

GREEN NANOTECHNOLOGY FOR MANGO PRESERVATION: ANTIOXIDANT AND ANTIFUNGAL PROPERTIES OF BIOWASTE- SYNTHESIZED NANOPARTICLES

Navya T N¹, V Ganesh², Raghavendra V³, Nagamani T. S⁴

^{1,2,3}Department of Biotechnology, Nrupathunga University, Bengaluru, Karnataka, India.

⁴Associate author Department of Biotechnology, Nrupathunga University, Bengaluru, Karnataka, India.

Corresponding author, Nagamani T. S

DOI: <https://www.doi.org/10.58257/IJPREMS35786>

ABSTRACT

Nanoparticles (AgNPs) have been used as a promising antibacterial, antifungal, antiviral, antioxidant, anti-inflammatory, anticancer, and anti-angiogenic agents. In this review, we focused on recent developments in synthesis, characterization, properties, and bio-applications of AgNPs in a single platform mainly on the antimicrobial, preservation, enhancing the shelf life of mango and antioxidant properties of *T.orientalis* (Cedar) leaf extract. The characterization of the synthesized AgNP was carried out by scanning electron microscopy (SEM), UV-Vis spectroscopy, and X-ray diffraction (XRD). The SEM results indicating particle sizes between 20 to 50 nm reveal a nanostructured material with potential applications in fields requiring precise control over particle dimensions. The antifungal activity of the nanoparticle was evaluated using the agar well diffusion method, and the anti-fungal activity was evaluated against *Aspergillus tubingensis* and *Scopulariopsis brevicaulis*. The in vitro antifungal activities of the biosynthesized NPs against pathogenic microorganisms, including pathogenic ones resistant to conventional antibiotics, highlight the superior antibacterial properties of the synthesis of silver nanomaterials. This review also emphasizes the increasing the shelf life of Mangoes using Silver nanoparticles.

Keywords: Silver nanoparticles, Shelf life, Mango, Antifungal, Antioxidant and Bio waste. .

1. INTRODUCTION

Mangoes, being one of the most consumed fruits worldwide, are highly susceptible to spoilage due to fungal infections, particularly during storage and transportation[2]. To address this issue, green nanotechnology offers a promising solution by using silver nanoparticles (AgNPs), which have strong antimicrobial properties[1]. These nanoparticles can be synthesized from plant-based bio-waste, providing an eco-friendly and sustainable method. The process begins with the collection of plant waste, which is rich in natural compounds that can act as reducing agents to synthesize AgNPs. This method not only makes use of waste material but also reduces the need for harmful chemicals[3]. Once synthesized, the AgNPs are carefully characterized to determine their size, shape, and effectiveness, where methods like UV-VIS spectroscopy, Scanning electron microscopy (SEM) and X-ray diffraction (XRD) are used[16,17,18]. In this study, we also focus on isolating and identifying the specific fungi that cause spoilage in mangoes. The AgNPs are also tested for the antioxidant activity[13,14,15]. The AgNPs will be tested for their antifungal activity to see how well they can inhibit the growth of these fungi[12]. The ultimate goal is to apply these AgNPs to extend the shelf life of mangoes, ensuring that they remain fresh for longer periods, thus reducing food waste and increasing their market value[23]. In this study, we focus on isolating fungi from infected mangoes to identify the main culprits behind the deterioration of the fruit. The process involves collecting samples from visibly infected mangoes, followed by culturing the fungi in a controlled environment to promote their growth. Once isolated, these fungal cultures are subjected to various identification techniques, including morphological examination and molecular analysis, to accurately determine their species. The fungus is isolated from infected mango and cultured on PDA media in suitable conditions. The DNA is isolated from fungus using CTAB method and amplification is done through PCR, amplified DNA is subjected to sanger sequencing for identification of fungal species. the DNA sequence obtained from Sanger sequencing is subjected to BLAST to identify similar sequences or potential matches within the database[24,25]. By aligning the query sequence with existing sequences, BLAST helps in identifying homologous genes, species, or related genetic elements. This process is crucial for confirming the identity of the sequence, understanding its function, or discovering relationships with other known sequences. After subjecting the Sanger sequence to BLAST analysis, the results revealed significant matches with two fungal species[28,30]: *Aspergillus tubingensis* and *Scopulariopsis brevicaulis*. The antifungal activity of silver nanoparticles (AgNPs) was evaluated against the fungal species *Aspergillus tubingensis* and *Scopulariopsis brevicaulis* using the agar well diffusion method. This technique involves introducing AgNPs into wells created in an agar plate that has been inoculated with the fungi[12]. As the AgNPs diffuse through the agar, their effectiveness in inhibiting fungal

growth is observed by measuring the zone of inhibition around each well[11]. This method provides a clear indication of the antifungal potency of the AgNPs, as a larger zone of inhibition suggests a stronger antifungal effect[12]. Testing against both *Aspergillus tubingensis* and *Scopulariopsis brevicaulis* is particularly important, as these fungi were identified in the earlier sequence analysis. The results from this assay help determine the potential of AgNPs as a treatment option for controlling fungal infections and extending the shelf life of mangoes[23].

2. MATERIALS AND METHODS

2.1 Sample collection and preparation of extract:

The plant sample was collected from RG Conventional Hall which is located in Malur, Kolar, Karnataka, India. *Thuja orientalis* widely grown as an ornamental plant in gardens and also used in functions. The used leaves from the above location were collected and thoroughly washed with tap water, followed by double distilled water, and cut into small pieces. 5g of cut leaves were boiled for 5 minutes in 50 ml ultrapure water and filtered through Whatman No. 1 filter paper. The filtered extract was refrigerated and used for the synthesis of silver nanoparticles[31].

2.2 Synthesis of Silver nanoparticles:

0.1M of aqueous solution of silver nitrate (AgNO₃) was prepared and used for the synthesis of silver nanoparticles. 5ml of leaf extract of *Nepeta cataria* was added to 45ml of 0.1M AgNO₃ solution was incubated in a dark chamber at room temperature to avoid photoactivation of silver nitrate. Bioreduction of Ag⁺ to Ag⁰ was confirmed by the color change of the solution from pale yellow to brown. The formation of AgNPs was confirmed by using UV-visible spectroscopy[2,3,31].

2.3 Characterization techniques

UV-visible Spectroscopy:

UV-vis spectrophotometry is one of the most commonly used technique for the characterization of synthesised nanoparticles which is also used to monitor stability and synthesis of AgNPs. In addition, UV-vis spectrophotometry is simple, easy, fast, sensitive and selective for different types of nanoparticles. It involves quantifying the amount of ultraviolet or visible radiation absorbed by a constituent in solution. UV-vis measures the ratio, or function of ratio, of the intensity of two beams of light in the UV-Visible region. In AgNPs, the valence band and conduction band lie very near to each other in which free movement of electrons[16]. These electrons produce a surface plasmon resonance (SPR) band due to the combined oscillation of electrons of AgNPs in resonance with the incident light wave. The absorption spectra of AgNPs depends on the dielectric medium, morphology, shape, size, and chemical surroundings of synthesised nanoparticles. Numerous studies have been shown that the AgNPs produces the absorption bands at around 200-800 nm, greater absorbance within the range of 460 and 540 nm are attributed to sizes of 10 to 30 nm[17].

X-ray diffraction analysis (XRD):

X-ray diffraction (XRD) is an analytical technique which has been utilised to investigate the crystal or polycrystalline structures, quantitative resolution of chemical compounds, qualitative identification of various chemical species, measuring the degree of crystallinity, particle sizes, etc. A beam of X-rays projected onto the crystal and the incident beam is scattered by the atoms it leads to the formation diffraction patterns[17]. The scattered x-rays interfere with each other. This interference could be observed by using Bragg's Law to find different characteristics of the crystal or polycrystalline material. Thus, XRD can investigate the structural features of various materials, such as biomolecules, polymers, superconductors, glasses, etc. Analysis of these materials largely depends on the formation of diffraction patterns. Formed diffraction patterns could reflect physical and chemical characteristics of the crystal. Generally, measurements are made in Angstroms (1 Å = 0.1 nm), thus X-ray diffraction is a primary characterization tool for obtaining critical features such as crystal structure, phase identification of crystal, crystallite size (information on unit cell dimensions), and strain. XRD spectra determine the crystalline nature of the silver nanoparticles by the overall oxidation state of the particles as a function of time[18].

