GREEN SYNTHESIS OF AG NANOPARTICLES AND ITS THERAPEUTIC POTENTIAL

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Abstract
Nano biotechnology is an emerging field in biotechnology and it has vast applications in all fields. In this study silver nanoparticles has been synthesized by using Cassia tora leaf aqueous extracts which was characterized by using UV-VIS spectroscopy and confirmed by SEM and TEM analysis. The synthesized nanoparticles have been evaluated for antioxidant and antimicrobial studies and effective results were found. In recent years, the major focus of researchers is to develop eco-friendly technique for production of well-characterized nanoparticles. Many methods are available for the production of metal nanoparticles using organisms viz. bacteria fungi and plants. Among these organisms plants are suitable for large-scale biosynthesis of metal nanoparticles. Nanoparticles produced by plants are more stable and the rate of synthesis is also faster and are more various in shape and size than produced by other organisms. The produced AgNPs from plant extract was confirmed by color change from transparent yellow to reddish brown which indicates formation of AgNPs on addition of AgNo3 solution to plant extract. In UV-visible spectra, the extract showed absorbance peak at 410nm. The TEM micrograph showed clustered nature of synthesized silver nanoparticles with size 30nm. The SEM images showed rod- shaped nanoparticles formed with diameter in the range of 30-60 nm from the aqueous extract of Cassia tora. The synthesized silver nanoparticles were investigated for bio catalytic and biological efficacy and found to have potent antioxidant and antimicrobial activity. The researchers are interested to investigate mechanisms of bio reduction of metal ions by plants because of many advantages of using plants and plant derived materials.

***Keywords**: Green synthesis, silver nanoparticles, Cassia tora, antioxidant activity, antimicrobial activity, UV-VIS., SEM ,TEM.*

Introduction
"Nanotechnology is the application of science to control matter at the molecular level" (S. Senapati 2005). major applications of silver nanoparticles such as antimicrobial effect (Khan et al., 2014; Kumar et al., 2014), antitumor effect (Jeyaraj et al., 2013), sensitivity to detect the presence of various pollutants such as metals (Balavigneswaran et al.,2014), dyes (Kumar et al., 2013), antibiotics (Singh et al.,2012) and nitro-aromatic compounds (Narayanan and Sakthivel, 2011) . It is found that the conventional methods of synthesis of silver nanoparticles have many limitations like slow process, a high cost and use of chemical reducing agents such as sodium borohydride, trisodium citrate and dimethyl formamide, and these chemicals cause environmental burden.

In contrast, the green synthesis of silver nanoparticles gained a lot of attention among researchers because of usage of natural resources, rapidness, eco-friendliness and benignancy. These appealing features are essential in medical applications. The other advantages of green synthesis include well-defined and controlled size of the nanoparticles. They

are lacking contaminants and the process is easy to scale-up (Mittal et al., 2013).

In the green synthesis of nanoparticles, various plants and their parts are utilized because biomolecules present in the plant extract such as enzymes, proteins, flavonoids, terpenoids and cofactors act as both reducing and capping agents (Tavakoli et al., 2015). In the present era, because of incredible applications of silver nanoparticles in all fields of science the development of nanoparticles through the green synthesis have been increased. A lot of work have been focused on the plant mediated synthesis of nanoparticles. AgNP has been synthesized by using various plants such as Bacopa monnieri[Krishnaraj C etal.,2012]. Catharanthus roseus [Mukunthan KS etal.,2011] and Coccinia grandis [Arunachalam R etal.,2012]. In this study, *Cassia tora* plant and its parts were used to study the antimicrobial and antioxidant activities of silver nanoparticles.

Cassia tora Linn. (Caesalpiniaceae) is a type of shrub which is used in Africa and India as a traditional medicine and In the rainy season it is extensively found like weed in the tropical region of India as an annual herb (Sirappuselvi S. and Chitra M. 2012**)**. It is also found in China and used as an edible medicine (A. Saravanakumar et al.,2015).

