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Green Synthesis of Silver Nanoparticle by *Ziziphus mauritiana* Leaf extract and its antifungal activity against *Aspergillus species*

Ashvanikumarchaudhari* & Shiv Kumar Dwivedi*

Affiliation: Department of Environmental Science, Babasaheb Bhimrao Ambedkar (A Central) University, Raebareli Road, Vidya Vihar, Lucknow-226025, U.P., India

***Corresponding Author's e-mail:**

Ashvanikumarchaudhari, krishnaanshu77gmail.com

Shiv Kumar Dwivedi, skdwivedibbau@gmail.com

Abstract:

In recent years, the use of biological agents, such as plant extracts, for the green synthesis of nanoparticles (NPs) has become increasingly popular. This study focuses on the bio synthesis of silver NPswith the help of leaf extract from *Ziziphus mauritiana*alog with its potential antifungal action against plant pathogenic fungi. The study investigated several parameters, including AgCl2 concentration (ranging from 1 to 100 mM), aqueous extract (100 to 900 µL), pH (4 to 10), incubation time (15 to 120 min), and temperature (30°C, 60°C, and 90°C) to determine their effect on the synthesis of AgNPs. The optimal conditions for synthesis were found to be a 100 mM concentration of AgCl2, a temperature of 30 °C, a pH of 6.0, and a duration of 120 min. The antifungal activity of the synthesized nanoparticles was evaluated against *Aspergillus terreus* and *Aspergillus niger*, and it was found to exhibit 53.5% and 65.1% inhibition rate, respectively, as measured by colony formation assay. The NPs were characterized by UV-Visible spectroscopy, which showed an absorption peak at around 460 nm. X-ray diffraction (XRD) revealed that the synthesized nanoparticles showed a crestline structure with a size extending from 10 to 100 nm, and SEM analysis demonstrated that they were nearly spherical in shape with no agglomeration.

Furthermore, the antifungal study demonstrated that these silver NPs had potent antifungal action.

Keywords: Nanoparticles: AgNPs: Characterization: Plant pathogenic fungus: Inhibition percentage

1.Introduction:

The production of metallic nanoparticles (NPs) is a thriving research field due to their breakthroughs and significant achievements in multiple areas, including engineering and biomedicine (Gaoet al.,2009,Vinci & Rapa 2019) The fascinating properties of metal NPs, such as drug delivery, imaging, bio-sensing, and catalysis, have led to successful applications in various fields(Ocsoyet al.,2018). Silver (Ag)NPs have been extensively utilized in biomedicine due to their exceptional biological characteristics, along with other types of nanoparticles(Khanet al.,2014,Chernousova&Epple 2013, Franciet al.,2015).

Nanoparticles possess novel and distinctive properties because of their small size and high surface-to-volume ratio, which makes them highly attractive for biomedical applications. Consequently, significant research efforts have been devoted to employing natural materials as safe and eco-friendly agents for synthesizing nanoparticles(Ahmadet al., 2003, Ahmadet al., 2005,Philip 2010, Thakkaret al., 2010).

Modern agriculture is confronted with significant challenges, including the attack of crops by insects and pathogens (such as fungi and bacteria), as well as the ability of these pathogens to adapt to stress conditions and develop resistance to traditional pesticides, resulting in substantial crop losses (Spagnolettiet al.,2019, Segorbe et al., 2017, Hartmann et al., 2017). The use of chemical pesticides is not only environmentally destructive, but also leads to persistent contamination of the soil and poses various health risks to humans. Therefore, innovative strategies are required for pathogen control and improving agricultural productivity. Nanoparticles have emerged as a promising approach to combat plant pathogens and enhance crop yields in the agricultural sector(Spagnolettiet al.,2019). However, the chemical synthesis of metallic nanoparticles using toxic organic solvents, reducing agents, and stabilizers is associated with numerous drawbacks at all stages (Wanget al., 2012, Wanget al., 2012).In contrast, green synthesis approaches can overcome these limitations. In the case of AgNPs, biologically active materials derived from a variety of sources, such as microbes (bacteria and fungi) and plants (plant extracts), are utilized for their synthesis, resulting in nanoparticles which work against different microorganism(Aliet al.,2017, Khanet al 2018, Chowdhury et al., 2018, Radhakrishnanet al., 2018, Huanget al., 2018).

