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ORIGINAL RESEARCH PAPER

Effect Iron Oxide (Fe_2O_3) nanoparticles synthesis by *Teucrium polium* L. on *Cryptococcus neformans* isolated from environmental sources in Kirkuk city, Iraq

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Abstract: This study isolated 60 samples from various locations in Kirkuk, including areas with high numbers of pigeons, and birds, residential backyards where chicken breeding takes place, and chicken selling markets where positive samples for *Cryptococcus neoformans* were identified. Five antifungals were used in different concentrations (Ketoconazole, Griseofulvin, Fluconazole, Nystatin, Clotrimazole), (0.02 g/mL, 0.04 g/mL, and 0.06 g/mL) the sensitivity of the drug to the spread method against isolates of the *Cryptococcus neformans*. The results showed that the fungal isolates were sensitive to antifungals. This study showed that nystatin has a greater inhibitory ability and a broad spectrum of activity than other antifungals, while griseofulvin did not show any inhibitory ability. Iron oxide nanoparticles (Fe_2O_3 NPs) are produced by the *Teucrium polium* L. extract as a reducing agent. We used an X-ray diffraction analysis and an SEM to confirm if these nano-oxides were present. The antifungal properties of Fe_2O_3 NPs against *Cryptococcus neformans* were investigated by the well diffusion method. With the increase in concentration of (Fe_2O_3 NPs). The diameter of inhibition growth zones of the fungal strains became greater; the *Cryptococcus neformans* growth inhibition zone was 32. 6 ± 0.6 mm, 31.2 ± 0.6 mm, 26 ± 0.5 mm, 24. 4 ± 0.4 mm and 21 ± 0.3 mm and 17.1 ± 0.0 for concentrations 6 mg/mL, 5 mg/mL, 4 mg/mL, 3 mg/mL, 2 mg/mL and 1 mg/mL respectively. Thus, we can conclude that the concentration of the solution of nanoparticles and antifungals directly affects the ability to inhibit the growth of fungi.

Keywords: *Cryptococcus neformans*, *Teucrium appolines*, Iron oxide, nanoparticles

1. Introduction

Teucrium polium L. (Lamiaceae family) is a sub-shrub plant from the Mediterranean region and the Middle East. It is widely distributed in the Republic of Macedonia and is traditionally used by native people as an herbal hypoglycemic tea. The decoctum of *T. polium* is usually taken as an appetizer, particularly among children, it is also used as a spice. Some biological and therapeutic uses of the plant include antioxidant, anti-inflamma-tory, anti-nociceptive, antipyretic, anti-microbial, hypolipidemic, hepato-protective, and cytotoxic (Stefkov et al., 2009).

Cryptococcus neoformans, a widespread fungal in the whole world, has a high frequency of human infections based on serological survey results. Many times, for the ones with a healthy immune system, the infection does not advance to disease, however, weakened immune systems related to diseases are at a greater risk of infection (Elhassan et al., 2019).

Affected populations include those that are exposed to aerosols generated by wind erosion, dust storms, and other means, as well as individuals who live close to the source and may breathe in fine particles carrying the fungus. Consequently, people with compromised immune systems, respiratory issues, or existing health conditions are at a higher risk of developing cryptococcosis caused by *C. neoformans*. On the other hand, the climaxing antimicrobial drug resistance has made it a challenge to the current treatment options

against cryptococcal infections, and therefore there is an urgent need for new therapies with enhanced efficacy that can successfully treat the pathogen (Liporagi Lopes et al., 2022).

The recent research studies into the possible uses of magnetic iron oxide nanoparticles (MNPs) in the treatment and diagnosis of a wide range of diseases is an area of great interest. The nanoparticles presented themselves as being biocompatible and respond to magnetic fields which makes them a more adequate potential for biomedical settings. When releasing MNPs into the blood system, the complexities of the host environment are interacted with, these MNPs may amass in certain organs or tissues based on their specific physical and chemical properties. Having knowledge of the involvement of nanoparticles in these interactions is essential for obtaining benefits to nanotechnology in medicine and for minimizing the risk of their toxicity (Damasco et al., 2020).

The fungal microbial infection treatment, for instance, *C. neoformans*, is what takes care of these diseases. One of the major importance of antifungal therapy is represented by the high mortality and morbidity linked with these infections, especially for people with compromised immunity systems with HIV as people with this pathology have more *C. neoformans*. Currently, there are difficulties with the existing cryptococcosis therapies. The fact that resistance to antibacterial drugs increases requires new treatment options to be developed (Sousa et al., 2023).

