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In vitro antifungal study of green synthesized silver nanoparticles from *Acacia nilotica* leaves extract against a plant and human pathogens

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ABSTRACT

Comparing to other nanoparticles, Silver nanoparticles have attained a special place in the field of nanotechnology due to their wider applications. This study was about to evaluate the green synthesized AgNPs for their effective Antifungal potential. The objective of this study was to evaluate the Antifungal efficacy of AgNps by using in vitro studies. In this investigation, aqueous extract of Acacia nilotica leaves was used as a reducing and stabilizing agent for the eco-friendly synthesis of silver nanoparticles. The Bio-reduction and stabilization of formed AgNps were monitored by UV-visible spectrophotometer. The surface Plasmon resonance of these particles was approximately 430 nm. The Structural, Optical and morphological properties of the synthesized AgNps were characterized by using SEM with EDAX and FTIR. The phytochemical analysis of Acacia nilotica leaves extract were also performed using various methods. The Anti-fungal activity of Plant extracts and synthesized AgNps were tested against five fungal pathogens such as Fusarium oxysporium, Aspergillus niger, Trichoderma reesei, Candida albicans and Rhizopus stolonifer using well diffusion method.

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INTRODUCTION

Medicinal plants represent a rich source of Antimicrobial agents. The use of higher plants as a source to cure various infections can be traced back over many years ago in India. There were several drugs which were produced from higher plants through the natural and artificial way. Among lakhs of plant species, only small numbers of plants were investigated for nanoparticle analysis. The unique features of silver nanoparticles had led to the development of various applications in the field of medicine. These nanoparticles were synthesized by chemical and biological synthesis. Green synthesis of silver nanoparticles was more advantageous then chemical synthesis because of its stability, non-toxicity and low cost effective. Acacia nilotica was a multipurpose medicinal plant. Many researches were carried out in this plant by various researchers due to its several properties like Antibacterial, Anti-fungal, Anti-diabetic, Anticancer, Antihypertensive, Antioxidant, etc., Dermatophytes were a common three types of fungi which causes skin infections in humans and animals. Some of these infections were Ringworm or Tinea. These infections were highly contagious which were cured by various topical and oral Antifungal agents like Miconazole, Ketoconazole, and Clotrimazole. These drugs sometimes cause inverse side

effects from minor to major, which includes liver and kidney failures through continuous uses. So, there is a need for the discovery of new effective and safer antifungal agents that overcomes these kinds of disadvantages. Treatment of these infections through nanoparticles was an alternative approach. So, any drugs which were developed using silver nanoparticles synthesized from Acacia nilotica may be effective for the treatment of fungal diseases in plants as well as animals. The synthesis of nanoparticles by green synthesis was another advantage which does not require any harmful chemical. This may be a novel approach for the development of Antifungal drugs and fungicides. This may also be very useful for the pharmaceutical industry and agricultural industry effectively for their cost-effectiveness, eco-friendliness, and no side effects.

MATERIALS AND METHODS

Plant material

The plant leaves were collected from the village of Ramanathapuram district, and it was identified as Acacia nilotica leaves by Prof.P.Jayaraman, Ph.D, Plant Anatomy and Research Centre, Chennai

Drug and Chemicals

Silver Nitrate ($AgNO_3$) was purchased from SUTHERLAND CHEMICALS. All other chemicals used were of analytical grade.

Plant Extract preparation

For the preparation of plant extract, 5 grams of Acacia nilotica leaves powder were weighed and added to the 100-ml distilled water. The extraction process C for 10-15 mins by using hot°was carried out by heating this mixture at 65 plates. After the plant sample completely dissolved to the water, the extract was filtered through muslin cloth. Then the filtered extract was stored in a refrigerator at 4°C.

Preparation of 1 mM Siver Nitrate ($AgNO_3$) Solution

0.0169 g of $AgNO_3$ was dissolved in 100 ml distilled water to prepare 1 mM aqueous solution of $AgNO_3$. This solution was kept in the dark place or wrapped the conical flask by using dark colour papers while doing a synthesis of AgNps by the use of plant extract because the silver is sensitive to light.

