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Green synthesis of Silver Nanoparticles Using *Withania coagulans* and its Antimicrobial and Anti-Oxidant Activity

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Abstract:

There is worldwide interest in silver nanoparticles (AgNPs) synthesized by various chemical reactions for use in applications exploiting their antibacterial activity, even though these processes exhibit a broad range of toxicity in vertebrates and invertebrates alike. To avoid the chemical toxicity, biosynthesis (green synthesis) of metal nanoparticles is proposed as a cost-effective and environmental friendly alternative. *Withania coagulans* extract is a medicinal agent with multiple properties including an antibacterial and anti oxidant activity. AgNPs were prepared by an eco-friendly hydrothermal method using a *Withania coagulans* plant extract solution as both a reducing and stabilizing agent. The synthesized nano particles was characterized using UV, IR XRD, TEM and SEM. Additionally, an agar disc diffusion method was used to screen for antimicrobial activity. Then the anti oxidant activity was investigated using DPPH assay method. The synthesized AgNPs were crystalline with sizes of 20-150nm as revealed using XRD and SEM. The particles were found to be Cubic and Spherical in shape.

These AgNPs were investigated for potential use as an antibacterial and anti fungal agent to inhibit growth of pathogenic bacteria and fungi. Very high bacterial zone inhibition activity was observed on *B Subtilis*, *B cereus* and *E coli*. Anong the two fungal strains observed, the AgNPs show very high activity against the growth of *A niger*. The LC50 for DPPH activity was observed at a very low concentration 50-100µg/mL. This confirms that the syntheisised nano particles were found to have high anti oxidant activity. These results indicated that AgNPs synthesized using *Withania coagulans* extract can be effectively utilized inpharmaceutical, biotechnological and biomedical applications

***Keywords*:** Green synthesis; *Withania coagulans*, Nanoparticle tracking analyzer, Transmission electron microscope; Energy dispersive X-ray spectra

1. Introduction:

There has been much recent interest in using silver nanoparticles (AgNPs) in new technologies owing to their drastically enhanced properties over bulk silver, especially particles of diameters 30 nm and smaller [1]. These NPs are increasingly being incorporated into consumer products [2] despite rising evidence suggesting AgNPs have toxic effects on humans and experimental animal models meant to mimic human bio- and neurochemistry such as mice, rats, and Drosophila [3]. Many studies also suggest that AgNPs are quite harmful to the aquatic environment should they be inadvertently released into wastewater [4]. However, toxicity studies of this nature are often hindered by the AgNPs themselves used in the studies. In lieu of fast and convenient synthesis methods, many studies have utilized AgNPs, but the specification by which these materials have been synthesized and/or purified may not be included. Many current protocols for the synthesis of AgNPs utilize harsh and/or toxic chemicals [5]. The presence of these harsh synthetic conditions or contaminants may confound sensitive toxicity studies. Furthermore, AgNPs available for purchase are

most often shipped as dry powders, and many studies fail to report the methods in which these AgNPs were redispersed in aqueous solutions [6]. The process of redispersion is quite crucial to the nature of the suspended AgNPs [7] and inconsistencies in this methodology may also confound toxicity studies. As an alternative to purchased powders, the method for synthesizing AgNPs reported here was done so in a cost- and time-efficient manner and keeping with the principles of green chemistry. The preparation of AgNPs using plant-based extracts [8] is widely growing in popularity; recently proposed syntheses use reagents such as many types of leaf extract [9] including menthol [10] aloe vera [11] clove extract [12] edible mushroom extract [13] and extracts from coffees and teas [14] AgNP synthesis using the extract of the navel orange (*Citrus sinensis*) was first proposed by Kaviya et al. in 2011 [15].

Green nanoparticle synthesis has been achieved using environmentally acceptable plant extract and ecofriendly reducing and capping agents. Plants and microbes are currently used for nanoparticle synthesis. The use of plants for synthesis of nanoparticles is rapid, low cost, eco-friendly, and a single-step method for biosynthesis process. Among the various known synthesis methods, plant-mediated nanoparticles synthesis is preferred as it is cost-effective, environmentally friendly, and safe for human therapeutic use. Hence in this work silver nano particles were synthesized using *Withania coagulans* extrat. Further the characterization and biological applications of the synthesized nano particles was studied.

