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A comparative study of the antifungal activity of green-synthesized ZnO nanoparticles, garlic extract, and conventional antifungal agents against *Cryptococcus spp.* isolated from pigeon droppings

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Abstract

Objective

Cryptococcus neoformans and Cryptococcus laurentii are opportunistic fungal pathogens associated with cryptococcosis, and pigeon droppings serve as a major environmental reservoir for these organisms. Increasing antifungal resistance and toxicity of conventional therapies have prompted the search for alternative antimicrobial agents. This study aimed to evaluate and compare the antifungal activity of green-synthesized zinc oxide nanoparticles (ZnO NPs), garlic (Allium sativum) extract, and conventional antifungal agents against Cryptococcus spp. isolated from pigeon droppings.

Materials and methods

A total of 40 pigeon dropping samples were collected from different locations in Diyala Governorate, Iraq. Fungal isolation was performed using Sabouraud dextrose agar supplemented with chloramphenicol, and identification was confirmed through microscopic examination and the VITEK 2 system. Antifungal susceptibility testing was conducted using the agar well diffusion

method and broth microdilution assay. Yeast cell viability was assessed using the resazurin-based Alamar Blue indicator. ZnO nanoparticles were synthesized via a green method using garlic extract as a reducing and stabilizing agent.

Results

Among the examined samples, 13 (32.5%) were positive for Cryptococcus spp., of which 61.5% were identified as *C. neoformans* and 38.4% as C. laurentii. Both ZnO nanoparticles and garlic extract exhibited significant, concentration-dependent antifungal activity against the tested isolates. ZnO nanoparticles demonstrated notable inhibitory effects, with variable inhibition zones and minimum inhibitory concentration (MIC) values ranging across tested concentrations. Similarly, garlic extract showed appreciable antifungal activity against both species, although with slightly lower potency compared to nanoparticles. Conventional antifungal agents, including itraconazole and fluconazole, also showed inhibitory effects; however, variability in susceptibility patterns was observed among isolates.

Conclusion

Overall, the results indicate that green-synthesized ZnO nanoparticles possess strong antifungal potential, likely due to their nanoscale size, high surface area, and ability to disrupt fungal cellular structures. Garlic extract also contributes antifungal bioactivity through its phytochemical constituents, particularly allicin. The combined findings suggest that both green nanotechnology and plant-derived compounds may serve as promising complementary or alternative strategies for managing Cryptococcus infections, particularly in the context of emerging antifungal resistance.

Keywords: antifungal activity, *Cryptococcus laurentii*, *Cryptococcus neoformans*, garlic extract, zinc oxide nanoparticles

Paper Type: Research Paper.

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Introduction

The genus *Cryptococcus* consists of yeasts that are found throughout the environment, and several of them have major medical and veterinary importance. These fungi occur abundantly in soil, decaying wood and plant debris and especially in pigeon dung. Humans and animals become infected mostly by inhalation of basidiospores or desiccated yeast cells from environmental

sources. Infection is primarily pulmonary, with central nervous system infection occurring in more severe cases, being able to be fatal (Kwon-Chung et al., 2014, DR Arora and Arora, 2024). Based on their form and structure, *Cryptococcus* species are encapsulated yeasts that can cause serious opportunistic infections (Briner et al., 2025). Cryptococcosis, a type of deep opportunistic mycosis, is found in most regions of the world but is more common in tropical and subtropical countries. Even though the disease is rare, cases have been recorded from various continents. For instance, in Iran, meta-analysis confirmed the rates of disease at 1.7% in animals and 2.8% in humans (Mahmoudi et al., 2019). *Cryptococcus neoformans* is considered the most virulent species within the genus. It has a strong association with pigeons which is an important environmental reservoir. Pigeon waste provides excellent nutritional conditions, especially high nitrogen content, which supports the survival and growth of fungi in the environment (Mahmoudi et al., 2019). Numerous studies have reported the recovery of Cryptococcus species, including *C. neoformans* sensu lato, from dried bird excreta, highlighting the epidemiological importance of avian habitats (Brito et al., 2019; Montagna et al., 2018). In recent decades, increasing attention has been directed toward non-neoformans *Cryptococcus* species, such as *C. laurentii*, *C. albidus* and *C. uniguttulatus*, which were previously considered non-pathogenic. Opportunistic pathogens from bird environments have frequently been isolated (Brito et al., 2019). According to environmental studies, pigeon droppings may contain up to 50 million yeast cells per gram. This highlights the resilience and adaptability of *Cryptococcus* species in such microhabitats (Soltani et al. 2013). The pathogenicity of Cryptococcus spp. and the extent of host damage depend on various virulence factors. These consist of capsule synthesis, melanin synthesis, heat tolerance, and discharge of more extracellular enzymes such as proteases, lipases, urease, and phospholipases (Al-Huthaifi et al., 2024; Yang et al., 2017). *C. neoformans* produces several virulence-associated enzymes which closely resemble those produced by various pathogenic fungi and bacteria that aids tissue invasion and immune evasion (Almeida et al., 2015). Urease is a nitrogen metabolism enzyme, which means it breaks down urea to produce ammonia and carbon dioxide. This is also an important diagnostic and pathogenic marker in cryptococcosis (Olszewski et al., 2004). At present, treatment of cryptococcosis predominantly relies on antifungal drugs. Amphotericin B, often in combination with flucytosine, is the mainstay of treatment. But increasing antifungal resistance and drug-related toxicity have created an urgent need for alternative and more effective therapeutic strategies (El-Saber Batiha et al., 2020; Wu et al., 2025). On the other hand, nanotechnology is a multidisciplinary scientific field that uses a set of tools and techniques derived from engineering, physics, chemistry and biology (Mohammadabadi et al., 2009). Advances in nanoscience and nanotechnology have routinely enabled the fabrication and identification of submicron bioactive carriers. The delivery of bioactive substances to target sites in the body and their release behavior are directly affected by particle size (Zarrabi et al., 2020). Compared to micrometer-sized carriers, nanocarriers provide more surface area and have the potential to increase solubility, increase bioavailability, improve controlled release, and enable precise targeting of entrapped substances (Mohammadabadi and Mozafari, 2019). Nanoparticle-based antifungal approaches are gaining emphasis because they favour drug delivery and penetration into fungal cells and biofilms with reduced toxicity. These properties may assist in

overcoming resistance mechanisms (Wu et al., 2025). In addition to nanoscale techniques, garlic (*Allium sativum*) is a natural product with broad-spectrum anti-microbial and -fungal activities. The principal bioactive constituent, allicin, has the ability to permeate the cell membranes of fungi and interrupt key enzymatic processes, leading to fungicidal activities toward an array of fungal pathogens including Cryptococcus species (El-Saber Batiha et al., 2020). Furthermore, the properties of garlic extracts such as antioxidant, anti-inflammatory, immunomodulatory, and neuroprotective activity support their use as additional antifungal agents. (Shang et al., 2019; Arreola et al., 2015). Due to increasing antifungal resistance and drug toxicity, there is an urgent need for alternative therapeutic strategies. Nanoparticles have emerged as promising antifungal agents due to their ability to enhance drug delivery and disrupt fungal cells. In addition, garlic (Allium sativum) contains bioactive compounds such as allicin, which exhibit broad-spectrum antifungal activity. Therefore, this study aimed to evaluate the antifungal effects of green-synthesized ZnO nanoparticles, garlic extract, and conventional antifungal agents against Cryptococcus spp. isolated from pigeon droppings.

