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GREEN SYNTHESIS OF SILVER NANOPARTICLES USING WILD MUSHROOM *LEPISTA SORDIDA* AND ITS ANTIFUNGAL ACTIVITY

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Abstract

Macrofungal-assisted synthesis of nanoparticles has been reported to produce a large amount of protein with high yield and low toxicity in many countries. However, it is a neglected filed in Pakistan. The aim of this research was to investigate the potential of wild neglected mushroom *Lepista sordida* occurring in Pakistan to synthesize silver nanoparticles (AgNPs) through economical and eco-friendly methods and to evaluate their antifungal potential against pathogenic fungi. This mushroom was examined first time for synthesis of its nanoparticles in Pakistan. *L. sordida* was also first time analyzed from Pakistan through molecular analysis. The synthesis of AgNPs (through Microwave assisted extraction method) was confirmed and characterized by ultraviolet (UV)- visible spectroscopy, Scanning electron microscopy (SEM), and X-ray diffraction analysis. UV-visible spectrum for silver nanoparticles exhibited a broad absorption peak between 200–800 nm and showed a maximum absorption spectrum at 345 nm, which indicated the existence of AgNPs. SEM micrographic studies confirmed that synthesized AgNPs from *L. sordida* extract had smooth hexagonal morphology in the nano-range. The antifungal activity was performed against the pathogenic micromycetes species (*Aspergillus flavus* and *Alternaria alternata*) to check the maximum inhibitory effect of AgNPs against these species. The AgNPs synthesized with fungus extract inhibited the growth of *A. flavus* and *A. alternata* by 97.3% and 91.75% at the concentration of 10µg/L. This indicated that the synthesized AgNPs using fungus had the capability to control pathogen's growth with low toxic rate and good biocompatibility mode. This work can be helpful in future projects related to the development of nanofungicides against fungal pathogens.

Key words: Basidiomycetes, Basidiospores, Mushrooms**,** ITS region, Macrofungi

Introduction

The use of nanotechnology, which manipulates matter at the molecular level, spans the fields of Science and Biotechnology, where it is used to novel delivery systems and diagnostics using a broad range of high-volume technologies. Because of their tiny size and large surface area, synthetic nanoparticles have increased catalytic activity (Bayda *et al*., 2020).

Mushrooms are fungi that have long been utilized as food and medicinal in many regions of the world and these have a long history of being consumed as food. For thousands of years, people have been collecting and eating mushrooms. The genus *Lepista* belongs to the order Agaricales. With 40 families, 374 genera, and over 16,000 species, the Agaricales is the biggest group of mushroom-forming fungi (Wijayawardene *et al*., 2020). pecies of *Lepista* called clitocyboid or tricholomatoid agarics, that easily detach from the cap and stem present in the field. This genus is extensively dispersed in Asia, North America, and Europe, has over 50 known species (De *et al*., 2022).

Many mushrooms, e.g., *Volvariella volvacea, Pleurotus* species, *Ganoderma* species, *Agaricus bisporus, Fomes fomentarius, Helvella* sp. and *Microporus xanthopus* have been successfully investigated for their nanoparticles for their beneficial use (Talie *et al*., 2020). AgNPs have become progressively popular as antifungal, antibacterial, antioxidant, and anti-inflammatory agents (Nandhini *et al*., 2023). The chemicals released by both therapeutic and edible mushroom result in the formation of nanoparticles with great stability and favorable dispersion properties. Nano-tests with macrofungi have opened up a new area of a green method to mycosynthesize eco-friendly, non-toxic, and stable nanoparticles, which thus far, seem to be quite promising (Moghaddam *et al*., 2015). They also release many extracellular proteins, which help to keep the nanoparticles stable (Netala *et al*., 2016; Du *et al*., 2015). The mass of mycelia has high pressure and resistance against agitation than that of plants, making it more suited for larger-scale synthesis (Velusamy *et al*., 2016).

Many methods have been in practice for the management of microbial pathogens, but these have some limitations (Abdullah, 2016). However, synthesized nanoparticles have great potential to be used as antimicrobial without limitations (Parveen *et al*., 2018). Considering current era's demand, the present environmental scenario and other safety measures, there is widespread interest in developing cost effective, benign, and eco-friendly approach for the synthesis of nanoparticles (Aswathi *et al*., 2023). The present study was carried out first time from Pakistan using wild mushroom that was previously neglected. These synthesized AgNPs were characterized and evaluated for their antifungal activity against fungal pathogens.

Material and Methods

Sample collection: Fruiting bodies of *Lepista sordida* were collected from field near University of Gujrat, Punjab, Pakistan, during rainy season in the Summer. Photographs and field notes of specimens in their native habitat were taken to preserve their natural state. After collecting and assigning numbers, the samples were dried and brought to lab for further analysis.

Identification of mushroom: The mushroom species (*Lepista sordida*) was identified through macroscopic, microscopic and molecular genetic analysis.