Green Synthesis of Silver Nanoparticles

Initially Optimization of synthesis of AgNps using plant extract was done by using different ratios such as 2:8 (2 ml of P. E + 8 ml of $AgNO_3$ solution),1:9, 7:3, 3:7, and 5:5 at different concentration of $AgNO_3$ (0.5 mM, 1 mM, and 1.5 mM). These three concentrations show a colour change at different intervals. Among this 1 mM concentration of $AgNO_3$ solution shows immediate colour change when compared to other concentration**,** For the 1 mM concentration of $AgNO_3$ solution in the 1:9 ratio was prepared as 10 ml aqueous plant extract (10 g in 100 ml distilled water) of Acacia nilotica leaves was added slowly to 90 ml 1 mM solution of silver nitrate in 250 ml conical flask and kept at room temperature for overnight. The other day the colour change of the solution from Green to Reddish brown was observed thus indicates the synthesis of AgNps. This solution was undergone further Analysis for confirmation of the presence of AgNps.

Qualitative Photochemical Analysis

The different qualitative chemical tests were performed for establishing the profile of a given extract for its chemical composition. The following tests were performed on the extracts to detect various phytoconstituents present in them.

Detection of alkaloids

Solvent-free extract (50 mg) was stirred with 2 ml of dilute hydrochloric acid (1 mL HCl + 1 mL H2O) and saturated picric acid was added. Yellow precipitate indicates positive for alkaloids (Evans, 1997).

Detection of a phenolic compound: Ferric chloride test

The extract (50 mg) was dissolved in 5 mL of distilled water. To this, a few drops of neutral 5% ferric chloride solution were added. A dark green colour indicated the presence of Phenol (Mace, 1963).

Detection of glycosides

To 2 mL of the filtrate, Sodium nitroprusside (Half pellet) and 3-5 drops of Pyridine was added. Blood red colour indicates the presence of glycosides (Evans, 1997).

Detection of terpenoids

Plant extract was added in 2 ml of chloroform. Concentrated H_2S0_4 (3 ml) was carefully added to form a layer. A reddish-brown colouration of the interface indicates the presence of terpenoids.

Detection of flavonoids

0.5 g of extract was dissolved in 5 mL of Distilled water, followed by the addition of half-pellet of sodium hydroxide. A yellow/red precipitate formation within a short period is positive for flavonoids.

Detection of saponins: Foam test The extract (50 mg) was diluted with 5 mL distilled water. The suspension was shaken in a graduated cylinder for 15 min. A 2-cm layer of foam indicated the presence of saponins (Kokate, 1999).

Detection of steroids

To the Plant extract, few drops of con sulphuric acid, followed by acetic anhydride, resulting in brown to red colour indicates positive for steroids.

Characterization of Silver Nanoparticles

UV-VISIBLE Spectroscopy

The sample of Acacia species seed was analysed in spectral studies by using double beam UV-VIS spectrophotometer, which was considered to be a very useful technique for the analysis of nanoparticles the peak at 434 nm obtained indicate the presence of silver nanoparticles (Prasad *et al.,* 2014).

Scanning Electron Microscopy

The morphology of the silver nanoparticles obtained from Acacia arabica bark was analysed by using SEM (JEOL-JSM 6360 MODEL, JAPAN). The powdered AgNps were uniformly spread and coated with platinum in an ion counter. The size distribution was obtained by an enlarged SEM image of 150 particles (Meka Venkateswara Rao *et al.,* 2016).

FTIR (Fourier Transform Infra-Red)

This FTIR measurement was carried out to identify the molecules responsible for capping and reducing agent for the silver nanoparticles synthesized from the sample. The bands in the region of 3450 cm-1 were assigned to hydroxyl stretching of polyphenols (Gangadhara *et al.,* 2014)

Anti-Fungal Activity

Test Pathogenic Fungi

An array of fungal type strains viz., Aspergillus niger (MTCC 404), Fusarium oxysporum (MTCC 7678) and Trichoderma reesei (MTCC 164), Candida albicans (MTCC 227), Rhizopus stolonifer (MTCC 2198) were used for in vitro antimicrobial activity. These selected pathogenic strains were obtained from Microbiological Division (Jayagen Biologics Analytical Laboratory, Chennai).

