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Evaluation of Antibacterial, Antioxidant, and Anti-Inflammatory Activities of Green Synthesized Silver Nanoparticles

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ABSTRACT: Kalanchoe pinnata (Syn: Bryophyllumpinnatum) medicinal plant belongs to the Crassulaceae family. This study aimed to investigate the antibacterial, antioxidant, and anti-inflammatory activities of silver nanoparticles synthesized from Kalanchoe pinnata. The nanoparticles were eco-friendly synthesized at room temperature and were characterized using various methods such as Visual interpretation, UV-visible spectroscopy, DLS, SEM, and FT-IR. The antibacterial activity of the nanoparticles was evaluated against two strains of Gram-positive bacteria (Bacillus subtilis and Staphylococcus aureus) and two strains of Gram-negative bacteria (Escherichia coli and Klebsiella pneumoniae) using the disc diffusion method. The results showed that the AgNPs-KP exhibited significant antibacterial activity against all tested bacteria. The in vitro antioxidant activity was assessed using DPPH and Phosphomolybdenum (PM) assays, and the AgNPs-KP showed potent antioxidant activity in both assays. Moreover, the in vitro anti-inflammatory activity of AgNPs-KP was evaluated by the Protein denaturation method, and the results showed that the AgNPs-KP exhibited a significant anti-inflammatory effect. Therefore, the green synthesized silver nanoparticles from Kalanchoe pinnata could be a potential candidate for the development of a new generation of antibacterial, antioxidant, and anti-inflammatory agents.

KEYWORDS: Green synthesis, Kalanchoe pinnata, Silver nanoparticles (AgNPs-KP).

I. INTRODUCTION

In recent years, the exploration of nanotechnology has opened up new avenues for biomedical research, leading to innovative approaches in medicine and health. One such promising area of investigation involves synthesizing and applying silver nanoparticles (AgNPs) for their potential antibacterial, antioxidant, and anti-inflammatory properties. Kalanchoe pinnata, a well-known medicinal plant with a rich history of traditional use, has drawnconsiderable attention in thiscontext. This study delves into the in vitro evaluation of silver nanoparticles' antibacterial, antioxidant, and anti-inflammatory activities derived from Kalanchoe pinnata, shedding light on their potential therapeutic applications in modern healthcare. [1] Such results hold promise for harnessing the therapeutic potential of natural resources in nanoscale formulations, intending to improve human health and well-being. [2]

Silver nanoparticles have garnered significant attention in the field of nanotechnology due to their unique properties and versatile applications. These nanoparticles, typically in the range of 1 to 100 nanometers in size, exhibit extraordinary optical, electrical, and antimicrobial characteristics. [3] In the medical field, silver nanoparticles have shown remarkable potential for a variety of applications. Their unique properties, including their small size and high surface area, have led to numerous innovations and breakthroughs. It can serve as a drug carrier, enhancing the delivery of therapeutic agents to specific targets in the body. Their controlled-release properties are invaluable for improving drug efficacy and reducing side effects.[4]

Plant profile

Kalanchoe pinnata, widely recognized as the "Life Plant" or "Bryophyllumpinnatum," is a succulent species native to tropical and subtropical regions, including Africa, Asia, and the Americas. [5] The leaves of Kalanchoe pinnata, often characterized by their distinctive notches and the ability to propagate new plants from these leaflets, are a rich source of phytoconstituents.Traditional and folk medicine systems have harnessed the potential of this plant for treating a wide spectrum of ailments, including wound healing, anti-inflammatory effects, anti-diabetic properties, and antimicrobial activities. The leaves of Kalanchoe pinnata contain a diverse array of phytochemical

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compounds, including Flavonoids, such as quercetin and kaempferol, which are widely found in the leaves and are known for their antioxidant and anti-inflammatory properties.Alkaloids, like bufadienolides,contribute to the plant's anti-inflammatory and potential anti-diabetic effects. [6]

Figure 1. Kalanchoe pinnata

Organic acids, including malic acid and citric acid, are present in the leaves and may have a role in the plant's antimicrobial properties. Glycosides, such as bryophyllin A, are among the bioactive compounds in Kalanchoe pinnata and have been associated with wound healing and anti-inflammatory actions. Triterpenes, like β-sitosterol and lupeol, are found in the leaves and may contribute to their diverse medicinal uses. Various polyphenolic compounds, such as epicatechin and catechin, are known for their antioxidant properties and are present in the leaves.

II. MATERIALS AND METHODS

Plant collection and Authentication

The plant Kalanchoe pinnata was collected from our medicinal garden, at PPG College of Pharmacy, Coimbatore, Tamil Nadu, India. It was taxonomically identified and authenticated by Dr. M. U. SHARIEF, SCIENTIST 'E' & HEAD OF OFFICE, Botanical Survey of India, TNAU Campus, Coimbatore, Tamil Nadu, India.