In this research, Cassia tora leaf extract were used to reduce the aqueous silver nitrate for the biosynthesis of AgNPs. It is demonstrated that some agents in plant extract act as cost effective, non-hazardous and eco-friendly capping and reducing agents causing bioreduction of $AgNO_3$.

Material and Methods

Sample collection

Cassia tora leaves were collected from the surroundings of Jaipur, Rajasthan India. Silver nitrate was obtained from Merck specialties Private Limited, Mumbai.

Preparation of leaf extract

The fresh *Cassia tora* leaves were collected from surroundings of Jaipur Rajasthan, India. The *Cassia tora* fresh leaves were washed with tap water and further with distilled water. 6gm fresh leaves of *Cassia tora* were crushed in 10ml d.w. and then it placed for centrifuge at 15000 rpm for 10 min. the supernatant of this extract was further used for formation of silver nanoparticles.

Synthesis of silver nanoparticles

In a typical experiment, the leaf extract (0.5 ml) was added to 10 ml of 1 mM AgNO3 aqueous solution. The bio reduced aqueous component (0.5 ml) was used to measuring UV-Vis spectra of the solution. The particle suspension was diluted 10 times with distilled water to avoid the errors due to high optical density of the solution In order to synthesize silver nanoparticles (SNPs), 10 mL of the leaf extract was mixed with 90 mL of 1 mM silver nitrate solution and heated in a water bath, set at 80 ^{0}C for 10 min. A color change into black brown designates the formation of colloidal SNPs.

Characterization of silver nanoparticles:

Transmission Electron Microscopy

The size and morphology of the nanoparticles were analyzed with the transmission electron microscope (JEOL). The sample was prepared by placing a drop of silver nanoparticles on carbon coated copper grid and it was dried before transferring to the microscope.

SEM analysis of silver nanoparticles:

Scanning electron microscopic (SEM) analysis was done using ZEISS machine. Thin films of the sample were prepared on a carbon coated copper grid by just dropping a very small amount of the

sample on the grid. Extra solution was removed using a blotting paper and then the films on the SEM grid were allowed to dry by putting it under a Mercury lamp for 5 mints.

Antibacterial activity

Agar well diffusion method were used to evaluate the antibacterial activity of biosynthesized Ag NPs . Both gram positive Staphylococcus aureus (NCIM 2079), Bacillus subtilis (NCIM 2063) and gram negative Escherichia coli (NCIM 2931), Pseudomonas aeruginosa (NCIM 5029) pathogenic bacteria were measured with the slight modifications in previous method [U.B. Jagtap, V.A. Bapat,2013]. Nutrient agar media (250ml) was prepared and 25–30 ml was transferred in to 10 sterile petri plates. When the media were solidified the bacterial culture (saline solution 8-10 ml + bacteria broth 10-15 ul) were added to the media. After drying for 10 min. holes were made with stainless steel cylinders. The different concentration of AgNPs (20ul, 40ul, 60ul and 80 ul) were filled and after 10 mint. the plates were incubated at 32 ^{o}C for 24 h.

Determination of antifungal activity

Antifungal activity of the AgNPs were investigated by agar well diffusion method (Perez etal.,1990). The fungi were subculture on to potato dextrose agar, PDA(Merck, Germany) and incubated at 37°C for 24 hr. and 25°C for 2-5 days.. The plates were dried at room temperature for 15 mints. wells of 6mm in diameter were punctured in the culture media using sterile glass tube. Several concentration of the sample (Ag NPs) as 20ul, 40ul, 60ul and 80ul with the standard ketoconazole 40ul were taken to make the wells fulfill. Plates were incubated at 37°C.After incubation of 24 hr. antifungal activity was determined by measuring the diameter of inhibition zone (in mm).

