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**Vinita Chawla**
Department of Biotechnology, Progressive Education Society's Modern College of Arts, Science and Commerce, Shivajinagar, Pune, Maharashtra, India

**Dr. Sangeeta Sathaye**
Department of Biotechnology, Progressive Education Society's Modern College of Arts, Science and Commerce, Shivajinagar, Pune, Maharashtra, India

**Correspondence**
**Vinita Chawla**
Department of Biotechnology, Progressive Education Society's Modern College of Arts, Science and Commerce, Shivajinagar, Pune, Maharashtra, India

# Biosynthesis of silver nanoparticles using methanolic extracts of *Acorus calamus*, and assessment of its antioxidant and antimicrobial activity

**Vinita Chawla and Dr. Sangeeta Sathaye**

**Abstract**
*Acorus calamus* (Sweet flag) is a wetland perennial monocot plant, the scented leaves and rhizomes of which have been traditionally used as medicine against different ailments like, fever, asthma, bronchitis, cough and mainly for digestive disorders such as bloating, colic, and poor digestive function. In addition, Ayurvedic medicine *Calamus* is also valued as a "rejuvenator" for the brain and nervous system. The present investigation was undertaken to prepare the plant extract, to analyze it for its phyto constituents, synthesize the Silver nanoparticles biologically, and to check the antimicrobial and antioxidant activity of the synthesized silver nanoparticles. The silver nanoparticles synthesized were characterized by UV Vis spectrophotometry, Fourier Emission Scanning Electron Microscopy (FESEM), X - Ray Diffraction (XRD) and Electron Dispersive Spectroscopy (EDS). The nanoparticles exhibited antioxidant activity and antimicrobial activity against Gram positive and Gram negative bacteria.

**Keywords:** *Acorus Calamus*, phytochemical analysis, Silver nanoprticles, antioxidant activity, antimicrobial activity

## Introduction
The use of plants as the production assembly of silver nanoparticles has drawn attention because of its rapid, ecofriendly, non-pathogenic, economical protocol and providing a single step technique for the biosynthetic processes. The reduction and stabilization of silver ions by combination of biomolecules such as proteins, amino acids, enzymes, polysaccharides, alkaloids, tannins, phenolics, saponins, terpinoids and vitamins which are already established in the plant extracts having medicinal values and are environmental benign, yet chemically complex structures [1]. Out of all nanomaterials, the metal nanoparticles have kindled much interest owing to their potential applications in catalysis, photonics, optics and biomedicine [2]. Nanotechnology has proven to open newer synthesis methods that can produce size controlled nanoparticles that can fight and stop the spread of disease. Metallic nanoparticles have large surface to volume ratios and crystallographic surface structure that make them excellent antibacterial materials [3]. The most commonly synthesized metallic nanoparticles are prepared from metals such as Gold, Silver, Platinum and Lead. Among these silver (Ag) has been used for ages in the field of biology and medicine for its antibacterial potency [4]. This biological method of synthesis is safe, economical and eco-friendly. Silver nanoparticles synthesis has been previously reported using extracts of *Aloe vera, alfalfa sprouts, Coleus amboinicus, Phytolacca decandra, Gelsemium sempervirens, Hydrastis canadensis, Thuja occidentalis, Jackfruit, Cycas, Azadirachta indica, Ocimum sanctum and Banyan* [5-11]. Silver nanoparticles are silver particles of size ranging from 1 nm and 100 nm. These particles get attached to cell wall thus disconcerting cell-wall permeability and cell respiration. Using a plant for synthesis of nanoparticles and characterizing its nanoparticles is an easier process as maintaining cell cultures and synthesizing its nanoparticles is an elaborate process. Green synthesis of nanoparticles is an emerging branch of nanotechnology, future these biologically synthesized nanoparticles were found highly toxic against different multi drug resistant human pathogens. Many methods have been used for the synthesis of silver nanoparticles, like chemical and photochemical reduction [12, 13], radiolysis methods [14] and electrochemical techniques [15]. The synthesis of nanoparticles using plant extracts is often termed as 'green synthesis' method that reduces or eliminates use and/or generation of hazardous substances.

