ORIGINAL ARTICLE

Synthesis of silver nanoparticles using ethanolic extract of *Annona squamosa* fresh leaves and investigation of antioxidant, anti-arthritic, and thrombolytic activities

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Abstract

Aim: Plant-mediated synthesis of nanomaterials has been rapidly gaining popularity due to its eco-friendly design and cost-effectiveness. In the present research, we synthesized silver (Ag) nanoparticles using ethanolic extracts of fresh leaves of *Annona squamosa* (family Annonaceae) medicinal plant as bioreducing agents. **Materials and Methods:** This method allowed the synthesis of nanoparticles, which was confirmed by ultraviolet-visible (UV-vis) spectrophotometry and transmission electron microscopy. UV-vis spectra and visual observation showed that the color of the fresh leaf extract of *A. squamosa* turned into grayish brown and brownish yellow, respectively, after treatment with Ag precursors. Moreover, ethanolic leaf extract of *A. squamosa* silver nanoparticles (AgNPs) was separately tested for their *In vitro* antioxidant, anti-arthritic, and thrombolytic activity. Thrombolytic activity was evaluated using the *in vitro* clot lysis model. Bovine serum albumin (BSA) was used to evaluate the antiarthritic potential. **Results:** Nitric oxide generation radical scavenging activity, reducing power, and Phosphomolybdenum assay of the synthesized AgNPs increased in a dose-dependent manner as compared to ascorbic acid the standard reference used. The maximum percentage inhibition by BSA method was observed as 71.4% at 200 µg/mL concentration for antiarthritic activity. During assay for thrombolytic activity, it revealed that 85.620 ± 2.6% lysis of clot, while standard streptokinase and water used as positive and negative controls, demonstrated 72.835 ± 1.702% and 2.725 ± 0.983% lysis of clot, respectively**. Conclusion:** This result confirmed that *A. squamosa* is a potential biomaterial for synthesizing AgNPs which can be exploited for its antioxidant activity, anti-arthritic, and thrombolytic activity.

Key words: *Annona squamosa*, ascorbic acid, bovine serum albumin, free radicals, thrombolytic

INTRODUCTION

A particle with a nanometer scale of 1–100 nm represents nanoparticles. Compared to its bulk structure, the nanoscale material has fresh, special, and superior physical and chemical properties due to an improvement in the surface area ratio per volume of the material/particle. Metal nanoparticles are the most widely studied nanoparticle materials because they are easier to synthesize.[1] In addition, there are a broad variety of uses for these materials: Detectors, catalysts, surface coating agents, antibacterial/antimicrobials, and among many others. Silver (Ag)[2,3] gold (Au),[4] platinum (Pt),[5-7] and palladium (Pd)[8] are some of the most studied metallic nanoparticles. The Ag nanoparticle, especially in the field of health and medicine, is an important metal to be studied. Ag is a potent antibacterial and toxic to cells as well. Due to the interaction between Ag ions with macromolecules in cells, such as proteins and deoxyribonucleic acid, Ag has the capacity to damage bacterial cell walls, inhibit bacterial

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cell growth, and disrupt cell metabolism (DNA).[9-11] Methods of chemical reduction are mostly used to synthesize silver nanoparticles (AgNPs) because they are simpler and more economical.[12] This process is carried out by the reduction of Ag salts by the reduction of agents such as sodium citrate or borohydride sodium.[13] However, in the synthesis of Ag nanoparticles, the use of chemicals results in the adsorption on the surface of the material of toxic chemicals (reducing agents and organic solvents) to have detrimental and harmful effects on its application.[13] The use of environmentally sustainable practices is, therefore, beneficial.

Green methods of synthesis for the synthesis of nanoparticles using natural products can be used by the use of plants or microorganisms to resolve the problem.[14] In the biosynthesis of nanoparticles, the use of plants requires the material of secondary metabolites as reduction agents.[15] Allegedly, in the process of forming nanoparticles, biological agents serve as reducers, stabilizers, or both.[16]

Annona squamosa is small shrub (or) well branched tree commonly known as Custard apple in English, Sitaphal in Hindi, and Sitaphalam in Telugu, it has excellent source in vitamins and high energy. AgNPs are increasingly used in various fields, including medical, food, health care, consumer, and industrial purposes, due to their unique physical and chemical properties and so far *A. squamosa* AgNPs were not prepared and they were evaluated for various pharmacological activities.