Materials and method

Sampling: A total of 40 pigeon dropping samples were collected in sterile containers and then transferred to the Microbiology Laboratory at the College of Veterinary Medicine, University of Diyala, for mycological examination. The samples were processed following the procedure reported by previous studies (Montenegro & Paula, 2000; Zarrin et al., 2010) with minor adjustments. For sample preparation, 1 g of weathered pigeon droppings was mixed with 10 mL of sterile distilled water to produce a 1:10 dilution. The mixture was thoroughly homogenized and filtered through sterile gauze to remove coarse debris. Aliquots from the 10^{-1} dilution were then aseptically inoculated onto Sabouraud dextrose agar supplemented with chloramphenicol to suppress bacterial contamination. The inoculated plates were incubated at 35 ± 2 °C for 48 to 72 hours (Walsh, 2018; Arora and Arora, 2024). The culture plates were examined daily for fungal growth. Colonies showing positive growth were sub cultured onto fresh SDA plates to obtain pure isolates.

Isolation and identification: In order to isolate *Cryptococcus* spp., the samples were inoculated on mycological media at 35 ± 2 °C and identified microscopically. Lactophenol cotton blue was used to stain fungal colonies to observe cellular morphology. A clean glass slide was placed on a drop of the stain. Following that, a small piece of the fungal colony was picked and mixed with the stain using a sterile inoculating loop, covered with a coverslip. Under the light microscope, the prepared slides were examined for the morphological features of the fungus. The procedure was repeated using India ink to visualize encapsulated cells (Kidd et al., 2026). The VITEK 2 Compact system was used to confirm species identification and determine antifungal susceptibility profiles.

McFarland standard solution: 0.5 McFarland standard (~1–5 × 10^6 CFU/mL for yeast) was acquired (bioMérieux) and utilized to adapt the turbidity of yeast suspensions. Microbial testing has a use of a standard that ensures cell density is not outside an acceptable range (CLSI, 2026).

Preparation of plant extract: The studied fresh garlic bulbs (*Allium sativum* L.) were purchased from the local market and identified by Prof. Dr. Kazal D. Wadi, College of Science, University of Diyala. The bulbs were employed to prepare an aqueous extract using a standard extraction method with a few alterations (Parekh and Canada, 2007). To start with, the garlic cloves were peeled manually, washed with tap water under running water followed by distilled water to remove dust, impurities and peel contents. The bulbs were thoroughly ground in a sterile mortar and pestle or electrically blended after cleaning. We mixed 25 g crushed garlic and 100 mL distilled water and thoroughly homogenized it. To increase the extraction of bioactive compounds the mixture was heated for 30 minutes at 50–60 °C using hot plates through magnetic stirring. The extract was filtered through sterile gauze / Whatman No. 1 filter paper to remove solids. The filtrate was collected in a sterile container and stored at 4 °C until used for antifungal assays.

Green synthesis of ZnO nanoparticles (ZnO-G): ZnO nanoparticles were synthesized applying the method of Bouttier-Figueroa et al. (2024) with slight modifications. Zinc acetate dihydrate is prepared by dissolving 4 g in 50 mL distilled water first. The garlic bulb extract in the volumes of 10 and 20 mL was gradually added while stirring the solution continuously for 10 minutes at room temperature.Subsequently, a 1 M NaOH solution was added dropwise until the pH attained 12. Heating at 50 °C for 2 hours was continued to form a precipitate. The precipitate was collected by centrifugation, washed, and dried at 60°C for 12 hours. The same procedure was followed for 20 mL and 30 mL extracts.

Antimicrobial activity of ZnNPs against *Cryptococcus spp:* The antimicrobial activity of ZnNPs was evaluated using the agar well diffusion method according to Gonelimali et al., (2018) and Daoud et al., (2015), A stock solution of ZnNPs was prepared at a concentration of 5 mg / ml and sterilized using a 0.22 µm Millipore filter. A suspension of yeast was obtained from 3 –5 well-isolated colonies which was suspended in normal saline and incubated at 37 °C for 2 hours till the turbidity was that of 0.5 McFarland standard (1.5 × 106 CFU/mL). Using a sterile swab, the prepared suspension was uniformly spread over Mueller-Hinton agar supplemented appropriately or RPMI 1640 medium to a thickness of 4 mm. Using a sterile cork borer, 6 mm diameter wells were made in the agar. In each well, a volume of 100 µL of ZnNP solution was added. The plates were subsequently placed in an incubator for 18 to 24 hours at a temperature of 35 ± 2 °C. Utilizing a digital vernier caliper, the diameter of the inhibition zone was measured in millimeters after incubation.

Minimum inhibitory concentration (MIC): The antimicrobial agent of lowest concentration which inhibits the visible growth of a yeast without killing the cells is the minimal inhibitory concentration (Balouiri et al., 2016) MIC values were determined using the broth microdilution method in 96-well polystyrene plates. A quantitative evaluation of the in vitro antimicrobial activity of ZnNPs against yeast isolates was performed according to the recommendations of the Clinical and Laboratory Standards Institute (CLSI, 2026) and the protocols described by Eloff (1998) and Balouiri et al. (2016) with some modifications.

Description of ZnO nanoparticles (ZnO-G): Field emission scanning electron microscopy (FE-SEM) was similar to an optical microscope except that it uses a focused beam of electrons

rather than light to focus on a specimen. The electrons are emitted from the field emission source and scan the surface of the sample in zigzag motion (Al-Shammary and Al-Mayaly, 2024). In the UV-visible spectroscopy occasioned a maximum absorbance of nanoparticles. This absorption-based technique identifies how electrons are excited from the ground state to upper energy levels when the particles absorb light (Rocha et al., 2018; Al-Shammary and Al-Mayaly, 2024). X-ray diffraction pattern was a non-destructive technique used to reveal the crystalline structure and estimate average particle size of nanoparticles (Bunaciu et al., 2015). Fourier Transform Infrared Spectroscopy used to identify the functional groups and chemical bonds present in the sample.For the above analyses approximately 0.001 g of nanoparticles was taken on a suitable sample holder and fed into the respective machines. The analysis of UV-vis was achieved in a two-phase system consisting of a blank and a sample.

Statistical analysis: ANOVA single factor was used to analyse the difference between variables.

