EVALUATION OF SILVER NANOPARTICLES OF *Averrhoa carambola* LEAF EXTRACT FOR ANTI-OXIDANT, ANTI-COAGULANT AND THROMBOLYTICACTIVITIES

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ABSTRACT

Biosynthesized silver nanoparticles are securing attention due to biologically active secondary plant metabolites that enable in green synthesis and also by virtue of their unique biological applications. Novel approach on green synthesis of silver nanoparticle using leaves of Averrhoea carambola have been carried out and evaluated for antithrombotic and anticoagulant activity. The silver nanoparticles were identified using FTIR, NMR, Mass Spectroscopy and X-ray diffraction method. In vitro activity of green synthesized silver nanoparticles were carried out for their antioxidant, anticoagulant and anti-thrombolytic activity. The antioxidant activity of the biosynthesized nanoparticles was determined using 2,2-diphenyl-1-picrylhydrazyl (DPPH).The concentration ranging from 25µg/ml to 800µg/ml of AgNPs sample were tested for the free radical scavenging activity by DPPH method. The percentage inhibition of AgNPs sample shows IC50 484.861 when compared to Ascorbic acid with IC50 24.589. For anticoagulant activity the test was performed at room temperature on the collected fresh blood. In control, the blood clot was formed within 5 minutes however, there is any mark of coagulation in the fresh blood sample added with AgNPs after 1 hour. This suggest that AgNPs synthesized from *Averrhoa carambola* might have anticoagulant activity. The thrombolytic activity was evaluated by adding AgNPs suspension to the clot, within 30 minutes the clot was completely lysed which indicate thrombolytic activity of the synthesized AgNPs from *Averrhoa carambola* leaves. Characterization of AgNPs of Avehoea carambola were done using UV-visible, FTIR spectral analysis and X ray diffraction. AgNPs showed reliable anticoagulant andthrombolytic and DPPH radical scavenging assay utilizing Ascorbic acid as standard. Thus, the results concluded that the synthesized AgNPs has comparable anticoagulant, thrombolytic and antioxidant activities.

Keyword: *Averrhoa carambola,* AgNPs, Anticoagulant, Thrombolytic activity

Graphical representation of Plan of Work

Collection of A*verrhoa carambola* leaves

Extraction of A*verrhoa carambola* leaves

Preliminary phytochemical screening

Green synthesis of silver nanoparticles

Characterization of silver nanoparticles

- **UV-Visible Spectroscopy**
- **FTIR Spectroscopy**
- **X-Ray Diffraction**

***In vitro* biological evaluation**

- **Antioxidant activity**
- **Anticoagulant activity**
- **Thrombolytic activity**

1. INTRODUCTION

Medicinal plants have been used in virtually all cultures as a source of medicine. Assurance of the safety, quality, and efficiency of medicinal plants and herbal products has now become a key issue in industrialized and in developing countries. The widespread use of herbal remedies and health care preparations is described in the Vedas and Bible. Medicinal plants have been used for thousands of years to flavor and conserve food, to treat health disorders and to prevent diseases including epidemics. The knowledge of their healing properties has been transmitted over the centuries within and among human communities. Active compounds produced during secondary metabolism are usually responsible for the biological properties of plant species used throughout the globe for various purposes including treatment of infectious diseases. (1)

1.1 *Averrhoa carambola*

FIG 1. *Averrhoa carambola* plant

Averrhoa carambola a tiny, rapidly growing, heavily branched, evergreen tree that can occasionally reach heights of 10 meters. It typically grows 3 to 5 meters tall. The short bole's diameter can reach 15 cm. A versatile tree with a very well-liked fruit as well as several medicinal and other applications. The plant *Averrhoa carambola* commonly seen in our local areas. Studies in this plant has reported such as antidiabetic, anti-hyperlipidemic, anti-hypertensive, anti-microbial, CNS depressant, anti-ulcer, anti-oxidant, anti-inflammatory, anti-tumour, immune boosting activities.

