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Optimization, Characterization and Green Synthesis of Iron Oxides Nanoparticles Using Nerium Oleander Extracts and their activities against *F. oxysporum f.sp. lycopersici* After Genetic Diagnosis

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Abstract

Background: Green-synthesized iron oxide nanoparticles may provide an eco-friendly approach for controlling fungal plant pathogens, such as *Fusarium oxysporum* f. sp. *Lycopersici*. **Aim:** This study aims at optimizing the synthesis and characterization of iron oxide nanoparticles utilizing *Nerium oleander* leaf extract and investigating its anti-fungal activity against the identified *Fusarium oxysporum f. sp. lycopersici*. **Methods:** Nanoparticles of iron oxide were prepared using a mixture of leaf extract of Nerium oleander. The optimization of synthesis parameters was done on the basis of pH (4, 7, and 10) and temperatures (30, 50, and 70°C). The nanoparticles were analyzed using ultraviolet-visible spectroscopy, atomic force microscopy, energy-dispersive X-ray analysis, field-emission scanning electron microscopy, and zeta potential analysis. The identification of fungus was done using PCR and sequencing of internal transcribed spacer (ITS). **Results:** The optimum conditions for nanoparticle formation were at a pH 4 and a temperature of 30°C, which resulted in the formation of spherical particles with an average size of 24.99 nm. The maximum absorbance was at 292 nm and the zeta potential was 20.9 mV. The percentage composition was made up of oxygen, carbon, and iron at 80.0%, 11.4%, and 8.6%, respectively. Internal Transcribed Spacer (ITS) sequencing showed 100% homology to *F. oxysporum f. sp. lycopersici* with GenBank Accession Number PQ060101. The inhibition zone diameters at 100, 200, and 400 µg/mL concentrations were 10, 14, and 19 mm. **Conclusions:** *N. oleander*-mediated iron oxide nanoparticles demonstrated concentration-dependent antifungal activity and may represent a potential green approach for controlling tomato Fusarium wilt.

Keywords: Nerium oleander, iron oxide nanoparticles (Fe3O4- NPs), *Fusarium oxysporum* f.sp. *lycopersici,* molecular diagnosis, Antimicrobial.

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Introduction

Nanotechnology is a study of enormously tiny structures, usually with sizes ranging from 1 to 100 nm (1) . Richard Feynman was credited with introducing the idea of nanotechnology in his remarks titled "There's Plenty of Room at the Bottom" (2). Since different quantum effects often develop, objects confined inside nanoscale zones frequently exhibit behaviors very different from those in the bulk phase (3). Numerous scientific disciplines have acknowledged that countless combinations of atoms and molecules arranged in different ways at the nanoscale can result in an infinite number of different types of materials (4). Two approaches are commonly used to synthesize nanomaterials: the top-down technique and the bottom-up approach (5). Using a top-down technique, a superstructure is broken down to the atomic or molecule level. It is a subtractive method, this one using mechanical or chemical techniques, a bulk substance is broken down and confined into small structures in this process. The researchers to focus on environmentally friendly nanoparticle production by incorporating readily available metabolites into biological and plant extracts, green synthesis of nanomaterials approach minimizing the harmful effects of nanoparticles on humans and the environment while enhancing their biocompatibility (6) .

Nerium oleander L. (Apocynaceae) is a plant that grows naturally, that has been extensively widespread over the Mediterranean area. It grows well in sunny, humid conditions close to water flows. The primary usage of oleander is as an ornamental plant. This species' toxicity and therapeutic qualities have been well documented since antiquity. In actuality, all plant parts are poisonous and can paralyze the heart, therefore causing death (7). It has some advantageous properties in addition to being toxic, such as being anticancerous, antimalarial, anti-inflammatory, hepatoprotective, and cardioprotetive (8) . The oxidation and reduction ability of the oleander plant has also been exploited in the manufacture of nanoparticles (9,10) .

