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GREEN SYNTHESIS OF SILVER NANOPARTICLES USING THE SEED OF *TRIBULUS TERRESTRIS* AND DEMONSTRATING ITS ANTIBACTERIAL ACTIVITY

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Abstract
The seed extract of *Tribulus terrestris* was used for the reduction of silver nitrate ($AgNO_3$) into silver nanoparticles. The colour change from colorless to dark brown in the solution confirmed the biosynthesis of silver nanoparticles (AgNPs) within few minutes of the commencement of the reaction. Furtherly AgNPs were characterised with the help of Ultraviolet-visible (UV-vis) spectroscopy, scanning electron microscopy (SEM), Fourier transform infrared spectroscopy (FTIR) and X-ray diffraction (XRD). The presence of AgNPs in the prepared solution was approved by 300 - 700 nm absorption spectra. XRD pattern indicated the crystalline structure of the nanoparticles (NPs) while the FTIR spectra confirmed the presence of plant residues adsorbed by these NPs. The antimicrobial activity of synthesized AgNPs showed positive results against *Escherichia coli, Staphylococcus aureus, Salmonella typhi, Vibrio hervayie, Streptococcus pneumoniae,* and *Pseudomonas aeruginosa*. AgNPs can be synthesized in a large scale in the way followed here.

Keywords: *Tribulus terrestris*, silver nitrate, nanoparticles, antibacterial

I. INTRODUCTION

Nanoparticles have various exhibits. Completely new and improved properties passed on specific characteristic such as size ranges from 1-100 nm, distribution and morphology (Jeong *et. al.* 2005). The recent interest in the development of new and novel strategies for the generation of silver nanoparticles stems from the potential application in the field of biology, medicine and material science. Nano size metals such as silver have been shown to exhibit size dependent optical properties (Hirst *et al.,* 2009). The synthesis of nanoparticles (NPs) has become a matter of great interest due to their different beneficial properties and applications in a variety of fields (Yallappa *et. al.*,2009). Several approaches have been proposed to generate metallic NPs, including electro-chemical, sonochemical, and photochemical processes; however, most of these methods suffer from the utilization of toxic, hazardous chemicals, and difficulty in purification (Iram *et. al.*,2014).

The first report of the plant employed in the synthesis of nanoparticles is attributed to *Medicago sativa* (alfalfa) which was capable of synthesizing gold and silver nanoparticles. Since then, more attention has been scattered on plants. Most of the studies confer the production of nanoparticles by plants that were known to be more stable than that of synthesized by microorganisms (Muangman *et al.,* 2006). Medicinal plants are rich in secondary metabolites hence used for the treatment of many diseases. Synthetic drugs used as immunomodulators have many side effects such as myelosuppression. Immunomodulator of herbal origin may replace such synthetic ones. Green synthesis of NPs has recently received widerspread attention because it is simple, speedy synthesis, inexpensive, eco-friendly, and size-controlling approach in the synthesis of metal NPs (MNPs) (Yallappa *et.al.,* 2015).

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*Tribulus terrestris***,** commonly known as Gokhru puncture vine and goathead, etc. is a shrub belonging to the family Zygophyllaceae. Historically, it was used in Indian and Greecian cultures as a rejuvenation tonic. It was also used as a therapy for a variety of health conditions affecting the liver, kidney, cardiovascular and immune systems. Today, *T. terrestris* in combination with a variety of herbal products is used for headaches, eye conditions such as itching, conjunctivitis and weak vision, and nervousness. This herb has also been used in connection with high blood pressure and rib pain. The inhibitory effect of flavonoids from *Boerhaavia diffusa* on Bcap-37 breast cancer cell line in vitro were also studied (Pandey *et. al*., 2005). They successfully demonstrated the immunomodulating activity of a combination of extracts of this plant. It has tonic and aphrodisiac properties. Its therapeutic properties have been attributed to the presence of active compounds saponins, alkaloids and flavonoids, etc.