Withania coagulans belongs to family Solanaceae, native to Afghanistan and the Indian subcontinent. The genus Withania Pauquy is having 10 species in the world, mostly in America and Africa; Withania coagulans was a lesser known plant which has drawn attention in the recent times because of the presence of important phytoconstituents like Withanolides in it which have immense pharmacological activities. The plant has been used in Unani system of medicine since many years

2. Materials and Methods:

2.1 Materials:

Silver nitrate of analytical grade was purchased from Indian Research Products, Chennai. Composition for the preparation of growth media for the growth of bacteria and fungi were purchased form Fisher Scientific Company, Mumbai. The standard antibiotic ciprofloxacin for antibiotic standard was obtained from Dr. Reddy's laboratories, Hyderabad. DPPH (2, 2-diphenyl-1-picrylhydrazyl) was purchased from Merck chemicals, Mumbai.

2.2 Collection of the plant:

The selected medicinal plant for the study W coagulans, was collected in Botanical garden at Dr N R S Ayurvedic Medical College, Bandar Road, Vijayawada. Primarily the collected plant was washed and the cleaned parts were dried with water absorbent paper (wet filter paper). Different parts of the plant were individually cut into small pieces, powdered used clean pestle and mortar. The plant powders were stored in an air tight container and were used for the extraction of plant components.

2.3 Preparation of plant extract:

5grams of selected plant part powder and equal volume mixture of selected parts were dispensed in 100 ml of sterile distilled water and boiled for one hour at 80ºC. Then individual extracts were collected in separate volumetric flasks by standard filtration method. The final volume in the volumetric flasks was made up to 100ml mark. The obtained plant extracts were used for the synthesis of nano particles.

2.4 Synthesis of Silver nanoparticles:

Various filtrates of the plant extract were collected separately.10-2 M Silver nitrate solution was prepared and stored in volumetric flask. Weigh accurately 95ml of $AgNO_3$ solution in a 100ml volumetric flask to this 5ml of plant extracts was added and incubated at room temperature. Same procedure was applied for all the parts and various combinations.

The color change of the extracts from pale yellow to dark brown was checked periodically. The brown color formation indicates the formation of silver nano particles from the plant extracts after incubation they were centrifuged at 5000 rpm for 15 minutes in order to obtain the pellet. The collected pellet was washed with double distilled water in order to remove the $AgNO_3$ solution. The collected pellet was air dried and the dry synthesized nano particles were studied for further characterization and activity studies.

2.5 Characterization of the Synthesized Silver Nanoparticles:

The optical absorbance was recorded on UV-Vis spectrophotometer (TECHOMP UV-2301 double beam model) in 340 - 800 nm wavelength range. FT–IR spectra of silver nanoparticles were performed using a BRUKER VERTEX 80/80v FT-IR spectrometer with KBr pellet in the range of 4000–400 cm–1. The crystalline nature of silver nanoparticles was investigated by XRD analysis. X–ray diffraction data of AgNPs were obtained using a Bruker- D4 ENDEAVOR with Cu kα radiation (λ = 1.54 Å) in the 2θ range of 20° to 80°, and with a steps size of 0.02° at 40 kV and 30 mA. The morphology of the AgNPs was examined by TEM (Hitachi H7500). Furthermore, SEM (LEO 1420 VP Compact variable pressure Digital SEM) study was carried out to investigate the shape, size and the surface area of the AgNPs.

2.6 Biological applications of synthesized silver nano particles:

2.6.1 Antibacterial Activity:

Antibacterial activity of the synthesized nanoparticles was determined by using the Kirby-Bauer disc diffusion method [14] against different pathogenic bacteria and fungi. The anti fungal zone inhibition activity was studied against *Aspergillus niger* (MTCC 282) and *Rhizopus oryzae* (MTCC 262).The anti bacterial zone inhibition activity was studied against five human pathogenic bacteria namely, *Bacillus Subtilis* (MTCC 10619), *Escherichia coli* (MTCC 443), *Pseudomonas aeruginosa* (MTCC 1688), *Staphylococcus aureus* (MTCC 3160), and *Bacillus cereus* (MTCC 1305). Stock cultures were maintained at 4°C on agar slants of nutrient media. Prior to the experiment, pure cultures were sub-cultured in Muller Hinton broth and incubated overnight at 37°C. The inoculums suspensions were swabbed uniformly in different Petri plates. Filter paper discs saturated with nanoparticles were placed aseptically in the plates with the help of sterile forceps and incubated at 37°C. 1% dimethyl sulfoxide was taken as positive and negative control respectively. After 24 hours of incubation, the zone of inhibition was observed and measured.