Results

Isolation and identification of *Cryptococcus* spp: Forty samples included in this study, 13 samples (32.5%) were positive for *Cryptococcus* species. Among the positive samples, 8 isolates (61.5 %) were identified as *Cryptococcus neoformans* and 5 isolates (38.4 %) as *C. laurentii*, from dropping of suspected animals having clinical symptoms in Diyala Province, were cultured on Sabouraud dextrose agar. The sample identified by convential macroscopic and microscopic methods (Figures 1 and 2). Confirmatory diagnosis the results of the VITEK2 technique showed that the two species had a 100 % identity to with a probability of 93% of *Cryptococcus neoformans* and 94% of *Cryptococcus laurentii*. Produced end confirmatory identification of the isolates with excellent accuracy.

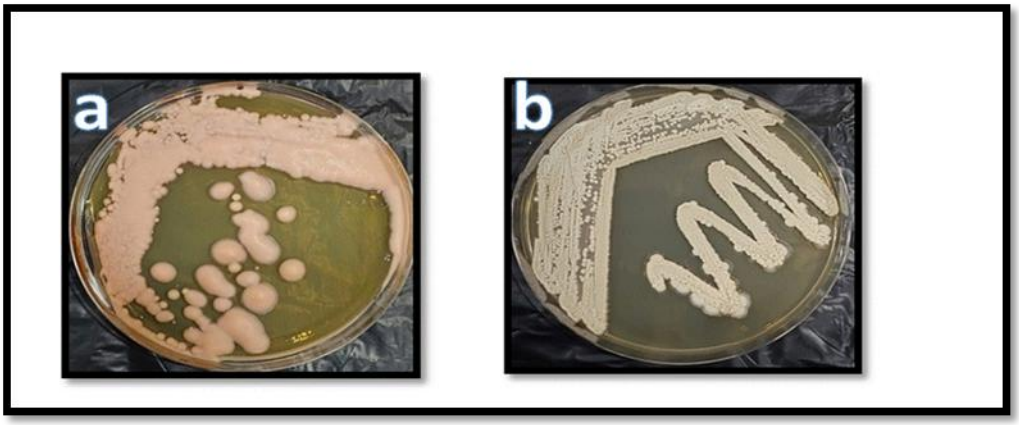

Figure 1. Macroscopically appearance of *Cryptococcus spp* cultured on SDA after 3 days at 35±2 °C; a, *C. laurentii* b, *C. neoformans*

The current study allows for the distinguishing of yeasts that cause respiratory infections in birds. Samples, which collected from the droppings of pigeons exhibiting clinical symptoms.

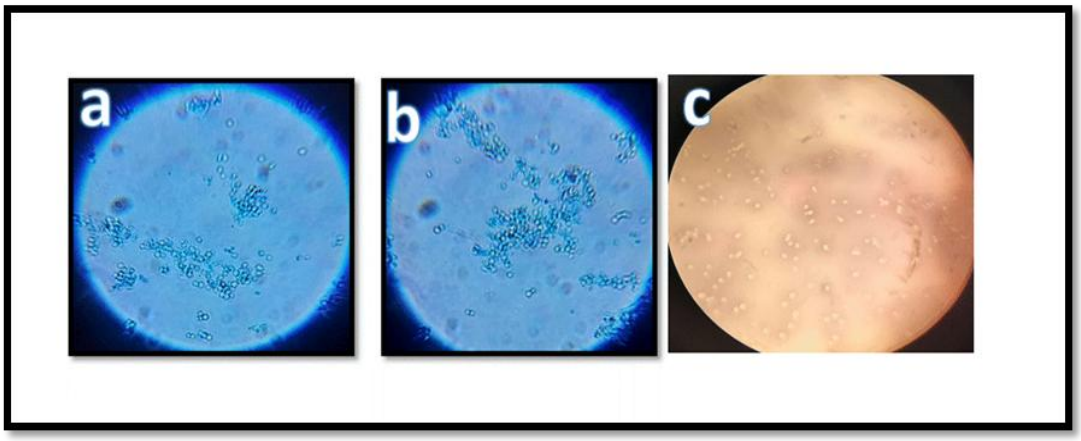

Figure 2. Microscopic appearance of *Cryptococcus* spp.: (a) *C. laurentii* stained with lactophenol cotton blue (×40); (b) *C. neoformans* stained with lactophenol cotton blue (×40); (c) *C. neoformans* visualized using India ink (×40)

Field emission scanning electron microscopy (FESEM) analysis: Figure 3 presents FESEM micrographs that illustrate the morphology of zinc oxide nanoparticles produced through a green synthesis route, where garlic extract acted as the reducing agent. The images reveal that the synthesized nanoparticles display a relatively consistent nanoscale organization, mainly composed of spherical particles that aggregate into porous clustered formations. By measuring the nanoparticles on the images provided, the diameters of the nanoparticles are within the range of ≈11-27 nm, which confirms the success of the biosynthesis method in producing nanoparticles of small size. This structural behavior of the nanoparticles is due to the contribution of the bioactive compounds in the garlic extract that were used as reducing and stabilizing agents in controlling the growth of the crystal and preventing agglomeration of the nanoparticles. However, some level of agglomeration of the nanoparticles is also evident due to the high surface energy of the nanoparticles. The small particle size and porous morphology suggest a high specific surface area, which makes them good candidates for photocatalytic, antimicrobial, and decontamination activities and further prove the efficiency of the green method in synthesizing nanoparticles of zinc oxide that have advanced structural and functional properties (Khadka et al., 2025). Figure 4 shows the UV-Vis absorption spectrum of the biosynthesized ZnO nanoparticles using garlic extract as the reducing agent, strong absorption peaks are observed in the ultraviolet region of the spectrum. The strong absorption peaks Confirmed the successful formation of ZnO NPs. The absorption peak at λmax 296 nm is attributed to the intrinsic band-edge absorption of ZnO nanoparticles. The band-edge absorption peak at λmax 296 nm arises from electron transition from the valence band to the conduction band. The shoulder peak at λmax ≈ 345 nm may arise from the surface states and organic molecules present on the ZnO nanoparticles. The absorption peaks slightly shift toward shorter wavelengths compared to those of bulk ZnO. The shift in the absorption peaks toward shorter wavelengths may arise from the quantum size effect. The absorption peak in the visible region may arise from defect states and phytochemical

functionalization. The UV-Vis absorption spectrum indicates that the ZnO nanoparticles have good optical activity.