2. MATERIALS AND METHODS

2.1. Sample collection

Collected *T. terrestrius* seeds were surface sterilized thoroughly with distilled water, shade dried for 10 days and grounded into fine powder using a kitchen blender. This powdered plant material (250 gm) was extracted with 500 ml solvents such as water, methanol, ethanol, dichloromethane and hexane in a Soxhlet apparatus and the extracts were evaporated to dryness under pressure at 45ºC using a rotary evaporator and stored under nitrogen at -20 ºC in amber glass bottle until those were used (Hussain *et al.* 2009).

For the synthesis of silver nanoparticles, 10 gm of this seed powder was taken and mixed with 100 ml of distilled water and kept in boiling water bath at 60^0 C for 20 minutes. After cooling, the extract was filtered with Whattman filter paper No.1 and stored in refrigerator at 4°C for further studies.

2.2. Preparation of Aqueous T. terrestris Seed Extract, and Synthesis of AgNPs

The aqueous solution of 1mM silver nitrate (AgNO3) was prepared and used for the synthesis of silver nanoparticles. 5ml of *T. terrestris* seed extract was added into 95 ml of silver nitrate for reduction into Ag+ ions and incubated in room temperature. The reaction mixtures were monitored spectrophotometrically at every 20 minutes interval for 0-60 minutes. The reduction of silver nanoparticles was observed by change in the colour of reaction mixture in different conditions. After the reaction period of 24 hrs it was observed that the maximum colour change was in 60 minutes and it was dark brown (Sadeghi and Gholamhoseinpoor, 2015).

2.3. Characterization Techniques

2.3.1. UV-Vis Spectroscopy

Synthesized silver nanoparticles in the biologically reduced brown color solution mixture was characterized by sampling small aliquots of the reaction mixture into UV-Visible spectrophotometer using the absorption spectra between 300 - 700 nm with the help of JASCO V-650 spectrophotometer. The reaction mixture of seed extracts and AgNPs was subjected to centrifugation at 8000 X g for 15 min, and, the resulting pellet was washed three times with deionized water and filtered. An aliquot of this filtrate was used for FTIR, XRD, FESEM and TEM analysis (Jayaseelan *et. al*., 2013).

2.3.2. Scanning Electron Microscope (SEM):

Jeol JSM-6480 LV SEM machine was used to characterize the surface morphology and size distribution of AgNPs nanoparticles. The AgNPs powder sample and freeze dried sample of AgNP solution were sonicated with distilled water and a small drop of this sample was placed on a glass slide and allowed to dry. A thin layer of platinum was coated to make the sample conductive. The SEM machine was operated at a vacuum of the order of 10-5 torr. The accelerating voltage of the microscope was kept in the range 10^{-20} kV.

2.3.3. Fourier Transform Infrared Spectroscopy

FTIR spectroscopic studies were carried out to find if there were any bio-reducing agents present in the plant seed. The wavelength spectrum of the seed extract was determined before and after the addition of $AgNO_3$, the samples were mixed with potassium bromide (KBr) powder and pelletized and after drying the spectra were recorded using Perkin Elmer make model RX1 (wavelength range between 4000 cm^{-1} and 400 cm^{-1}) (Jayaseelan *et. al*., 2013).

2.3.4. X-ray diffraction measurement

The synthesized AgNPs sample was drop-coated onto an aluminum plate by just dropping a small amount of sample on the plate frequently, allowed to dry and finally a thick coat of sample was prepared. The XRD measurement was performed on a Shimazdu, model LabX-XRD-6000 instrument operated at a voltage of 20 to30 KeV and a current of 30mA with Cu K α radiation with a wavelength of 1.5418 Å.

