INNSPUB

International Journal of Biosciences | IJB |
ISSN: 2220-6655 (Print) 2222-5234 (Online)
http://www.innspub.net
Vol. 15, No. 2, p. 600-611, 2019

RESEARCH PAPER OPEN ACCESS

Phytosynthesis of silver nanoparticles using *Tamrixa phylla* and biological applications

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Key words: Green synthesis, Silver nanoparticles, Characterization, *Tamarix aphylla*

http://dx.doi.org/10.12692/ijb/15.2.600-611 Article published on August 30, 2019

Abstract

Due to biocompatibility, antimicrobial activities and other medicinal properties, silver nanoparticles (Ag-NPs) have drawn significant attention for bio-medical applications. The present study focused on eco-friendly synthesis of Ag-NPs using *Tamarix aphylla* aqueous extract. The synthesized Ag-NPs were characterized by UV-Vis spectrophotometer and their characteristic Surface Plasmon Resonance (SPR) peak was observed at (435nm). Fourier Transform Infrared (FT-IR) spectrophotometer was used to confirm formation of the Ag-NPs and to find out the specific functional groups responsible for reduction of $AgNO_3$. The morphology of Ag-NPs was characterized by Scanning Electron Microscopy (SEM) and find out that nanoparticles were spherical and in the size range of 4-48nm. Energy Dispersive X-ray (EDX) was used for elemental analysis to detect the presence of elemental silver. X-Ray Diffraction (XRD) was used to determine the crystalline nature, purity and average particle size of nanoparticles. The average particle size was observed to be approximately 22nm. Moreover, the antibacterial potential of synthesized Ag-NPs was tested against six selected pathogenic bacteria, and five fungal strains. The nanoparticles showed strong anti-bacterial and antifungal activities. Nonetheless, in culture cells Ag-NPs bound to outer proteins of viral particles and inhibited the binding site and also replication.

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Introduction

Nanotechnology is the design, characterization, production and application of structures, devices and systems by controlling shape and size at the nanoscale (1-100nm) where unique phenomenon brings about diverse features (Khan *et al.,* 2019b; Ratner and Ratner, 2003). Nanotechnology is further classified in to two classes i.e. wet and dry nanotechnologies. The first one deals with living Biosystems such as enzyme, membrane and cellular component, while second one deals with the objects made by man such as surface science, physical chemistry which focus on fabrication of structure in carbon, silicon and inorganic materials at nanoscale structures (Keat *et al.,* 2015). In the recent past, nano science and technology have got more attention of scientists all over the world working in many fields like medicine, materials, energy, catalysis and sensors because of its tremendous attributes in diverse anthropogenic applications (Vertegel *et al.,* 2004).

Generally, nanomaterials are classified into different types on the basis of shape, size and morphology (Zargar *et al.,* 2014). Depending on the conditions, nanoparticles may have single crystalline or polycrystalline structure (Arico *et al.,* 2011). They have electrical (Zhang *et al.,* 2000) and magnetic properties (Shankar *et al.,* 2003) and are used in the field of catalysis (Rashid *et al.,* 2006), drug delivery (De Jong and Borm, 2008), inflammation against cancer (Kim *et al.,* 2001), electrodes (Yang *et al.,* 2010), food and cosmetics industries (Salata, 2004). Within the different metal Nano particles, due to more surface area silver nanoparticles (Ag-NPs) are having more reactivity (Tolaymat *et al.,* 2010). Ag-NPs have been reported for their diverse various applications in different fields due to the distinctive features of higher surface plasmon resonance (SPR) (Behera and Debata, 2011) and surface enhanced Raman scattering (SERS), these properties make Ag-NPs more potent in the healing of wounds, diabetic socks, pharmaceutical industries, sterilization materials in hospitals etc. Ag-NPs also used as antibacterial, antiviral, antifungal, anti-inflammatory, cancer therapy, diagnosis, orthopedics, anesthesiology, dentistry, eye caring, neurosurgery, and drug delivery (Awwad *et al.,* 2013). Among the several approaches for the synthesis of metal nanoparticles such as physical methods, chemical methods and biological methods, the biological methods using plant part/s or extracts is the most suited method for the synthesis of stable, regular and viable nanoparticles (Khan *et al.,* 2016; Khan *et al.,* 2015). Plant extracts provide a variety of chemical compounds collectively called as plant secondary metabolites, which act as efficient reducing as well as stabilizing agents during synthesis of nanoparticles. However, in planta synthesis of metal nanoparticles depends on the nature of the plant extract and the relative concentrations of the extract and metal salt(s) reacting, pH, temperature, and time of reaction, as well as the rate of mixing of plant extract and metal salt (Noruzi *et al.,* 2011). Green synthesis methods employing either biological microorganisms or plant extracts have emerged as a simple and alternative to chemical synthesis (Kazmi *et al.,* 2019; Khan *et al.,* 2019a; Khan *et al.,* 2019b). Plant mediated Ag-NPs has got more attention globally, because of its low cost, simple and has no any bad effects on environment and human health are anti-fungal anti-inflammatory (Safaepour *et al.,* 2009). The plant based synthesis of Ag-NPs provide suitable platform for the production of most effective silver nanomaterials which are more stable with confined size and regular shapes result in exceptionally higher activity in diverse biological applications (Khan *et al.,* 2016; Khan *et al.,* 2015). The present study was therefore attempted for the following objectives of this proposed work were to synthesize Ag-NPs using aqueous extract of *Tamarix Aphylla*, to optimize the reaction conditions and to characterize the synthesized Ag-NPs. Finally to evaluate the antifungal, antibacterial and antiviral applications of the green synthesized Ag-NPs.