In various studies, different types of PEs have been utilized effectively for the production of various metallic nanomaterials, including AgNPs. For example, researchers have used aqueous extracts of coffee and tea leaves to prepare polydispersed Ag NPs of spherical shape, ranging in size from 5 to 100 nm. Additionally, biocompatible Ag NPs were obtained using the fruit extract and stem bark of *Terminalia chebula* and *Callicarpa maingayi* respectively in other investigations (Nadagouda& Varma 2008, Looet al., 2012)The present research utilized leaf extract of *Ziziphus mauritiana*(ZM-LE), which is generally know as jujube, to synthesized silver NPs (ZM-AgNPs).Ziziphus plants belongs to the *Rhamnaceae* family and is being used traditionally in the area of medicine for its exceptional biological properties and nutritional value(Parmaret al., 2012). The plant grows in arid region, and there are more than 40 diverse species of *Ziziphus* found globally (Mahajan &Chopda 2009). The plant secondary metabolites such asproteins, triterpenes, alkaloids and flavonoids as well as saponinsand many vitamins are present in high quantity in leaf extract of ZM. As a result, it has excellent antioxidant, antimicrobial, antitumor, and anticancer properties (Choiet al.,2011, Gao et al., 2013)

The application of nanoparticles as antimicrobial agents is an emerging field, but their exact mechanism of action remains unknown. Nevertheless, some researchers suggest that the production of reactive oxygen species (oxidative stress) by AgNPs within the microorganism's cell is the main cause of its toxicity and antimicrobial activity (Christensenet al.,2010, Chenet al., 2014). Several studies have reported that exposure to AgNPs induces oxidative stress in microorganisms, leading to cell death. For instance, (Spagnolettiet al.,2019)demonstrated that AgNPs cause oxidative stress in E. coli by causing the formation of reactive oxygen species (ROS). Similarly, in *Phanerochaetechrysosporium*, low concentrations of AgNPs stimulate an antioxidant effect (peroxidase; POD, catalase; CAT,glutathione; GSH and superoxide dismutase; SOD), which results in oxidative stress, whereas higher concentrations of AgNPs cause apoptosis (Huang et al., 2018).In addition to rats, studies have also shown that oxidative stress mediated by ROS can occurs in various organism such as higher plants, algae and Zebrafish(Choiet al., 2010, Aroraet al., 2009, Oukarroumet al., 2012, Jianget al., 2014).

This study focuses on the synthesis of AgNPs utilizing *Z. mauritiana* leaf extract. The synthesized sample was subjected to various analytical techniques including SEM-EDS (scanning electron microscope-energy dispersive X-ray spectroscope), FTIR (Fourier transform infrared spectroscopy), and XRD for characterization. Additionally, the antifungal activity of the sample was assessed. To gain insight into the antifungal mechanism, mycelia of selected plant pathogenic fungi were exposed to AgNPs, and the resulting oxidative stress was analyzed. Significant changes in the antioxidant response were observed.

2. Experimental:

2.1. Materials:

Silver Chloride (AgCl2) and the phytochemical screening reagents were purchased from Sigma Aldrich. The plastic wares used were obtained from Tarsons Products Pvt. Ltd., India. All solutions were prepared using sterile Milli-Q water.

2.2. Collection and Preparation of *Z. mauritiana* Leaves Extract:

Z. mauritiana (Jujube) fresh leaves were collected from the BBAU campus, Lucknow, Uttar Pradesh, India. After being washed twice with water, they were dried for 3 days and then ground into a fine powder using an electric blender. The aqueous extract of the leaves was prepared by mixing 10 g of leaf powder with 100 mL of Milli-Q (100 mg/mL) in a 300 mL conical flask. The mixture was stirred and heated to 70-90 ◦C for an hour. The mixture was then allowed to cool to room temperature. The leaf extract was obtained by centrifuging the resultant mixture at 6500 rpm for 20 minutes. The residual powder was collected by simple decantation and stored in a refrigerator at 4°C for later use. The resulting powder is known as leaf extract.

2.3. Leaf Extract Based Synthesis of Silver (Ag) Nanoparticles

In this experiment, a 10 mL aqueous solution of ZM-LE (100 mg/mL) was added to 90 mL of water containing a 0.1M AgCl2 solution. The resulting mixture was then stirred and heated to 95°C for 30 minutes. After this, the mixture was incubated at 85°C for 15 minutes. The solution was then centrifuged at 10,000 rpm for 10 minutes to obtain the precipitate (AgNPs), which was washed twice with double-distilled water and centrifuged again at 10,000 rpm for 10 minutes. The resulting mass was collected and dried at 30-43°C in a hot air oven. The dried AgNPs were then scraped and stored.