2.3.5. Anti bacterial activity using in-vitro method

The antibacterial activity of green synthesized AgNPs was performed by agar well diffusion method against different Gram-positive and Gram-negative pathogenic bacteria (Saha *et. al*., 2007). A total of six bacterial strains namely *E.coli, Staphylococcus aureus, Salmonella typhi, Vibrio hervayie, Streptococcus pneumoniae,* and *Pseudomonas aeruginosa* were stock cultured and maintained at 4° C on nutrient agar slants. Prior to the experiment, pure cultures were created using nutrient broth and incubated over night at 37°C. Each strain was swabbed uniformly into individual nutrient agar plate impregnated with standard antibiotic solution (Streptomycin 100mg/ml) using sterile cotton swap and they were punctured for wells and loaded with different quantities of test sample of AgNPs (10µl, 20µl, 30µl, 40µl). After 24 hrs of incubation at 37^0C, the different ranges of zone of inhibition were measured. *T. terrestris* seed extract was used as a control. Tests were performed in triplicate, and mean values of the diameter of the zones were recorded.

A minimum inhibitory concentration (MIC) test was conducted parallely with the seed-extract synthesized AgNPs with various concentrations prepared with dimethyl sulphoxide (DMSO) in roughly 0.5 mL increments, and subsequently mixed with 50 µL of 24 hours-old individual bacterial pathogens. Each mixture was incubated at 37°C for 48 hours, and the visible turbidity was observed in each concentration in order to calculate MIC (Chudasama, *et.al.*, 2010).

3. RESULT & DISCUSSION

The seed extract of *T. terrestris* was used for the reduction of silver nitrate ($AgNO_3$) into silver nanoparticles. The biosynthesis reaction was confirmed based on the colour change from colorless to dark brown started within few minutes of the commencement of the reaction. The intensity of brown colour kept on increased during the reaction which was a temperature dependent approach to fabricate nanoparticles by reducing silver nitrate ($AgNO_3$) solution (Fig.1). It has been observed that the increase in temperature resulted in the rapid biosynthesis of nanoparticles. The control $AgNO_3$ solution (without seed extract) showed no colour change. Absorption spectrum of reaction mixture at different wavelengths ranging from 200-800 nm revealed a peak at 420 nm. The characteristic of $AgNO_3$ absorption peak at 2.991 nm (Fig.2a), characteristic of plant extract *T. terrestris* absorption peak at 2.217 (Fig.2b) characteristic of synthesis nanoparticles absorption peak at 459 nm (Fig.2c). The characteristic UV-Vis spectrum confirmed the formation of AgNPs.