2.6.2 Antioxidant activity by DPPH method:

The free radical scavenging activity of the synthesized AgNPs was determined by using DPPH method described by Marsden S. Blois et al 1958 [15]. Briefly, DPPH solution of 0.1 mM was prepared in 95% methanol and 1 ml of this solution was added to 3.0 ml of synthesized AgNPs solution of 5–250µg/ml. The solution was incubated for 30min at dark conditions at room temperature and absorbance was measured at 517 nm using a UV–Vis spectrophotometer.

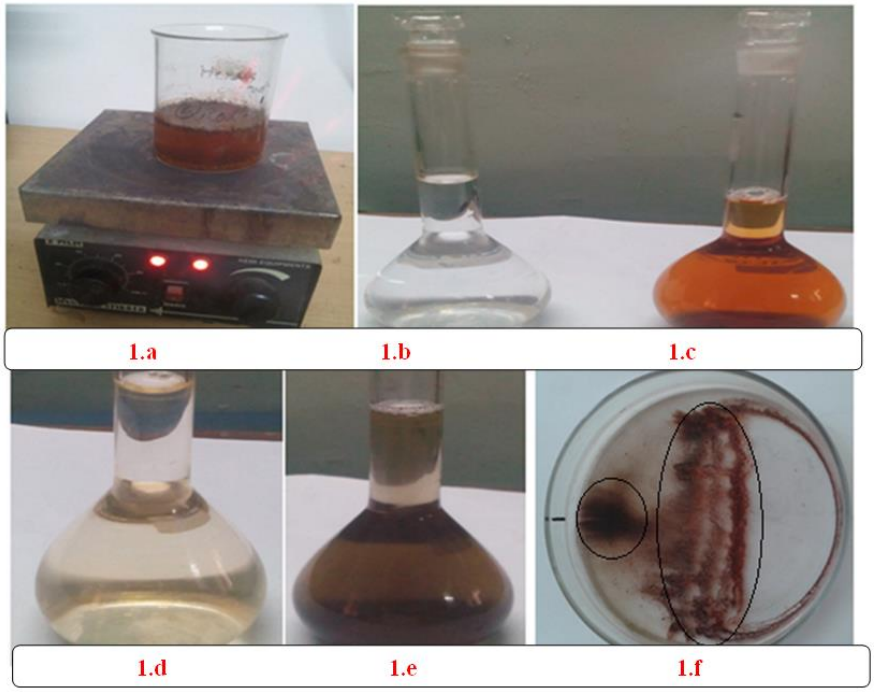
Ascorbic acid was used as a standard. The experiment was repeated triplicate and the DPPH scavenging activity was calculated by using the formula

$$\% \text{ Inhibition} = \frac{A0 - A1}{A0} \times 100$$

Where A_o is the absorbance of the control and A1 the absorbance of the AgNPs solution

3. Results and Discussions:

The biosynthesis of nanoparticles has been proposed as a cost effective and environmental friendly alternative to chemical and physical methods. Plant mediated synthesis of nanoparticles is a green chemistry approach that intercomnects nanotechnology and plant biotechnology. Hence silver nano particles were synthesized using *Withania coagulans* extract. It was observed that upon addition of the extract into the flask containing the aqueous silver nitrate solution, the color of the medium changed to brown within 1.5H. This indicated the formation of silver nanoparticles. The solution containing the signatory colour of AgNPs was then poured out into petri-dishes and left in the oven for drying at 250°C for 24 h. the Process of synthesis of Silver nanoparticles by using *Withania coagulans* was given in figure 1.