In vitro antimicrobial activity

The antifungal activity was determined by well diffusion methods (Holder and Boyce 1994). About 25 mL of molten potato dextrose agar was poured into a sterile Petri plate (Himedia, Mumbai, India). The plates were allowed to solidify, after which 72 h grown pathogenic fungal cultures were transferred onto a plate and made culture lawn by using sterile L-rod spreader. After five min setting of the pathogenic fungi, a sterile cork borer was used to make 5 mm well on the agar. The test samples were dissolved in 5% DMSO with sterile saline and loaded into wells with various concentrations such as 50 g/well. The solvent DMSO loaded µag/well, and 200 µg/well, 150 µg/well, 100 µ well served as negative control and Clotrimazole amended (20 µl) well served as positive control. The plates were incubated at 37°C in a 40 W fluorescent light source (~ 400 nm) for 72 h. The antifungal activity was determined by measuring the diameter of the zone of inhibition around the well using antibiotic zone scale (Himedia, Mumbai, India).

RESULTS AND DISCUSSION

Quantitative Physiochemical Analysis

Table 1: Result of phytochemical analysis in the aqueous extracts of Acacia nilotica leaves

Sl.No	TEST	RESULT
1	Phenols	+++
2	Flavonoids	+++
3	Tannins	+++
4	Alkaloids	+++
5	Saponins	-
6	Terpenoids	+++
7	Glycosides	+++
8	Steroids	+++

The Result of phytochemical analysis is shown in Table.1. In this investigation, aqueous extract of Acacia nilotica shows a positive result in all the subjected phyto constituents except saponin.

Characterization of synthesized silver nanoparticles

UV-VIS Spectroscopy

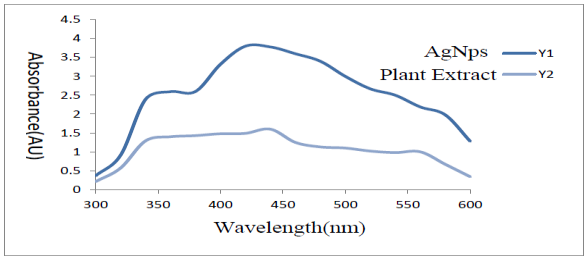

Figure 1: UV-Vis absorption spectra of AgNps synthesized from Acacia nilotica and the aqueous leaf extract

The sample of *Acacia species* seed was analyzed in spectral studies by using double beam UV-vis spectrophotometer, which was considered to be a very useful technique for the analysis of nanoparticles the peak at 434 nm obtained indicate the presence of silver nanoparticles (Prasad *et al.,* 2014)

In this investigation, the leaves extract of *Acacia nilotica,* and the AgNps were undergone the UV analysis for the confirmation of the presence of AgNps synthesized from the leaves extract. The fig of the sample solution containing silver nitrate and plant extract shows an appearance of dark green to reddish brown which confirms the existence of silver nanoparticles. This colour is due to the excitation

Table 2: Interpretation of FT-IR profile for *Acacia nilotica* plant Extract

S.NO	Peak Values	Possible Functional groups	Intensity of peak	Possible Compounds
1	1037.70	C-N	Strong	Amines
2	1103.28	C-N	Medium	Amines
3	1151.50	C-O	Strong	Carboxylic acids/Ethers/Esters
4	1240.23	N-H	Medium	Amines
5	1321.24	N-H	Medium	Amines
6	1375.25	C-H	Strong	Alkanes
7	1446.61	C-H	Medium	Alkanes
8	1539.20	NO2	Strong	Nitro compounds
9	1651.07	C-H	Strong	Phenyl ring substitution Overtones
10	1734.01	C=O	Strong	Ketones
11	2357.01	O-H	Strong	Alcohols/Phenols
12	2850.79	C-H	Strong	Alkanes
13	2918.30	C-H	Strong	Alkanes
14	3304.06	C-H	Strong	Alkanes