In this regard, investigation of iron oxide nanoparticles (Fe_2O_3 NPs) as a promising antifungal candidate has become crucial (Batool, 2022). These nanoparticles possess beneficial properties, which make them the potential vaccines against these diseases. They are rich in diversity of the host and ability to target certain strains of fungi, thus, eliminating the undesired reactions and embellishing the therapy (Sousa et al.,2023).

The bactericidal effectiveness of Fe_2O_3 NPs was determined against a simple pathogenic range which included bacteria such as *Escherichia coli* and fungi such as *Candida albicans*. The actions demonstrated promising bactericidal and myelosuppression competencies with MICs (minimum inhibitory concentration) values portraying efficiency against a wide range of strains. Furthermore, the photocatalytic ability of Fe_2O_3 NPs which is manifested through their clearance of crystal violet dye was discovered (Shakoor et al., 2023).

Thus, to develop new technologies to get improved results in the eradications of pathogenic fungi, this study seeks to synthesize nanoparticles from a plant extract that would be effective as antifungal agents against pathogenic fungi.

2. Material and Methods

2.1 Preparation of extract Teucrium polium L.

The aerial part of the plant was cleaned and dried in the shade. In a 500 mL glass beaker, weigh 10 g of dried and crushed *Teucrium polium* L. *with* a glass rod, then add 200 mL of sterile distilled water to the glass beaker, then boil it for 20 min, or until the aqueous solution shows a yellowish change The solution was pushed using Whatman filter paper (n. 1), centrifuged for thirty minutes at 3000 rpm, the supernatant was separated, and *T. polium* extract was kept at a temperature of 15 °C (Burange et al., 2021).

2.2. Preparation of (Fe_2O_3) nanoparticles using Teucrium polium extract

0.1 mM (Fe_2O_3) was dissolved in 100 mL of distilled water and stirred for a short period. Then drop by drop add 5 mL of Teucrium polium L. extract. The solution composition was stirred in a magnetic stirrer at a speed of 650-800 rpm for 10 min after 72 h and the color changes of the reaction mixture were monitored continuously by changing the color from colorless to light yellow and then to dark brown (Luengo et al., 2020).

2.3. Iron oxide nanoparticles characterization

The study contains the synthesis of iron oxide nanoparticles by *T. polium* extract which acts as a taking-off agent. The synthesis of Fe_2O_3 using the chemical method was investigated and the morphology was studied via scanning electron microscopy (SEM). The inorganic structure of the obtained Fe_2O_3 was determined using X-ray diffraction (XRD).

2.4. Isolation and diagnosis of Cryptococcus neformans from different locations in Kirkuk, Iraq

As a result, we collected 60 samples from different parts of Kirkuk, Iraq with high populations of birds (pigeons), private chicken keepers' places, and markets where chicken is sold. The others came from dumpsites within the city, and the media used for primary plate culture were EBM (Essential Broadcast Media) and BSA(bovine serum albumin), which were able to support growth due to the activity of phenoloxidase of *C. neoformans*. All the *Petri* dishes were kept at 30 ºC for 2-10 days. All brownish colonies were transferred to SDA(Sabouraud dextrose

agar) media for further identification. First, the isolates were authenticated to provide assimilation properties such as sugar assimilation as detected by the API20C AUX system, a positive urease test result on Christensen's broth, and incapability for reducing nitrate and growing at 37 °C (Kwon-Chung et al., 2002).

On the other hand, the shift in colour from a pale yellow-green to blue Cobalt represented a positive CGB test outcome, and no colour change indicated a negative result which in turn meant that the test was from *C. neoformans* (Kangogo et al., 2014).

2.5. Preparation of antifungal drugs

five antifungal drugs were used in this work. They are Ketoconazole and Griseofulvin and Fluconazole and Nystatin and Clotrimazole. The antifungal tablets were ground into fine powdery form using mortar and pestle. The ground tablets were measured in different concentrations: 0.02 g/mL, 0.04 g/mL, and 0.06 g/mL, and were dissolved in 95% ethanol and diluted with distilled water to make the different concentrations. A rotary shaker was used to homogenized the mixture. (Adetitun et al., 2015).

2.6 sensitivity of fungal antibiotics test

A sensitivity test was then performed using the spread method on the solid medium and based on formula (9) that indicates only 6 types of antifungal processed.