Materials and methods

Plant material, chemicals and preparation of solvents

The plant *Tamarix aphylla* was collected from local area of district lakki marwat Khyber Pakhtunkhwa, Pakistan in the month of July 2017. Bark of *Tamarix aphylla* was taken and washed with doubly distilled

water. Further it was dried in shade to avoid photochemical reactions then the dried sample was grinded converted to the powder as shown in fig. 1 The 5g of plant material was taken and heated it in 100mL of double distill water for 8mins at 80°C. Sample was then cooled and doubly filtered. It was then stored at 4°C for further use. Silver Nitrate ($AgNO_3$) was purchased from Sigma Aldrich Germany. Deionized water (doubly distilled water) was used as solvent for plant extraction, and in all other experiments during nanoparticles synthesis. A 2mM solution of $AgNO_3$ was prepared by dissolving 17 mg of $AgNO_3$ in limited amount of de-ionized water and then diluted to a total volume of 100mL. The solution was kept at 4°C for further use in Ag-NPs synthesis.

Fig. 1. (a). Drying of *Tamarix Aphyllai* (b): Powdered form for extraction (c): Steps involved in the synthesis of Ag-NPS.

Synthesis of silver nanoparticles

Ag-NPs of *Tamarix aphylla* were synthesized using classical Turkevich method. The plant extract (2mL) was stirred with 2mL solution of $AgNO_3$ (2mM) at room temperature for 30 minutes. Bio-reduction and formation of the Ag-NPs was indicated by appearance of brown color. The resulting suspension was centrifuged at 8000rpm for 30 minutes, and the residue was washed with cold ethanol to get purified Ag-NPs. UV-visible spectroscopy was used as a primary tool for nanoparticles characterization. The steps involved in the synthesis of Ag-NPs from plant extract are listed in fig. 1.

Characterization

After synthesis, nanoparticles were characterized with some spectroscopic and microscopic techniques to confirm their shape, size and morphology. The following characterization techniques were used.

UV-Visible Spectroscopy (Uv-Vis)

The reduction of silver ions (Ag+) was observed by measuring the UV-Visible spectrum of the reaction medium. UV-Visible spectroscopic analysis was carried out by double beam UV-Visible Spectrophotometer Lambda 25, Perkin Elmer in the spectral range of 300-700nm using deionized water as solvent (Çakmak *et al.,* 2003).

Fourier Transform Infrared (FT-IR) Spectroscopic Analysis

The detection of functional groups that involved in nanoparticles synthesis was carried out by Fourier-transform Infrared Spectrophotometer, Prestege-21 Shimadzu, Japan, in the range of 4000-400cm-1 using a KBr pellet technique (Wu and Chen, 2010).

Scanning Electron Microscopic (SEM) Analysis

Scanning electron microscopic analysis was carried out by using JSM-5910, England SEM instrument. Thin film of Ag-NPs was made on a carbon coated Cu grid by only dropping a little quantity of Ag-NPs sample on the grid, extra solution was removed by using of blotting paper and then thin film of sample on the SEM grid could become dry by placed it under the Hg lamp for 5 minutes (Yu *et al.,* 2004).

Energy Dispersive X-ray (EDX) Analysis

The elemental composition of Ag-NPs was finding out by using EDX spectrometer INCA-200, England (Çakmak *et al.,* 2003).

X-Ray Diffraction (XRD) Analysis

The composition and structure of the purified Ag-NPs after freeze drying were investigated by XRD diffractometer, RX- III, Shimadzu, Japan (Hall *et al.,* 2000).

Thermo Gravimetric Analysis (TGA)

The thermal profile of the nanoparticles was examined by thermo gravimetric analyzer (TGA). Shrinkage of the silver nanoparticle compacts during the sintering process was observed by thermo mechanical analysis (TMA). Sintering of the nanoparticle pellet led to a significant increase in density and electrical conductivity. The size of the sintered particles and the crystallite size of the particles increased with increasing sintering temperature (Varberg and Skakuj, 2015).