Macroscopic and microscopic analysis: Morphological identification was based on previous studies (Singer, 1986; Bon, 1987; Li *et al*., 2015). The pileus, stipe, and hymenium were all possibly visible sections of macrofungi that were examined during the macroscopic investigation. To conduct microscopic examinations, slides of macrofungus portion were prepared and stained with Congo red, Melzer's reagent, and potassium hydroxide (KOH). Each prepared slide was examined under Labomed compound light microscope at various powers, and all apparent microscopic features (Hyphae, Cystidia, Basidia, and Basidiospores) were captured and measured (length x breadth) in micrometers.

Molecular genetic analysis: DNA was isolated using a modified CTAB technique from dried sporocarps (Gardes & Bruns, 1996). For PCR and Sanger sequencing, the primer pairs ITS1F/ITS4 (White *et al*., 1990) for the ITS region of nuclear ribosomal DNA were selected. Gel electrophoresis was used to analyze the PCR results. Sequencing services of Macrogen Korea were taken for sequencing of samples. Sequencing chromatograms were edited by analyzing overlapping reads with BioEdit and matching them to GenBank entries with the Basic Local Alignment Search Tool (BLAST). For phylogenetic analysis, ITS sequences of closely related species were retrieved from GenBank. The MUltiple Sequence Comparison by Log-Expectation (MUSCLE) software was used for aligning the sequences (Edgar, 2004). The MEGA5 software's Model Testing tool was used to perform phylogenetic analysis using the probabilistic technique and the Jukes & Cantor (1969) model of sequence evolution (Tamura *et al*., 2011). From 1000 replicates, a bootstrap consensus tree was inferred; the tree includes matching bootstrap values > 50%.

Preparation of mushroom extract from microwave assisted extraction (MAE): By using a sterile knife, mushroom samples were cut into pieces. In a microwave at a power level of 600 W, 5g of the sample was heated for 3 minutes at 70°C with 100 ml of ethanol by following Ashraf *et al*., (2020) The aqueous mushroom extract was filtered using. Whatman filter paper No. 1 and stored at 4°C.

Biosynthesis of silver nanoparticles: For synthesis purpose, 1 mM solution of silver nitrate was prepared to be used as a basic source of silver ions. 10mL of this solution was mixed with 30 mL of mushroom extract. A change in color indicated the possible silver nanoparticle synthesis. Further confirmation of AgNPs synthesis was done by their characterization using various techniques, as described below.

Characterization of silver nanoparticles: Following techniques were applied for the characterization of synthesized AgNPs following Ghaffar *et al*., (2020). Services were taken from Central Lab, LCWU, Lahore.

UV-visible spectroscopy analysis: For this purpose, a UV-visible absorption spectrophotometer with a resolution of 2.0 nm between 200 and 800 nm and a scanning speed of 300 nm/min was used. The progress of the reaction between metal ions and the mushroom extract was seen using the UV-visible spectra of silver nanoparticles in an aqueous solution.

Scanning electron microscopy: This facility was available in the Central Lab, Lahore College for Women University, Lahore. The size and surface morphology of silver nanoparticles was measured by using scanning electron microscope (JSM-6480) at 20 KV for characterization. For this purpose, a small amount of silver nanoparticles was taken and a smear was formed on copper grid and were coated with carbon for SEM. Slides were then dried. Slides prepared in this way were then subjected to SEM analysis and images were taken at different magnifications.

X- Ray diffraction analysis: The structure and content of the purified silver particles were examined by X-ray diffraction (XRD) after freeze-drying in central Lab, Lahore College for Women University, Lahore. An X-ray diffractometer was used to gather the dried mixture of silver nanoparticles to determine how silver nanoparticles are formed. XRD analyzed the colloidal suspensions of AgNPs to establish its crystalline character and to corroborate the accuracy of the UV spectral study findings.

Antifungal potential of synthesized AgNPs against fungal pathogens: *Aspergillus flavus* and *Alternaria alternata*, two fungal isolates (identified cultures), were used to test AgNPs' antifungal activity. Fungal cells were grown at 26°C in PDA liquid medium for five days; after that, cells with 1×10^6 colony-forming units/mL were cultured on new PDA solid media. The agar well diffusion method was used. 8 mm diameter holes were punched into the PDA medium, 50µl of various AgNPs concentrations (10µg ml^{-1}, 8µg ml^{-1}, 6µg ml^{-1} and 2 µg ml^{-1}) were added, after which the wells underwent a five-day incubation period at 26°C. The same conditions were used to incubate controls made of silver-free plates.

Statistical analysis

All the data recorded was a mean of 5 replicates with ±SD. Whereas one-way ANOVA was applied to compare the significance of results at 5% level of significance (p=0.05). BioEdit, Muscle Alignment and MEGA 5 software were used for phylogenetic analysis.

Results

Identification of selected mushroom

Macroscopic analysis (Fig. 1)

Pileus brown in color, having broad central part in dark yellow to golden, convex to flat, slightly depressed from center, smooth, context thick, fleshy, soft and dry, wavy margin decurved, brown to blackish brown in color. **Hymenium** closely packed gills, fimbriate edges, average thickness, adnate to decurrent, dry and equal misc, and with shade of brown color. **Stipe** thick, central, brownish, longitudinally striate, subcylindrical, flattened, smooth surface; **Annulus** absent.