To optimize the reaction conditions, various reactions were carried out by varying parameters such as time, temperature, and precursor concentration. For example, to optimize the reaction time, the incubation time was varied between 20 to 120 minutes while keeping other conditions constant. Similarly, for temperature optimization, the incubation temperature was varied between 25 to 95°C. Additionally, reactions with different concentrations of $AgCl_2$ and leaf extract were carried out to investigate their effect on the quality of AgNPs produced. The concentration of $AgCl_2$ ranged between 0.25 to 0.1 mM, while the concentration of leaf extract ranged from 1 to 10 ml.

2.4 Isolation of fungus:

The cultures of *Aspegillusterreus* and *Aspergillus niger* was collected from the laboratory(Singh & Dwivedi.(2020,2022))

2.5 Fungi and their molecular characterization:

The molecular characterization of both test fungus was done by(Singh & Dwivedi(2020,2022))

2.6 Characterization of Synthesized AgNPs:

2.6.1 Electron Microscopy Analysis:

The JEOL JSM-6490LV model was utilized for electron microscopy to analyzeAgNPs, which were coupled with an Energy Dispersive X-ray Spectrophotometer (EDS). The AgNPs were dried in an oven at 40°C for 10 hours, mounted on aluminum studs using carbon tape, and coated with platinum before analysis to ascertain their shape. To determine the size dimension of the AgNPs, a copper grid was used for FE-SEM analysis using a Scanning Electron Microscope (JEOL)

2.6.2 XRD Analysis

X-ray diffraction was used to analyze the crystalline structure of synthesized AgNPs which were dried in an oven at 40°C for 10 hours. The XRD was performed via a Bruker D8 Advance Eco model from Germany.

2.6.3 FTIR Spectroscopy:

Fourier transform infrared spectroscopy (FTIR) was employed in the USA to examine the IR spectrum of silver nanoparticles. The sample was prepared for FTIR analysis by blending it with solid KBr (potassium bromide) and pressing the mixture with a hydraulic press to form thin, see-through pellets. The IR absorbance of the sample was measured across a wavelength range of 4000 to 400 cm-1, as stated in references (Babu& Gunasekaran 2009).

2.7 Antifungal Activity Against Plant Pathogenic Fungi

Antifungal Assay

To determine the antifungal activity of AgNPs, the plate dilution method was employed. Different concentrations of AgNPs (0.25, 0.50, 1, 2, 4 mg/L) were introduced into PDA medium and poured into 90 mm diameter petri plates. The AgNPs-modified plates were then inoculated with a 0.5 cm diameter of 7^{th}-day-old culture of *Aspergillus niger* and *Aspergillus terrius* GPB and incubated at 28 ± 1°C for six days. Colony diameter was measured at regular intervals to track growth in the plates. The inhibition percentage was measured by following formula:

$$IP = \frac{A - a}{A} \times 100$$

where **Ip:** inhibition percentage, **A:** radial growth of fungal colony on without AgNPs amended PDA plate, **a:** radial growth of fungal colony on AgNPs amended PDA plate.

2.8 Analysis of Lipid Peroxidation and Antioxidant Response in Fungi Exposed to AgNPs

To evaluate the degree of lipid peroxidation, *Aspergillus niger* and *Aspergillus terreus* mycelial biomass was collected after six days of growth in PDB medium containing different concentrations of

AgNPs (0, 0.25, and 1 mg/L). The extent of lipid peroxidation was determined by measuring the levels of malondialdehyde (MDA), a known marker for lipid peroxidation, using the method described by(Zhanget al.,2007). In addition, we assessed the antioxidant response by analyzing the activity of catalase (CAT) (Xuet al.,2010) and peroxidase (POD)(Zhuet al., 2004) in both fungi grown under different concentrations of AgNPs.

2.9 Data Analysis:

The evolutionary analysis of isolate GPB was carried out using MEGA X(Kumar et al., 2018) The obtained data were subjected to analysis of variance (ANOVA) using SPSS software version 20.0. Post hoc analysis was performed using the Duncan Multiple Range Test (DMRT) with a significance level set at $P \leq 0.05$ to compare the means of the data.

