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ORIGINAL ARTICLE **OPEN ACCESS**

Green synthesis of zinc hydroxide nanoparticles using culture filtrate of *Macrophomina phaseolina* and *Sclerotium rolfsii*, and evaluating their antimicrobial activities

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ABSTRACT
Two isolates of soil-borne pathogenic fungi, Macrophomina phaseolina and Sclerotium rolfsii infecting a wide range of host plants (about 500 plant species) were used for zinc hydroxide nanoparticles (Zn(OH)2-Nps) production naturally by simple and low cost methods. The produced nanoparticles were investigated for their possible antifungal applications. The produced nanoparticles were characterized by UV-Vis, transmission electron microscope (TEM), Fourier transform infrared (FTIR) and X-ray diffraction (XRD). The antimicrobial activity of Zn(OH)2-Nps were investigated by agar disc diffusion method to determine the minimal inhibitory concentration (MIC). The nanoparticles exhibited potent antifungal activity against two air and soil borne pathogens, Exserohilum rostratum and Fusarium solani. The biosynthesized Zn(OH)2-Nps inhibited growth of the investigated microbial strains at all tested concentrations. Additionally, the recorded inhibition zones increased proportionally on increasing the used concentrations of the Zn(OH)2–NPs. The results showed that the biosynthesized Zn(OH)2-NPs can be used as a promising antimicrobial agent against various fungal pathogens.
Keywords: Macrophomina phaseolina, Sclerotiumrolfsii, Zinc hydroxide, nanoparticles, agar disc diffusion, antifungal activity

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INTRODUCTION

The soil-borne fungus *Macrophomina phaseolina* (Tassi) (the pycnidial stage of *Sclerotium bataticola*Taub) has a large host range of over 500 cultivated and wild plant species worldwide [1] Collar rot, damping off, charcoal rot, stem rot, root rot, and seedling blight are all diseases produced by *M. phaseolina* in economically significant crops [2].*Sclerotium rolfsii* (a soil-borne plant disease) is a deadly pathogen which causes root rot, stem rot, wilt, and foot rot in over 500 plant species, involving practically whole agricultural and horticulture crops [3].Chemical nanoparticle production methods are widely used, however their use is limited.

Where naturally benign organic molecules do not represent harm to human health or the environment, the bio-genic synthesis is thus the ideal option. Microbes have a promising function in nanoparticle creation because of their inherent method for metal ion detoxification via reduction, which can occur extracellularly or intracellularly via bioaccumulation, precipitation, bio mineralization, and bio sorption [4-6]. Because some fungi are pathogenic, extreme caution should be exercised when working with them in research. When given the identical conditions, fungus biomass grows quicker than bacterium biomass [7]. Although bacteria are commonly used to make metal nanoparticles, fungi are preferable because of their tolerance and ability to accumulate metals [8]. Furthermore, because their mycelia provide a wide surface area for interaction, fungi are an excellent option for the creation of metal nanoparticles [9]. As a result, fungi are more efficient than bacteria in converting metal salts to metal nanoparticles.

Nanotechnology has been the potential to have a huge impact on society because to its many applications and unique optical, chemical, photoelectron chemical, and biological capabilities [10]. Inorganic metal

nanoparticles, such as zinc, are increasingly used as antimicrobial agents because they are more stable and have a longer shelf life than organic antibacterial agents [11]. The goal of this research is to develop a simple method for green-synthesis of Zn(OH)2-Nps and to test the antifungal activity of the nanoparticles generated.

MATERIAL AND METHODS

Fungal Isolation and Cultivation

Pepper plant tissues were gathered and evaluated from diseased pepper root fields in the BeniSuef governorate, and processed according to the manner outlined by Qin et al. [12]. In a nutshell, the samples were thoroughly cleaned in running water for 30 minutes before being air-dried at room temperature for 2 hours. The plant samples were surface-sterilized after drying using the process described by Tan et al. [13] with minor changes. Surface-sterilized air-dried plant samples were washed in 70% ethyl alcohol for one minute, 2.5 percent sodium chlorite for two minutes, and 70% ethyl alcohol for one minute. The leaves were sterilized and then washed 3 times in sterile distilled water. After that, whole samples were cut to 555 pieces of 1–2 mm in length. Using 90 mm petri plates, segments were put on a PDA medium [14]. To prevent bacteria from growing, the medium was treated with tetracycline (100 U/mL). Each petri dish (90 mm) was filled with four tissue segments and cultured for one week at 28°C. For further identification, emerging fungal colonies were isolated and purified in a PDA medium.