1.a: Process of Aqueous Stem+Root extraction of *Withania coagulans*, **1.b**: 10^{-2}M Silver Nitrate solution, **1.c:** Quantified Plant extract solutions, **1.d:** Mixture of silver nitrate solution and plant extract immediately after mxing, **1.e**: Solution containing sysnthesised silver nano particles before centrifugation, **1.f:** Cluster of Silver Nanoparticles after centrifugation

Figure 1: Process of synthesis of Silver nanoparticles by using *Withania coagulans*

The color change of solution after incubation due to the bio-reduction of silver using the selected plant extracts was centrifuged separately and the supernatant was discarded and the pellet was collected and dried. The dry weight of the silver nano particles was measured as was observed that very high amount of silver nano particle formation was observed with Stem and root combination aqueous extract of *Withania*

coagulans was used as biological reducing agent. The time required for the synthesis was also found to be less for this sample. Hence further studies were carried for the nano particles synthesized using stem and root combination aqueous extract of *Withania coagulans* as biological reducing agents.

3.1 Characterizations of synthesized nano-particles:

The chemical characterization of the synthesized nano-particles synthesized from the selected plant extracts were carried out using UV, IR spectroscopy, XRD, ¸TEM and EDX.

3.1.1 UV spectral analysis:

The synthesis of AgNPs by the reduction of aqueous metal ions during the exposure of different individual and combined extracts of *Withania coagulans* was observed. Stem and root mixture extract of *Withania coagulans* was reported to be best choice for nano silver synthesis and monitored by using UV-Visible Spectrophotometer. Efficient synthesis was noticed and the peak absorbance was noticed at 429nm confirming the formation of silver nanoparticles. Figure 2 shows the UV-Visible spectra recorded from the reaction medium. The obtained wavelength maxima observed was compared with previous reports and the reports support the obtained wavelength was found to be for the bio-reduced silver nano-particles.

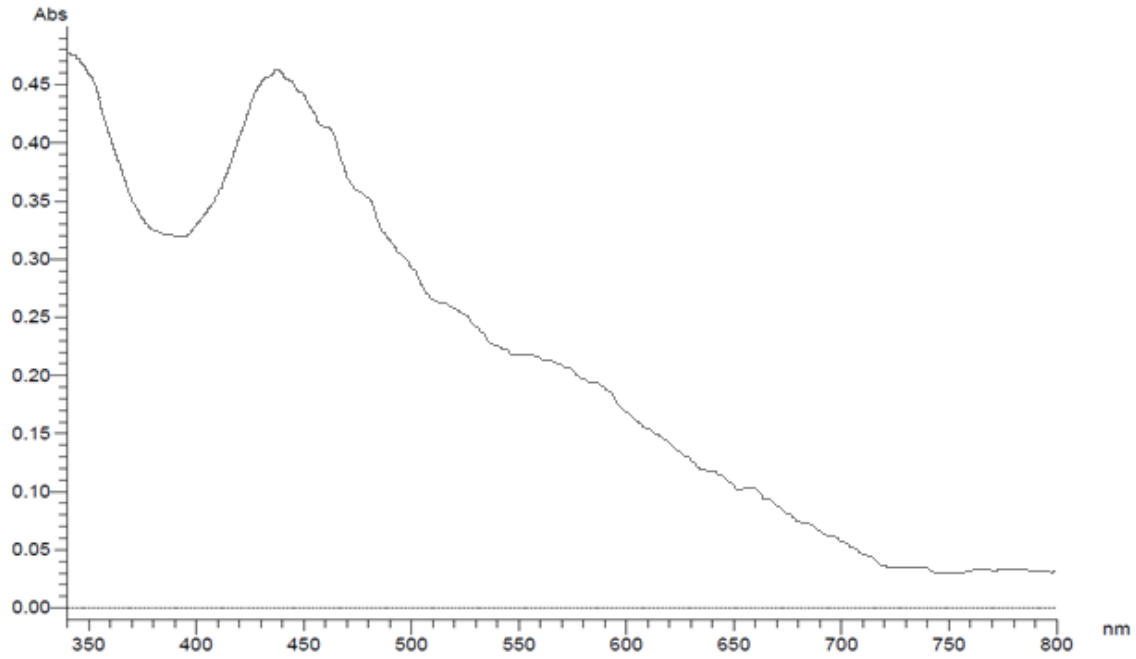
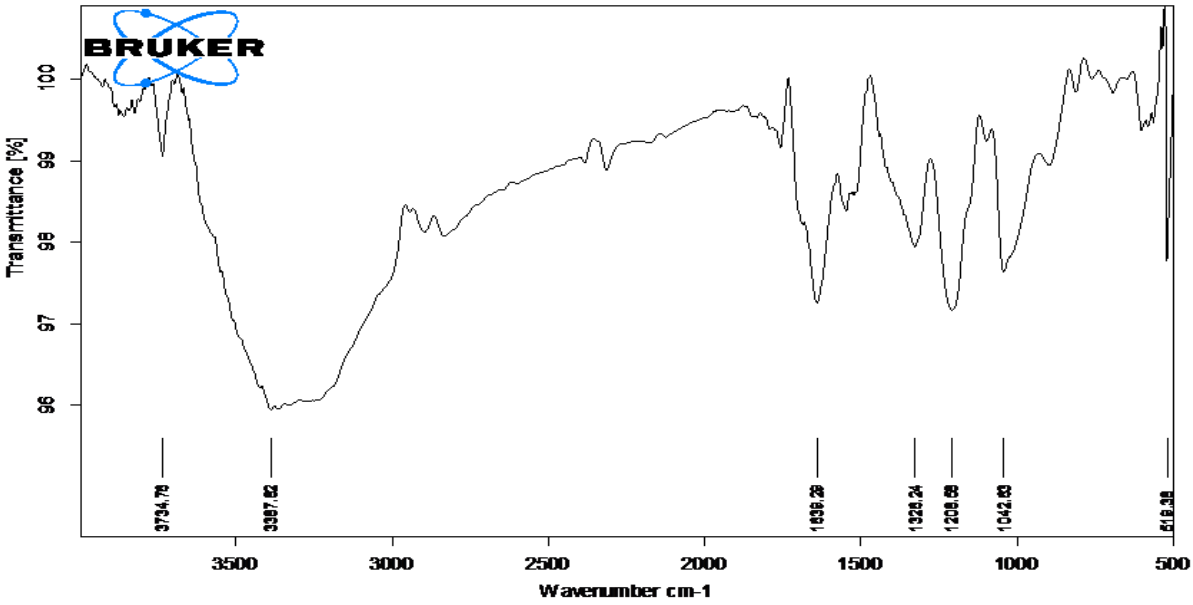


