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Anti-candidal and antibacterial influence of zinc oxide nanoparticles biosynthesized by *Penicillium crustosu*m AUMC15766

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Abstract: Bio-nanotechnology have attracted strong interest due to their valuable bioactivities and eco-friendly features. In this study, *Penicillium crustosum* AUMC15766 isolated from soil, molecularly identified, was used to reduce zinc nitrate to zinc oxide nanoparticles (ZnONPs) using fungal filtrate. Zinc oxide nanoparticles, visually slimy white, were subjected to characterization using UV-vis spectrophotometry (peak at 314 nm), X-ray diffraction determined its crystallinity (2θ values of 31.77°, 34.44°, 36.26°, 47.54°, 56.61°, 62.86°, 66.39°, and 67.96°), transmission electron microscopy established the form and size (hexagonal diameters ranging from 8 to 25 nm), and functional groups acting as capping agents were illustrated using Fourier transform infrared spectroscopy. Assessment of anti-candidal and antibacterial potency of ZnONPs at several concentrations presented significant activity against tested pathogens. Thus, myco-genesis of ZnONPs represents promising approach to introduce sustainable agents available for biotechnological applications.

Keywords: Zinc oxide nanoparticles, *Penicillium crustosum*, Characterization, Anti-candidal, Antibacterial.

1. Introduction

Nanoparticles are defined as manufactured materials containing particles in a free state or as aggregates and having dimensions ranged between 1 to 100 nm (Pulit-Prociak & Banach, 2016). The metallic nanoparticles have unique characteristics including optical and magnetic properties, surface plasmon resonance, quantum confinement, and large surface energies (Dreaden *et al*., 2012), in addition to other characteristics such as size, shape, chemical compositions and high surface area to volume ratio (Balashanmugam *et al*., 2013). So, they have utilized in a wide range of applications in medicine *(Nosrati et al*., 2021), agriculture (Thul *et al*., 2013), electronics (Boisselier & Astruc, 2009), catalysis, chemical sensing, environmental remediation, biological labeling, and drug delivery (Prabhu & Poulose, 2012).

Metallic nanoparticles are synthesized by chemical, physical, and biological methods (Pulit-Prociak & Banach, 2016), where physical methods gain many disadvantages comprising high cost, high energy, pressure, and temperature requirements, less yield and stability, and high waste production. While, chemical methods are unsafe due to the involvement of hazardous chemical substances that are attached to the surface of metal nanoparticles, thus has side effects in medical applications (Ovais *et al*., 2018). Despite the developments in chemical and physical synthetic approaches of metallic nanoparticles (Abdel-Rahim *et al*., 2024; Kamal *et al*., 2024), biological methods are eco-friendly and cost efficient. Biological methods include using of plants, bacteria, cyanobacteria, algae and fungi for the synthesis of nanoparticles (Salem, 2022).

Among biological systems, fungi offered a variety of biological activities and biotechnological applications.

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This encompasses nutraceutical, medical, and biotechnological polysaccharides (Giavasis, 2014), lipids and fatty acids (Mohamed *et al*., 2022a), and enzymes and peptides (Al Mousa *et al*., 2022a) as well as low molecular weight secondary products exhibiting different antibacterial (Baazeem *et al*., 2021), antifungal (Abdelrahem *et al*., 2023, 2024), anticancer (Al Mousa *et al*., 2022b), antioxidant (Hassane *et al*., 2022a), wound healing (Al Mousa *et al*., 2024_b), and/or even toxigenic (mycotoxins) properties (Abo Dahab *et al*., 2016; Saber *et al*., 2016).

Fungi have been frequently reduced the metal salts to produce metal nanoparticles due to their characteristics such as high biomass production, ease of handling and cultivation, the ability to produce a lot of quantities of enzymes, metabolites, and extracellular proteins (Azmath *et al*., 2016). These molecules are responsible for synthesis nanoparticles and forming capping agents responsible for stability and activity of nanoparticles (Zhao *et al*., 2018).

