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Original Article

SYNTHESIS AND CHARACTERIZATION OF SILVER NANOPARTICLES USING *TABERNAEMONTANA DIVARICATA* AND ITS CYTOTOXIC ACTIVITY AGAINST MCF-7 CELL LINE

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ABSTRACT

Objective: *Tabernaemontana divaricata* a common garden plant on tropical countries has been used as a traditional medicine. There is an increasing commercial demand for nanoparticles due to their wide applicability in various areas such as electronics, catalysis, chemistry, energy, and medicine. Metallic nanoparticles are traditionally synthesized by wet chemical techniques, where the chemicals used are quite often toxic and flammable. In this work, we describe a cost effective and environment friendly technique for green synthesis of silver nanoparticles (AgNPs) from *T. divaricata* leaf extract.

Methods: Biosynthesized AgNPs were characterized using UV– Vis absorption spectroscopy, TEM, FTIR and SEM analysis. Cytotoxicity of AgNPs was tested in human breast cancer cell line (MCF-7).

Results: TEM analysis showed the average particle size of 22.85 nm as revealed in their structure. The qualitative assessment of reducing potential of leaf extract has also been carried out which indicated presence of significant amount of reducing entities. Green synthesized AgNPs by *T. divaricata* leaf extract show cytotoxicity to human breast cancer cell line (MCF-7).

Conclusion: The most important outcome of this work will be the development of value-added products from *T. divaricata*. The characteristics of the obtained AgNPs were analysed which could be used potentially in various human contacting areas such as cosmetics, foods, and medical applications.

Keywords: *Tabernaemontana divaricata*, AgNPs, TEM, Cytotoxic analysis.

INTRODUCTION

Tabernaemontana is a genus of 100-110 species of flowering plants in the family apocyanaceae. It has a pan-tropical distribution. These plants are shrubs and small trees growing to 1-15 m tall. The leaves are evergreen, opposite, 3-25 cm long, with milky sap; hence it is one of the diverse plant genera commonly called "milk wood". The flowers are fragrant, white, 1-5 cm in diameter. Crape jasmine (*T. coronaria)* is also popular as an ornamental plant. Some members of the genus *Tabernaemontana* are used as additives to some versions of the psychedelic drink ayahuasca; the genus is known to contain ibogaine, conolidine and voacangine (namely in *T. africana*). *T. sananho* preparations are used in native medicine to treat eye injuries and as an anxiolytic, and *T. heterophylla* is used to treat dementia in the elderly. Plants are well known as a major source of modern medicines. Conolidine may be developed as a new class of pain-killer. Caterpillars of the oleander hawk-moth (*Daphnis nerii*) have been found to feed on pinwheel flower (*T. divaricata*). From ancient times, humans have utilized plants for the treatment or prevention of diseases, leading to the dawn of traditional medicine [1]. *Tabernaemontanum* is one of the genera that are used in Chinese, ayurvedic and Thai traditional medicine for the treatment of fever, pain and dysentery. *T. divaricata* belonging to apocyanaceae family is traditionally used by people in many parts of the world to treat various disorders [2].

Biological methods of synthesis have paved way for the “greener synthesis” of nanoparticles and these have proven to be better methods due to slower kinetics, they offer better manipulation and control over crystal growth and their stabilization. This has motivated an upsurge in research on the synthesis routes that allow better control of shape and size for various nanotechnological applications. The use of environmentally benign materials like plant extract, bacteria, fungi and enzymes for the synthesis of silver nanoparticles offers numerous. Chemical synthesis methods lead to presence of some toxic chemical absorbed on the surface that may have adverse effect in the medical applications. Green synthesis provides advancement over chemical and physical method as it is cost effective, environment friendly, easily scaled up for large scale synthesis and in this method there is no need to use high pressure, energy, temperature and toxic chemicals. The synthesis of nanocrystals is in the limelight in modern nanotechnology. Biosynthesis of nanoparticles by plant extracts is currently under exploitation [3], [4]. Nanotechnology is currently employed as a tool to explore the darkest avenues of medical sciences in several ways like imaging, [5] sensing, [6] targeted drug delivery, [7] gene delivery systems [8] and artificial implants [9].

