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Phyto-synthesis of Manganese Oxide Nanoparticles for the Mitigation of Phytopathogenic Fungi *Sclerotinia sclerotium*

ABSTRACT

The green synthesis approach was used to fabricate manganese oxide nanoparticles (MnO NPs) using an aqueous extract of *Russelia equisetiformis* leaves as the study's primary aim. The biosynthesized MnO NPs were monitored using various techniques such as UV-visible spectroscopy (UV), dynamic light scattering (DLS), scanning electron microscopy (SEM), energy-dispersive X-ray spectroscopy (EDX), transmission electron microscopy (TEM), and Fourier transform infrared spectroscopy (FTIR). The latter was conducted to determine the organic ingredients in the leaves extract that could be responsible for the bioreduction and stabilization of MnO NPs that were further tested for their antifungal activity against tomato pathogenic fungus *Sclerotinia sclerotiorum*. Results indicated the successful formation of MnO NPs, as confirmed by peak absorbance of the UV–Vis spectra at 325.09 nm. The SEM and TEM analysis showed the presence of spherical nanoparticles, while the EDX analysis revealed intense signals of Mn. FTIR indicated the presence of phenol and protein that might contribute to the stability of MnO NPs as confirmed by the negative zeta potential, −0.014 mV, for particles of 211.9 nm size with a polydispersity index of 0.29 indicating good dispersion. The study also explored the potential use of biosynthesized MnO NPs against *Sclerotinia sclerotiorum* since treated fungus showed a remarkable decrease in mycelial growth, thin, deformed, and lysed hyphae when viewed under the light microscope. The promising antifungal activity results provided an important perspective for using biosynthesized MnO NPs in various applications.

KEYWORDS: Nanostructure, *Russelia equisetiformis*, Tomato, Antifungal

INTRODUCTION

The stability of the ecosystem and agriculture is threatened by various environmental factors, such as microbial pathogens, pests, chemical pollutants, and weeds (Moore et al., 2020). As a result of the factors mentioned above, crop production has seen a severe and continuous decline (Moore et al., 2020; Prakash, 2022). Various factors, including natural disasters, pests and diseases, climate change, and human activities, can significantly impact crop yield and quality. Among these factors, pests and diseases are considered cause of crop loss.

Fungal and bacterial diseases like blights and wilts can also negatively affect crops. The spread of pests and diseases can be rapid, leading to significant crop loss in a short period (Erlee, 2023; Richard et al., 2022). Phytopathogens may even further amplify losses through poor agricultural practices and overuse of pesticides, increasing the chances of toxin production in cereal crops within the food chain (Prakash, 2022). Phytopathogens, like fungi, pose a significant threat due to their saprophytic lifestyle and the limited availability of antifungal agents. Still, their harm will further increase with the progression of global warming (Nnadi and Carter, 2021). Standard management and control methods for fungi include chemical pesticides; however, this presents multiple drawbacks as they contain petrochemical derivatives, which pose extreme health hazards when accumulated in soil or groundwater.

Overuse and the lack of compliance with usage regulations also pose more significant risks due to the possible induction of resistance within fungi (Hernandez-Diaz et al., 2021). According to a survey conducted by the World Health Organization on 56 countries, 32% lack legislation covering public health pesticides, and 65% lack provisions restricting their overuse (World Health Organization and Food and Agriculture Organization of the United Nations, 2019). This could eventually lead to further repercussions through the development of pesticide-resistant and mycotoxin infection (Wang et al., 2022). Such outcomes encourage the spread of various plant phytopathogen diseases and mycotoxins, such as multiple species of *Alternaria* sp., *Sclerotinia* sp., and *Fusarium* sp. These fungi are known to cause leaf blight, withering, and decay in tomato plants, giving a high potential risk (Antwi-Boasiako et al., 2022; Chen et al., 2021; de Chaves et al., 2022). However, they could be managed by fungicide applications.