This biological method for synthesis of nanoparticles uses plant based extracts, products, enzymes, reducing factors, proteins, peptides, antioxidants, triglycerides, saponins, glycoproteins, antioxidants, pigments, latex, gums, polysaccharides, phytochemical constituents like terpenoids, flavonoids, tannins, vitamins and cofactors for reduction and/or stabilization of nanoparticles [16, 17].

Both roots and leaves of *Acorus calamus* have shown antioxidant, antimicrobial and insecticidal activities [18].

**Fig 1:** *Acorus calamus* plant

## Methodology

### Preparation of Plant Extract

The plant of *Acorus calamus* was collected from More Garden Nursey, Manjri Pune. The plant sample was authenticated by Botanical Survey of India (BSI), Pune Voucher No. BSI/WRC/100-1/Tech./ 2017/1. The leaves of *Acorus calamus* were initially rinsed twice in distilled water and dried using a paper towel, Samples of 10 gm size were cut into fine pieces and dried. Later the methanolic extract was prepared by Soxhlet extraction method. The extract was filtered through Whatman No.1 filter paper and the filtrates were stored at $4^0$C for further use [19].

### Phytochemical Analysis

The phytochemical analysis was performed as per experimental method described by Prashant Tiwari [20].

**1. Test for steroids**

Crude extract was mixed with 2ml of chloroform and concentrated H2SO4 added sidewise, a red color produced in the lower chloroform layer indicated the presence of steroids.

Another test was performed by mixing crude extract with 2ml of chloroform. Then 2ml of each of concentrated H2SO4 and acetic acid were poured into a mixture. The development of a greenish coloration indicated the presence of steroid.

**2. Test for Terpenoids**

Crude extract was dissolved in 2ml of chloroform and evaporated to dryness. To this 2ml of concentrated H2SO4 was added and heated about 2 minutes. A greyish color indicated the presence of Terpenoids.

**3. Test for Alkaloids**

Dragendroff's Test: Filtrates were treated with Dragendroff's reagent (solution of Potassium Bismuth Iodide). Formation of red precipitate indicated the presence of alkaloids

**4. Test for Flavanoids**

Alkaline Reagent Test: Extracts were treated with few drops of sodium hydroxide solution. Formation of intense yellow colour, becoming colourless on addition of dilute acid, confirmed the presence of flavonoids

**5. Test for glycosides**

Modified Borntrager's Test: Extracts were treated with Ferric Chloride solution and immersed in boiling water for about 5 minutes. The mixture was cooled and extracted with equal volumes of benzene. The benzene layer was separated and treated with ammonia solution. Formation of rose-pink colour in the ammonical layer indicates the presence of anthranol glycosides.

**6. Test for Phytosterols**

Salkowski's Test: Crude extract was mixed with 2ml of chloroform. Then 2ml of concentered H2SO4 was added carefully and shaken gently. A reddish brown color indicated the presence of steroids rings glycine portion of glycoside.

**7. Test of phenols and tannins**

**Ferric Chloride Test:** Extracts were treated with 3-4 drops of ferric chloride solution. Formation of bluish black colour indicates the presence of phenols. presence of phenols and tannins.

**Gelatin Test:** To the extract, 1% gelatin solution containing sodium chloride was added. Formation of white precipitate indicates the presence of tannins.

**8. Test for detection of proteins and aminoacids**

Ninhydrin Test: To the extract, 0.25% w/v ninhydrin reagent was added and boiled for few minutes. Formation of blue colour indicates the presence of amino acid.

**9. Test of Carbohydrates [Benedicts Test]**

Benedict's Test: Filtrates were treated with Benedict's reagent and heated gently. Orange red precipitate indicates the presence of reducing sugars.

**10. Test for Saponins**

Froth Test: Extracts were diluted with distilled water to 20ml and this was shaken in a graduated cylinder for 15 minutes. Formation of 1 cm layer of foam indicates the presence of saponins.