Table 3: Interpretation of FT-IR profile for silver nanoparticles

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14	3304.06	C-H	Strong	Alkanes

of surface plasmon resonance. As shown in the below fig the surface plasmon resonance of the AgNps has centered at 420 nm approximately, thus indicates the presence of AgNps in the solution. The UV-vis absorption spectra were shown in the fig.1. Gangadhara *et al.,* 2014 reported that the UV analysis was a powerful technique for the analysis of nanoparticles. The surface plasma on a resonance of the silver nanoparticles they synthesized from *Acacia* species was up to 430-440 nm.

SEM

The morphology of the silver nanoparticles obtained from Acacia arabica bark was analysed by using SEM (JEOL-JSM 6360 MODEL, JAPAN). The powdered AgNps were uniformly spread and coated with platinum in an ion counter. The size distribution was obtained by an enlarged SEM image of 150 particles (Meka Venkateswara Rao *et al.,* 2016).

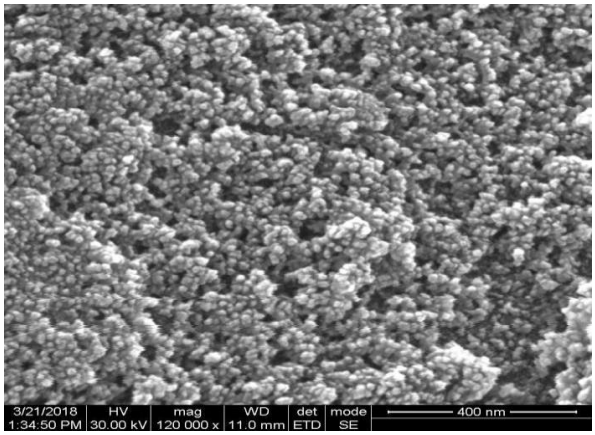

Figure 2: SEM micrograph of silver nanoparticles synthesized from Acacia nilotica leaves

In this SEM analysis, the AgNps were observed that spherical in shape ranging between 200-400 nm that were shown in fig.2 (Meka Venkateswara Rao *et al.,* 2016) was observed the spherical shaped nanoparticles of Acacia arabica bark in the SEM micrograph.

Table 4: Antifungal Activity of aqueous extract of *Acacia nilotica* leaves

Name of the organism Concentration	Zone of Inhibition (mm) Test samples				ZOI (mm) Clotrimazole 30µl /well
	50 µl/well	100 µl/well	150 µl/well	200 µl/well	
Aspergillus niger	-	-	8	9	15
Candida albicans	8	9	10	11	19
Trichoderma reesei	-	7	8	9	15
Fusarium oxysporum	-	-	-	-	12
Rhizopus stolinifer	-	-	-	-	10

Table 5: Anti-fungal Activity of Silver nanoparticles

Name of the organism Concentration	Zone of Inhibition (mm) Test samples				ZOI (mm) Clotrimazole 30µl /well
	50 µl/well	100 µl/well	150 µl/well	200 µl/well	
Aspergillus niger	7	8	9	11	16
Candida albicans	10	11	12	13	20
Trichoderma reesei	-	8	8	9	16
Fusarium oxysporum	-	-	-	8	10
Rhizopus stolinifer	-	-	-	-	10

EDAX

The elemental analysis of the silver nanoparticles obtained from the plant sample was conducted using an EDAX detector (EDS, EDAX Inc., Mahwah, NJ, USA) attached to the SEM machine (Subrahmanya Sastry *et al.,* 2016).

In this EDAX analysis was confirmed the elemental composition of the synthesized nanoparticles primarily with silicon with the highest composition, whereas silver and oxygen comes to secondary. This confirms the presence of elemental silver, which indicates the reduction of silver ion to silver nanoparticles. (Meka Venkateswara Rao *et al.,* 2016) Reported that the EDX quantitative analysis confirmed that the synthesized particles contain silver as a highest composition nest to carbon, whereas silicon and oxygen are in traces. The EDX spectra and Elemental composition of the synthesized silver nanoparticles were shown in the following Figure 3.

