Harnessing *Albizzia lebbeck* Leaf Extract for Phytofabricated Silver Nanoparticles with Thrombolytic, Anticancer and Antioxidant Properties

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Abstract

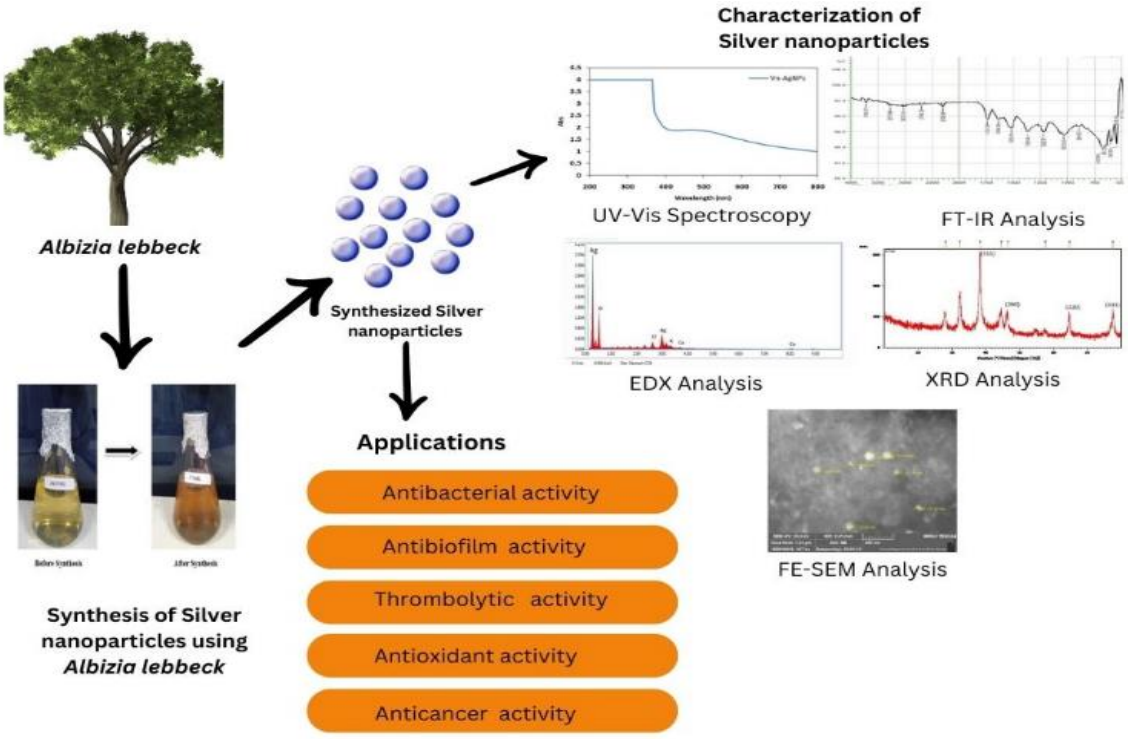
The aqueous extract of Albizzia lebbeck was used to synthesize Ag NPs and was characterized using UV-visible spectroscopy, FTIR analysis, XRD and FE-SEM, respectively. The agent proves to be an effective antibacterial, antioxidant and anticancer treatment. UV-visible spectroscopy analysis demonstrates a peak at λmax 422 nm, confirming the successful reduction of silver ions to elemental Ag nanoparticles (Ag NPs). Furthermore, FTIR analysis unequivocally validates the presence of biological moieties involved in the synthesis process. XRD and FESEM inferred that the crystalline size of the synthesized Ag NPs was 28nm and was spherical in appearance.

The nanoparticles showed promising inhibitory and dose-dependent activity on the Gram-positive and Gram-negative bacteria. A simple and rapid in vitro clot lysis model was employed to conduct the thrombolytic activity which revealed 47% clot lysis. Ag NPs exhibited cytotoxic effects on prostate cancer cells at concentrations (12, 25 and 55 µg/mL), with an IC_{50} value of 51.5 µg/mL. The results of this study suggest that A. lebbeck Ag NPs have remarkable potential multipotent drug activities and could be considered for future therapeutic applications after further analysis.

Keywords: *Albizzia lebbeck*, Ag NPs, Thrombolytic activity, Antibacterial, Antioxidant, Anticancer activity.

Introduction

Prostate cancer is a form of cancer that specifically affects the prostate gland in males. The prostate gland produces seminal fluid to nourish and transport sperm. It is a prevalent form of cancer that ranks second in terms of frequency of diagnosis. It affects approximately 15% of all male cancer cases, making it a significant health concern. Moreover, prostate cancer stands as the sixth leading cause of cancer-related deaths among men on a global scale[38]. It may require minimal or even no treatment as they are asymptomatic early. Prostate-specific antigen (PSA) testing facilitates cancer detection but does not decrease mortality[11]. Treatments may include a combination of surgery, radiation therapy, hormone therapy, or chemotherapy.