Preparation of plant extraction

The fresh leaves of Kalanchoe pinnata were collected from the medicinal garden and then washed thoroughly with tap water to remove the dust and dirt particles. Leaves were weighed and crushed. Add 100ml deionized water and heat at 80^{o}C for about 10 minutes. The aqueous leaf extract was taken and filtered using the Whatman No. 1 filter paper. The obtained leaf extracts were used as reducing and capping agents for the green synthesis of AgNPs. [7]

Preparation of 1mM Silver Nitrate solution

0.1699g of Silver Nitrate ($AgNO_3$) was added into 1000ml of deionized water and stirred continuously for 1-2 minutes to get 1mM Silver Nitrate solution.

Green synthesis of Silver Nanoparticles (AgNPs)

10 ml of plant extract was added to the 90 ml of 1mM $AgNO_3$ solution at room temperature and stirred continuously for 15 minutes using a magnetic stirrer. Slow reduction takes place and is kept for 24 hours to obtain the colour change. [7] [8]

Qualitative characterization techniques

The characterization study of synthesizedSilvernanoparticles was done by the examining size and shape of nanoparticles. Several techniques are used for this purpose, including Visual interpretation, UV-visible spectroscopy, Scanning Electron Microscopy (SEM), Dynamic Light Scattering (DLS), and Fourier Transform Infrared (FT-IR). [7] [8]

Antibacterial Activity

The antibacterial activity of silver nanoparticles wascarried out by the standard disc diffusion method. The antibacterial activity was tested against both Gram +ve(Staphylococcus aureus, Bacillus subtilis) and Gram –ve(Klebsiella pneumoniae, Escherichia coli) bacteria. Nutrient agar (1g beef extract, 1g peptone, 0.5 g NaCl dissolved in 100 ml of double distilled water) was used to cultivate bacteria. The media was autoclaved and cooled. The media was poured into the petri dish and kept for 30 minutes for solidification. After 30 minutes the fresh overnight cultures of inoculum of four different cultures were spread onto solidified nutrient agar plates. Sterile paper discs made of Whatman filter paper, 5 mm diameter. The disc dipped in silver nanoparticles at different concentrations of 10µg and 20µg,and plant extract, and standard solution (20µg) were placed in each plate. Ciprofloxacin was used as standard. The cultured agar plates were incubated at 37°C for 24 h. The zone of inhibition was calculated by measuring the diameters of the inhibition zone around the well. [8]

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Antioxidant Activity

2, 2-Diphenyl-1-picrylhydrazyl (DPPH) Assay

In DPPH free radical scavenging activity, different concentrations of standard solution (ascorbic acid) were prepared. To 1 ml of methanolic solution of DPPH (0.2 mM), different concentrations of Silvernanoparticles (50, 100, 150, 200, 250 µg/ml) were added. To serve as a control, instead of the nanoparticle sample, an equivalent amount of standard phosphate buffer was added to the reaction mixture. The mixture was shaken and allowed to stand at room temperature for 30 min and the absorbance of reaction mixtures measured at 517 nm. The percentage scavenging activity of different concentrations was determined and the formula for percent scavenging activity is:

DPPH scavenging effect (%) = A_0-A_1/A_0 × 100,

WhereA_0 is theabsorbance of the control and $A_1$is the absorbance of the sample. [9]

Phosphomolybdenum Assay (PM Assay)

A reagent mixture was prepared by combining 16.7 ml of H_2SO_4, 5.3 g of sodium phosphate, and 2.5 g of ammonium molybdate with different concentrations (50, 100, 150, 200, 250 µg/ml) of both AgNPs and the standard (ascorbic acid). A 4 ml reagent solution was then mixed with the AgNPs solution and standard, maintaining the combination at 95°C for 1 hour and 30 minutes in a water bath, with the reagent solution in a distinct vial. Subsequently, the absorbance was measured at 695 nm after cooling, compared to a blank of the reagent suspension. The percentage scavenging was calculated using the formula:

% Scavenging = (A_c - A_s) / A_c × 100,

Where, $A_s$is the absorbance of the sample and $A_c$is the absorbance of the control. [10]

Anti-inflammatory activity

Protein denaturation assay

The assay was by the reaction mixture (5 ml) consisting of 0.5 ml of egg albumin and 4.78 ml of phosphate-buffered saline (pH 6.4) and different concentrations of (50, 100, 150, 200, 250 µg/ml) AgNPs and standard (Diclofenac sodium) the mixture was mixed, and incubated in a water bath (37 ◦C) for 15 min. Then the reaction mixture was heated at 70^{o}C for 5 min. After cooling, the turbidity was measured at660 nm. A phosphate buffer solution wasused as the control.The percentage inhibition of protein denaturation was calculated by using thefollowing formula:

Percentage of inhibition = (V_c - V_s / V_c) x 100,

Where $V_s$is the absorbance of the sample and $V_c$is the absorbance of the control. [11]

III. RESULTS & DISCUSSION

Visual interpretation

Visual identification of the colour change is used as a preliminary confirmatory test of nanoparticle synthesis. Color change occurs due to Surface Plasmon Resonance (SPR) which involves the oscillation of free electrons in the reaction mixture.The colour change of the reaction mixture from white to different shades of brown indicates the synthesis of silver nanoparticles. The colour change appeared after 3 hours of incubation of the plant extract with $AgNO_3$ solution. The intensity of the color increased till 22 hours and after 22 hours nanoparticles started to settle down at the bottom of the flask indicating the completion of the synthesis process. Figure 2 represents the colour change obtained after 22 hours of incubation of the reaction mixture. [7]

Figure 2. SynthesizedAgNPs after 22 hours

UV-Visible Spectroscopy

UV-visible spectroscopy was used to characterize the Kalanchoe pinnata-mediatedSilver nanoparticles to find the structural properties ofAgNPs. The absorbance spectra range from 345-450 nm after 24 hours of synthesized silver nanoparticles as shown in Figure 3. The absorption peak of Kalanchoe pinnata intervenedsilver nanoparticles was obtained at 448 nm which denotes the intense absorption in the visible light region. It states the reduction and stabilization capability of Kalanchoe pinnata extract. [8]

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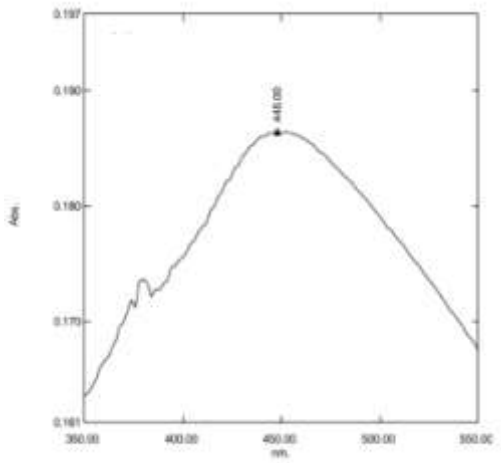

Figure 3. Absorbance peak of green synthesized AgNPs

Dynamic Light Scattering (DLS)

Particle Size Analysis

The particle size determination of green synthesized AgNPs was shown based on intensity. Figure 4 shows the average particle size (z-average) of AgNPs is found to be 552.2 nm. Particle size analysis showed the presence of nanoparticles with a polydispersity index (PDI) value of 0.791 with an intercept of 0.916.

Zeta Potential

Zeta potential ofAgNPs was found to be -14.9 mV in Figure 5. A negative value in the zeta potential of silver nanoparticles might be possible due to the presence of hydroxyl (OH^-) functional groups adsorbed on the surface of both nanoparticles during capping which are responsible for increasing stability owing to electrostatic repulsion between each nanoparticle. [7] [8]