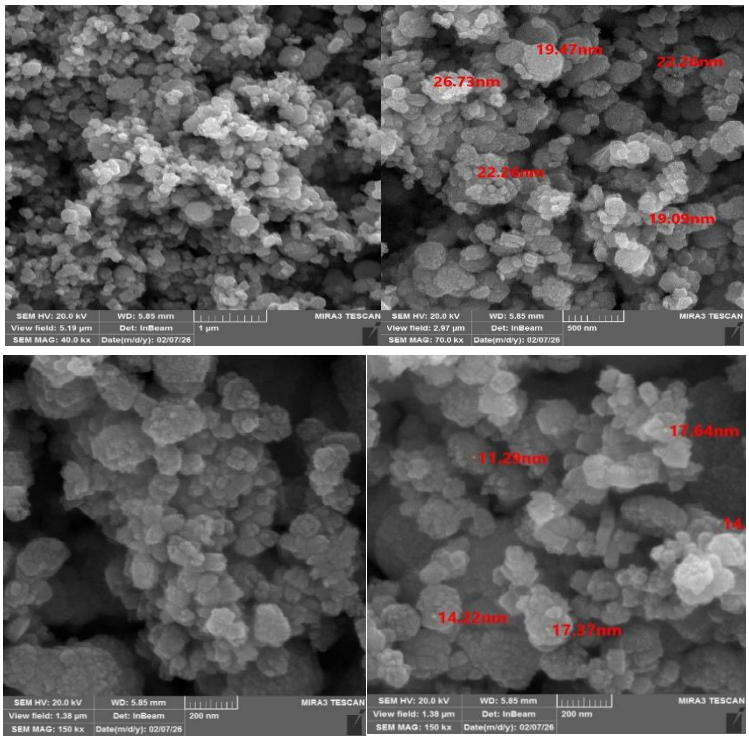

Figure 3. The FESEM of ZnO NPs synthesized via the green method

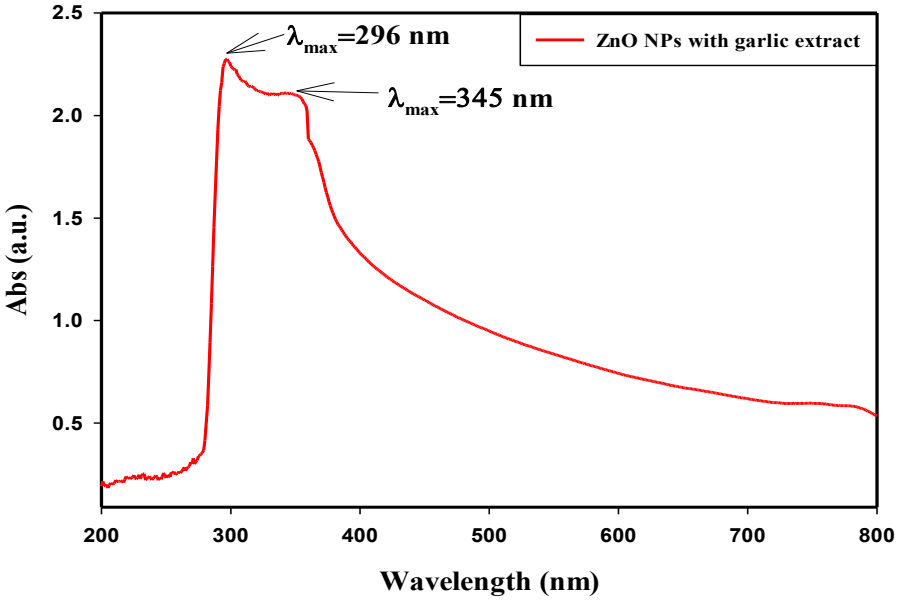

Figure 4. The UV-Vis spectrum of ZnO NPs

Figure 5 shows the X-ray diffraction pattern of ZnO nanoparticles synthesized using garlic extract as a green reducing agent. The diffraction pattern shows several sharp peaks at 2θ values of around 31.62°, 34.31°, 36.18°, 47.41°, 56.50°, and 62.79°. The observed peaks at (100), (002), (101), (102), (110), and (103) lattice planes confirm the existence of ZnO in a hexagonal wurtzite

crystal structure. Furthermore, minor peaks seen at higher angles of diffraction further support the presence of the same crystalline phase. This indicates that the synthesized product does not have any secondary phase or impurity. The strong intensity of the (101) reflection indicates that preferential crystal growth occurred in this direction. The recognition of the nanocrystalline nature of the studied particles is agreed upon, as evidenced by the broadening of peaks, whose full width at half maximum values range from approximately 0.29° to 0.57°. The d-values obtained were in agreement with the ZnO reference data which shows that the new biological method utilizing garlic extract does not alter the crystal phase but can be used for the synthesis of stable, crystalline ZnO nanoparticles for advanced functional properties.

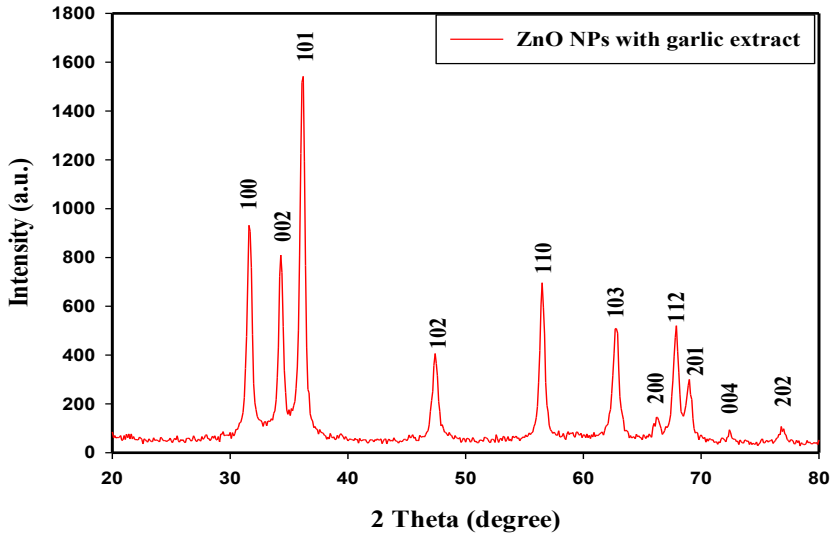

Figure 5. X-ray diffraction profile of ZnO nanoparticles produced through a garlic-extract-mediated green synthesis approach

Table 1 clearly documented parameters deduced from XRD analysis. These parameters reveal that the fresh synthesized ZnO nanoparticles indeed have a hexagonal wurtzite crystal structure. Also, interplanar spacing is found consistent. The crystallite size calculated using Scherrer equation ranges in nanoscale which is approximately 17 to 33 nm. The results confirmed the formation of ZnO nanoparticles by the green synthesis method using garlic extract.

Fourier transform infrared spectrum: The FTIR spectrum of zinc oxide nanoparticles synthesized by garlic extract is shown in Figure 6. The spectrum shows several characteristic absorption bands that are a clear confirmation of biosynthesized material and they also reveal the involvement of a plant source in the use of reducing and stabilizing agents. A strong and wide band between 3445 and 3360 cm^{-1} is attributed to broad and intense stretch vibrations due to hydroxyl and amine groups present in the phenolic and protein constituents of the extract. It is likely that these functional groups make contact with and bind to the nanoparticles. The absorption peak at around 2932 cm^{-1} corresponds to the stretching vibrations of aliphatic C–H. Band near 1789 cm^{-1} assignable to carbonyl groups of oxidized organic molecules. The attribution of peaks noticed at 1574 cm^{-1} and 1506 cm^{-1} can be due to either C=C stretching or N–H bending vibrations. A band around 1408 cm^{-1} can be assigned to C–N or COO^- functional group. The

absorption band located at almost 1123 cm^{-1} is linked to the stretching modes of C–O and C–O–C. We can observe a characteristic band at the lower region around 445 cm^{-1} which is assigned to Zn–O stretching confirming zinc oxide formation. The organic functional groups present on the nanoparticle surface indicate that bioactive compounds from garlic act as phytochemical stabilizing capping agents. The green synthesis method is thus effective as per these results. Results from the above are summarized in Table 2.