The present study synthesized Ag nanoparticles using ethanolic extracts of fresh leaves of *A. squamosa* then evaluated its *In vitro* antioxidant, anti-arthritic, and thrombolytic activity.

MATERIALS AND METHODS

Collection of Plant Material

The fresh leaves of *A. squamosa* were collected December 2020 near Rajahmundry, Andhra Pradesh, India country. The plant was authenticated by Dr. T. Raghuram, Taxonomist, Maharani College, Peddapuram and voucher specimen number given is 22,126.

Preparation of Ethanolic Leaf Extracts

The freshly collected leaves of *A. squamosa* were washed with water to remove dirt and sand particles and dried under shade for 40 days and they were grounded into powder using a mechanical grinder. The powder was extracted with 95% ethanol for 3 days, followed by hot percolation for 3 h. Then, it was filtered and distilled at 80°C. Then, it was transferred into the empty china dish and evaporated to get an ethanolic extract and kept in anhydrous calcium chloride containing desiccators.[17]

Chemicals

All chemicals and reagents used are of analytical grade procured from S.D. Fine Chemical Limited, India.

Synthesis of Ag Nanoparticles

A 10 mM $AgNO_3$ stock solution was prepared by dissolving $AgNO_3$ powder in distilled water and producing a series of 1 mM, 2 mM, 3 mM, 4 mM, and 5 mM $AgNO_3$ solutions. In a flask with a volume of 50 mL, the $AgNO_3$ solutions were combined with an ethanolic extract of *A. squamosa* fresh leaves at a ratio of 1: 1 (v/v). The flask was covered in aluminum foil and heated for 5 h in a water bath at 60°C. For the antibacterial activity test, the mixture was also held in the refrigerator and analyzed using a ultraviolet-visible (UV-Vis) spectrophotometer. The ethanolic extract of *A. squamosa* fresh leaves underwent the same treatment.

In vitro Antioxidant Activity

Phosphomolybdenum antioxidant assay

The antioxidant activity of the ethanolic leaf extract of *A. squamosa* AgNPs was evaluated by the phosphomolybdenum method according to the procedure.[18] The assay is based on Mo (VI)–Mo (V) reduction by the extract and at acid pH leads to formation of a green phosphate/Mo (V) complex. 0.3 mL of extracts (0.05, 0.1, 0.3, and 0.5 mg/mL) were combined with 3 mL of reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate, and 4 mM ammonium molybdate). The reaction solution was incubated at 95°C for 90 min. The absorbance of the solution was measured at 695 nm.

Nitric Oxide Generation and Assay of Nitric Oxide Scavenging

Sodium nitroprusside in aqueous solution at physiological pH spontaneously generates nitric oxide which interacts with oxygen to produce nitrite ions that can be estimated by Greiss reagent. Scavengers of nitric oxide compete with oxygen leading to reduced production of nitric oxide.[19] Sodium nitroprusside (5 mM) in phosphate-buffered saline was mixed with different concentrations of the extracts (0.05, 0.1, 0.3, and 0.5 mg/mL) dissolved in the suitable solvent systems and incubated at 25°C for 150 min. The samples above were reacted with Greiss reagent (1% sulfanilamide, 2% H3PO4 and 0.1% naphthylethylenediamine dihydrochloride). The absorbance of the chromophore formed during the diazotization of nitrite with sulfanilamide and subsequent coupling with naphthylethylenediamine was read at 546 nm and referred to the absorbance of standard solutions of ascorbic acid was treated in the same way with Griess reagent. The formula to calculate the percentage inhibition was

$$\text{Nitric oxide scavenged (\%)} = [(A_o - A_1)/A_o] \times 100$$

where A_o is the absorbance of the control reaction (containing all reagents except the sample extract), and A_1 is the absorbance of the sample extract. Ascorbic acid was used as positive controls.