1.2 Silver Nanoparticles

AgNPs growing production and application in various areas including catalysis, consumer products, food technology, textiles as well as medical products and devices. Silver nanoparticles are the particles in the size ranging between 1-100 nm. The two major methods used for synthesis of silver nanoparticles are physical and chemical methods. Biological method is being used as an expedient alternative, as this approachis environment friendly and less toxic and it includes plant extracts, microorganisms, fungi, etc. The major application of silver nanoparticles in the medical field includes diagnostic applications and therapeutic applications, apart from its antimicrobial activity. (3) The biochemical properties of silver nanoparticles like anti-coagulant, thrombolytic, anti-oxidant activities can also be explored to assess their potentiality in the field of hematology.

Plants are envisaged and easily available source of bioactive plant secondary metabolites such as polysaccharides, proteins, polyphenols, flavonoids, terpenoids, tannins, alkaloids, amines, ketones, and aldehydes, which act as reducing, stabilizing, and capping agents in the conversion of metal ions to metal nanoparticles, leading to the production of desirable nanoparticles with predefined characteristics. Among various biosynthesized metal nanoparticles, silver nanoparticles (AgNPs) have emerged as the champion in the last two decades due to their unique biological, chemical, and physical properties

2. METHODS AND MATERIALS

2.1Collection of *Averrhoa carambola* Leaves

The fresh leaves of *Averrhoa carambola* was used for the preparation of *Averrhoa carambola* leaf extract. The fresh leaves were collected from the *Averrhoa carambola*plant in the surroundings and this specimen sample was identified and authenticated (Authentication number: BOT/KUBH/137/23) by the Department of Botany, Kerala University, Trivandrum.

2.2 Extraction of *Averrhoa carambola* Leaves

Fresh leaves were washed thoroughly with distilled water and weighed 60g of leaves, 240ml of distilled water was added, and the mixture was heated at 80^0C for 3 hours with continuous stirring and resultant extract was then filtered through Whatmanfilter paper. (8)

2.3 Preliminary Phytochemical Screening

Preliminary phytochemical screening of alkaloids, tannins, saponins, phenols, flavonoids,glycosides were carried out in *Averrhoa carambola* leaf extract.

2.4 **Green synthesis of Silver Nanoparticle**

30 mL of aqueous leaf extract of *Averrhoa carambola* was added to 270 ml of 1mM aqueous silver nitrate solution, followed by heating at 80^0C for 3 hrs with constant stirring. The formation of AgNPs was preliminary detected by the change in colour from yellowish green to dark brown. [8] Obtained AgNPs were separated by centrifugation at 10,000 rpm for 25 minutes.

2.5 Characterization of Silver Nanoparticles

2.5.1 UV-Visible Spectroscopic Analysis

UV-Visible spectroscopy is a quantitative technique used in analytical chemistry to measure the amount of light absorbed by a substance. The principle of UV-Visible spectrum is based on the production of distinct spectrum by the absorption of ultraviolet light or visible light by chemical compounds, which results in the production of distinct spectra. When light falls upon a substance it absorbs and reflects a certain amount of radiation.1 mg of AgNPs were dissolved in 1 mL of distilled water then absorbance was taken at 300-800nm.

2.5.2 FTIR Spectroscopic Analysis

The FTIR spectrum was used to identify the functional groups of active components in the AgNPs based on the peak value in the region of infrared radiation. The wavelength of light absorbed in characteristic of the chemical bond as can be seen in the annotated spectrum by interpreting the infrared absorption spectrum, the chemical bonds in a molecule can be determined. [9]

The AgNPs were encapsulated separately in KBr pellet, to prepare translucent sample discs. The sample was loaded in FTIR spectroscopy with a scan range from 4000-500 cm^{-1} on Shimadzu FTIR spectrometer. [9]

2.5.3 X-RAY Diffraction

Principle

X-Ray diffraction is now a common technique for the study of crystal structure and atomic spacing. It is based on construct an interface and monochromatic X-Rays froma crystalline sample, this X-rays are generated by a cathode ray tube filtered to producemonochromatic radiation collimated to concentrate and directed towards the sample. The interaction of the generated X-rays on incidence with sample produces constructive interface, producing diffracted X-ray satisfying Bragg's law of diffraction given by,

$$n\lambda = 2d \sin\theta$$

n = Integer

λ = Wavelength of X-Ray

d = Interplanar spacing

Θ = Diffraction angle

Method

- Clean the sample well, spatula, glass microscope with isopropyl alcohol.
- Place a sheet of white paper under the sample well.
- Place the sample.
- Then the sample well placed into an appropriate sample space.
- Insert the prepared sample into the desired sample analysis location to the instrument.
- Set X-Ray generation voltage and volt 40kv - 60kv in diffractometer.

