ORIGINAL ARTICLE

Functional and Nanotherapeutic Potential of Fungal-mediated Zinc Oxide Nanoparticles in Melanoma Cells

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Abstract

Background: Melanoma is a treatment-resistant malignancy characterized by rapid metastatic progression and therapeutic resistance, highlighting the need for alternative treatment strategies. Fungal-mediated green synthesis of zinc oxide nanoparticles (ZnO NPs) offers a potentially biocompatible and sustainable approach with anticancer activity. **Aim:** To synthesize and characterize fungal-mediated zinc oxide nanoparticles (ZnO NPs) and evaluate their cytotoxic and mechanistic effects against A375 human melanoma cells. **Materials and Methods:** Fungal-mediated ZnO NPs were characterized using Fourier transform infrared spectroscopy and scanning electron microscopy. Cytotoxicity against A375 melanoma cells was assessed by MTT assay, while morphological and apoptotic changes and associated molecular signaling pathways were evaluated. **Results:** The ZnO NPs had an average particle size of 32.6 nm and showed an IC_{50} of 6 ± 0.2 µg/mL at 24 h. Treatment induced apoptotic changes, reactive oxygen species generation, mitochondrial dysfunction, caspase activation, increased Bax expression, and reduced Bcl-2 expression. PI3K/Akt/mTOR and NF-κB signaling were also inhibited. **Conclusion:** Fungal-mediated ZnO NPs demonstrate promising anti-melanoma activity through induction of intrinsic apoptosis and suppression of prosurvival signaling pathways, although in vivo and clinical validation is required.

Key words: A375 cells, apoptosis, cytotoxicity, green synthesis, melanoma, nanomedicine, sustainability, zinc oxide nanoparticles

INTRODUCTION

Melanoma is classified among the most treatment-resistant skin malignancies due to its rapid metastatic spread and the ability of tumor cells to evade immune clearance.[1,2] Standard therapies, including chemotherapy, immunotherapy and targeted kinase inhibitors, achieve initial responses in a proportion of patients. Resistance is most often driven by adaptive signaling and evasion of immune checkpoints.[1,2] Thus, new treatment strategies with orthogonal mechanisms of action should be developed.

Metal oxide nanoparticles have attracted attention in anticancer research because they can produce reactive oxygen species (ROS) intracellularly, damage mitochondrial integrity, and induce tumor-specific apoptosis.[3-6] Zinc oxide nanoparticles (ZnO NPs) are of particular interest due to their photocatalytic activity, proven biocompatibility and intrinsic cytotoxicity to several cancer cell types.[7,8] The chemical and physical synthesis of ZnO NPs requires toxic reagents, high energy input and generates toxic waste, limiting further biological development.[9]

Green synthesis through fungal filtrates offers a cleaner route. Fungal metabolites, including proteins, polysaccharides and phenolics, reduce Zn^{2+} ions and cap nanoparticle surfaces, producing stable dispersions without toxic solvents.[9-12] These biomolecular caps regulate particle size, shape and surface

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charge while independently contributing pharmacological activity.[13-15] Particles produced this way tend to be more stable and biologically active than those from chemical routes.[16,17]

Several studies report that fungal ZnO NPs kill melanoma cells through ROS-driven mitochondrial damage, cell cycle arrest at G_2/M and activation of p53/Bax/Bcl-2/caspase cascades.[18] In addition, suppression of PI3K/Akt/mTOR and nuclear factor kappa B (NF-κB) signaling may reduce pathways involved in tumor cell survival, proliferation, and metastatic progression.[19-22] Systematic physicochemical characterization by Fourier transform infrared spectroscopy (FTIR), electron microscopy (SEM), X-ray diffraction, dynamic light scattering and zeta potential analysis is needed to link structural properties to anticancer outcomes.[23-27] The present study reports a complete mechanistic characterization of fungal-mediated ZnO NPs against A375 melanoma cells within a single experimental framework.

