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Green synthesis of silver Nanoparticles using Azadirachta indica Leaf Extract and studying their activity against Alternaria alternata Associated with Potato Early Blight

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I. Abstract

Background: Alternaria filamentous fungi are worldwide. Most Alternaria species are widespread in both natural and human-dominated habitats. Alternaria was the most prevalent plant pathogen in a global assessment of soilborne fungi**. Aims of the study:** The goal of this study is to assess Alternaria alternata antifungal activity of Azadirachta indica ethanolic leaf extract and silver nanoparticle activity. It was also the goal of the study to isolate the active phytochemical components of the extract and find out what concentrations of the extract were necessary to prevent fungal growth. **Methods:** Alternaria alternata, a phytopathogenic fungus, was used to assess the antifungal properties of neem (Azadirachta indica). Three plant extract dosages (25, 50, and 100 mg/ml) were tested on A. alternata by quantifying the fungal mycelial growth inhibition zone using the disc-diffusion method on potato dextrose agar (PDA). Plant extracts' minimum fungicidal concentration (MFC) and minimum inhibitory concentration (MIC) were ascertained. The extract was used for silver nanoparticle preparation with concentrations of 6, 12 and 18 mg/ml with distilled water as a negative control, which then was used to study the inhibitory effect on plant pathogenic fungi. The AgNPs were characterised by UV-spectrum analysis. **Results:** Plant extracts had an inhibitory impact on A. alternata that varied between 37±0.8 and 52±1.2 and 100 mm. Major chemicals that are biologically active, including alkaloids, flavonoids, saponins, tannins, and terpenoids, are present in neem plant extracts. Plant extracts had MICs and MFCs of 17–36, respectively. The high inhibitory zone of silver nanoparticles (72±1.2 mm) on A. alternata was shown in a of 18 mg/ml, followed by a concentration of 12 mg/ml with an inhibition zone (63±1.1 mm). While the smallest inhibition zone (50±1 mm) was shown in 6 mg/ml of concentration. **Conclusion:** This research demonstrated that the beneficial phytochemicals found in neem extract (Azadirachta indica)—including flavonoids, alkaloids, saponins, tannins, and terpenes—were critical in preventing the pathogenic fungus Alternaria alternata from growing. Confirming the high efficacy of neem extract as a natural antifungal agent, the inhibitory impact increased with increasing extract concentration. Also, silver nanoparticles enhanced the crude neem extract's effectiveness against plant pathogenic fungi.

Keywords : ***Azadirachta indica, flavonoids, Alternaria alternata, mycelial growth***

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II. Introduction

The potato (Solanum tuberosum) ranks as the fourth most significant food crop globally by production volume, following maize, wheat, and rice. It is primarily a crop of temperate climes, however it is cultivated in approximately 100 nations under temperate, subtropical, and tropical environments. Asia and Europe are the predominant potato-producing regions globally, contributing over 80% of total production (1). The potato is an important crop for world food security because of its high productivity and economic importance, but it is also susceptible to a broad variety of plant diseases that can reduce output and quality (2).

In agriculture, crop loss attributable to plant infections has emerged as a significant issue, with Alternaria spp. being one such pathogen. Alternaria is a soil-dwelling, airborne pathogen responsible for early blight, a significant chronic foliar disease mostly affecting the Solanaceae family, including tomatoes (Lycopersicon esculentum) and potatoes (Solanum tuberosum) (3).

Alternaria spp. was filamentous fungus which distributed worldwide. most Alternaria species are widespread in both natural and human-dominated habitats. Alternaria was the most prevalent plant pathogen in a global assessment of soilborne fungus (4). A field warming experiment indicates that the genus Alternaria becomes more abundant and important under climate change scenarios. Alternaria generally live as saprophytes in soil and rotting plants (5). Alternaria species, notably A. alternata, can cause allergies or pathogenicity in immunocompromised patients. These necrotrophic infections on plants produce economically significant crop diseases and post-harvest rots. Alternaria can exist as an endophyte in plants without producing illness (6).