FRAP assay: Benzie and Strain (1996) procedure was followed to measure the antioxidant power of AgNPs biosynthesized by *Cassia tora* leaf extract. This method is based on the principle of reduction of a ferric-tripyridyl-triazine complex to its ferrous, colored form by the antioxidants found in the extract. The FRAP reagent contained 2.5 mL of a 10 mmol/L TPTZ (2,4,6- tripyridyl-s-triazine, Sigma) solution in 40 mmol/L HCl plus 2.5 mL of 20 mmol/L FeCl3 6H2O and 25 mL of 0.3 mol/L acetate buffer, pH 3.6 and was prepared freshly and warmed at 37^{o}C. Aliquots of 0.5 mL sample were mixed with 1.5 mL FRAP reagent and the absorbance of reaction mixture at 593 nm was measured spectrophotometrically after incubation at 37^{o}C for 10 min. As the standard Gallic acid or ascorbic acid were used and final result was expressed as the concentration of antioxidants having a ferric reducing power equivalent to that of mg of standard used per gram AgNPs.

Lipid peroxidation activity:

Lipid peroxidase was measured by Hodges *et al.*,1998 with some modifications. 1ml sample with AgNPs mixed with 4ml of (5%) TBA, (10%) TCA and left for heat in water bath at 95^{o}C for 25 mints. lipid per-oxidation was allowed to induce.. The mixture was further allowed to cooling, after cooling OD of the sample was measured spectrophotometrically at 532 nm and subsequently at 600 nm. Values were taken by the difference of both the absorbance. Control was taken with 10% TCA only.

Peroxidase

For the determination of peroxidase activity of AgNPs 2.4 ml phosphate buffer, 0.3 ml pyrogallol, 0.2 ml H_2O_2 and 0.1 ml

sample were taken. The bioactivity was determined by taking absorbance at 420 nm.

Catalase:

The antioxidant potential through catalase was measured by Aebi *et al.,* 1984 with the slight modifications. 0.2ml of plant extract was taken and mixed with 2 ml of phosphate buffer and 0.8 ml H_2O_2. The catalase activity of Ag NPs was measured spectrophotometrically and OD was taken at 240nm for the duration of 1 minute. For determination of the activity of catalase molar extinction capacity was measured as unit/mg of protein.

Results and discussion:

Synthesis of silver nanoparticles:

When the *Cassia tora* leaf extract added to the colorless AgNO3 solution it was turned to a greenish black color (fig.1) indicates the synthesis of AgNPs. After further incubation of sample it was found that color intensity has increased. *Cassia tora* leaf extract did not show any color change without $AgNO_3$ solution which indicates reduction of silver ions and biosynthesis of AgNPs.

Characterization of silver nanoparticles:

Ag NPs were further characterized by TEM and SEM analysis. TEM image of Ag NPs was obtained (size 30nm) and it was showed clustered particles due to aggregation of nanoparticles. The TEM micrographs suggested that the synthesized Ag-NPs were of spherical shape. It was clearly visible by high resolution scanning electron microscope that particles were more or less spherical in size with the diameter (size) 30-60nm. UV–VIS spectroscopy is used as a first technique in the characterization of metallic nanoparticles due to surface plasmon resonance (SPR) phenomenon shown by metallic nanoparticles. UV-visible spectroscopy is a simple and quite a sensitive technique that is applied to detect the formation of silver nanoparticles. UV-Visible spectroscopy is a straightforward technique for the detection of nanoparticles. By the UV-visible spectra (Fig.3) of the bio reduced Ag NPs solution, it was clearly showed an absorbance peak at 410 nm for Ag NPs from *Cassia tora,* which is a characteristic band (400-500nm) for silver nanoparticles and in accordance with a previous report (Shameli etal.,2013). Absorbance peak strongly suggests that *Cassia tora* could be a better biological source in synthesis of silver nanoparticles.