Limitations in current solutions for this problem guided us to the implementation of nanotechnology in the development of efficient compounds for treatment. Nanotechnology has gained immense attraction in recent years. It is the utilization and modification of materials on an atomic level to achieve advantageous properties that could be used in multiple desired applications (Gleiter, 2000). Nanoparticles within the 1 to 100 nm size range and of diverse shapes present distinct chemical, physical, and optical characteristics. This has broadened multiple interdisciplinary research fields in chemistry, biology, environmental science, and medicine (Scholes, 2008; Singh et al., 2018; Yilmaz and Yilmaz, 2020). Using metal nanoparticles (MNPs) is a viable option for controlling phytopathogenic fungi (Cruz-Luna et al., 2021). MNPs can be synthesized using physical, chemical, and biological methods (Chen et al., 2008); however, such approaches could have high negative environmental impacts. In contrast, green synthesis of nanoparticles utilizes plants, bacteria, fungi, and algae and has the advantages of environmental friendliness, low cost, high feasibility, and ease of production. Although nanoparticles' variable size and aggregation tendency could be expected (Zhang et al., 2023).

In the current study, the perennial shrub *Russelia equisetiformis* was used since previous studies reported its ability to form silver nanoparticles (Mohammed and Al-Megrin, 2021; Sabiha Sulthana et al., 2022). This plant, native to South America, belongs to the family Scrophulariaceae (recently Plantaginaceae). It is a ritualistic medicine used to treat diabetes, malaria, and inflammation in various parts of Nigeria (Kolawole and Kolawole, 2010). Besides, multiple anti-inflammatory, antimicrobial, and antioxidant properties were recorded (Muhammad Riaz, 2012). Despite many advantageous properties, *R. equisetiformis* has only been used as a biogenic agent in nanoparticle synthesis in limited studies (Mohammed and Al-Megrin, 2021; Sabiha

Sulthana et al., 2022). Our current study focused on Manganese oxide nanoparticles (MnONPs) that have been proven to have effects against fungi. MnONPs are emerging as agents for biomedical applications such as drug delivery, antimicrobial, photothermal therapy, and anti-angiogenic, having different mechanisms of action (Haque et al., 2021). Previous studies have also proven MnONPs to be efficient antifungal agents against many types of fungi like *Candida albicans*, *Curvularia lunata*, and *Aspergillus niger* (Gillani et al., 2021). The antifungal activity of nanoparticles is influenced by various factors, including their shape, size, distribution, composition, crystallinity, agglomeration, and surface chemistry (Cruz-Luna et al., 2021). The antifungal mechanism of NPs is mainly attributed to their ability to inhibit the growth of fungi by degrading their cell walls and membranes, disrupting protein synthesis, and influencing their metabolism, signal transduction, and genetic information processing. Additionally, NPs produce reactive oxygen species (ROS), further contributing to their antifungal properties (Al-Otibi et al., 2022; Jian et al., 2022). The purpose of the current study was to utilize *R. equisetiformis* for the biosynthesis of MnONPs and assess their antifungal potential against *S. sclerotiorum*, the causal agent of Sclerotinia stem rot of tomato plants. The morphology of the fungi was observed under light microscopic after the MnONPs treatment.

MATERIALS AND METHODS

Materials

Russelia equisetiformis leaves were collected from the nursery of the Royal Commission for Riyadh City (RCRC), Riyadh, Saudi Arabia (Potato Dextrose Agar (PDA) and, Magnesium (II) sulfate were obtained from the Laboratory of Princess Nourah bint Abdulrahman University, Riyadh, Saudi Arabia. The strain of *S. sclerotiorum* (ON876490) was isolated from infected tomato (*Solanum lycopersicum*) at Health Sciences Research Center at Princess Nourah bint Abdulrahman University

Biosynthesis of MnONPs

A 50 mL of 1 mM aqueous $MnsO4.H_2O$ solution was combined with 2.5 g of powdered plant extract. The resulting solution was then heated for 15 minutes at 90°C until the color changed from green to purple, indicating the reduction of metal ions. Afterward, the solution was filtered and centrifuged at 14,000 rpm for 1 hour. The precipitate was washed thrice with distilled water and centrifuged at 14,000 rpm for 30 minutes.

Characterization:

UV-Vis spectral analysis

The optical properties of MnONPs were measured using a UV-visible spectrophotometer (Thermo Fisher Scientific, USA) with an absorption spectrum between 200-500 nm.

Fourier Transform Infrared Spectroscopy (FT-IR)

FTIR spectroscopy (Perkin-Elmer, USA) analyzed the functional groups responsible for reducing and capping nanoparticles in phytoconstituents. The measurements were performed in the transmission range of 400 cm^{-1} to 4000 cm^{-1} with 64 scans.