2.6 *Invitro* Biological Evaluation

2.6.1 Determination of Anticoagulant activity

Principle

Anticoagulant activity, which means the prevention or reduction of coagulation of blood or prolonging the clotting time. Commonly using anticoagulants such as Heparin, Warfarin etc. are known as blood thinners.

Methods

The anti-coagulant activity of the obtained silver nanoparticles was evaluated on freshly collected human blood at room temperature. 1 ml suspension of AgNPs (Concentration 50 μg/mL) was added to 10 mL fresh human blood (Vial B) another 10 mL of blood without addition was taken as control (Vial A). The two blood samples were then observed for next 1 hour at room temperature for any noticeable changes. [10]

2.6.2 Determination of Thrombolytic Activity

Principle

Thrombolysis, also called as fibrinolytic therapy, which means the breakdown ofblood clots formed in blood vessels using medications. It is used in ST elevation myocardial infarction, stroke, and in case of severe venous thromboembolism. It also plays an important part in reperfusion therapy that deals specifically with blocked arteries.

Method

Thrombolytic activity for the obtained AgNPs was evaluated by dissolving fresh human blood clots. 2-3 drops of fresh blood were spread on a clean glass slide and allowed to form clot. 0.5 ml suspension of AgNPs (Concentration 50μg/mL) was then added to it. The blood clot sample was then observed visually at room temperature for next 1 hour to observe various changes of thrombolysis. [10]

2.7 Determination of Antioxidant Activity

Principle

Radical scavenging activity of the test sample against stable 2, 2- diphenyl 2-picrylhydrazyl hydrate (DPPH) was determined according to the method of Brand-William et al., (1995) with slight modification. DPPH reacts with an antioxidant compound, which can donate hydrogen, and reduce DPPH. The change in color (from deep violet to light yellow) was measured at the optical density 515 nm on a UV visible spectrophotometer.

Method

For the DPPH assay the Ascorbic acid was used as reference standard. The Ascorbic acid stock solution was prepared in distilled water (1 mg/ ml; w/v). A 60µM solution of DPPH in methanol was freshly prepared and a 200µl of this solution was mixed with 50µl of test sample at various concentrations (25, 50, 100, 200, 400 and 800µg/ml). The plates were kept in the dark for 15 minutes at room temperature and the decrease in absorbance was measured at 515 nm. Control was prepared with DPPHsolution only, without any extract or Ascorbic acid. 95% methanol was used asblank.(6)

$$\textbf{Percentage inhibition} = \frac{\textbf{Absorbance of Control - Absorbance of test}}{\textbf{Absorbance of control}} \times 100$$

3. RESULTS

3.1 EXTRACTION OF *Averrhoa carambola* LEAVES

Averrhoa carambola leaf extract was obtained after 3 hours continuous heating at 80^0C.

Fig 2: *Averrhoa carambola* leaf extract

3.2 Preliminary Phytochemical Screening

The preliminary phytochemical screening of *Averrhoa carambola* leaf extract.

The results are summarized in table 1

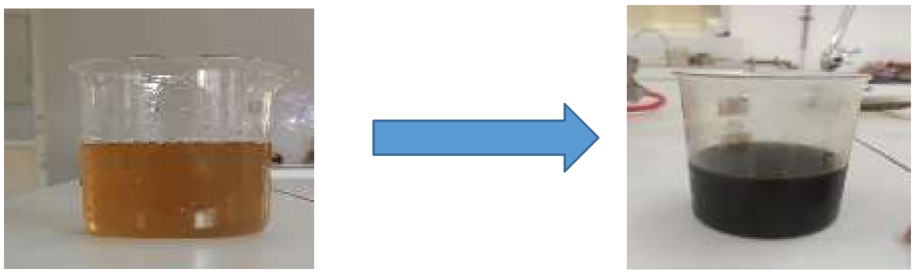
QUALITATIVE TESTS	RESULT
TEST FOR ALKALOIDS	
Dragendroff's test	+
Mayer's test	+
Hager's test	+
Wager's test	+
TEST FOR TANNINS	
Ferric chloride test	-
Lead acetate test	+
Potassium dichromate test	+
TEST FOR SAPONINS	
Foam test	+
Liebermann Burchard's test	+
TEST FOR PHENOLS	
Lead acetate test	+
Potassium dichromate test	+
TEST FOR FLAVONOIDS	
Shinoda test	-
Lead acetate test	+
Sodium hydroxide test	+
Pew's test	+
TEST FOR GLYCOSIDES	
Ferric chloride test	-
Bromine water test	+