Figure 1: Synthesis of silver nanoparticles

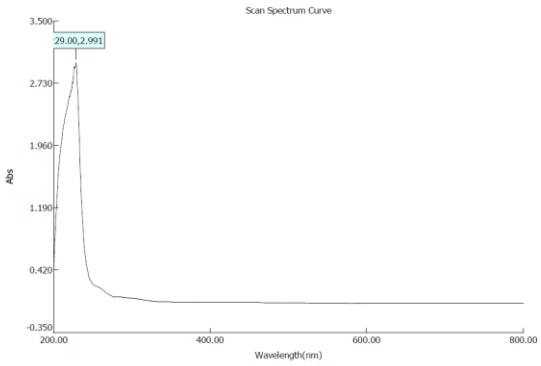

Figure 2a: UV-Vis spectrum analysis of $AgNO_3$

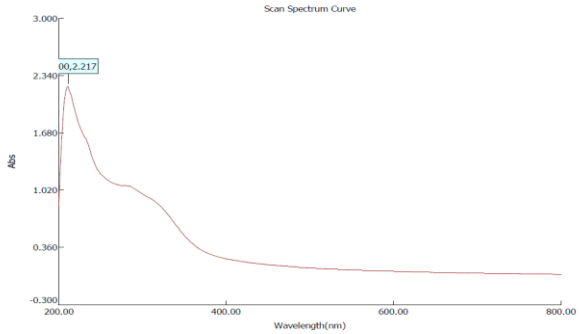

Figure 2b: UV-Vis spectrum analysis of plant extract

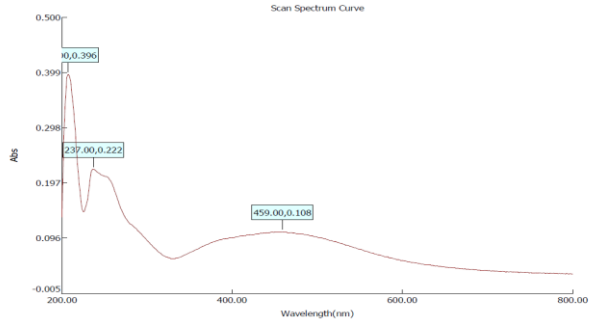

Figure 2c: UV-Vis spectrum analysis of synthesis of nanoparticles

3.1. FTIR- analysis

FTIR has become an important tool in understanding the involvement of functional group in relation between metal particles and biomolecules. The FTIR measurement of biosynthesized silver nanoparticles was carried out to identify the possible interaction between plant proteins and AgNPs. The FTIR result shows sharp absorption peaks as follows 3377.90, 2923.72, 2855.54, 2361.86, 1739.46, 1647.85, 1511.37, 1426.74, 1374.26, 1322.18, 1245.98, 1159.26, 1056.78, 668.23, 610.81 (Fig.3).

The FTIR spectra of dried *Tribulus terrestris* seed extract showed strong absorption band at 2923.72 cm which can be assigned to the absorption peaks of N-H of stretching vibration in the amide linkage. The band at 2361.86 cm can be assigned to the absorption peaks of SH structure (organo silicon compound), on the other hand the shift of band from 1647.85 cm has been attributed to the bonding of C=C (Alkanes) with nanoparticles. Band at 1374.26 cm can be assigned to absorption peaks of S=O stretching vibration (Sulfonamide). The band at 1159.26 cm in silver nano may be attributed to C-O (thiocarbonyl group). Band at 1056.78 cm can be assigned to O-C-C (Aromatic ester of primary alcohols). The bands at 610.81 cm can be assigned to absorption peaks of C=S stretching vibration sulfide.

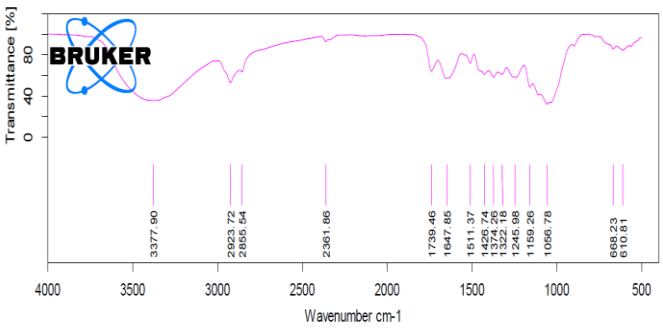

Figure 3: FTIR-Spectrum analysis of synthesis nanoparticles

3.2. Scanning electron microscopy

For the understanding of the surface morphology of silver nanoclusteres the scanning electron microscope was used. SEM images of synthesized nanoparticles using *T. terrestris* plant seed extract were found to be spherical in shape with agglomeration (Fig.4). Some nanoparticles got aggregated when the reaction mixture was incubated for 48 hours. The size of silver nanoparticles was found to be 115 nm with an average of 120 nm. It is accepted as true that, higher concentration of bioactive compounds in the colloidal solution might cause the formation of nanoclusters. Moreover the result suggested that the silver nanoparticles are synthesized due to the action of *T. terrestris* (seed) plant extract which act as good bioreductant for biosynthesis.