Dynamic Light Scattering (DLS) and Zeta Potential

For size and distribution measurement of the biomolecules and determination of the stability of their colloidal properties, zeta (ζ) potential and dynamic light scattering (DLS) were used. Four measurements per sample were performed with the Zetasizer Ultra (Malvern Panalytical, UK).

Scanning Electron Microscopy (SEM) and Transmission Electron Microscopy (TEM)

The morphology and topography of MnONPs were determined using a scanning electron microscope (JSM-IT500HR, JEOL, Japan) equipped with an X-ray energydispersive spectroscopy (EDX) (STD-PC80, JEOL, Japan) operated at 15 kV and were utilized. Further information about the size, shape, and crystallography of the MnONPs was obtained through a transmission electron microscope (JEM-1400 flash, JEOL, Japan).

Identification of fungal strain isolated from infected tomato fruit

The isolation process of the fungal strain was applied according to Nizamani et al. (2021). Infected tomato fruits were thoroughly washed with tap water and dried, then a piece from the infected part (2-3 mm in length) was cut at the junction of the diseased area with the help of an alcohol-sterilized sharp blade. These pieces were aseptically located at Petri plates containing sterile PDA and incubated at 25°C. The plates were monitored regularly for the development of the colonies. After that, fungal growth was purified using a single spore culture technique. Further, for fungal identification, 18S RNA gene sequencing was used. The molecular identification of fungus, DNA extraction, PCR amplification, and sequencing were done according to Mohammed et al. (2021).

Antifungal Activity of MnONPs

The agar dilution method tested biosynthesized MnONPs as antifungal agents against *S. sclerotiorum* (ON876495). A 10 mg/mL concentration was prepared from MnONPs and added to a Petri plate. Then, 9 mL of sterilized potato dextrose agar was added before solidification. An inoculum of 9 mm diameter of *S. sclerotiorum* was taken from a 7-day-old culture and placed aseptically at the center of the solidified agar. The plates were then incubated at 25°C for 3 days.

Microscopic observation of fungal growth

To investigate the impact of treatments on fungal growth. The cells from treated and control plates were collected using a sterilized loop from the Petri plates' surface containing *Sclerotinia sclerotiorum.* The morphology of the fungal cells was observed under a light microscope (LABOMED, SLA2000).

Statistical analysis

Statistical analysis for antifungal investigation was carried out using GraphPad Prism and Image J 1.54d. Statistical comparisons of multigroup data were collected using Oneway (ANOVA), and only values of $p < 0.01$ were considered significant.

RESULTS AND DISCUSSION

The current study evaluated the ability to fabricate MnNOPs from *R. equisetiformis* leaf extract, and the final product was assessed for its anti-fungal properties against tomato pathogenic fungi *S. sclerotiorum*. The phyto-produced MnONPs were characterized using different approaches as follows:

UV-Vis Analysis

UV-Vis analysis was performed to investigate the optical properties and absorption maxima (λmax) of MnONPs. The concentration of NPs is a determining factor of UVVis absorption intensity. Usually, increased absorption of MNOs within the UV region implies better solubility and dispersion of the nanomaterials, meaning more efficient applicability (Souri et al., 2018). The absorption range of MnONPs is critical for their reactivity to the biological and chemical systems (Selim et al., 2020). Figure 1 shows the absorption spectrum of MnONPs suspended in the aqueous solution using UVVis. The Absorption maximum (λmax) of green synthesized MnONPs was 325.09 nm, corresponding to the characteristic band of MnONPs, confirming that the leaf extract can reduce the metal to its nanoforms. Compared to other studies, a slight shift was observed in the absorption maxima (Khan et al., 2020; Roy et al., 2018).