Results and Discussion

3.1 Molecular identification of selected fungus:

Molecular identification of both the test fungus was done by (Singh & Dwivedi(2020,2022))

3.2 Characterization of biosynthesized AgNPs UV–Visible Spectroscopic Analysis

At the beginning of the AgNP synthesis experiment, no color difference was seen in the solution incubated in the darkened condition even after 2 hours, while the solution which was incubated in the presence of sunlight turned dark brown, indicating the synthesis of Ag nanoparticles. To confirm the synthesis of AgNPs, the difference in color from transparent to dark brown in the sunlight-incubated sample was scanned with the help of UV-Visible spectrophotometer in the spectral band of 300-700 nm. A sharp peak at 460 nm was noticed(Fig.1), which is indicative of the synthesis of AgNPs because of the Surface Plasmon Resonance (SPR) phenomenon in the NPs(Verma et al., 2016,Kumar et al., 2016). It should be noted that the color change caused by the SPR phenomenon is dependent on the size, concentration, and reducing material of the nanoparticles (Kumar et al., 2016, Lee et al., 2016).

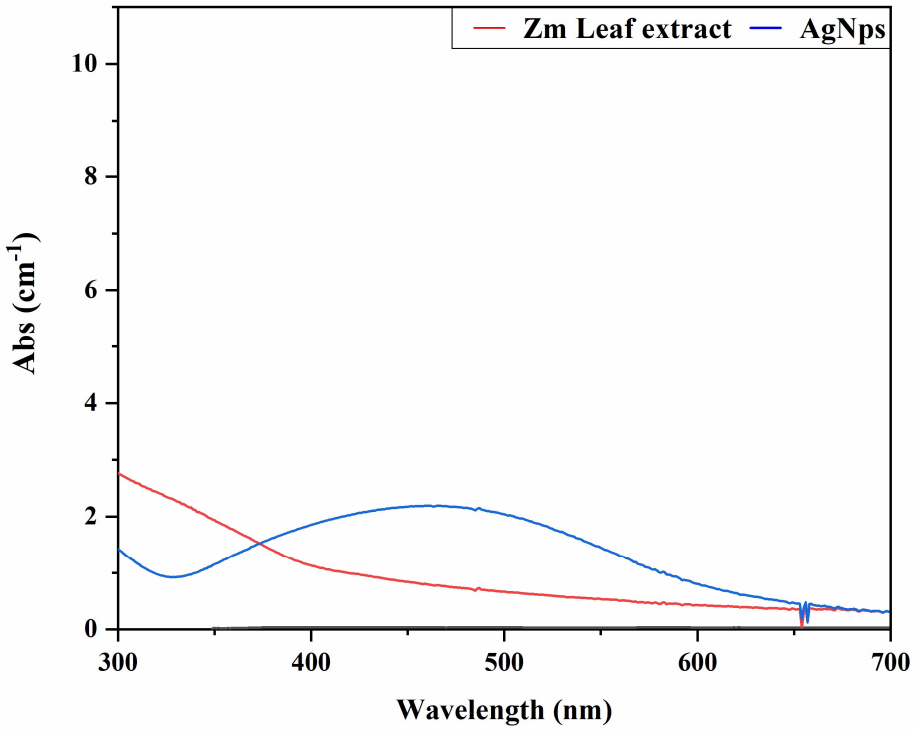
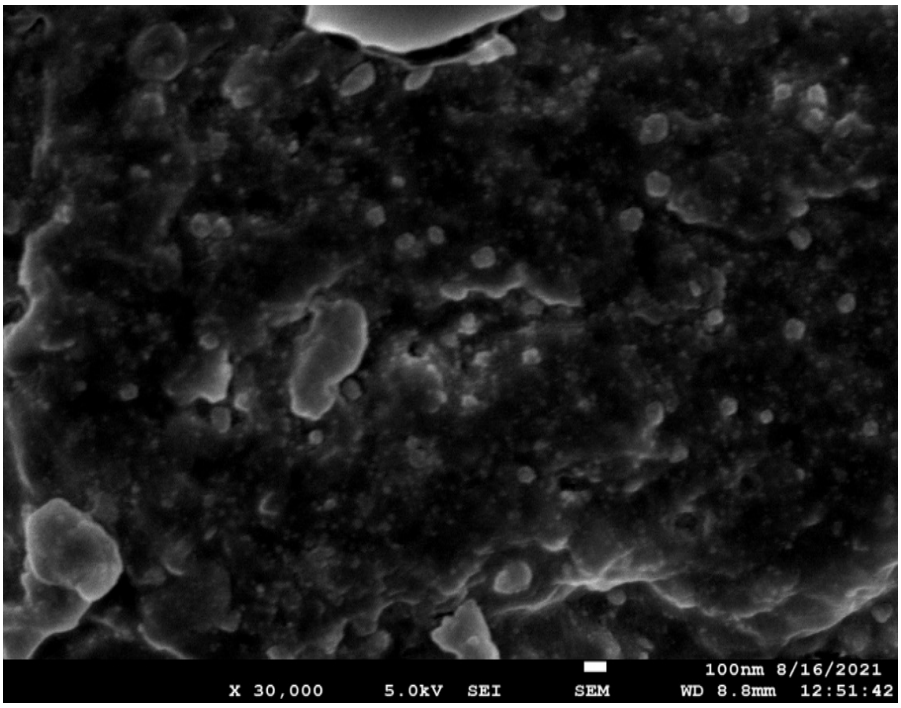
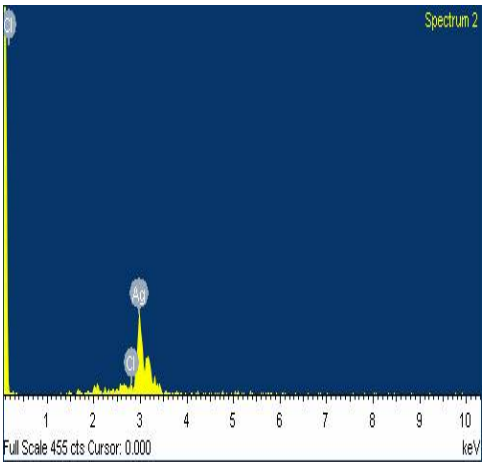