Biological application of biosynthesized Ag-NPs

Anti-bacterial assay:

Agar method diffusion method was used which have nutrient agar, nutrient broth and soft agar (composed of solid medium, liquid medium and semi-solid medium respectively). The green synthesized silver nanoparticles were dissolved in dimethylsulfoxide (DMSO) and kept for using as a stock solution. In liquid medium single colony of bacterial culture were inoculated and kept under 37°C for 24h. Freshly prepared 100µL bacterial culture were added to soft agar tube after that poured it on the solid medium having plate and allowable it to solidify. Then making wells with the help of sterile borer having 6mm-diameter and added sample in to agar well plate. The Ciprofloxacin and Levofloxacin were used as standard drugs against the pathogenic bacteria and DMSO were also inserted to other wells. The plates were kept at 37°C for 24 with continuous observation of inhibition (Carron *et al.,* 1987).

Anti-Fungal Assay

Agar tube diffusion method was also used for the determination of anti-fungal activity. The Fluconazole were used as standard drug in this method. The green synthesized silver nanoparticles were dissolved in dimethylsulfoxide (DMSO) and kept for using as a stock solution. In screw caps tubes the Sabouraud's Dextrose agar (SDA) medium was placed and inoculated with sample solution. Then tubes were allowable at room temperature to solidify in slanting position and were inoculated with seven day old culture of fungus piece of 4mm diameter. The samples were kept at 28°C for 7 days with continuous observation of inhibition (Choudhary *et al.,* 1995).

Antiviral Assay

Dulbecco's Modified Eagle's medium and Ag-NPs were mixed with no addition of serum. Different ratios were taken such as 0.1,0.5, 1 and 6µg/mL. Inside cell monolayers no antiviral compounds were added and final percentage inhibition was noted (Selvam *et al.,* 2017).

Statistical analysis

Mean values from the triplicate data were taken, where also one-way ANOVA at significant level $P<0.05$ was determined using computer software Graph pad Prism 5.01 and statistics 8.1.

Results and discussion

Reduction of silver ion into Ag-NPs during reaction mixture through *Tamarix aphylla* extract was indicated by color change. Absorption spectra of Ag-NPs formed in the reaction media had an absorbance peak at 435nm, broadening of peak indicated that the particles are polydispersed. Different ratios of plant extract: silver solution was taken such as 1:1, 1:2, 1:3 1:4 and were mixed to get the optimal conditions. Wherein, the 1:2 gave the more intense peak. This indicates that more nanoparticles formation was taking place. Hence 1:2 was the optimized condition for Ag-NPs synthesis as indicated in fig. 2, showing the presence of biomolecules in the plant extract that resulted in the broad stretching band of O-H at 3282cm^{-1}, a mini peak appeared at 2162cm^{-1} due to the presence of C≡C, a medium peak present at the region of 1560cm^{-1} that

confirmed the stretching band of the C=C due to the presence of *Tamarix aphylla* flavonoids, and also a small peak was observed at 648 cm^{-1} due to the bending vibration of C-H out of the plane.

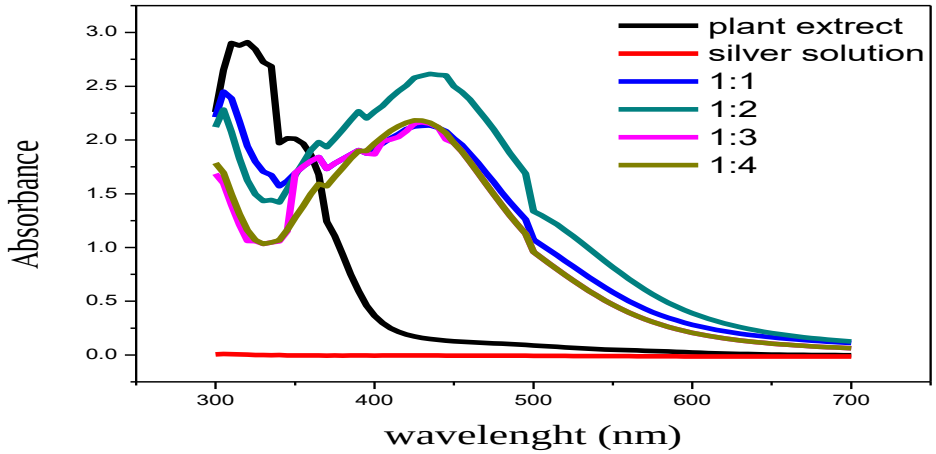

Fig. 2. UV-Vis spectra of different mixing ratios of sample.