### Synthesis of Silver Nanoparticles

For the synthesis of silver nanoparticles, 45ml of methanolic extract of *Acorus calamus* was added to 45ml of 1mM of aqueous AgNo3 solution in 100ml conical flask and this setup was incubated in dark room, at $37^0$C under static condition. Suitable controls were maintained throughout the control of experiment. Reduction of silver nitrate to silver ions was confirmed by the colour changes to light yellow colour to brown colour the fully reduced solution was centrifuged at 5000 rpm for 30mins. The supernatant was discarded and the pellet obtained was re-dispersed in deionized water. The centrifugation process was repeated for 2-3 times to wash off any absorbed substances on the surface of the silver nanoparticles.

### Characterization

**1. UV Vis Spectrophotometry**

The silver nanoparticles were characterized by UV-Vis spectroscopy, one of the most widely used techniques for structural characterization of silver nanoparticles [21]. UV-Visible absorption spectrophotometer with a resolution of 1nm between 300 to 700 was used. The reduction of pure Ag+ ions was monitored by measuring the UV-Vis spectrum of the reaction medium after diluting small aliquot of the

sample into deionized water. 1mL of the sample was pipetted into test tube and diluted with 4ml of deionized water and subsequently analyzed at room temperature, it is done by using a Schimadzu (UV-2450, Japan)

**2. X ray Diffraction (XRD) Analysis**

The crystalline nature of silver nanoparticles was checked by X-ray diffraction (XRD) analysis using an X-Ray diffractometer (Bruker AXS D8 ADVANCE).The size, shape, lattice parameter determination and phase fraction analysis of the unit cell for any compound can be determined easily by XRD. The information of translational symmetry-size and shape of the unit cell are obtained from peak positions of Diffraction pattern [22].

**3. FESEM (Fourier Emission Scanning Electron Microscopy)**

The morphology and topography of the nanoparticles was analysed using Field Emission Scanning Electron Microscope (FESEM) (FEI Nova Nano, Lincoln, SEM 450).

**4. Energy Dispersive X-ray Spectroscopy (EDX or EDS)**

is a technique used to identify the elemental composition of as little as a cubic micron of material[23]. This digital quantum counting technique makes the energy dispersive spectrometry exceedingly reliable [17]. A semiconductor material is used to detect the X-ray together with processing electronics to analyses the spectrum EDX observations were carried out by a Bruker X-Flash 6130.

The XRD Analysis, FESEM and EDS Analysis were performed at Savitribai Phule University of Pune.

**DPPH assay**

Using Blois (1958) approach with minor modification [24], free radical scavenging activity of different dry and fresh extracts was estimatied. The fresh extracts of *Acorus calamus* at different concentrations (50, 100, 150and 200 ppm equivalent to 50, 100 and 200 µg, respectively) were taken in separate test tubes. One millilitre of DPPH (2, 2-diphenyl-1-picrylhydrazyl) solution (0.1 Mm) was dissolved in methanol and added each to test tube and shaken vigorously [24]. After the addition of DPPH solution, all of the test tubes were shaken gently and allowed to stand at 27 °C in a dark place for 45 min. The blank and positive controls were prepared in the same way without any extract. Ascorbic acid was used as standard at the concentration of 50 ppm. The absorbance of the prepared samples was measured using UV spectroscopy at a wavelength of 517 nm. Each method in this experiment was replicated three times. Radical scavenging activity of the tested crude extract samples was estimated as an inhibition percentage and was calculated by using the following formula,

Measurement of radical scavenging activity (%) = $A^{control} - A^{extract} / A^{control} * 100$

**Antimicrobial Activity**

The disc diffusion method was used to screen the antimicrobial activity[25]. The invitro antimicrobial activity was screened by using Nutrient Agar. The Nutrient Agar plates were prepared by pouring 15 ml of molten media into sterile plates. The plates were allowed to solidify for 5 min and 0.1% inoculums was allowed to dry for 5 mins. The anti microbial activity was studied against *E.coli, Bacillus subtilis* and *S. aureus* by using the synthesized nanoparticles of methanolic extracts of *Acorus calamus*. The methanolic extract (20µl) were loaded on 4mm sterile discs. The loaded discs were placed on the surface of the medium and the compound was allowed to diffuse for 5 minutes and the plates were incubated at 37 ˚C for 24 hours and the zone of inhibition obtained was measured and recorded. The positive control was Gentamycin and the negative control was Distilled water.