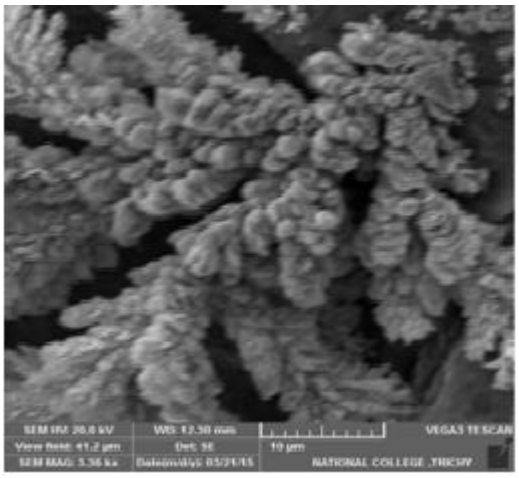

Figure 4. SEM image of synthesis nanoparticles

3.3. X-ray diffraction measurement

The characteristics peak observed in X-ray diffraction pattern of the synthesized silver nanoparticles confirmed the presence of silver nanoparticles (Fig.5). The XRD peaks at 37.430°, 77.640° and 64.560° correspond to the 111, 113 and 080 planes are observed which may be indexed as the band for centered cubic structure of silver. The XRD pattern thus clearly illustrates that the silver nanoparticles synthesized by the present green method are crystalline in nature.

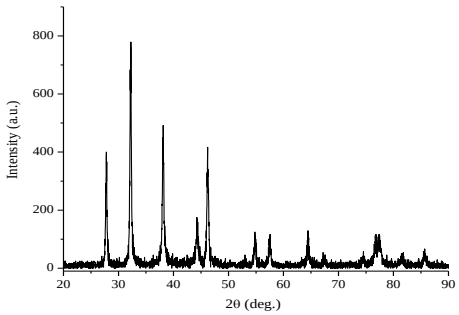

Figure 5: X-ray image of the nanoparticles

3.4. Antibacterial activity

For the analysis of antibacterial potentiality, both gram positive and gram negative bacteria were used. In this study the silver nanoparticles showed more than 10 mm zone of inhibition against *E.coli*, *Staphylococcus aureus*, *Salmonella* sps, *Vibrio hervayi*, *Streptococcus pneumoniae*, *and Pseudomas* sps (Fig.6).

Comparatively Streptomycin (antibiotic) was used as a positive control. A zone of less than 10 mm was inferred in *E.coli* and *V.hervay*. It is clear from the study that the green synthesized nanoparticles can compete with commercial antibacterial agents (antibiotic) used for the treatment of bacterial infection.

T. terrestris has been known to contain protein, sterol, flavonoids and alkaloids which may possibly helpful in the synthesis of nanoscale value particles hence in this study its seed was used to evaluate for the same in a short reaction time. Its seed extract when used for the synthesis of silver nanoparticles resulted in a color change in the solution from transparent to dark brown due to the production of silver nanoparticles while no color change was observed with the control of 1mM $AgNO_3$ solution without plant seed extract. Several approaches have been employed to obtain a better synthesis of silver nanoparticles by both chemical and biological methods. Recent report of biological synthesis of AgNPs was also achieved using the plant *Garcinia mangostana* (Veerasamy *et.al.*, 2011).

There are several reports published regarding the synthesis of AgNPs by both chemical and biological reduction, but still we need a standard strategy for the synthesis of AgNPs with good stability and almost neutral toxicity (Badri and Sekthivel, 2011). In this study an attempt is made to synthesize the NPs with good stability with *T. terrestris* seed extracts.