Microscopic analysis (Fig. 2)

Basidiospores 4.5-7 × 4-5 µm, smooth, thin walled, guttulate, hyaline in 10% KOH, thin walled, globose to ovoid. **Basidia** thin walled, clavate, 24-48 × 8-10 µm, hyaline in KOH. **Cystidia** 24.5-34 × 5-6 µm, thin walled and clavate. **Pileipellis** 19-29 × 2-4 µm, thin walled, non-septate, branched, hyaline in 10% KOH solution, at terminal hyphae rounded at the tip. Cuticle 6-8 × 5-6 µm, thin walled, cellular, and slightly hexagonal to circular shape.

Material examined: Pakistan, Punjab, District Gujrat, from fields near University of Gujrat, at 243 m.a.s.l, on the soil LCWUBOT.MM.TK03082113.

Molecular genetic analysis (Fig. 3)

The sporocarp's amplified ITS region produced a consensus of bp 653. The initial blast search showed 99% identity with *Lepista sordida* (ON876174). 36 nucleotide sequences were included in the aligned ITS data set for phylogenetic analysis. There were 638 positions over all in the final data set after trimming the aligned sequences from both ′ and ′ ends. Of these, 165 were variable, 103 were parsimony-informative and 62 were singletons. As outgroups, *Tricholoma matsutake* (AB699640) were used. Phylogenetic tree was constructed in MEGA5 software with default settings. Our Pakistani collection fell in the same clad with *Lepista sordida* (MK116605, MK116606, MK116607, MK116608, MK116609, and MK116610) that supported already described species with a high bootstrap value.

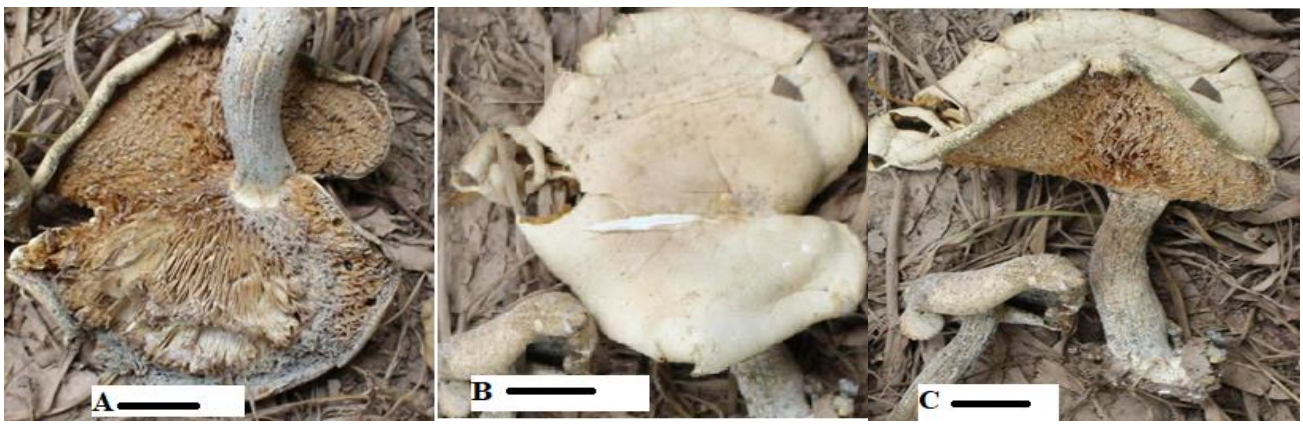

Fig. 1. Macroscopic features of *Lepista sordida* (A) Hymenium, (B) Pileus, (C) Stipe. Bars = 1.5 cm.

