Terminalia chebula Retz. gallic acid – biased silver nanoparticles and their antiphytopathogenic activity

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ABSTRACT

Owing to the unique properties, nanomaterials play a major role in many areas of science and technology. In this paper the antiphytopathogenic activity of gallic acid reduced *Terminalia chebula* Retz. silver nanoparticles (Ag NPs) was studied. $AgNO_3$ (10^{-3} M) stock solution was prepared by dissolving 17mg of silver nitrate in 100ml of double distilled water. 10ml of gallic acid solution isolated from *Terminalia chebula* Retz. (Combretaceae) was added to 90ml of 10^{-3} M $AgNO_3$ solution for reduction of Ag^+ ions. The reduction of pure Ag^+ ions was monitored by measuring in the UV-Vis Spectroscopy at 426nm. Determination of the shape and structure of silver nanoparticle was characterized by Transmission Electron Microscopic (TEM) and X-ray Diffraction studies (XRD). The plasma resonance of the gallic acid reduced silver particle is brownish yellow. In the light of these studies, the shape of the silver nanoparticle (spherical) and face centered cubic (FCC) structure were explained. The antibiotic experiment conducted in the present study revealed the antiphytopathogenic activity of gallic acid reduced *Terminalia chebula* Retz. silver nanoparticles against the phytopathogen *Xanthomonos axonopodis* pv. *malvacearum* and also confirmed the antiphytopathogenic activity studies based on "Broth microdilution method" against *Xanthomonos axonopodis* pv. *malvacearum.*

MS History: 05.11.2013 (Received)-05.03.2014 (Revised)-17.03.2014 (Accepted)

Key words: *Terminalia chebula*, gallic acid, silver nanoparticles, TEM, XRD, antiphytopathogenic activity.

INTRODUCTION

Silver has been in use since time immemorial in the form of metallic silver, silver nitrate, silver sulfadiazine for the treatment of burns, wounds and several bacterial infections (Mahendra Rai *et al*., 2009). But due to the emergence of several antibiotics, the use of these silver compounds has declined remarkably. However silver nanoparticles could be used as a potential alternative therapy to reduce severity of disease due to *Pseudomonas aeruginosa* infections (Navindra Kumari Palanisamy *et al*., 2014). Nanotechnology is gaining tremendous impetus in the present century due to its capability to modulate metals into their nanosize, which drastically changes their chemical, physical and optical properties. Hence metallic silver in the form of silver nanoparticles has made a remarkable comeback as a potential antimicrobial agent. The use of silver nanoparticles is also important, as several pathogenic bacteria have developed resistance against various antibiotics. It has diverse medical applications ranging from silver based dressings, silver coated medicinal devices, such as nanogels (Mahendra Rai *et al*., 2009), nanolotions (Mahendra Rai *et al*., 2009) etc. The nanocrystalline silver dressings, creams and gel effectively reduce bacterial infections in chronic wounds (Richard *et al.*, 2002; Leaper, 2006; Ip *et al*., 2006). Silver impregnated medical devices like surgical masks and implantable devices show significant antimicrobial efficacy (Furno *et al*., 2004). There are numerous works related to green synthesis of metallic nanoparticles using higher plants. Gardea-Toroesday *et al*., (2003) first reported the formation of silver nanoparticles by living plants. Shankar *et al*. (2004) reported pure metallic silver nanoparticles synthesis by the reduction of Ag^+ and Au^+ ions using neem (*Azadirachta indica* Juss.) leaf broth. For instance, some of the renowed reports on phytosynthesis of silver nanoparticles by employing lemon grass extract (Shankar *et al*., 2004, 2005), green tea, *Camellia sinensis* (L.) Kuntze (Vilchis-Nestor *et al*.,2008), Aloe vera (L.) Burm. Plant extract (Chandran *et al*., 2006), sundried