Silver has long been recognized as having inhibitory effect on microbes present in medical and industrial process. The most important application of silver and silver nanoparticles is in medical industry such as topical ointments to prevent infection against burn and open wounds. In recent years, noble metal nanoparticles have been the subject of focused research due to their unique optical, electronic, mechanical, magnetic, and chemical properties that are significantly different from those of bulk materials.

These special and unique properties could be attributed to their small sizes and large surface areas. For these reasons, metallic nanoparticles have found uses in many applications in different fields, such as catalysis, photonics, and electronics. Preparation of silver nanoparticles has attracted particularly considerable attention due to their diverse properties and uses, like magnetic and optical polarizability [10], electrical conductivity, catalysis [10], antimicrobial and antibacterial activities [11], [12], DNA sequencing [13], and Surface-enhanced Raman scattering (SERS) [14]. Many techniques of synthesizing silver nanoparticles, such as chemical reduction of silver ions in aqueous solutions with or without stabilizing agents, thermal decomposition in organic solvents, chemical reduction and photo-reduction in reverse micelles, and radiation chemical reduction have been reported. Most of these methods are extremely expensive and also involve the use of toxic, hazardous chemicals, which may pose potential environmental and biological risks. Since noble metal nanoparticles are widely applied

to areas of human contact, there is a growing need to develop environmentally friendly processes for nanoparticle synthesis that do not use toxic chemicals. A quest for an environmentally sustainable synthesis process has led to a few biomimetic approaches [15]. In the present work, we investigated the synthesis of stable silver nanoparticles with the biosynthesis method using plant leaves extract. The application of nanoscale materials and structures, usually ranging from 1 to 100 nanometers (nm), is an emerging area of nanoscience and nanotechnology. Nanomaterials may provide solutions to technological and environmental challenges in the areas of solar energy conversion, catalysis, medicine, and water treatment. This increasing demand must be accompanied by "green" synthesis methods. In the global efforts to reduce generated hazardous waste, "green" chemistry and chemical processes are progressively integrating with modern developments in science and industry.

In recent years, plant-mediated biological synthesis of nanoparticles is gaining importance due to its simplicity and eco-friendliness. Although biosynthesis of gold and silver nanoparticles by plants such as Alfalfa [16], *Aloe vera* [17], *Cinnamomum camphora* [3], *Geranium* [18], neem [19], *Euphorbia hirta* [20], [21] and lemongrass [22] have been reported, the potential of the plants as biological materials for the synthesis of nanoparticles is yet to be fully explored. In the present study the green synthesis of silver nanoparticles from the *T. divaricata* leaf extract has been carried out and characterized by UV-Vis spectra, SEM, TEM and FTIR analysis. The cytotoxicity activity of synthesized AgNPs against MCF -7 breast cancer cell line was determined.

MATERIALS AND METHODS

Collection of plant Materials

Leaves of *T. divaricata* were collected from SRM University campus, Kattankulathur, Kancheepuram, Tamil Nadu, India. The leaves were washed and processed for the preparation of extract. Three different extracts were prepared namely aqueous extract, solvent extract and the fresh leaves extract.

Preparation of extracts

Aqueous Extract

10g of dried powder of *T. divaricata* with 100ml of distilled water is boiled for 6hrs at slow heat. Every 2hrs it was filtered through 8 layers of muslin cloth and centrifuged at 5000rpm for 15mins. The supernatant was collected. This process was repeated twice and after 6hrs, the supernatant was concentrated to make the final volume one-fourth of the original volume. It was then autoclave at121°C and 15lbs pressure and then stored at 4°C.

Solvent extract

10g of dried powder of *T. divaricata* was extracted with 100ml of ethanol, acetone, chloroform, petroleum ether kept on a rotatory shaker at 190-220rpm for 24hrs. These were then filtered through 8 layers of muslin cloth and centrifuge at 5000rpm for 15mins. The supernatant was collected and the solvent was evaporated to make the final volume one-fourth of the original volume. It was stored at 4°C in airtight bottles for further studies.