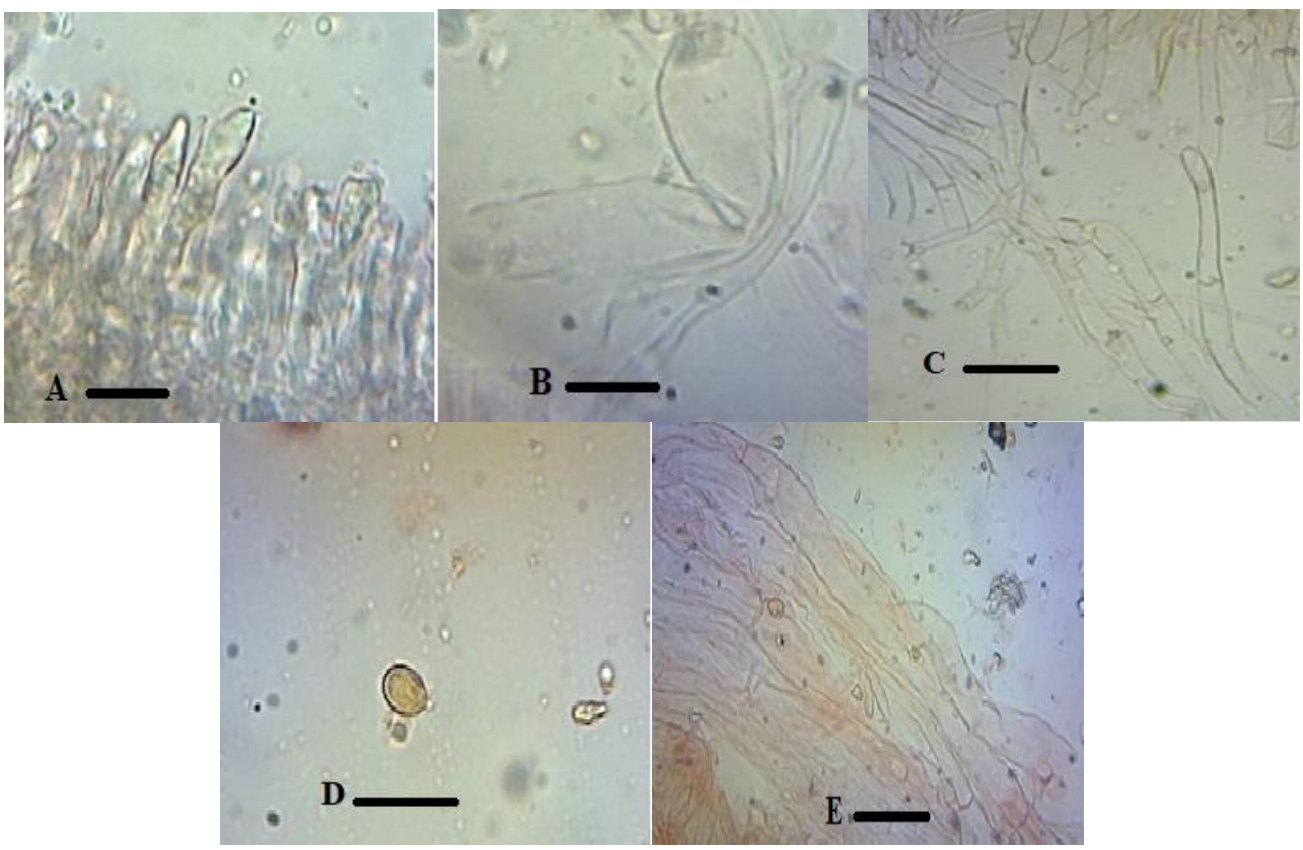

Fig. 2. Microscopic features of *Lepista sordida* (A) Basidia, (B) Cystidia, (C) Pileipellis (D) Basidiospores (E) Stipitipellis. Bars = A -E: 10 µm.

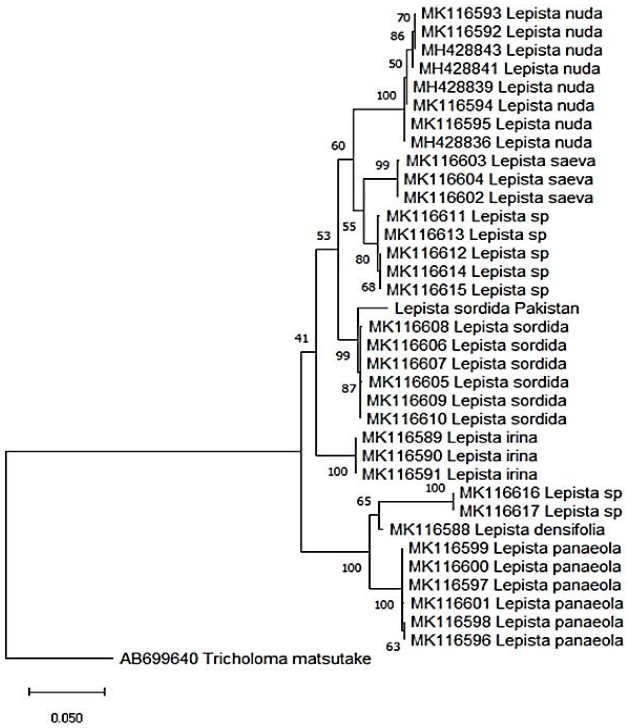

Fig. 3. Molecular genetic analysis of *Lepista sordida*.

Biosynthesis of silver nanoparticles and their characterization (Fig. 4)

Synthesis of AgNPs by using extract of *Lepista sordida* with the help of microwave assisted extraction (MEA) technique, in which extract was formed having brownish yellow color. After the addition of $AgNO_3$ salt with mushroom extract, the color of the solution was changed. This distinct change in color from brownish yellow to greyish brown showed the formation of AgNPs. These results were correlated to earlier work of Skottrup *et al*., (2007) which explained that the surface plasmon resonance in metals was linked to the characteristic brown colour that resulted from the bio-reaction of cell-free extract of fungus and Ag+ ions, demonstrating production of silver nanoparticles.

UV Visible Spectroscopy (Figs. 5-6)

A major and reliable method for the precise characterization of metal nanoparticles is UV-visible spectrophotometry. The absorbance spectra (Surface Plasmon bands) are influenced by the sizes, shapes, and dielectric constants of the nanoparticles as well. UV visible spectrophotometer was used to check the maximum absorption between the wavelength range of 200-800 nm. The UV-vis spectra of mushroom extract before adding the $AgNO_3$ salt was 340nm and after adding the $AgNO_3$ salt was 345nm.