Table 1. Parameters derived from XRD analysis

No.	2θ (°)	d (Å)	FWHM (°)	(hkl)	Crystallite Size D (nm)
1	31.623	2.827	0.462	(100)	17.5
2	34.306	2.6118	0.385	(002)	21.5
3	36.178	2.4808	0.461	(101)	18.0
4	47.410	1.916	0.512	(102)	17.0
5	56.505	1.6273	0.457	(110)	19.5
6	62.792	1.4786	0.574	(103)	16.5
7	66.216	1.4102	0.292	(200)	33.0
8	67.882	1.3796	0.373	(112)	26.0
9	68.993	1.3601	0.486	(201)	20.0
10	72.408	1.3041	0.342	(004)	30.0
11	76.828	1.2397	0.399	(202)	27.0

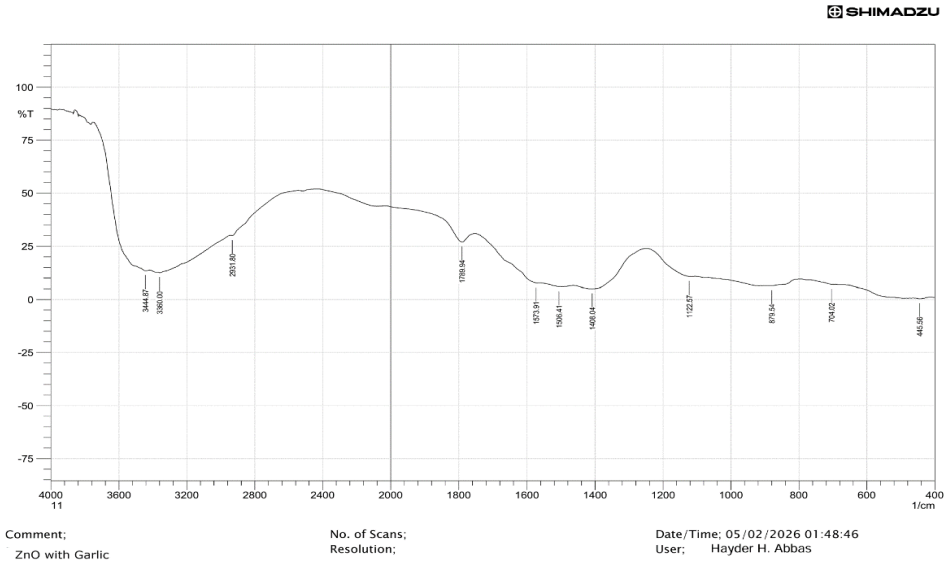

Figure 6. FTIR spectrum of ZnO nanoparticles synthesized through a garlic-extract-mediated green approach

Determination of minimum inhibitory concentration (MIC) of ZnO NPs, Garlic extract and antifungal activity: The assay was performed using sterile 96-well microtiter plates, where Alamar Blue reagent, a resazurin-based indicator, was introduced into each well. A shift in color from blue to pink reflected cellular metabolic activity and indicated the presence of viable cells.

The minimum inhibitory concentration was identified as the lowest concentration at which no color transition occurred (Figure 7).

Table 2. FTIR analysis results of ZnO nanoparticles obtained using garlic extract

No.	Peak Position (cm^{-1})	Assigned Group / Compound	Vibration Mode	Scientific Interpretation and Significance
1	3445–3360	O–H, N–H	Stretching	Broad band attributed to hydroxyl and amine groups from phenolic and proteinaceous compounds in garlic extract, indicating their role as bioreducing and stabilizing agents for the nanoparticles
2	2932	Aliphatic C–H	Stretching	Corresponds to aliphatic carbon chains from organic phytochemicals attached to the nanoparticle surface
3	1789	C=O	Stretching	Assigned to carbonyl groups (aldehydes/ketones/esters) from oxidized organic constituents involved in the green synthesis process
4	1574	N–H or C=C	Bending / Stretching	Related to amine vibrations or aromatic C=C bonds, suggesting the presence of bio-organic residues bound to the surface
5	1506	Aromatic C=C	Stretching	Indicates aromatic ring structures from plant metabolites acting as capping agents
6	1408	C–N or COO^-	Stretching	Associated with amine or carboxylate groups contributing to nanoparticle stabilization and anti-aggregation effects
7	1123	C–O, C–O–C	Stretching	Assigned to alcohol or ether groups from biomolecules coating the nanoparticles
8	875	Aromatic C–H (out-of-plane)	Bending	May indicate aromatic structures or minor surface carbonate traces
9	704	Ring vibrations	Bending	Additional evidence of aromatic organic compounds from the extract
10	445	Zn–O	Stretching	Characteristic Zn–O vibration band confirming the successful formation of ZnO nanoparticles

The antifungal activity of green-synthesized ZnO nanoparticles was evaluated against both Cryptococcus species. The nanoparticles exhibited concentration-dependent inhibitory effects against *C. laurentii* and *C. neoformans*. The measured inhibition zone diameters are presented in Table 3, while the MIC values are summarized in Table 4. Statistical analysis showed no

significant difference between the susceptibility of the two species to ZnO nanoparticles (P = 0.53696).

Table 3. Mean inhibition zone diameter (mm) of green-synthesized ZnO NPs (ZnO-G) at different concentrations against *Cryptococcus laurentii* and *C. neoformans*

ZnO-G	*Cryptococcus laurentti*	*Cryptococcus neoformans*	P value
1	26.95	25.12	0.656394
2	27.31	16.92	
3	8.17	9.12	

Table 4. MIC ranges for *Cryptococcus laurentii* and *Cryptococcus neoformans*

	ZnO-G (MIC g/mL)	
Sample	*Cryptococcus laurentii*	*Cryptococcus neoformans*
1	0.07	0.07
2	0.07	0.07
3	0.03	0.03
4	0.07	0.03
T test P value	0.53696	