Antioxidant activity of synthesized silver nanoparticles:

Antioxidant activity of synthesized silver nanoparticles was assessed and profiled in table 3. The higher antioxidant activity of silver nanoparticles is might be possible due to adsorption of antioxidant materials from Cassia tora leaf extract to the surface of nanoparticles. The amount of formation of Fe2+-TPTZ complex decides the antioxidant effect on the FRAP method. SNPs were able to reduce TPTZ-Fe(III) complex to TPTZ-Fe(II). Increasing absorbance indicated an increase in reductive ability (Vijayakumar etal., 2013). The calculated FRAP values revealed that the SNPs synthesized from *Cassia tora* extract showed a higher antioxidant capacity. The ferric reducing power of silver nanoparticles assessed more (1.04 µM/l/gm FW) in *Cassia tora.* In lipid peroxidation assay, the highest antioxidant activity was assessed in SNPs synthesized from *Cassia tora* leaf extract i.e. (40.14µM/l/gm FW). Peroxidase content was found 1.764µM/l/gm FW in *Cassia tora* SNPs. Catalase activity was

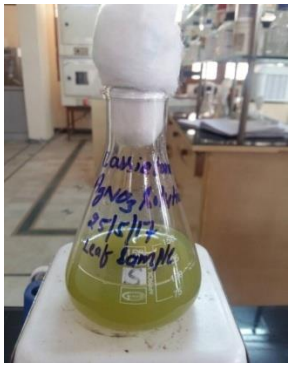
determined in *Cassia tora* AgNPs i.e. (1.0µM/l/gm FW).

Antimicrobial activity of synthesized silver nanoparticles:

The present study reveals the antibacterial activity of synthesized SNPs from aqueous extract of *Cassia tora* at different concentrations. The results were obtained from disc diffusion method against eight different microorganisms (four reference and four clinical) indicated that SNPs inhibited growth of many of the microorganisms tested and it was observed that the amount of inhibition highly dependent on the concentration of SNPs. The antibacterial activity of biologically synthesised AgNPs from Cassia tora were tested against Gram-negative bacteria E. coli, Pseudomonas aeruginosa, and Gram positive bacteria Staphylococcus aureus and Bacillus subtilis. Silver nanoparticles exhibit better antibacterial potential due to their small size and high surface area. The exact mechanism of silver nanoparticles to inhibit the growth of bacteria is not fully understood. Generally it is accepted that silver nanoparticles penetrate the cells. It was reported that the AgNPs are found more efficient in controlling Gram-negative bacteria in comparison to Gram-positive (Jung etal.,2008). This difference in activity is due to the difference in the membrane of gram positive and gram negative bacteria. It is relevant to connect this difference with peptidoglycan layer which is more thick and stable in gram positive bacteria and did not allow negatively charged AgNPs to enter the cell through providing sufficient binding site. Whereas AgNPs can bind to Gram negative cell wall easily and thus exhibit more efficiency in controlling them(Ramalingam etal.,2016). In this research it was observed that the biologically synthesised nanoparticle show enhanced antibacterial potential. It was demonstrated that the AgNPs synthesized from Cassia tora extract exhibited maximum activity with zone of inhibition of 13mm against E.coli bacteria at the concentration of 80ul. Silver nanoparticles from *Cassia tora* extract found efficient towards Bacillus subtilis also with the zone of inhibition of 10mm and other bacterial strains did not found susceptible towards synthesized nanoparticles by using *Cassia tora* leaf extract. The antifungal potential of biological synthesised nanoparticles was tested against four phytopathogenic fungus *Penicillium funiculosum, Trichoderma reesei, Candida albicans* and *Aspergillus niger*. The mode of action of silver nanoparticles against the fungus is still under investigation but it is widely accepted that silver nanoparticle attached to fungal membrane to penetrate the cell and cause cell death either by blocking respiratory enzymes or after entering the cell silver nanoparticles releases silver ions which disrupt DNA replication pathways and lead to cell death (Narayanan and Park 2014). Biologically synthesised silver nanoparticle exhibited moderate to good antifungal activity. The biologically synthesized silver nanoparticle from *Cassia tora* extract was found most active against *T. reesei* and *P. funiculosum.* It was observed that the activity is not much influenced by concentration of AgNPs. Other fungal strains (*A. Niger* and *C. albicans*) did not found susceptible towards AgNPs of *Cassia tora* leaf extract. Maximum antifungal potential of *Cassia tora* AgNPs was observed against Trichoderma reesei at the conc. Of 80ul and Penicillium funiculosum at the conc. of 60ul with inhibition zone as 10mm,