Scanning electron microscopy (SEM) (Fig. 7)

SEM was performed to observe size and morphology of the particles produced. When AgNPs were formed using cell-free filtrate, SEM analysis showed that the size of particles ranged from 65-75 nm. Further, analysis showed that the shape of fungal silver nanoparticles was hexagonal, having a size range 65 to 75nm. These results indicated that silver nanoparticles were synthesized extracellularly. SEM micrographic studies confirmed that synthesis of AgNPs by *Lepista sordida* extract had smooth hexagonal morphology in the nano-range.

X-ray diffraction analysis (XRD) (Fig. 8)

In order to analyze crystalline materials both quantitatively and qualitatively, X-ray powder diffraction is a quick, adaptable, and non-destructive method. Using this method, it was possible to ascertain the general structure of a single crystal as well as its particle size, average bulk composition and texture. An estimation of nanoparticle's particle size might be determined using the X-ray diffraction technique. The surface plasmon resonance's blue shift indicated a reduction in particle diameter.

The peaks at 2θ values of 38.3◦, 46.4◦, 63.45◦ and 77.8◦ corresponded to lattice plane clusters (101), (200), (220) and (311) were produced during synthesis. XRD pattern showed that AgNPs had a hexagonal structure attained by the green synthesis. These results confirmed the presence and formation of silver ions (Ag^+) in the solution. With clear bands of Bragg peaks corresponding to crystalline AgNPs were stabilized by reducing agents in the process, which might be the consequence of the AgNPs. These results of the X-ray diffractogram further supported the biosynthesis of AgNPs. The current results clearly demonstrate that AgNPs produced by using this environmentally friendly process were nano-crystalline in nature.

Antifungal activity against micromycetes (fungal pathogens) (Figs. 9-11)

Silver nanoparticles were examined for antifungal activity against a number of pathogenic fungi. The fungus growth inhibition was increased for all of the observed fungi compared to the control. Two pathogenic fungi, *Aspergillus flavus* and *Alternaria alternata*, were inhibited by the biosynthesized AgNPs. These particles were considered as having effective antifungal agents which had broad-spectrum and demonstrated substantial antifungal potential, suggesting a diverse variety of medicinal uses. The growth of *Aspergillus flavus* and *Alternaria alternata* were apparently inhibited by higher concentration of AgNPs as compared to control group (no silver nanoparticles). The colonies' sizes decrease with the increase of the concentration of silver nanoparticles. A large number of inhibition effect was noted on the plates, when the concentrations of silver nanoparticles were >10 µg/ml. The AgNPs showed minimum inhibition effect on the growth of colony, when the concentration was <2 µg/ml.

Minimum inhibitory concentrations (MICs) of silver nanoparticles against isolated fungi: The Minimum inhibitory concentrations of AgNPs were estimated to 2.0-10.0 µg/ml against isolated fungi. According to the results, isolated fungi, *Aspergillus flavus*, and *Alternaria alternata* might be inhibited by mycosynthesized AgNPs, which had zones of inhibition shown in (Table 1). Recent results showed that in *A. flavus*, at the 10 µg/ml AgNPs concentration, higher zone of inhibition was 9.7± 0.5.

While, at 2 µg/ml concentration, zone of inhibition was seen to be 5.6 ± 0.11 which was decreased by the decrease AgNPs concentration. While, in the case of *A. alternata*, at the 10 µg/ml concentration of AgNPs, the zone of inhibition was 9.1 ± 0.5. At 2 µg/ml concentration, zone of inhibition measured 3.75 ± 0.04.

A. flavus was inhibited (97.3%) when exposed to silver nanoparticles at a 10µg/ml concentration and inhibitory percentage of *A. alternata* was 91.75%. With 2 µg/ml concentration of AgNPs, the minimum inhibitory effect was 37.5% against *A. alternata*. Hence, these results demonstrated that the rate of inhibition increased with the rise of AgNPs concentration (Table 2). These results of the solution were present in high density, which allowed it to saturate and attached to fungal hyphae while killing harmful pathogens.

Table 1. Diameter of growth inhibitory zones in presence of synthesized AgNPs by *Lepista sordida* extract against *Aspergillus flavus* and *Alternaria alternata* isolates.

Isolated fungi	Zone of inhibition (mm)			
	Dilutions (µg/ml)			
	2 µg/ml	6 µg/ml	8 µg/ml	10 µg/ml
	Mean ± Standard deviation			
Aspergillus flavus	$5.6^{a} \pm 0.11$	$6.87^{b} \pm 0.11$	$8.7^{a} \pm 0.5$	$9.7^{a} \pm 0.5$
Alternaria alternata	$3.75^{b} \pm 0.04$	$7.8^{a} \pm 0.12$	$8.4^{a} \pm 0.12$	$9.1^{b} \pm 0.5$

Table 2. Inhibitory rate (%) of silver nanoparticles against fungal strains. Inhibition rates were measured by five replicates of each experiment; Rate of inhibition of control=0%.