The morphological properties of the isolated fungi were used to confirm their identification at the Assiut University, Mycological Center. Plant pathology department, Sids Agricultural Research Station verified a representative isolate of *Sclerotium rolfsii* and *Macrophomina phaseolina* based on colonial characteristics on culture plates incubated at 28oC for 6–7 days, morphological characteristics, and microscopic features in slide cultures using the method of Dhingra and Sinclair [15] Prasad [16].

Preparation of fungal filtrate

Fungi were cultivated in 250 mL Erlenmeyer flasks with 50 mL liquid potato dextrose medium in each flask. After 5 days of incubation at 28°C and 150 rpm, the biomass was filtered using Whatman filter paper No. 1 and rinsed with distilled water. The fungal biomass was transferred to 100 mL of deionized water and cultured in an orbital shaker for 72 hours at 140 rpm. Each fungal biomass was filtered once more with Whatman filter paper No. 2 before being used in the manufacture of zinc hydroxide nanoparticles [17].

Synthesis of $Zn(OH)_2$-Nps

Biosynthesis of Zn(OH)2 was obtained by mixing 10 ml of 3Mm Zinc nitrate with 10 ml of fungal filtrate, lowering the pH to 6.5, and incubating for 72 hours at 32 C in an orbital shaker at 150 rpm. Centrifugation at 10.000 rpm for 10 minutes removed the white precipitate that had accumulated at the bottom of the flask [18]. Each fungal mycelium was incubated individually with deionized water and Zinc nitrate solution to create positive and negative controls [19].

Characterization of Zinc hydroxide NPs

UV–Visible spectroscopy analysis

Visual examination revealed a change in colour in the reaction mixture, indicating the formation of Zn(OH)2-Nps. Periodic sampling of aliquots 3–5 ml and subsequent measurement of the solution's UV–Vis spectra at 300–700 nm in a 1cm path length quartz cuvette were used to track the bio-reduction of Zinc ions in aqueous solution. A spectrophotometer (UV-2600, SHIMADZU, Japan) was used to measure the UV–Vis spectra of these aliquots as a function of reaction time. At room temperature, all of the tests were carried out.

TEM analysis of $Zn(OH)_2$-NPs

Transmission electron microscopy was used to examine the nanoparticles' morphology (TEM.(Via submersion of hydrous sample solution on a carbon covered grid with an accelerating voltage of 80 kV, TEM micrograph pictures were captured. The samples were inspected by (JEOL JEM- 2100 electron microscope, Japan.) In various magnification levels, clear microscopic images with good clarity were observed and described.

X-ray diffraction analysis

Using a CuKá1-radiated X-Ray powder diffractometer (202964 P analytical Empyrean), the crystallinity and elemental composition of biosynthesized Zn(OH)2-NPs were examined and identified. The X-ray resource had a 40 kV voltage and a 30 mA current. The sample was drop-covered onto a silica plate using layering tiny amounts of the sample on the plate and desiccating intermittently, resulting in a dense coat of the sample.

Fourier Transform InfraRed analyses

The biomolecules dependable for the decrease of ions and covering the zinc hydroxide nanoparticles synthesized using fungal species were identified using FT-IR. The ATR-Technique was used to assess the

functional groups and their likely participation in the production of Zinc hydroxide nanoparticles (ATR permits qualitative or quantitative analysis of materials with little or no sample preparation, considerably speeding up sample analysis). The major advantage of ATR sampling is the short sampling route length and shallow penetration depth of the IR beam into the sample. This is in opposite to standard transmission FTIR sampling, which requires the sample to be diluted with IR transparent salt, crushed into a pellet, or compressed to a thin film before the analysis to avoid completely absorbing bands in the infrared spectrum) utilising (VERTEX 70 Spectroscopy, Japan).