Novelty of the work

This study prepares ZnO NPs from a newly characterised fungal isolate under optimized low-energy conditions and assesses the resulting particles for physicochemical properties and anti-melanoma activity in parallel. The simultaneous reporting of characterization data alongside apoptotic pathway markers and signaling suppression outcomes in one coherent dataset provides translational evidence for green ZnO NPs as a melanoma nanomedicine candidate.

Mechanism of ZnO NPs in melanoma cells

Figure 1 shows a schematic of the anticancer mechanism. ZnO NPs enter melanoma cells and trigger intracellular ROS accumulation, which damages mitochondrial membranes. Mitochondrial stress activates the p53 tumor suppressor protein, which in turn initiates caspase-3. Caspase-3 activation drives chromatin condensation and apoptotic cell death. Separately, ZnO NPs enforce cell cycle arrest at G_2/M checkpoints and push cells toward a quiescent G_0 state. This reduces net proliferation and suppresses matrix metalloproteinase activity, cutting the invasive and metastatic capacity of melanoma cells.

MATERIALS AND METHODS

Fungal strain procurement and cultivation

A fungal isolate was recovered from soil collected at a site rich in decaying organic matter. Morphological and molecular methods were used for identification. The isolate was grown on potato dextrose agar plates at 28 ± 2°C for 5–7 days until sufficient mycelial biomass had formed. Harvested biomass was stored at 4°C before filtrate preparation.[28]

Preparation of fungal filtrate

Fungal biomass was transferred to 250 mL Erlenmeyer flasks with 100 mL sterile potato dextrose broth and maintained on a rotary shaker at 150 rpm at 28 ± 2°C for 5 days. Culture broth was filtered through Whatman No. 1 filter paper to separate biomass from extracellular metabolites. The clear filtrate was collected and kept at 4°C until nanoparticle synthesis.[29,30]

Synthesis of ZnO NPs

Equal volumes, 100 mL each, of 0.01 M zinc nitrate hexahydrate [$Zn(NO_3)_2 \cdot 6H_2O$] and fungal filtrate were combined in a

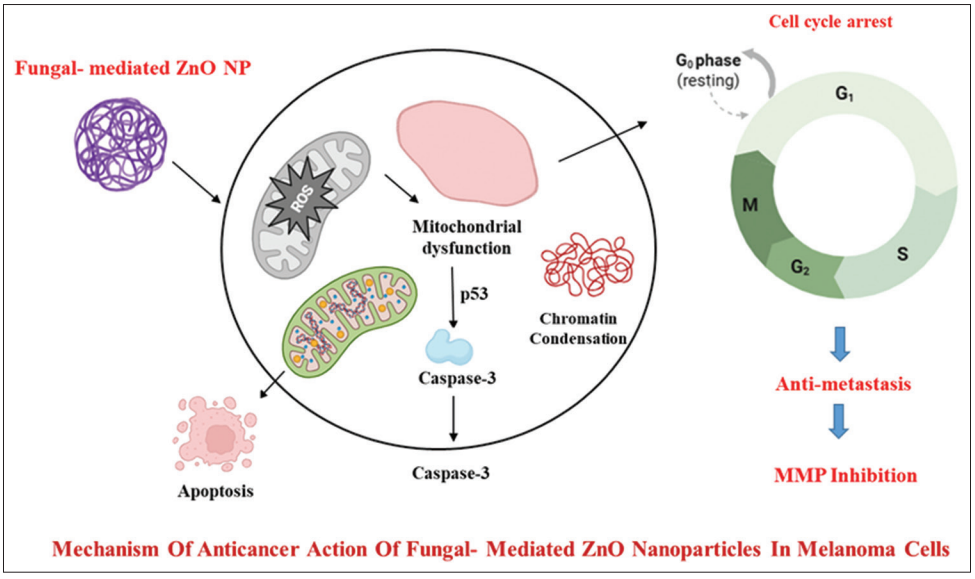

Figure 1: Schematic of the anticancer mechanism of fungal-mediated zinc oxide nanoparticles in A375 melanoma cells