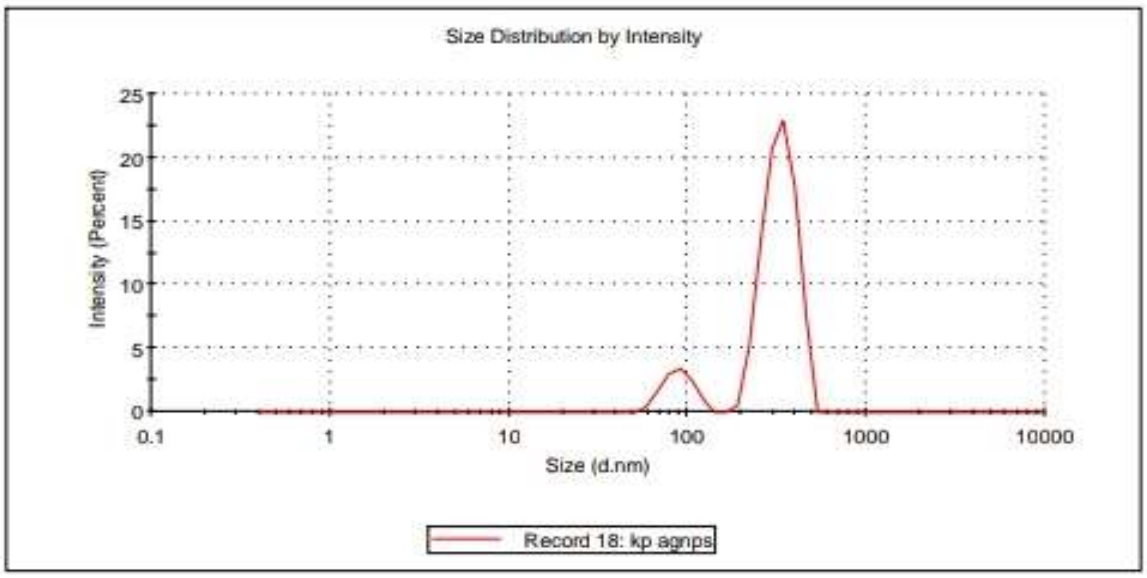

Figure 4. Size distribution of AgNPs

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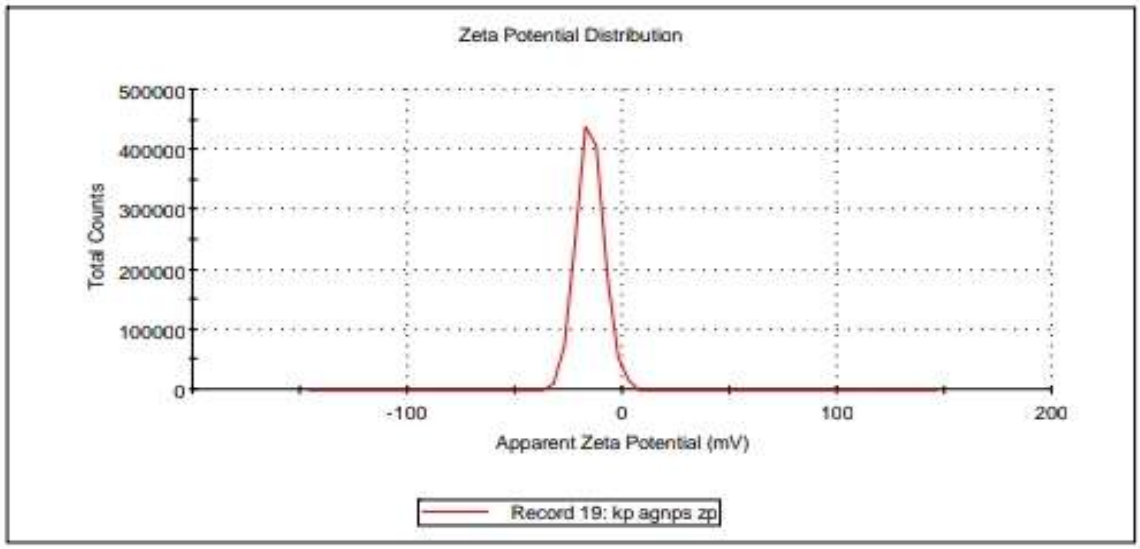

Figure 5. Zeta potential of AgNPs

Scanning Electron Microscopy (SEM)

The nanoparticles were characterized for their structure and morphology by SEM analysis. The SEM images of the silver nanoparticles were taken using EDAX-FEI, QUANTA 200. Figure 6 shows the size of silver nanoparticles (AgNPs) is less than 100 nm and the shape is spherical.

FTIR spectroscopy

Figure 7 shows the FTIR of synthesized AgNps peaks at 3865.35, 2978.09, 2368.59, 2314.58, 1550.77 and 1396.46 cm^{-1}. The interactions between Kalanchoe pinnata and the supporting materials were characterized by analyzing the samples with FTIR spectroscopy at room temperature. In the FT-IR spectrum of AgNPs the sharp and intense peak at 3865.35cm^{-1} and 2978.09 is due to the –OH group in Kalanchoe pinnata. It is well known that H_2O and CO_2 molecules are easily chemisorbed onto the Ag surface when exposed to the atmosphere. In the spectrum of the sample shown in Figure 7, the sharp band at 3865.35 $cm^{-1}$and 2978.09 is associated with the OH stretching vibrations of surface-adsorbed water molecules. For the AgNPs, the 2978.09 cm^{-1},2368.59 cm^{-1} and 2314.58 cm^{-1}sharp peak due to C–H stretching. For the Silver nanosized particles existence was confirmed. A strong band was observed at 2368.59 cm^{-1} and 2314.58 cm^{-1} along with two other shoulder peaks at 1550.77 cm^{-1} and 1396.46 cm^{-1}. When the metal nanoparticles form in the solution, they must be stabilized against the Van der Waals forces of attraction which may otherwise cause coagulation. FTIR analysis data confirms the presence of O–H stretching (around 3865.35 and 2978.09cm^{-1}) which may be responsible for reducing metal ions into their respective nanoparticles