The synthesis of ZnONPs has attracted considerable interest due to its eco-friendliness, broad antibacterial action, and involvement in a variety of industries, including the rubber, pharmaceutical, textile, biosensor, and cosmetics industries (Singh *et al*., 2014). Okaiyeto *et al.* (2024) recorded a promising result through investigating the antibacterial activity of ZnONPs against diverse bacterial species including *Escherichia coli*, *Salmonella typhi*, *Bacillus subtilis*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa*. The present study aimed to myco-synthesize, characterize and assess the antimicrobial activities of zinc oxide nanoparticles on variety of bacterial and *Candida* strains.

2. Materials and methods

2.1. Isolation and molecular identification of the fungus

Soil isolate *Penicillium crustosum* was used for myco-synthesis of ZnONPs. The isolate identification was depended on its macro- and micro-morphological culture characteristics on Czapek's (Cz) agar medium ($NaNO_3$, 2 g; KH_2PO_4, 1 g; $MgSO_4.7H_2O$, 0.5 g; KCl, 0.5 g; glucose, 10 g; agar, 20 g per liter of distilled water with pH value 6.5 and chloramphenicol, 1 mg/mL), established by phylogenetic analysis according to Hassan et al. (2019) and Mohamed et al. (2020), and kept with an institutional number at Assiut University Mycological Center (AUMC) culture collection.

2.2. Biosynthesis of ZnONPs

After incubation in Cz broth under shaking (150 rpm/min) at 28 °C for 5 days, the fungal biomass was filtered and rinsed repeatedly by sterilized double-distilled H_2O. After that, 10 g of fungal were immersed in 200 millilitres of sterile double-distilled water and kept under shaking for 2 days at 28 °C. A stock solution of 2.97 g zinc nitrate ($Zn(NO_3)_2$) was dissolved in 50 mL of deionized water to obtain salt solution with 200 mM concentration. The filtrate was then employed for producing ZnONPs through mixing with 3 mM $Zn(NO_3)_2$ at ratio 1:1 and incubated at room temperature in dark conditions for 24 h. Formed ZnONPs were centrifuged at 7000 rpm for 15 min and repeated rinsing was performed to remove impurities and then ZnONPs were harvested for further analysis.

2.3. ZnONPs characterization

Myco-synthesized zinc oxide nanoparticles were characterized using UV-visible (UV-Vis) spectroscopy analysis (Jasco V-530, Japan), Transmission Electron Microscopy (TEM) (JEOL/JEM-2100, HRTEM, Tokyo, Japan) Fourier transform infrared spectroscopy (FTIR) (6100, Perkin-Elmer, Germany), X-ray diffraction (XRD) analysis (Panalytical X'PERT PRO, UK) as described by Khalaf *et al*. (2024).

2.4. Antimicrobial susceptibility assay

Gram-positive bacteria (*Bacillus subtilis* ATCC6633 and *Staphylococcus aureus* ATCC6538) and Gram-negative bacteria (*Escherichia coli* ATCC8739, *Klebsiella pneumonia* ATCC43816, *Proteus vulgaris* AUH123, *Pseudomonas aeruginosa* ATCC9027, *Salmonella typhi* AUH71, and *Serratia marcescens* AUH98) in addition to fungal isolates (*Candida albicans* ATCC10231, *C. krusei* TU87, *C. glabrata* TU52, *Geotrichum candidum* TU65 [ON430507], and *Rhodotorula mucilaginosa* [ON459714]) were used as test pathogenic microbes (Al Mousa *et al*., 2021, 2024a; Mohamed *et al*., 2021).