Antimicrobial activity of biosynthesized $Zn(OH)_2$-NPs

Target microorganisms and culture conditions

Fusarium solani and *Exserohilum rostratum* are two pathogenic fungus. They were collected from the Plant Pathology Research Institute (PPRI), Agricultural Research Center (ARC), Giza, Egypt's Diseases Research Department of Vegetable Diseases. The fungi were cultivated in PDA medium with the following ingredients: 20 g Dextrose, 4 g Potato extract, and 15 g Agar ml in 1L distilled water for 72 hours at 30 °C.

Evaluation of the antimicrobial activities of $Zn(OH)_2$-NPs

The antibacterial activity of biosynthesized Zn(OH)2-NPs was determined using the agar-disc diffusion method described byNehal et al.[20]. Different amounts of biosynthesized Zn(OH)2-NPs were tested using 5 mm diameter discs collected separately from fungal cultures and put in the centre of each Petri dish to demonstrate the minimum inhibitory concentration (MIC).

Antifungal activity

Three different concentrations of Zn(OH)2-NPs, namely 10, 50, and 100 g/disc, were used to test antifungal activity. Negative and positive controls, respectively, were deionized water and Moncut & Tebuconazole (chemical fungicides). Pathogenic fungi and Zn(OH)2-NPs discs were planted on plates and cultured for 72 hours at 30°C. The emergence of a clear region surrounding the hole was used to get inhibition zone readings. Two alternative incubation times were used to collect data. Triplicates of all measurements were taken.

RESULTS

UV–visible spectroscopy analysis

Prominent and sharp absorption bands were observed at 367 and 366 nm of the biosynthesized $Zn(OH)_2$-Nps for *M. phaseolina* and *S. rolfsii*respectively, confirming the biosynthesis process of $Zn(OH)_2$-NPs by their characteristic peaks shown in (Fig 1).

Transmission Electron Microscopy (TEM)

The morphology and sizes of the biosynthesized zinc hydroxide nanoparticles were focused using transmission electron microscopy (TEM) analysis are given in (Fig 2). The TEM images of biosynthesized $Zn(OH)_2$-NPs revealed the plate shape with diameter about 30 nm.

X-ray diffraction (XRD) pattern of ($Zn(OH)_2$-NPs)

The X-ray diffraction XRD pattern of the purified nanoparticles powder allowed clear identification of the cubic crystalline phase of $Zn(OH)_2$-NPs in the spectrum of 2θ value are shown in (Fig 3). The XRD spectrum of purified nanoparticle powder of *M. phaseolina* and *S. rolfsii* exhibited 6 distinct peaksat 2θ values of (19.24^o, 20.6^o, 27.1^o 28.14^o 40.4^o and 41.06^o) was matched with the standards set by the Joint Committee on Powder Diffraction standards (JCPDS), card file no. (74-0094).

FTIR analysis

FTIR analysis was performed to identify the existence of functional groups within the biomolecules linked to zinc hydroxide nanoparticles which might be dependable for decreasing zinc ions and stabilization of the biosynthesized Zn(OH)2-NPs as shown in (Fig 4). In the FTIR spectrum of *M. phaseolina*, the major absorption peak detected was 3260 cm^{-1} which is attributed to primary amide linkage in the protein and hydroxyl group (O-H).

Peaks located at 1339 and 1043 cm^{-1} might be assigned to the existence of stretching vibrations of carboxylic acids and amino groups.The wide absorption band at 602 cm^{-1} indicated (C-H bend alkyne). With respect to the FTIR spectrum of *R. rolfsii*, the major infrared absorption bands were 3302 cm^{-1} which is attributed to primary amide linkage in the protein and hydroxyl group (O-H). A band of 2107 cm-1 could be allocated to CO2. The sharp peak observed at 1635 cm-1 indicated the possible sample as, wave numbers related to amines, amides and amino acids are exhibited in FTIR.Peaks located at 1341and 1042 cm-1 might be assigned to the existence of stretching vibrations of carboxylic acids and amino groups. The wide absorption band at 606 cm^{-1} indicated (C-H bend alkyne).