2.7 Antifungal activity of iron oxide nanoparticles

The test was done by the well diffusion method (Abid et al., 2021; Eslami et al., 2017). The *C. neoformans* fungi populations were collected using a cotton swab and then separately spread on a culture medium. In the previous culture, media was ready by the user according to the manufacturer's instructions. We cut the forms with a cork borer. In the study, the samples were prepared which having 100 µL of concentration (1, 2, 3, 4, 5, and 6 mg/mL^{-1}) of iron oxide nanoparticles which, then incubated at an incubator that has been set at 37 °C for 24 h. An inhibition diameter size was measured after recording the produced inhibition diameter measuring the zones of growth inhibition around all the discs (millimeters) using a meter ruler.

2.8 Statistical analysis

The results were analyzed using a one-way analysis of variance and *t*-test. A *p-value* < 0.05 was statistically considered significant.

3. Results

3.1. Isolation and identification of fungi

The environmental isolates of *C. neoformans* were retrieved from different sources and sites as shown above in (Table 1 and Figure 1). *C. neoformans* which is present in bird droppings (6) and chicken droppings (6) and contaminated soil (4) and tree swabs (2) and decaying vegetation (1). In a total of 19 samples, dark-brown colors were determined in both EBM and BSA media through the use of India ink preparations and helped us differentiate *C. neoformans* colonies. All the presumptive cultures were found to be *C. neoformans* according to the results of the analysis done using the API 20C kit® system, biochemical ability to grow at 37 ºC, and a positive urease test. All five isolates of *C. neoformans* showed agglutination as expected based on them being classified as serotype A by using the Cryptochek® typing system. The serotype for all isolates was *C. neoformans* serotype A, which showed no color change on CGB (Oh et al., 2005).

Table 1. *Cryptococcus neoformans* environmental isolates in Kirkuk, Iraq.

Sample	Samples*	Positive samples**
birds droppings	15	6
Chicken droppings	15	6
Contaminated soil	15	4
Tree swabs	8	2
Decaying vegetation	7	1
Total	60	19

Note: *Total number of samples. **Positive samples of *Cryptococcus neoformans*. Source: Authors, 2024.

Figure 1. Colony morphology of the *Cryptococcus neoformans* isolate. Source: Authors, 2024.

3.2 Antifungal susceptibility test

The antifungal sensitivity of *C. neoformans* isolates was tested against 5 types of antifungal solutions and Most of the different concentrations of antifungal

tablets have effects on the isolates. However, as the concentration of the antifungal drugs increased, the diameter of the zone of inhibition also increased (Table 2).

Table 2. Antibiotic sensitivity pattern on the *Cryptococcus neoformans* isolates.

Fungal	Concentrations (g/mL)	Diameter of zone of inhibition (mm)					
		Nystatin	Ketoconazole	Fluconazole	Clotrimazole	Griseofulvin	Control (water)
C. neoformans	0.02	26.00±0.5	24.00±0.5	15.00±0.5	9.00±0.5	0.00±0.0	0.00±0.0
	0.04	29.00±0.5	26.00±0.5	18.00±0.5	11.00±0.5	0.00±0.0	0.00±0.0 0
	0.06	33.00±0.5	27.00±0.5	20.00±0.5	15.00±0.5	0.00±0.0	0.00±0.0

Source: Authors, 2024.

3.2. Characterisation of (Fe_2O_3) nanoparticles

The SEM image of the (Fe_2O_3) nanoparticles shown in (Figure 2) indicates that the product has almost uniform spherical morphology. The sizes of the majority of them are in the range of 30-120 nm. While the volume of NP with a size greater than 100 nm is very small. The SEM image demonstrates the same size distribution of the (Fe_2O_3) nanoparticles. The mean diameter in the data of the (Fe_2O_3) nanoparticles is 10 nm (Abod et al., 2017).

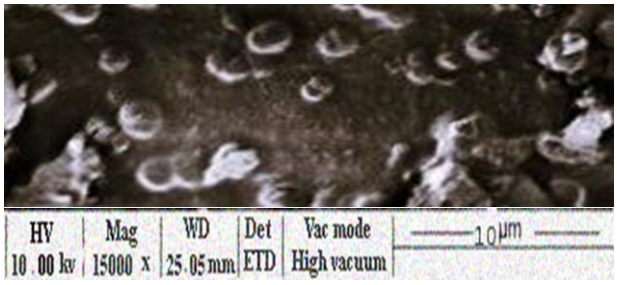

Figure 2. Scanning electron microscope (SEM) of (Fe_2O_3) nanoparticles. Source: Authors, 2024.