**Result and discussion**

**Synthesis of AGNPS (Visual inspection)**

After 12 hours of incubation the colour of the plant extract with silver nitrate solution in the flask turns from light green to brown. It is inferred that Silver nanoparticles exhibit bark brown colour in an aqueous solution. This brown colour arises due to excitation of Surface Plasmon Vibrations in the metal nanoparticles [26]. This important observation indicates that the reduction of Ag+ions takes place extracellularly. The appearance of dark brown colour in the solution containing the *Acorus calamus* aqueous extract is a clear indication of the formation of silver nanoparticles in the reaction mixture (Fig 2).

**Fig 2:** Bioreduction of silver nitrate by *Acorus Calamus* Leaf extract (a) Aqueous extract (b) colour change after addition of silver nitrate to it.

**Phytochemical Analysis**

**Table 1:** Results of Phytochemical analysis

| Sr. No. | Test | Result |
|---|---|---|
| 1 | Test for Steroids | + |
| 2 | Test for Terpenoids | + |
| 3 | Test for Alkaloids | + |
| 4 | Test for Flavanoids | + |
| 5 | Test for Glycosides | + |
| 6 | Test for Phenols and Tannins | + |
| 7 | Test for Proteins | + |
| 8 | Test for Carbohydrates | + |

+ = Positive/ Present, - = Negative/ Absent

**Characterisation of Nanoparticles**

**UV-Visible Spectrometry**

Silver nanoparticles suspension exhibits an intense dark brown colour due to the surface Plasmon resonance (SPR), which form collective oscillations of their conduction band electrons in response to electromagnetic waves. Consequently, absorbance peaks can be used as tools to predict size and stability. A strong broad peak located between 425 and 435nm was observed for the silver nanoparticles prepared using the *Acorus calamus* aqueous extract. Observation of this peak, assigned to a surface Plasmon, is well documented for various metal nanoparticles with sizes ranging from 2 to 100 nm. The mean absorbance peak was observed at 446nm (Fig 3).

| Absorbance | Wavelength |
|---|---|
| 1 | 190 |
| 3.2 | 296 |
| 4 | 375 |
| 4.4 | 446 |
| 5 | 321 |
| 5.6 | 175 |

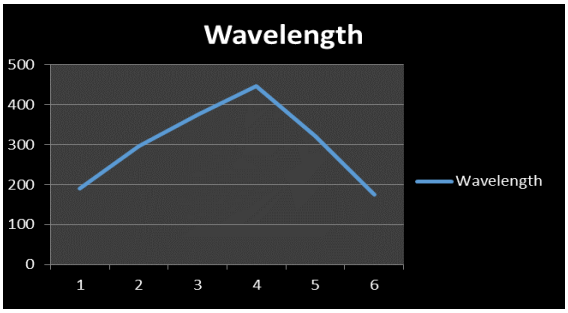


**Fig 3:** Graph indicating the conversion of silver nitrate to silver nanoparticle (446nm)

**Xray Diffraction XRD Analysis**

The XRD of silver nanoparticles shows the presence of peaks (at 2θvalue) that correspond to (161), (137) and (25) planes of silver. This confirms the crystalline nature of the silver nanoparticles and the size of the nanoparticles from Scherrer's formula as well as FEG-SEM data ranges from 120-240 nm.