The antimicrobial susceptibility assay was carried out as described by Jahangirian *et al.* (2013) using well diffusion technique with 8 mm well diameter hold with 100 µL of well dispersed, by sonication, 5000 µg/mL ZnONPs. Clotrimazole and chloramphenicol, at concentration of 1 mg/mL, were used for fungi and bacteria as positive control, respectively. Muller-Hinton and Sabouraud dextrose plates impregnated with broth cultures of the assayed bacterial and fungal species were used for evaluating ZnONPs antimicrobial potency. The MICs of ZnONPs antibacterial potency, in nutrient broth, were assessed using the *p*-iodonitrotetrazolium chloride (INT) micro-dilution colorimetric approach as depicted by Gebreyohannes *et al.* (2019). For determining the MIC for antifungal activity, Sabouraud dextrose broth dilution assay was utilized without the use of INT.

3. Results

This study dealt with biosynthesis of ZnONPs using

Aspergillus terreus extracellular filtrate, followed by ZnONPs characterization utilizing UV-Vis, XRD, TEM, FTIR, and DLS analysis. Biosynthesized ZnONPs antimicrobial potency were assessed against a variety of fungal and bacterial pathogens.

3.1. Phylogenetic analysis of fungal isolate

Identification was performed through sequencing of ITS loci and sequences were undergone to BLAST within NCBI database. The isolate was identified as *Penicillium crustosum* AUMC15766 (GenBank accession no. OR840516) aligned with closely relevant strains from the GenBank, exhibited 99.20% - 99.80% identity and 100% coverage with multiple strains of the same species encompassing the type material *P. crustosum* FRR 1669 strain (NR_077153) (Figure 1).