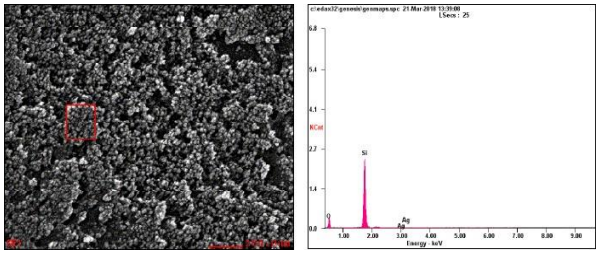

Figure 3: EDAX spectra of Silver nanoparticles with their respective SEM image

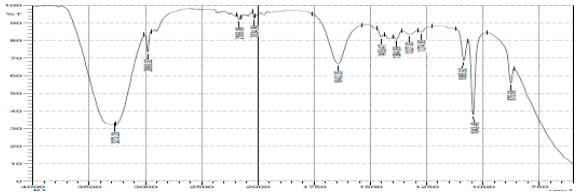

Figure 4: FT-IR spectra for *Acacia nilotica* plant extract

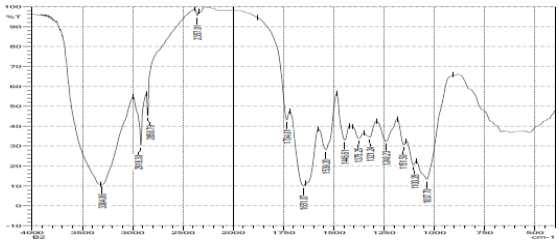

Figure 5: FT-IR spectra for silver nanoparticles

Anti-Fungal Activity

Test Pathogenic Fungi

An array of fungal type strains viz., *Aspergillus niger* MTCC 404, *Fusarium oxysporum* MTCC 7678 and *Trichoderma reesei* MTCC 164, *Candida albicans* MTCC 227, *Rhizopus stolonifer* (MTCC 2198) were used for *in vitro* antimicrobial activity. These selected pathogenic strains were obtained from Microbiological Division (Jayagen Biologics Analytical Laboratory, Chennai).

***In vitro* antimicrobial activity**

The antifungal activity was determined by well diffusion methods (Holder and Boyce 1994). About 25 mL of molten potato dextrose agar was poured into a sterile Petri plate (Himedia, Mumbai, India). The plates were allowed to solidify, after which 72 h grown pathogenic fungal cultures were transferred onto a plate and made culture lawn by using sterile L-rod spreader. After five min setting of the pathogenic fungi, a sterile cork borer was used to make 5 mm well on the agar. The test samples were dissolved in 5% DMSO with sterile saline and loaded into wells with various concentrations such as 50 µg/well, 100 µg/well, 150 µg/well and 200 µg/well. The solvent DMSO loaded well served as negative control and Clotrimazole amended (20µl) well served as positive control. The plates were incubated at 37°C in a 40 W fluorescent light source

(~ 400 nm) for 72 h. The antifungal activity was determined by measuring the diameter of the zone of inhibition around the well using antibiotic zone scale (Himedia, Mumbai, India).