Reducing Power Method

Electron donating activity is indicated by Fe (III) reduction, which is an important mechanism of phenolic antioxidant action.[20] Different concentration of the ethanolic leaf extract of *A. squamosa* AgNPs (0.05, 0.1, 0.3, and 0.5 mg/mL) extract in 1ml of distilled water was mixed with phosphate buffer (2.5 mL, 0.2 M, and pH 6.6) and potassium ferricyanide [$K_3Fe(CN)_6$] (2.5 mL, 1%). The mixture was incubated at 50°C for 20 min. A portion (2.5 mL) of trichloroacetic acid (10%) was added to the mixture, which was then centrifuged at 3000 rpm for 10 min. The upper layer of the solution (2.5 mL) was mixed with distilled water (2.5 mL) and $FeCl_3$ (0.5 mL, 0.1%) and the absorbance was measured at 700 nm. Ascorbic acid was used as standard. Increased absorbance of the reaction mixture indicates increase in reducing power.

Evaluation of Antiarthritic Activity

Bovine serum albumin (BSA) method

Test solution (0.5 mL) consists of 0.45 mL of BSA (5% w/v aqueous solution) and 0.05 mL of test samples of different concentrations (50 µg/mL, 100 µg/mL, 300 µg/mL, and 500 µg/mL). Control solution of test (0.5 mL) mainly consists of BSA of 0.45 mL (5% w/v aqueous solution) and 0.05 mL of distilled water. Product control solution (0.5 mL) consists of 0.45 mL of distilled water and 0.05 mL of test samples of different concentrations (50 µg/mL, 100 µg/mL, 300 µg/mL, and 500 µg/mL). Standard solution consists of 0.45 ml of serum albumin (5% w/v aqueous solution) and 0.05 mL of diclofenac sodium of concentrations 100 µg/mL and 200 µg/mL. The above solutions were adjusted to a pH of 6.3 using 1 N HCl. The samples were incubated for 20 min at 37°C and the temperature was raised to 57°C for 3 min. After cooling, 2.5 mL of phosphate buffer was added to the above solutions.[21,22] The absorbance was measured using UV-vis spectrophotometer at 255 nm. The percentage inhibition of protein denaturation was calculated as,

% Inhibition of protein denaturation = 100–[{(O.D of test solution–O.D of product control)/O.D of test control} × 100]

The control represents 100% protein denaturation. The results were compared with diclofenac sodium.

Thrombolytic Activity

The thrombolytic activity of this extractive was evaluated by the *in vitro* thrombolytic test[23] using SK as standard. The ethanolic leaf extract of A. squamosa AgNPs (10 mg) was suspended in 10 mL of distilled water, and it was kept overnight. Then, the soluble supernatant was decanted and filtered. The blood was withdrawn from healthy volunteers and was distributed into five different pre-weighed microcentrifuge tubes and is incubated at 37°C and then the serum was removed completely without disturbing the clot, then tubes with clot were weighed to determine clot weight (clot weight = weight of clot containing tube-weight of tube alone). To each pre-weighed clot in microcentrifuge tube, along with the crude extract and 100 µL aqueous solutions of different partitionates was added separately. 100 µL of distilled water was separately added to the control tubes. As a positive control 100 µL of SK was used. All the tubes were then incubated at 37°C for 90 min and observed for clot lysis. After incubation, the released of fluid was removed, and tubes were again weighed to observe the difference in weight after clot disruption. The percentage of clot lysis is shown.

% of clot lysis = (weight of released clot/clot weight) × 100

Characterization of Ag Nanoparticles

The reduction of pure Ag^+ ions was monitored by measuring the UV-Vis spectrum of the reaction medium after diluting a small aliquot of the sample into distilled water. The color change in the reaction mixture (metal ion solution + ethanolic leaf extract of *A. squamosa* AgNPs) was recorded through visual observation. UV-Vis spectral analysis was done using UV-Vis spectrophotometer UV-1800 (Shimadzu) at the wavelength of 200–800 nm.

Statistical Analysis

Using one-way analysis of variance followed by Dunnett's multiple tests statistical analysis was performed. Results are expressed as Mean±SEM for animals in each group. Differences among groups were considered significant at $P < 0.001$ level.

RESULTS

The ethanolic AgNPs leaf extract of *A. squamosa* change their colors when warmed. The *A. squamosa* extract changes color from colorless to brownish yellow.