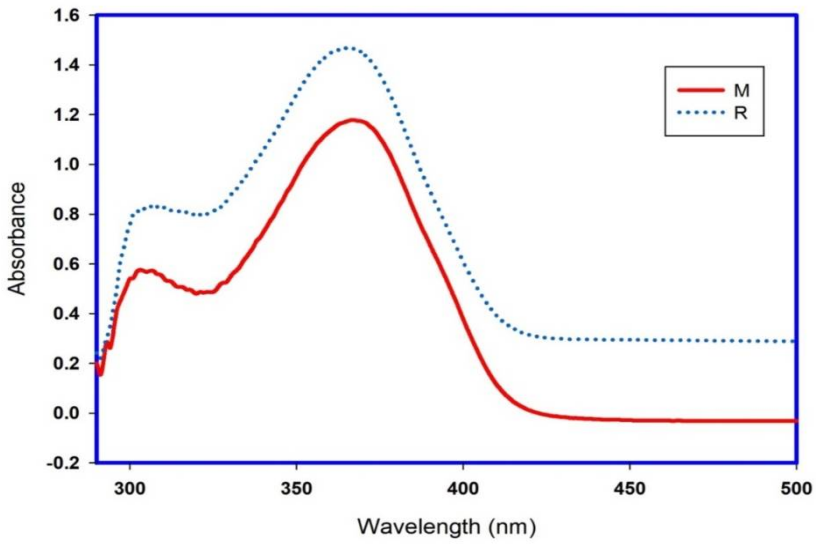

Fig. 1- UV- visible spectroscopy analyses of biosynthesized $Zn(OH)_2$-Nps using *Macrophomina phaseolina* (M) and *Sclerotium rolfsii* (R).

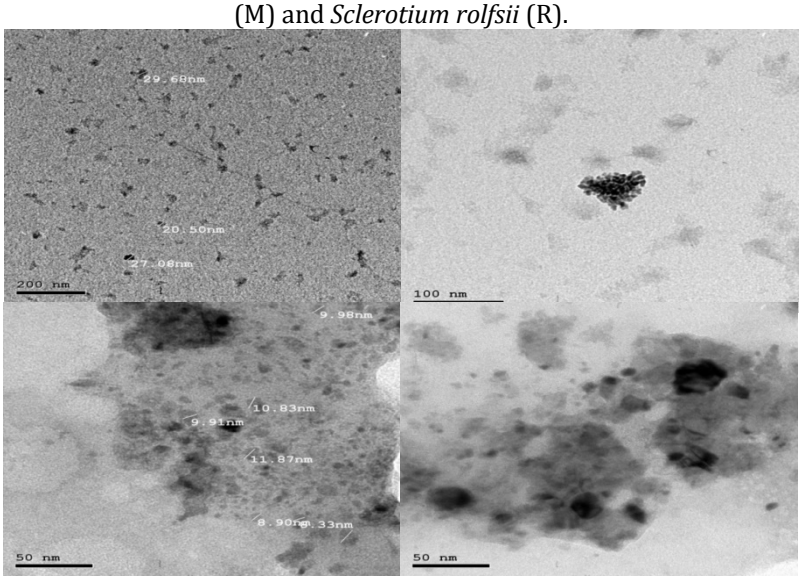

Fig. 2- Transmission electron microscopy (TEM) of the biosynthesized $Zn(OH)_2$-Nps using *Macrophomina phaseolina* and *Sclerotium rolfsii.*

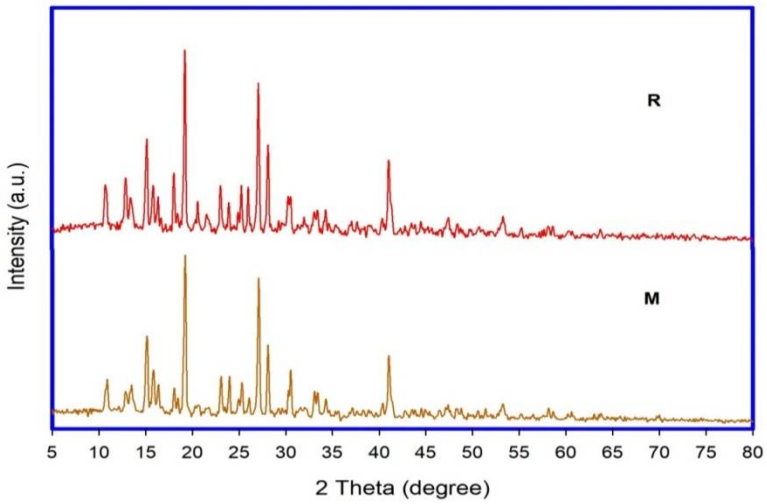

Fig. 3- X-ray diffraction pattern (XRD) of $Zn(OH)_2$-Nps using *Macrophomina phaseolina* (M) and *Sclerotium rolfsii* (R).