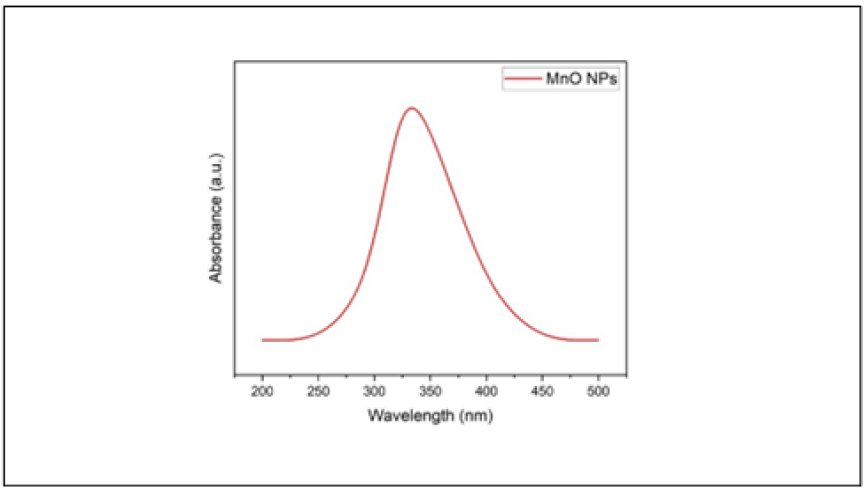

Figure 1. UV- vis spectra showing absorbance peaks of MnONPs prepared by *R.equisetiformis*

FTIR Analysis

In Figure 2, the FTIR spectrum of *R. equisetiformis* leaves extract, and the phytofabricated MnONPs provided information about the functional groups and biomolecules involved in the fabrication process (Mohammed and Al-Megrin, 2021). The major peak at 3314 cm^{-1} was noted in the spectra of *R. equisetiformis* and MnONPs, which indicate the presence of polyphenolic-OH groups. A peak at 1640 cm^{-1}, which belongs to C = O, was also detected, indicating amide 1 and carbonyl (C = O) stretching of proteins. Various biomolecules, such as proteins and polyphenolics, were noticed in both tested materials, demonstrating their role as capping and stabilizing agents in the NPs (Mohammed and Al-Megrin, 2021). Recent findings indicated similar peaks ranging between 3550 and 3500 cm^{-1} and 1639.2 cm^{-1} for AgNPs prepared by the same plant extract; however, different parts were used, thus confirming that the functional group determination is dependent on the plant part used (Sabiha Sulthana et al., 2022).

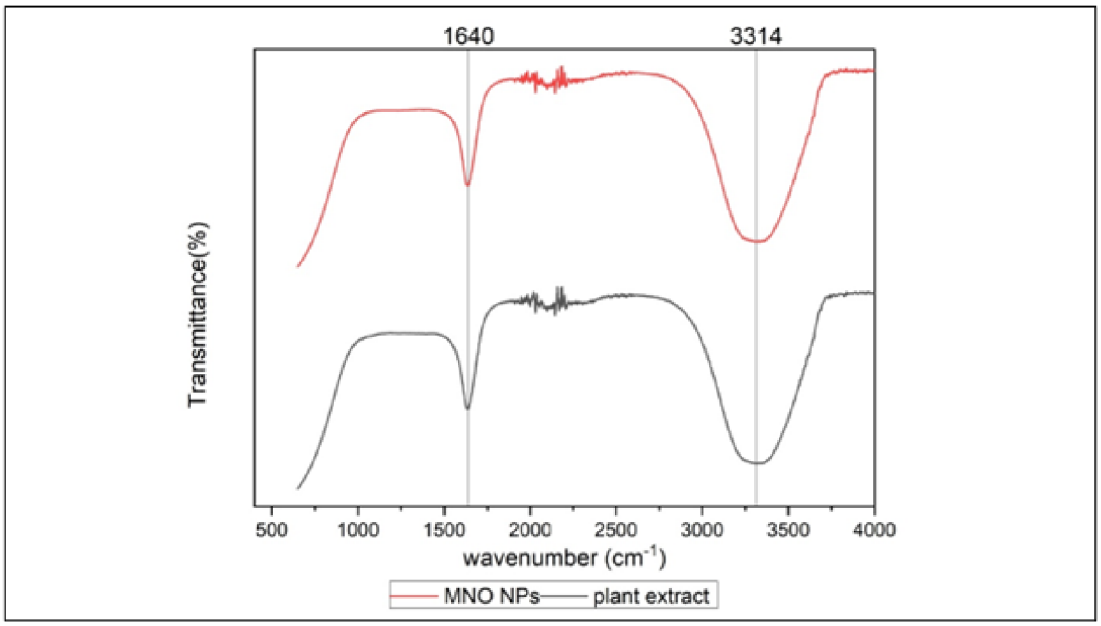

Figure 2. The FTIR spectra of *R. equisetiformis* extract, and MnONPs.