One of the most important diseases that affect the plants is Fusarium wilt, which is one of the most dangerous and widespread diseases that affect tomatoes and is caused by the pathogen Fusarium *oxysporum f.sp. Lycopersic*i (11,12). *Fusarium* wilt is an economically important disease that affects tomato plants wherever they are planted, whether in the field or greenhouses. Because the disease thrives in warm, humid environments, it lowers fruit quality and decreases yields by 50–60%; for susceptible cultivars, the loss may be as high as 100% (13). In order to identify the relationships between the fungal *F. oxysporum* and formae specials, differentiate them, detect their races, and assess the susceptibility and resistance of tomato varieties to disease, molecular techniques have emerged as one of the most effective methods. Through DNA sequence comparison, internal transcribed spacer (ITS) requirements can be identified for specific forms of the disease. To address these issues, newer technologies and strategies are constantly evolving. In view of this, one of the steps being taken is the introduction of nanomaterial-based products to revolutionize modern agricultural practices as these materials possess novel physical and chemical properties that provide a distinct advantage in plant disease management. (14,15) . Hence, studies have been directed towards the use of bio-based green nanopexhibitss such as Iron oxide nanoparticles because eco-friendly, high reaction rate and high surface area to mass ratio and the smaller size that they have the highest penetrating power to inhibit the against plant pathogens and for protection of various crops as an alternative to commercially available synthetic chemical fungicide, which exhibit higher toxicity to humans

Material and methods

Plant material

Nerium oleander leaves were collected locally from the capital, Baghdad, and diagnosed by PhD. Majid R. Majeed assistant professor. University of Baghdad, College of Science, Department of Biotechnology.

Preparation of leaves extract

According to Shawuti *et al*. (16) with certain modifications, dried leaves of oleander that had been collected were thoroughly rinsed (three times) in distilled water. Using a mortar and pestle, the leaves was powdered after being shade-dried, 100 g of leaves powder dissolved in 1L of distilled water. The mixtures stirred for 24 h at 40 °C, then allowed cooling to room temperature before being filtered through Whatman no. 42 filter paper and centrifuged for 20 minutes at around 4000 rpm. After that, the prepared were put in a sterile container and kept at 4 °C.

Synthesis of Iron oxides nanoparticles

Iron oxide nanoparticles was prepared according to Alden and Yaaqoob (17) with certain modifications, the co-precipitation technique was used to create iron oxide nanoparticles (Fe_3O_4 NPs). It was preparation by use oleander extracts for the biosynthesis of iron oxide nanoparticles, which involved adding ferric sulfate to oleander extract in a 0.5:10 g/ml ratio. The mixture was shaken overnight in a dark room. The solution, which includes iron nanoparticles, was separated, centrifuged at 5000 rpm for 10 minutes to concentrate it, washed twice with ethanol, and then precipitated for another 30 minutes at a comparable speed. After that, it was oven-dried at 40 ˚C for 30 minutes to produce a brown-black powder, and then kept in a dark container, further investigation and use.

Optimization iron oxide nanoparticles

One hundred millilitres of oleander extract was mixed with five grammes of ferric sulphate. After that, we made sure that each solution was created at a distinct pH range (4.0, 7.0, 10.0) by adjusting the pH using 0.1N HCL and 0.1N NaOH. The liquid was kept in the darkroom overnight after being heated to a certain temperature (ranging from 30,50 and 70 degrees) using a stirring hot plate for each pH. Particle sizes in each of the resulting solutions were measured using an atomic force microscope and an AFM system model (AA-3000, USA).

Characterization of iron oxide nanoparticles

The synthesized nanoparticles were characterized through a UV-Vis spectrophotometer, atomic force microscopy, Energy-dispersive X-ray analysis, Scanning electron microscope, Zeta Potential and Transmission Electron Microscope (FE-SEM).