Cinnamomium camphora (L.) Prest. Leaves (Huang *et al*., 2007), *Cinnamon zeylanicum* (L.) (Satishkumar *et al.*, 2009), Phyllanthin extract (Kasthuri *et al*.,2009a), purified apilin compound extracted from henna leaves (Kasthuri *et al*., 2009b), *Acalypha indica* (L.) (Krishnaraj *et al.*, 2010), *Curcuma longa* (L.) (Sathishkumar *et al.*, 2010), *Hibiscus rosasinensis* (L.) (Philip, 2010), *Rosa rugosa* Thunb. (Dubey *et al.*, 2010), *Piper betle* (L.) (Usha Rani and Rajasekhara Reddy, 2011), *Stevia rebaudiana* Cav. (Yilmaz *et al*., 2011.,), *Citrus Limon* (L.) Burm. (Prathna *et al*., 2011), *Ficus benghalensis* (L.) (Saxena *et al.*, 2012), and *Ocimum basilicum* (L.) (Sivaranjini and Meenakshisundaram, 2013) have been suggested as possible ecofriendly alternatives to both chemical and physical methods. A detailed review on the biological synthesis has been provided by Sahayaraj and Rajesh (2011). An overview of novel Biosilver nanoparticles and their biological utility has been provided by Sahayaraj (2014) using plant extract or plant bioactive molecules through bioreduction procedures. Bacterial blight, caused by *Xanthomonas axonopodis* pv. *malvacearum* can be a serious disease in most cotton growing areas of the world (Bayles and Verhalen, 2007). Biological control of *Xanthomonas axonopodis* pv. m*alvacearum* using marine macroalgae, *Sargassum wightii* (Raghavendra *et al.*, 2007) and terrestrial plants (Mohana and Raveesha, 2006; Raghavendra *et al*., 2006; Babu *et al.*, 2007; Fatima *et al*., 2012), endophytic bacterial agents (Mandal *et al.*, 2001, Salaheddin *et al.*, 2010; Ingole *et al.*, 2011; Jagtap *et al*., 2012a) and fungal agents (Rashid and Khan, 2000) and nimbolik 60 EC formulation (Khan *et al.*, 2000) were reported in the past. The hypothesis of *Xanthomonas axonopodis* pv. *malvacearum* inhibition may be due to the collective mechanism involving formation of pits in the cell wall thereby release of intracellular materials including reducing sugars and proteins, binding with the proteins containing SH group thereby affecting the hydrolytic enzymes required for pathogenecity and damaging DNA which affects protein synthesis leading to cell death. The inhibitory effect of silver is probably the sum of distinct mechanisms of action. Previous reports suggest that the silver ions react with SH groups of proteins (Liau *et al*., 1997; Feng *et al*., 2000; Duran *et al*., 2010) and play an essential role in bacterial inactivation (Morones *et al.*, 2005; Duran *et al.*, 2010). It is also reported to uncouple respiratory electron transport from oxidative phosphorylation which inhibits respiratory chain enzymes or interferes with membrane permeability to protons and phosphate (Feng *et al.*, 2000). The presence of silver ions and sulphur in the electron dense granules observed after silver ion treatment in the cytoplasm of bacterial cells suggests an interaction with nucleic acids that probably results in the impartment of DNA replication (Feng *et al.*, 2000). Thus it is reasonable to infer that the biosynthesized silver nanoparticles can be used to manage the cotton diseases; there is a high probability of generating a new antimicrobial agent. In this context the present study was carried out to synthesize and characterize silver nanoparticles using gallic acid isolated from *Terminalia chebula* Retz. fruit using UV-Visible spectroscopy, XRD, TEM and to evaluate the bioactivity of silver nanoparticle against *Xanthomonos axonopodis* pv. *malvacearum.*

MATERIALS AND METHODS

Collection and extraction

Terminalia chebula fruits were obtained from Asthagiri Herbal Research Institute, Chennai and were authenticated by Dr. P. Jayaraman, Plant Anatomy Research Center, Chennai. Gallic acid was isolated from *Terminalia Chebula* fruits by column chromatography method and was used to synthesize silver nanoparticles.