Dynamic Light Scattering (DLS) and Zeta Potential

Dynamic light scattering was performed to obtain the measurement of particle size distributions (Babick, 2019). The size distribution of the nanomaterials is described by the polydispersity index (PDI), which specifies their uniformity. The PDI value of 0.1 to 0.25 indicates a narrow size distribution, while a PI greater than 0.5 refers to a broad distribution (Hoseini et al., 2023). MnONPs (Figure 3A) indicated stable nanoparticle synthesis through DLS with a size of 211.9 nm and a polydispersity index of 0.29. In another investigation, the solution and thickness of the stabilizing compounds surrounding metallic particles of MnO2-NPs determined its average size distribution. This investigation presented results slightly different from those of the current study; DLS Showed the average particle size was 500 nm due to possible swelling of particles in an aqueous medium and a polydispersity index of 0.34 (Khan et al., 2019). Overall, the DLS result of MnONPs confirms the formation of well-defined dimensions with efficient monodispersity due to their PDIs presenting less than 0.5. Zeta potential measurements were also performed to define the samples' colloidal stability and surface charge (Faisal et al., 2021). Particles with zeta potentials that are more positive than +30 mV or more negative than -30 mV are typically considered stable (Clogston and Patri, 2011). Currently, the zeta potential of the MnONPs in distilled water was −0.014 mV, as shown in Figure 8B, indicating particles with low negative zeta potential that may lead to coagulation over a short period. However, they are still deemed stable given their zeta values remain within the negative range but cannot remain in suspended form within solutions due to surface charge (Seidel et al., 2022). Chemically synthesized MnO2-NPs showed a high surface charge at a zeta potential of − 20.4 mV (Khan et al., 2019).

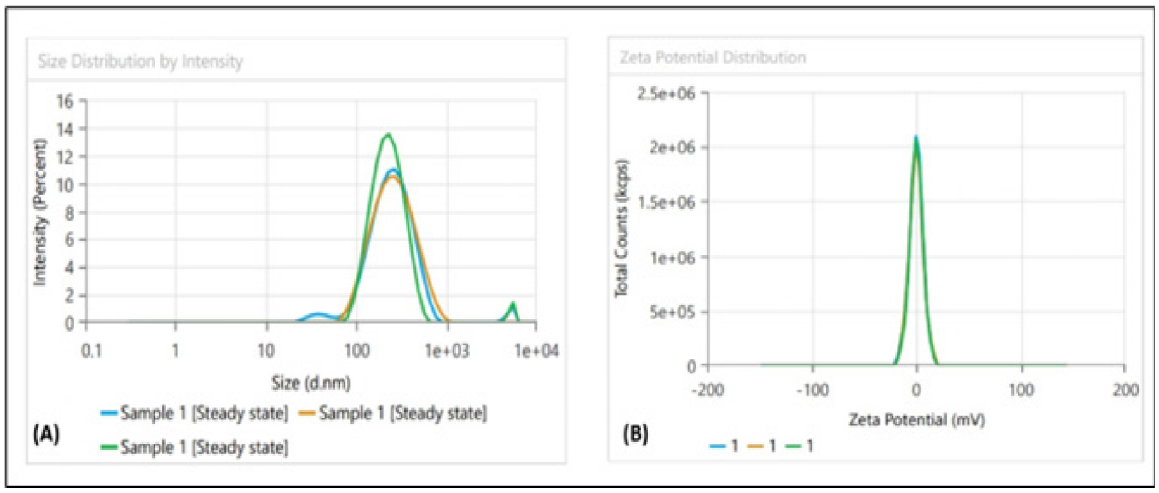

Figure 3. A) Size distribution and B) zeta potential distribution for the MnONPs fabricated by *R. equisetiformis*

SEM and TEM Imaging and EDX analysis

TEM and SEM were employed to investigate the surface morphology and porous structure of MnONPs further. Using EDX, we further studied the purity and chemical composition of the samples. TEM micrographs of MnONPs are indicated in Figure 4. Particles appeared to agglomerate as a spheroid. SEM analysis of MnONPs showed spherical, moderately dispersed, and slightly agglomerated particles (Figure 5A). Mapping (Figure 5B) revealed the presence of multiple elemental compositions like 0.22 wt% of Mn, 33.85wt% of O, and 65.92 wt% of C. Green synthesized MnONPs showed spectral signals at 0.4 keV, 5.9keV and 6.5 keV for Mn and 0.5 keV for Oxygen in Figure 5C. Similar EDX spectral signals of previous studies showed MnONPs synthesized from Indian abutilon (Ekinci et al., 2023). Overall, it can be concluded that *R. equisetiformis* leaf extract is a promising substance for effectively synthesizing nanomaterials; however, aggregation of the biomolecules produced is common in green synthesis (Zhang et al., 2023).