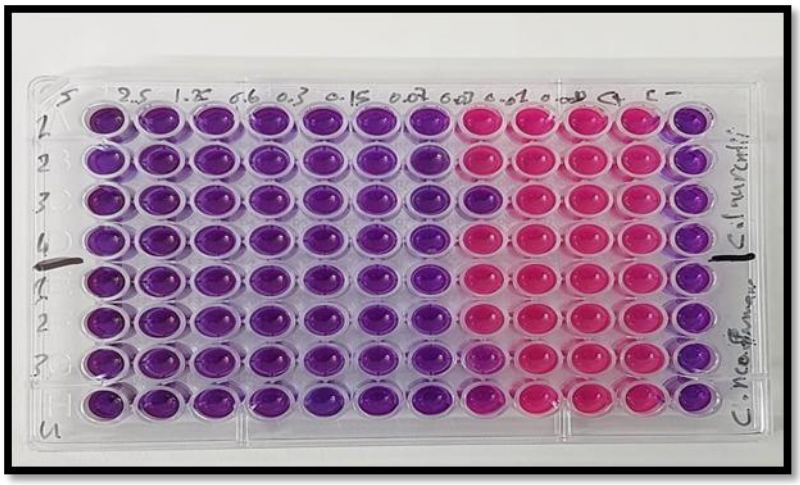

Figure 7. Comparison of MIC values of green-synthesized ZnO nanoparticles against Cryptococcus neoformans and Cryptococcus laurentii (P = 0.53696)

Tables 5 and 6 summarize the antifungal activity of garlic extract against Cryptococcus laurentii and Cryptococcus neoformans. The inhibition zone diameters ranged from 8.78 to 20.24 mm for *C. laurentii* and from 8.27 to 19.23 mm for *C. neoformans*. No statistically significant difference was observed between the two species (P = 0.766260). Figure 8 also shows the effect of garlic extract against the two species of *Cryptococcus.*

Table 5. Mean inhibition zone diameter (mm) of garlic extract against Cryptococcus laurentii and Cryptococcus neoformans

Garlic extract	*Cryptococcus laurentti*	*Cryptococcus neoformans*	P value
1	20.24	19.23	
2	17.02	16.12	0.8725964
3	8.78	8.27	

Table 6. MIC ranges for *Cryptococcus neoformans* and *Cryptococcus laurintii*

Garlic extract (MIC mg/mL)		
Sample	***Cryptococcus laurentii***	***Cryptococcus neoformans***
1	0.15	0.07
2	0.07	0.07
3	0.07	0.07
4	0.03	0.15
T test p value	0.766260	

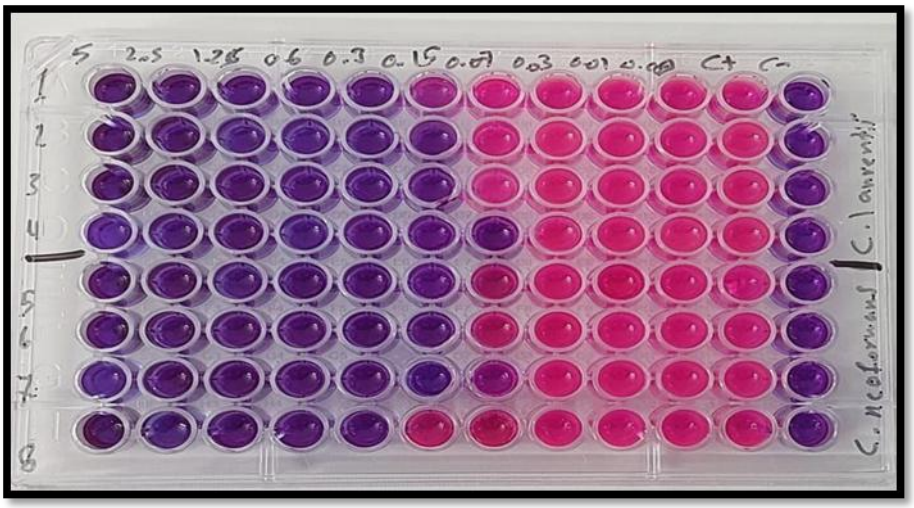

Figure 8. The effect of different concentration of Garlic extract against Cryptococcus *neoformans* and *Cryptococcus laurentii* (0.766260)

Tables 7, 8, and 9 demonstrates the antifungal effects against *Cryptococcus laurintii* and against *Cryptococcus neoformans*. A statistically significant difference was observed in fluconazole MIC values between *C. laurentii* and *C. neoformans* isolates (P = 0.024). Figure 9 and 10 show the activity of two antifungals against *Cryptococcus* spp.

Table 7. Strength of Antifungal against *Cryptococcus neoformans* and *Cryptococcus laurintii*

Antifungal	Traconazole	Fluconazole	P value
1	16.97	12.77	0.415639
2	17.40	19.29	
3	15.22	15.48	
4	12.36	23.50	

Table 8. MIC ranges of Antifungal for *Cryptococcus neoformans* and *Cryptococcus laurintii*

MIC Itraconazole (µg)		
Sample	***Cryptococcus laurentii***	***Cryptococcus neoformans***
1	0.125	0.125
2	0.125	0.125
3	0.125	0.125
4	0.125	0.125
T test, p value	t = 1.94318, P = 0.10	

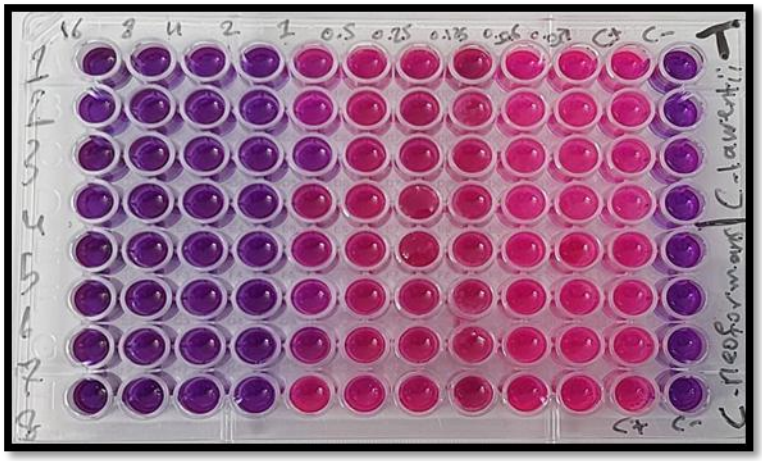

Figure 9. Comparison of itraconazole MIC values against Cryptococcus neoformans and Cryptococcus laurentii

MIC fluconazole (µg)		
Sample	***Cryptococcus laurentii***	***Cryptococcus neoformans***
1	1	0.5
2	1	0.5
3	1	0.5
4	0.5	0.5
T test p value	0.024008*	

Discussion

This study focused on the detection and characterization of *Cryptococcus* species obtained from pigeon droppings in Iraq and evaluation of antifungal activity of nanoparticles, garlic extract and conventional antifungal agents. The result indicated that humid conditions and reduced sunlight exposure in pigeon-dropping habitats were favourable for the survival and growth of fungi. The standard procedure for fungal diagnosis usually relies upon culture-based techniques, allowing for identification of species and antifungal susceptibility testing. *Cryptococcus species* are a group of clinically important pathogenic fungi that produce serious infections and potentially lethal diseases, especially in immunocompromised individuals (Kwon-Chung et al., 2014).