Fungal strains	Rate of inhibition (%)			
	2µg/ml	6µg/ml	8µg/ml	10µg/ml
Aspergillus flavus	56.25^{a}	68.75^{b}	87.5^{a}	97.3^{a}
Alternaria alternata	37.5^{b}	78.00^{a}	84.45^{b}	91.75^{b}

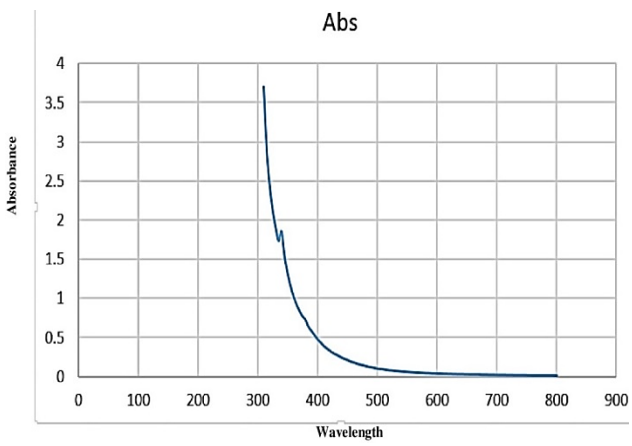

Fig. 5. The Ultraviolet-visible spectra of mushroom extract before adding the $AgNO_3$ salt.

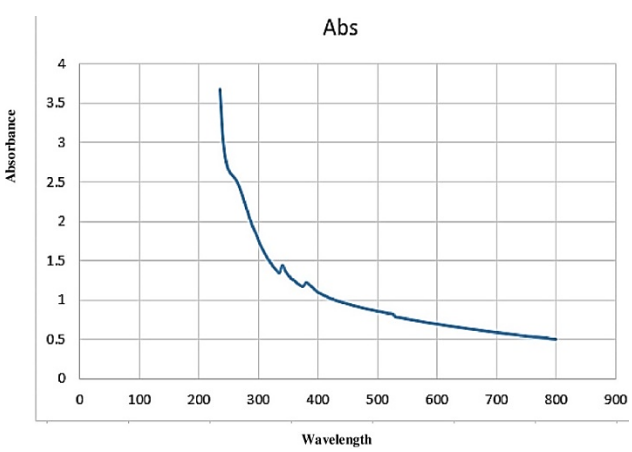

Fig. 6. UV- visible spectra of mushroom extract after adding the $AgNO_3$ salt.

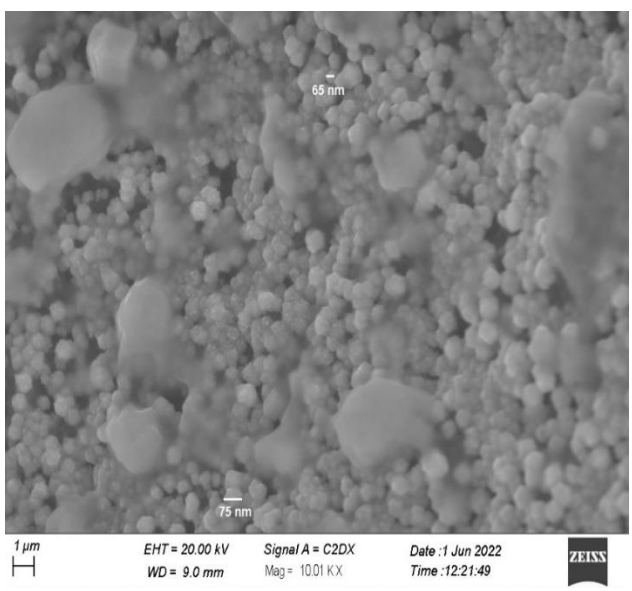

Fig. 7. SEM Micrographs of synthesized AgNPs from *Lepista sordida*.

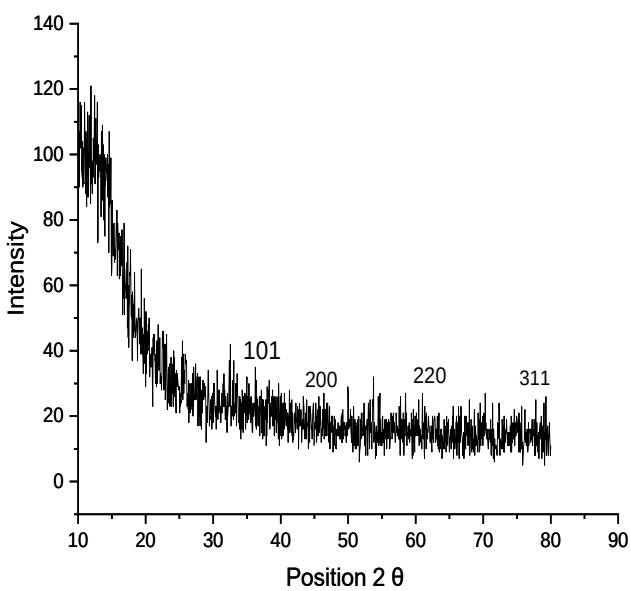

Fig. 8. XRD Analysis of synthesized AgNPs.