Synthesis of Silver nanoparticle

Silver nitrate stock solution (10^{-3} M) was prepared by dissolving 17mg of silver nitrate in 100ml of double distilled water. 10ml of gallic acid isolated from *Terminalia Chebula* was added to 90ml of 10^{-3} M $AgNO_3$ solution for reduction. The formation of brownish yellow colour shows the formation of silver nanoparticles (Kannan *et al*., 2012). The reduction of pure Ag^+ ions was monitored by measuring in the UV-Vis Spectroscopy at 426 nm. The silver nanoparticle was characterized by Transmission Electron Microscopic (TEM) and X-ray Diffraction studies (XRD) and the shape and structure were determined. The activity against *Xanthomonos axonopodis* pv. *malvacearum* was done by broth microdilution method and MIC was calculated.

UV-Vis Spectral analysis

Synthesis of AgNP's by $AgNO_3$ metal ion solution with gallic acid reduced *Terminalia chebula* Retz. silver nanoparticles can be observed by UV–vis spectroscopy at 426 nm. The absorption spectra was taken using a Shimadzu UV-1601 spectrophotometer with samples in Quartz cuvette operated at a resolution of 1 nm. The absorbance was recorded at 426 nm.

Powder X-Ray diffraction

The X-ray powder diffraction data was acquired by Cu Kα radiation (1.5406 Å; 45 kV, 30 mA). XRD patterns were analyzed to determine peak intensity, position and width. The particle size was calculated using Scherrer formula, $d = 0.9\lambda/ \beta\cos\theta$ where, d is the mean diameter of the nanoparticles, λ the wavelength of X-ray radiation source and β, the angular FWHM of the XRD peak at the diffraction angle (Culity, 1978). Sample powders for the analysis were prepared by centrifugation at 13,000 rpm for 15 minutes, re-dispersed in sterile distilled water to get rid of any uncoordinated biological molecules. Centrifugation and re-dispersion were repeated thrice in order to ensure better separation.

TEM and SEM analysis

The size and shape of the biosynthesized nanoparticles were observed and analyzed under TEM and the measurements were made. Samples for Transmission Electron Microscopy (TEM) were prepared by drop-coating the Ag nanoparticle solution onto carbon-coated copper grids. The films on the TEM grids were allowed to stand for two minutes, following which the extra solution was removed using a blotting paper and the grid was allowed to dry prior to the analysis. TEM analysis was performed on a JEOL model 3010 and Philips CM-200, Japan instrument operated at an accelerating voltage at 120 kV. Selected Area Electron Diffraction Pattern (SAED) was obtained using TEM. The size and shape of nanoparticles were manually interpreted by counting 50 particles randomly for their different shapes and sizes.

Antimicrobial activity

The antiphytopathogenic activity of gallic acid reduced *Terminalia chebula* Retz. silver nanoparticles was carried out against the phytopathogen *Xanthomonos axonopodis* pv. *malvacearum*. The broth microdilution method was carried out to determine the minimum inhibitory concentration (MIC) at 10 different concentrations of compounds viz., 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100 µg/ml, diluted in Mueller Hinton broth and the final volume was made upto 100 µl. One microlitre (1 µl) of an overnight grown bacterial culture was added to the test medium to bring the final inoculum to 1×10^{-7} cfu/ml. The agar plates were incubated at 37°C for 48 h and the absorbance was read at 600nm. The growth of the inoculum in the broth was indicated by turbidity or cloudiness of the broth and the lowest concentration of the compound which inhibited the growth of the test organism was considered as the MIC, which was expressed in µg/ml. A negative control was set up by taking only Mueller Hinton broth, while a positive control with Streptomycin was maintained with Mueller Hinton broth inoculated with the test bacterial cell suspension. (Bibhuti Bhushan Sahu *et al*., 2012).