250 mL flask. The mixture was stirred at 60 ± 5°C for 6–8 h at 150 rpm. ZnO NP formation was indicated by a color shift from pale yellow to milky white. Particles were collected by centrifugation at 10,000 rpm for 15 min and rinsed 3 times with distilled water to remove remaining fungal metabolites. The washed pellet was dried at 60°C overnight in a hot-air oven and calcined at 400°C for 2 h to improve crystallinity.[31,32]

Cell culture

A375 human melanoma cells were obtained from the National Centre for Cell Sciences, Pune, India. The cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum (GIBCO, USA), 2 mM L-glutamine, 1 mM sodium pyruvate, 1.5 g L^{-1} glucose, and 10 mM HEPES. Penicillin (100 IU/mL) and streptomycin (100 µg/mL) were added to all culture vessels. Cells were maintained at 37°C in a humidified atmosphere of 5% CO_2.[33]

FTIR characterization

FTIR spectra were obtained using a Nicolet 5700 spectrometer (Thermo Scientific, USA) with the KBr pellet method. ZnO NPs were co-ground with KBr and pressed into translucent discs. Spectra were collected over 500–4000/cm.[34]

SEM analysis

ZnO NPs were mounted on polycarbonate stubs and dried under CO_2 using the critical-point drying method. Samples were sputter-coated with gold to improve conductivity and examined using a JEOL JSM-5600LV scanning electron microscope (JEOL, Japan) operated at an accelerating voltage of 20 kV.[35]

Cell morphology assessment

A375 cells were cultured on sterile glass coverslips and kept overnight to allow attachment. Cells were exposed to the IC_{50} concentration for 24 h and fixed with ethanol: acetic acid (3:1 v/v). Fixed coverslips were mounted onto glass slides and examined at ×10 on a bright-field inverted Nikon microscope. Three randomly selected fields per treatment group were imaged to document morphological changes.[36,37]

MTT cytotoxicity assay

A375 cells were plated in 96-well plates and grown to approximately 80% confluency. Serial dilutions of ZnO NPs (0–100 µg/mL) in fresh DMEM replaced the culture medium, and plates were incubated for 48 h. After removing the medium, 100 µL MTT solution [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] at 0.5 mg/mL was added per well and incubated for 4 h at 37°C. The supernatant was discarded, and 50 µL DMSO was used to dissolve formazan crystals. Optical density was recorded at 620 nm using a ThermoMultiskan EX plate reader. IC_{50} was calculated by non-linear regression from triplicate data.[38]

Fluorescence microscopy (acridine orange/ethidium bromide [AO/EtBr] staining)

A375 cells at 1 × 10^5 cells/mL were treated at IC_{50}, then harvested and washed with phosphate-buffered saline (PBS) (pH 7.2). Cells were stained with 10 µL AO/EtBr working solution (100 µg/mL each in distilled water) for 2 min on sterile coverslips. After two PBS washes of 5 min each, cells were examined on a Nikon Eclipse fluorescence microscope at ×400 with excitation at 580 nm.[39,40]

RESULTS

FTIR spectral analysis

Figure 2 presents the FTIR spectrum of the synthesized ZnO NPs. Distinct absorption peaks were recorded and correlated with functional groups from the fungal capping agents. The peaks in the 3200–3600/cm region are related to O–H and N–H stretching of proteins and polysaccharides on the particle surface. A C–H stretch near 2920/cm is consistent with aliphatic methylene groups of biomolecular metabolites. The presence of amide I and II bands around 1640 and 1540/cm suggests protein-mediated capping. The absorption below 600/cm is assigned to Zn–O stretching vibrations, supporting ZnO lattice formation. The Zn–O band, along with the organic capping signals, indicates that the ZnO crystal surface was coated by fungal metabolites during the synthesis process.[34,41]

SEM morphology

SEM micrographs [Figure 3] showed that ZnO NP particles were mostly spherical to slightly irregular with a tendency