Graphical Abstract

The first cancer treatments were surgeries to relieve urinary obstruction[22]. The first systematic chemotherapy (cyclophosphamide and 5-fluorouracil) for prostate cancer was in the 1970s, followed by multiple regimens[42]. There are many problems with current cancer treatments such as unfavorable side effects, poor selectivity and drug resistance. As a result, the challenge is to find sensitive lead compounds that are efficient, economical and cell-targeted, as well as to improve their sensitivity[26]. Metal nanoparticles have found extensive utility across diverse industries due to their unique properties.

The physical, chemical and optical characteristics of metallic nanomaterials are intrinsically associated with their specific shapes, sizes and compositions. This correlation has paved the way for using nanoscale materials in many fields, from chemistry to medicine[24]. In recent years, there has been a significant emphasis on conducting extensive research on cytotoxicity using silver nanoparticles (Ag NPs). Numerous studies have investigated the impact of Ag NPs on various cell lines to understand their potentially harmful effects. Ag NPs have emerged as a promising tool in cancer therapy. These tiny particles have shown remarkable potential in targeting cancer cells selectively while minimizing the adverse effects on healthy cells. Furthermore, Ag NPs exhibit powerful anticancer properties, making them a valuable asset in the fight against cancer. Their effectiveness and low toxicity make them an attractive option for developing novel treatments in the battle against this devastating disease[20].

Albizia lebbeck is a tree that proliferates and sheds its leaves seasonally. It has a broad, expansive crown that typically reaches 15 to 20 meters, although some exceptional specimens can grow as tall as 30 meters. The trunk of this tree is straight and cylindrical and the largest specimens can have a diameter of 50 to 100 cm with some reaching an impressive 300 cm^2. It possesses several medicinal properties such as anti-asthmatic, anticancer, anti-inflammatory, anti-fertility, anti-diarrheal, antiseptic, anti-dysenteric, anti-tubercular and anti-leprosy effects. It also treats paralysis, helminth infections, allergic rhinitis and eye-related issues. It has psychoactive properties and can alleviate flu symptoms, lung problems, pectoral problems, cough, gingivitis and abdominal tumors[6].

The plant is also effective in treating ringworms and wounds when applied topically and it can be used to address conditions like Gonorrhea, Leucorrhea and other genital diseases[49]. Recently, it has been reported that zinc oxide nanoparticles derived from *Albizia lebbeck* stem bark exhibit noteworthy properties such as antimicrobial and antioxidant activities. Additionally, these nanoparticles have been found to have a cytotoxic effect on human breast cancer cells[9]. In this study, we have investigated the environmentally friendly production of Ag NPs using an aqueous extract from *A. lebbeck* to find an alternative approach to conventional chemical methods. Also, we examined the potential of these Ag NPs to inhibit the growth of prostate cancer cells by an ethnopharmacological approach.

Material and Methods

Sample collection and preparation of extracts: The leaves of *A. lebbeck* were collected in and around Karamadai, Coimbatore. The fresh leaves were washed with tap water to remove dirt or impurities and then rinsed with deionized water. After that, they were dried in the shade for 6–7 days and turned into powder using an electric blender. The leaf powder of 2 g was suspended in 100 mL of sterile distilled water. Then, the mixture was boiled for 5 minutes. Once boiled, it was allowed to cool and filtered using Whatmann no. 1 filter paper. The filtered solution was then stored in a refrigerator at a temperature of 40°C for future use[32].

Biosynthesis of Ag NPs: The leaves of *A. lebbeck* were mixed with a solution containing 1 mM $AgNO_3$ in a ratio of 1:9. The mixture was then placed under sunlight for 3 minutes to accelerate the reaction and turn it brown. After that, it was left at room temperature for 1 hr to finish the reduction process. The samples were centrifuged at 12,000 rpm for 15 minutes to remove the liquid on top and then washed with deionized water. This centrifugation process was repeated two to three times to obtain pure Ag NPs. Finally, the samples were dried in an oven at 50°C for further analysis[1].

Characterization of synthesized Ag NPs: The absorbance value of Ag NPs in a solution was determined using a UV-visible spectrophotometer within the wavelength range (λ) of 300-600 nm[31]. The specific functional group of biosynthesized Ag NPs was examined using FTIR in the wavelength range of 400 - 4000 cm^{-1} to determine the particular functional group of biosynthesized Ag NPs. The preparatory process involved dispersing Ag NPs in a dry KBr matrix and compressing them to form a transparent disc. A KBr pellet served as a reference standard[50]. The sample's crystalline nature was detected by analyzing the diffracted X-ray 2Θ degree, generated using a Cu anode at 40 kV and 30 Mv[3].

The Field Emission Scanning Electron Microscope (FE-SEM) was utilized to examine and to analyze the surface morphology and shape[40]. Energy-dispersive X-ray spectrometers use the photon properties of light. To analyze the material, we performed EDX analysis by measuring the energy and intensity distribution of X-ray signals. This was done by focusing an electron beam on a specimen operating at 120 kV using an EDX instrument. The collected data were used to determine the element composition of the material[15].

Antibacterial activity: The biosynthesized Ag NPs were tested for their antibacterial activity against Gram-positive and Gram-negative bacteria using the well-diffusion method. Bacterial species were swabbed on Mueller-Hinton agar. Wells of 6 mm diameter were created and filled with different concentrations of Ag NPs (25, 50, 100 and 150

µg/mL). The plates were incubated at 37°C for 24 hours. The diameter of the inhibition zone was measured to assess the antibacterial activity of the Ag NPs. Vancomycin and gentamycin were positive controls for Gram-positive and Gram-negative bacteria respectively[8].

