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GREEN SYNTHESIS OF SILVER NANOPARTICLES USING *Annona Squamosa*: AND THEIR BIOLOGICAL ACTIVITY EVALUATION

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ABSTRACT
The present study focuses on the green synthesis of silver nanoparticles (AgNPs) using the aqueous leaf extract of *Annona squamosa*, a sustainable and ecofriendly approach that avoids the use of hazardous chemicals. The phytochemicals present in the plant extract act as both reducing and stabilizing agents, leading to the efficient formation of stable silver nanoparticles. The synthesized AgNPs were characterized using UV-Vis spectroscopy, FTIR, XRD, and SEM to determine their optical, structural, and morphological properties. Biological evaluations were conducted to assess the antimicrobial and antioxidant activities of the synthesized nanoparticles. The AgNPs exhibited significant antioxidant activity against DPPH radical scavenging assay as well as promising Alzheimers potential in Anticholinesterase inhibition using Galantamine as standard. The study highlights the potential of *Annona squamosa* as a natural, green resource for nanoparticle synthesis and supports the application of these biogenic AgNPs in biomedical and environmental fields.

KEYWORDS: AgNP, Alzheimers, Antioxidant, Insilico studies, *Annona squamosa*.

INTRODUCTION
In recent years, nanotechnology has emerged as a dynamic and interdisciplinary field with promising applications in medicine, agriculture, and environmental science. Among various nanomaterials, silver nanoparticles (AgNPs) have gained significant attention due to their potent antimicrobial, antioxidant, anticancer, and wound healing properties. Traditionally, chemical and physical methods have been employed for the synthesis of AgNPs; however, these methods often involve hazardous reagents and energy-intensive processes. In contrast, green synthesis has emerged as an ecofriendly, cost-effective, and sustainable alternative, utilizing biological entities such as plants, bacteria, and fungi to produce nanoparticles.

Annona squamosa L., commonly known as custard apple, is a tropical fruit-bearing plant belonging to the family Annonaceae. It is widely cultivated in regions such as India, the West Indies, South and Central America, Ecuador, Peru, Brazil, Mexico, the Bahamas, Bermuda, and Egypt. Beyond its nutritional value being rich in vitamin C, potassium, dietary fiber, and thiamine—the plant has been traditionally recognized for its medicinal properties. Notably, the leaves of *Annona squamosa* are abundant in bioactive phytochemicals, including flavonoids, alkaloids, and proanthocyanidins. These compounds exhibit various pharmacological activities, such as hepatoprotective, antidiabetic, lipid-lowering, antibacterial, antioxidant, and anticancer effects.[1,2]

Given the rich phytochemical profile of *Annona squamosa* leaves, they represent a viable candidate for the green synthesis of silver nanoparticles. In this biosynthetic approach, plant-derived compounds act as natural reducing and stabilizing agents, leading to the formation of AgNPs under mild conditions without the use of toxic chemicals. Such green-synthesized AgNPs not only exhibit enhanced biocompatibility but also demonstrate promising biological activities.[3]

The present study aims to synthesize silver nanoparticles using *Annona squamosa* leaf extract via an environmentally benign route and to evaluate their biological potential through antioxidant and anticholinesterase assays. Additionally, the synthesized nanoparticles are characterized using spectroscopic techniques to determine their structural and functional attributes. Molecular docking studies are also employed to gain insights into the interaction mechanisms of key bioactive compounds with target proteins, further

validating the therapeutic relevance of the synthesized nanoparticles. This integrative approach underscores the significance of green nanotechnology in developing sustainable solutions with potential biomedical applications.[4,5]

MATERIALS AND METHODS

Synthesis of Silver Nanoparticles

Leaf Extract Preparation

The leaves were washed thoroughly with distilled water. Leaves were dried in shade for 7 days separately. Then leaves were crushed using a mixer. A total of 60 g of powdered leaves is added with 240ml of distilled water and the resulting mixture was heated at 80°C with continuous stirring for 3 hours. [6]

Figure 1: Leaf extract preparation.