In the formation of Ag-NPs difference occurred in FT-IR peaks of plant extract and its Ag-NPs due to the involvement of secondary metabolites in reduction and capping process. As indicated in fig. 3, during Ag-NPs synthesis the broad stretching band of O-H shifted from 3282cm-¹ to lower frequency 3269 cm-¹ and this revealed that O-H group of biomolecules was involved in the stabilizing and reduction process of Ag-NPs synthesis. A mini peak of C≡C also shifted from 2162 cm-1 to 2099 cm^{-1} (from higher to lower frequency) which also indicated the involvement of C≡C in alkynes or because of cumulative double bond in ketones in the stabilization process. While the medium peak of C=C at 1560 cm^{-1} was also shifted toward lower band at 1628 cm^{-1} this indicated that flavonoids were involved in the capping and reduction process. Similarly, the FT-IR results showed that biomolecules (such as O-H, C≡C and C=C) of plant extracts not only reduced the silver (Ag) metal but also acted as a stabilizing agent (Gardea-Torresdey *et al.,* 2003). In this study, the images of the SEM indicated that the green synthesized Ag-NPs were in the range of 4-48 nm as shown in fig. 4. It was noted that the particles were predominantly spherical in shape with different sizes but a minute number of anisotropic nanostructures such as nano-triangles, nanorods and many hexagonal and polygonal nanomaterials were also observed. In this work the uniform size distribution of (Ag-NPs) indicated the well-organized stabilization of Ag nanoparticles while some have anisotropic shapes and larger size were due to the combination of smaller nanoparticles, these observations are in accordance to the work reported by Bar *et al.,* (2009). Moreover, strong signals of Ag were recorded from Ag-NPs at 2.6 keV. However, other strong signals were also observed for carbon and oxygen owing to the existence of various secondary metabolites that were involved in Ag-NPs stabilizations. It can be seen in fig. 5. The signals for magnesium (Mg), calcium (Ca), potassium (K) chlorine (Cl) oxygen (O) and sulphur (S) were due to X-rays emitted from different secondary metabolites of the plant extract. In a similar fashion, elemental analysis of nanoparticles in the EDX confirmed the reduction of elemental forms of metal cations (Vidhu *et al.,* 2011).

The XRD patterns of synthesized silver nanoparticles indicated that the structure of Ag-NPs is face centered cubic (FCC) as shown in fig. 6, the XRD pattern of the synthesized Ag-NPs. clearly indicated that the plant extracts are crystalline in nature. In addition to the Bragg peaks representative of FCC silver nanocrystals, unassigned peaks were also observed suggesting that the crystallization of bio-organic

phase occurs on the surface of the Ag-NPs**.** The line broadening of the peaks is primarily due to small particle size (Mukunthan *et al.,* 2011). During thermo gravimetric analysis ceramic crucible (Al_2O_3) was used for heating and measurements were carried out in air atmosphere (Kalidindi and Jagirdar, 2009). Nonetheless, curves of TGA of synthesized Ag-NPs using *Tamarix aphylla* extract as shown in the fig. 7 highlighted no weight loss at the temperature range 50 to 600°C. *Tamarix aphylla* is an important medicinal plant and has shown presence of different phytochemicals produced as secondary metabolites. These important secondary products include flavonoids, phenols and volatile essential oils. This is the important attribute of this plant that the presence of such highly active compounds in the plant extract acted as efficient reducing and stabilizing agents. We have proposed the mechanism of synthesis of Ag-NPs using *Tamarix aphylla,* wherein the role of the biologically active secondary metabolites can be read in the reduction, stabilization and capping of the Ag-NPs (Fig. 8).

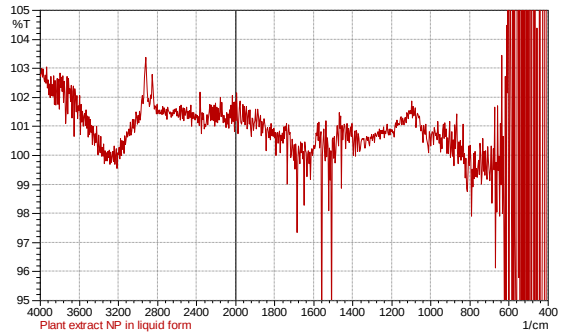

Fig. A

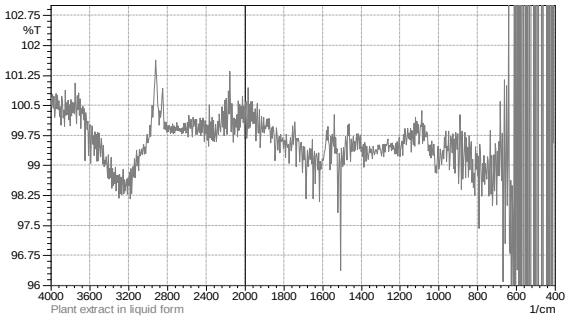

Fig. B

Fig. 3. FT-IR spectra of plant extract (A) Nanoparticles (B).