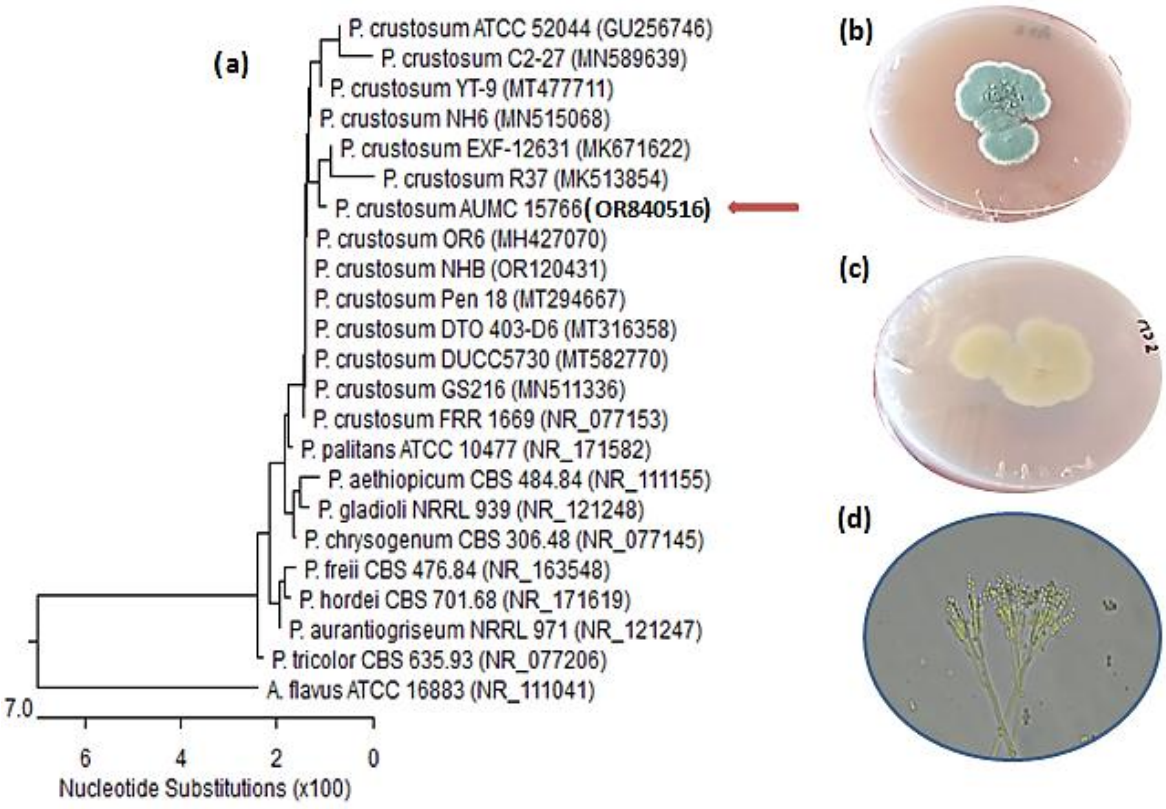

Figure 1: *Penicillium crustosum* AUMC15766; **(a)** Phylogenetic tree depended on the ITS sequences of rDNA, and **(b-d)** cultural characteristics; microscopic magnification 400x

3.2. Characterization of biosynthesized nanoparticles

The color of fungal filtrate turned into creamy white after treatment with $Zn(NO_3)_2$ and incubation in dark under shaking for one day (Figure 2A). UV–Visible wave analysis of ZnONPs manifested surface plasmon resonance (SPR) band at 314 nm (Figure 2B). Zinc oxide nanoparticles pure crystalline characteristic was established using XRD analysis which revealed at 2θ values diffraction peaks of 31.77°, 34.44°, 36.26°, 47.54°, 56.61°, 62.86°, 66.39°, and 67.96° that were compatible with 100, 101, 110, 122, 111, 200, 201, and 210 ZnONPs reflection planes, respectively (Figure 3). Transmission electron microscopy showed hexagonal ZnONPs with size ranging from 8 to 25 nm in diameter (Figure 4).

The spectral FTIR analysis of myco-synthesized ZnONPs exposed a number of diversified bands comprising strong bands appeared at 2986.67 and 2906.19 cm^{-1} which indicated aliphatic C–H stretching, while carbonyl group C=O stretching at 1638.64 and 1533.07 cm^{-1} was indicated. Moreover, bands centered at 1395.54^{-1} and 1006.01cm^{-1} were owed to S=O and C-O functional stretching, respectively (Figure 5).

3.3. Antimicrobial activity and MICs of ZnONPs

Antimicrobial activity of ZnONPs at different concentration range were evaluated against diverse pathogenic bacterial and fungal species (Figure 6). Different concentrations of ZnONPs (5000 to 500 µg/mL) exhibited antibacterial activity against *B. subtilis*, *S. aureus*, *S. typhi*, and *S. marcescens* with zone of inhibition ranged from 21.00 to 10.60 mm diameter. Zinc oxide nanoparticles at 5000 µg/mL showed inhibition of bacterial growth with diameters of 21.00, 20.50, 19.50, and 18.10 mm against *B. subtilis*, *S. marcescens*, *S. typhi*, and *S. aureus*,

respectively. while *E. coli*, *K. pneumonia*, *P. vulgaris*, and *P. aeruginosa* showed resistance to ZnONPs. Chloramphenicol, at concentration 1 mg/mL, showed antibacterial activity against whole bacterial species with diameters of inhibition zones ranged from 38.00 to 14.00 mm (Figure 7). Zinc oxide nanoparticles exhibited MICs values of 416.66, 416.66, 208.23, and 208.23 µg/mL against *B. subtilis*, *S. marcescens*, *S. aureus*, and *S. typhi*, respectively (Figure 8).

Antifungal activity of ZnONPs at 5000 µg/mL inhibited the growth of *C. krusei*, *C. glabrata*, and *C. albicans* with inhibition zone diameters 16.60, 16.50, and 15.80 mm, respectively. On the other hand, ZnONPs had no effect on the growth of *G. candidum* and *R. mucilaginosa*. Clotrimazole inhibited of the growth of all fungal species with inhibition zone diameters ranged from 20.60 to 27.00 mm (Figure 9). Zinc oxide nanoparticles, using well diffusion, exhibited MICs values of 700, 1000, and 1000 µg/mL against *C. albican*, *C. krusei*, and *C. glabrata*, respectively, while broth dilution assay offered of 166.6,166.6, and 333.33 µg/mL, respectively (Figure 10).