Nanoparticle Synthesis

10ml of aqueous extract was treated with 90ml of 1mM silver nitrate solution taken in the conical flask and the mixture is stirred at 50°C for 3hour. The resulting solution was then centrifuged to collect the nanoparticles. Subsequently the residue of silver nanoparticles was dried, collected, and stored in the refrigerator at 4°C.[6,7]

Spectral Characterization

The Synthesized Silver Nanoparticles Were Characterized by UV, IR, Scanning electron microscopy and X-ray diffraction.[11,12]

UV-visible Spectra

To observe the full bioreduction of AgNO3 to silver nanoparticles the spectra of the sample or medicament were checked using a Shimadzu U.V. probe1800 in the scanning series 400 to 800 nm with a resolution of 1nm.

IR Spectra

IR spectra of the synthesized silver nanoparticles were recorded in the range of 4000-300cm^{-1} on Shimadzu FTIR spectrophotometer using KBr pellet method. FTIR involves in the verification of bioactive agents covalently grafted onto silver, copper, and zinc together with any other NPs.[13]

Scanning electron microscopy (SEM)

For the analysis of the nanostructures of the samples, thin films are prepared in glass slides and then observed in Scanning Electron Microscopic (SEM, Hitachi, S-3000N). Thin films of the sample are prepared by using spin coater (Delta spin) and the films are dried by putting it under the IR lamp (Philips) for 5 min.

X-ray diffraction (XRD)

Any extra particles introduced to X-ray radiation reflected it, resulting in various redirection patterns that reflected the physicochemical characteristics of the crystal structure. The architectural characteristics of a powder illustration or metallic nanoparticle are frequently reflected in diffraction patterns from a specimen.[14]

Docking Methodology

AM Dock is a software application designed for automated docking and virtual screening in the realm of computational drug discovery. It facilitates the screening of compound libraries against specific targets. The software identifies the optimal orientation of molecules, ensuring they achieve the lowest energy state as determined by a predefined scoring function. This capability is instrumental in accurately predicting the positioning of drugs or ligands within the binding pocket of the target receptor. For Antioxidant activity X-Ray crystal structure of Human Heme Oxygenase-1 (Ho-1) from oxidoreductase family with (PDB ID 1N45) and Anticholinesterase the Recombinant human acetylcholinesterase from hydrolase family with (PDB ID 3LII).[9]

Biological Evaluation

***In vitro* Antioxidant activity**

DPPH radical scavenging assay

Radical scavenging activity of the test sample against stable 2, 2- diphenyl-1- 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 colour (from deep violet to light yellow) was measured at the optical density 515 nm on a UV visible spectrophotometer.[14,15]

Procedure

For DPPH assay the ascorbic acid used as reference standard. The ascorbic acid stock solution was prepared in distilled water (1mg/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 (1.56, 3.12, 6.25,12.5,25,50,100,200,400,800 µg/ml). The plates were kept in the dark for 15 minutes at room temperature and the decrease in absorbance was measured at 515nm. Control was prepared with DPPH solution only, without any extract or ascorbic acid. 95% methanol was used as blank and Ascorbic acid used as standard drug.

***In vitro* Anticholinesterase activity**

Ellman technique

The Ellman technique, also known as the Ellman's assay, is a spectrophotometric method used to quantify the concentration of sulfhydryl (SH) groups in a solution by reacting them with a chemical called 5,5'-dithiobis-2-nitrobenzoic acid (DTNB, also known as Ellman's reagent), which produces a yellow colored product that can be measured at a specific wavelength (typically 412nm) using a spectrophotometer; essentially, the intensity of the yellow colour directly correlates to the amount of free sulfhydryl groups present. This technique is widely used in biochemistry to measure the activity of enzymes like acetylcholinesterase (AChE) by detecting the thiocholine produced upon hydrolysis of acetylcholine substrate.[16,17]

