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SYNTHESIS OF SILVER NANOPARTICLES USING FLAVONOID: APIGENIN AND ITS ANTIBACTERIAL EFFECT

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ABSTRACT

Apigenin, a dietary plant derived flavonoid was isolated from the leaves of *Smilax perfoliata*. The separation was carried out by different chromatographic techniques and their structures were elucidated by IR spectra, MS, ^{1}HNMR, and ^{13}CNMR spectroscopic methods. Further, the isolated flavonoid – apigenin was used for the synthesis of silver nanoparticles (AgNPs). Aqueous solution of pure flavonoid mixed with 1mM $AgNO_3$ solution were exposed to bright sunlight to prepare the nanoparticles. Characterization of the synthesized nanoparticles by UV–Visible spectrophotometer, X-ray diffraction and scanning electron microscopy revealed that the synthesized silver nanoparticles were 10–80 nm in size and polydispersed in nature. Bactericidal effect against common pathogens of the synthesized silver nanoparticles was investigated. It is concluded that AgNPs synthesized using Apigengin as reducing agent showed higher stability and better antibacterial activity.

KEYWORDS: Apigenin, Flavonoids, Silver Nanoparticle, Bio-synthesis, *Smilax perfoliata.*

INTRODUCTION

Smilax (Family -*Smilacaceae*) is a large genus of climbing shrub distributed in tropical and temperate regions of the world. *Smilax perfoliata Lour* is found in various parts of India and has tuberous rhizomes. It is a robust more or less strongly armed climber. Stem is used as tooth brush to strengthen the gums. Tender shoot is taken in curries and is useful as blood purifier.[1] Roots and stems are used as anticancer, anti-dysenteric and in urinary complaints.[2] The leaves and fruits of *Smilax perfoliata* are traditionally used for treatment of various ailments such as rheumatism, lumbago, nourishing the functions of spleen, stomach, muscle and bone.[3]

The nanoparticles (Nps) of noble metals are nontoxic to human cells and have high thermal stability.[4] In addition to non-toxicity, their unique characteristics include optical, electrical.[5] and magnetic properties.[6] These have made AgNPs to own applications ranging from catalysts and sensing to optics, antibacterial agents, antioxidant agents and data storage.[7,8] In particular, silver nanoparticles (AgNPs) exhibit fabulous antimicrobial potential and can be used in medicine for dental materials, burn treatment, water treatment and coating stainless steel materials.[9,6] For the synthesis of AgNPs, chemical, physical and biological methods have been used and among those, the chemical method is extensively employed owing to its high yield and quick throughput.[10,11] Yet, the chemical synthesis of NPs frequently necessitates the utilization of toxic reagents such as reducing and stabilizing substances. Hence, biological synthesis of AgNPs has been suggested as an ecofriendly and less toxic approach.[12] The present study is an attempt to test the antibacterial efficacy of AgNPs synthezied by using a flavanoid –apigenin, which is isolated from the leaf extract of *Smilax perfoliata*, which have been using in traditional medicine without any validation.

MATERIALS AND METHODS

Plant Material

Fresh leaves of *Smilax perfoliate* were collected from Siluvinippatti village, Sivagangai District, Tamil Nadu during June 2016 and were dried under shade for several days.

Isolation of Total Flavonoids by Soxhlet Extraction Method

Before extraction, *S. perfoliata* was crushed into powder. The powdered sample was degreased by soxhlet extractor with petroleum ether until the colour of elute becomes colourless. The same powder sample was accurately weighed and placed in soxhlet extractor by adding 80 mL of ethanol: water (70: 30) solvent, followed by the extraction for up to 5 h, and then extract solution was concentrated. The extract was centrifuged for 30 min; supernatant was taken for further use

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Separation of Flavonoids by Column Chromatography

The total flavonoids which are isolated can be purified and separated by column chromatography separation method. The 45 cm length and 3 cm width of the column were used and it is filled with the slurry of silica gel-H of mesh size 60–120 μ (Hi media, Mumbai) to 1/3 portion using n-hexane. Set the column by the solvent n-hexane. 10 g of total flavonoid extract was bound with silica gel and loaded on the top of the column. The column was eluted with gradient solvent system of n-butanol: water: acetic acid system 12: 2 : 1 v/v/v until the colour of the column is colourless. The total flavonoid of ethanol extract of *S. perfoliata L.* of about 20 g were fractionated on a silica gel-H (60–120 mesh) column. The total 32 fractions of 30 mL of each were collected. Sub fractions were concentrated and monitored by TLC using n-butanol-water-acetic acid (12: 2 : 1 v/v/v) solvent system as mobile phase. Similar fractions combined together and recrystallized with chloroform. The purified compound was subjected to its spectral analysis for structural elucidation.