Isolation of pathogen

The fungal isolate used in this study (*Fusarium oxysporum*) was obtained from Ministry of Agriculture / Plant

Protection directorate / Agricultural Pest Diagnosis Department. The isolate was activated and grown on Potato dextrose agar (PDA) medium, characterization morphological and restored Molecular characterization using PCR technique.

DNA extraction

The pathogenic fungus was activated on PDA medium at 25°C by cultivating the dish with a fungal disc (0.5 cm) and after 7 days. the fungal hyphae were collected then placed in test tubes. All this work was done inside the isolation chamber (Laminear) and then stored in the refrigerator. Later, 100 mg of mycelium was used to extract DNA using the (ZR FUNGAL/BACTERIAL/ YEAST DNA MINIPREP)-kit according to the manufacturer's instructions.

Molecular characterization

Taking 1.5 µL of DNA and 2 µL of primers (1 µL of Forword and 1 µL of Reverse). The volume was then completed to 25 µL by adding 16.5 µL of sterile deionized water into a small tube containing 5 µL of Taq PCR PreMix. The tube was then mixed by Vortex. The tube was then placed in a Thermocycler to perform the Polymerase Chain Reaction (PCR). The amplification conditions included the following: Initial denaturation 94°C (1 cycle for 5 minutes), Denaturation-2 94°C (35 cycles for 40 second), Fixation Annealing 52°C (35 cycles for 40 second) Extension-1 72°C (35 cycles for 40second) and Final extension-2 72°C (1 cycle for 7 minutes). In order to diagnose DNA contamination in reagents. A standard DNA molecular weight marker was used to examine the amplicons on 1% agarose gels.

Antimicrobial activity

Agar well diffusion was used to assess the antimicrobial activity of biogenic iron oxide nanoparticles against Fusarium oxysporum. The McFarland turbidity standard of 0.5 was applied to the test microbial cultures, resulting in a microbial suspension of 1.5*108 colony-forming units (CFU/ml). Sterile L-rods were used to disseminate 100µl of bacterial cultures on Mueller-Hinton agar plates, which were then placed into sterile petri dishes for the antimicrobial investigations. Once the agar plates were seeded, three wells were pierced to allow biogenic nanomaterials to be put into them at three different concentrations: 100 µg/ml, 200 µg/ml, and 400 µg/ml. The plates were incubation for 48 h at 25 °C. Measurements in millimeters were made of the inhibition zones against the test organisms (18,19).

Results

Crude extracts of *Nerium oleander* leaves

Following the leaves' washing, drying, and grinding into a powder, *Nerium oleander* leaf extract was made. The final extract was made by mixing the powder with distilled water. The description of the final extract revealed that the aqueous extract displayed a brown color.

Optimization of iron oxide nanoparticles

The natural substance of Nerium oleander extract acts as a reducing and stabilizing agent during the biological manufacturing of iron oxide nanoparticles, ensuring their stability. The results determined within Table (1) demonstrated that the optimal pH was (4.0) at (30) °C. The mixture was exposed to completely different hydrogen ion concentrations (4.0, 7.0, and 10.0) and every different pH exposure to varied temperatures (30, 50, and 70) °C. In order to synthesize Fe3O4-NPs, using AFM the typical size is 24.99 nm in diameter.

Table (1): Effect of pH and temperature of iron oxide nanoparticles.

PH	Temperature (°C)	Nanoparticles size (nm)
4	30	24.99
	50	54.68
	70	147.7
7	30	37.07
	50	91.97
	70	161.6
10	30	89.71
	50	55.07
	70	94.90

Characterization of iron oxide nanoparticles

Ultraviolet-visible (UV-VIS) spectroscopy

Nanoparticles made of iron oxide UV-VIS scanner spectrophotometry, which covers a wavelength range of 200-1000 nm, were used to measure the absorbance. The scan of the Fe3O4-NPs spectra with the observed spectra at 292 nm is shown in Figure (1).