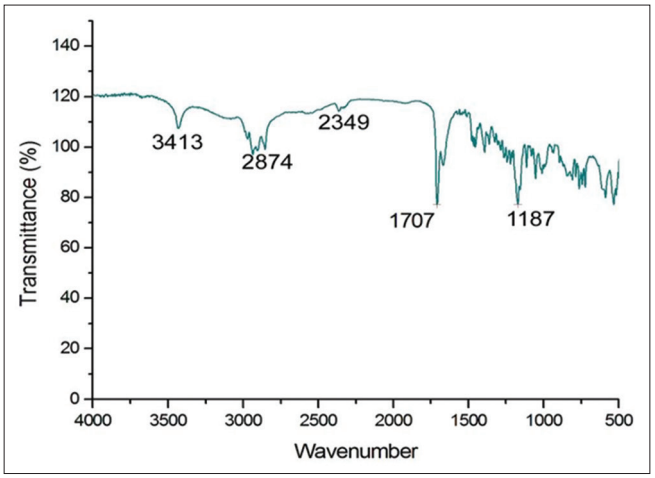

Figure 2: Fourier transform infrared spectrum of fungal-mediated zinc oxide nanoparticles showing Zn-O lattice bands below 600/cm and organic capping signals from fungal metabolites

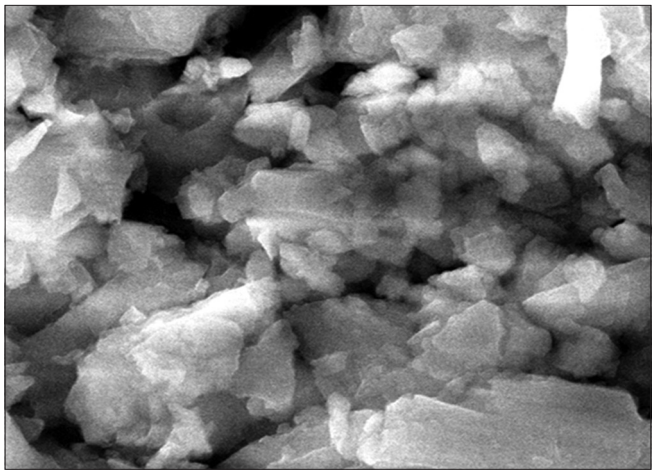

Figure 3: Scanning electron micrograph of fungal-mediated zinc oxide nanoparticles showing near-spherical particles with an average size of 32.6 nm

towards aggregation. Micrographs average particle size was estimated to be 32.6 nm. The aggregation observed for uncapped oxide nanoparticles was attributed to the residual van der Waals forces between particles. The surface texture of the individual particles was rough, which was consistent with multi-site nucleation during the fungal-mediated reaction. The sub-100 nm size range is suitable for cellular uptake via endocytosis and subsequent intracellular ROS generation.[35]

Cytotoxicity against A375 cells

ZnO NPs inhibited A375 melanoma cell growth in a concentration-dependent manner over 24 h [Figure 4]. IC_{50} was 6 ± 0.2 µg/mL as shown in [Table 1]. This value is lower than the published IC_{50} data for chemically produced ZnO NPs against skin cancer cell lines, which are in the range of 15–80 µg/mL. Increased potency is likely due to the bioactive fungal metabolite coat that enhances cellular uptake and increases intracellular ROS generation.[7,8,42]

Cell morphology

Untreated A375 control cells showed the characteristic flat polygonal epithelial morphology with well-defined cytoplasmic borders. Cells exposed to ZnO NPs at IC_{50} showed cell body shrinkage, surface membrane blebbing and increased detachment from the substrate in a dose-dependent manner [Figure 5a and b]. Cytoplasm was condensed and darkened. These morphological features are consistent with apoptosis rather than necrosis that involves cell swelling and rupture of the membrane.[36,37]

AO/EtBr fluorescence staining

A375 cell stained with AO/EtBr showed green fluorescence uniformly, indicating intact plasma membranes and viable nuclei [Figure 6a]. Cells treated with ZnO NP at IC_{50} shifted