Figure 2: UV scanning spectra for the synthesized nano particles using *Withania coagulans*

3.1.2 FT-IR spectral analysis:

FT-IR measurement was carried out to identify the possible biomolecules for capping and efficient stabilization of the metal nanoparticles synthesized by *Withania coagulans* broth. The FT-IR Spectrum of AgNPs is shown in Figure 3. The peak value at 519.35 cm-1 corresponds to alkyl halides (C–Br stretch). The peak value at 1042.53 cm-1 corresponds to aliphatic amines and alcohols, carboxylic acids, esters, ethers respectively (C–N stretch and C–O stretch). The peak value 1208.52cm^{-1} corresponds to aliphatic amines, alkyl halides, alcohols, carboxylic acids, esters, ethers respectively (C–N stretch, C–H wag (–CH_2X), C–O stretch).

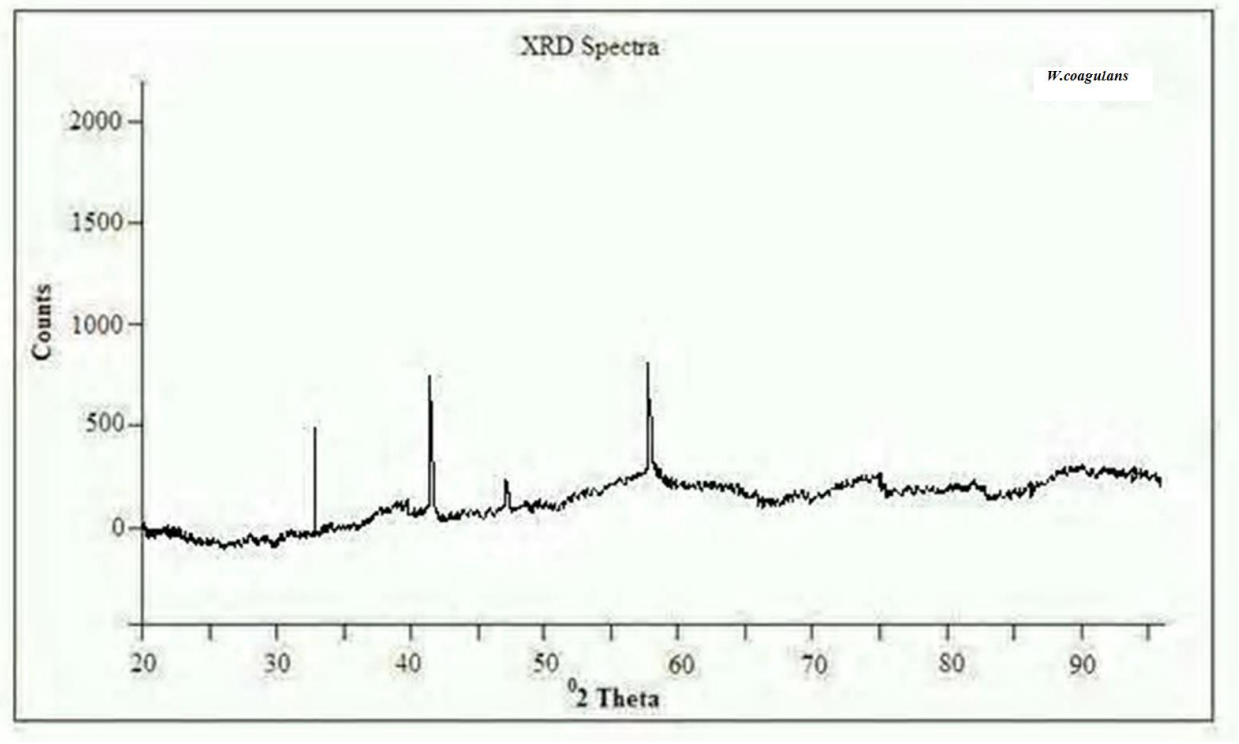
The peak value at 1630.29 cm-1 corresponds to 1° amines (N-H bend). Peak value at 3387.82 cm-1 corresponds to 1°, 2° amines, amides (N–H stretch) and alcohols, phenols (O–H stretch, H–bonded). The peak at 3734.76 cm-1 corresponds to alcohols, phenols (O–H stretch, free hydroxyl). Analysis of FTIR studies were confirmed that the carbonyl group from the amino acid residues and proteins has stronger ability to bind metal indicating that the proteins could