4. Discussion

The myco-synthesis of zinc oxide nanoparticles encompasses advantages including high biomass production, facility of handling and cultivation, and the ability to produce elevated quantities of enzymes end bioactive metabolites. These molecules responsible for synthesis nanoparticles and forming capping agent responsible for stability and activity of nanoparticles (Zhao *et al.*, 2018). Biosynthesis of ZnONPs was carried out by biomolecules of microorganism such as those mated with the complex pathways including electron transport during the transformation of NADPH/NADH to $NADP^+/NAD^+$ (Gudikandula *et al.*, 2017).

In the present study, molecular identification using ITS genetic sequences of 18S rDNA and phylogenetic characterization of *P. crustosum* AUMC 15766 was carried out. Both ends of the ITS region disclosed nucleotide sequence variations in the multiple alignment (Hassane et al., 2022b). Lazreg et al. (2013) reported that the 5.8S rDNA nucleotide sequences were proved to have ideal homology, while Mohamed et al. (2022b) stated that the ITS loci represent essential effective markers for confirming identification of fungal strains at the species level. Visagie et al. (2014) reported that *Penicillium* phylogenetic analysis is depended on the integration of β-tubulin gene and ITS of rDNA sequences in addition to others markers.

Regarding characterization of myco-synthesized ZnONPs by *P. crustosum* extracellular reducing agent filtrate, Baymiller *et al.* (2017) reported that NAD-dependent nitrate reductase enzyme is essential in biosynthesis of metallic nanoparticles. Our findings were compatible with preceding studies, where UV–Vis analysis revealed SPR band at 314 nm, hexagonal particles with size in diameter between 8 and 25 nm by TEM analysis, crystalline nature by XRD, and functional coating groups by FTIR. The presence of ZnONPs in the *A. niger* culture filtrate of was indicated by absorption bands at 320 nm (Talam *et al.* 2012). Kalpana *et al.* (2018) reported that myco-synthesized zinc ZnONPs revealed a 320–380 nm peak range. Similar XRD diffraction peaks were reported by Moghaddam *et al.* (2017) at 100, 002, 101, 102, and 110. According to our FTIR findings, Kavitha *et al* (2017) confirmed a band at 3334.71 cm^{-1} corresponding to O-H stretching and at 1656.36 cm^{-1} correspondance to -O vibrations, while at 1637.56 cm^{-1} Raghunandan *et al*. (2010) indicated correspondence with C-C aromatic stretching. Our results were in agreement with Kalpana *et al.* (2018) who detected ZnONPs O–H stretch absorption peak at 3199.91 cm^{-1} and C-C stretch at 1587.42 cm^{-1}.

Biosynthesized ZnONPs showed activity against Gram-positive (*B. subtilis* and *S. aureus*), Gram-negative (*S. typhi* and *S. marcescens*), and *Candida* spp. In this regard, Kalpana *et al*. (2018) reported zone of inhibition of ZnONPs against pathogenic *E. coli* and *S. aureus*. The ZnONPs synthesized by *Daedalea* sp. Mushroom revealed an efficient zone of inhibition of 10, 7, 7, and 7 mm for *E. coli* and *K. pneumonia*, *P. aureginosa*, and *S. aureus*, respectively. Kamal *et al.* (2023) synthesized ZnONPs using mushroom, which showed antimicrobial activity against *P. aeureginosa*, *E. coli*, *S. aeururs*, and *K. pneumonia* with inhibition zone diameters 7, 10, 7, and 7 mm, respectively. The myco-synthesized ZnONPs bactericidal influence was observed to be higher against Gram-ve than Gram+ve because of variations among their structures (Kamal *et al.*, 2023). Madkour *et al.* (2017) indicated ZnONPs' antimicrobial efficacy against *C. albicans*, *A. flavus*, *P. aeruginosa*, *E. coli*, *S. aureus*, and *B. subtilis*. Feris *et al.* (2010) reported that, even at low concentrations, ZnONPs afforded elevated level of antibacterial and antifungal activities. However, Fouda *et al.* (2018) proved suppression zone formation of ZnONPs at 2000 µg/mL of 14.1, 14.2, 19.1, 20.2 mm for *P. aeruginosa*, *E. coli*, *S. aureus*, and *B. subtilis*, respectively.