Scanning electron microscope (SEM):

Among several electron microscopy techniques, SEM is a surface imaging technique, capable of determining different particle shapes, surface morphology, sizes and size distributions of the synthesised nanoparticles at the micro (10⁻⁶) and nano(10⁻⁹) scales. SEM utilises a high energy electron beam which is scanned over the surface of the AgNPs sample and then backscattered electrons observation provides characteristic features of the sample. Energy Dispersive X-Ray Spectroscopy (EDX) is a chemical analysis method used in combination with SEM to know the elemental composition AgNPs sample[17]. The EDX technique detects x-rays emitted from the sample during the bombardment by an electron beam and ED x-ray detector quantifies the relative abundance of discharged x-rays vs their energy. The drawback of

SEM is that it is not able to investigate the interior structure of the sample, but it can deliver valuable info regarding the degree of particle aggregation and purity[18].

2.4 Isolation of Fungus from Mango

The infected Mango fruit was cut out into small segments (3mm diameter) using sterilized scalpel, the segments of the infected fruits were then plated on solidified Potato Dextrose Agar plates aseptically. Inoculated plates were incubated at $25\pm30^{\circ}\text{C}$ for 7days. Actively growing fungal tips emerging from plant tissues were subcultured on PDA Petri plates for further identification [32].

2.5 Morphological and Microscopical Characteristics.

Morphological identification was based on morphological characteristics such as pattern, hyphae, colour of the colony, surface texture, marginal character, aerial, mycelium, mechanism of spore production and characteristics of conidia. The identification of fungal strains isolated was done by the observation of cultural characters and by the morphology of the strains isolated on PDA. Microscopical identification was done by using cotton blue lactophenol staining and the stained slides are prepared from stock culture using a microscope [24.25]. The microscopic examination of fungal colony consist of taking a sample from a petridish after a 2-3days of incubation, using a sterilized platinum loop and performing a spread between slide and coverslip with cotton blue as mounting liquid. observation was done under optical microscope with 10X and 40X objectives. The structures observed are the mycelium (septate or aseptate), nature of the conidiophore(brush or aspergillus head) , conidiogeneous cells (biseriate or uniseriate), the presence or absence of zygosporae and the spores[32].

2.6 Molecular identification

There are many protocols used for molecular characterization of fungal isolates. This requires a following steps:

- a. Genomic DNA extraction
- b. PCR amplification of conserved DNA sequences (rDNA) using specific or universal oligonucleotide primers forward and reverse.
- c. Sanger Sequencing of PCR products.
- d. Sequence processing and comparison with related sequences deposited in DNA database.
- e. Data interpretation [27]

CTAB Extraction Buffer (2%)

To prepare 100 ml of extraction buffer, 2 g of CTAB dissolved in 28ml of 5M NaCl, 4ml of 0.5M EDTA (pH 8.0) and 10 ml of 1 M Tris HCl (PH 8.0) were mixed together, 1% polyvinyl pyrrolidone, and the volume was made up to 100ml with double distilled water and autoclaved. 100 μl of 0.1% w/v Mercaptoethanol was added to the buffer freshly before the extraction prepared.

Chloroform: Isoamylalcohol (24:1).

To prepare 100 ml of the mixture, 96 ml of chloroform was mixed with 4ml of isoamylalcohol.

TE buffer: 1M Tris HCl (pH 8.0), 0.5M EDTA (pH 8.0). 1ml of 1M Tris HCl and 1 ml of 0.5M EDTA was added to 98.9 ml of distilled water and autoclaved.

1M Tris HCl: To prepare 100 ml of 1 M Tris HCl, 15.76 g of Tris base was dissolved in 70ml of distilled water, pH was adjusted to 8.0 with 10 N HCl and the volume was made up to 100 ml and autoclaved.

0.5M EDTA: To prepare 50 ml of 0.5M EDTA, 9.305 g of EDTA was dissolved in 30 ml of distilled water, pH was adjusted to 8.0 with 10 N NaOH and the volume was made up to 50ml and autoclaved.

5M NaCl: To prepare 100 ml of 5 M NaCl, 29.2 g of NaCl was dissolved in 70 ml of distilled water and final volume was made up to 100 ml.

3. METHOD

Fungal sample can be prepared by cryogenically grinding tissue in a mortar and pestle after chilling in liquid nitrogen. Freeze dried fungus can be ground at room temperature.

1. For each 100 mg homogenized tissue use 500 μl of CTAB Extraction Buffer. Mix and thoroughly vortex. Transfer the homogenate to a 60°C bath for 30 minutes.
2. Following the incubation period, centrifuge the homogenate for 5 minutes. at 14,000 x g.
3. Add an equal volume of chloroform/isoamyl alcohol (24:1). Vortex for 5 seconds then centrifuge the sample for 5 mins at 14,000 x g to separate the phases. Transfer the aqueous upper phase to a new tube.
4. Precipitate the DNA by adding 0.7 volume cold isopropanol and incubate at -20°C for 15minutes.
5. Transfer the mixture to the silica-based DNA column (750 μl each time) and Spin at 12000 rpm for 1 minute.
6. Add wash buffer 750 μl and spin at 12000 rpm for 1minute.

7. Repeat the wash buffer step
8. Dry spin for 2 minutes
9. Add 30 pl of elution buffer, spin at 12000 rpm for 1 minute
10. 1ul RNAase should be added and incubated at 37°C for 30 minutes
11. The DNA thus obtained has to be quantified [28,33].

Quantification of DNA.

After isolation of DNA, quantification and analysis of quality are necessary ascertain the approximate quantity of DNA obtained and the suitability of DNA sample for further analysis. This is important for many applications including digestion of DNA by restriction enzymes or PCR amplification of target DNA. The most commonly used methodologies for quantifying the amount of nucleic acid in a preparation are;

- Electrophoretic run along with standard DNA
- Spectrophotometric estimation and DNA quantification using NanoDrop.

Polymerase Chain Reaction

Polymerase chain reaction (PCR) is a technique used in the molecular biology to amplify a single copy or a few copies of segment of DNA across several orders of magnitude, generating thousands to millions of copies of a particular DNA sequence. Developed in 1983 by Kary Mullis. It is an easy, cheap and reliable way to repeatedly replicate a focused segment of DNA. PCR is probably the most used technique in molecular biology [34].

Essential components of PCR

Table 1: PCR components with their concentrations

Sl. no	Components	Concentrations
1.	10X PCR Buffer	1x
2.	25mM MgCl ₂	2.5mM
3.	10mM dNTPS	100μM
4.	Forward primer	5PM
5.	Reverse primer	5PM
6.	Taq polymerase	0.5 UNIT
7.	Nuclease free water	For Volume Making
8.	DNA Template	25ng

Primers

PCR primers are short, single-stranded segments of DNA that are designed to be complementary to the beginning and end of the target sequence that will be amplified. In a PCR, it is the primers that dictate exactly what sequence of DNA gets copied.

To design primers, you first need to know the gene or DNA sequence that will be your PCR target sequence. A standard PCR uses two primers, often called the “forward” and “reverse” primers. The forward and reverse primers are oriented on opposite strands of the DNA. During a PCR run, the primers will bind to the DNA, bookending the sequence you wish to amplify. DNA polymerase then copies the part of the target sequence that falls between the primers, selectively amplifying your sequence of interest. The spacing of your primers determines the size of your “amplicon” or PCR product. PCR can amplify fragments that range from a few dozen base pairs to several thousand base pairs long. Most PCRs, though, especially for diagnostic purposes, aim to amplify fragments in the hundreds of base pairs [34].

The primers depend upon

- Primer length
- Melting point
- Specificity
- Complementary primer sequence

Table 2: Examples of primers

Primer	Sequence	Annealing Temperature
ITS1-F	ATGCGATACTTGGTGTGAAT	53°C
ITS4-R	GACGCTTCTCCAGACTACAAT	51°C

Process Involved In PCR:

1. Initial denaturation 95°C for 2 minutes.
2. Final denaturation 95°C for 30 seconds
3. Annealing 50°C for 30 seconds
4. Elongation 72°C for 1 minute
5. Repeat steps 2,3 and 4 for 30 cycles
6. Final Elongation 72°C for 10 minutes
7. Hold at 4°C forever

The obtained PCR product is run on gel and compared the gene size with standard ladder.