Cultural methods play a crucial role in disease control. These activities include using resistant cultivars and foliar fungicides, rotating crops, tilling the soil, removing and burning diseased plant waste, and eliminating weed hosts to lower the inoculum level for future plantings. In order to combat early blight, foliar fungicides are applied most often and are quite effective (7).

Maneb, mancozeb, chlorothalonil, and triphenyl tin hydroxide are some of the protective fungicides that are recommended for late blight control. They also work well against early blight. Nevertheless, there are risks associated with pesticide treatment. Chemicals pollute the environment, endanger human health, and endanger all forms of life when they enter the food chain (8). To control harmful fungi, botanical byproducts including flavonoids, tannins, and phenols offer an eco-friendly alternative to commercial fungicides (9).

Neem (Azadirachta indica) has attracted conserible attention of modern medicine due to its widespread usage in Ayurvedic, Unani, and homoeopathic therapy. In addition to their usage as insecticides in agriculture, extracts from seeds and leaves have anti-inflammatory, anti-hyperglycemic, anti-ulcer, anti-malarial, antifungal, antibacterial, antiviral, antioxidant, ant mutagenic, and ant carcinogenic qualities (10). Neem extracts have potential fungicidal qualities and considerably decreased conidial germination in a number of fungi(11). The goal of this study is to assess Neem's (Azadarachta indica) antifungal qualities against Alternaria alternata, a damaging plant disease that causes wilt in potatoes.

Due to global environmental issues, ‘green’ chemistry and chemical technologies are becoming more popular and necessary. Silver is one of the most commercialised nanomaterials, producing 500 tonnes of nanoparticles per year and expected to rise shortly. In addition to its importance in high-sensitivity biomolecular detection, catalysis,

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biosensors, and medicine, it has strong inhibitory, bactericidal, anti-fungal, anti-inflammatory, and anti-angiogenesis properties(12).

There has been scant research into the antifungal effects of neem leaf extracts on Alternaria alternata, a fungal pathogen identified from potato plants grown in the area, despite the growing interest in plant-based antifungal medicines. Consequently, the objective of this work was to assess Alternaria alternata antifungal activity of Azadirachta indica ethanolic leaf extract and silver nanoparticles activity. It was also the goal of the study to isolate the active phytochemical components of the extract and find out what concentrations of the extract were necessary to prevent fungal growth.

III. Methods

2.1Isolation of Fungi

Isolation was given in spring 2025 by infected potato crops. We collected samples of potatoes from the affected fields. They were placed in sterile plastic bags before being sent to the lab. We collected the crown and root sections after rinsing the samples under running water for 30 minutes to eliminate dirt. cut into pieces that are four to five millimeters in size. These components underwent a three-minute surface sterilization with 1% NaClO before being rinsed with sterile DW to eliminate any surplus. After the components had dried between the sterile folds of filter paper, they were evenly spread onto 9 cm diameter Petri plates with dextrose PDA (HiMedia Laboratories Pvt. Ltd., Mumbai, India) and a sterile potato (autoclaved at 1.5 kg/cm2 pressure and 121C for 20 minutes). The pressure decreased following the end of the sterilization process. After allowing the flasks to cool, 250 milliliters of Amoxicillin (0.5 mg/ml) was added per liter to stop the growth of bacteria. After five days of incubation at 25 ° C, the plates were prepared for pathogenicity testing by purification. Using the incomplete fungal key, the researchers in this study were able to isolate and identify Alternaria alternata by looking at their colonies, mycelium, and conidia (13).

2.2 Collection of medicinal plant

Then Azadirachta indica leaves were harvested. After vigorous tap water washing, 1% sodium hypochlorite solution and distilled water surface sterilized the plant material. The plants were dried at 40 °C in an electric oven and ground into powder. Continuous Soxhlet extraction of 200 g of powder in 1 Liter of 70% ethanol at 50°C for 48 hours. Rotary evaporators filtered and concentrated ethanol extract at 40°C under reduced pressure. The residue (active principles) was stored at -4 until use.