Antibiofilm activity: The impact of extracts on biofilm formation was assessed using 96-well polystyrene flat-bottom plates. The bacterial cultures were grown overnight and mixed with LB broth at 1-2 x 10^{-6} CFUs/mL. These samples were then put into a 96-well plate and kept at 37°C for 5 hours. After the incubation period, the old culture medium was replaced with a new medium that contained varying amounts of Ag NPs and then incubated at 37°C for 24 hours. After removing the medium, the samples were washed twice with sterile water. Then, they were dried at room temperature for 30 minutes. The biofilms were stained with a 0.1 % solution of crystal violet for 20 minutes. The excess stain was washed away with sterile water and the stained biofilms were dried for 1 hour at room temperature. Each stained biofilm sample was treated with 200 mL of absolute ethanol and agitated vigorously for 15 minutes to extract the stain. The optical density (OD) was measured at 590 nm[44].

***In vitro* thrombolytic analysis:** Approximately 3 mL of fresh blood was collected from volunteers and promptly divided into 5 sterile microfuge tubes, placed in an incubator at 37°C for 45 minutes. After incubation, the tubes were carefully removed and weighed again to determine the weight of the clots formed. 100µL of Ag NPs were added to the clot tube at various concentrations. The tubes were then placed in an incubator at 37°C for 90 minutes to observe the lysis of the clots. The fluid released after incubation was again weighed to observe the difference in weight after clot disruption[43].

$$\%\ of\ clot\ lysis = \left(\frac{Weight\ of\ released\ clot}{Clot\ weight}\right) \times 100$$

Antioxidant activity

DPPH Radical Scavenging Activity: The antioxidant activity of the sample was evaluated using the stable radical DPPH technique, which measures its ability to donate hydrogen or scavenge radicals. To perform the test, different concentrations of Ag NPs (ranging from 20 to 100 µL) were mixed with 100 µL volume of 0.1 mM DPPH solution in methanol. The mixture was then allowed to stand at 27°C for 20 minutes. The absorbance of the sample was measured at a wavelength of 517 nm[39]. The percentage of radical scavenging activity in the sample was determined as follows:

$$\%\ DPPH\ radical\ scavenging\ activity = \left(\frac{Control\ OD - Sample\ OD}{Control\ OD}\right) \times 100$$

Hydrogen peroxides radical scavenging activity: 0.1 mL of Ag NPs in phosphate buffer was combined with 0.3 mL of phosphate buffer and 0.6 mL of H_2O_2 solution. The mixture was mixed and after 10 mins, the absorbance was measured at 230 nm using a UV-Vis spectrophotometer. Vitamin C was used as a standard, while phosphate buffer was blank. The percentage of H_2O_2 scavenging activity was calculated[16]. The rate of H_2O_2 radical scavenging activity was determined as follows:

$$Scavenging = \frac{\mathrm{Oc} - \mathrm{Os}}{Oc} \times 100$$

where Oc represents the absorbance of the control and Os represents the absorbance of Ag NPs/Vitamin C.

Ferrous Ion Chelating Activity: The ferrous ion chelating activity was performed with slight modifications[21]. Different concentrations (200-100 µL) of Ag NPs were combined with 30 µL of 5 mM ferrozine and 50 µL of 2 mM ferrous sulfate. The mixture was left at room temperature for 10 minutes and the absorbance of the sample was measured at 562 nm. The percentage of ferrous ion chelating activity was determined as follows:

$$\%\ Ferrous\ ion\ chelating\ activity = \left(\frac{Control\ OD\ sampleOD}{Control\ OD}\right) \times 100$$

Cytotoxicity assay of Ag NPs: The prostate cancer cell line was selected for the MTT assay. The cells were grown in DMEM with 10% FBS, 0.2% $NaHCO_3$ and 1 mL/100 mL of antibiotic-antimycotic solution. They were placed in 96-well tissue culture plates and incubated overnight in a CO_2 incubator. Then, the medium was aspirated and replaced with media containing different concentrations (12, 25 and 55 µg/mL) of the synthesized nanoparticles. MTT (10 µL/ well containing 100 µL of the cell suspension; 5 mg/mL of the stock in PBS) was added to each well and incubated for four hrs. After carefully removing the reaction mixture, 200 µL of DMSO was added to each well and the contents were homogenized by pipetting up and down numerous times. An ELISA reader was used to read the color at 570 nm after 10 minutes. The untreated control was also run simultaneously under identical conditions serving as control[10].