El-Rafie *et al.* (2010) suggested that mode of ZnONPs inhibition potency might be attributed to combination with the thiol groups, thus led to deactivation of microbial proteins thus retarded metabolic functions, growth, and proliferation. Agarwal *et al.* (2018) proposed that antibacterial efficacy of ZnONPs is caused by the production of reactive oxygen species

(ROS) which resulted in cell death due to generated oxidative stress. Jayaseelan *et al.* (2012) illustrated that ZnONPs could be, through electrostatic forces, attached to bacterial membrane and subsequent penetrate into the cells leading to destruction of bacterial cell integrity.

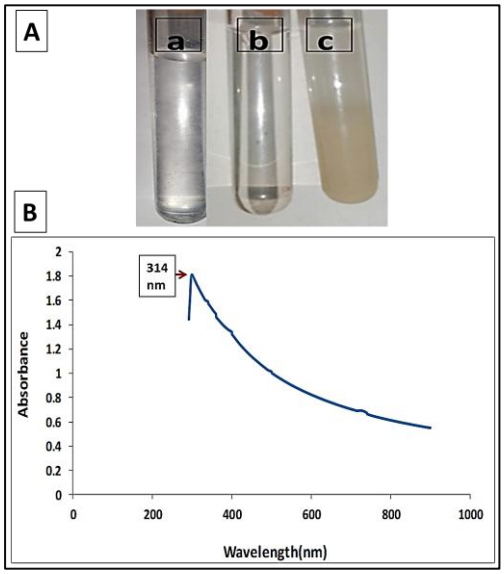

Figure 2: (A) Flasks containing **a)** zinc nitrate, **b)** fungal filtrate, and **c)** fungal filtrate combined zinc nitrate = (ZnONPs). **(B)** UV–Vis absorption band of zinc NPs.

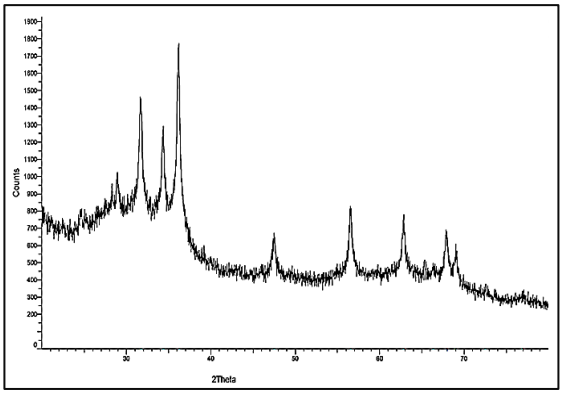

Figure 3: XRD micrograph of biofabricated ZnONPs.

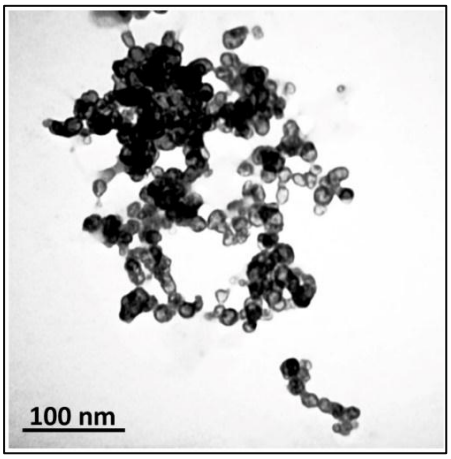

Figure 4: TEM image of biogenic ZnONPs.