Fig.1 Detection of biosynthesized AgNPs using extract of ZM leaf extract by UV–visible spectrophotometer

3.3 SEM/EDX Analysis of Silver Nanoparticles

The shape and size of AgNPs were determined using SEM analysis. The images displayed both individual AgNPs and aggregates, with particle sizes ranging from 67-120nm. The dominant size was 120nm for individual cubical particles (refer to Fig. 2). The AgNPs had a spherical and cubical shape with an uneven surface, and the images of SEM displayed that they were stable and in close contact with each other. Some aggregated nanoparticles were also present with slightly larger, uneven structures and their morphology was not so distinct. The analysis of EDX indicated a significant rise in peak near 3.0 keV, corresponding to Ag's binding energies (refer to Fig. 2), suggesting that the high-purity Ag were present in synthesized nanoparticles. Chlorine was detected too in the EDX analysis. The weight % of Ag was determined to be 98.20, confirming the existence of AgNPs in the synthesized sample of NPs. Similar findings have been reported for synthesized silver nanoparticles from various sources, further supporting the presence of Ag (Francis et al., 2017,Dhand et al., 2016, Mohammed et al., 2018, Bocate et al., 2019).





Element	Weight%	Atomic%
Cl K	1.80	5.27
Ag L	98.20	94.73
Totals	100.00	

Fig. 2(a) SEM analysis of synthesized AgNPs, (b) EDX Spectrum of silver nanoparticles

3.4 FTIR Analysis:

The presences of functional groups on the synthesized nanoparticle of Ag were analyzed by FTIR. The results are revealed in Figure 3 and in table 1the corresponding functional groups are mentioned. The observed peak at 3387.0 cm-1 is indicative of the stretching vibrations of N-H and O-H. The

prominent peak observed at 1610.0 cm-1 corresponds to the extending vibrations of C=O(Kumar et al., 2019, Neethu et al., 2018). The peak at 1382.6 cm-1 represents the stretching vibrations of –COOH, while the band of absorption at 1036.2 cm-1 indicates the stretching vibrations of the phosphate group. The bending vibrations of SO2 are represented by the absorption band at 635.8 cm-1. The presence of functional groups likes C=O, N-H and COOH may have played a significant part in the bio-reduction of Ag+ to AgNPs (Saravanakumar et al., 2017).Previous studies have suggested that carbonyl groups and amino acids in peptide bonds of proteins may bind strongly to metals and form a coating on the synthesized metallic nanoparticles (Basavaraja et al., 2008).