10mm respectively. It was observed that AgNPs from *Cassia tora* showed highest activity (13mm) against E.coli. In this study antifungal activity of AgNPs was less when compared with anti-bacterial activity. Among the bacteria the zone of inhibition was found less on gram positive bacteria when compared with gram negative bacterial strains. In the present study it was strongly revealed that both bacterial strains as gram positive and gram negative were found more susceptible to AgNPs than the fungus strain.

a



b

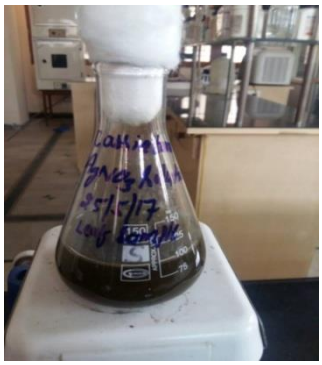


Fig.1 (a) Cassia tora leaf extract before AgNPs synthesis (b) after AgNPs synthesis

Change in color shows the synthesis of AgNPs

Table 1: Antibacterial activity of biosynthesized AgNPs

Bacterial strain	Sample 20ul ZOI (mm)	Sample 40ul ZOI (mm)	Sample 60ul ZOI (mm)	Sample 80ul ZOI (mm)	Standard ciprofloxacin
Escherichia coli	NA	NA	NA	13±0.52	22
Staphylococcus aureus	NA	NA	NA	NA	22
Bacillus subtilis	10±0.36	NA	NA	NA	22
Pseudomonas aeruginosa	NA	NA	NA	NA	22

Table 2: Antifungal activity of AgNPs

Fungal strain	Sample 20ul ZOI (mm)	Sample 40ul ZOI (mm)	Sample 60ul ZOI (mm)	Sample 80ul ZOI (mm)	Standard ketoconazole
Trichoderma ressei	NA	NA	NA	10±0.51	31
Aspergillus niger	NA	NA	NA	NA	31
Penicillium-funiculosum	NA	NA	10±0.44	NA	31
Candida	NA	NA	NA	NA	31