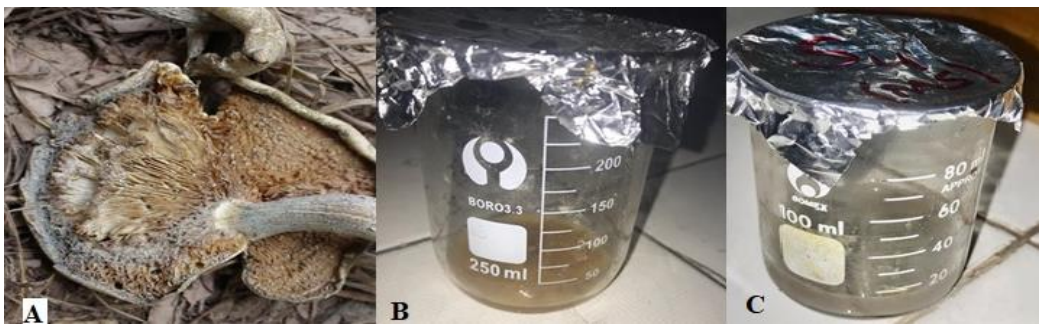

Fig.4. (A) *Lepista sordida*; (B) *Lepista sordida* extract and $AgNO_3$; (C) synthesized silver nanoparticles (AgNPs).

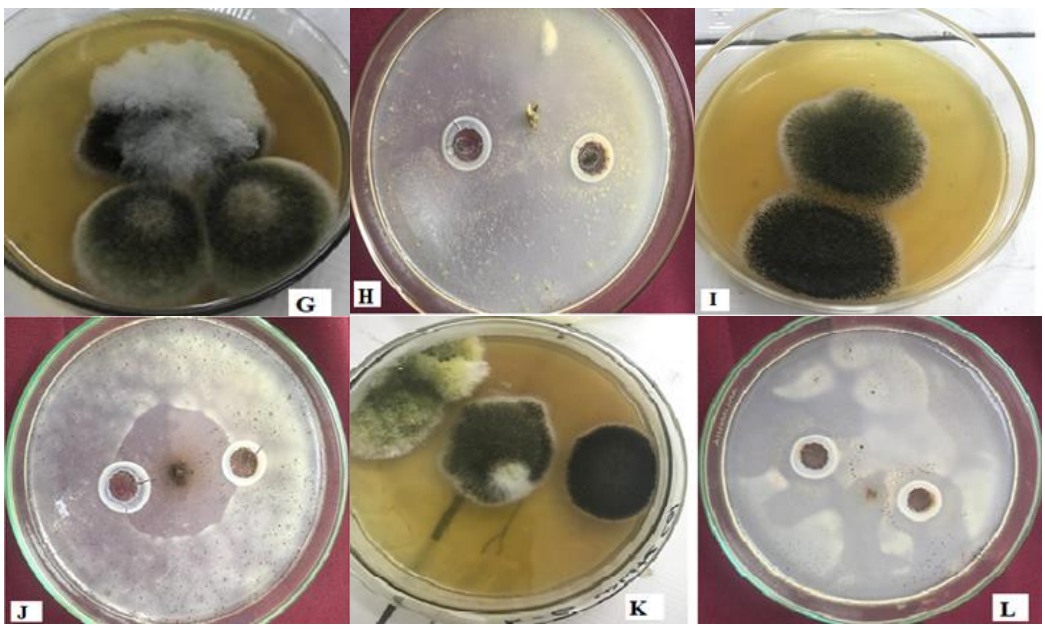

Fig. 9. Antifungal Effect of synthesized AgNPs against pathogenic fungi (A, C & E). Inhibitory effect of fungal isolates produced by biosynthesized AgNPs (B, D & F) post inoculation of five days with concentrations of 10 µg/ml, 8 µg/ml, 6 µg/ml and 2 µg/ml AgNPs respectively. Positive control of (A) *Aspergillus flavus*.