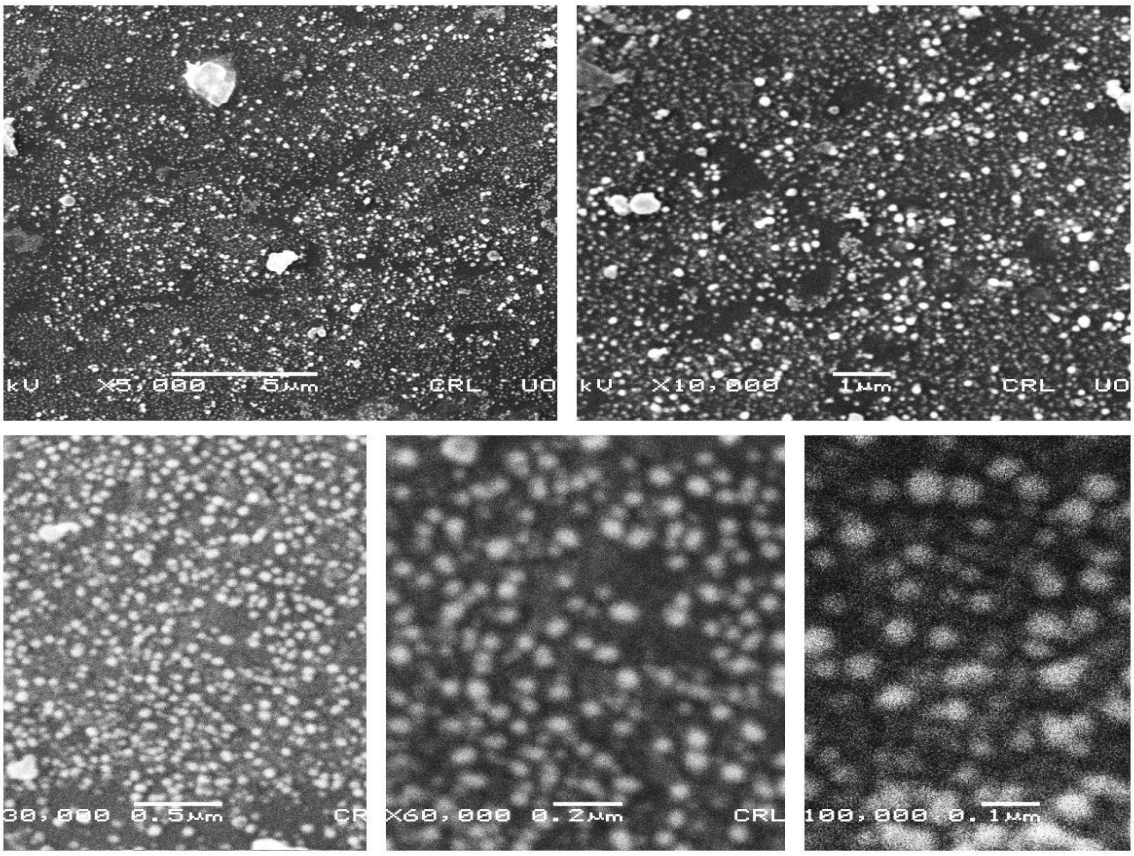

Fig. 4. SEM images of Ag-NPs.

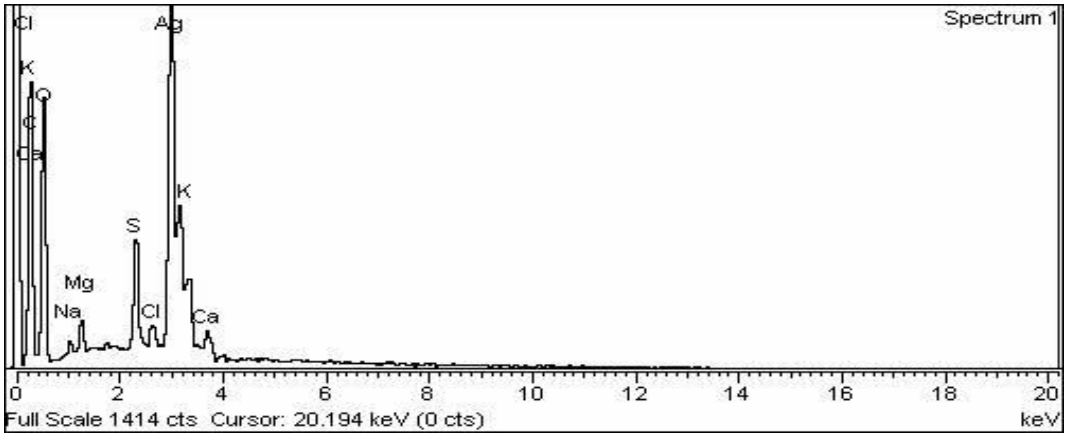

Fig. 5. Elemental analysis of Ag-NPs through EDX.