2.3 Determination of chemical constituents of the ethanolic plant extracts

The chemical makeup of the plant extract was ascertained by means of Gas Chromatography-Mass Spectroscopy (GC-MS) analysis, specifically AGLENT GC-MS and the NIST Library (14).

2.4antifungal activity of plant extracts

According to (15), disc diffusion experiment was used to assess plant ethanolic extract antifungal activity. Alternaria alternata were grown in PDA-medium Petri plates. On agar that had already been inoculated with the fungus being tested, paper discs (6 mm in diameter) were covered with 20 µl of plant extract (25, 50, and 100 mg/ml). A distilled water disc that had been sterilized was used as a control. Plates were kept at 28°C for 5 days. Measurement of disc

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inhibition zone diameter in millimeters determined antifungal activity. Three replications of inhibitory zone measurements were used to calculate averages.

2.5Determination of MIC and MFC of plant extract

A broth dilution approach was also used to assess the minimum fungicidal concentration (MFC) and minimum inhibitory concentration (MIC) (16). Potato dextrose broth (PDB) was used for all antifungal tests. Plant extracts in serial dilutions ranging from 1 to 60 mg/ml were employed. One milliliter of the tested fungus' spore suspension was added to the tubes, which were then incubated for seventy-two hours at 28°C. Then, using a UV spectrophotometer (Model AE-450, 2003, Japan), the MICs and MFCs were calculated based on the change in optical density after conidial germination, which occurred 72 hours after the inoculation. Turbidity and a "pellet" on the tube bottom were signs of fungal growth. The concentration at which 99% or more of the original inoculum was killed was known as the MFC, whereas the MIC value was defined as the lowest concentration of plant extract that prevented the pathogenic fungus from growing visibly. Every assay was made three times.

-The experiments were carried out in duplicate. The negative control was sterile distilled water. The inhibition zone was evaluated by millimetre and expressed as the mean with standard deviation.

2.6silver nanoparticles synthesis

silver nanoparticles (AgNPs) **Synthesis** was conducted by combining 250 ml of deionized water with 2.00 g of silver nitrate ($AgNO_3$) and heating using a hot plate with magnetic stirring at 40°C. Subsequently, 10 ml of the peel extract was incrementally introduced into the silver ion solution. The hue of the combination was subsequently modified to yellow. Following four hrs. of agitation at ambient temp., the mixture exhibits a transformation in color to a deep red or brown, indicating the formation of colloidal silver nanoparticles. The colloid samples, stored in dark bottles, exhibited color variations over five days, with the AgNPs generated by the mixing processes and the active reduction of Ag^+ displaying either a brown or deep red hue(17).

2.7Characterization of silver nanoparticles

A- UV- Visible spectroscopy

The sample was produced for the absorption spectroscopy analysis of Ag-NPs and conjugated materials separately. 2 ml of each solution was separately extracted following filtration through sterile filter paper and thorough agitation to ensure homogeneity. It was analyzed using a Visible Spectroscopy UV instrument, calibrated with sterile deionized water prior to measurement. Vis-UV spectroscopy is regarded as a significant method for identifying nanostructures, as the analysis is conducted within a wavelength range of 200-800 nm (18).

2. 8Inhibitory effect of biosynthesized silver nanoparticles against A. alternata

In vitro tests assessed biosynthesized silver nanoparticles' antifungal efficacy against A. alternata. Individually, 600, 1200, and 1800 mg of pre-prepared dry plant extract were dissolved in 10 mL of sterile distilled water. To synthesize silver nanoparticles, 90 mL of a 1 mmol silver nitrate ($AgNO_3$) solution was combined with these solutions in a sterile glass flask in a 10:1 ratio. After that, 2 mL of each concentration was applied to Petri dishes containing 18 mL of PDA medium to generate final concentrations of 6, 12, and 18 mg/mL, respectively, and a negative control treatment (sterile distilled water only). Post-solidification, plates were infected with a 0.5 cm disc from 7-day-old active cultures and incubated at 36 ± 1 °C.