Gel Purification.

1. Cut the required DNA band on gel
2. Add 400µl of Gel solubilization buffer and heat at 55°C until gel dissolves completely.
3. Add 200µl of Isopropanol, Mix and transfer to DNA column
4. Spin at 12000 rpm for 1 minute
5. Add 700µl of wash buffer, spin at 12000 rpm for 1 min.
6. Dry Spin for 2 minutes
7. Add 20µl of Elution Buffer, Spin at 12000 rpm for 1 minute[34].

Sanger Sequencing

DNA sequencing enables us to perform a thorough analysis of DNA because it provides us with the most basic information of all: the sequence of nucleotides. Sanger's method, which is also referred to as dideoxy sequencing or chain termination, is based on the use of dideoxynucleotides (ddNTP's) in addition to the normal nucleotides (NTP's) found in DNA. Dideoxynucleotides are essentially the same as nucleotides except they contain a hydrogen group on the 3' carbon instead of a hydroxyl group (OH). These modified nucleotides, when integrated into a sequence, prevent the addition of further nucleotides. This occurs because a phosphodiester bond cannot form between the dideoxynucleotide and the next incoming nucleotide, and thus the DNA chain is terminated[29,26].

Sequencing PCR

Initial denaturation 95°C for 2 minutes.

Final denaturation 95°C for 30 seconds.

Annealing 50°C for 30 seconds.

Termination 60°C for 4 minutes.

Repeat steps 2,3 and 4 for 30 cycles.

Hold at 4°C forever.

Post Sequencing and PCR Purification

1. Add 125 Mm 2.5µl EDTA to each well and give a short spin.
2. Add 35µl of ethanol using multichannel pipette
3. Vortex for 10 minutes at 2000 rpm, Centrifuge at 3510 rpm for 30 minutes.
4. Using tissue bed decant ethanol at 300 rpm (for 30 seconds invert the plate)
5. Add 40uL of 80% Ethanol to the wells and Centrifuge at 3510 rpm for 12 minutes
6. Repeat the above mentioned invert spin
7. Air dry for 30-45 minutes covering the plate with lint free tissue
8. Add 13uL of HiDi Formamide and give a short spin
9. Denature at 95°C for 5 minutes.
10. Place the plate in sequencer.

The Bidirectional sequencing of PCR products were carried in Sequencer-Applied Biosynthesis (Hitachi) 3130x1 Genetic Analyzer. The data from the sequencer was taken in storage drive and processed in the software Finch TV. The electropherogram files which were in ab1 format was converted to pdf and fasta files using a sequence scanner software. The sequence data generated during this study were subjected to BLAST searches in the nucleotide database of GeneBank to determine their most probably closely related taxa[29].

Finch TV

FinchTV is a versatile chromatogram viewer developed by Geospiza, tailored for analyzing and editing DNA sequence files generated from automated sequencers. Renowned for its user-friendly interface and compatibility with various operating systems, FinchTV allows researchers to visualize and edit electropherograms, making it an essential tool for

molecular biologists and geneticists. It supports multiple file formats, including ABI and SCF, and offers features like quality score visualization, sequence trimming, and annotation. FinchTV's intuitive navigation and robust functionality streamline the process of examining sequencing data, enabling users to easily interpret chromatograms, identify base-calling errors, and ensure the accuracy of their DNA sequences. Its capacity to handle large datasets and facilitate data sharing further enhances its utility in both academic and clinical research settings[29].

Electropherogram Peak in Finch TV

An electropherogram, also known as a chromatogram, is a visual output of DNA sequencing that displays the signal intensity of each nucleotide base (A, T, G, C) along the sequence. In Finch TV, the electropherogram is represented by a series of peaks, each corresponding to a nucleotide.

Key Components of an Electropherogram Peak:

1. Peaks:

- Each peak in the electropherogram corresponds to a nucleotide in the DNA sequence
- Peaks are color-coded for each base: typically, adenine (A) is green, cytosine (C) is blue, guanine (G) is black, and thymine (T) is red.

2. Height of Peaks:

- The height of each peak indicates the signal strength of the corresponding nucleotide. Taller peaks represent stronger signals.
- Consistently tall peaks suggest high-quality sequencing data, whereas variations in peak height may indicate sequencing issues.

3. Width and Shape of Peaks:

- The width and shape of the peaks provide insights into the resolution and accuracy of the sequencing run.
- Ideal peaks are sharp and well-defined, whereas broad or overlapping peaks can signal problems such as contamination or sequencing errors.

2.7 Determination of Antifungal activity.

The method used to check the antifungal activity is agar well diffusion method. Agar well diffusion method is used for antimicrobial tests for plant extracts, Nanoparticles, and secondary metabolites, to check the inhibiting ability against fungus. This method is frequently used to evaluate the antimicrobial activity. Firstly, prepare the media (PDA). then the PDA is poured into the plates to grow test fungus. The agar plate surface is inoculated by spreading a volume of the microbial inoculum over the entire media surface. Then, a hole with a diameter of 6 to 8mm is punched aseptically with a sterile cork borer or a tip, and a volume (20-100 μ l) of the antimicrobial agent or extract solution at desired concentration is introduced into the well. Then, agar plates are incubated under suitable conditions depending upon the test microorganism.

The antifungal activity of the synthesized AgNPs was evaluated using the agar well diffusion method. A spore suspension of the fungi, **Aspergillus tubingensis** was aseptically spread on the surface of a potato dextrose agar plate. Wells were prepared using a sterile well borer. The wells were filled aseptically with Aqueous plant extract, AgNPs, water (negative control), and an Antibiotic Fluconazole (positive control). These plates were incubated at 28 degrees Celsius for 48h. The antifungal activity was evaluated by measuring the zone of inhibition[26,29].

2.8 Assessment of antioxidant activity:

Antioxidant activity was determined using methods like the H₂O₂ scavenging method, and the Phosphomolybdenum method.

1. H₂O₂ scavenging method:

1 ml of AgNPs was mixed with 3.6 ml of phosphate buffer and 5.4 ml of Hydrogen peroxide solution. The mixture was incubated for 10 minutes at room temperature. The absorbance was recorded at 230nm using UV-Vis spectrophotometry and the phosphate buffer was taken as blank[13]. The percentage antioxidant capacity were calculated using the following formula:

$$\% \text{Scavenging (H}_2\text{O}_2\text{)} = 1 - \frac{\text{Abs(sample)}}{\text{Abs(extract)}} \times 100$$

2. Phosphomolybdenum method:

An aliquot of 0.5ml of AgNP solution was combined with 3ml of phosphomolybdenum reagent. The tubes were incubated in a boiling water bath at 95 degrees Celsius for 90mins. In the presence of extracts, Mo (VI) is reduced to Mo (V) and forms a green colored phosphomolybdenum(V) complex. After the tubes were cooled to room temperature, the absorbance was measured at 695nm taking water as blank the higher absorbance value indicated higher antioxidant

activity[13,14]. The percentage antioxidant capacity were calculated using the following formula: %Antioxidant Capacity = $1 - \frac{\text{Abs}(\text{sample})}{\text{Abs}(\text{extract})} \times 100$

2.9 Increasing the Shelf life of Mango by AgNPs

The Study on quality parameters and storage stability of Mango coated with silver nanoparticles. The details of materials and methods used during the course of the increasing the Shelf life of Mango by AgNPs are as follows:

Materials Required

The materials required are AgNPs nanoparticles. Fresh and firm mangoes were procured from the agriculture farms of Nisarga, Kolar. Bright colored Mangoes with almost uniform size and shape, free from blemishes, apparent diseases, and injuries, were selected for coating[21,23].

Procedure for coating of mango

After selecting the Mango fruits of uniform size and ripeness, they were then cleaned by washing with distilled water for 1 minute followed by air drying at room temperature. Fruits were coated with the film forming solution by spraying them for 1 minute. These coated fruits were then air dried and stored at room temperature. Control fruits were also stored under same conditions and the quality parameters of all the coated and uncoated samples were evaluated during the storage[20,23].