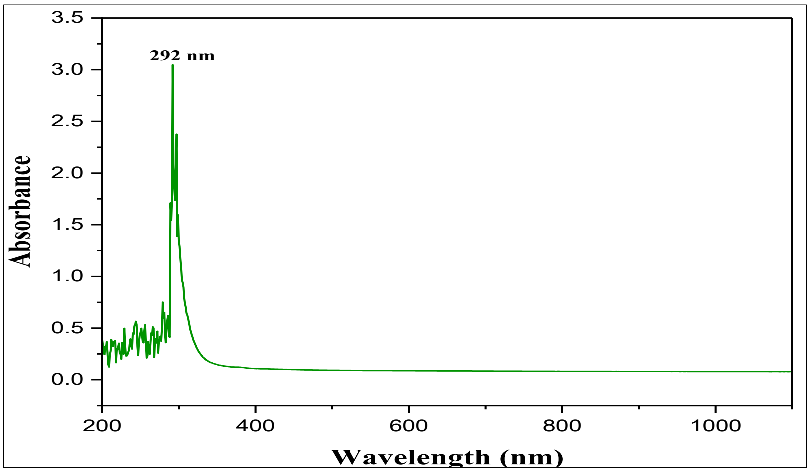

Figure (1) Spectra scan of iron oxide nanoparticle.

Atomic force microscopy (AFM)

Atomic force microscopy was used to investigate the surface shape formation of the Iron oxide nanoparticle, the result demonstrating that the Fe_3O_4-NPs are spherical, as revealed by AFM images, 2D and 3D in Figure (2). The results also showed the average diameters of Fe3O4-NPs 24.99 nm as shown in Table (2).

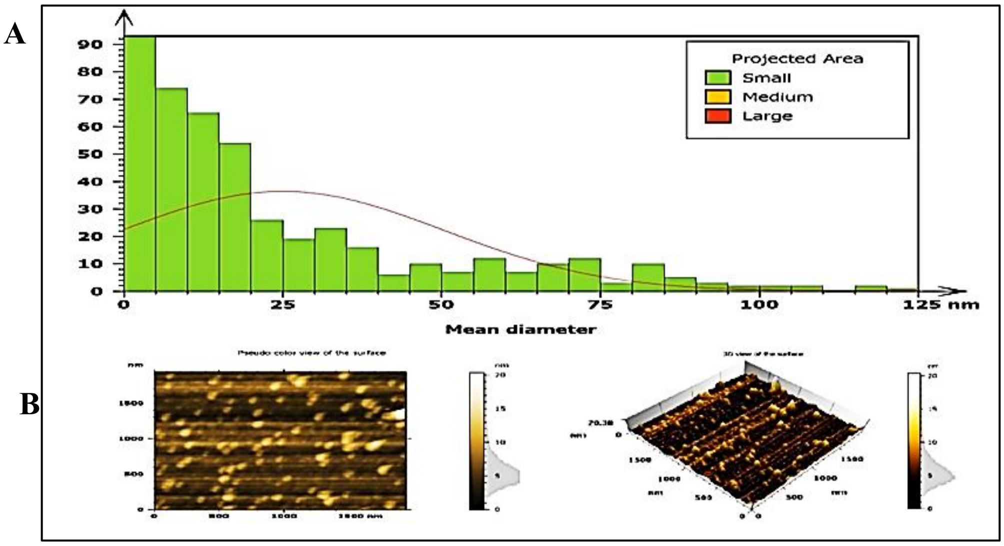

Figure (2) Atomic Force Microscopy of iron oxide NPs (A: Histogram of iron oxide NPs, B: 2D and 3D of iron oxide nanoparticles).

Table (2) the global statistical of iron oxide nanoparticles.

Global statistical	mean diameter
Mean	24.99
Min	2.820
Max	122.4

Energy diffraction X- ray (EDX)

This analysis indicated that the majority of atoms (80 %) was oxygen, followed by (11.4%) were carbon atoms and (8.6 %) were Fe atoms.as shown in Figure 3).