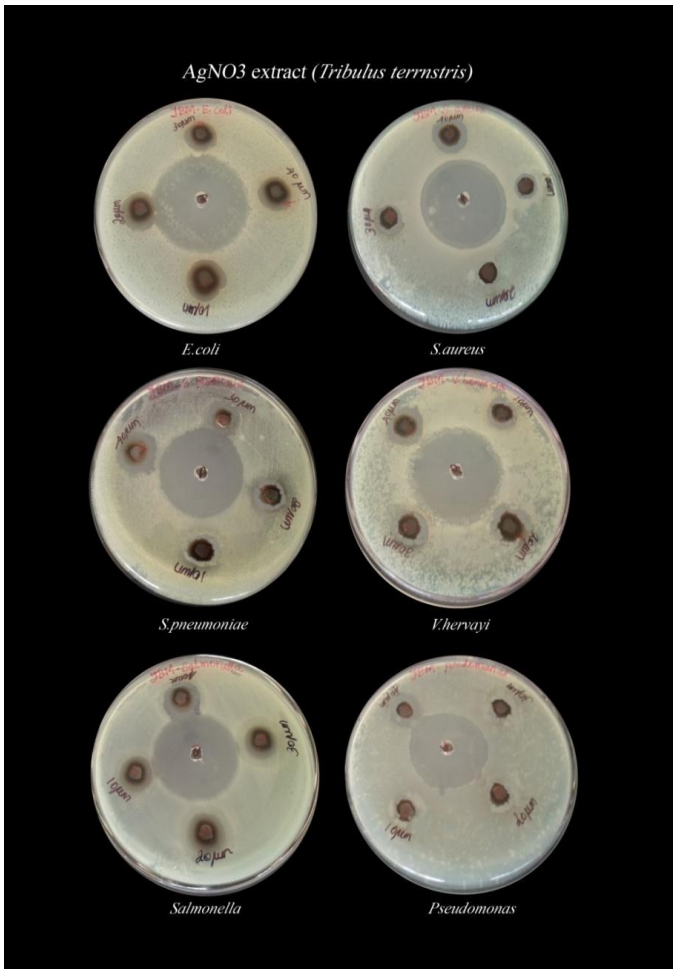

Figure 6: Antibacterial activity of Tribulus terrestris (seed)

The FTIR measurement of biosynthesized silver nanoparticles (AgNPs) was carried out to identify the possible interaction between protein and AgNPs, (room temperature). FTIR results show sharp absorption peaks (Fig.3). The result indicates the presence of phenolics compounds such as flavonoids, tri terpenoids present in the seed extract which may possibly influence the reduction and stabilization of silver nanoparticles due to interaction of biomolecules with them.

Table 1: Zone of inhibition on nutrient agar plates

S.NO	ORGANISM	Zone of inhibition (mm) in Seed extract				
		10µl	20µl	30µl	40µl	Antibiotic
1	*E. coli*	8	9	10	10	21
2	*S.aureus*	8	8	9	9	22
3	*Pseudomonas*	4	6	8	8	20
4	*Salmonella*	9	9	10	10	20
5	*S. pneumonia*	5	7	9	10	23
6	*V. hervayi*	9	10	10	10	22

Silver nanoparticles pretend to have strong bactericidal activity against gram-negative and gram-positive bacteria including multidrug resistant strains (Rai *et al*., 2012). The enhanced antibacterial effects of novel silver nanoparticles is characterized and also stated that once inside the cell, nanoparticles would interfere with the bacterial growth signaling pathway by modulating tyrosine phosphorylation of putative peptide substrate critical for cell viability and division and the nanoparticles were not in direct contact even within the aggregates, indicating stabilization of the nanoparticles by a capping agent (Shrivastava *et al*., 2007 and Duran *et al*., 2005).

3.5. Conclusion

The suggested plant-mediated synthesis method is an inexpensive approach capable of producing AgNPs at room temperature. Characterization with UV-vis, FT-IR, SEM and XRD has been an evidence for the formation of nanoparticles. The present study demonstrated that AgNPs are capable of rendering high antibacterial results, and therefore show great potential for the preparation of antibacterial drugs. The synthesized silver nanoparticles showed promising antimicrobial activity against human pathogenic bacterial strains. Results confirmed that the *T. terrestris* seed extract is a higher quality eco-friendly and safe source for AgNPs synthesis than conventional chemical or physical methods, and its utility invites further investigation.

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