Keller Killiani's test	+

Table 1: Preliminary phytochemical screening of Averrhoa carambola leaf exact

3.3 Green synthesis of Silver Nanoparticle

As the aqueous leaf extract of *Averrhoa carambola* was added to silver nitrate solution, the colour change of the solution changed from light brown to blackish brown, after 3 hours continuous stirring at 80^0C, and this indicate the biosynthesis of *Averrhoa carambola* AgNPs.



0 min 3 hours

FIG 3: Color changes of reacting solution with time

3.4 Characterization of Silver Nanoparticles

3.4.1 UV-Visible Spectroscopic Analysis

UV-visible spectroscopy is one of the most widely used technique for structural characterization of nano-particles. The absorbance was taken at 300-800 nm. A peak was obtained at 420 nm corresponding with the standard value between 400-450 nm.

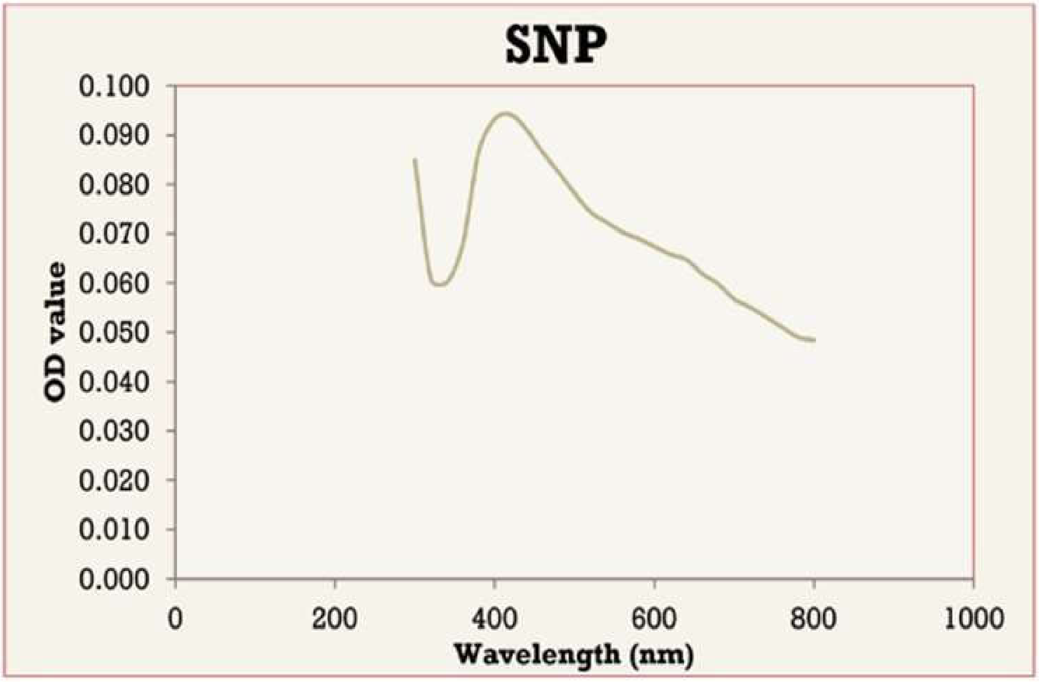


FIG 4: UV visible spectra of obtained AgNPs

3.4.2 FTIR Spectroscopic Analysis