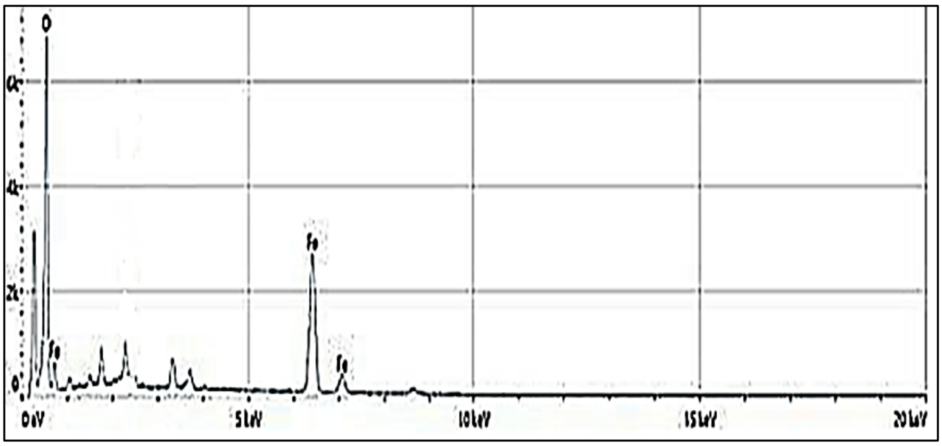

Figure (3): Energy diffraction X- ray (EDX) of iron oxide nanoparticles.

Field Emission Scanning Electron Microscopy (FE-SEM)

Analysis of FE-SEM images is generally appropriate for studying particle form and surface morphology. Figure (4) displays the FESEM images of Fe3O4-NPs. which validate that the nanoparticles are grown and display a well-defined shape, it possessed a round or spherical form.

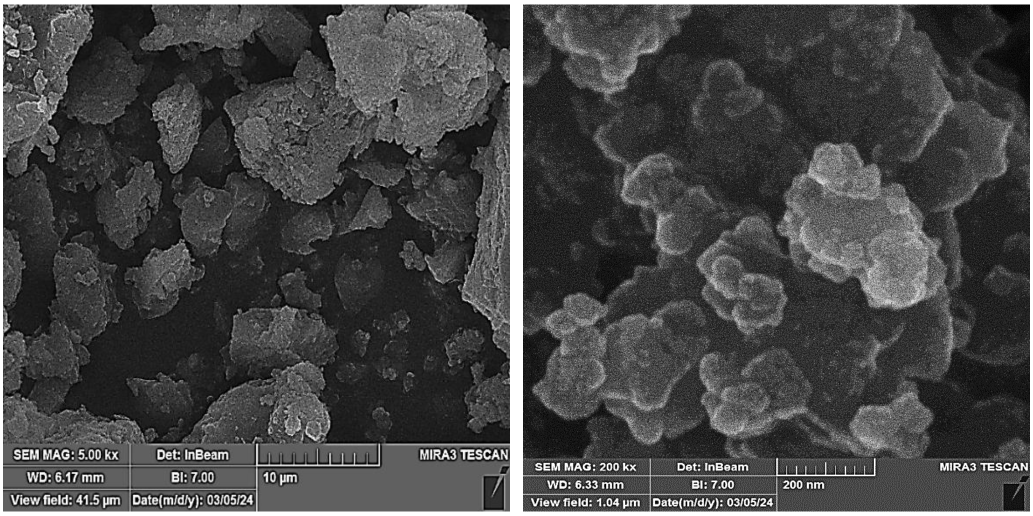

Figure (4): FE-SEM image of iron oxide nanoparticles.

Zeta potential

One common way to measure the strength of an electrical charge is via the zeta potential. As seen in Figure (5), the mean zeta potential of Fe_3O_4-NP was determined to be 20.9 mV.