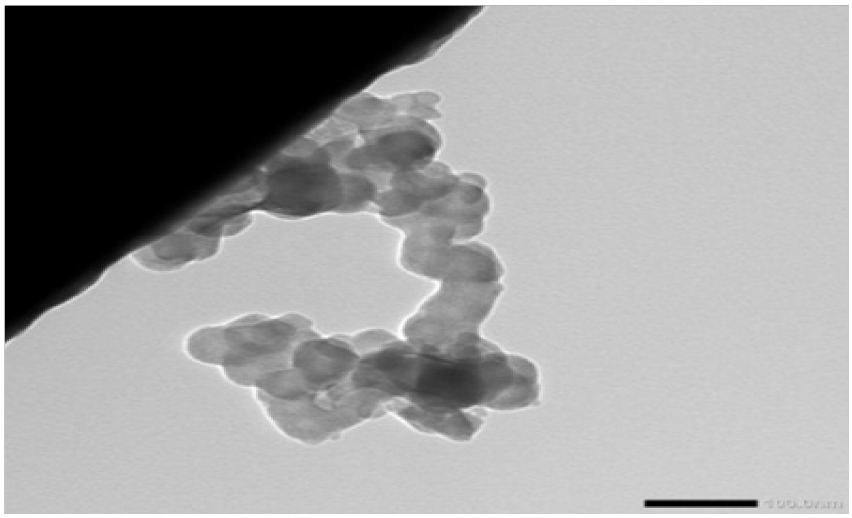

Figure 4. The TEM images of MnONPs

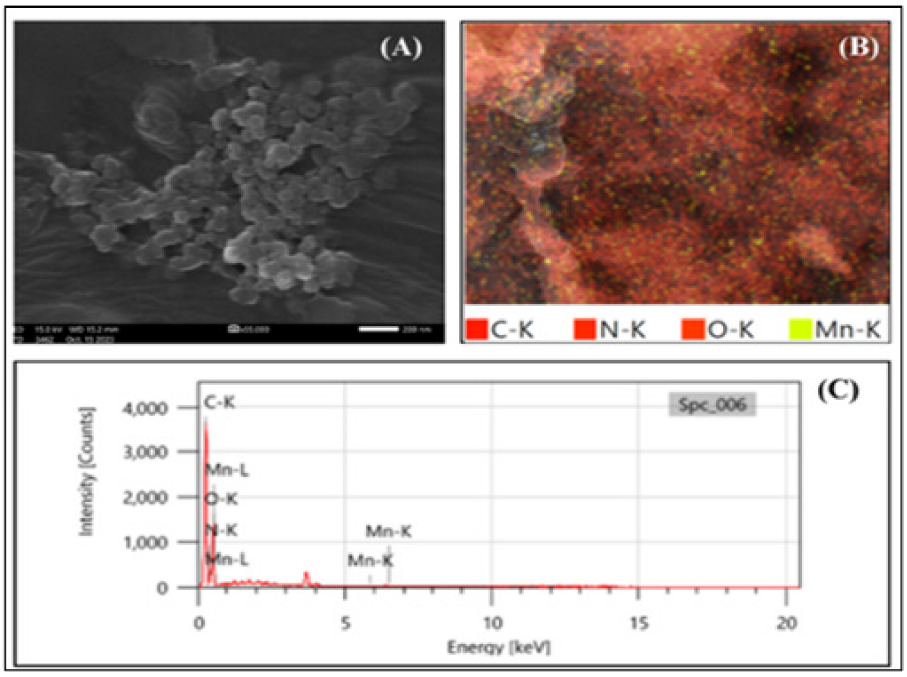

Figure 5. A) SEM image; B) element mapping and C) EDX pattern and elemental composition of MnONPs