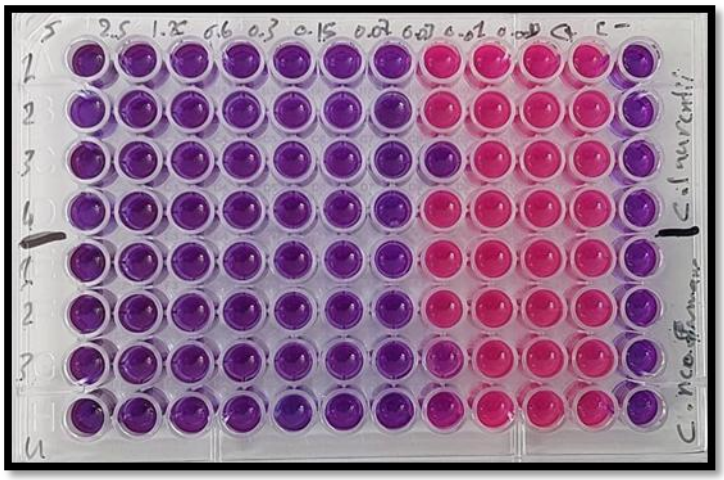

Figure 10. The effect of fluconazole against Cryptococcus *neoformans* and *Cryptococcus laurintii* (0.024008)

The association between *Cryptococcus* neoformans and pigeon droppings is mainly due to the presence of nitrogenous compounds such as urea, uric acid, creatinine and xanthine that support the growth of this fungus (Arora and Arora, 2024). The current findings suggest that pigeon excreta may play an important environmental reservoir for other species of *Cryptococcus*, including *C. neoformans*, *C. albidus*, and *C. laurentii*, that are associated with human cryptococcosis. These findings are in accordance with earlier published studies of Canónico-González et al. (2013) as well as studies conducted in other countries (Kamal & Al-Haddad, 2022), which supports pigeons' epidemiological role in the environmental dissemination of Cryptococcus species.

Antifungal activity of green-synthesized nanoparticles: The present study demonstrated that the combination of *Allium sativum* extract with nanoparticles exhibited significant antifungal activity against *C. laurentii* and *C. neoformans*. The inhibition zones show that nano-formulated garlic is better than the garlic component alone. The likely cause of that improvement is attributed to the beneficial synergistic interactions between bioactive materials from garlic and the physicochemical properties of nanoparticles, which have enhanced the antifungal effect. Combining ZnO nanoparticles with Allium sativum extract is an efficient way of developing new antimicrobial agents that may help in overcoming antimicrobial resistance. The natural origin of these compounds also meets the increasing demand for sustainable and environmentally friendly antimicrobial solutions. However, their use in biomedical applications requires careful evaluation of biocompatibility to ensure safety. Compounds like terpenoids that come from plants can stabilise the synthesized nanoparticles. They perform capping to reduce direct interactions of metal ions thereby also improving the intrinsic biological resistance of these nanoparticles (Jain et al., 2021). Moreover, studies have revealed that the combined antimicrobial properties of essential oils and nanoparticles can effectively inhibit microorganisms; for example, mixing *Cinnamon*, *Citrus*, and ***lava*** essential oils with silver nanoparticles exhibited synergistic antifungal and antibacterial action against *Mucor circinelloides Aspergillus*, *E. coli*, and

Penicillium chrysogenum (Dorjee et al., 2023). In accordance with the purposes of the present work, the antifungal potential of nanoparticles was assessed to be a possible alternative or complement to the control of *Cryptococcus* infection. Nanotechnology is quickly becoming a viable approach due to its several antifungal mechanisms of action. Nanoparticles can disrupt capsule integrity, improve intracellular drug delivery, and interfere with virulence factors, notably those involved in capsule biosynthesis and tissue invasion. Silver and selenium nanoparticles are known to interact with the capsular structures and generate oxidative stress leading to membrane damage and release of intracellular materials eventually causing cell death in fungi (Na Pombejra et al., 2017). Also, nanoparticles with irregular or sharp-edged surface features show enhanced antifungal activity due to their ability to penetrate and disrupt fungal cell wall and membrane (Baigorria et al., 2024). Besides the chemical interaction, nanoparticles can also exert mechanical stress on the fungal membrane. According to Pincus & Qi (2025), a biophysical model that was proposed indicates that the deformation of the membrane caused by the adhesion of a nanoparticle occurs due to stretching and compressing of the structures that results in structural instability of the membrane and rupture of the membrane. Upon combined evaluation of minimum inhibitory concentration and effector concentration, the combined treatment demonstrated synergistic antifungal effects.

Antifungal effects of garlic and its bioactive compounds: Garlic offers antimicrobial activity, with a strong antifungal action against such pathogens as *Cryptococcus* spp. and other fungal pathogens. (Davis et al., 1994). Garlic extracts are known to have fungicidal and fungistatic properties under in vitro conditions (Yoshida et al., 1987). In China, people have used garlic-based preparations to treat cryptococcosis, a systemic fungal infection, for many years (Davis et al., 1994). The main bioactive compound in garlic that has antifungal activity is allicin which is a sulfur-containing compound. (El-Saber Batiha et al., 2020). Allicin has good antifungal activity on Cryptococcus species in vitro at low concentrations with minimum inhibitory concentration values ranging from 1.57 to 6.25 µg/mL (Yamada & Azuma, 1977). It stops germination of spores and hyphal development. In addition, the antifungal mechanisms of allicin and garlic oil involve the disruption of fungal cell membranes and intracellular organelles, including the mitochondria. This damage causes impairment of organelles and ultimately cell death (Borlinghaus et al., 2014). These findings lend more support to the use of garlic as a natural antifungal agent.

Antifungal drug susceptibility patterns: The antifungal susceptibility testing was performed in this study revealed varied responses by *Cryptococcus* spp. to classic antifungal agents. The determination of minimum inhibitory concentrations (MICs) is still an important method for evaluating antifungal activity. While fluconazole usually produces higher plasma levels than itraconazole. The elevated MIC values found in certain isolates may be due to a phenomenon known as tolerance rather than more stable genetic resistance. Tolerance is the ability of fungal cells to survive transiently antifungal stress without permanent resistance mechanisms. Reports indicate that resistance to fluconazole, amphotericin B, itraconazole, voriconazole, nystatin, and other drugs is growing in several isolates of Cryptococcus. Results obtained conform with Sionov et al. (2013), which reported the growing resistance of C. neoformans to azole treatment. The pattern indicates a major global threat which is accompanied

with prolonged or incorrect use of antifungal in poorer regions. Past studies have indicated that fluconazole has low MIC_{40} and MIC_{90} values against *C. Neoformans* (Chong et al., 2010). In vitro activity of itraconazole shown to be more potent, which is evident with lower MIC values of ≤0.25 µg/mL as quoted in earlier reports (Herkert et al., 2018). Yet in the current study 14.13% of C. The neoformans isolates were classified as susceptible–dose dependent to itraconazole, consistent with findings reported for Serbian isolates (Arsic Arsenijevic et al., 2014). Moreover, colored C. The presence of melanin in the cell wall of neoformans cells most probably reduced the penetration of fluconazole to intracellular targets, rendering the neoformans cells less sensitive (Ikeda et al., 2003). The study also found a positive correlation between MIC values of fluconazole and itraconazole which indicates a simultaneous development of resistance to azole antifungals as supported by the work of Trpković et al. (2012). The simultaneous increase in MIC values of both drugs which was significant at level 0.01 indicates the need to alter treatment protocols. There are nanoparticle-based therapies and plant-derived substances that potentially improve cryptococcal treatment.

