-), and per-hydroxyl radicals (HO_2*) are generated from the surface of ZnO-NP. When interaction occurs between cells and nanoparticles, cellular preservation mechanisms at this time are activated to decrease damage.

However, if the highly active free radical production exceeds the anti-oxidative defensive ability of the cell, it results in oxidative harm of biomolecules which can lead to cell death[33,84].

Treatment with natural-synthetic ZnO-NP resulted in significant changes in cell morphology at 1000 and 500 µg/ml concentrations. Therefore, a microscopic examination was performed. As shown in Table 7 and Figure 17 & 18, the control cells remained normal. These results show high cytotoxicity values at high concentrations of ZnO-NP, as reported by Brunner et al., using lower concentrations and similar particle sizes in chemically prepared ZnO-NPs. However, they also reported significantly less cytotoxicity (with a particle size larger or smaller than this study) using the same concentration and cell line[83].

After treatment of cells with lower concentrations of ZnO-NPs in Figure 17 & 18, and Table 7. The Cells ' morphology was not different from that of the control, and most of the cells could adhere and spread.

CONCLUSION

The current study shows that zinc oxide (ZnO) nanoparticles have been successfully biosynthesized using fungi as a biological system can be done easily. *Aspergillus niger* can be operated under controlled conditions and has great potential for synthesizing metal oxide nanoparticles outside and inside the cell. The synthesized nanoparticles are stabilized by the protein released by the fungus during the synthesis of NP from outside the ZnO cell. Through different characterization techniques, such as ultraviolet-visible light absorption, morphological structure using TEM and XRD, the physical characteristics of the prepared zinc oxide (ZnO) nanoparticles were studied. Then, the colloidal stability uses zeta meter technology and the surface function uses FT-IR spectroscopy. The average particle size is approximately 20 ± 5 nm and it has a hexagonal structure. Furthermore, the prepared particles show significant antimicrobial activity against different bacterial and fungal isolates. They exhibited different levels of cytotoxicity dependent upon the concentration of ZnO NPs using the MTT assay with an IC_{50}˜800.42.

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None.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHORS' CONTRIBUTION

All authors listed have made a substantial, direct, and intellectual contribution to the work, and approved it for publication.

FUNDING

None.

DATA AVAILABILITY

All data included in this study were presented in the form of tables and Figures.

ETHICS STATEMENT

The protocol of this study was approved by the Faculty of Science, Al-Azhar University, Cairo, Egypt (2018).

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