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2.9 Statistical analysis

We used one-way analysis of variance to do the statistical analysis on the data. In order to differentiate between the means and standard deviations, the statistical analysis program Biostat 2008 was used in conjunction with Tukey's honest significant difference test.

3.Results and discussion

3.1Morphological and microscopic diagnosis of Alternaria alternata

Colonies of this fungus appeared growing on SDA agar on the third day of incubation. They were initially white, then changed color to dark green and finally to black. The opposite side was black. The A. alternata colonies were characterized by the presence of large conidia with multiple subdivisions, as shown in Figure (1).

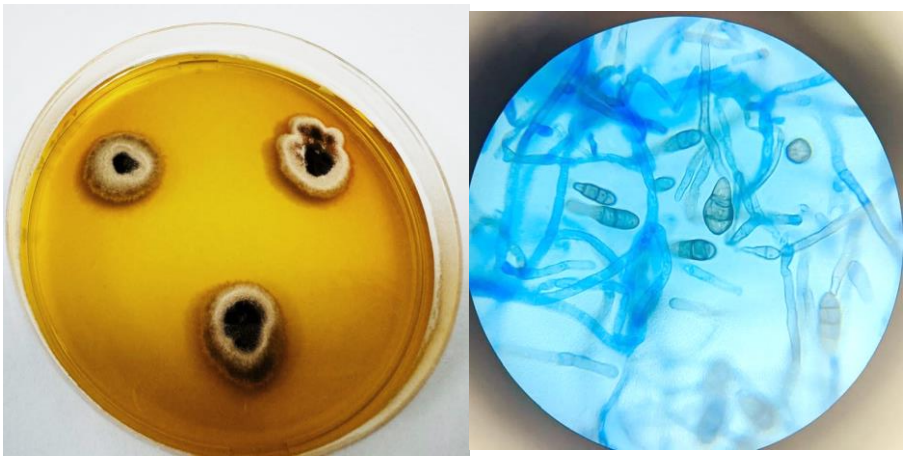

Figure (1) Morphological and Microscopic Characteristics of Alternaria alternata on SDA agar. A - Fungus Colony B - Under a Microscope (x100)

3.2Phytochemical analysis of neem extracts

Phytochemical examination of neem extracts via Gas Chromatography-Mass Spectroscopy (GC-MS) demonstrated the presence of physiologically active primary compounds, including alkaloids, flavonoids, saponins, tannins, and terpenoids (Table 1).

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Table (1): The phytochemical study revealed the presence of various phytochemicals in the ethanolic extracts of different medicinal plants

Compounds	phytochemical analysis
alkaloids	+
Flavonoids	+
Saponins	+
Terpenoids	+
Glycosides	-
Polyphenols	+
tannins	+
Steroids	+
Coumarins	-

3.3Antifungal activity of neem plant extract against A. alternata

The results in table(2) showed differences in zone of inhibition of neem plant extract. The high concentration (100 mg/ml) exhibited high zone of inhibition compared with low concentration(25 mg/ml).

Table(2):zone of inhibition of neem plant extract against A. alternata

Concentration	Zone of inhibition (mean±SD)
25 mg/ml	37±0.8c
50 mg/ml	52±1.2b
100 mg/ml	75±1 a
P value	0.03

- **Different letters mean presence of significance between groups**

25 mg/ml **50 mg/ml** **100 mg/ml**

figure (2): zone of inhibition of neem plant extact against A. alternata

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3.4Minimum inhibitory concentration and minimum fungicidal concentration of neem extract against A. alternata

The Minimum inhibitory concentration against fungi pathogen was (17) while minimum fungicidal concentration of neem extract against A. alternata was (36) as shown in table (3).