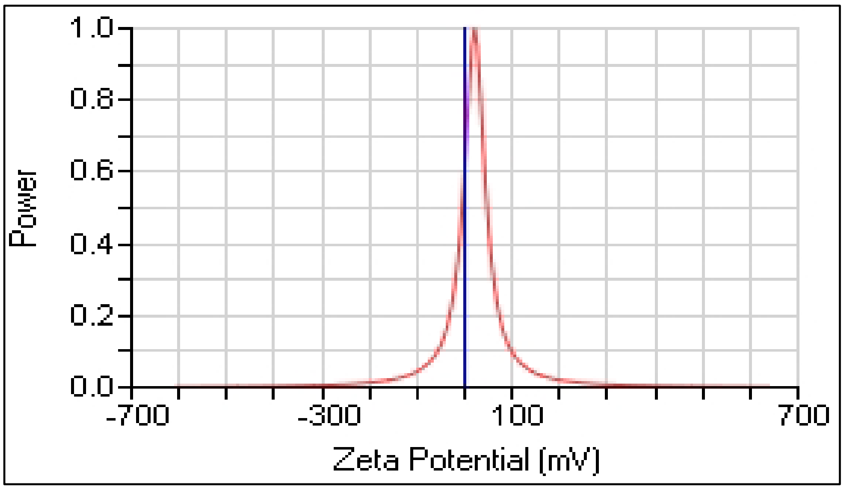

Figure (5): Zeta potential images of iron oxide nanoparticles.

Molecular characterization

Figure 6 shows the results of polymerase chain reaction (PCR) amplification of the isolate's Internal Transcribed Spacer (ITS) region. In order to determine the strain's identification, the Internal Transcribed Spacer (ITS) region was sequenced. The results showed that the strain was identical to *F. oxysporum f.sp. lycopersici*, with a 100% match in the NCBI GenBank database. The ITS rDNA sequencing of the strain has been submitted to the National Centre for Biotechnology Information GenBank database (GenBank Accession numbers PQ060101).

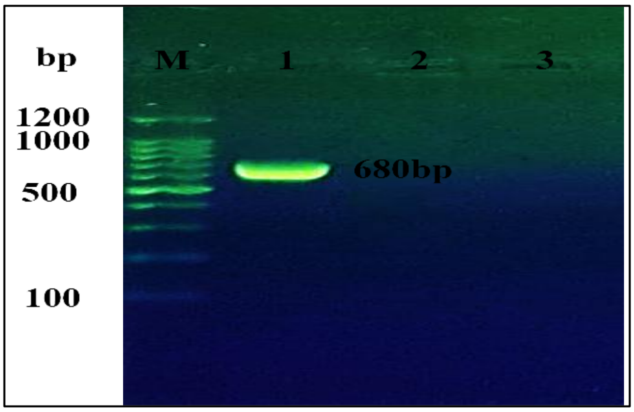

Figure (6): Electrophoresis of PCR product from pathogenic fungal DNA

Estimation of antifungals effect of iron oxide nanoparticles

The antifungals activity of iron oxide nanoparticles was shown in Figure (7). It was directly influenced by the concentration of NPs. The concentration of iron oxide at 400 µg/ml produced a maximum inhibition zone of 19 mm. On the other hand, the minimum inhibition zone was found at 100 µg/ml iron oxide 10 mm. As shown in Table (3), the results demonstrated that the antifungal activity of each investigated NP increased significantly with concentration.