S.no.	Peak(cm^{-1})	Assignment
1.	3387.0	–OHand–NHstretching
2.	1610.0	C=Ostretching
3.	1382.6	COOHstretching
4.	1036.2	Phosphatestretching
6.	635.8	SO_2stretchinginsulphones

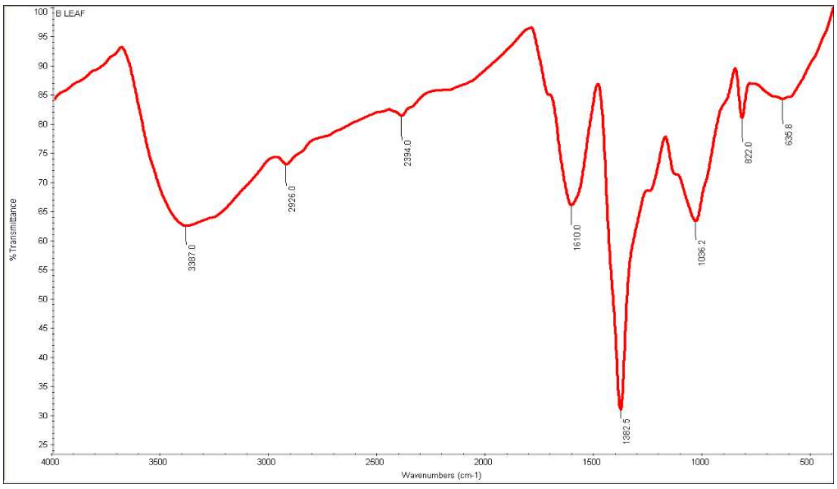


Fig. 3 Detection of biosynthesized AgNPs using extract of ZM leaf by FTIR spectra.

3.5 XRD Analysis

XRD was utilized in order to confirm the crystallinity of AgNPs obtained via ZM-LE as displayed in Figure (4). The prepared ZM-AgNPs had a cubic lattice, the presence of 5 distinct peaks in the pattern of XRD provides evidence like, 27.77° (111), 32.29° (200), 37.10° (111), 43.44° (200), 46.15° (220), 50.10° (311), and 57.44° (222) as well as some major peaks at 2h position 27.77°, 32.29° and 35.10° denoting the different plane (111), (200), (111) respectively (Wojnicki et al.,2019, Yang et al., 2016,Rajaram et al., 2015). These results showed that AgNPs are crystalline in nature. Some recent

studies found similar results and reached the same conclusion(Wojnicki et al.,2019, Yang et al., 2016, Anandalakshmi et al., 2016).

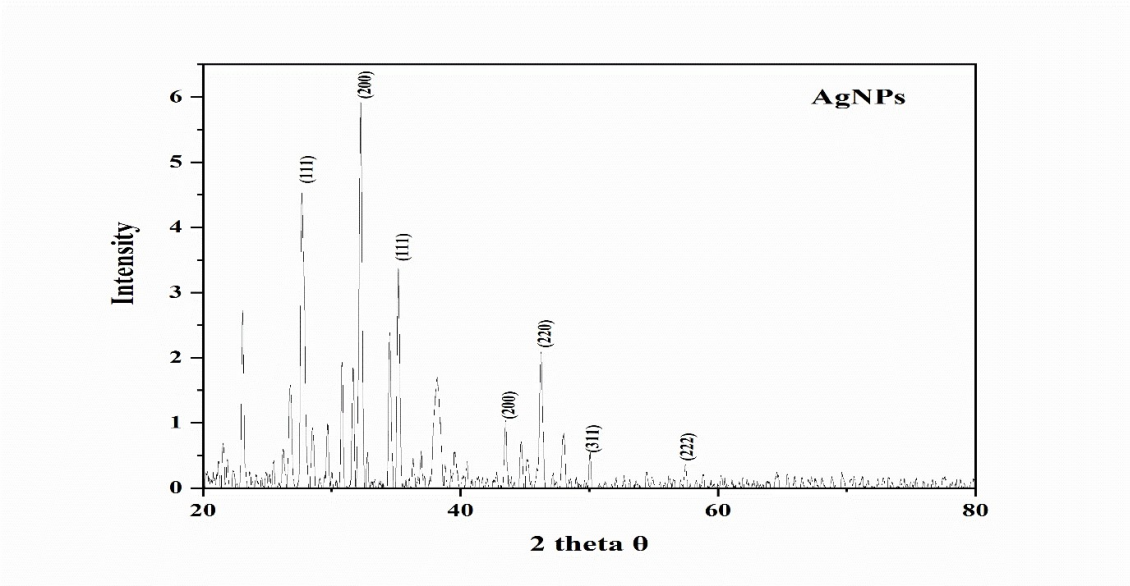
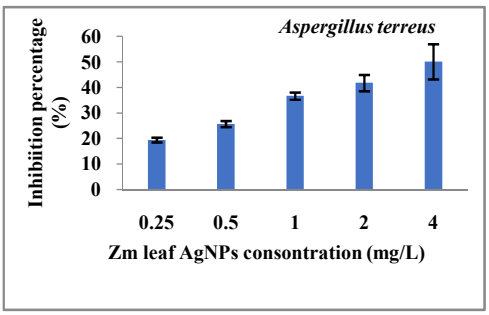