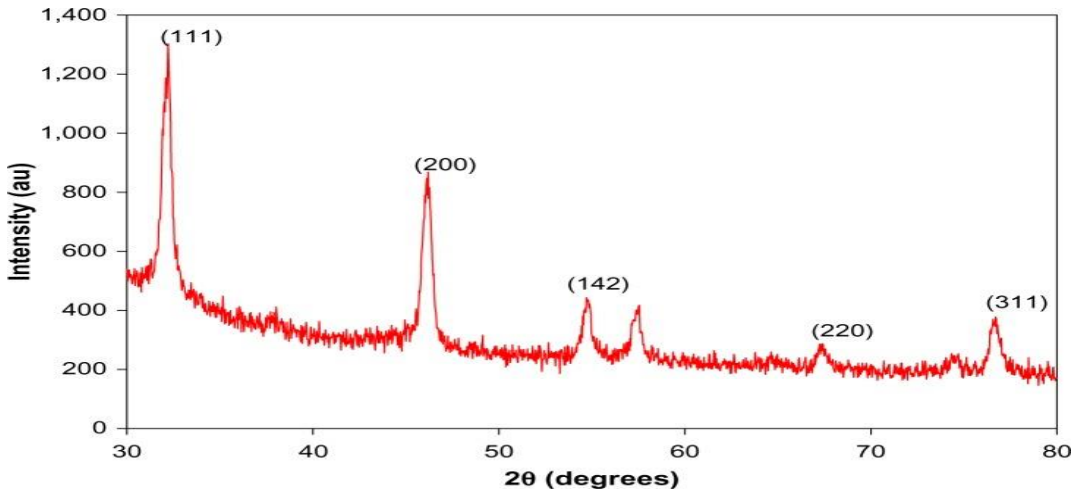

Fig. 6. XRD spectrum of Ag-NPs.

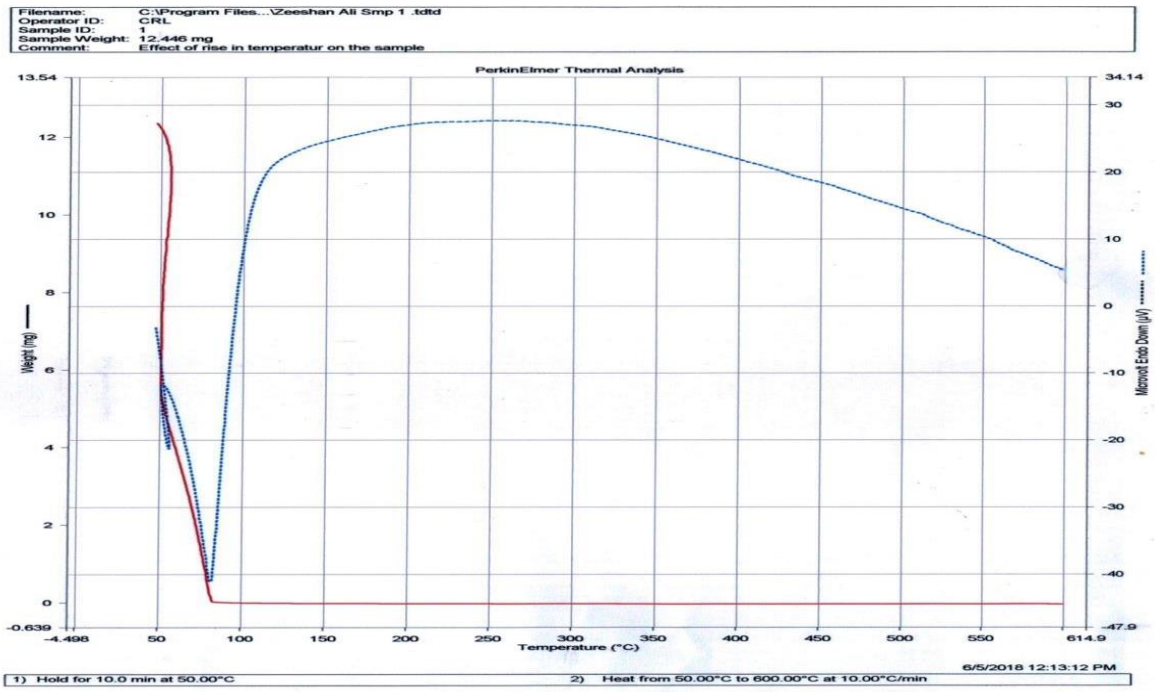

Fig. 7. Thermogram of Ag-NPS.