Procedure

The Ellman technique, with slight changes, was used to measure the inhibitory potential of AC-AgNPs on cholinesterase enzymes (AchE and BchE) spectrophotometrically Amounts of 130 Ml of sodium phosphate buffer (Ph 8.0, 100 Mm) and 10 Ml of sample solution were combined in various quantities. The combination was mixed and incubated at 25 °C for 15 min before being added 20 Ml of 0.5 Mm DTNB (5,5′-dithiobis(2-nitrobenzoic) acid). This was done after adding 20 Ml of enzyme (AchE or BchE) buffer solution. The reaction was started by adding 20 Ml of the substrates acetylthiocholine iodide (0.71 Mm) or butyrylthiocholine chloride (0.2 Mm). Using a 96-well microplate reader, the absorbance and appearance of the yellow 5-thio-2-nitrobenzoate anion produced by the reaction of DTNB with thiocholine were determined at 412 nm. The outcomes were expressed as an enzyme inhibition percentage (percent) at a concentration of 200 g/Ml and an inhibitory concentration for nanoparticles (IC50) of 50%.[24,25]

RESULTS AND DISCUSSION

Phytochemical screening of *Annona squamosa* leaf extract.

Table 1: Result of Phytochemical Screening.

SL NO	PHYTO-CONSTITUENT	TEST	RESULT
1	Flavonoids	NaOH test	+
2	Alkaloids	Wagner's test	+
3	Reducing sugars	Fehling's test	+
4	Phenol	FeCl3 test	+
5	Saponins	Froth test	-
6	Tannins	KOH test	+
7	Steroids	Liebermann–Burchard test	+

Spectral Characterization

AgNP were characterized by UV, FTIR, SEM and XRD analysis.

UV-visible Spectra

The AgNP was subjected to UV-Vis analysis and the absorption peak of AgNP given below.

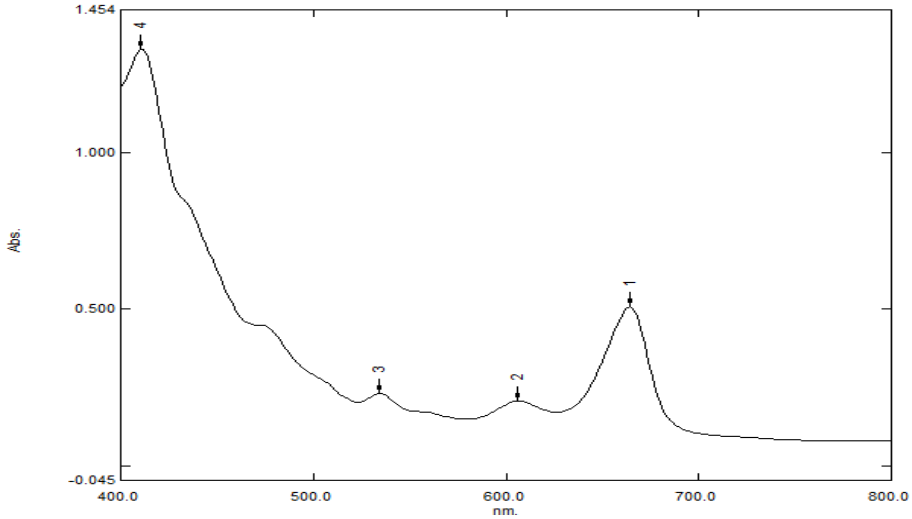


Figure 2: UV-Vis spectrum of AgNP.

The UV-Vis analysis displays a graph with the wavelength along the x-axis and absorbance along the y-axis, where a peak at 410 nm indicated the presence of AgNPs in the sample solution due to the surface plasmon resonance effects.[25]

Infrared Spectral Analysis

The silver nanoparticles were subjected to infrared spectroscopic analysis using Shimadzu FTIR spectrometer and the characteristic absorption band of sample are given in the table 2.