4. RESULTS AND DISCUSSION

4.1 Collection of Sample and preparation of extract:

Plant sample was collected as a Bio-waste sample which was thrown in the degradable wastes collection bin. Further the plant sample was kept in water for 5 days for the extraction purpose. Use of leaves extract for synthesis of nanoparticles has an added advantage of environmental friendly. Leaves are normally thrown away into the environment, so evaluating therapeutic value of discarded material is a novel idea. In this research, an attempt was made to synthesize silver nanoparticles from *T.orientalis* extract.



Figure 1: Plant Sample (*T. orientalis*)

Bio waste collected leaves of *T.orientalis* were boiled for 5 minutes in 50 ml ultrapure water and filtered through Whatman No. 1 filter paper. The obtained water extract was refrigerated and used for the synthesis of silver nanoparticles. The different parts of plant extracts are ecofriendly, economical and safe for the synthesis of nanoparticles. Use of leaves extract for synthesis of nanoparticles has an added advantage of environmental friendly. Leaves are normally thrown away into the environment, so evaluating therapeutic value of discarded material is a novel idea. In the present study, an attempt was made to synthesize silver nanoparticles from *T.orientalis* extract.



Figure 2: Plant Extract (*T. orientalis*)

4.2 Synthesis of Silver nanoparticle:

Initially, silver nitrate was colorless without the extract that was taken as the control. During the synthesis of silver nanoparticles (AgNPs), it was visually observed that after the addition of the leaf extract resulted in the colour change of the silver nitrate solution from pale yellow to dark brown indicating the synthesis of AgNPs (Fig.3). The addition of silver ions into the filtered cell free filtrate of the sample, changes its colour from almost colorless to brown with intensity increasing during the period of incubation. It is well known that AgNPs produces a yellowish-brown color in the solution due to excitation of Surface Plasmon Resonance (SPR) vibrations that in turn is due to the presence of free electrons. For synthesis of silver nanoparticles standardization was done in respect to addition of extract amount and boiling time for the preparation of plant extract. Optimization of these two parameters was essential as both had a profound effect on the formation of silver nanoparticles also

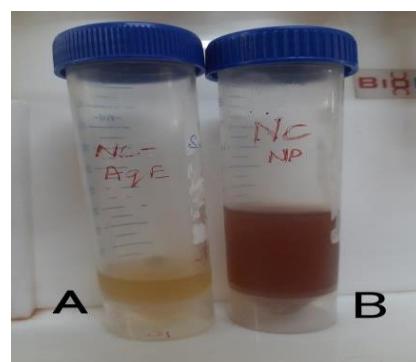


Figure 3: a) T.orientalis leaf extract reported effect of extract amount on AgNPs formation. b) Synthesized AgNP

4.3 Characterization

1) Visual Observation and UV-Vis Spectroscopy:

The synthesized silver nanoparticles were analyzed by UV-Vis spectra at 200-1000nm in comparison with the extract. The peak was obtained at 400nm. Then at 400nm UV-Vis spectra were recorded after different time intervals of 2, 4, 12, 24, 48, and 72 hours.

Table 3: Values of extract and nanoparticles at different ODs

Absorbance(nm)	Extract	Nanoparticle
200	-	-
300	-	-
400	2.306	3.638
500	1.306	2.682
600	1.182	2.106
700	0.988	1.548
800	1.088	1.394
900	1.044	1.224
1000	1.018	1.134

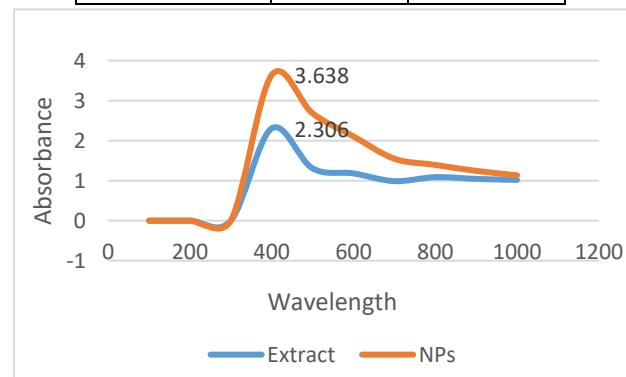


Figure 4: Line graph of extract and nanoparticles at different ODs

Table 4: Values of AgNPs at different time interval @400nm

Time Interval	AgNPs
2hrs	3.638
4hrs	4.123
8hrs	4.645
12hrs	5.092
24hrs	5.373
48hrs	5.842
72hrs	4.092

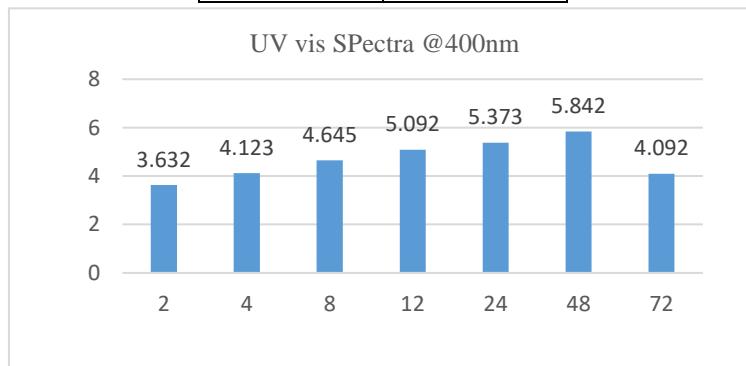


Figure 5: Column graph of AgNPs at different time intervals @400nm.

UV-spectra revealed maximum absorption peak at 400 nm and the intensity of absorption increased with time, absorption for Extract at 400nm is 2.302 and the absorption for Nanoparticle at 400nm is 3.638. Followed by the highest amount of Nanoparticle was synthesized at 48 hrs. The increase in intensity could be due to the increasing number of nanoparticles formed as a result of reduction of silver ions present in the aqueous solution with the help of phyto-constituents present in *T.orientalis* leaf extract.

2) Scanning Electron Microscopy (SEM)

The scanning electron microscope (SEM) uses a focused beam of high-energy electrons to generate a variety of signals at the surface of solid specimens. The signals that derive from electron-sample interactions reveal information about the sample including external morphology (texture), chemical composition, and crystalline structure and orientation of materials making up the sample. Areas ranging from approximately 1 cm to 5 microns in width can be imaged in a scanning mode using conventional SEM techniques.

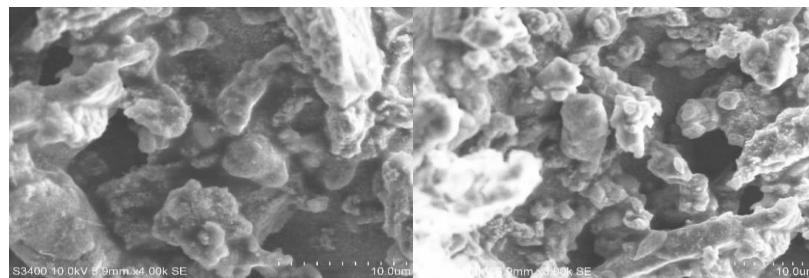


Fig 6: SEM images of Silver Nanoparticles

The SEM results indicating particle sizes between 20 to 50 nm reveal a nanostructured material with potential applications in fields requiring precise control over particle dimensions. This size range suggests a high surface area to volume ratio, which can enhance reactivity and performance in various applications such as catalysis, drug delivery, or electronic devices. The uniformity of particle size, if observed, could imply a well-controlled synthesis process, while any deviations might point to process inconsistencies or the need for optimization. Overall, these nanometer-sized particles could significantly impact the material's functional properties and performance.

2) X-Ray Diffraction

The XRD pattern of biosynthesized silver nanoparticles shows characteristic peaks at 38.27 °, 42.71 °, 64.32 ° and 77.65 °, which correspond to the (111), (200), (220) and (311) crystallographic planes, respectively, of face-centred cubic (FCC) silver nanoparticles.