Table (3) The inhibition zone of Antifungal effect for iron oxide nanoparticles against *F. oxysporum f.sp. lycopersici.*

Zone inhibition (in mm)		
NO	**Concentrations µg/ml**	**iron oxide nanoparticles**
1	100	10
2	200	14
3	400	19

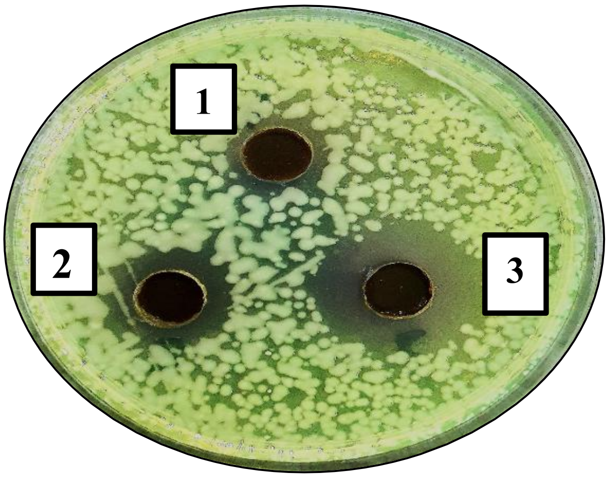

Figure (7): Antimicrobial activity of different concentrations of iron oxide nanoparticles against *Fusarium oxysporum f.sp. lycopersici.*

Discussion

The results of extraction of *Nerium oleander* extremely agreed with previous studies (20) . After adding ferric sulfate for biosynthesize Fe3O4 nanoparticles aqueous extract displayed a black color. agreed with (21). The findings suggest that *Nerium oleander* may undergo oxidation and reduction reactions, and that it is possible to synthesise green nanoparticles using plant extracts that are safe for the environment and non-toxic (22).

The present work studied many parameters, including pH and temperature, to determine the perfect conditions for the manufacturing of iron oxide nanoparticles. pH has an important impact on the synthesis of nanoparticles and can affect the compounds used in the reaction by changing their charge. The production of nanoparticles is significantly influenced by PH (23). On the other hand, the thermal conductivity of nanofluids increases with increasing temperature, mostly as a result of Brownian motion acting on the nanoparticles. Researchers discovered that 30°C was the sweet spot for steady NP production. Even while prolonged exposure to high temperatures may cause proteins to decompose, they found no discernible impact on synthesis when temperatures were raised from 30, 50 and 70°C (24).

The finding of UV-vis analysis was in perfect harmony with prior research (25). A different research demonstrated that Fe3O4-NPs, when produced biologically, had an absorbance range of 230–290 nm (26).

The FESEM analysis of Fe3O4 nanostructures demonstrates the formation of well-defined structures in the shape of mostly spherical morphology (27).

This result of zeta potential extremely agreed with previous studies (28). Another study showed that the stability range of metal oxide (20 to 40 mV) (29).

The identification of the strain was achieved through sequencing of the Internal Transcribed Spacer (ITS). The data revealed that the strain belongs to the F. oxysporum f.sp. lycopersici strain with an identity percentage of 100%. This conclusion is consistent with earlier research on the detection and transportation of harmful fungal isolates (30,31).

Antifungal properties of iron oxide nanoparticles depend on the concentration of NPs. The highest inhibition zone of 19 mm was achieved when the concentration of nanoparticles was 400 µg/mL; the lowest zone was 10 mm at the concentration of 100 µg/mL. Antifungal properties of all NPs under study increased with their concentrations. Iron oxide, frequently referred to as hematite, has been demonstrated to eliminate microbe by osmosis and to be resistant to the body's acidity. In addition to reactive oxygen species, interaction between iron oxide nanoparticles and hydrogen peroxide in the cells surrounds results in the production of hydroxyl and peroxide free radicals (32). The deterioration of biological elements including DNA, lipids, and proteins in particular adds to the toxicity of ROS on the cell membrane. This ultimately causes oxidation, which either kills or inhibits the microbes (17). Nanoparticles possess the ability to penetrate membranes, interfere with DNA replication, and act as a catalyst to deactivate enzymes necessary for microbes' metabolism. Higher surface-to-volume ratio metal nanoparticles displayed maximum antimicrobial activity (33).

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