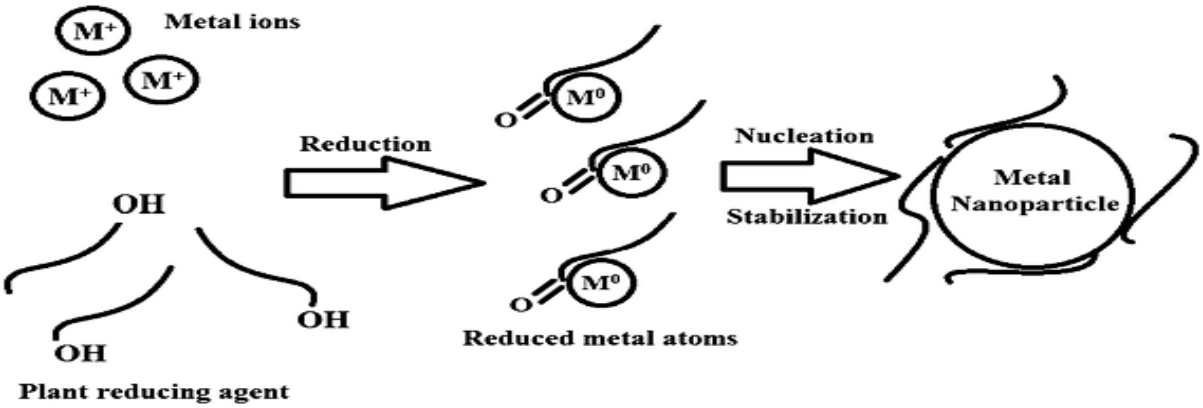

Fig. 8. Mechanism for the synthesis of Ag-NPS using plant extract.

The antibacterial potential of synthesized Ag-NPs were tested against six selected pathogenic bacteria, three gram positive bacteria such as Staphylococcus aureus, Staphylococcus epidermidis, Bacillus subtilus and three gram negative bacteria include Escherichia coli, Pseudomonas aeruginosa and Salmonella typhi by agar tube diffusion method. Two concentrations of silver nanoparticles (0.1mg/ml and 0.2mg/ml) were used against micro-organism by agar tube diffusion method (Table. 1). In this method the micro-organism were diffused on nutrient agar medium plates and kept at 37°C for 24hrs. The zone of inhibition against bacterial growth was measured in millimeter (mm) around the well. Levofloxacin and Ciprofloxacin were used as standard drugs against the selected pathogenic bacteria (Kumar *et al.,* 2012). The nanoparticles showed strong anti-bacterial activity against gram negative bacteria as compared to gram positive bacteria. The synthesized nanoparticles of *Tamarix aphylla* showed strong zone of inhibition against the bacteria than the plant extract as reported in the literature. The Ag-NPs showed efficient antibacterial activities than the other salts due to their particularly enormous surface area, which provides greater contact by micro-organisms (Saxena *et al.,* 2012). In the literature reported that the Ag-NPs become attached to the cell membrane and also penetrated into bacteria. The cell membrane of bacteria having sulfur (S) containing proteins and the Ag-NPs interrelate in the cell with these proteins and also the compounds having phosphorus such as DNA. When silver nanoparticles penetrate into the bacterial cell it shaped a low molecular weight area in the centre of the bacteria in which bacteria reproduce thus shielding the DNA from the silver ions. The nanoparticles rapidly assault the respiratory sequence then cell division and at last leading to cell death. The NPs discharge Ag ions in the bacterial cells which increase their antibacterial activities (Rastogi and Arunachalam, 2011).

The green synthesized Ag-NPs were tested for antifungal activity against selected five fungal strains such as *Rhizophus, Trichoderma, Candida albicans, Acromonium and Aspergilus nigar* by agar tube diffusion method. Sabouraud's dextrose agar (SDA) medium were used for the culturing of fungal strains (Lok *et al.,* 2007). Fluconazole (positive control) and DMSO (negative control) was used as standard. In this method all species of fungus were splashed on plates of agar medium and kept at 37°C for 7 days. Against micro-organism two types of concentrations of silver nanoparticles (0.1mg/ml and 0.2mg/ml) were subjected by agar tube dilution method. The zone of inhibition against each fungus was measured in millimeter (mm). The silver nanoparticles showed strong anti-fungal activities against each fungus as listed in the table 2. The synthesized silver nanoparticle of *Tamarix Aphylla* exhibited strong antifungal activity than the plant extract as already reported in the literature (Anandalakshmi *et al.,* 2016)]. The silver nanoparticles show high antimicrobial activity and this property is very useful against microorganisms resistant to conventional antimicrobials. It has been found that silver nanoparticles have shown good activity against the