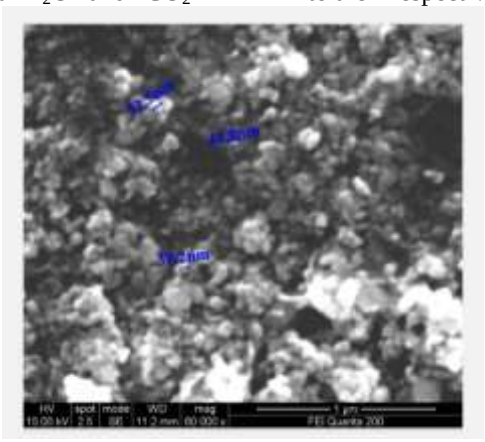

Figure 6. SEM image of AgNPs

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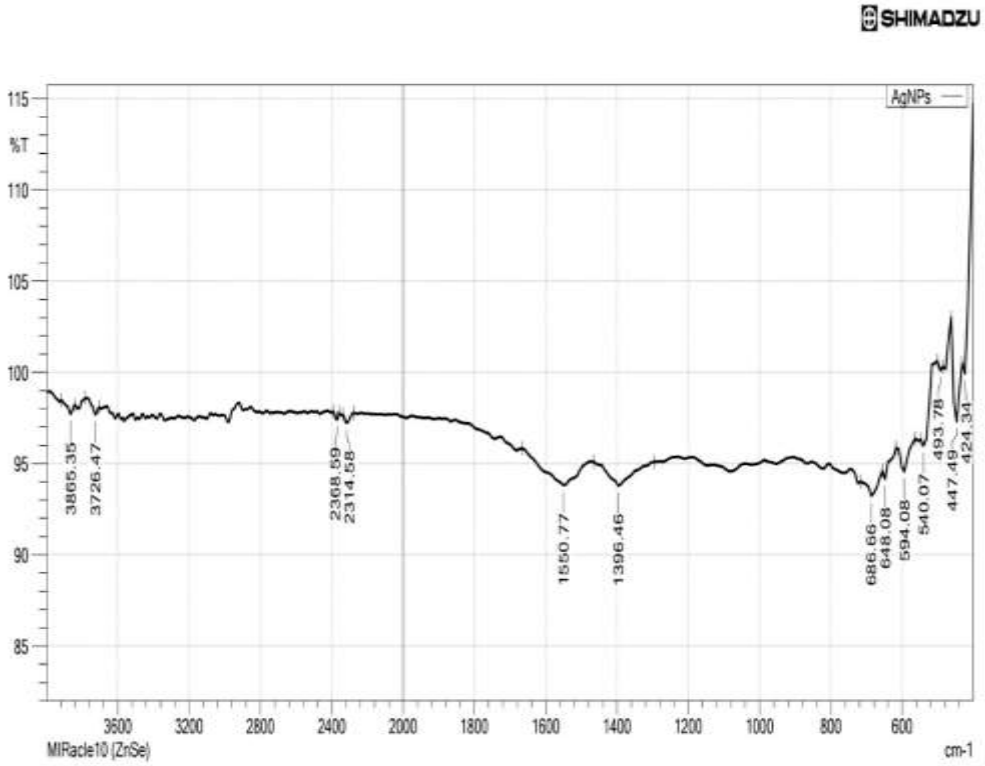

Figure 7.FT-IR of AgNPs

Antibacterial Activity

The antibacterial activity of synthesized nanoparticles was checked against both Gram +ve(Staphylococcus aureus, Bacillus subtilis) and Gram -ve (Klebsiella pneumoniae, Escherichia coli) bacteria. Silver nanoparticles showed a significant inhibitory effect on both Gram-positive and Gram-negative bacteria shown in Figure 8 and Table 1. The zone of inhibition observed by nanoparticles was greater than a plant extract shown in Figure 9. Some of the mechanisms reported for the antibacterial effect of nanoparticles were disruption of the cell membrane by reactive oxygen species (ROS), binding to the cell membrane, lipid peroxidation, and disruption of DNA, RNA, and protein synthesis process.

Antioxidant Activity

DPPH (2, 2-Diphenyl-1-picrylhydrazyl) Assay

Unpaired electrons in free radicals lead to cellular damage, making them extremely unstable. One free radical can start a chain reaction that damages other atoms or molecules. This is how free radicals are formed. A free radical is created when an electron is lost similarly. Subsequently, this free radical interacts with the subsequent molecule. The activity goes on in this manner. To lessen the negative effects of free radicals, antioxidants are molecules with sufficient stability to donate an electron to produce a stable complex. The DPPH test is used in this study to evaluate the biologically produced AgNPs' ability to function as an antioxidant. DPPH is often reduced to DPPH-H by the antioxidants in the sample. The level of discolouration was used to measure the antioxidant compound's scavenging capacity. Deep purple in hue, DPPH exhibits high absorption at 517 nm. Afterreacting with the antioxidant, this colour becomes colourless or pale yellow. The results of the scavenging ability of the standard (ascorbic acid) and Green synthesized silver nanoparticles are listed in Table 2, silver nanoparticles scavenge31.66, 43.11, 50.23, 60.74, and 65.04percent of conventional ascorbic acid at concentrations of 50, 100, 150, 200, and 250 µg/ml, respectively, while standard ascorbic acid scavenges 49.18, 54.16, 60.66, 69.57, and 74.59 percent at comparable concentrations (Figure 10).