possibly form the metal nanoparticles. These are derived from water soluble compounds such as flavonoids, alkaloids, and polyphenols present in the selected plants. Biological components present in the plant are known to interact with metal salts via these functional groups and mediate their reduction to nano-particles.



Figures 3: FT-IR Specta for the synthesized nano-particles using *Withania coagulans*

3.1.3 X ray diffraction analysis

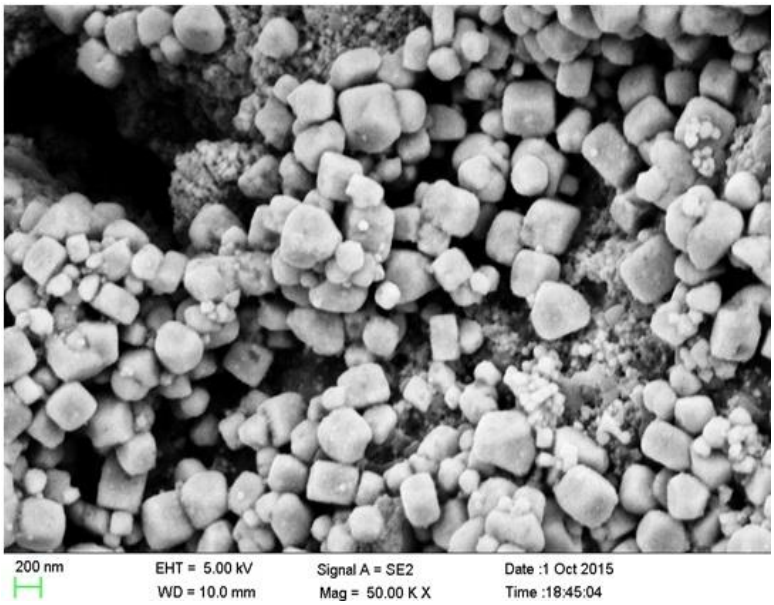
The XRD pattern showed three intense peaks in the whole spectrum of 2θ value ranging from 20 to 125. Average size of the particles synthesized was 83nm with size range 20 to 125nm with cubic and spherical shape. The typical XRD pattern revealed that the sample contains a mixed phase (cubic and spherical) structures of silver nanoparticles. The peaks observed in the spectrum at 2θ values of 33.17°, 42.66° and 59.54° corresponds to 111, 200 and 220 planes for silver, respectively. Some unidentified peaks were also observed near the characteristic peaks. A peak at 48º is possibly due to crystalline nature of the capping agent. This clearly shows that the AgNPs are crystalline in nature due to reduction of Ag+ ions by *Withania coagulans* stem and root mixture extract.