Structure Elucidation of Flavonoid by Spectral Analysis

The pure compound of *S. perfoliata L.* isolated was subjected to IR, MS, ^{1}HNMR and ^{13}CNMR studies to obtain the structure of the isolated compound.

Silver nanoparticles synthesis

1 mM solutions of $AgNO_3$ and 0.02 % flavonoid were used as stock solutions for the preparation of AgNPs. The flavonoid was dissolved in water by slightly increasing the pH using NaOH as they are sparingly soluble in water. After complete dissolution, the pH was neutralized using HCl. 10 mL of aqueous flavonoid solution was mixed with 90 mL of 1 mM $AgNO_3$ solution separately. The reaction mixtures were immediately placed in bright sunlight and observed for change in colour of the solution. After 5 minutes, the resulting solution was turned out from yellow to dark brown colour indicating the synthesis of silver nanoparticles. After the incubation, the solution was centrifuged at 18,000 rpm for 25 minutes. The pellet was resuspended in distilled water and stored in the freezer for the further study.

Characterization of synthesized silver nanoparticles

UV-Vis and XRD Studies: The reduction of pure Ag^{+} ions were monitored by measuring the UV-Vis spectrum of the reaction medium at 5h after diluting a small aliquot of the sample into distilled water. UV-Vis spectral analysis was done by using UV-Vis spectrophotometer UV-1800. For XRD studies, dried nanoparticles were coated on XRD grid, and the spectra was recorded by using philips PW1830 X-ray generator operated at a voltage of 40kV and a current of 30mA with Cu Kα1 radiation.

SEM analysis of silver nanoparticles: SEM analysis was done by using VEGA3 TESCAN machine, Japan. Thin films of the sample were prepared on a carbon coated copper grid by a dropping a very small amount of the sample on the grid. Extra solution was removed using a blotting study and then the films on the SEM grid were allowed to dry by putting it under a mercury lamp for 5 min.

Assay of Antibacterial Activity

Disc Preparation: The 6mm (diameter) discs were prepared from Whatmann No. 1 filter paper. The discs were sterilized by autoclave at 121°C.After the sterilization the moisture discs were dried on hot air oven at 50°C.Then discs were mixed with chemical compounds separately and control discs were prepared.

Collection of test microorganisms: The Bacterial strains of *Staphylococcus aureus* , *Bacillus subtilis, Escherichia coli* , obtained from Microbial Type culture Collection Centre (MTCC), Chandigarh.

Antibacterial activity test was carried out following the modification of the method originally described by Bauer *et al.*, (1966).[13] Muller Hinton agar was prepared and autoclaved at 15 lbs pressure for 20 minutes and cooled to 45 ºC. The cooled media was poured on to sterile petri plates and allowed for solidification. The plates with media were seeded with the respective microbial suspension using sterile swab. The various solvents extract prepared discs individually were placed on the each petri plates and also placed control and standard (*Ampicillin*) discs. The plates were incubated at 37 ºC for 24 hrs. After incubation period, the diameter of the zone formed around the paper disc were measured and expressed in mm.