Conclusion: The results of this study showed that pigeon droppings, as one of the most important environmental reservoirs of Cryptococcus species, especially Cryptococcus neoformans and Cryptococcus laurentii, play a significant role in the spread of these pathogens. The relatively high isolation rate of these yeasts from the studied samples further highlights the importance of sanitary monitoring of environments contaminated with bird droppings. Investigation of the antifungal activity of various agents showed that zinc oxide nanoparticles synthesized by a green method using garlic extract have a significant and concentration-dependent inhibitory effect against both Cryptococcus species. The physicochemical properties of these nanoparticles, including nanometer size, suitable crystal structure, and high specific surface area, probably played a fundamental role in increasing their antifungal efficacy. Also, garlic extract showed favorable antifungal activity due to the presence of bioactive compounds, especially allicin, although its effectiveness was slightly lower compared to ZnO nanoparticles. On the other hand, although conventional antifungal drugs such as fluconazole and itraconazole were still effective, the difference in susceptibility patterns and the increase in MIC values in some isolates highlight the need to search for alternative or complementary therapeutic strategies. The findings of this study indicate that the use of greenly synthesized nanoparticles and natural plant compounds can be considered as a promising, safe and environmentally friendly approach for the control and treatment of cryptococcal infections, especially in the context of increasing drug resistance. In summary, green nanotechnology based on garlic extract and zinc oxide nanoparticles has great potential for the development of a new generation of antifungal agents. However, further studies in vivo, investigating biotoxicity, molecular mechanisms of action and evaluating their clinical applications are necessary to confirm the efficacy and safety of these compounds.

Author contributions

Noor Their Talib: Investigation, Formal analysis, Validation, Writing - original draft. Hiba Yaseen Abbass: Data curation, Formal analysis, Validation, Writing - original draft. Shahad

Abdul Majeed Aswad: Conceptualization, Methodology, Supervision, Writing - review & editing. Noor Ali Faleh: Data curation, Formal analysis, Validation, Writing - original draft. Anaaam Faud Hussain: Resources, Validation.

Data availability statement

The data contributing to the findings of this study are available from the investigating researcher upon request.

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Ethical considerations

The authors avoided data fabrication, falsification, plagiarism, and misconduct.

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Conflict of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper. No financial support was received from any organization or agency for the research conducted. The authors alone are responsible for the content and writing of this manuscript.

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چکیده

هدف: *Cryptococcus neoformans* و *Cryptococcus laurentii* قارچ‌های بیماری‌زای فرصت‌طلبی هستند که با بیماری کریپتوکوکوزیس ارتباط دارند و فضولات کبوتر یکی از مهم‌ترین مخازن محیطی این میکروارگانیسم‌ها محسوب می‌شود. افزایش مقاومت قارچی و سمیت ناشی از درمان‌های ضدقارچی متداول، ضرورت جستجوی عوامل ضدمیکروبی جایگزین را برجسته ساخته است. هدف این مطالعه، ارزیابی و مقایسه فعالیت ضدقارچی نانوذرات اکسید روی سنتزشده به روش سبز، عصاره سیر و داروهای ضدقارچی رایج علیه گونه‌های *Cryptococcus* جداشده از فضولات کبوتر بود.

مواد و روش‌ها: در مجموع، ۴۰ نمونه فضولات کبوتر از مناطق مختلف استان دیالی عراق جمع‌آوری شد. جداسازی قارچ‌ها با استفاده از محیط کشت سابورو دکستروز آگار حاوی کلرامفنیکل انجام شد و شناسایی آنها از طریق بررسی میکروسکوپی و سامانه

VITEK 2 تأیید گردید. حساسیت ضدقارچی با استفاده از روش انتشار در چاهک آگار و آزمون رقت‌سازی متوالی در محیط مایع ارزیابی شد. زنده‌مانی سلول‌های مخمری با استفاده از شناساگر آلامار بلو مبتنی بر رزازورین بررسی گردید. نانوذرات اکسید روی به روش سنتز سبز و با استفاده از عصاره سیر به‌عنوان عامل احیاکننده و پایدارکننده تولید شدند.

نتایج: از میان نمونه‌های بررسی‌شده، ۱۳ نمونه (۵/۳۲ درصد) از نظر گونه‌های *Cryptococcus* مثبت بودند که از این میان، ۵/۶۱ درصد به عنوان *C. neoformans* و ۴/۳۸ درصد به عنوان *C. laurentii* شناسایی شدند. نانوذرات اکسید روی و عصاره سیر هر دو فعالیت ضدقارچی معنی‌دار و وابسته به غلظت علیه جدایه‌های مورد مطالعه نشان دادند. نانوذرات اکسید روی اثرات مهاری قابل توجهی ایجاد کردند و در غلظت‌های مختلف، هاله‌های عدم رشد و مقادیر متفاوت حداقل غلظت مهارکنندگی مشاهده شد. به‌طور مشابه، عصاره سیر نیز فعالیت ضدقارچی مطلوبی علیه هر دو گونه نشان داد، اگرچه قدرت اثر آن اندکی کمتر از نانوذرات بود. داروهای ضدقارچی متداول از جمله ایتراکونازول و فلوکونازول نیز اثرات مهاری قابل توجهی داشتند، اما الگوهای حساسیت در میان جدایه‌های مختلف متفاوت بود.

نتیجه‌گیری: به طور کلی، نتایج این مطالعه نشان داد که نانوذرات اکسید روی سنتزشده به روش سبز دارای پتانسیل ضدقارچی قوی هستند که احتمالاً ناشی از اندازه نانومتری، سطح ویژه بالا و توانایی آنها در تخریب ساختارهای سلولی قارچها است. عصاره سیر نیز به واسطه ترکیبات فیتوشیمیایی خود، به‌ویژه آلیسین، فعالیت ضدقارچی قابل توجهی از خود نشان داد. این یافته‌ها بیانگر آن است که فناوری نانوی سبز و ترکیبات مشتق‌شده از گیاهان می‌توانند به عنوان راهبردهای مکمل یا جایگزین امیدوارکننده برای کنترل و درمان عفونت‌های ناشی از *Cryptococcus*، به‌ویژه در شرایط افزایش مقاومت ضدقارچی، مورد استفاده قرار گیرند.

کلمات کلیدی: عصاره سیر، فعالیت ضدقارچی، نانوذرات اکسید روی، *Cryptococcus laurentii*، *Cryptococcus neoformans*

نوع مقاله: پژوهشی

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