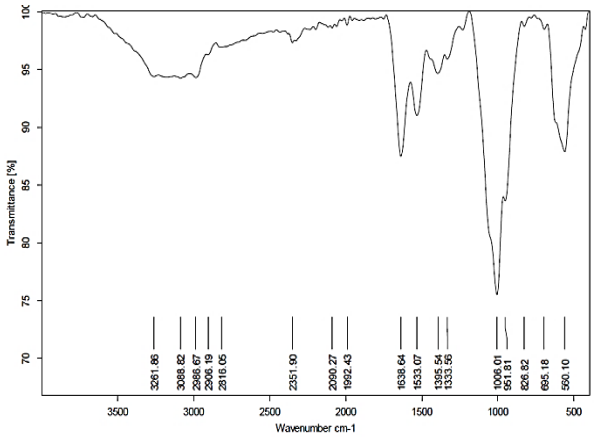

Figure 5: FTIR analysis of biosynthesized ZnONPs.

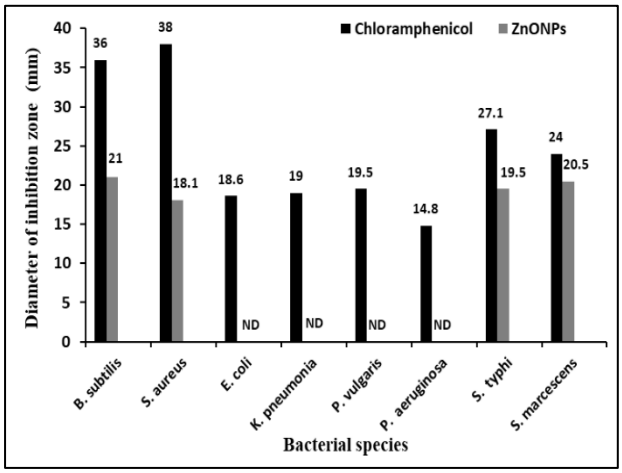

Figure 6: Antimicrobial activity of ZnONPs against **(A)** *B. subtilis*, **(B)** *S. marcescens*, **(C)** *C. albicans*, and **(D)** *C. glabrata*.

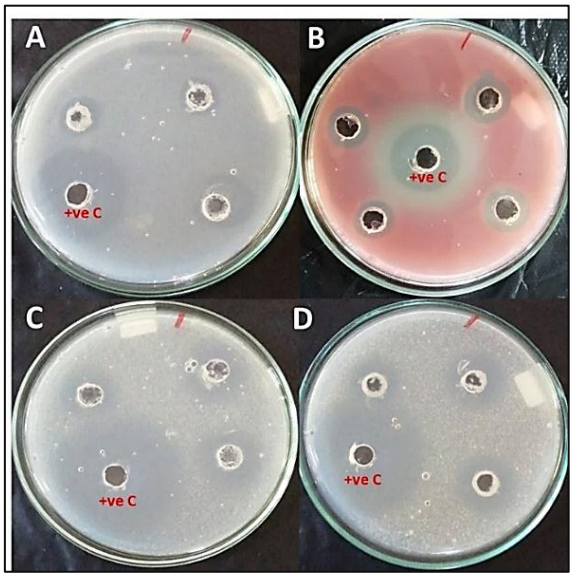

Figure 7: Antibacterial activity of ZnONPs (5000 µg/mL) and chloramphenicol (1 mg/mL).