Antimicrobial bioassay

Antifungal activity

The antifungal activity of $Zn(OH)_2$-Nps was tested against *Exserohilum rostratum* and *Fusarium solani* at two incubation periods. The MIC results of the $Zn(OH)_2$-Nps are represented in (Table 1) and (Fig 5 & 6). The results confirm that all the used concentrations of the biosynthesized $Zn(OH)_2$ nanoparticles inhibited significantlythe growth of all the tested microbes *F. solani* and *E. rostratum*. Data focused on the potentialities of the 100 µg treatment of $Zn(OH)_2$-NPsrecorded the highest effect in *E. rostratum*and *F. solani* growth 3.0 and 3.4 cm respectively followed by 50 µg recorded 2.1 & 3.1 cm respectively. On the other hand, 10 µg treatments recorded the lowest effect in the same parameters 1.8 & 2.2 cm respectively compared with the fungicide which recorded in the two pathogens growth 2.0 & 2.8 cm respectively. Studies on the antifungal effects of ZnO and MgO nanoparticles, with average size of approximately 30 ± 10 nm and approximately 50 ± 10 nm, respectively

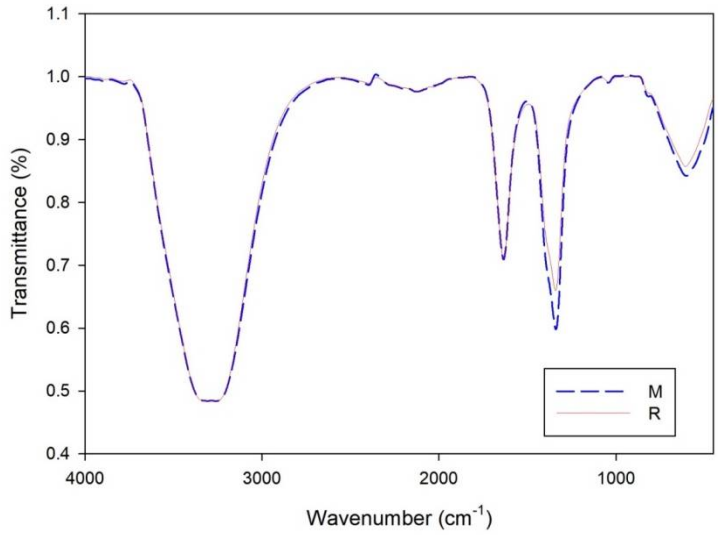

Fig. 4- FTIR spectrum of $Zn(OH)_2$-Nps biosynthesized by using *Macrophomina phaseolina* (M) and *Sclerotium rolfsii* (R).

Table (1): Antifungal activities of $Zn(OH)_2$-NPs against some soil-borne pathogenic fungi using agar diffusion method. Data points are the mean average of three replicates ± SE.

Treatments	Isolates	Inhibition zone (cm)			
		48 h	72 h	Mean	Overall mean
10 µg	*Exserohilum rostratum*	1.8	1.7	1.8	2.0
	Fusarium solani	2.2	2.1	2.2	
Mean		2.0	1.9	---	
50 µg	*Exserohilum rostratum*	2.2	2.0	2.1	2.6
	Fusarium solani	3.1	3.0	3.1	
Mean		2.7	2.5	---	
100 µg	*Exserohilum rostratum*	3.0	2.9	3.0	3.2
	Fusarium solani	3.5	3.3	3.4	
Mean		3.3	3.1	---	
Fungicide	*Exserohilum rostratum*	2.1	1.9	2.0	2.4
	Fusarium solani	2.9	2.7	2.8	
Mean		2.5	2.3	---	
Control	*Exserohilum rostratum*	0.0	0.0	0.0	0.0
	Fusarium solani	0.0	0.0	0.0	
Mean		0.0	0.0	---	
Mean of isolates	*Exserohilum rostratum*	1.8	1.7	1.8	---
	Fusariumsolani	2.3	2.2	2.3	
Overall mean		2.1	2.0	---	
L.S.D. at 1% for: T x I = 0.28 Treatments (T) = 0.11 T x R = 0.11 Isolates (I) = 0.12 I x R = 0.07 Reading time (R) = 0.05 T x I x R = 0.16					