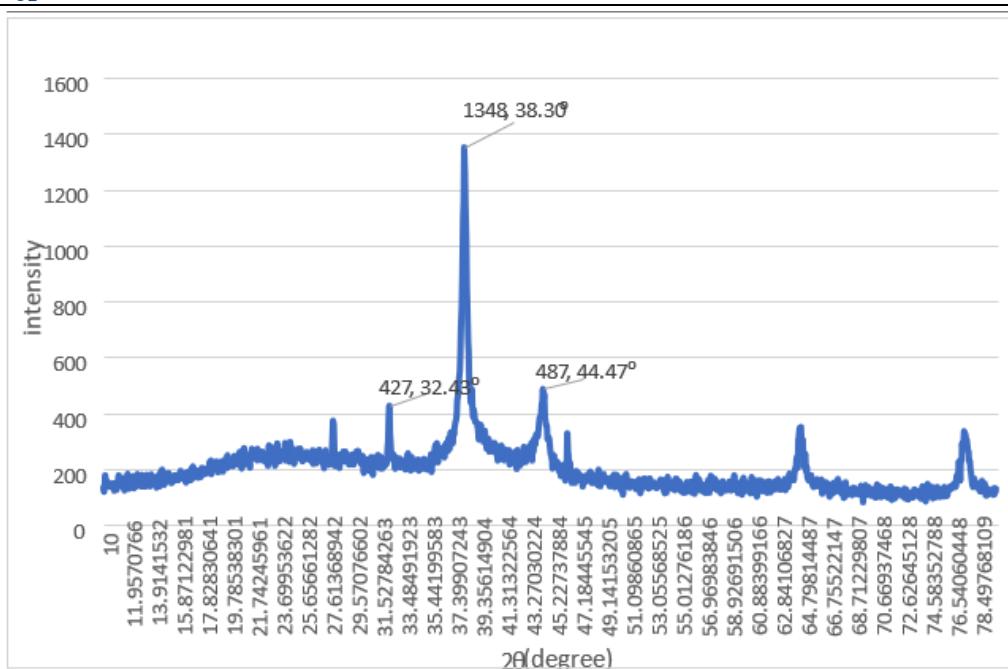


Fig.7: Line graph of Silver Nanoparticles from XRD

XRD analysis

The average crystalline size of synthesized AgNPs is 14.5 nm

The XRD peaks at 427, 1348, and 487 suggest specific crystallographic planes. Peaks typically correspond to the diffraction angles of distinct lattice planes. The ultimate goal is to match these peaks to known patterns to identify phases or structural characteristics. Delving into the relative intensities and peak widths can further reveal information about crystallinity and phase purity. Consider comparing your data to standard references to interpret the material composition and structure more accurately.

Antimicrobial and Antioxidant Activity:

Antifungal activity:

Antifungal activity of AgNPs towards *Aspergillus tubingensis* and *Scopulariopsis brevicaulis* was evaluated using the agar well diffusion method. Results illustrated that biosynthesized AgNPs exhibited promising antifungal activity by inhibiting the growth of the fungi. In comparison with Control, Antibiotic, Extract and Nanoparticles, the nanoparticles showed highest Zone of Inhibition against *Aspergillus tubingensis*

Table 5: Antifungal activity by Zone of Inhibition

Sample	Zone of Inhibition (cm)
Antibiotic (Fluconazole)	3.8
Control	0.0
Nanoparticle	2.5
50% Nanoparticle	1.6
Extract	0.0



Fig 8: Visible Zone of inhibition obtained by Well diffusion method for *Aspergillus tubingensis*

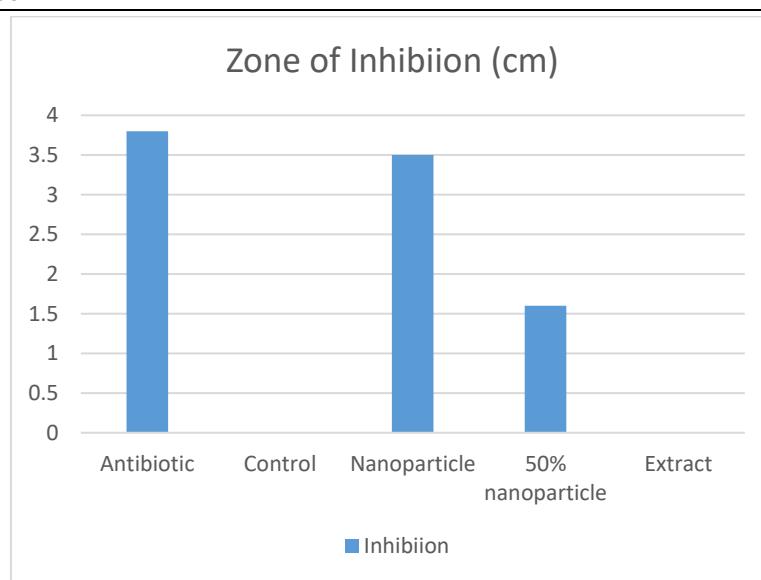


Fig 9: Column graph exhibiting Zone of inhibition for Antifungal activity against *Aspergillus tubingensis*

Table 6: Antifungal activity by Zone of Inhibition

Sample	Zone of Inhibition (cm)
Antibiotic (Fluconazole)	2.8
Control	0.0
Nanoparticle	1.9
50% Nanoparticle	1.7
Extract	0.0



Figure 10: Visible Zone of inhibition obtained by Well diffusion method for *Scopulariopsis brevicaulis*

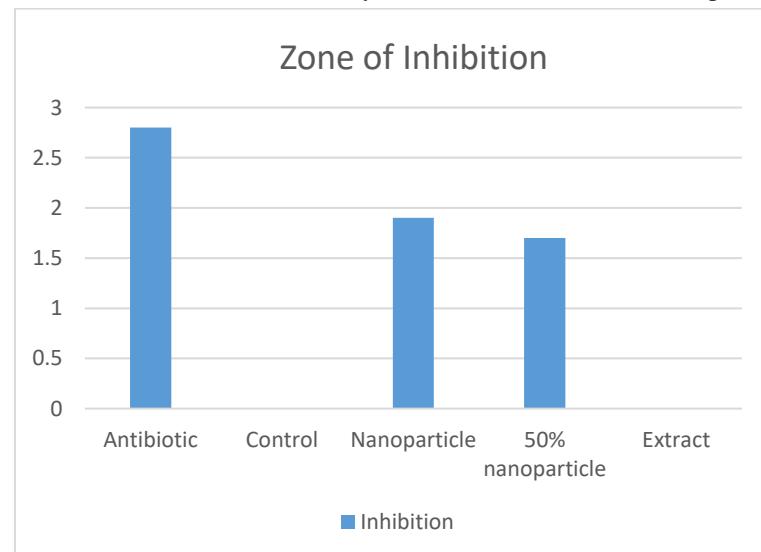


Fig 11: Graph exhibiting Zone of inhibition for Antifungal activity against *Scopulariopsis brevicaulis*

The results of the present work clearly showed that antifungal activity of AgNPs was more than water and extract. The AgNPs successfully inhibited fungal strain, even better than wa extract. Some studies have reported that positive charge on the silver ion is significant for its antimicrobial activity through the electrostatic attraction between negative charge on cell membrane of microorganism and positive charged nanoparticles. Over all, it can be concluded that AgNPs showed more inhibitory activity than antibiotics alone and water extract. The inhibition was more against pathogenic fungal strains. This is very interesting because they both are very resistant pathogenic microbial strains causing incurable infectious diseases and there is always a look out for alternative novel approach to treat them. To date, synthesis of AgNPs with leaf extracts is scanty and synthesis of AgNPs with *T.orientalis* leaf extract is reported for the first time. The reduction of silver ions occurred due to the watersoluble phytochemicals like flavonoids, tannins, triterpenes, cardiac glycosides and alkaloids present in the leaf sample of *T.orientalis*. The results clearly demonstrated that AgNPs synthesized by green route can definitely compete with commercial antibiotics used for the treatment of microbial infections and sometimes are even better. Thus, these ecofriendly silver nanoparticles can be used as an excellent antimicrobial agent against multi drug resistant pathogenic microorganisms. However, more research work especially on animal models needs to be done before they can be used as antimicrobial agents. Finally the therapeutic use of nanoparticles synthesized from leaf, otherwise thrown away as useless material into environment is noteworthy.

5.4Antioxidant activity

The synthesized AgNPs were reported to have antioxidant property through its hydrogen peroxide scavenging and phosphomolybdenum activity. This is due to the presence of certain bioactive molecules which are present on the surface of silver nanoparticles.