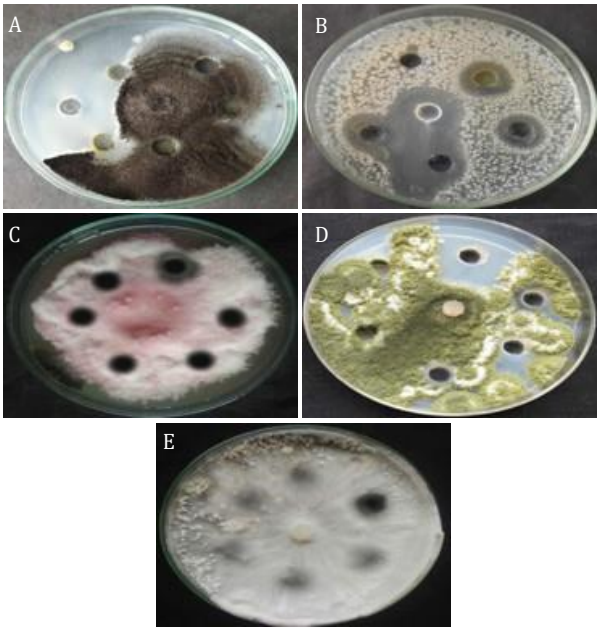

Figure 4: Antifungal Activity of aqueous extract of *Acacia nilotica* leaves *A-Candida albicans, B-Aspergillus niger, C-Trichoderma reesei, D-Fusarium oxysporum, E-Rhizopus stolinifer*

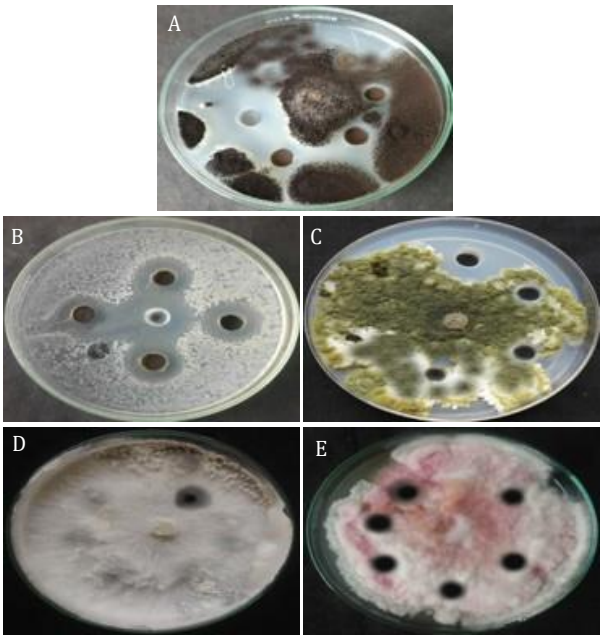

Figure 5: Anti-fungal Activity of Silver nanoparticles *A-Candida albicans, B-Aspergillus niger, C-Trichoderma reesei, D-Fusarium oxysporum, E-Rhizopus stolinifer*

In this study, the Antifungal potential of studied plant and silver nanoparticles were evaluated with varying concentrations as shown in the below Tables 4 and 5. The efficacy of the extract based on the zone of inhibition was compared with the standard antibiotic such as clotrimazole observed that the plant extract was less than those standard drugs, but the silver nanoparticles show somewhat equal zone of inhibition when compared with this drug.

(Rwarinda U Angelo *et al.,*2015) Reported that the acetonic extract of *Acacia nilotica* shows the minimum inhibition against *Aspergillus niger* which was 10 mm. Here the Aqeeous extract of *Acacia nilotica* shows 9 mm zone of inhibition against *Aspergillus niger* without using any chemical solvent, which was shown in the below Table.6. When compared to the plant extract the silver nanoparticles shows a maximum zone of inhibition against the same *Aspergillus niger* which was 11 mm as shown in Table.7

CONCLUSION

A Novel approach for the synthesis of silver nanoparticles by using an aqueous extract of *Acacia nilotica* leaves was demonstrated. In this research, *Acacia nilotica* leaves extract which is environmentally benign, act as a reducing and stabilizing agent. The

SEM result shows the morphology of silver nanoparticles using this plant extract. UV shows that the presence of silver nanoparticles. The synthesized AgNps by this green chemistry approach showed best Anti-fungal efficacy against five fungal pathogens such as *Fusarium oxysporium*, *Candida albicans*, *Aspergillus niger*, *Trichoderma reesei*, and *Rhizopus stolonifer*. The present study has opened up a possible way for synthesizing Multi-drug resistant antifungal AgNps using natural biomolecules which could be used in the pharmaceutical and agricultural industry for effective Antifungal agent. This investigation may encourage the development of Antifungal drug & fungicide. This study may also be the eco-friendly, cost-effective and has no side effects**.**

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