RESULTS AND DISCUSSION

Structure Elucidation of Flavonoid by Spectral Analysis

Yellow crystalline solid obtained (R_f 0.83). The molecular formula and molecular weight were found to be $C_{15}H_{10}O_5$ and 270.2375 respectively. IR spectrum showed the band in the region of 3300 cm^{-1} shows probably the result of υ_{OH} vibration of phenol OH groups. The intensive band at approximately 1715cm^{-1} is most probably the result of $\upsilon_{C=O}$ vibration of C=O group from central heterocyclic ring, while the υ_{C-O} vibration occurs at approximately 1160 cm^{-1}. ^{1}H-NMR (500 MHz, DMSO-d6) Singlet protons available at δ 6.79. Doublet protons available at 6.20, 6.48, 6.93, 7.93. –OH protons(singlet) available at 10.35 , 10.82 , 12.97 . ^{13}C-NMR (125 MHz, DMSO-d6) : The signals at 162.08, 162.37, 164.65 and 165.04 indicate the equivalent carbon atoms on benzene ring. MS: The mass spectrum (**Fig. 2**) that displayed a molecular ion (M^{+}) peak at *m/z* 270 indicates the molecular weight of the compound corresponds to the molecular weight of apigenin and also corresponds to molecular formula $C_{15}H_{10}O_5$. This further

confirms that the structure of the isolated compound is apigenin (**Fig. 1**).

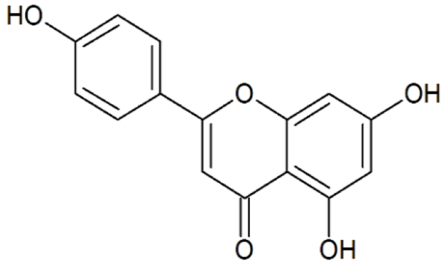

Fig. 1. Structure of Apigenin

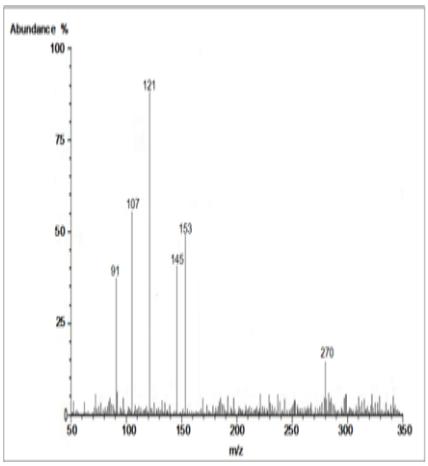

Fig.2. Mass spectrum of isolated compound

Physical characterization of AgNPs

UV-Visible analysis: Mixing of flavonoid solution with silver nitrate solution brought immediate change in colour of reaction mixture. Initially the colour changed into faint white from transparent, which turned into dark brown within 5 min of exposure to sunlight. The instant colour change could be due to reaction between Ag ions and flavonoid solution that resulted in faint white colour of solution. Placing the reaction solution in bright sunlight reduced the Ag ions therefore, the colour of the solution changed into dark brown **(Fig. 3)** which indicated the formation of AgNPs.[14,15] UV-Visible spectroscopy indicated that the phytosynthesized silver nanoparticles had an absorbance at about 435 nm **(Fig. 4)** with a narrow peak width at half maximum, suggesting the nanoparticles narrow size distribution. The UV-Visible spectra also revealed the silver nanoparticles occurrence rapidly within five minutes.

Fig. 3. Addition of flavanoid resulted in the colour change of $AgNO_3$ solution to dark brown

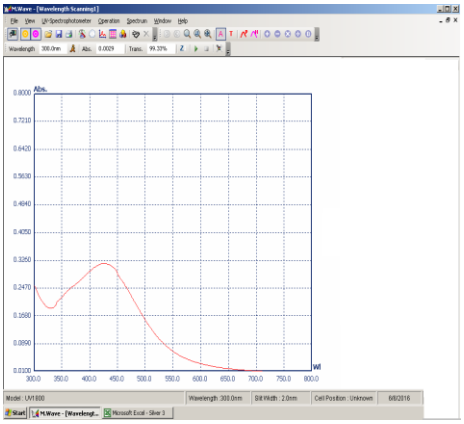

Fig. 4. UV vis spectra of synthezied AgNPs

SEM analysis: Scanning electron microscope analysis indicated the polydispersed AgNPs with particle size range from 10-80 nm **(Fig. 5)**. This is further supported by XRD studies. XRD study confirmed the existence of silver colloids in the sample. The Braggs reflections were observed in the XRD pattern at 2 Ø = 27.960, 32.500, 38.160 and 46.300. These Braggs reflections clearly indicated the presence of (111), (200), (220) and (331) sets of lattice planes, which can be indexed as face-centred-cubic (FCC) structure of silver. Hence, XRD pattern evidently illustrated the silver nanoparticles as crystalline in nature **(Fig. 6)**.