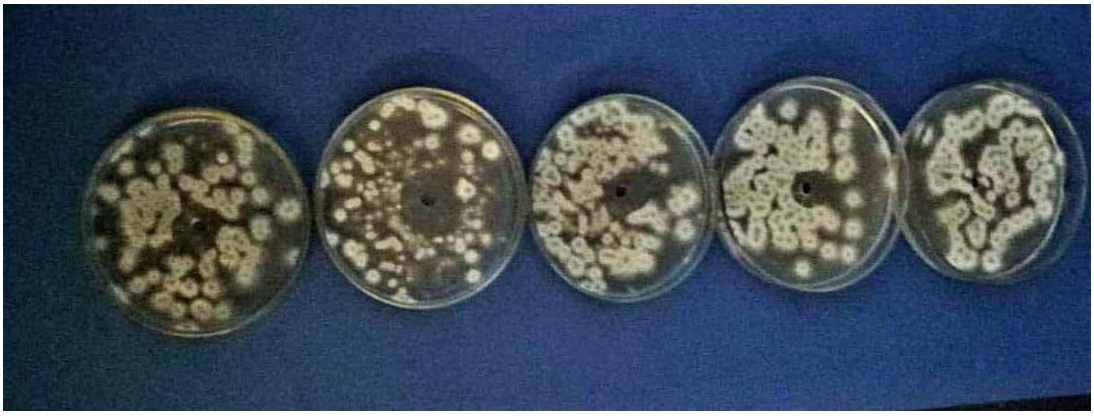

Fig. 5- Antifungal activity of $Zn(OH)_2$-Nps concentrates (10,50, and100 µg) and chemical fungicide (Moncut) against *Exserohilum rostratum* .