Results and Discussion

In the current investigation, the extract derived from *A. lebbeck* leaves were employed as a reducing agent in the synthesis of Ag NPs. The UV-visible absorption spectrum of Ag NPs is depicted in figure 1. Ag NPs exhibited an absorption peak at 422 nm, attributed to surface plasmon resonance (SPR) resulting from the stimulation of the metal's unbound electrons during the Ag NP formation process. This is comparable to the studies previously documented regarding the reduction of Ag NPs through the utilization of the extracts and the examination of Ag NPs for their potential therapeutic uses[32,37]. The determination of the presence of various functional groups in a biosynthesized plant extract was conducted through FTIR analysis. As depicted in figure

2, the FTIR spectra of an Ag NPs biosynthesized plant extract were obtained. The measured absorption peaks were compared with reference values, revealing multiple functional groups. This observation suggests that the extract comprises a variety of phytochemicals[46]. The FTIR spectrum exhibited a prominent peak at 1639 cm^{-1}, attributed to the C-C stretching in alkene compounds.

The CBr alkyl halides are assigned to the medium intensity band 678 cm^{-1}. The band at 2704 cm^{-1} is attributed to the C-H stretching vibration of the methyl, methylene and methoxy groups. The peak at 1739 cm^{-1} indicates the presence of C-O stretching in the alcohols, amides, ester and ether groups. The extract spectra revealed several comparable absorption peaks at 3278, 2925, 1519 and 1033 cm^{-1}. The observed aromatic vibrations correspond to the peak at 3032 cm^{-1}. The constant vibrational bands such as -OH, -CH, -C = C and -C = O in *A. lebbeck* are sourced from flavonoids and terpenoids. Various functional groups suggest that certain phytochemicals have transferred from the plant extract to the Ag NPs, where they have played a significant role as possible reducing and capping agents during biosynthesis[35].

The X-ray diffraction (XRD) pattern of the synthesized Ag NPs is presented in figure 3. The XRD technique is advantageous in providing information about the geometric arrangements and interatomic distances, thereby supporting the formation of nanoparticles[48]. The intense peaks observed at 38.25°, 46.53°, 64.74° and 77.55° correspond to (111), (200), (220) and (311) planes respectively. These results are consistent with previously reported values associated with the spherical structure of photosynthesized Ag NPs. The Scherrer formula was utilized to calculate the crystallite size which was determined to be an average of 28 nm for the spherical nanoparticles[16].

The EDX spectrum depicted in figure 4 illustrates the presence of metallic Ag NPs, as evidenced by the intense peak detected at 3 keV. Additionally, the EDX scan revealed the interaction of Cl with the nanoparticles. It is plausible that the plant extract, which is known to contain abundant amounts of chloride, may have produced these chloride ions[47]. Furthermore, the C and O peaks observed on the EDX spectrum may be attributed to the various metabolites in the plant extract[29]. The surface morphology (precisely the shape and size of the synthesized Ag NPs) was investigated *via* FESEM. The resulting FESEM image depicted in figure 5 distinctly displayed the spherical shape of the synthesized Ag NPs.

Additionally, the electron micrograph revealed that the size of the Ag NPs was distributed within an average diameter range of approximately 18 to 54 nm. Notably, numerous other studies have reported the biosynthesis of Ag NPs with extracts of various plants, yielding particles of nearly identical size range[36].