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Bacteria	Minimum Inhibitory Concentration (MIC) in mm			
	Ciprofloxacin	Kalanchoe pinnata Plant extract	AgNPs (10µg/ml)	AgNPs (20µg/ml)
Staphylococcus aureus	32	7	16	20
Bacillus subtilis	38	6	15	23
Klebsiella pneumoniae	36	6	19	22
Escherichia coli	38	7	18	25

Table 1. Antibacterial activity of Green synthesized silver nanoparticles

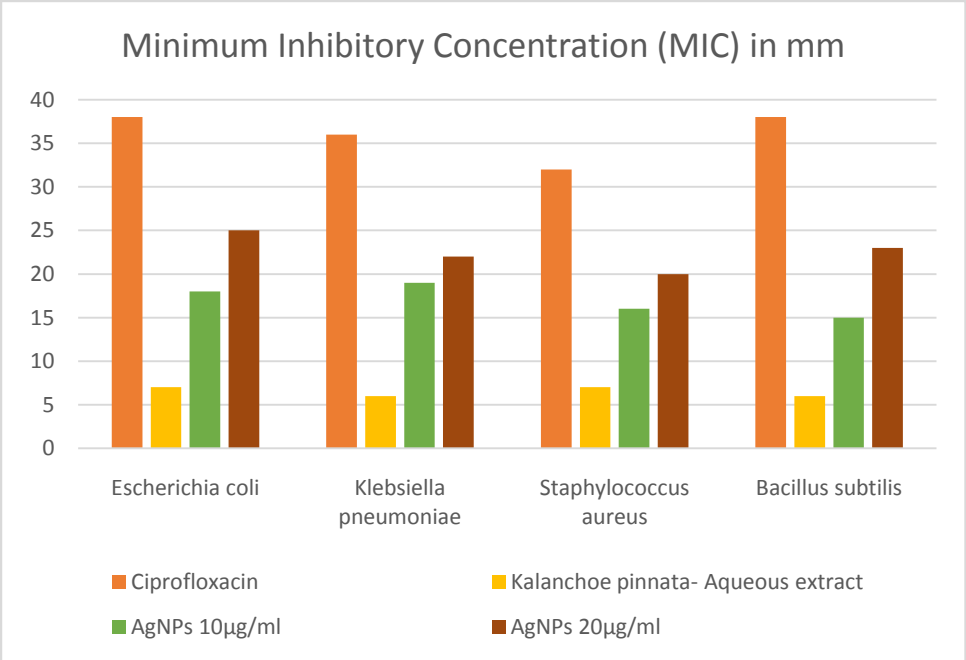

Figure 8. MIC of Silver nanoparticles against pathogenic bacteria

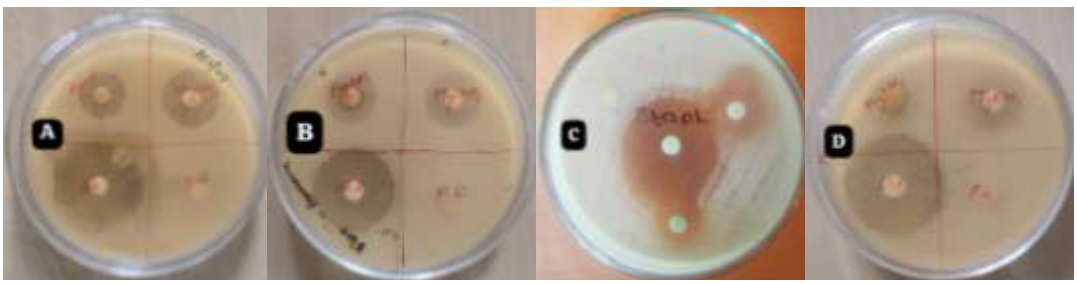

Figure 9. Antibacterial activity against pathogenic bacteria: (A) E. coli. (B) K. pneumoniae. (C) S. aureus. (D) B. subtilis.

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Table 2. DPPH % Scavenging of Standard and sample

Concentration (µg/ml)	% of Scavenging	
	Standard (Ascorbic Acid)	Sample (AgNPs)
50	49.18	31.66
100	54.16	43.11
150	60.66	50.23
200	69.57	60.74
250	74.59	65.04

Phosphomolybdenum Assay(PM Assay)

The scavenging capacity of green synthesized silver nanoparticles is also assessed using this technique. This procedure is called a phosphomolybdenum (PM) assay because it uses a phosphomolybdate reagent. The phosphomolybdate reagent changes colour when the plant extract is applied, indicating a decrease in phosphomolybdenum. Table 3 shows the percentage of scavenging for both the standard (ascorbic acid) and sample silver nanoparticles (AgNPs). Ascorbic acid is scavenged up to 13.8 percent at a content of 50µg/ml and up to 75.71percent at a dose of 250µg/ml. Silver nanoparticles scavenged up to 8.57 percent at 50µg/ml and 53.3percent at 250 µg/ml.