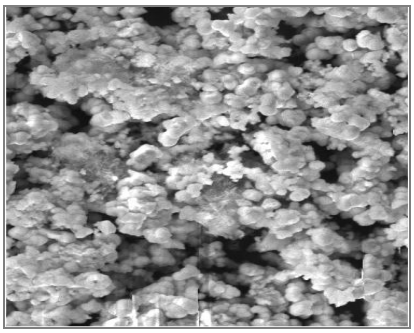

Fig. 5. Scanning Electron Microscope image of synthezied AgNPs

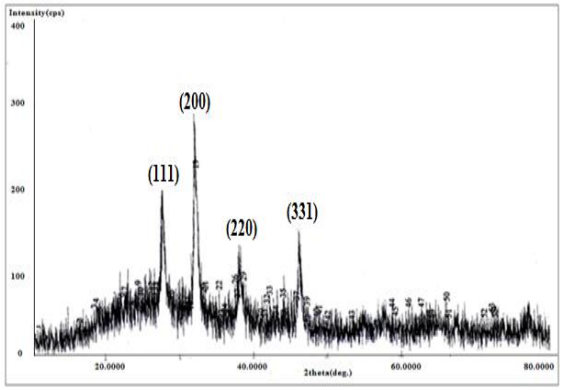

Fig. 6. X-Ray Diffraction pattern of synthezied AgNPs

In this study synthesized AgNPs showed antimicrobial activity against *B. Subtilis, E. Coli* and *S. aureus* . The size of zone of inhibition by synthesized nanoparticles is presented in **Table 1**and **Fig. 7**. AgNPs showed good antimicrobial activity against *E. coli*, the inhibitory effect of AgNPs was mild in *S. aureus.* Capping agents of NPs also affect the antimicrobial activity of AgNPs. In this study, flavonoid might also act as capping agents of synthesized AgNPs, therefore, affecting the antimicrobial activity of AgNPs.[15]

Table 1: Antimicrobial activity of the Plant Extract, Isolated Flavanoid and Silver Nano Particles

S. No.	Bacteria	Zone of Inhibition (mm in diameter)				
		Control	Standard	Plant Extract	Flavanoid	AgNPs
1	***Bacillus subtilis***	-	15	18	18	20
2	***Escherichia coli***	-	12	21	20	22
3	***Staphylococcus aureus***	-	15	17	16	18

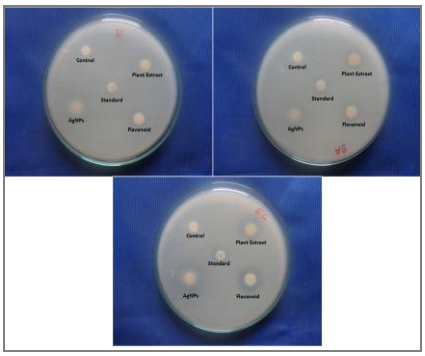

Fig.7. Antibacterial activitity of synthesized AgNPs against common pathogens

CONCLUSION

In this study silver nano particles were synthezied by using apigenin, which is isolated from the leaves of *Smilax perfoliata*. Silver nano particles (AgNPs) were characterized by using ultraviolet-visible (UV-Vis) spectrophotometry, scanning electron microscopy (SEM), and X-ray diffraction. The ultraviolet and visible absorption spectroscopy results show a strong resonance centred on the surface of silver nanoparticles at 435 nm. SEM showed the formation of silver nanoparticles were polycrystalline with nano sized grains. X-ray diffraction analysis showed that the particles were crystalline in nature with face centered cubic structure of the bulk silver with the broad peaks at 27.960, 32.500, 38.160 and 46.300. The bactericidal properties of the synthesized AgNPs were investigated. The results conclude that silver nanoparticles have good antibacterial activity against different microorganisms such as *B.subtilis, E. coli and S. aureus*. The synthesized silver nanoparticcles and the leaf extract the plants were found to exhibit antibacterial property.

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