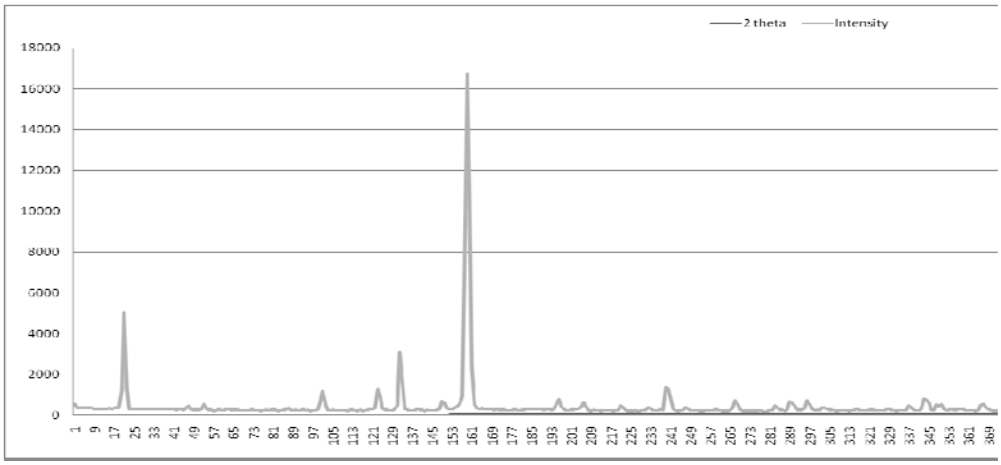


**Fig 4:** XRD of Silver nanoparticles produced by *Acorus Calamus* Plant.

**Fourier Emission Scanning Electron Microscopy (FESEM)**

Morphological and structural studies were investigated using Fourier emission scanning electron microscopy was utilized to characterize the particle and their shapes, size and distribution by taking micrograph from drop coated films of the silver nanoparticles. The size of nanoparticles was found to be ranging between 10nm – 17nm (Fig 5). They appeared to be spongy, rhombic in shape and stable.

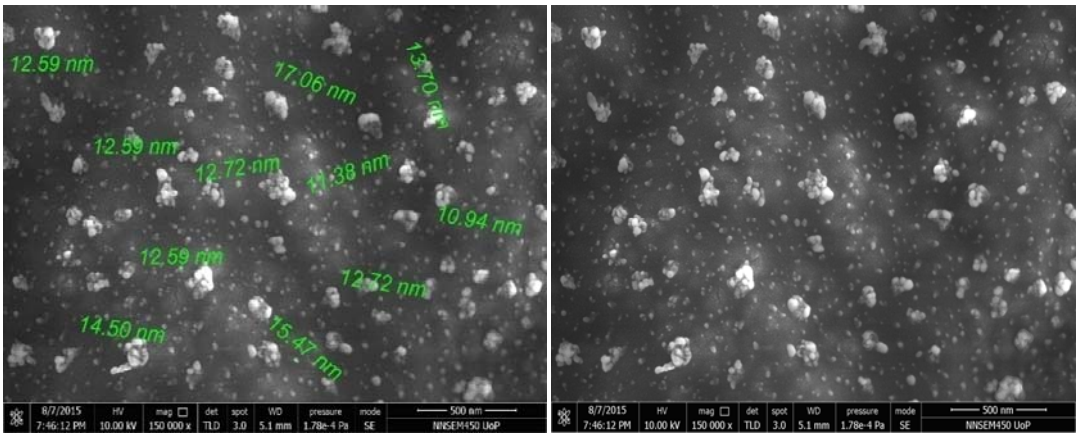


**Fig 5:** The Fourier Emission Scanning Electron Microscopy (FESEM) image that confirms the formation of silver nanoparticles capped with biomoites.

**Energy Dispersive Analysis (EDS)**

The EDS of nanoparticles ascertained the presence of 65% silver ion in the particles. The EDS shows a characteristic strong peak, indicating the binding energy of silver. The EDS sprectrum also indicated the presence of weak signals from Oxygen, Nitrogen and Carbon in the matrix, which might have appeared due to the X-Ray emissions from the peptides, phytochemicals, liposomes and enzymes present in the plant extract of the plant that have coated the nanoparticles(Fig No 6).

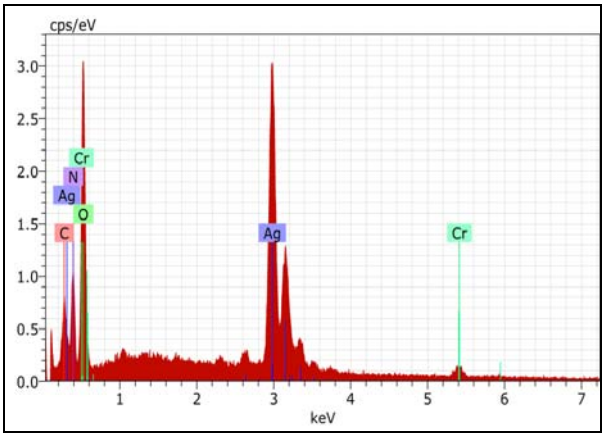


**Fig 6:** EDS of silver nanoparticles produced by *Acorus calamus* leaves.

**Antioxidant activity**

The antioxidant activity of the plant extract was studied using DPPH assay and the results are presented in Fig. 7. The antioxidant activity was found to be better for the higher concentration of the leaf extract. At 250 ppm 69% Radical scavenging.