The synthesized AgNPs were identified using FTIR spectral analysis. The observed intense bands were compared with standard values to identify the functional groups. The FTIR spectrum shows absorption bands at 3431, 2917, 2849, 1650, 1141, 1327, 1033, 775 cm^{-1}. The bands at 3431 cm^{-1} in the spectra corresponds to O-H stretching vibration indicating the presence of alcohol and phenol. Band at 2917 cm^{-1} indicating the presence of methyl C-H stretching. The band 2849 cm^{-1} indicate the presence of alkyl C-H stretching. The band at 1650 cm^{-1} corresponds to the double bond (C=C, C=O) and aromatic stretching. The band at 1441 cm^{-1} corresponds to the hetero aromatic C-C ring stretching. The band at 1327 cm^{-1} corresponds to O-H bending. Theband at 1033 cm^{-1} corresponds to O-H stretching of phenol. The band at 775 cm^{-1} corresponds to aromatic C-H bending. The band at 668 cm^{-1} indicates the strong bindingaffinity with the metal suggesting the encapsulation of metal nanoparticles.

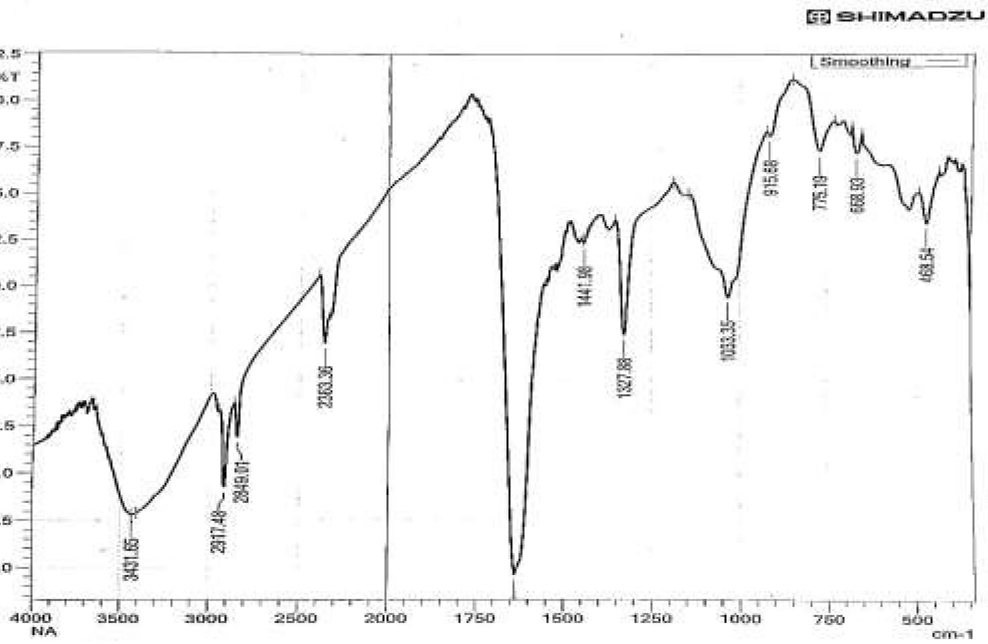


Fig 5: FTIR spectra of obtained AgNPs

3.4.3 X-RAY Diffraction

X-Ray diffraction peaks of synthesized AgNPs were obtained diffraction angles at 38^o,44^o,69^o and 77^o which corresponds to the silver nanoparticles.