3.3. X-ray diffraction patterns (XRD)

The XRD patterns were collected to assess the IONPs (Figure 3). XRD mapping provides information on a great number of peaks of significant intensity in the range of 20-80º. From the XRD plot aligned with the standard sample data for Fe_2O_3, we have confirmed the structure of the Fe_2O_3 phase. The detected peaks at a 2θ value reached their maximum at (30.30°, 33.89°, 45.59°, 58.69°, 63.79°) which leads to Bragg reflections at (220), (311), (400), (422), (440) (Salih et al., 2017).

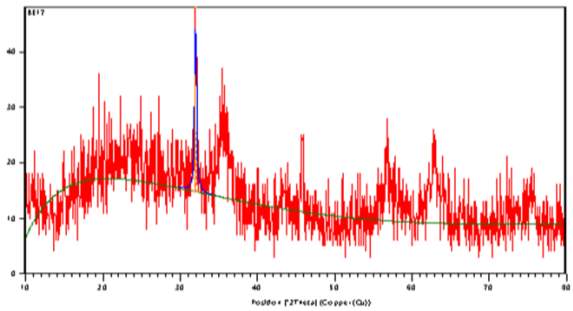

Figure 3. X-ray diffraction (XRD) of Fe_2O_3 IONPs. Source: Authors, 2024.

Antifungal activities of Fe_2O_3 NPs were observed against *C. neoformans* which were diffused at excellence; see (Table) (1). The stopping action on C. neoformans by Fe_2O_3 NPs was much stronger than the control group with deionized water. This was seen in the comparison of the average zone where growth was stopped for Fe_2O_3 NPs with deionized water. The differences were clear when comparing the zone diameters for these concentrations with deionized water. The P-value was found to vary between 0.0001 for high Fe2O3 NPs concentrations to less than 0.05 for low concentrations of Fe_2O_3 NPs.

Table 2. The results of descriptive statistics are zones of inhibition of Fe_2O_3 NPs in *Cryptococcus neoformans*.

Fe2O3 NPs concentrations	Zone of Inhibition *C. neoformans* (mm)	Confidence 95%	T-test	*P* value
6 mg/mL	32.6 ± 0.678**	-8.126 to -7.821	17.639	< 0.001 [HS]
5 mg/mL	31.2 ± 0.689**	-10.592 to -9.51	29.581	< 0.001 [HS]
4 mg/mL	26 ± 0.562**	-7.301 to -6.194	21.528	< 0.001 [HS]
3 mg/mL	24.4 ± 0.444**	-10.065 to -9.365	38.032	< 0.001 [HS]
2 mg/mL	21 ± 0.369*	-3.371 to -2.212	11.074	< 0.001 [HS]
1 mg/mL	17.1 ± 0.285**	-1.571 to -1.191	16.392	< 0.001 [HS]
D.W (control)	5 ± 0.0	-	-	< 0. 1 N.S

Note: **Compared with control group $p < 0.01$, * Compared with control group $p < 0.05$, HS refers to high statically significant $p < 0.0001$, NS refers to non-statically correlation $p > 0.05$, and Standard deviation (SD). Source: Authors, 2024.

In this research, the level of fungi growth inhibition zone size increased with increasing iron oxide nanoparticle concentrations 6, 5, 4, 3, 2, and 1 mg/mL^{-1} respectively (Figure 4).

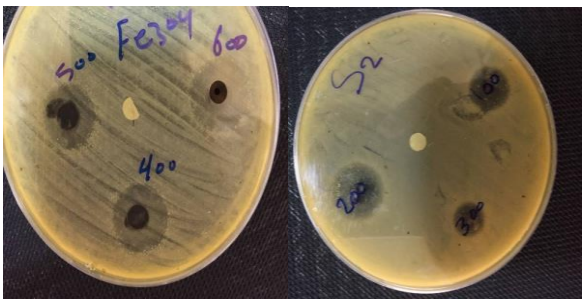

Figure 4. Antifungal activities of the Fe_2O_3 NPs. Source: Authors, 2024.