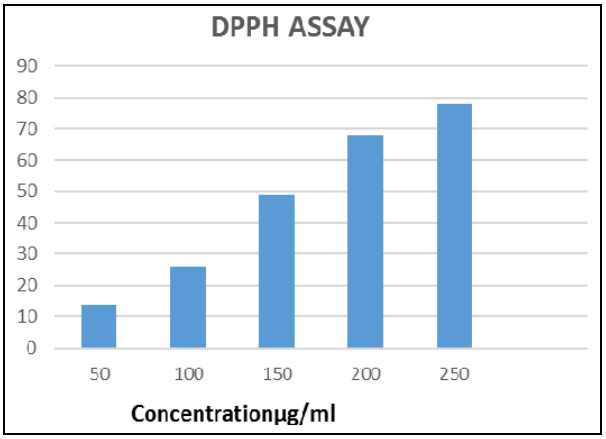


**Fig 7:** Graph indicating the Radical Scavenging Activity (Antioxidant activity) at increasing concentrations.

**Antimicrobial Activity**

Silver and its compounds are effective antimicrobial agents [28-30]. The antibacterial activity of silver nanoparticles was studied against *E. coli*, *B. subtilis* and *S. aureus*. The silver nanoparticles demonstrated a zone of inhibition against all the test organisms with maximum inhibition against Gram negative E. coli (12 mm), followed by antimicrobial activity against Gram positive B. subtilis (9 mm) and S. aureus (8 mm). Several mechanisms have been proposed for the mode of action of silver nanoparticles against bacteria. Silver nanoparticles have demonstrated bactericidal activity by inhibiting cellular respiration and membrane permeability [31]. The nanoparticles bind to the sulphur containing proteins on bacteria and inactivate them. It is reported that the attachment of silver nanoparticles to the bacterial cell wall dissipates proton motive force, destabilizes the outer membrane and ruptures the cell causing depletion of intracellular ATP[32]. Besides the activity of the silver nanoparticles, the antimicrobial compounds and phytoconstituents present in the plant extract may also contribute to the antimicrobial activity of the biostabilized silver nanoparticles. Biologically synthesized silver nanoparticles could be of immense use in medical for their efficient antimicrobial properties

**Conclusion**

In conclusion, the *Acorus calamus* methanol extract has shown potential for extra cellular synthesis of fairly monodispersed, silver nanoparticles in the range of 10 to 17nm. Silver nanoparticles were biologically synthesized from *Acorus calamus* leaf methanol extract. The phytochemical analysis revealed the presence of Steroids, Terpenoids, Alkaloids, Flavanoids, Glycosides, Phenols, Tannins, Proteins and Carbohydrates. The detail characterization of the nanoparticles was carried out using UV-Vis spectroscopy, Scanning Electron Microscopy (SEM), X-Ray Diffraction (XRD) analysis and Energy Dispersive Spectroscopy (EDS). The XRD analysis revealed the presence of crystals. From FESEM image analysis, the average particle size was found to be 10nm and 17nm. The reduction of silver nitrate to silver nanoparticles with high stability and without any impurity was confirmed. Comparison of experimental results showed that the average size of synthesized silver nanoparticles was about 14 nm. The antioxidant activity was confirmed by DPPH assay and found to increase with concentration of plant extract. At 250 ppm 69% radical scavenging activity was analysed. The nanosilver was found to have antimicrobial activity in Bacillus subtilis, E coli and S aureus organisms. Biologically synthesized silver nanoparticles could be of immense use in medical for their efficient antimicrobial and antimicrobial properties We believe that the silver nanoparticle has great potential for applications in catalysis, biomedical, and pharmaceutical industries. The preliminary work carried out and the observations recorded need further studies to conclusively prove better antimicrobial active in biologically synthesized silver nanoparticles.

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