Table (3):MIC and MFC of neem extract against A. alternata

Pathogenic fungi	MIC	MFC
A. Alternata	17	36

Characterization of Ag- nanoparticles using UV-visible analysis

To confirm the presence of biosynthesized silver nanoparticles from the plantextract, UV–Vis spectroscopy was performed. The peak was determined using the Origin software (v. 2018). The extract showed the highest absorbance at a wavelength of 268 nm.As for the silver nanoparticle solution synthesized from neem plant extract, a peak appeared at 434 nm, as shown in figure (3). Silver nanoparticles possess optical properties that depend on their shape and size. The wavelength of silver nanoparticles can be observed in the range of 393 to 738 nm.

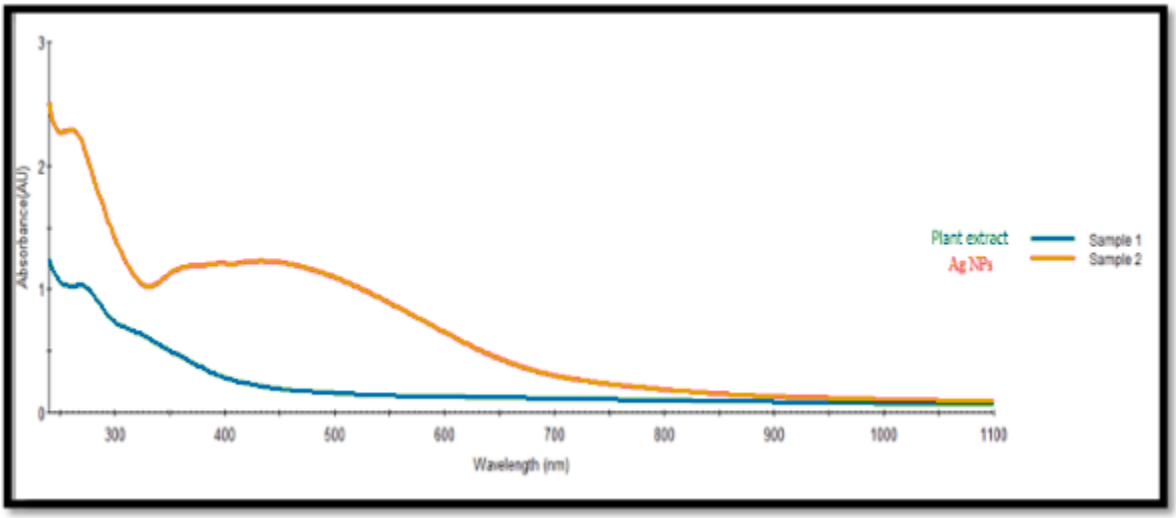

Figure (3): UV specrum of plant extract and the biosynthesized nanoparticles

Inhibitory effect of AgNP with Citrus extract on A. alternata

The inhibitory effect of silver nanoparticles was shown in table (4), in which different concentrations of AgNPs were applied on agar (6, 12 and 18 mg/ml) with water as a control. The high inhibitory zone (72±1.2 mm) on A. alternata was shown in a conc. of 18 mg/ml, followed by 12 mg/ml of concentration with an inhibition zone (63±1.1 mm). While the smallest inhibition zone (50±1 mm) was shown in 6 mg/ml of concentration.

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Table(4):zone of inhibition of neem plant extact and silver nanoparticles against A. alternata

Treatments	Concentration	Zone of inhibition (mean±SD)
Neem leaf extract	25 mg/ml	37±0.8
	50 mg/ml	52±1.2
	100 mg/ml	65±1
Neem mediated Ag-NPs	6mg/ml	50±1.0
	12mg/ml	63±1.1
	18mg/ml	72±1.2
Control(D.W)	0	
P value	0.03	

IV. Discussion

Phytopathogens cause illnesses in both field-grown and harvested potato plants. Plant disease protection is crucial for addressing the growing food demand of the population. Growing antibiotic resistance drives interest in discovering novel antimicrobial drugs. Fungi can infect crop plants, producing diseases in the stem, leaves, and flowers(12,19).