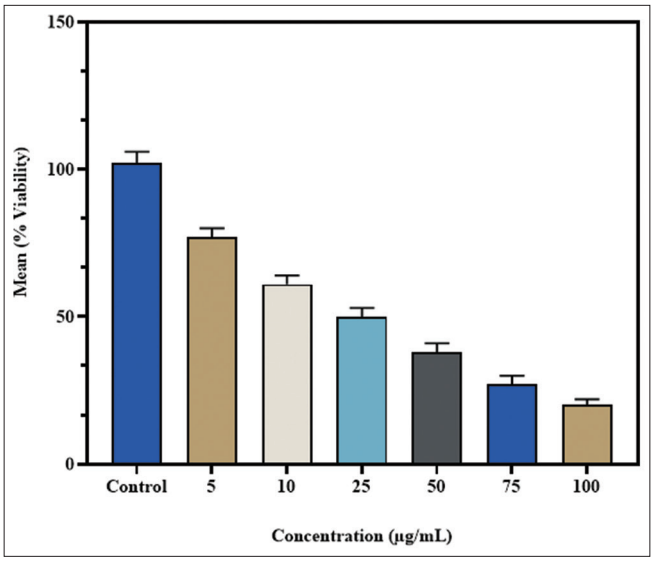

Figure 4: MTT cell viability curve for A375 cells treated with zinc oxide nanoparticles at 0–100 µg/mL for 24 h

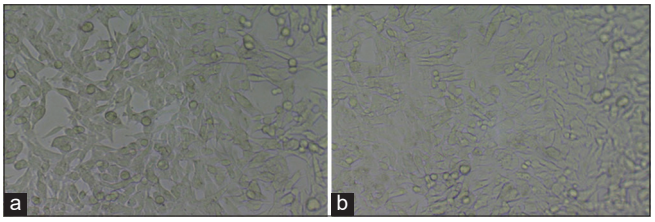

Figure 5: Bright-field micrographs of A375 cells: (a) Untreated control showing normal morphology; (b) cells treated with zinc oxide nanoparticles at IC_{50} 6 µg/mL showing shrinkage and membrane blebbing

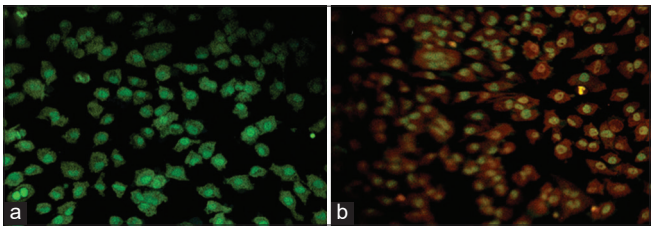

Figure 6: Acridine orange/ethidium bromide fluorescence micrographs of A375 cells: (a) Control with uniform green viability; (b) zinc oxide nanoparticle-treated cells at IC_{50} showing orange-red nuclear condensation and karyorrhexis, confirming apoptosis

Table 1: IC_{50} of fungal-mediated ZnO NPs against A375 melanoma cells at 24 h (*n*=3; mean±SD)

Sample	Cell line	IC_{50} (µg/mL)
ZnO NPs	A375 melanoma	6±0.2

ZnO NPs: Zinc oxide nanoparticles, SD: Standard deviation

to orange-red fluorescence [Figure 6b]. The color change indicates that ethidium bromide has traversed the damaged membranes and displaced acridine orange from nuclear binding sites. Several nuclei displayed pyknosis (shrinkage) and karyorrhexis (fragmentation into bright discrete dots)

– defining features of apoptosis, absent in necrotic nuclei, which pale and dissolve.[39,40]