This warm extract solution changed color again after adding $AgNO_3$ solution. Color changes are possible because some of the Ag ions begin to be reduced due to the effects of heat and produces Ag^+ complex. This color change indicates the formation of Ag nanoparticles.[24]

The Ag nanoparticles synthesized in each extract solution was analyzed using UV-visible spectroscopy. This was done to determine the characteristics of the peak spectrum of the

Ag nanoparticle wavelength prepared for each different $AgNO_3$ concentrations (1 mM–5 mM) [Figure 1].

In vitro Antioxidant Activity

Scavengers of nitricoxide compete with oxygen leading to reduced production of nitric oxide. *Ethanolic Silver Nanoparticles leaf extract of Annona Squamosa* had comparably more nitric oxide radical scavenging activity than Ascorbic acid.

DISCUSSION

The characteristics of Ag nanoparticles normally appear at a wavelength interval of 400–600 nm.[25] UV-vis spectra of Ag nanoparticles synthesized using the *A. squamosa* ethanolic extract evince the blue shift of the absorption band with increasing $AgNO_3$ concentration. For 1 mM, 2 mM, 3 mM, 4 mM, and 5 mM samples, the absorption peak is centered around 450–420 nm. This information shows that the Ag nanoparticles have formed in the extract, where the Ag^+ has been reduced to Ag^0. Proteins and all secondary metabolites of extract play a critical role in both the reducing and capping mechanism for nanoparticle formation.[24] The Ag nanoparticles contained in the ethanolic extract of the *A. squamosa* also exhibit similar characteristics, where the shift of the absorption band with increasing $AgNO_3$ concentrations. However, the shift of the absorption peak was a little narrower than that of the Ag nanoparticles synthesized with ethanolic extract of the *A. squamosa*, where the absorption peak is centered on 450–440 nm. The peak wavelength of Ag nanoparticles in ethanolic fresh leaf extracts is shown in Table 1, Figure 1.

The reducing power assay measures the electron-donating ability of antioxidants using potassium ferricyanide reduction method. Antioxidants reduce the ferric ion/ferricyanide complex to the ferrous form, the Perl's Prussianblue complex.[18] The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity.[26] The antioxidant activity of extract *and* ascorbic acid has been attributed to various mechanisms, among which are prevention of chain initiation, binding of transition metal ion catalysts, decomposition of peroxides, prevention of continued hydrogen abstraction, reductive capacity, and radical scavenging.[27] The reducing capacity of the ethanolic AgNPs leaf extract of *A. squamosa* and ascorbic acid indicates their potential antioxidant activity Figure 2.

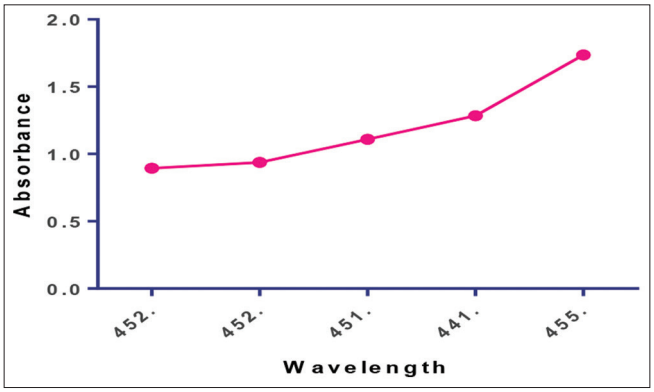

Figure 1: Peak wavelength and absorbance of Ag nanoparticles in ethanolic extract of fresh leaves of *Annona squamosa*

Nitric oxide radical generated from sodium nitroprusside at physiological pH was found to be inhibited by ethanolic AgNPs leaf extract of *A. squamosa* and ascorbic acid. Sodium nitroprusside in aqueous solution at physiological pH spontaneously generates nitric oxide.[28] which interacts with oxygen to produce nitrite ions that can be estimated by use of Griess reagent. Scavengers of nitric oxide compete with oxygen leading to reduced production of nitric oxide. Ethanolic AgNPs leaf extract of *A. squamosa* had comparably more nitric oxide radical scavenging activity than ascorbic acid Table 2, Figure 3.