Fig. 4 Detection of biosynthesized AgNPs using extract of ZM leaf by XRD analysis.

3.6 Antimicrobial Properties:

The antifungal assay was conducted using various concentrations of ZM-AgNPs (0.25, 0.50, 1, 2, 4 mg/L) against *A. terreus* and *A. niger* fungal strains, and the resulting inhibition zones are shown in Fig. (4). The biosynthesized AgNPs at a dilution of 4 mg/L showed the highest zone of inhibition against both *A. terreus* (53.5%) and *A. niger* (65.1%) compared to other dilutions. The decrease in ZM-AgNPs concentration lead to the decreased in the zone of inhibition. However, the observed findings (Fig. 5) were lower than the control value (100%) (Fig.5(a) and 5(b)). These findings suggest that ZM-AgNPs exhibit selective antimicrobial activity against pathogenic fungal species.



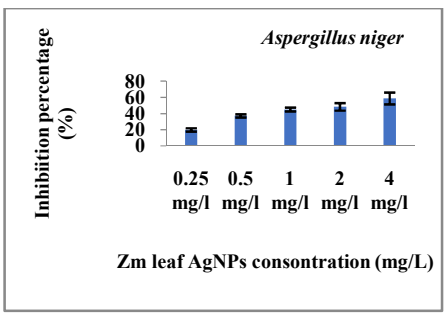


Fig.5 Inhibition percentage of (**a)***Aspergillus terreus* and (**b)***Aspergillus niger* at different concentration of biosynthesized AgNPs (mean ± standard deviation of three replicates followed by the same letter are not significantly different at the level of p <0.05DMRT)

3.7 Lipid Peroxidation:

In previous studies, it was observed that exposure to AgNPs increased lipid peroxidation in green algae, higher plants, and zebra fish (Choi et al., 2010, Oukarroum et al.,2012, Jiang et al., 2014). The current study found that the accumulation of melondialdehyde (MDA) in *A. niger* and *A. terreus* significantly increased as the concentration of AgNPs increased from 0 to 0.25 and 1 mg/L (Table 2). MDA accumulation in *A. niger* was consistently more than in *A. terreus* across all treatments, which may have contributed to the higher inhibition at less AgNPs concentrations and the severe inhibition of both fungi when the concentration is increased gradually. Similar findings were reported in *Phanerochaetechrysosporium,* where rise in MDA accumulation was observed with increase AgNPs concentration(Huang et al., 2018). It has been reported that ROS production in the presence of AgNPs inhibit *Candida albicans*, *Alternaria brassicicola*, and *Fusarium oxysporum*(Radhakrishnan et al., 2018, Kumari et al., 2019). In microbes and plants, under environmental stress condition like heavy metals, drought and osmotic stress, MDA i.e the end product of Lipid peroxidation is produced by ROS(Huang et al., 2018, Chen et al., 2014,Choi et al., 2010,Jiang et al., 2014,Xu et al.,2012) .Therefore, it can be concluded that the major inhibition in the growth of *A. niger*GPB and *A. terreus* is due to ROS are produced in the presence of AgNPs.