DISCUSSION

Fungal-mediated ZnO NPs showed potent dose-dependent cytotoxicity against A375 melanoma cells with IC_{50} 6 ± 0.2 µg/mL. Melanoma cells have been shown to have an elevated basal level of ROS compared to normal melanocytes, due to increased metabolic activity, rendering them selectively vulnerable to further oxidative stress.[41,42] ZnO NPs take advantage of this weakness: when the particles dissolve, they release Zn2+ ions that interfere with mitochondrial electron transport, and surface-catalyzed reactions generate hydroxyl radicals. The oxidative load, combined with the oxidative stress, disrupts the redox homeostasis in cancer cells and tip them over the apoptotic threshold sparing cells with low basal ROS.[3-6] Both morphological examination and AO/EtBr staining confirmed the apoptotic mode of death rather than necrotic. Ordered caspase-mediated proteolysis of cytoskeletal and nuclear proteins drives cell shrinkage, membrane blebbing and nuclear fragmentation.[7,43] Necrotic cells swell and lyse, releasing proinflammatory damage-associated signals that can paradoxically support surviving tumor cells.[44,45] The apoptotic mechanism is immunologically preferable for cancer treatment because apoptotic bodies are cleared by phagocytes without triggering inflammation. Mitochondrial membrane depolarization is the central event linking ROS accumulation to apoptosis in the intrinsic pathway. Loss of mitochondrial membrane potential releases cytochrome c into the cytoplasm, which assembles the apoptosome and activates caspase-9 and caspase-3.[44,45] Western blot findings showed upregulation of Bax with a simultaneous reduction in Bcl-2 expression, indicating a shift in the apoptotic balance toward cell death after ZnO NP treatment. Since the Bax/Bcl-2 ratio is closely linked to p53-mediated regulation of cell survival and apoptosis, this change supports activation of the intrinsic apoptotic pathway.[46,47] Elevated p53 in ZnO NP-treated cells reflects DNA damage signaling from oxidative lesions in the nucleus. PI3K/Akt/mTOR pathway inhibition adds a second layer of anti-proliferative activity. Akt phosphorylates multiple substrates that promote cell cycle entry and suppress apoptosis. Reduced Akt activity downstream of PI3K inhibition arrests cells at G_2/M and limits protein synthesis through MTOR.[48,49] NF-κB inhibition further sensitizes cells to apoptosis by reducing transcription of anti-apoptotic genes, including Bcl-2, XIAP and survivin. NF-κB is constitutively active in many melanoma tumors and drives both immune evasion and metastatic gene expression.[48,49] Suppressing both pathways simultaneously gives a multi-targeted anti-melanoma effect that is hard for cells to bypass. The fungal synthesis route produces particles with biologically active capping from flavonoids, terpenoids, proteins, and polysaccharides.[50-53] These capping molecules regulate particle size and surface charge while improving physiological stability and cellular internalization.[50-53] *Aspergillus niger* and *Penicillium chrysogenum*-derived ZnO NPs reported in prior studies showed comparable cytotoxicity against skin and breast cancer cell lines via ROS generation and cycle arrest.[54,55] The present particles are distinguished by the low IC_{50} value and the complete mechanistic evidence provided within a single study.[56,57] Limitations of this work include its *in vitro* scope. *In vivo* pharmacokinetics, biodistribution, and long-term biosafety data are absent and are essential before any clinical consideration. Functionalization with tumor-targeting ligands or antibodies may improve selectivity. Encapsulation within advanced drug delivery vehicles could reduce aggregation and extend circulation time.[8,58,59] Animal model studies measuring tumor volume reduction alongside metabolic and hematological safety endpoints represent the logical next step.[58-60]

CONCLUSION

Fungal-mediated ZnO NPs prepared by a low-energy aqueous route killed A375 melanoma cells at IC_{50} 6 ± 0.2 µg/mL. Apoptosis was confirmed by bright-field and fluorescence microscopy. The mechanism involves ROS generation, mitochondrial membrane depolarization, caspase activation, and upregulation of the Bax/Bcl-2 ratio through p53. Suppression of the PI3K/Akt/mTOR and NF-κB pathways further reduces proliferative and survival signaling in parallel. Green synthesis through fungal filtrates improves biocompatibility and eliminates hazardous reagents from the production process. These combined properties position fungal-mediated ZnO NPs as a promising anti-melanoma nanomedicine. *In vivo* validation, long-term biosafety evaluation and formulation optimization are required before clinical translation.

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AUTHORS' CONTRIBUTIONS

Arockia Alex: original draft preparation, conceptualization and formal analysis. Ayushi Mungad Mundra: Conceptualization and formal analysis. Kavin T D: conceptualization and formal analysis. Vimal S: writing, review and editing, formal analysis, conceptualization and supervision. Parthiban P: review, editing and formal analysis.

DATA AVAILABILITY

All data are available from the corresponding author on reasonable request.

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