The total antioxidant capacity of ethanolic AgNPs leaf extract of *A. squamosa* was determined by phosphomolybdenum assay and the highest absorbance was recorded at 0.5 mg/mL Figure 2. The antioxidant capacity of the ethanolic AgNPs leaf extract of *A. squamosa* was measured by phosphomolybdenum method, which is based on the reduction of Mo (IV) to Mo (V) by the sample analyte and the subsequent formation of green

Table 1: Peak wavelength and absorbance of Ag nanoparticles in the ethanolic extract of fresh leaves of *A. squamosal*

Concentration (mM)	Wavelength (nm)	Absorbance
A. squamosal		
1	452	0.894
2	452	0.937
3	451	1.109
4	441	1.285
5	455	1.736

A. squamosal: Annona squamosal

Table 2: *In vitro* antioxidant potential of ethanolic AgNPs leaf extract of *A. squamosa* and ascorbic acid against nitric oxide radicals

Conc. (ug/ml)	Nitric oxide (%)	IC_{50} (ug/ml)		IC_{50} (ug/ml)
	Eth acetate .Ext	89.125	STD	7.94
50	40.19±0.35		58.45±0.2 3	
100	55.88±0.13		70.07±0.22	
300	61.76±0.18		71.76±0.32	
500	73.52±0.13*		74.84±0.13	

All the values are expressed as Mean±SEM, n=3 ; *P<0.001 when compared with standard values. *A. squamosal*: *Annona squamosal*, AgNPs: Silver nanoparticles

phosphate/Mo (V) compounds with a maximum absorption at 695 nm. The antioxidant capacity of extracts was found to increase with increase in concentration.

In inhibiting heat-induced albumin denaturation, it was effective. Maximum inhibition at 200 µg/mL was observed as 71.42%, a standard anti-inflammatory drug showed the maximum inhibition of 45.52% at the concentration of 100 µg/mL which was compared with control. Hence, from the results of our study reveal that the ethanolic AgNPs leaf extract of *A. squamosa* is capable of controlling the production of autoantigen and also inhibits albumin denaturation in rheumatic disease. Our present studies indicate that ethanolic AgNPs leaf extract of *A. squamosa* exhibits strong antiarthritic property could be potential source of antiarthritic property. The inhibition of albumin denaturation was studied to establish the mechanism of antiarthritic activity of ethanolic AgNPs leaf extract of *A. squamosa.* Therefore, our *in vitro* studies on extract of the ethanolic AgNPs leaf extract of *A. squamosa* demonstrate the significant antiarthritic activity [Figure 4].

Addition of 100 µL SK (Durakinase, Dongkook Phama. Co. Ltd., South Korea), a positive control (30,000 I.U.) to the clots along with 90 min incubation at 37°C, showed 72.83% clot lysis. On the other hand, with 100 µL sterile distilled water (negative control), it showed negligible

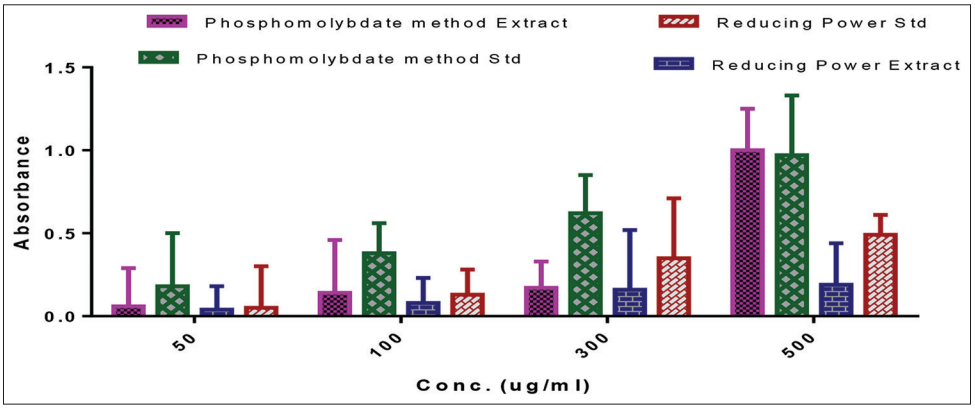

Figure 2: *In vitro* antioxidant potential of the ethanolic silver nanoparticles leaf extract of *Annona squamosa* and ascorbic acid by phosphomolybdate and reducing power assay