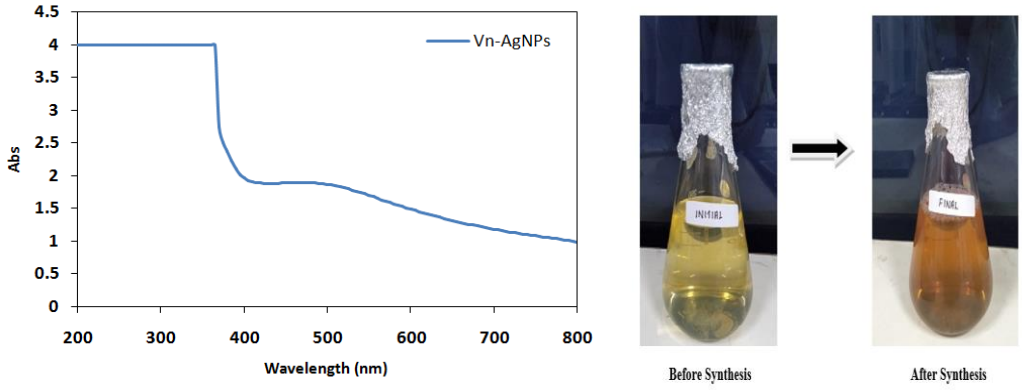


Fig. 1: UV-Vis spectra of Ag NPs synthesized with *A. lebbeck* leaf extract

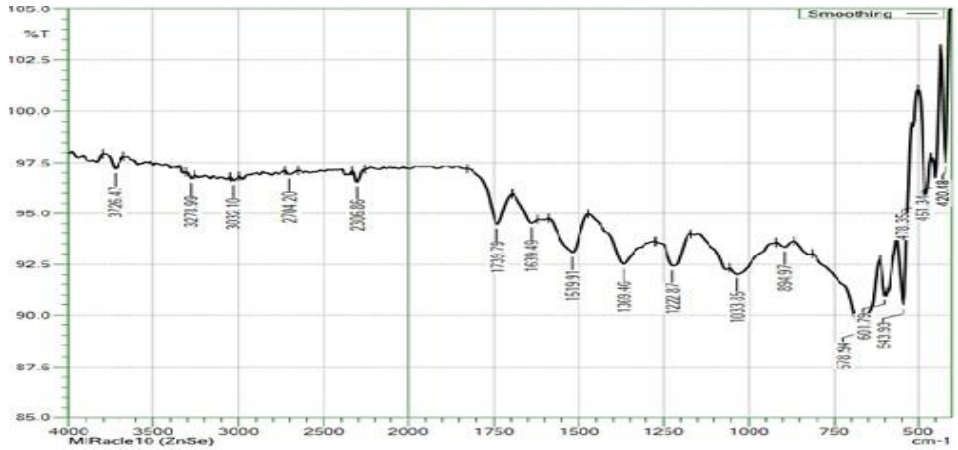


Fig. 2: FTIR analysis of Ag NPs synthesized with *A. lebbeck* leaf extract

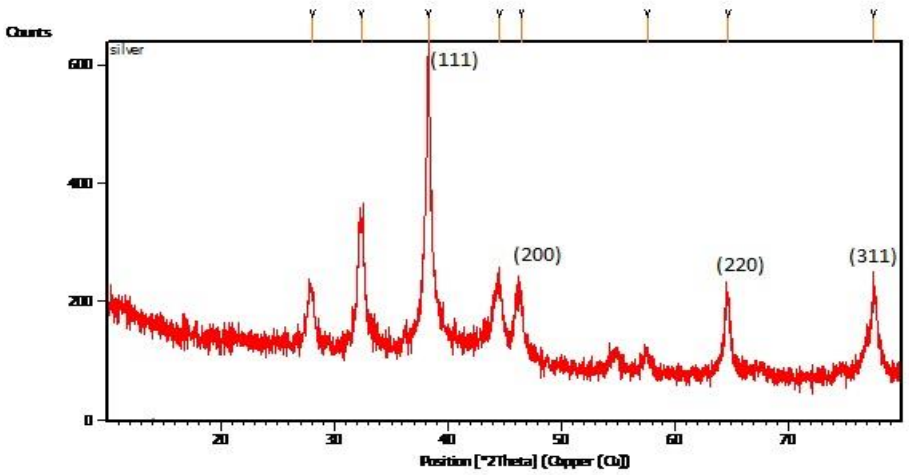


Fig. 3: XRD analysis of Ag NPs synthesized with *A. lebbeck* leaf extract

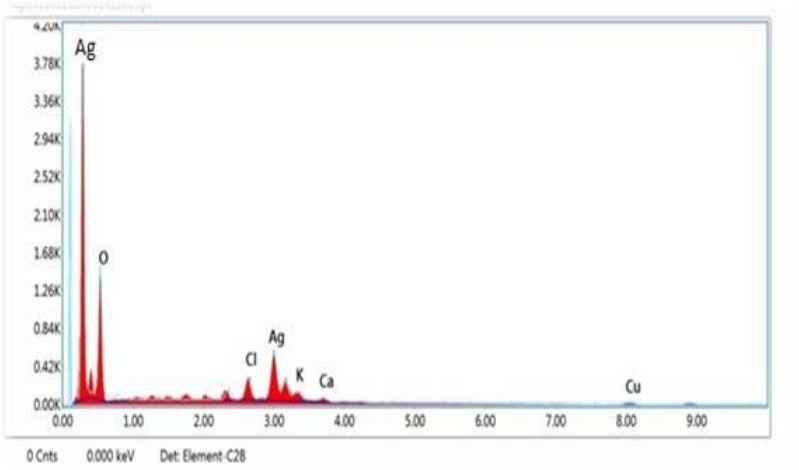


Fig. 4: EDX analysis of Ag NPs synthesized with *A. lebbeck* leaf extract

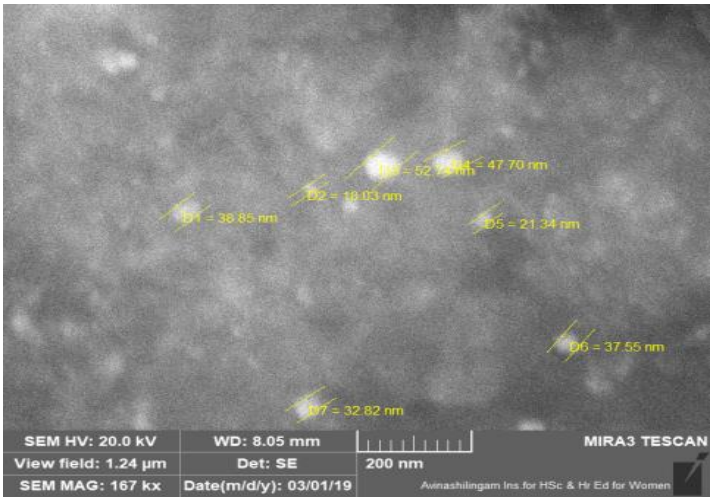


Fig. 5: FESEM analysis of Ag NPs synthesized with *A. lebbeck* leaf extract

Antibacterial Activity: The study aimed to assess the antibacterial efficacy of *A. lebbeck* Ag NPs through the agar well diffusion method. Various concentrations of plant-derived Ag NPs were employed to determine their impact on bacterial growth. The results indicated that an increase in the concentration of Ag NPs led to the suppression of bacterial growth across all strains. Notably, the most significant inhibition was observed at a concentration of 0.14 mg/mL, which showed the highest antibacterial activity against the Gram-negative bacterium *E. coli*. Notably, even a minute concentration of Ag NPs has been reported to suppress bacterial growth effectively[14,19]. It is widely held that the

antibacterial efficacy of Ag NPs is predominantly attributed to the destruction of cellular membranes.