Molecular identification of the isolated fungi and the antifungal Effect

The isolated fungal strain was detected using an 18S rRNA gene sequence compared to the sequence database at GenBank for genera or species detection by BLASTn. The presence of the fungus presented 100% similarity to *S. sclerotiorum* and the sequence has been deposited in gene Bank (ON876495). The antifungal properties of MnONPs were tested against *S. sclerotiorum* in a laboratory setting. The average growth area of the fungus from the plates treated with MnONPs was analyzed and compared to untreated control ones. The results showed that the average growth area of the fungus from treated plates was 0.02 ± 0.001 Cm, while from untreated plates, it was 0.07 ± 0.001 Cm. Findings reported the significant impact of MnONPs on the growth rate of the *S. sclerotiorum*. Similar findings were also reported for Pd-doped Mn_3O_4NPs, which showed antimicrobial activity against *S. sclerotiorum* (Vikal et al., 2023). Several theories exist concerning the mechanisms through which NPs demonstrate antifungal properties. However, despite extensive research on the antifungal activity of MNPs, the exact mechanisms remain unknown. MNPs exhibited antifungal properties by interacting with the fungal cell wall, causing structural damage and cell death (Nguyen et al., 2022). The antifungal properties of NPs can be divided into three stages: firstly, NPs attach themselves to the cell walls of fungi and enter the fungal cell using various pathways. Secondly, once inside, they spread to different locations within the cell. Lastly, they interact with various biomolecules, setting off cellular reactions that ultimately result in the death of the fungal cell (Gurunathan et al., 2022; Li et al., 2022; Rana et al., 2023).

Microscopic observation of fungal growth

Due to their suppression ability, the impact of MnONPs on the growth of the *S. sclerotiorum* was studied under the light microscope in an atrial to find any morphological changes in spore and fungal mycelia. A remarkable decrease in mycelial growth was observed (Figure 6). Thickened and septate hyphae of *S. sclerotiorum* mycelium are displayed in Figure 6A. However, thin, deformed, and lysed hyphae were observed from

treated plates (Figure 6B). Similar findings were also noted for *S. sclerotiorum* treated by the biogenic AgNPs fabricated using Trichoderma Isolates, where small fragments and damage of hyphae were reported (Tomah et al., 2020).

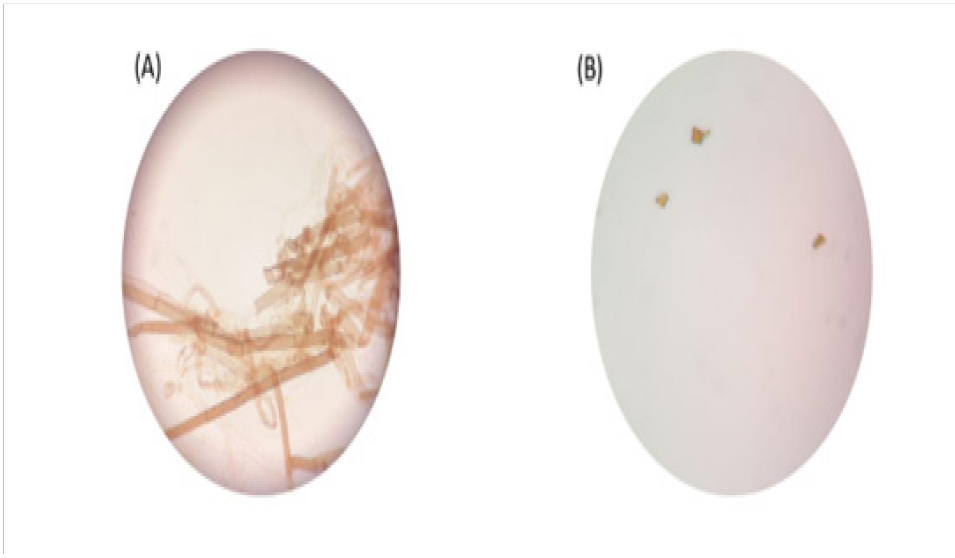

Figure 6. Morphology of *S. sclerotiorum* mycelia. A) is the control and B) is the treated fungi

CONCLUSIONS

The current study has proposed a cost-effective, eco-friendly way of producing stable MnONPs. The method involved using the leaves of *R. equisetiformis*, which are abundant in South America. The polyphenolic compounds present in this leaf extract helped form and stabilize the MnONPs. The biosynthesized MnONPs were found to be spherical. The study also found that the biosynthesized MnONPs had excellent antifungal activity against *S. sclerotiorum*, indicating their potential as an effective treatment for pathogenic fungi. More fungal strains should be tested at different concentrations of MnONPs to better understand their effectiveness as anti-fungal nanomaterials.

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Declaration of interests

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.

Availability of data

All data supporting our findings are contained within the manuscript. Further details can be provided upon written request to the corresponding author.

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