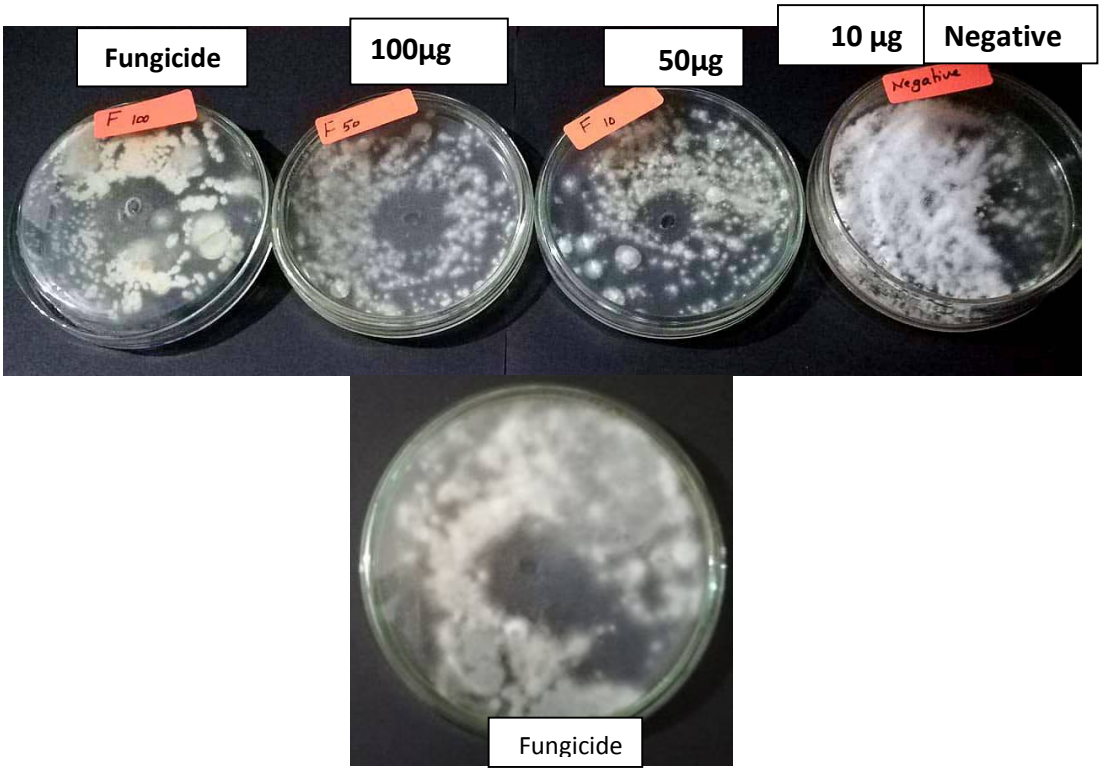

Fig. 6- Antifungal activity of $Zn(OH)_2$-Nps concentrates (10,50, and100 µg) and chemical fungicide (Tebuconazole) against *Fusarium solani*.

DISCUSSION

The success of the biosynthesis process of Zn(OH)2-NPs was validated by our findings, particularly in the examination of visible spectroscopy, similar findings reported by Mingsong *et al.* [21]. Transmission electron microscopy was used to examine the shape and sizes of the biosynthesized Zn(OH)2-NPs, which were dispersed without aggregation [22].

The pure Zn(OH)2-NPs generated by *M. phaseolina* and *S. rolfsii* have an XRD pattern that is similar to that of zinc hydroxide, as described by Hossein et al. [22].

The existence of functional groups inside biomolecules linked with Zn(OH)2-NPs was revealed by FTIR analysis, which might be dependable for the decreasing of zinc ions and stability of bioformed Zn(OH)2-NPs.The largest absorption peaks found in the FTIR spectra of *M. phaseolina* were 3260 cm-1, which are attributed to the protein's primary amide linkage and hydroxyl group (O-H), and these results were accepted with confidence [23-26]. According to the findings of FTIR investigation, proteins and polysaccharides function as reducing and stabilizing agents in the production of zinc hydroxide nanoparticles [24].

The antagonistic impact of zinc hydroxide nanoparticles on *Exserohilum rostratum* and *Fusarium solani* throughout two incubation periods according to the established technique on *Exserohilum rostratum* and

Fusarium solani [20]. The findings show that biosynthesized Zn(OH)2 strongly reduced the development of all examined microorganisms, including *F. solani* and *E. rostratum*, at all doses studied. On harmful fungi such as *Alternaria alternata*, *Fusarium oxysporum*, *Rhizopus stolonifer,* and *Mucor plumbeus*, our results with Waniand Shah [27] are satisfactory.*Penicilliumexpansum* (28), *Fusarium oxysporum*, and *Peronospora tabacina*[29].In another study, ZnO NPs were shown to limit *Botrytis cinerea* growth by interfering with cell activity and causing distortion in fungal hyphae [30]. In contrast, ZnO NPs at concentrations more than 6 mmol l1 entirely stopped *Peronospora expansum* from growing by preventing the formation of conidiophores and conidia.ZnO NPs may be employed as an effective fungicide in agricultural and food safety applications, based on these findings. A substantial reduction of pathogen development was seen in plate tests in another investigation, indicating the efficiency of zinc titanium oxide (ZnTiO3) nano-powder as a biocidal against the fungus *Aspergillus niger* [31]. Finally, the actions of both structurally distinct nano-zinc oxide substances, SG4 and SG6, versus *Xanthomonas citri* subsp. citri, the bacteria that causes citrus canker, were verified in the same field [32].

CONCLUSIONS

$Zn(OH)_2$ nanoparticles has been biosynthesized successfully by *Macrophomina phaseolina*, *Sclerotium rolfsii*. The biosynthesized $Zn(OH)_2$-Nps were characterized by using UV-Vis, FTIR, TEM and XRD analyses. The biosynthesized $Zn(OH)_2$ nanoparticles showed excellent antifungal activities against *Exserohilum rostratum* and *Fusarium solani*.

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