4. Discussion

Most of the yeast isolates under consideration were relatively resistant to many of the antibiotics in use different concentrations. Nystatin was found to be one of the most effective antifungals with the highest inhibitory because nystatin acts by important Ergosterol synthesis in the cell membrane of the yeast. This is in agreement with the study which showed that out of all the antifungal agents, nystatin was rated among the best in inhibiting the growth of *C. neoformans* (Sheehan et al., 1999). The growth of *C. neoformans* was least inhibited by clotrimazole compared to other used antifungal agents and explained by (Khan et al.,2018) that this antibody plays a defensive role in inhibiting the growth of *C. neoformans*. Fluconazole was the fourth most significant inhibitor among the growth of the types of *C. neoformans* under consideration. This may be attributed to the high usage of antibiotics and the nature of resistance of these isolates to most of the used antibiotics (Sibanda et al., 2007).

In the last few years Fe_2O_3 nanoparticles (Fe_2O_3 NPs) have attracted a lot of attention for their suitability and safety in different types of biomedical applications. The nanoparticles associated with them (maghemite and magnetite) are biodegradable, and have very low cytotoxic effects, and their surfaces can be modified by using biocompatible materials. Although there is much research related to their magnetic nature for medical imaging and drug delivery, a lot of attention has been given to the possibility of their antifungal use (Pourmadadi et al., 2022).

The studies have revealed that tannic acid-modified Fe_2O_3NPs have presented remarkable antifungal acts. The particles from these sizes having MIC of 16-63 µg/mL^{-1} exhibited anti-fungal activities against various fungi including *Alternaria alternata*, *Aspergillus niger*, and *Penicillium chrysogenum* (Gámez et al., 2020).

Moreover, the Fe_2O_3 NPs displayed a capability for fungal biofilm formation inhibition. For example, at 50 g/mL^{-1} Fe_2O_3 NPs significant inhibition (approximately 70%) was observed on the formation of biofilm of Candida albicans and it was completely inhibited at 200 g/mL^{-1} (Harandi et al., 2022).

Additionally, the nanosized particles Fe_2O_3 modified with a metallocarbonate group significantly reduced the mass of the biofilm created by the fluorinated *Candida albicans*. Data from experiments described herein suggest that an involved process could involve such nanoparticles increasing the sensitivity of cells within the biofilm matrix to appropriate complexes, leading to a reduced cell survival rate and biofilm formation (Mi et al., 2018).

The specific antifungal mechanisms of Fe_2O_3 NPs have not yet been discovered and further research is required, but they give hope for the potential of Fe_2O_3 NPs in case of fungi diseases. Assessment of the toxicity of these nanoparticles against mammalian cells concerning their antifungal purpose is critical to

provide the safety and efficacy of the final product in the clinical stage (Huang et al., 2023).

5. Conclusion

Fe_2O_3 NPs have special features like their physical and chemical stability, biocompatibility, as well as a good environmental security, which makes them welcome in many biomedical areas. The nanoparticles have, however, shown an outstanding immunomodulating ability by enhancing or reducing inflammation following the administration of the dosage. Investigating Fe_2O_3 NPs functioning in cryptococcosis models establish a new healing pathway offering new prospects in management of cryptococcosis related infectious disease.

In addition, there is a need to explore the mechanisms of how Fe_2O_3 NPs interfere with *C. neformans* infections. By explaining the complicated pattern of iron oxide nanoparticle dosage, immunomodulation, and inflammation, future studies may provide a basis for novel therapeutic ideas that capitalize on the peculiar characteristics of iron oxide nanoparticles to allow for better solutions to cryptococcosis.

In addition to this, there is also a need for the development of standard test protocols that can quantitatively measure the antifungal ability of Fe_2O_3 nanoparticles. The knowledge of exact matter compounds and properties that lead to their anti-fungal activity will help make more effective and safer anti-fungal particles in the future. Moreover, the analysis of the chemical compositions on the surface of the nanoparticles would provide huge information on their operability and possible mechanisms of action.

Furthermore, novel methods of improving the therapeutic efficiency of antifungal drug nanoparticles, like injection of imaging elements for nanoparticle in vivo imaging, could offer an alternative to conventional treatment methods. Designing nanoparticles that can identify the places of infection and decrease the bad impacts is what makes the researchers do the job. Therefore, they will revolutionize the treatment of systemic fungal infections.

In summary, future research should be focused on the discovery of new antifungal mechanisms particular to the fungal cells, tuning nanoparticle compositions and characteristics to further improve effectiveness, and incorporating imaging into the drug delivery systems. These technological developments can drastically affect clinical practice through increasing the safety and effectiveness of fungal infection treatment.

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Ethics

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