The antibacterial activity of Ag NPs can interfere with intracellular signaling pathways. Moreover, research has demonstrated that the antibacterial mechanism of biogenic Ag NPs is linked to the disruption of cellular membranes, followed by the generation of reactive oxygen species that can disturb normal cellular processes, leading to DNA damage and protein denaturation. Additionally, Ag^+ ions produced by Ag NPs interfere with the bacterial cell cycle, induce mitochondrial dysfunction and trigger apoptosis[28,40].

Antibiofilm activity: The present study aimed to evaluate the *in vitro* anti-biofilm activity of Ag NPs against *S. aureus*, known to form biofilm. The inhibitory effect of Ag NPs on biofilm formation was assessed in a dose-dependent manner with concentrations ranging from 20-100 g/mL. Following a 24-hour culture of the bacteria in 96-well microtiter plates, each well was treated with individually produced Ag NPs.

The results demonstrated that the biosynthesized Ag NPs effectively hindered the ability of *S. aureus* to form a biofilm (Fig. 6). Notably, at 100 µg/mL, it exhibited an impressive antibiofilm action of approximately 64.5% (Table 1). Exopolysaccharides (EPSs) are essential for bacterial biofilm and are synthesized within 24 hours and secreted by bacterial cells in response to external stimuli[21]. The control of biofilm development can be achieved by inhibiting EPS production. Our study on the anti-biofilm activity of Ag NPs was based on this principle. Lateef et al[23] also demonstrated that the suppression of EPS production is associated with anti-biofilm activity. Our findings suggest that the sensitivity of the bacteria to Ag NPs may be linked to the intricate biofilm signaling process and cell viability.

Thrombolytic Activity: Utilizing nanoparticles in thrombolysis presents a solution to the challenges posed by conventional anti-thrombotic treatments such as neutralization by antibodies, short half-lives and excessive bleeding risk, particularly with streptokinase[4]. Furthermore, metallic nanoparticles have been reported to possess anticoagulant and thrombolytic properties[22]. This study aimed to investigate the thrombolytic activity of *A. lebbeck* Ag NPs using a simple and rapid *in vitro* clot lysis model with a 90-minute incubation period at 37°C. The results indicated that 100 µL of *A. lebbeck* Ag NPs led to 47% clot lysis, as demonstrated in figure 7. Conversely, clots treated with 100 µL of sterile distilled water (a negative control) showed negligible clot lysis. Notably, the study revealed that the highest percentage of clot lysis was achieved with a high concentration of synthesized *A. lebbeck* leaf extract.

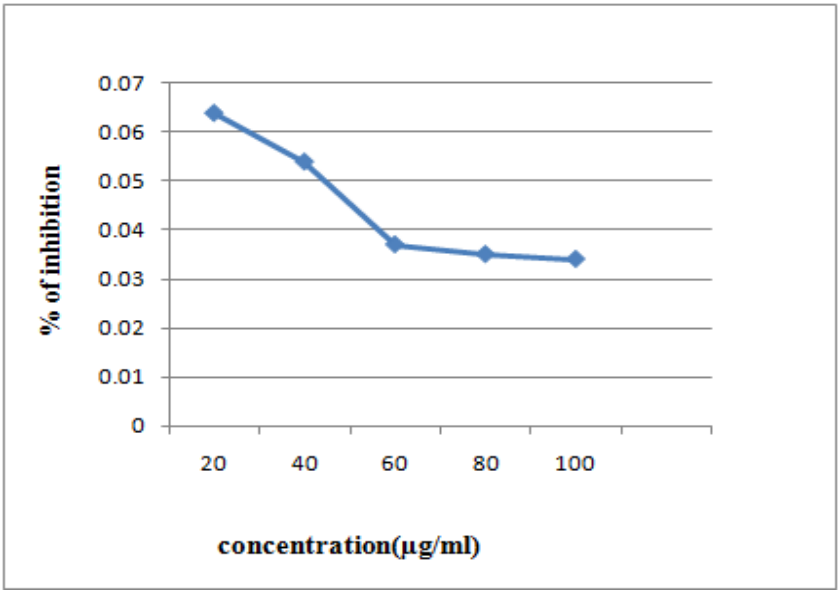


Fig. 6: Antibiofilm activity of Ag NPs synthesized with *A. lebbeck* leaf extract

Table 1
Biosynthesized Ag NPs effective against *S. aureus* to form a biofilm

Concentration (µg/mL)	% of inhibition	Biofilm activity (%)
Control	0.096	0
20	0.064	33.3%
40	0.054	48%
60	0.037	61.4%
80	0.035	63.5%
100	0.034	64.5%

Antioxidant Activity

DPPH radical-scavenging activity: The biosynthesized nanoparticles exhibit significant potential for scavenging DPPH radicals, as evidenced by their IC_{50} value of approximately 75.47-80 g/mL, as depicted in figure 8. This value is comparable to Bhakya et al[7] for the scavenging activity of Ag NPs. Furthermore, the free radical scavenging capabilities of nanoparticles are attributed to the functional groups of the bio-reductant molecules. Additionally, the ability of the nanoparticles to adhere to their surfaces may result in increased surface areas for activity[34].