Fresh leaf extract

T. divaricata leaves were used to make the aqueous fresh leaf extract. *T. divaricata* leaves weighing 35g were thoroughly washed in distilled water, cut into fine pieces and were boiled with 100 ml sterile distilled water for 5 mins and filtered through Whatmann no.1 filter paper (pore size 25 µm) the filtrate was used as the leaf extract for the studies[23], [24].

Synthesis of AgNPs

1mM aqueous solution of silver nitrate ($AgNO_3$) was prepared and used for the synthesis of silver nanoparticles. 10 ml of *T. divaricata* leaf extract was added into 90 ml of aqueous solution of 1 mM silver nitrate for reduction into Ag+ ions and kept at room temperature for 4 hours.

Confirmatory test for AgNPs

After boiling of the solution 10 ml of the extract was mixed to the 90 ml of 1mM aqueous silver nitrate ($AgNO_3$). Solution was heated at 80º C. Color of the solution was changed from colorless to yellowish color. Color change showed the positive test for synthesis of AgNPs.

Characterization of AgNPs

UV-absorption spectra of synthesized AgNPs by using *T. divaricata* leaf extract were measured using UV-visible spectrometer (Shimadzu UV-2700). Scanning electron microscopy (SEM) analysis of synthesized AgNPs was done using a Hitachi S-4500 SEM machine. The size and shape of the synthesized AgNPs were determined by transmission electron microscopy (TEM). The TEM images of synthesized AgNPs were obtained by using TECHNAI 10 Philips. Prior to analysis, AgNPs were sonicated for 5 minutes, and a drop of appropriately diluted sample was placed onto a carbon-coated copper grid. The liquid fraction was allowed to evaporate at room temperature. Fourier transform infrared (FTIR) spectral measurements were carried out to identify the potential biomolecules in *T. divaricata* leaf extract which is responsible for reducing and capping the bioreduced silver nanoparticles.

Cytotoxicity of AgNPs

The cytotoxicity of synthesized AgNPs against MCF-7 cells was measured by MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5- diphenyl tetrazolium bromide) assay. The MTT assay is a colorimetric, non-radioactive assay for measuring cell viability through increased metabolization of tetrazolium salt [25]. MCF-7 cells were seeded at a density of 5 x 10^4 cells/well into 96-well plates. Then, the cells were treated with different concentration of synthesized AgNPs (0–100 µl/ml) and incubated in the presence of 5% CO_2 and 95% humidity at 37°C for 24 h. MTT (5 mg/ml) was added to the incubated-cells, then further incubated for another 4 h. The crystals were dissolved in 200 µl of DMSO and the absorbance was measured in a colorimetric at 570 nm with reference filter as 655 nm [26].

RESULTS AND DISCUSSION

Of all three extracts aqueous, solvent and fresh leaf extract, fresh leaf extract showed the positive result for the green synthesis of the AgNPs. The color change of the extract from colorless to yellow color (Figure 1) by the addition of $AgNO_3$ solution indicated the formation of the AgNPs [27]. The obtained AgNPs is further processed for the analysis after pelletizing the nanoparticles.

Fig. 1: (A). *Tabernaemontana divaricata*. Linn (crape jasmine) belongs to the family apocyanaceae. (B). Fresh leaf extract and (C). Color changes to yellowish.

UV- VIS spectra analysis

The UV-Vis Spectra of aqueous component as a function of time variation of leaf broth with 1 mM aqueous AgNO3 solution. Metal nanoparticles have free electrons, which gives surface plasmon resonance (SPR) absorption band, due to the combined vibration of electrons of metal nanoparticles in resonance with light wave. In the present study reduction of silver ions present in the aqueous solution of silver complex during the reaction with the ingredients present in the Tabernaemontana divaricata plant leaf extract have been seen by the UV-Vis Spectroscopy range from 300-600nm. The maximum absorption was obtained at 430nm value (figure. 2).