H₂O₂ Scavenging method:

Table 7: Samples observed in UV-Vis Spectroscopy for H₂O₂ Scavenging method

Sample	O.D @ 230nm
Blank	0.0
Aqueous extract	0.379
Nanoparticle	0.162



Fig 12: Antioxidant activity by H₂O₂ Scavenging method

The observed antioxidant effect percentage of AgNPs and the extract was 57.26% indicating that AgNPs are antioxidant source.



b) Phosphomolybdenum method:

Fig 13: Antioxidant activity by Phosphomolybdenum method

Table 8: Samples observed in UV-Vis Spectroscopy

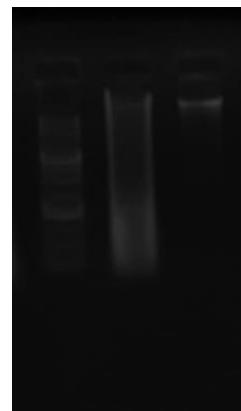
Sample	OD @ 695nm
Blank	0.0
Aqueous extract	2.048
Nanoparticles	0.996

48.6% antioxidant activity

The synthesized AgNPs also showed antioxidant activity of by H_2O_2 Scavenging method and Phosphomolybdenum method revealed the efficiency of AgNPs as a source of good antioxidant and to combat the free radicals that are the potent cause of oxidative stress.

5.5DNA Barcoding: Molecular identification

The DNA band pattern captured was analyzed using a standard ladder (100bp). As shown in Fig-23, the resulting sequence was subjected to Sanger sequencing and was viewed with the Finch TV tool, which depicts electropherogram peaks. After BLAST, we identified a similar sequence by analyzing the similarity percentage and E value.



a) Aspergillus tubingensis

Fig14: Genomic DNA isolated from fungus, compared with 100bp ladder

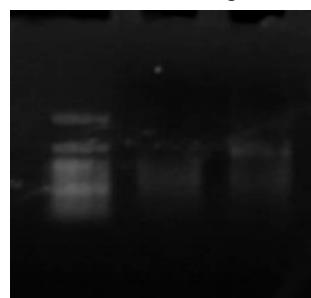


Fig 15: ITS gene (600bp) amplified from fungus, compared with 1000bp ladder

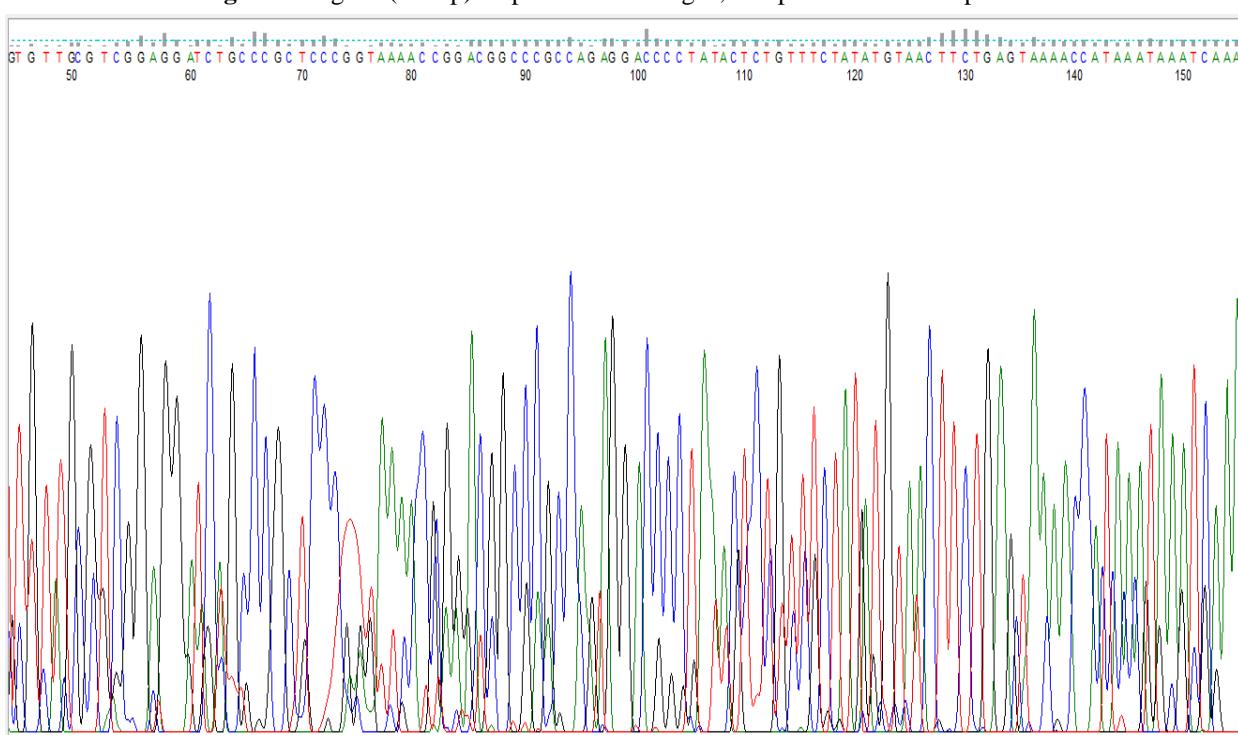


Fig 16: Sanger sequence was viewed with the Finch TV tool, which depicts Electropherogram peaks

Fungal fasta sequences:

>MFB_ITS

```
ACGATCGATCATAACGTAACGAATTGTTCCGGTCCTTACCTCTATCCGAGGTCAACCTGGAAAAAA
TGGTTGGAAAACGTCGGCAGGGCGCCGTTCAATCCTACAGAGCATGTGACAAAGCCCCATACGCT
CGAGGATCGGACGCGGTGCCGCCGCTGCCCTTCGGGCCGTCGGGGGGAGAGGGGGACGGCGA
CCCAACACACAAGCCGGCTTGAGGGCAGCAATGACGCTCGGACAGGCATGCCCGGAATAC
CAGGGGGCGCAATGTGCGTTCAAAGACTCGATGATTCACTGAATTCTGCAATTCACATTAGTTATC
GCATTCGCTGCGTTCTGCTTCATCGATGCCGAACCAAGAGATCCATTGTTGAAAGTTTAAC TG
ATTGCATTCAATCAACTCAGACTGCACGGTTTCAGAACAGAGATCGTGTGGGGTCTCCACACGT
ACGA
```

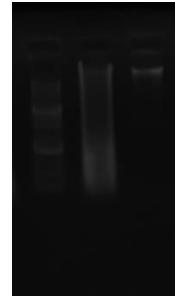
Hit: *Aspergillus tubingensis* strain wxm77 18S ribosomal RNA gene, partial sequence; internal transcribed spacer

Percent identity: 96.88%

E value: 0.0

Query coverage: 90%

Accession no.: HM037959.1



Scopulariopsis brevicaulis

Fig17: Genomic DNA isolated from fungus, compared with 100bp ladder

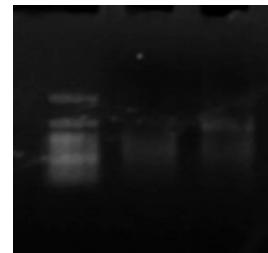


Fig 18: ITS gene (600bp) amplified from fungus, compared with 1000bp ladder

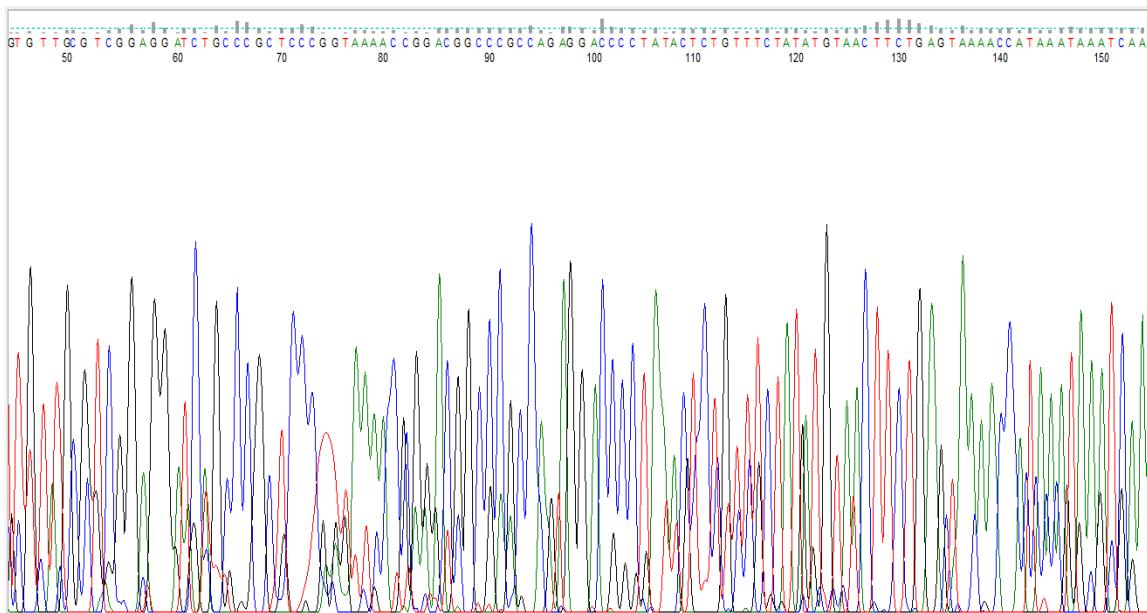


Fig19: Sanger sequence was viewed with the Finch TV tool, which depicts Electropherogram peaks.