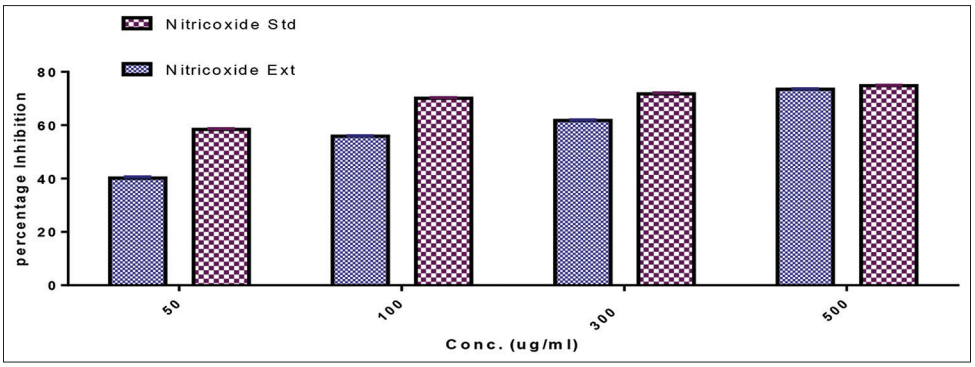

Figure 3: *In vitro* antioxidant potential of the ethanolic silver nanoparticles leaf extract of *Annona squamosa* and ascorbic acid against nitric oxide radicals

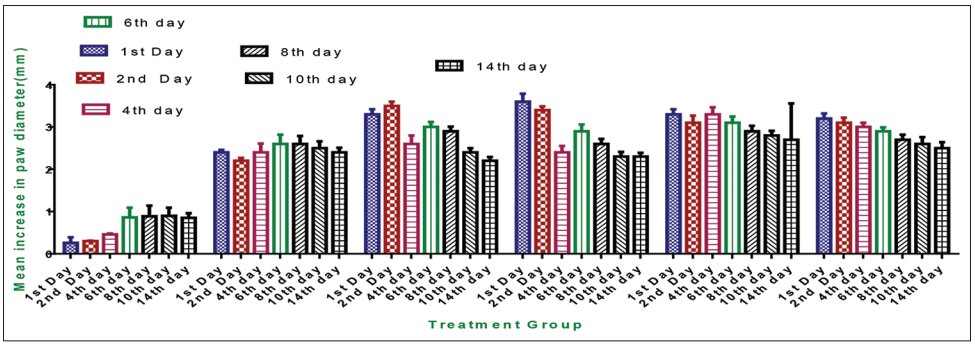

Figure 4: *In vitro* anti-arthritic activity of the ethanolic silver nanoparticles leaf extract of *Annona squamosa* and diclofenac sodium by bovine serum albumin method

Table 3: Thrombolytic activity of the ethanolic leaf extract of the ethanolic AgNPs leaf extract of *A. squamosa* and standard drug

Extracts/drugs	Mean±SEM (% clot lysis)
Water (negative control)	2.725±0.983%
SK (positive control)	72.835±1.702%***
Ethanolic silver nanoparticles leaf extract of *A. squamosa*	85.620±2.6%***

*Values are expressed as mean±SEM, *n*=3. *A. squamosa*: *Annona squamosa*, AgNPs: Silver nanoparticles

clot lysis which was only 2.72%. The mean difference in percentage clot lysis between positive and negative control was found to be very statistically significant (***$P < 0.001$). However, when 100 µL sample formulation was added to three different clots, 85.620% clot lysis was obtained and when compared with the negative control (water) the mean clot lysis percentage difference was found to be statistically significant (***$P < 0.001$) [Table 3].

CONCLUSION

From the result of the study, it can be concluded that the ethanolic AgNPs leaf extract of *A. squamosa* possessed anti-oxidant, antiarthritic, and thrombolytic activities. However, one should try to further figure out extract more as having much better activity in quest of active candidate or chemical molecule that is mainly responsible for this activity through detailed experimentation.

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