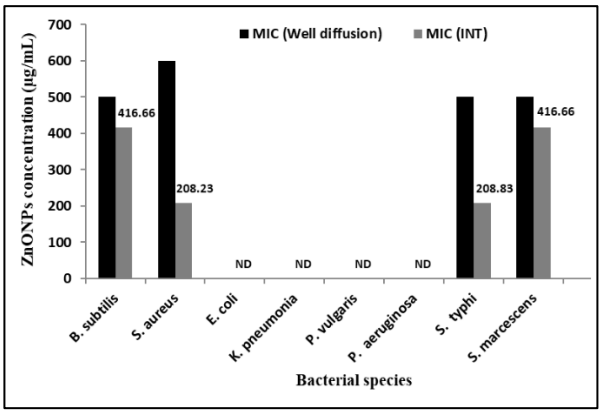

Figure 8: Antibacterial MICs values of ZnONPs by well diffusion and INT methods.

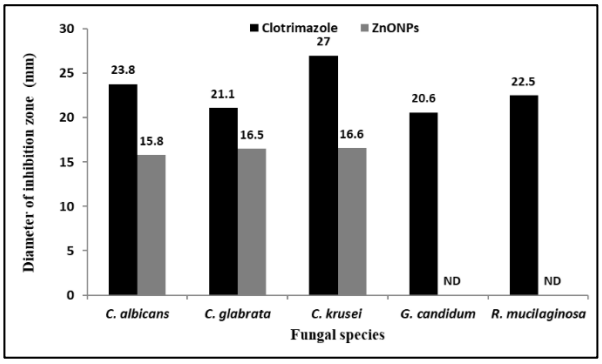

Figure 9: Antifungal activity of ZnONPs (5000 µg/mL) and clotrimazole (1 mg/mL) against tested fungal strains.

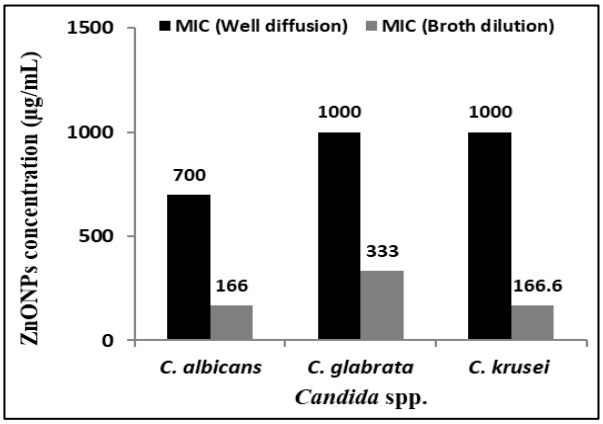

Figure 10: Anticandidal MICs values of ZnONPs using well diffusion and broth dilution assays.

5. Conclusion

Zinc oxide nanoparticles, ZnONPs, were bio-fabricated via a green synthesis using *P. crustosum* extracellular filtrate. Various characterization tools were utilized, including UV-Vis, TEM, XRD, DLS, and FTIR. TEM analysis revealed hexagonal forms with average size of 8 to 25 nm ZnONPs, while XRD affirmed crystallinity nature. Biosynthesized ZnONPs offered reasonable antimicrobial activity against diverse G+ve and G-ve bacteria and *Candida* spp. These findings suggest that ZnONPs have significant potential for treating infections and could be further optimized for safe, convenient, and biocompatible clinical use. Further study will be conducted to ensure more efficiency of ZnONPs regarding stability and bioactivity and to be introduced into other biotechnological applications.

Conflict of interests

The authors declare that there are no conflicts of interest.

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Ethical approval

None *applicable.*

Authors' Contributions

Nourhan H. Khalaf: Methodology, Investigation, and Writing original draft; **Abdallah M. A. Hassane**: Conceptualization, Supervision, Writing, Review & Editing original draft, and Formal analysis; **Nageh F. Abo-Dahab and Bahig A. El-Deeb**: Supervision, and reviewing final draft.

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