Anti-inflammatory Activity

Protein Denaturation Assay

The anti-inflammatory assessment of green synthesized AgNPs was evaluated to check their ability to reduceinflammation. The findings were then compared to those of acommon anti-inflammatory medication (diclofenac sodium). Table 4 shows thepercentage inhibition of both the standard drug (diclofenac sodium)andsilver nanoparticles. Diclofenac sodium reduced protein denaturation by up to24.31% at 50 µg/ml and up to 86.03% at 250 µg/ml.Silver nanoparticles reduced protein denaturation up to31.96 percent at a cncentration of 50 µg/ml and up to 65.30 percent at a concentration of 250µg/ml.

Concentration (µg/ml)	Absorbance of Control	Absorbance of Standard (L-Ascorbic acid)	Absorbance of Sample (AgNPs)	% Scavenging of Standard	% Scavenging of Sample
50	0.210	0.181	0.192	13.8	8.57
100	0.210	0.155	0.175	26.19	16.66
150	0.210	0.111	0.144	47.14	31.42
200	0.210	0.082	0.117	60.95	44.28
250	0.210	0.051	0.098	75.71	53.3

Table 3. PM Assay – Percentage Scavenging of Standard and Sample

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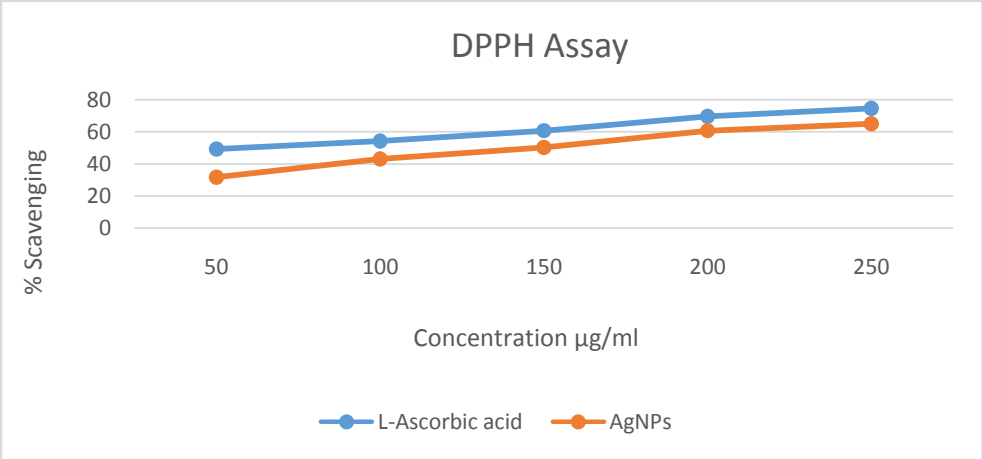

Figure 10. DPPH Assay of AgNPs& Standard

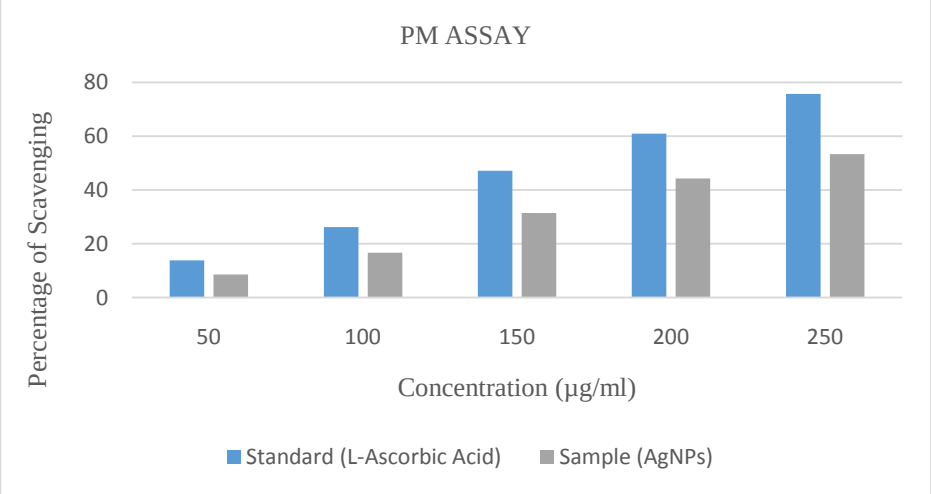

Figure 11. PM Assay – Percentage Scavenging of Standard and Sample

Concentration (µg/ml)	Absorbance of Control	Absorbance of Standard (Diclofenac sodium)	% Protein inhibition of Standard	Absorbance of Sample (AgNPs)	% Protein inhibition of Sample
50	0.366	0.277	24.31	0.249	31.96
100	0.366	0.218	40.43	0.192	47.57
150	0.366	0.162	55.73	0.176	51.91
200	0.366	0.0862	76.44	0.159	56.55
250	0.366	0.0511	86.03	0.127	65.30