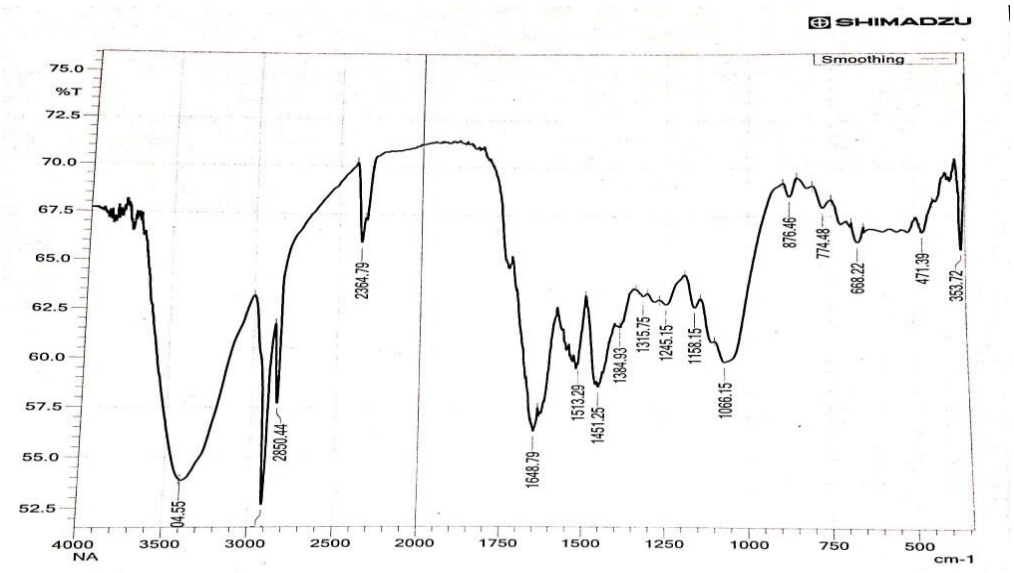


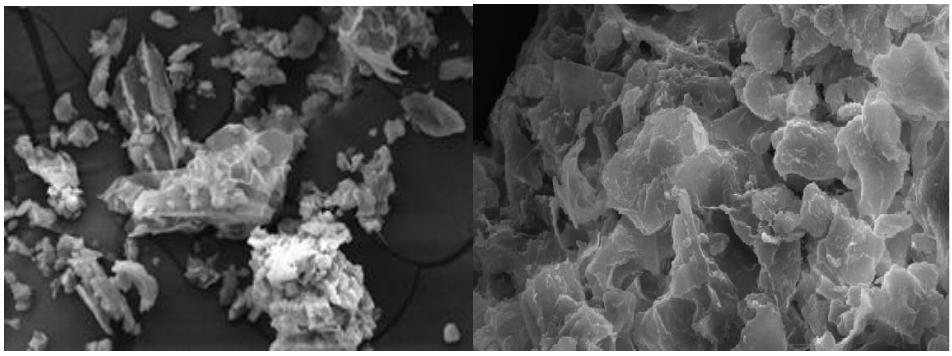
Figure 3: FTIR spectrum of AgNP.

Table 2: FTIR Peak values.

PEAKS(cm^{-1})	FUNCTIONAL GROUPS
1648	C=O stretch
1513	N-O asymmetric stretch
1384	C-H rock
1245	C-O stretch
1066	C-N stretch
876	O-H bend
2850	C-H stretch
471	Ag-O stretch

Scanning Electron Microscopy (SEM)

AgNP was subjected to Scanning electron microscopy (SEM) and the image obtained is given in Fig 4.



Particle size: 50µm **Particle size 100µm**

Figure 4: SEM image of AgNP.

Particle size ranging from 50nm to 100nm.

X-ray Diffraction (XRD)

AgNP was subjected to X-ray diffraction and the data obtained is given below in table 3

Table 3: hkl values of AgNP.