Fungal fasta sequences:

>MFW_ITS

```
AGTTACCCCTCTAAACCCATTGTGAACCTTACCTCTGCCGCGCGTGCCTCGGCAGGGAT
CGCGCGCCCTCTGCCGGCCGCTCCCGCCCCGCTCCCGCCGGCGCCAAACTCTAAA
GCAAAGCGGACTGCACGTTCTGATTTAAACAAAAACAAGTAAAAACTTTAACACGGATCTCTT
GGTTTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCACTGAA
TCATCGAATCTTGACCGCACATTGCGCCCGCAGCAATCTGCCGGCATGCCTGTCCGAGCGTC
ATTCTCCCTCGAGCGCGCTAGCCCTACGGGGCCTGCCGCCCGGTGTTGGGGCTTACGG
GTGGGCTCGTCCCCCCCCGAGTCCCCGAAATGTAGTGGCGGTCCAGCCCGGGCGCCCCCTCGTA
GTAGATCCTACATCTGCATGGGTCCGGGAAGGCCAGCCGTCGAACCTTACTCATGGTT
TGACCTCGGATCAGGTAGGGTTACCCGCTGAACCTTAAGCATATCAATAA
```

Hit: *Scopulariopsis brevicaulis* strain wxm77 18S ribosomal RNA gene, partial sequence; internal transcribed spacer

Percent identity: 95.53%

E value: 0.0

Query coverage: 98%

Accession no.: ON624335.1

The study was designed to identify the microorganisms using a growingly popular DNA barcode method. The extracted DNA was performed PCR for amplification. After successful amplification the BLAST result showed a high rate of similarity with its homologous sequence. The highest similarity was found with *Aspergillus tubingensis* and *Scopulariopsis brevicaulis*.

5.6 Increasing the Shelf life of Mango by AgNPs

Increasing the shelf life of mangoes through the use of silver nanoparticles involves applying nanotechnology to enhance preservation methods. Silver nanoparticles are known for their antimicrobial properties, which can inhibit the growth of bacteria, fungi, and other pathogens that cause spoilage in fruits like mangoes. By coating mangoes with silver nanoparticles researchers aim to extend the fruit's shelf life by preventing microbial growth and maintaining freshness.

Table 9: 1st Day of Observation

Sample	Weight	Color	Texture	Size (cm)
Control	190g	Greenish Yellow	Smooth	21
Coated (AgNPs)	215g	Greenish Yellow	Smooth	21.5

Table 10: 10th Day of Observation

Sample	Weight	Color	Texture	Size (cm)
Control	188g	Yellow	Wrinkled	20.5
Coated (AgNPs)	210g	Yellow	Smooth	21.0

Control Coated(NPs)



Day 1



Day 3



Day 5



Day 7



Day 10

Fig 20: Comparison of Mangoes after coating Nanoparticles

The synthesized AgNPs are used to extend the Shelf life of Mango fruits during postharvest storage of both Organic and Commercial mangoes. It has been shown to be efficient in preventing the of spoilage microorganisms and delaying the ripening and senescence processes in mangoes. It has been demonstrated that, depending on the kind and concentration of the AgNPs, applying them as coatings on the mangoes to keep fresher longer than they would under normal storage circumstances. Mangoes sensory and nutritional qualities can be preserved through-out extended storage by using AgNPs that successfully maintain the fruits firmness, color, and general quality features. The increase in shelf life by nanoparticles may also decrease food waste increase supply chain effectiveness. The use of silver nanoparticles shows potential in enhancing food safety and reducing food waste by extending the shelf life of mangoes. As compared to Commercial mangoes, Organic mangoes gives effective result to Silver Nanoparticles. Because, Organic mango observes the Coated AgNPs and increases the shelf life, which was shown in the fig 20

5. CONCLISION

The study reports a simple, cost effective and efficient synthesis of Silver nanoparticles from leaves of *T.orientalis*(Cedar) and their Antifungal, Antioxidant properties and Extending Shelf life. The synthesis of AgNPs using *T.orientalis* leaf extract was successfully achieved by optimizing the amount of extract and boiling time, critical parameters that influenced the nanoparticle formation. The transition of the solution from colorless to brown was indicative of nanoparticle formation, with the brown color serving as a clear spectroscopic signature. UV-Vis spectroscopy revealed a peak at 400 nm and an increase in peak intensity over time, which suggests an ongoing reduction of silver ions facilitated by the phyto-constituents in the leaf extract.

The antifungal activity of the synthesized AgNPs was noted higher than that of the water extract alone. The enhanced inhibitory effect against fungal strains, including pathogenic ones resistant to conventional antibiotics, highlights the superior antimicrobial properties of AgNPs. The positive charge on silver ions contributes significantly to antimicrobial activity by attracting to the negatively charged microbial cell membranes. This finding is particularly relevant given the persistent challenge of treating multi-drug resistant pathogens. The synthesis of AgNPs using *T.orientalis* leaf extract, a novel approach, presents a compelling alternative to traditional antibiotics, especially against resistant strains.

In addition to its antimicrobial properties, the AgNPs exhibited significant antioxidant activity. The H_2O_2 scavenging method and the Phosphomolybdenum assay confirmed the efficacy of AgNPs in neutralizing free radicals, which are known to cause oxidative stress and contribute to various diseases. This dual functionality of AgNPs—both as an antimicrobial and antioxidant agent—broadens their potential applications in health and food preservation.

The application of AgNPs in extending the shelf life of mango fruits represents a practical and valuable use of these nanoparticles. The ability of AgNPs to prevent spoilage, delay ripening, and maintain the sensory and nutritional qualities of mangoes is a significant advancement in postharvest technology. By reducing spoilage and food waste, AgNPs contribute to increased supply chain effectiveness and sustainability in food storage practices.

6. REFERENCES

- [1] Sarkar S, Jana AD, Samanta SK, Mostafa G (2007) Facile synthesis of silver nanoparticles with highly efficient anti-microbial property. *Polyhedron* 26: 4419-4426.
- [2] Sastry M, Ahmad A, Khan MI, Kumar R (2003) Biosynthesis of metal nanoparticles using fungi and actinomycetes. *Curr Sci* 85: 162-170.
- [3] Ankamwar B, Damle C, Ahmad A, Sastry M (2005) Biosynthesis of gold and silver nanoparticles using Emblica officinalis fruit extract and their phase transfer and transmetallation in an Organic Solution. *J Nanosci Nanotechnol* 5: 1665-1671.

[4] Gurunathan, S.; Park, J.H.; Han, J.W.; Kim, J.H. Comparative assessment of the apoptotic potential of silver nanoparticles synthesized by *Bacillus tequilensis* and *Calocybe indica* in MDA-MB-231 human breast cancer cells: Targeting p53 for anticancer therapy. *Int. J. Nanomed.* 2015, 10, 4203–4222.

[5] Mukherjee, P.; Ahmad, A.; Mandal, D.; Senapati, S.; Sainkar, S.R.; Khan, M.I.; Renu, P.; Ajaykumar, P.V.; Alam, M.; Kumar, R.; et al. Fungus-mediated synthesis of silver nanoparticles and their immobilization in the mycelial matrix: A novel biological approach to nanoparticle synthesis. *Nano Lett.* 2001, 1, 515–519.