The sharp bands of silver colloids were observed at 436 nm. The intensity of absorption band increases with increasing time period of aqueous component and consequent color changes were observed from without color to reddish yellow. These characteristic color variation is due to the excitation of the of the surface plasmon resonance in the metal nanoparticles [28]. The synthesized Ag nanoparticles were confirmed by visual observation. The color was changed into reddish brown due to reduction of silver ions. It was well known that Ag nanoparticles exhibits reddish brown color in aqueous solution due to excitation of surface plasmon vibrations. The synthesized Ag nanoparticle using *E.hirta* and *N.indicum* plant extracts were detected by UV-Vis spectrophotometer at various nm. Absorption spectra of silver nanoparticles formed in the reaction mixture at different nm. i.e. 340,380,420,460,500,540,580 and 620nm, the particle has increasingly sharp absorbance maximum peak at 380nm and gradually decreased while nanometer increased [21]. Silver complex during the reaction with the ingredients present in the *Cleome viscose* plant leaf extract have been seen by the UV-Vis spectroscopy and found that UV-Vis spectrograph of the colloidal solution of silver nanoparticles has been recorded as a function of time by using a quartz cuvette with water as reference. Maximum absorbance was seen at 455nm,indicating that the formation of spherical silver nanoparticles in majority or anisotropic particles whose appearance and ratio increases with time but the UV-Vis spectra for the leaf extract alone showed no absorption in the spectral window between 400-700nm [29].

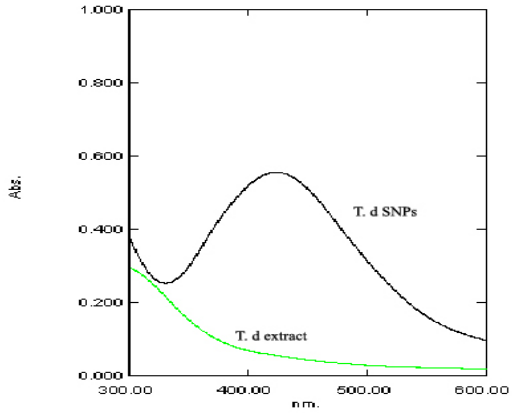

Fig. 2: UV-Vis absorption of Spectrum of AgNPs synthesized from *T. divaricata* leaf extract.

SEM Studies

After the reduction of silver ions by the *Tabernaemontana divaricata* seed extract, the solution was centrifuged at 10,000rpm for 15minutes. Repeat the centrifugation process to separate silver nanoparticle free from other organic compounds present in solution. The Ag Nanoparticle pellet obtained after centrifugation were redispersed in distilled water and washed for 2 or 3 times. As shown in the figure 3, the size of the synthesized nanoparticles from *T. divaricata* leaf extract was 32.85nm was observed.

The synthesized Nanoparticles morphology were characterized by scanning electron microscope, this micrograph was taken using a Philips model CM 200 instrument. The silver Nanoparticle formed was predominantly spherical with uniform shape. It is known that the shape of metal Nanoparticle considerably change their optical and electronic properties. The SEM image exposed that the formed nanoparticle was spherical in shape formed with the size range of 40-70 nm [30]. The SEM analysis of the sample after reduction will show the presence of the AgNO3 in the sample and are spherical shaped, well distributed without aggregation in the solution with the average size of about (5- 50nm). Synthesis of AgNO3 is quite fast and Nanoparticles formed within few hours because AgNO3 come and contact with the plant leaf sample filtrate. Mostly spherical and near spherical shape is obtained, also obtained more nanoparticles by increasing the interaction time. Some time non spherical polyhedral particle also be found, increase with the interaction time the aggregation and anisotropy shape is also increased. The result shows the silver ions formed should have Skew spherical in shape and they have the range of 60nm - 80nm. The result shows the silver ions formed should have Skew spheroid in shape and they have the range of 5-50nm [31]. Scanning electron microscopy provided further insight into the morphology and size details of the silver nanoparticles. Comparison of experimental results showed that the diameter of prepared nanoparticles in the solution was about 29-68 nm. The scanning electron micrograph of the plant extract as a positive control (incubated with deionized water for 48 h), and the scanning electron micrograph of silver nanoparticles obtained from the proposed bioreduction method at various magnifications [15], [26].