Table 4. Protein denaturation assay

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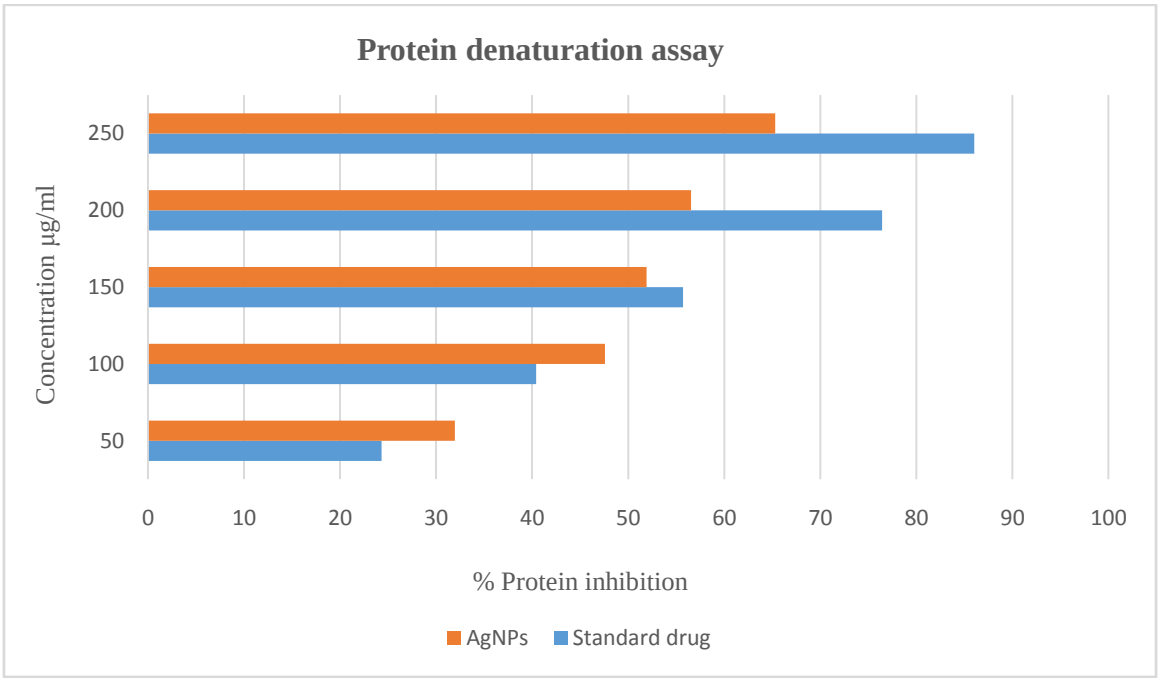

Figure 12. Protein denaturation assay

IV. CONCLUSION

The green synthesis of silver nanoparticles using plant extracts has many advantages over other methods as they are cost-effective, easily scaled up, and environment-friendly. Kalanchoe pinnata showed great capability to synthesize AgNPs at optimum temperature conditions. The formation of Ag nanoparticles in the extract was observed by the visual interpretation. The UV absorption peak at 448 nm indicates the synthesis of AgNPs.FTIR for characterizing nanoparticle surfaces. DLS and SEM studies validated the size and charge of the nanoparticles in the colloidal system without any aggregation in which nanoparticles are mostly spherical and with a size range of 1-100 nm. The plant itself acts as a capping and reducing agent in the formation of nanoparticles. Silver nanoparticles have great Antibacterial activity in selected bacteria. The anti-inflammatory activity of Ag nanoparticles was performed by using diclofenac sodium as a standard. The percentage inhibition was calculated, and it was observed that the AgNPs showed 65.30% inhibition, while at a similar concentration standard (diclofenac sodium), they showed 86.03% inhibition.The phosphomolybdenum technique and DPPH were the two methods used to measure antioxidant activity.The percentage of standard and silver nanoparticles that were scavenged increased with concentration. The DPPH & PM assay scavenging potential ofthe green synthesized AgNPs was 65.04% and 53.3% whereas ascorbic acid demonstrated a 74.59%and 75.71% scavenging potential at a comparable dose. In all techniques, conventional ascorbic acid and silver nanoparticles demonstrated comparable levels of free radical scavenging.Therefore, the studyconcludes that biologically synthesizedAgNPs might be excellent topical antibacterial, antioxidant, and anti-inflammatory medications.

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Conflict of Interest: None.
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