Hydrogen peroxides radical scavenging activity: Superoxide dismutase is an enzyme that plays a role in H_2O_2 production in living organisms. H_2O_2 possesses the ability to traverse biological membranes. Despite its relatively low reactivity, it causes a threat to cells due to the significant generation of hydroxyl radicals, which can lead to cellular damage and various severe disorders[51]. The efficacy of the synthesized Ag NPs in scavenging OH was assessed and the results indicate that the sample displays robust radical scavenging activity with an IC_{50} value of 87.7 g/mL, as depicted in figure 9.

Ferrous-ion radical scavenging activity: Figure 10 illustrates the concentration-dependent reducing power of Ag NPs (20-100 g/mL) derived from *A. lebbeck*. The results indicate a decrease in reducing power as the concentration of Ag NP increases. Notably, a significant increase in reducing power was observed at 20 g/mL, with an IC_{50} value of 98-100 g/mL. The reducing power value of Ag NPs demonstrates their ability to reduce the Fe^{3+}/ferricyanide complex to the ferrous form. The presence of phenolic functional groups in the plant extract is crucial for the antioxidant action[13,45]. The phenolic antioxidant effect is primarily attributed to the electron-donating activity, as evidenced by Fe III reduction. The reduction ability of any compound depends on the reduced ones and reductions play a vital role in breaking the free radical chain and donating an H atom, thereby exerting an essential antioxidant function.

Cytotoxic Assay of Ag NPs: Following a 24-hour treatment, it was observed that *A. lebbeck* Ag NPs exhibited a dose-dependent suppression of prostate cancer cells. Cytotoxicity testing was conducted at 48 and 72 hours. However, no significant differences were observed in comparison to the results obtained at 24 hours. The cytotoxicity of synthesized Ag NPs was demonstrated with an IC_{50} value of 51.550 g/mL out of the three concentrations (12, 25 and 55 g/mL) examined, as shown in figure 11. The tumor-inhibiting effects of the synthesized nanoparticles were validated by the IC_{50} values of the Ag NPs against PC3 (Table 3). According to reports, the cytotoxicity of Ag NPs to cell lines is attributed to their cellular absorption through pinocytosis and endocytosis.

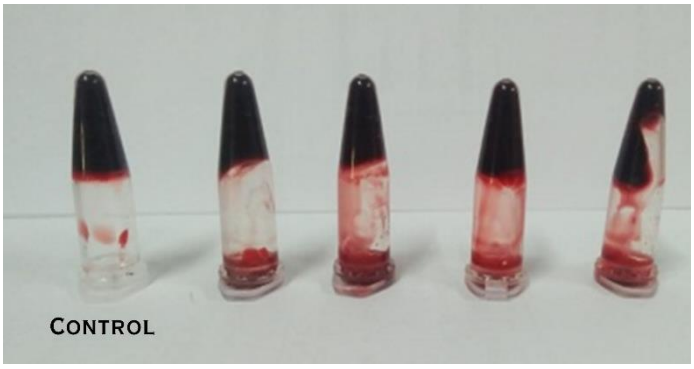


Fig. 7: Thrombolytic activity of Ag NPs synthesized with *A. lebbeck* leaf extract

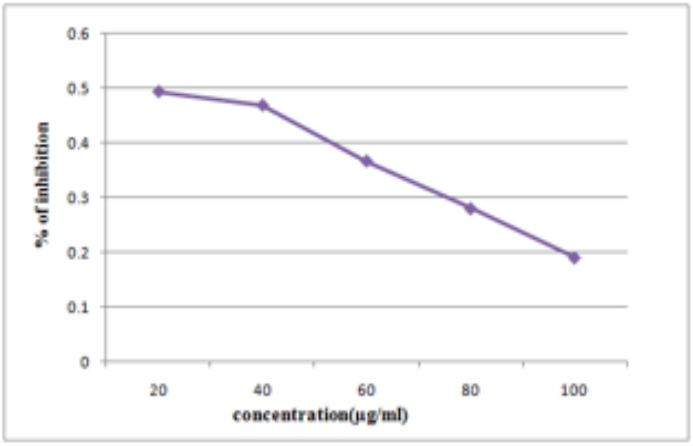


Fig. 8: DPPH radical-scavenging activity of Ag NPs synthesized with *A. lebbeck* leaf extract