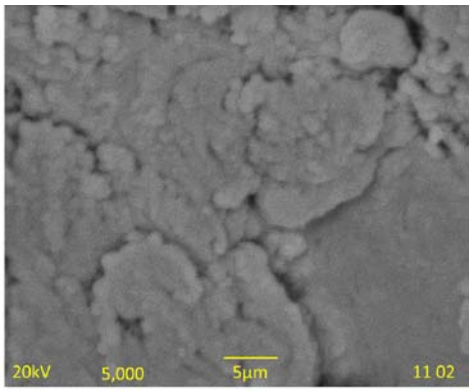

Fig. 3: SEM micrograph of AgNPs synthesized by *T. divaricata* leaf extract.

TEM Analysis

Transmission Electron Microscopy was utilized to characterize the particles and their sizes and distribution by taking micrograph from drop coated films of the silver nanoparticles shows that most of them are spherical with the average size range from 10 nm to 30 nm (figure 4) which could be correlated with the morphology of the nanoparticles which is highly variable, with spherical and occasionally triangular nanoparticles observed in the micrograph. In the present study, the mean size of synthesized nanoparticles from *T. divaricata* leaf extract 22.85 nm was observed.

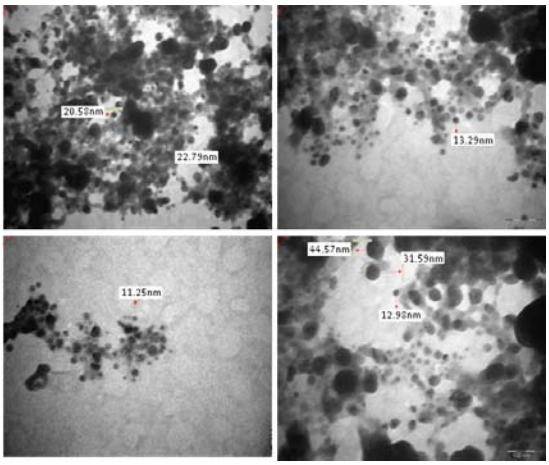

Fig. 4: Transmission electron microscopy images of AgNPs from *T. divaricata* leaf extract with different size distribution.

The nanoparticles were not in direct contact even within the aggregates, indicating stabilization of the nanoparticles by a capping

agent. This corroborates with the previous observation by in their study on *Cleome viscose* [29]. The AgNPs are predominantly spherical in shape and are not in physical contact with each other. Lower magnification image reveals the nanoparticles are embedded in a dense matrix which may be the organic stabilizing components of Tulsi leaf extract with the mean particle size of AgNPs is 18 nm [24].

FTIR Analysis

Different stretches of bonds are shown at different peaks: 3475.33- N-H stretch; 2071.22- C≡C; 1637.89- C=C; 1069.43- C=O. In the present work FTIR spectral measurements were carried out to identify the potential biomolecules in *T. divaricata* extract which is responsible for reducing and capping the bioreduced silver nanoparticles. As shown in the figure.5 FTIR spectra indicate various functional groups present at different positions. The appearance of peaks in the amide I and amide II regions characteristic of proteins/enzymes that have been found to be responsible for the reduction of metal ions when using the plant extract for the synthesis of silver nanoparticle similar to the use of microorganisms such as fungi for the synthesis of metal nanoparticles indicates the binding of the nanoparticles with proteins. The IR peaks for amide I and amide II arise owing to carbonyl stretch and –N–H stretch vibrations in the amide linkage of the proteins. IR spectroscopy study has confirmed that the carbonyl group of amino acid residues and peptides of proteins has a stronger ability to bind metal, so that the proteins could most possibly form a coat covering the metal nanoparticles (i.e. capping of AuNP) to prevent the agglomeration of the particles, and thus, the nanoparticles are stabilized in the medium [29]. FTIR measurement was carried out to identify the possible biomolecules responsible for capping and efficient stabilization of Ag Nanoparticle synthesized using *Elettaria cardamomom*. This spectrum shows lot of absorption bands indicates the presence of active functional groups in the synthesized silver Nanoparticles. The intensity peaks are slightly increased for the period of silver nanoparticle synthesis like 3429, 1637, 1382, 595 cm-1 as well as some intensity peaks decreased like 1045, 2080, and 2359 cm-1. The band at 3429 corresponds to N-H, O-H Stretching vibrations of alkanes, amide, alcohol and H–bonded to phenols. The peak at 1637 indicate to C=C, C=O stretching vibrations to alkenes and amide. The peak at 1382 represents to C-H in plane bend to alkenes. The peak at 595 corresponds to C- Cl, C-Br stretching vibrations to alkyl halides. The band at 2080 corresponds to C-N stretching vibration. The weak band at 1045 indicates C-O, C-N stretching vibrations and it corresponds to the presence of alcohols, carboxylic, acids, ethers, esters and aliphatic amines in the seed extract. The presence of active functional groups in seed extract results in the swift reduction of silver ions to silver Nanoparticle. To obtain good signal to noise ratio of silver nanoparticle were taken in the range 500–3400 cm−1 [30].