RESULTS AND DISCUSSION

UV-Visible Spectroscopic Analysis - Surface Plasma Resonance

The formation of nanoparticles was detected and characterized by UV-Visible spectroscopy. Owing to the Surface Plasmon Resonance (SPR) in the interaction of electromagnetic radiation and the electrons in the conduction band around the nanoparticles, an optical absorption band of 426 nm λ max value which is a typical feature of the absorption of metallic silver NP's due to the Surface Plasmon Resonance (SPR), indicating the presence of AgNP's in the solutions. (Fig. 1).

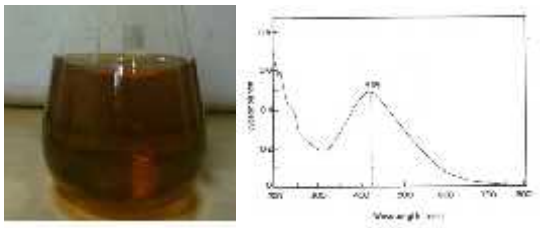

Fig.1. *Terminalia chebula* gallic acid –based liquid silver nanomaterial (right) and its UV-visible spectrum (left)

XRD analysis

The crystalline nature of AgNP's was further confirmed from X-ray Diffraction (XRD) Analysis. The typical XRD patterns of gallic acid reduced

Terminalia chebula Retz. silver nanoparticles shows that the number of Bragg reflections at 2θ values for the extract observed are 38.19°, 44.31°, 64.52°, 77.44°. These results illustrate that the silver nanoparticles formed in this present synthesis are crystalline in nature. The peaks at 38.19°, 44.31°, 64.52°, 77.44° correspond to 111, 200, 220 and 311 planes of face centered cubic (fcc) geometry of silver nanoparticles. (Fig. 2).

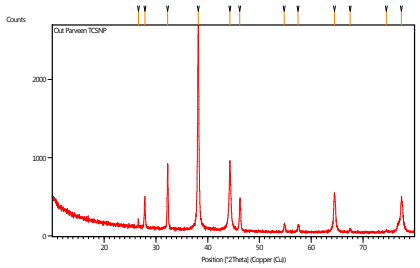

Fig. 2. XRD spectrum of the bionanomaterial of *T. chebula* gallic acid

Transmission Electron Microscopic Studies (TEM) The morphology and size of synthesized AgNPs were also determined by Transmission Electron Microscopic (TEM). TEM images obtained for colloids clearly showed the formation of silver nanoparticles and their size. The size and shape of bio-silver nanoparticles were observed by TEM. The size was found to range from 14 to 60 nm. Spherical shaped particles were predominant besides oval, hexagonal and triangular particles. (Fig. 3).

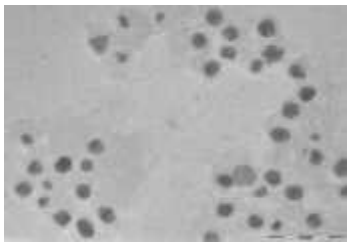

Fig. 3. TEM image of silver nanomaterial synthesised using gallic acid of *Terminalia Chebula*

Antibacterial activity

The gallic acid reduced *Terminalia chebula* Retz. silver nanoparticles were further subjected to the broth microdilution method to determine the MIC. The maximum activity was observed against *Xanthomonos axonopodis* pv. *malvacearum* at a concentration of 70µg/ml. The present study showed antibacterial activity at a low concentration compared to the control streptomycin.

The plasma resonance of the gallic acid reduced *Terminalia chebula* Retz. silver nanoparticles is brownish yellow. Shape and size were characterized by TEM analysis, the shape was spherical and the size was found to range from 14 to 60 nm and face centered cubic (FCC) structure was explained by XRD studies. Broth dilution method revealed antiphytopathogenic activity of the gallic acid reduced *Terminalia chebula* Retz. silver nanoparticles against *Xanthomonos axonopodis* pv. *malvacearum* at a concentration of 70µg/ml.

ACKNOWLEDGEMENT

We thank the management of St. Xavier's College (Autonomous) and Sadakathullah Appa College (Autonomous), Palayamkottai for offering laboratory facilities and encouragement.

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