2θ	$Sin^2\theta$	$\frac{1\times Sin^2\theta}{Sin^2\theta}$	$\frac{2\times Sin^2\theta}{Sin^2\theta}$	$\frac{3\times Sin^2\theta}{Sin^2\theta}$	$h^2+k^2+l^2$	hkl
27.18	0.0552	1	2	3	3	111
32.27	0.0771	1.396	2.793	4.188	4	200
38.63	0.1094	1.981	3.962	5.943	6	211
47.22	0.1604	2.905	5.81	8.715	9	221
55.80	0.2189	3.96	7.92	11.88	12	222

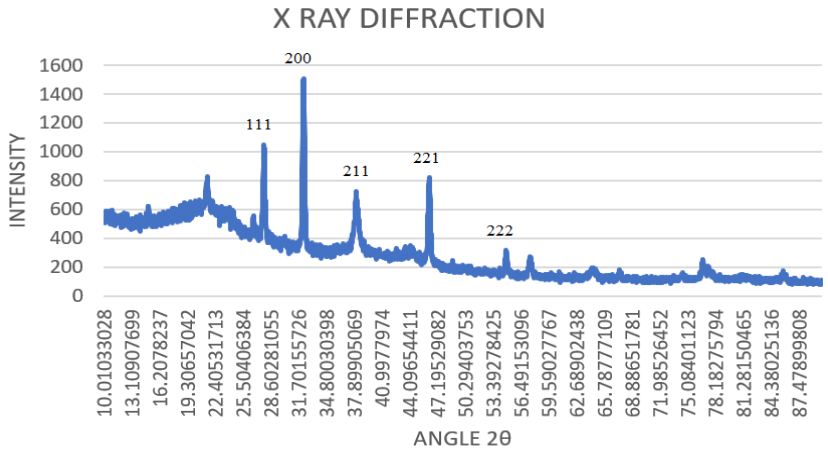


Figure 5: XRD of AgNP.

XRD analysis showed five peaks at 2θ values of 27.18°, 32.27°, 38.63°, 47.22° and 55.80° degree in the experimental diffractogram have been identified to be due to silver metal and corresponding to (hkl) values - (111), (200), (211), (221)and (222) silver crystalline planes of face centered cubic structure and nanoparticles are crystalline in nature.

Molecular docking using AM Dock

The docking studies were carried using AM Dock and the results are shown below.

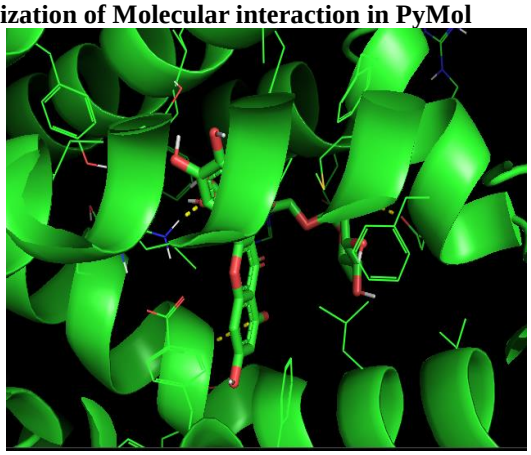
Docking profile of flavanoids for Antioxidant Activity.

The docking study of flavonoids for antioxidant activity was carried out by using the X-Ray crystal structure of Human Heme Oxygenase-1 (Ho-1) from oxidoreductase family with PDB ID 1N45 were retrieved from Protein Data Bank (PDB) having a resolution of 1.50 A°. For Antioxidant activity, of the flavonoids rutin showed a binding score of -9.2 when compared with the standard drug ascorbic acid with binding score of -5.2 indicating good binding interaction of rutin.[28,29]

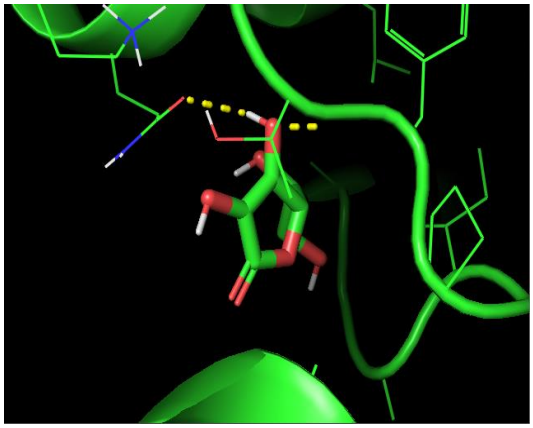
Table 4: Docking profile of flavonoids for antioxidant activity.