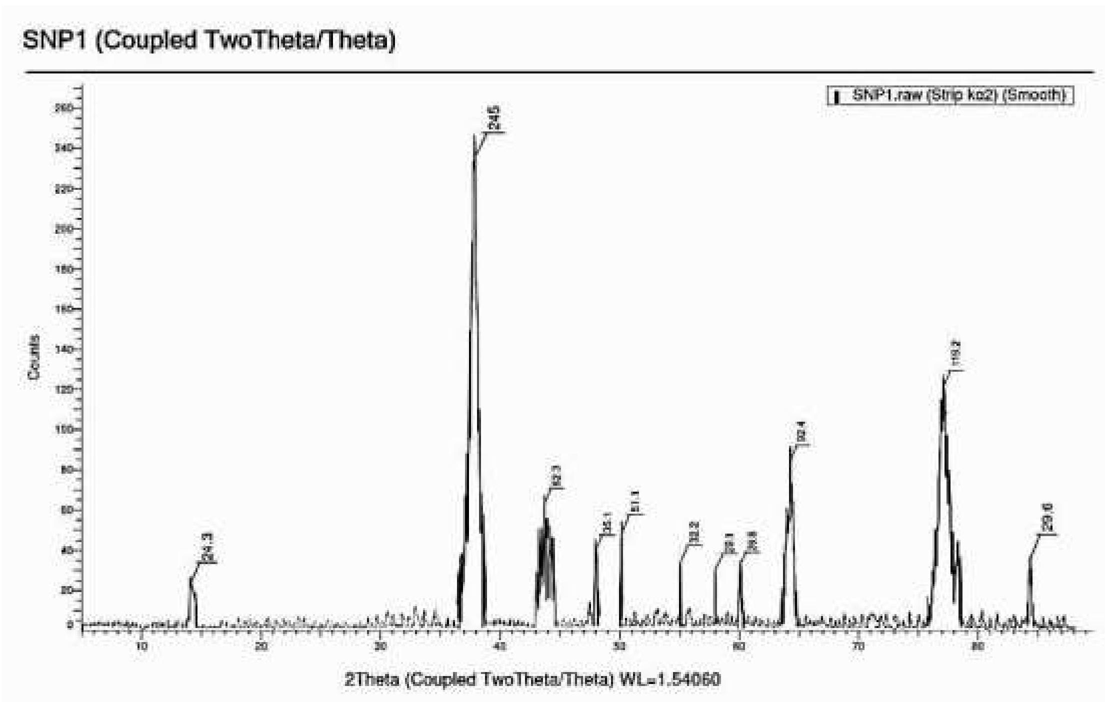


Fig 6: X-Ray diffraction spectra of obtained AgNPs

4. *IN-VITRO* Biological Evaluation

4.1 Determination of Anticoagulant Activity

Anticoagulant activity of green synthesized AgNPs was tested by addition of AgNPs suspension to human blood samples and further observed. The control in vial A without addition of AgNPs began to coagulate within 5 minutes at room temperature. The blood sample thickened over time and finally formed thick blood clot after 60 minutes at room temperature. On the other hand, the blood sample with addition of silver nanoparticle in vial B underwent no significant changes and eventually no mark of coagulation was observed after 60 minutes at room temperature.[18,19]

0 min

30 min

60 min

Fig 7: Anticoagulant activity of obtained AgNPs

4.2 Determination of Thrombolytic Activity

During the assessment of thrombolytic property, the suspension of AgNPs was added to the formed blood clot for the further observation. Immediately after addition the blood clot on the glass slide began to be liquefied gradually and was dissolved completely after 30 minutes[22]

Fresh blood

Blood clot formed at 4min

0 min (50µg/ml AgNPs)

15 min

30 min

Fig 8: Thrombolytic activity of obtained AgNPs

4.3 Determination of Antioxidant Activity

Various concentration of AgNPs sample ranging from 25µg/ml to 800µg/ml were tested for the free radical scavenging activity by DPPH method. Percentage inhibition of free radical concentration of AgNPs sample was determined and the result were compared with same concentration of Ascorbic acid as standard. The percentage inhibition of AgNPs sample shows IC_{50} 484.861 when compared to Ascorbic acid with IC_{50} 24.589. The results are shown in the table 3.

CONCENTRATION (µg/ml)	**PERCENTAGE INHIBITION**	
	ASCORBIC ACID	**AgNPs**
25	40.04±2.900	21.47±0.5554
50	56.75±1.6947	28.65±1.3559
100	60.82±1.356	32.71±1.4119
200	68.86±2.3636	38.98±0.8751
400	82.69±1.5106	45.66±1.1895
600	97.74±2.8096	55.60±1.4956
800	118.90±2.4275	65.04±1.0686