albicans					

Fig.2 Shows antimicrobial activity of AgNPs

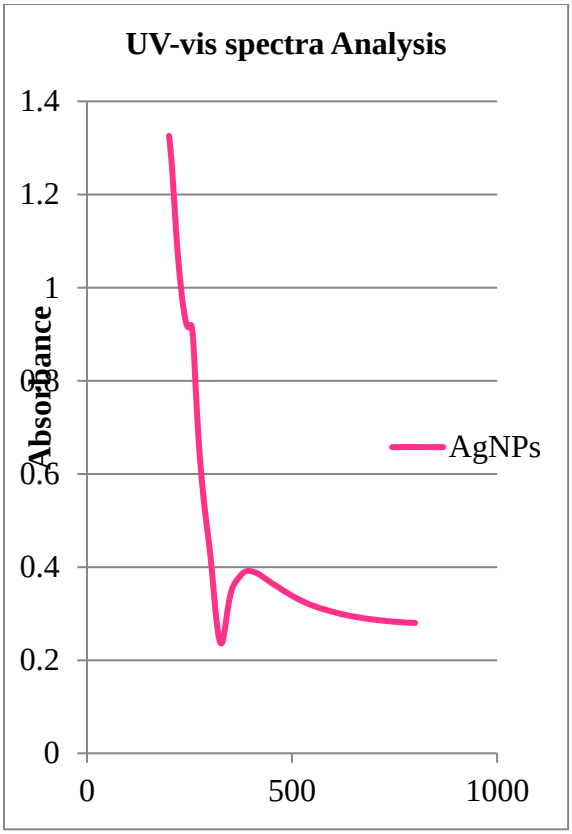


Fig.3 UV-VIS spectral analysis of Ag NPs synthesized from Cassia tora leaf

Table 3:Invitro antioxidant activity of AgNPs

Serial no.	Assay	OD (nm)	Bioactivity (um/l/gm-fresh weight)
1.	FRAP	593	1.0458±0.05
2.	CAT	240	1.00±0.02
3.	LPO	532	40.145±0.13
4.	PO	420	1.764±0.21

Note-Bioactivity values are calculated as OD x ε (OD is optical density , ε is molar extinction coefficient)

CAT- 40 , FRAP-1.8 , Peroxidase-2.8 , LPO-155 (values of ε)

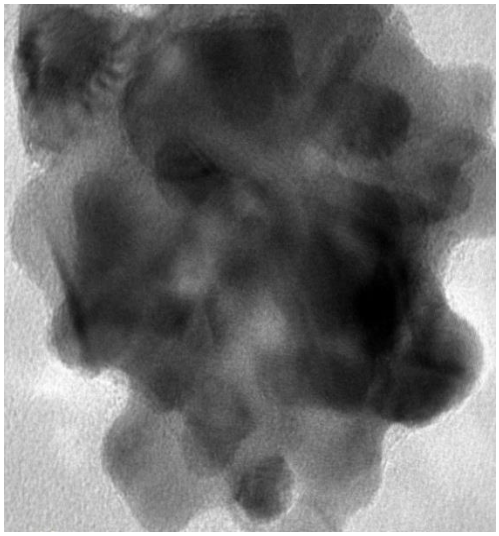


Fig.3 TEM micrograph of synthesized AgNPs.

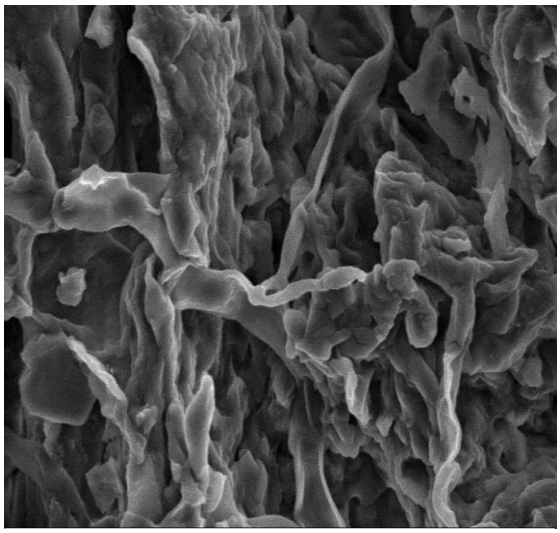


Fig. 4 SEM micrograph of synthesized AgNPs

Conclusion:

It was concluded that the green synthesis of silver nanoparticle is easy, ecofriendly and non-toxic also. AgNPs of *Cassia tora* plant exhibited moderate to good antioxidant and antimicrobial activity. Found more susceptible towards gram negative bacteria than gram positive ones and fungal strains. SNPs can be useful for the development of newer and more potent antioxidants. Because of vast applications and easy scale up process of silver nanoparticles, these particles can use for large scale production of pharmaceuticals.

Acknowledgement:

I would like to thanks to Dr.Ajit kumar **(**Department of Biotechnology Seminal applied Science Pvt. Ltd. Jaipur, Rajasthan, India) and Dr. Ikram qureshi (Department of Biotechnology, JJT University Chudela, Jhunjhunu, Rajasthan, india) for his continue support and guidance during this research.

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