COMPOUND NAME	BINDING AFFINITY
Rutin	-9.2
Quercetin	-7.0
Kaempferol	-7.1
Standard Drug : Ascorbic acid	-5.2

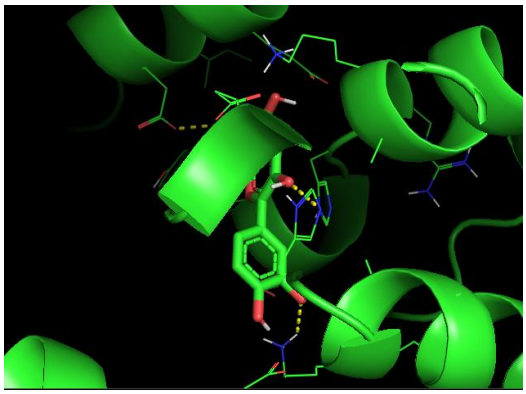
Visualization of Molecular interaction in PyMol



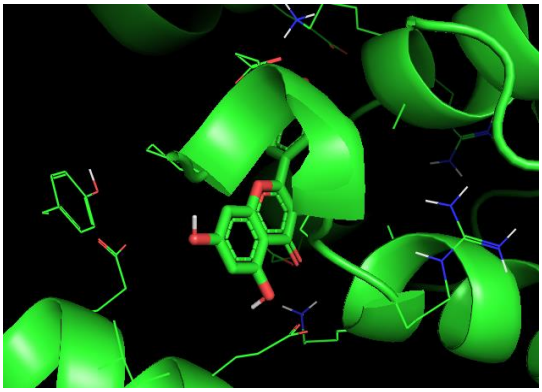
RUTIN



ASCORBIC ACID



QUERCETIN



KAEMPFEROL

Figure 6: Interaction of molecules with 1N45.

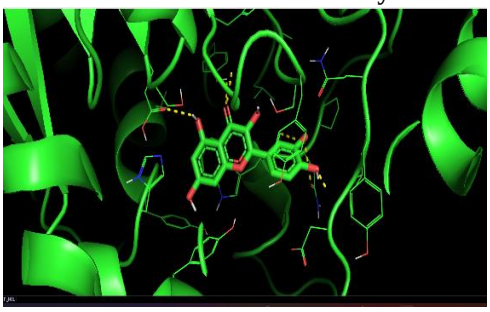
Docking profile of flavanoids for Anticholinesterase Activity

The docking study of flavonoids for anticholinesterase activity on Acetylcholinesterase was carried out by using the structure of recombinant Human Acetylcholinesterase from Hydrolase family with PDB ID 3LII were retrieved from Protein Data Bank (PDB) having a resolution of 3.20 A°. For Anticholinesterase activity, of the flavonoids Quercetin showed a binding score of -9.4 when compared with the standard drug Galantamine with binding score of -7.7 indicating good binding interaction of Quercetin.[30,31]

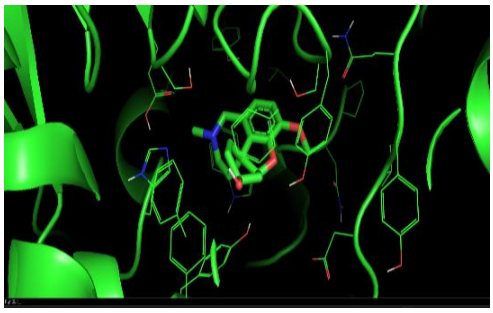
Table 5: Docking profile of flavonoids for anticholinesterase activity.