Table 3: Antioxidant activity of Ascorbic acid and AgNPs

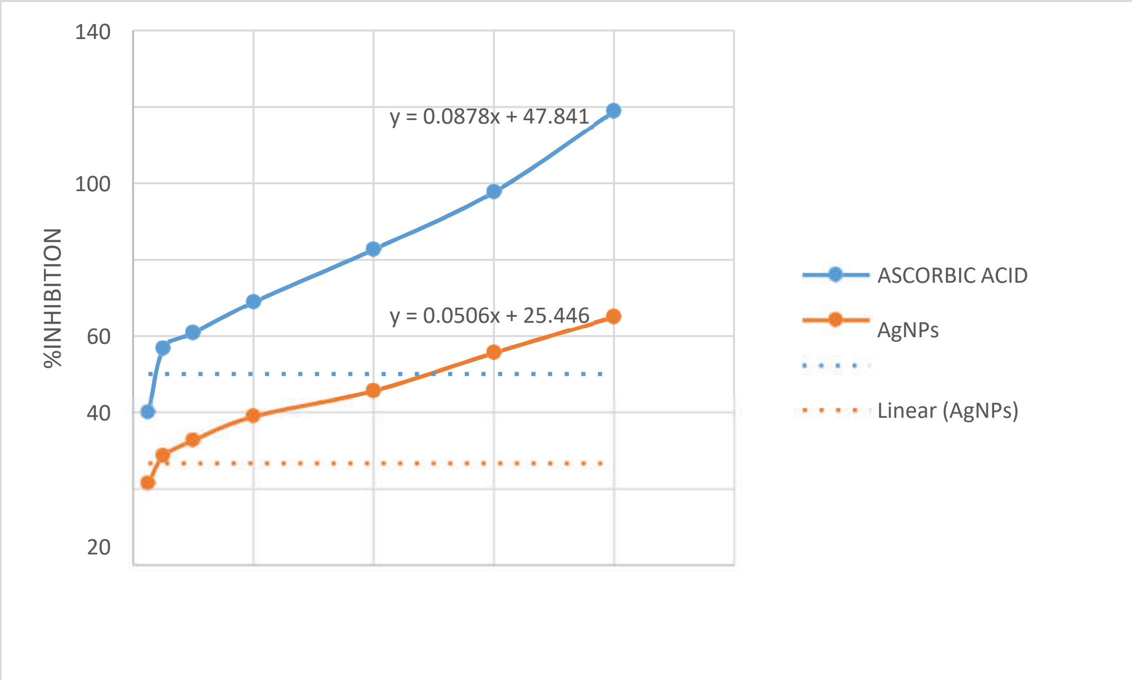


Fig 9: Graphical representation of antioxidant activity of AgNPs and Ascorbic acid

5. DISCUSSION

Silver nanoparticles were synthesized using a green method with 1 mM $AgNO_3$ and fresh leaves of *Averrhoa carambola,* heated at 80°C for 3 hours. The color change from light brown to blackish brown indicated nanoparticle formation. Phytochemical screening of the leaf extract revealed the presence of alkaloids, tannins, glycosides, flavonoids, and saponins. The formation of AgNPs was confirmed by UV-Vis spectroscopy, FTIR analysis, and X-ray diffraction. The UV-Vis absorbance spectrum showed a peak at 420 nm, indicating nanoparticle formation. FTIR analysis revealed functional group peaks consistent with those of silver nanoparticles in other studies. X-ray diffraction displayed peaks at diffraction angles of 38°, 44°, 69°, and 77°, corresponding to silver nanoparticles.

In-vitro evaluations demonstrated the biological activities of the AgNPs. Anticoagulant activity was tested using fresh blood at room temperature. While the control sample formed a clot within 5 minutes, the blood sample with AgNPs showed no coagulation even after 1 hour, suggesting anticoagulant properties. Thrombolytic activity was assessed by adding AgNPs to a clot, which was completely lysed within 30 minutes, indicating thrombolytic activity. Antioxidant activity was measured using the DPPH radical scavenging assay, with Ascorbic acid as the standard. The AgNPs exhibited antioxidant activity, with an IC50 of 484.861 compared to ascorbic acid's IC50 of 24.589.

6. SUMMARY AND CONCLUSION

This research focused on the green synthesis of silver nanoparticles (AgNPs) from *Averrhoa carambola* leaf extract. The AgNPs were characterized using UV-visible and FTIR spectroscopy, as well as X-ray diffraction. The AgNPs demonstrated significant anticoagulant and thrombolytic effects and antioxidant activity using the DPPH radical scavenging assay, with ascorbic acid as the standard. The in-vitro studies highlight the relevance of the work, suggesting that this analogue warrants further investigation in future studies.

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