The present study showed the high effect of neem extract on pathogenic fungi , A. alternata, which is consistent with the study of (20), in which they investigated that leaf spot caused by Alternaria alternata represents a significant hazard to potato cultivation; however, the specific effects of its primary toxins remain unclear. This pathogen produces a variety of secondary metabolites that disrupt normal leaf function and hasten the progression of the disease, leading to symptoms such as wilting, yellowing, and reduced crop yield. Also agreed with the study of (21) which showed that Alternaria spp. cause different diseases in potato and tomato crops. Early blight caused by Alternaria solani and brown spot caused by Alternaria alternata are most common, but the disease complex is far more diverse.

The wide range of bioactive chemicals in neem (Azadirachta indica) and their complex modes of action are responsible for its strong antifungal effects. According to Oliveira et al. [23], these processes include rupture of fungal cell membranes, suppression of fungal enzyme activity, interference with the formation of fungal cell walls, and modification of fungal gene expression.

The A.indica plant does contain alkaloids, flavonoids, saponins, glycosides, steroids, terpenoids, polyphenols, and tannins. The study concurred with the findings of (22, 23), who concluded the same things when they examined the phytochemical composition of the leaves, alkaloids, flavonoids, saponins, terpenoids, polyphenols, and tannins. This study contradicts (24) because it found that neem bark extract contains steroids, polyphenols, terpenoids, saponins, flavonoids, and alkaloids. Plant components display pharmacological and therapeutic properties due to the presence of phytochemicals including alkaloids, flavonoids, saponins, glycosides, steroids, terpenoids, polyphenols, and tannins (25). Compared to the stem-bark and root, the leaves of A. indica contained the highest concentrations of alkaloids, flavonoids, terpenoids, and saponins (26). A study by (27) found that A. indica aqueous leaf extract had a high saponin content and a low alkaloid content, which is consistent with our findings.

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The leaf extract contains phytochemicals such as phenol, tannins, saponins, and others that contribute to its antibacterial activity. The 100% concentration had the highest antibacterial activity, perhaps due to its higher phytochemical content. The control has the lowest antifungal activity due to its lack of phytochemicals (28). The Ali et al.,(11) study's findings will assist in formulating a natural fungicide that will revolutionise plant disease control. A. indica extracts inhibited mycelium, spore germination, mycelium dry weight, lesion width, peach rot incidence, and disease severity against post-harvest R. stolonifer and M. fructicola.

The antifungal activity of neem extract was studied by (29) on the two saprophytic fungi, Rhizopus and Aspergillus. Results demonstrated that both fungal and yeast development were inhibited by the use of crude alcoholic and water-based neem leaf extracts of different ages. Further in vitro research confirmed that both fungal species were inhibited more effectively by the ethanolic leaf extract, and this finding held true even for the more aged leaf extracts. Another study, (30)reported that neem cake extract has antifungal effects against Sclerotium rolfsii and powdery mildew in balsam when sprayed over leaves.

The silver nanoparticles showed efficient antimicrobial properties compared to others due to their extremely large surface area, providing better contact with the cell walls of microorganisms(31). Neem-mediated silver nanoparticles had more effectiveness than crude neem extract because of their small size and big surface-to-volume ratio, which interact better with fungal cells. It penetrates the fungal cell wall, affects membrane permeability and destroys cell components. They also produce ROS, which causes oxidative stress, protein denaturation, and fungal cell death (32).

The results agreed with(33), who investigate in their study the effectiveness of silver nanoparticles that are synthesized from A. indica extract on pathogenic fungi such as Alternaria , Aspergillus, and Fusarium species. AgNPs also shrank, distorted, and inhibited spore germination in fungal hyphae(34).

V. Conclusion

This research demonstrated that the beneficial phytochemicals found in neem extract (Azadirachta indica)—including flavonoids, alkaloids, saponins, tannins, and terpenes—were critical in preventing the pathogenic fungus Alternaria alternata from growing. Confirming the high efficacy of neem extract as a natural antifungal agent, the inhibitory impact increased with increasing extract concentration. Also, silver nanoparticles enhanced the crude neem extract effectiveness against plant pathogenic fungi.

Author Contributions

The whole manuscript is done by Eman Mohammed

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Conflicts of Interest

The authors declare no conflict of interest.

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