COMPOUND NAME	BINDING AFFINITY
Quercetin	-9.4
Rutin	-8.6
Kaempferol	-8.7
Standard Drug : Galantamine	-7.7

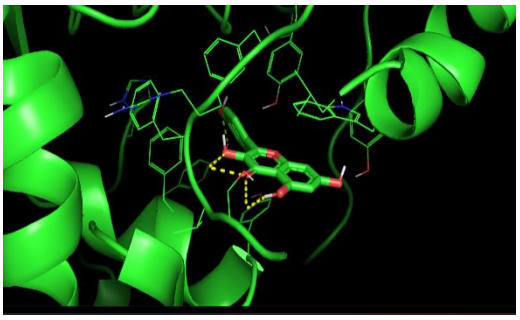
Visualization of Molecular interaction in PyMol



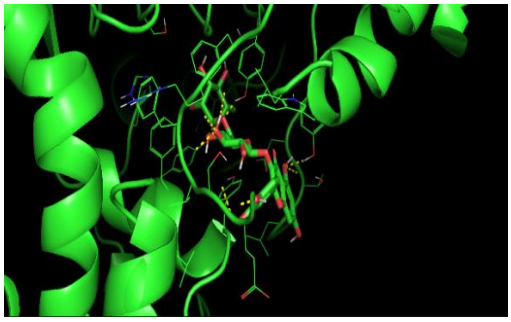
QUERCETIN



GALANTAMINE



KAEMFEROL



RUTIN

Figure 7: Interaction of molecules with 3LII.

BIOLOGICAL EVALUATION

In vitro Antioxidant activity

In vitro antioxidant activity of AgNP was evaluated by DPPH radical scavenging assay. In assessing Antioxidant activity, DPPH radical scavenging assay was conducted, revealing that AgNP exhibited an IC50 value of 322.53µg/ml in comparison to standard ascorbic acid with IC50 value 184.94 µg/ml.[32,33]

Table 6: Antioxidant analysis by DPPH radical scavenging assay.

CONCENTRATION	PERCENTAGE INHIBITION	
	Ascorbic acid	AgNP
6.25	11.45 ±2.454	12.16 ±3.621
12.5	20.40 ±2.586	18.87±3.819
25	35.85 ±3.853	27.56±2.534
50	42.79±6.359	32.77±4.567
100	52.82±4.157	39.47±5.317
200	58.07±3.621	40.12±3.263
400	77.29±2.154	59.25±17.887
800	115.19±4.534	87.23±3.234
IC50	IC50=184.94	IC50=322.53

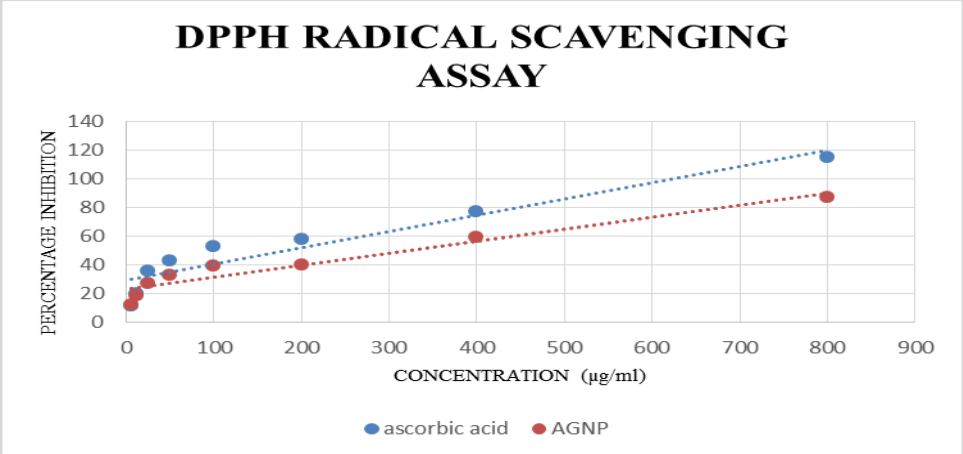


Figure 8: Antioxidant activity of AgNP.