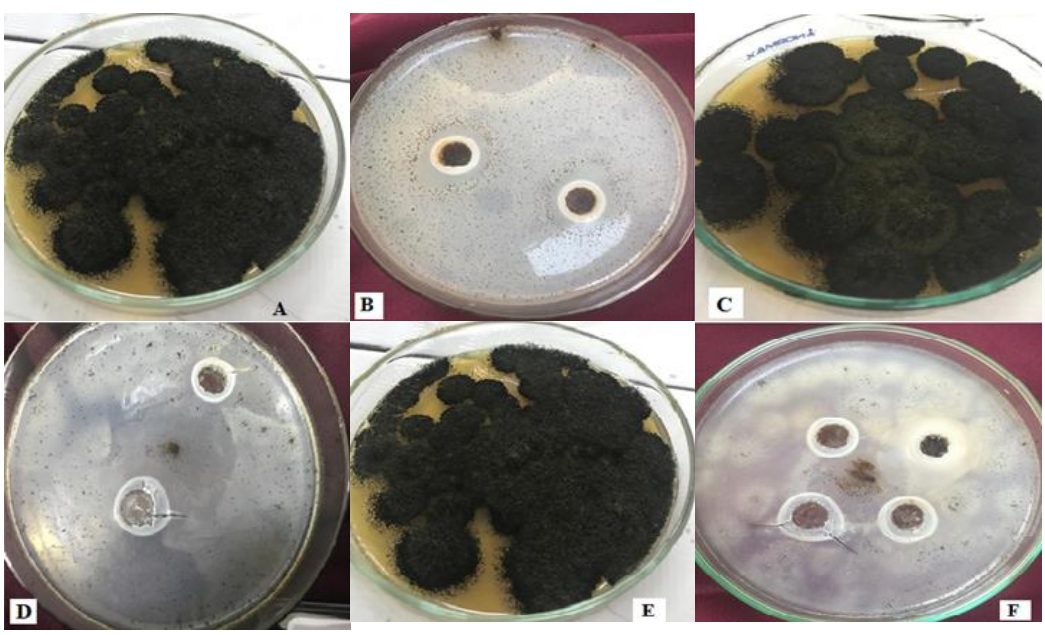

Fig. 10. Antifungal effect of synthesized AgNPs against pathogenic fungi (G, I & K). Inhibitory effect of fungal isolates produced by biosynthesized AgNPs (H, J & L) post inoculation of five days with the concentrations of 10 µg/ml, 8 µg/ml, 6 µg/ml and 2 µg/ml AgNPs respectively. Positive control of (G) *A. alternata*.

Discussion

The present research was conducted by using wild mushroom (*Lepista sordida*), its identification and antifungal activity of AgNPs synthesized by using this mushroom against micromycetes (*Aspergillus* and *Alternaria* spp.). Such type of study has been carried out for the first time in Pakistan. The selected mushroom belongs to genus *Lepista* that is well known with number of edible and medicinal species (Stott, 1998). The regular synthesis of silver nanoparticles involves biological, chemical and physical techniques that have drawbacks also (Singh *et al*., 2016). During this investigation, AgNPs were synthesized by green method using mushroom extract of *Lepista sordida* with the help of microwave assisted extraction (MEA) technique. Silver nanoparticles synthesized were characterized and compared with literature. The results were correlated to early work of Skottrup *et al*. (2007), Bawaskar *et al*. (2010), Mohanta *et al*., (2018), Moharrer *et al*., (2012) and Talie *et al*., (2020).

It has also been found in different studies that green synthesis of nanoparticles offers a simple, clean, nontoxic, and environmental-friendly approach of synthesizing nanoparticles with a wide range of morphological and physiochemical properties (Talie *et al*., 2020). The synthesis of AgNPs was confirmed by characterization of silver nanoparticles which included SEM analysis. SEM micrographic studies confirmed that synthesis of AgNPs by *Lepista sordida* extract had smooth hexagonal morphology in the nano-range. Similar techniques were also used for characterization of other biosynthesized nanoparticles by different workers (Mohanta *et al*., 2018; Talie *et al*., 2020). Synthesis of silver nanoparticles was confirmed by XRD analysis. The peaks at 2θ values of 38.3◦, 46.4◦, 63.45◦ and 77.8◦ corresponded to plane angles (101), (200), (220) and (311), produced during synthesis. These results confirmed the presence and formation of silver ions (Ag^{+}) in the solution. These results were quite similar to the earlier work of Prakash *et al*. (2013), in which the diffracted intensities were recorded from 20◦ to 80◦. The planes of four strong Bragg reflections at 38.45°, 46.35°, 64.75°, and 78.05°, respectively; corresponded to the sites of the structure of silver shown face-centered cubic crystals and could be indexed as (111), (200), (220), and (311). The average crystallite size of AgNPs was estimated 25 nm.

Furthermore, it was revealed from the results that synthesized AgNPs at different concentrations brought about significant spore germination inhibition and reduction in zone of inhibition against both tested pathogenic fungi (*Aspergillus flavus* and *Alternaria alternata*) indicating their antifungal activity. The inhibitory effect of the silver nanoparticles was higher against *A. flavus* at the higher concentrations. Similar results were reported by Talie *et al*., (2020) by using mushroom *Helvella leucopus*. In another similar study, Rajeshkumar *et al*., (2017) reported antifungal activity of synthesized AgNPs against *A. niger, A. fumigatus, A. flavus, Fusarium* sp., and *Candida albicans*. Pulit *et al*., (2013) also studied the antifungal activity of synthesized AgNPs against *A. niger* and *Cladosporium cladosporioides* and reported that these nanoparticles had potent biocidal activities even at lower concentrations as were found in the present study.

Conclusion

From the present investigation, it is concluded that AgNPs have good potential to be used as antifungal agents against many fungal pathogens. The successful synthesis of AgNPs by using *L. sordida* mushroom extract as bioreductants can be developed an imperative new green technology solution, where related mushrooms could be used actively for nanoparticles synthesis. These nanoparticles can be used in pharmaceutical industry for synthesis of medicines having antifungal actions and can also replace chemical fungicides to control plant fungal pathogens as cost effective and environmental friendly method.

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