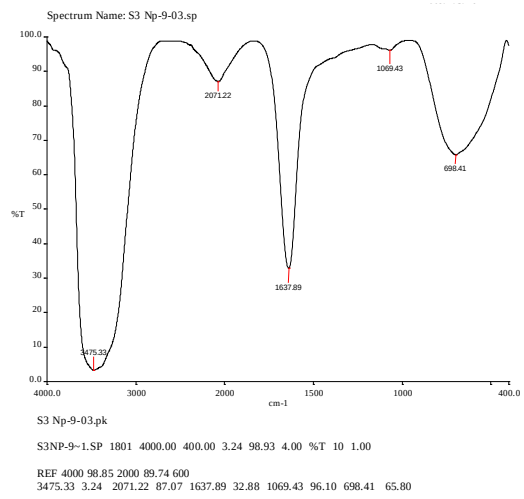

Fig. 5: FTIR spectra of silver nanoparticles synthesized from *T. divaricata* extract.

Cytotoxic activity

The cytotoxic activity of AgNPs synthesized by using *T. divaricata* leaf extract was determined by MTT assay (Figure 6). In the present study, the minimum inhibitory concentration (IC_{50}) of AgNPs on MCF-7 cells was obtained at 20 µl/ml at 24 hours. Exposure to increasing concentration of AgNPs shows dose-dependent cytotoxicity on MCF-7 cells.

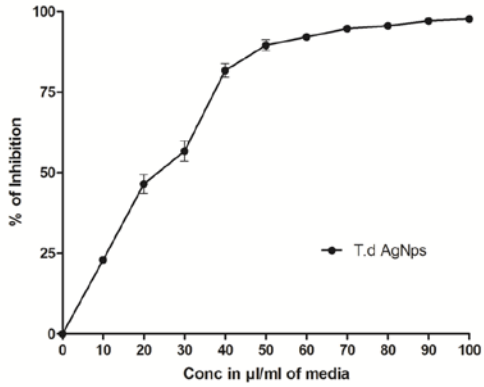

Fig. 6: Cytotoxic activity of *T. divaricata synthesized AgNPs* against MCF-7 cell line. IC50 concentration – 20µl/ml

CONCLUSION

The fresh leaf extract of the *T. divaricata* reduced the silver ions and the nanoparticles were synthesized. The synthesis is found to be efficient in terms of reaction time as well as stability of the synthesized AgNPs. This nanoparticulate solution exhibits excellent stability for six months from the date of synthesis. The green synthesized nanoparticles were analyzed UV- VIS Spectra at 430nm and the SEM and TEM analysis was performed and silver nanoparticle of size around 22.85nm was observed. Further FTIR analysis of the sample showed the 3475.33-N-H stretch, 2071.22-C≡C, 1637.89-C=C, 1069.43-C=O. The nanoparticles were tested for cytotoxicity analysis by MTT assay on MCF 7 breast cancer cell line and IC50 was obtained at 20µl concentration. The current study reveals that the AgNPs synthesized from *T. divaricata* can be used for the medicinal applications and further it can be analyzed for the anticancer profiling.

CONFLICT OF INTERESTS

Declared None

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