***In vitro* Anticholinesterase activity**

In vitro Anticholinesterase activity of AgNP was evaluated by Ellman technique. In assessing Anticholinesterase activity, Ellman technique was conducted, revealing that AgNP in AChE exhibited an IC50 value of 83.28 µg/ml in comparison to standard Galantamine with IC50 value 91.65 µg/ml.[32]

Table 7: Anticholinesterase analysis by Ellman method.

CONCENTRATION (µg/ml)	PERCENTAGE INHIBITION	
	AChE	
	AgNP	GALANTAMINE
6.25	54.75±1.26	43.08±1.283
12.5	67.08±0.971	59.22±1.732
25	86.13±2.753	84.75±1.328
50	114.67±1.682	110.58±1.091
100	167.01±2.10	153.96±2.826
IC50	IC50= 83.28	IC50=91.65

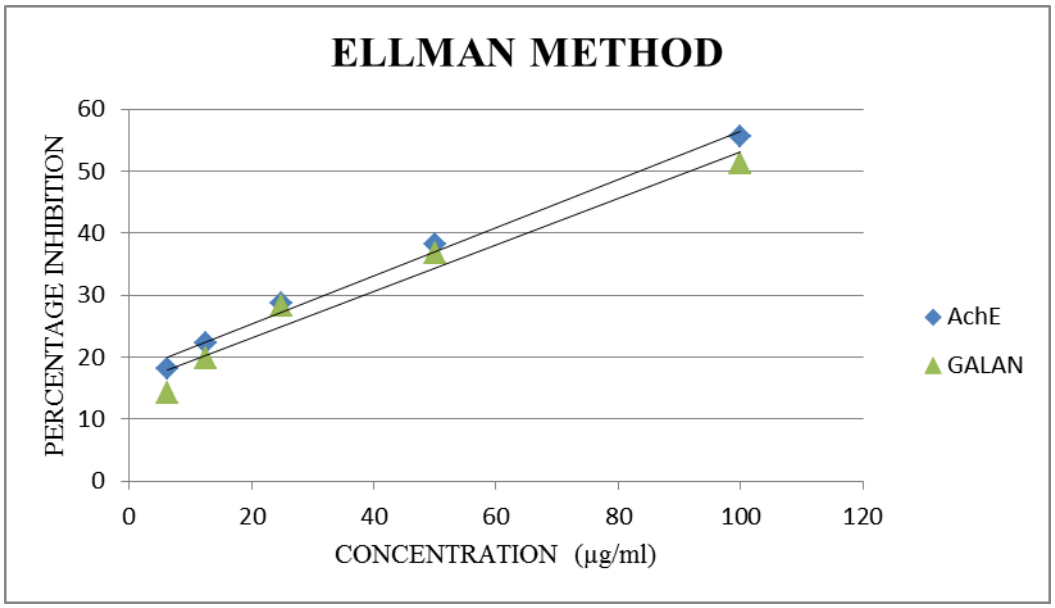


Figure 9: Anticholinesterase activity of AgNP.

CONCLUSION

This study focused on the synthesis, phytochemical screening, characterization, and evaluation of silver nanoparticles (AgNPs) for their potential antioxidant and anticholinesterase activities of *Annona squamosa* leaf extract. Spectral characterization of the synthesized AgNPs was performed using UV-Vis spectroscopy, FTIR, SEM, and XRD analyses. Molecular docking studies were conducted using AM Dock software. The AgNPs were evaluated for antioxidant and anticholinesterase activities, showing results comparable to standard drugs—Ascorbic acid (for antioxidant activity) and Galantamine (for anticholinesterase activity).The AgNPs demonstrated significant biological activity, particularly in terms of antioxidant and anticholinesterase effects. These findings suggest that the synthesized AgNPs may serve as promising candidates for therapeutic applications related to oxidative stress and neurodegenerative conditions.

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