Figures 4: XRD Spectra for the synthesized nano-particles using *Withania coagulans*.

3.1.4 Scanning Electron microscopy analysis

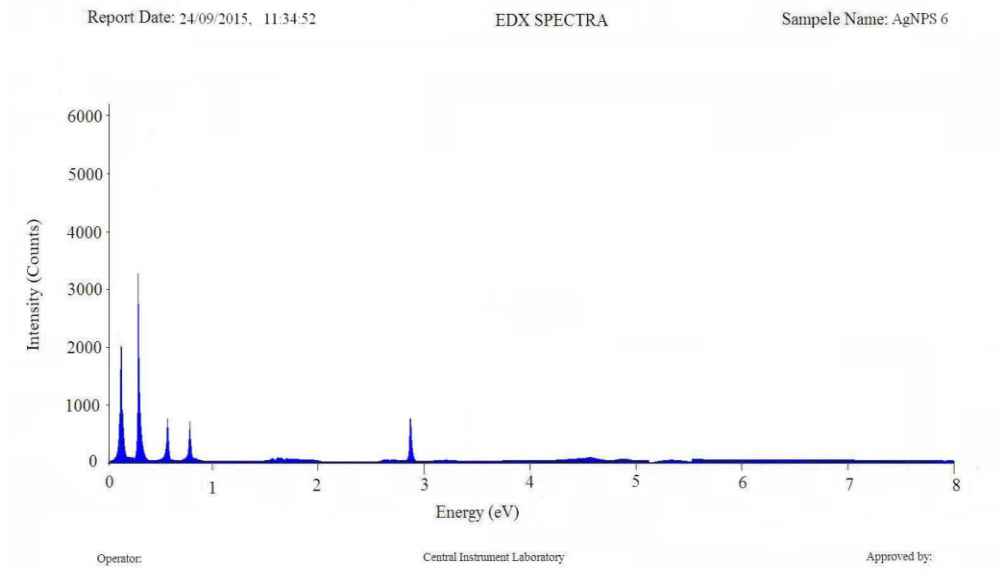
Electron microscopy analysis revealed the size and shape of silver nano-particles. The synthesized nano-particles were found to be cubic in shape and were in the form of clusters. The size nanoparticles with SEM image found less than 200nm. Individual particles are 20-125nm in size. Silver nanoparricles were synthesized from plant extract were assembled on to the surface due to the interactions such as hydrogen bond and electrostatic interactions between the bio-organic capping molecules bound to the Ag nanoparticles. The SEM results of the synthesized nanoparticles were given in figure 5.



Figures 5: SEM images for the synthesized nano-particles using *Withania coagulans*