[6] Sharma, V.K.; Yngard, R.A.; Lin, Y. Silver nanoparticles: Green synthesis and their antimicrobial activities. *Adv. Colloid Interface* 2009, 145, 83–96.

[7] D.S. Balaji, S. Basavaraja, R. Deshpande, D. Bedre Mahesh, B.K. Prabhakar, A. Venkataraman Extracellular biosynthesis of functionalized silver nanoparticles by strains of *Cladosporium cladosporioides* fungus *Colloids Surf. B Biointerf.*, 68 (2009), pp. 88-92

[8] S.P. Chandran, M. Chaudhary, R. Pasricha, A. Ahmad, M. Sastry Synthesis of gold nanotriangles and silver nanoparticles using *Aloe vera* plant extract *Biotechnol. Prog.*, 22 (2006), pp. 577-583

[9] K.A. Jha, K. Prasad Green synthesis of silver nanoparticles using *Cycas* leaf *Int. J. Green Nanotechnol. Phy. Chem.*, 1 (2) (2010), pp. 110-117

[10] Li, W.R.; Xie, X.B.; Shi, Q.S.; Zeng, H.Y.; Ou-Yang, Y.S.; Chen, Y.B. Antibacterial activity and mechanism of silver nanoparticles on *Escherichia coli*. *Appl. Microbiol. Biotechnol.* 2010, 8, 1115–1122.

[11] Sondi, I.; Salopek-Sondi, B. Silver nanoparticles as antimicrobial agent: A case study on *E. coli* as a model for Gram-negative bacteria. *J. Colloid Interface Sci.* 2004, 275, 177–182

[12] Gupta A.K, Renaud H.J, Quinlan E.M, Shear N.H, Piguet V. The growing problem of antifungal resistance in onychomycosis and other superficial mycoses. *Am. J. Clin. Dermatol.* 2021;22(2):149–157.

[13] T. Thilagavathi, G. Kathiravan, K. Srinivasan Antioxidant activity and synthesis of silver nanoparticles using the leaf extract of *Limonia acidissima* *Int J Pharm Bio Sci*, 7 (4) (2016), pp. 201-205,

[14] P. Kanchana, V. Hemapriya, N. Arunadevi, S.S. Sundari, I.M. Chung, M. Prabakaran Phytofabrication of silver nanoparticles from *Limonia acidissima* leaf extract and their antimicrobial, antioxidant and its anticancer prophecy *Indian Chemical Society*, 99 (10) (2022), Article 100679,

[15] A. Abida, M.H. Almutairi, N. Mushtaq, M. Ahmed, N. Sher, F. Fozia, I. Ahmad, B.O. Almutairi, Z. Ullah Revolutionizing nanotechnology with *filago desertorum* extracts: biogenic synthesis of silver nanoparticles exhibiting potent antioxidant and antibacterial activities *ACS Omega*, 8 (38) (2023), pp. 35140-35151, 10.1021/acsomega.3c04373.

[16] Lin P.C., Lin S., Wang P.C., Sridhar R. Techniques for physicochemical characterization of nanomaterials. *Biotechnol. Adv.* 2014;32:711–726. doi: 10.1016/j.biotechadv.2013.11.006.

[17] Albanese A., Tang P.S., Chan W.C. The effect of nanoparticle size, shape, and surface chemistry on biological systems. *Annu. Rev. Biomed. Eng.* 2012;14:1–16. doi: 10.1146/annurev-bioeng-071811-150124.

[18] Johnson I, Prabu HJ (2015) Green synthesis and characterization of silver nanoparticles by leaf extracts of *Cycas circinalis*, *Ficus amplissima*, *Commelina benghalensis* and *Lippia nodiflora*. *Int Nano Lett* 5:43–51

[19] The scientific world journal, review article-Comprehensive Literature Review on Metal Nanoparticle for Enhanced Shelf Life of Mango Fruit. Yalew Yiblet, Indiris Abdu, Basaznew Belew, First published: 24 June 2024 <https://doi.org/10.1155/2024/4782328>

[20] Food Science & Nutrition Volume 10, Issue 7Jul 2022-Nanotechnology-enhanced edible coating application on climacteric fruits by Temitayo Odetayo, Samson Tesfay, Nomali Ziphorah Ngobese, <https://doi.org/10.1002/fsn3.2557>

[21] Zhang, J.; Cao, C.; Wang, Y.; Xie, L.; Li, W.; Li, B.; Guo, R.; Yan, H. Magnesium oxide/silver nanoparticles reinforced poly(butylene succinate-co-terephthalate) biofilms for food packaging applications. *Food Packag. Shelf Life* 2021, 30, 100748.

[22] T. Adhikari et al. Zinc delivery to plants through seed coating with nano-zinc oxide particles *Journal of Plant Nutrition* (2016)

[23] Praveen Kumar Dubey, Rama Nath Shukla, Gaurav Srivastava, Atul Anand Mishra and Ashutosh Pandey. 2019. Study on Quality Parameters and Storage Stability of Mango Coated with Developed Nanocomposite Edible Film. *Int.J.Curr.Microbiol.App.Sci.* 8(04): 2899-2935.

[24] J.-A.H. van Burik, R.W. Schreckhise, T.C. White, R.A. Bowden, D. Myerson *Medical Mycology*, Volume 36, Issue 5, January 1998, Pages 299–303, <https://doi.org/10.1080/02681219880000471> Published: 01 January 1998

[25] J.-A.H. van Burik, R.W. Schreckhise, T.C. White, R.A. Bowden, D. Myerson *Programs in Infectious diseases and Pathology*, Fred Hutchinson Cancer Research Center; *Medical Mycology* 1998, 36, 299–303

- [26] Rapid Sanger sequencing of the 16S rRNA gene for identification of some common pathogens Linxiang Chen, Ying Cai, Guangbiao Zhou, Xiaojun Shi, Jianhui Su, Guanwu Chen, Kun Lin PLoS one 9 (2), e88886, 2014
- [27] Amin, S., Ghosh, S., Biswas, B., Arifuzzaman, M., Azad, M. A. K., & Siddiki, A. Z. (2020). Molecular identification of four medicinal plants using DNA barcoding approach from Chittagong, Bangladesh. Journal of Advanced Biotechnology and Experimental Therapeutics, 3(3), 268-272.
- [28] Kaur, S. (2015). DNA barcoding and its applications. International Journal of Engineering Research and General Science, 3(2), 602-604.
- [29] Crossley, B. M., Bai, J., Glaser, A., Maes, R., Porter, E., Killian, M. L., ... & Toohey-Kurth, K. (2020). Guidelines for Sanger sequencing and molecular assay monitoring. Journal of Veterinary Diagnostic Investigation, 32(6), 767-775.
- [30] Purty, R. S., & Chatterjee, S. (2016). DNA barcoding: an effective technique in molecular taxonomy. Austin J Biotechnol Bioeng, 3(1), 1059
- [31] Laura García-Hernández*, Diana Arenas-Islas, Pedro Alberto Ramírez-Ortega, Mizraim Uriel Flores-Guerrero and Damian Neri-Enriquez García-Hernández et al., J Nanomater Mol Nanotechnol 2016, 5:6 DOI: 10.4172/2324-8777.1000198
- [32] Isolation and Identification of Fungal Post-harvest Rot of Some Fruits in Yemen Qais Abdullah1, Ahmed Mahmoud1, Amira Al-harethi11Department of Biology, Faculty of Science, Sana'a University, Sana'a, Yemen PSM Microbiology 2016; 1(1): 36-44
- [33] Swapan K. Tripathy, Manasmita Maharana, Dinesh Manohar Ithape, Devraj Lenka, Dayanidhi Mishra, Arjun Prusti, Digbijaya Swain, Mihir Ranjan Mohanty and Reshma Raj K.R. 2017. Exploring Rapid and Efficient Protocol for Isolation of Fungal DNA. Int.J.Curr.Microbiol.App.Sci. 6(3): 951-960. doi: <https://doi.org/10.20546/ijcmas.2017.603.113>
- [34] A universal DNA extraction and PCR amplification method for fungal rDNA sequence-based identification A. M. Romanelli, J. Fu, M. L. Herrera, B. L. Wickes First published: 27 May 2014 <https://doi.org/10.1111/myc.12208>