fungi which is resistant to antifungal agents like Fluconazole, miconazole and Micafungin. They have high activity against Acromonium and *Aspergilus nigar* (Nasrollahi *et al.*2011). The synthesized Ag-NPs showed significantly higher antiviral activity (Table. 3). In culture cells Ag-NPs bound to outer proteins of viral particles and inhibited the binding site and also replication. Ag-NPs experience size dependent interaction with in the range of 1-12nm.They interacts with HIV-1 virus and open Sulphur bonds of glycoprotein. Small sized nanoparticles were able to inhibit the virus infection (Pan *et al.,* 2011). Silver nanoparticles have an anti-HIV activity at an early stage of viral replication, such as a virucidal agent or as an inhibitor of viral entry. They bound to gp120 which prevent CD4- i.e. virion binding, fusion, and infectivity, acting as an effective virucidal agent against cell-free virus (Galdiero *et al.,* 2011). Silver nanoparticles also blocked the post-entry stages of the HIV-1 life cycle. Hence silver nanoparticles has a broad-spectrum agent and do not have resistance that can be use against circulating HIV-1 strains (Gaikwad *et al.,* 2013).

Table 1. Antibacterial activities of Ag-NPs.

Zone of Inhibition (mm) Against Gram Positive Bacteria							Zone of Inhibition (mm) Against Gram Negative Bacteria					
Sample	S. a.		S. e.		B. s.		E. c.		p. a.		S.t.	
	0.1mg/ml 0.2mg/ml		0.1mg/ml 0.2mg/ml		0.1mg/ml 0.2mg/ml		0.1mg/ml 0.2mg/ml		0.1mg/ml 0.2mg/ml		0.1mg/ml 0.2mg/ml	
Ag-NPs	13	15	10	13	16	18	17	20	13	17	16	20
DMSO (-)	00	00	00	00	00	00	00	00	00	00	00	00
Ciprofloxacin	.....	.....	.....	.....	.....	.....	22	24	20	23	21	22
Levofloxacin	24	28	22	25	24	27	.....	.....	.....	.....	.....	.....

Key words: S. a. = Staphylococcus aureus, S. e. = Staphylococcus epidermidis, B. s. = Bacillus subtilus, E. c. = Escherichia coli, p. a. = Pseudomonas aeruginosa, S.t. = Salmonella typhi.

Table 2. Anti-fungal activities of silver nanoparticles.

Anti-fungal assay of Ag-NPs										
Sample	R.		T.		C.a.		A.		A.n.	
	0.1mg/ml 0.2mg/ml		0.1mg/ml 0.2mg/ml		0.1mg/ml 0.2mg/ml		0.1mg/ml 0.2mg/ml		0.1mg/ml 0.2mg/ml	
Ag-NPs	11	14	16	18	12	14	8	12	13	17
DMSO (-)	00	00	00	00	00	00	00	00	00	00
Fluconazole	17	18	19	21	14	17	13	16	17	19

Key words: R. = Rhizophus, T. = Trichoderma, C.a. = Candida albicans, A. = Acromonium, A.n. = Aspergilus nigar.

Table 3. Antiviral activities of silver nanoparticles.

Type of virus	Family	Action of virus on host	Silver nanoparticles interaction with virus
Monkey pox virus	Poxviridae	Host cell blocked and penetrated	Ag-NPs aggregate and disturb intercellular pathway with plaque formation.
Influenza virus	Orthomyxoviridae	Viruas replicate and stop binding site for plasma membrane of cell	Ag-NPs facilitates the protein fusion of endosomal membrane which in return discharge the nucleoproteins and genome fragments into cytoplasm.
HIV-1	Herpesviridae	Interact with glycoprotein and open Sulphur bonds	Ag-NPs inhibit HIV-1 infection and blocked the entry of virus in to cell hence stop cell to cell spread of virus.

Conclusion

The rapid biological synthesis of Ag-NPs using bark of *Tamarix aphylla* is an eco-friendly, straightforward, efficient and simple method for the synthesis of nanoparticles. The synthesized Ag-NPs were characterized by using spectroscopic and microscopic techniques such as UV-Visible, FT-IR, SEM, EDX, TGA and XRD. The size of nanoparticles was found out to be in the range of 4-48nm. These synthesized nanoparticles were surrounded by a thin layer of

secondary metabolites. These results recommended that Ag-NPs can be used as powerful growth inhibitors for different microorganisms, make them useful to different medical devices and anti-microbial agents. Thus the present studies revealed that the using of smaller size Ag-NPs have diverse applications in the field of nano-medicine.

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