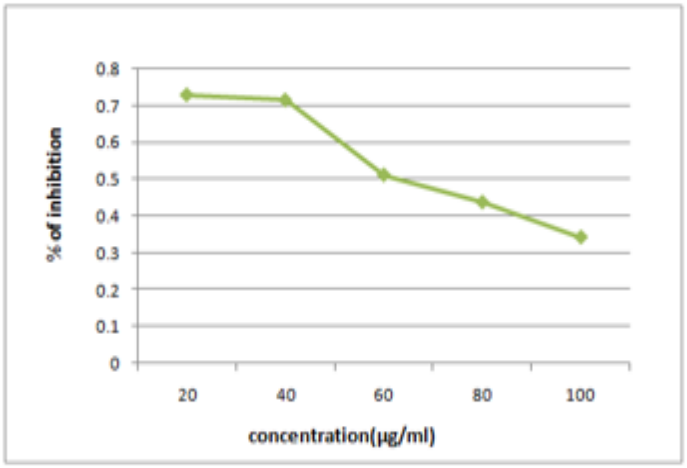


Fig. 9: Hydrogen peroxide radical scavenging activity of Ag NPs synthesized with *A. lebbeck* leaf extract

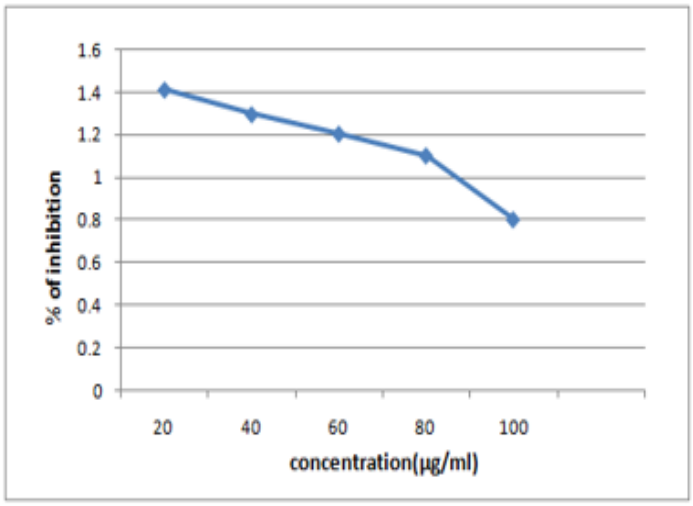


Fig. 10: Ferrous-ion radical scavenging activity of Ag NPs synthesized with *A. lebbeck* leaf extract

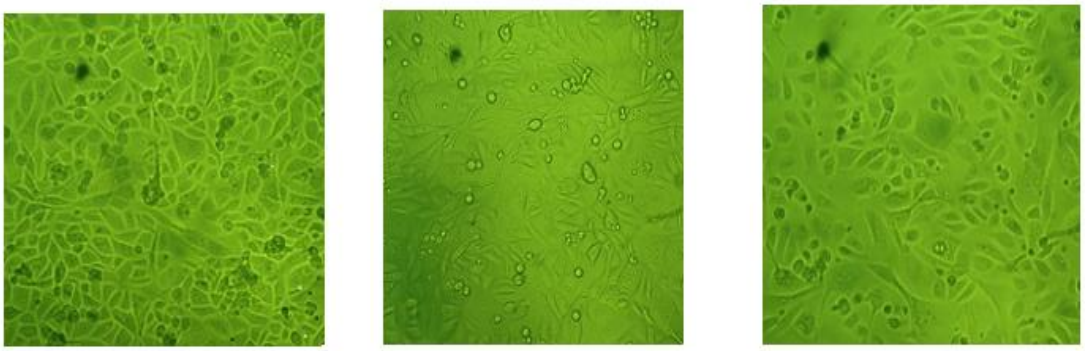


Fig. 11: Anticancer activity of Ag NPs synthesized with *A. lebbeck* leaf extract

Table 2
Cytotoxic Assay of Ag NPs synthesized with *A. lebbeck* leaf extract

Sample concentration (µg/mL)	OD value at 570nm	% of inhibition
Control	0.568	0
12	0.565	0.52%
25	0.385	32.21%
55	0.265	53.34%

NPs are considered cytotoxic when cell viability falls below 50%[47,48]. The cytotoxicity of Ag NPs is influenced by the plant extract surface coating and nanoparticle size[5,17,27,33]. Ag NPs cause an increase in intracellular ROS and cell death occurs as reactive hydroxyl radicals damage DNA. Ag NPs coated with plant constituents cause oxidative stress, which triggers death through mitochondrial and caspase-mediated pathways. Nanoparticles induce the production of hydroxyl radicals and are highly detrimental to cancer cells[12].

Conclusion

A. lebbeck is a versatile and beneficial plant with numerous applications and advantages. Its ornamental value, ecological benefits, traditional medicinal properties and timber potential highlight its significance in various domains. It contains saponins, macrocyclic alkaloids, tannins and flavanols. Recently, the anticancer properties of flavanols have garnered the attention of numerous researchers in various cancer types, including prostate cancer.

The utilization of Ag NPs derived from *A. lebbeck* plant extract has been proposed as a potential anticancer agent against prostate cancer cell lines. The present study aims to elucidate the characteristics of Ag NPs from *A. lebbeck* and establish a fundamental basis for their use in *in vitro* studies and as an anticancer agent against prostate cancer cells. Ag NPs from *A. lebbeck* have been reported to possess excellent antibacterial, anti-thrombolytic, anticancer and antioxidant properties, indicating their potential therapeutic value. In the future, extensive research may lead to the discovery of valuable medicinal drugs from this source.

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