3.8Antioxidant Response:

The table (Table 2) illustrates the effect of varying concentrations of AgNPs on the activity of CAT in *A. niger* and *A. terreus*. When exposed to AgNPs, the CAT activity in both fungi increased significantly. In *A. niger*, the CAT activity increased from 0.015 ± to 0.0177 ± 3.51 lM H2O2/min/mg protein with the rise in concentration of AgNPsranging from 0-0.25 and to 1 mg/L. Similarly, in *A. terreus*, the CAT activity increased from 0.0114 ± 2.0 to 0.0161 ± 3.51 l M H2O2/min/mg of protein with the rise in concentration of AgNPs from 0-0.25 and to 1 mg/L.

A study conducted by (Huang et al., 2018) on the impact of AgNPs on *Phanerochaetechrysosporium* showed an increment in CAT activity in the presence of AgNPs. It has been reported that CAT plays a vital function in scavenging H2O2, that is produced under stressful conditions(Kumar & Dwivedi 2019,Prasad et al ., 2018, Xu et al., 2012, Kumar et al., 2019, Kumar & Dwivedi 2019The presence of AgNPs has been shown to produce H2O2 and ROS in both the pathogenic fungi, supporting the hypothesis that AgNPs induce oxidative stress and death of cell. However, at lower concentrations of AgNPs, both *A. niger* and *A. terreus* showed a strong antioxidant response. Higher concentrations of AgNPs may result in the induction of high amounts of H2O2 and ROS, which may be greater than the antioxidative potential of the fungi, resulting in major inhibition of fungal growth.

The variation in POD activity is connected to metabolizable constituents' degradation in microscopic organism and also their growth(Serra-Wittling 1995). During this research, the trend in POD action was identical to that of CAT action in fungi exposed to AgNPs. At 1 mg/L of AgNPs, POD activity in *A. niger* increased considerable to 0.065U mg-1 protein, accompanied by 0.0012 U mg-1 protein in *A. terreus* (Table 2). An identcal variation in POD was observed in *P. chrysosporium* after AgNPscontact(Huang et al., 2018). Antioxidant responses such as changes in SOD,POD, CAT, and GSH after nanoparticle exposure have also been described in various microbes and plants(Huang et al., 2018, Spagnoletti et al., 2019, Gupta et al., 2018,Kanaujiya et al., 2020, Kumar& Dwivedi 2019).

The impact of AgNPs on lipid peroxidation, as well as the activity of CAT and POD enzymes in *A. niger* and *A. terreus,* was assessed. The obtained results are expressed as the mean of three replicates ± standard deviation. Any two groups denoted by similar letter indicate no significant change at the level of $p<0.05$ (determined using DMRT).

AgNPsconcentration(mg/L)	LipidPeroxidation(MDA)(mU/mgofprot)	Catalase(1MH_2O_2/min/mgprot)	Peroxidase(U/mgprot)
Aspergillus niger			
0	3.83±0.35[a]	0.015±2.64[a]	0.0161±0.15[a]
0.25	25.23±0.90[b]	0.0154 ±3.60[b]	0.0153±0.32[b]
1	32.2±0.95[c]	0.0177 ±3.51[c]	0.065±0.30[c]
Aspergillus terreus			
0	1.53±0.20[a]	0.0114±2.0[a]	0.0011±0.15[a]
0.25	13.10±0.52[b]	0.0133±3.21[b]	0.0007±0.15[b]
1	18.67±1.01[c]	0.0161±3.51[c]	0.0012±0.35[c]

4.Conclusion

Biosynthesized AgNPs produced from the leaf extract of *Z. mauritiana* have a high potential for inhibiting the growth of *Aspergillus niger* and *Aspergillus terreus*, which are plant pathogenic fungi. This is due to their crystalline nature, size range of 65-130 nm, and high functional group availability that leads to the production of ROS. Various techniques such as UV-Vis spectroscopy, FTIR, XRD, SEM, and FE-SEM analysis were used to characterize the green synthesized ZM-AgNPs, revealing spherical shaped AgNPs with an average size of 65-129 nm.Additionally, biosynthesized ZM-AgNPs exhibited efficient antifungal activity against *A. terreus* and *A. niger* in a short time, as demonstrated by the inhibition zone and minimum concentration of inhibition. As a result, these AgNPs can be used on a large scale in an eco-friendly and cost-effective manner to control the aforementioned test fungal species.

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