3.1.5 Energy dispersive X-ray (EDX) spectra analysis

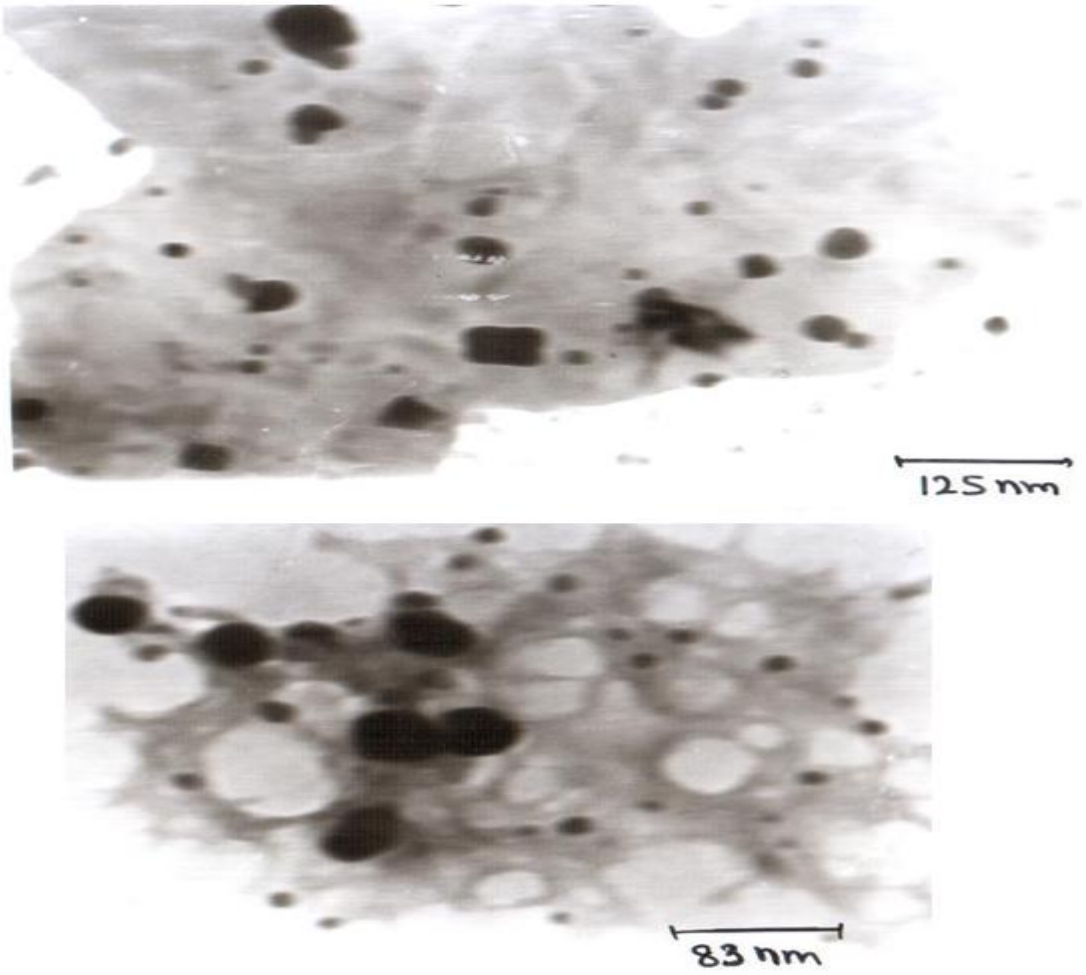
The elemental silver in the synthesized silver nano particles was confirmed by EDX analysis. Analysis through EDX spectrometers confirmed the presence of elemental silver signal at 2.89eV. Identification lines for the major emission energies for silver (Ag) are displayed and these correspond with peaks in the spectrum, thus giving confidence that silver has been significantly identified. In addition to silver, prominent signal for carbon, nitrogen and oxygen were also observed. The molecules in the plant extract were responsible for the observation of prominent signal for C, N and O. hence the particles formed in the synthesis were of silver and molecules present in the plant extract. The EDX spectra were given in figure 6



Figures 6: EDX images for the synthesized nano-particles using *Withania coagulans*

3.1.6 Transmission electron microscopy (*TEM*) analysis

The Stem + Root combination extract of *Withania coagulans* was used for the synthesis of silver nano particles. The particle size, shape and diameter of nano-particles synthesized using Stem + Root combination extract of *Withania coagulans* was determined using TEM micrograph analysis. A typical TEM image of biologically synthesized AgNPs suggests that the particles are uneven in shape. Some are spherical, rod, and triangular shaped particles with a varying size below the range of 20-125nm. A size of 20, 80 and 125 nano meters with different shapes mostly cubic in nature were observed for nano particles synthesized by using *Withania coagulans*. The TEM results of the synthesized nano-particles were given in figure 7.



Figures 7: TEM images for the synthesized nano-particles using *Withania coagulans*

3.2 Biological applications of synthesized silver nano particles:

3.2.1 Anti microbial Activity:

Silver nano particles were applied as a best anti microbial agent and shows growth resistance activity against different pathogenic micro organisms. For this the applicability of synthesized silver nano particle as anti microbial agents, the agar plate disc diffusion method was followed against different pathogenic

microorganisms.

The antibacterial activity of biosynthesized silver nanoparticles synthesized using *Withania coagulans* extract was studied against five bacterial *B Subtilis*, *E coli*, *P aeruginosa*, *S aureus*, and *B cereus* and two fungal strain *A niger* and *R oryzae* using the agar well diffusion assay, and the zone of inhibition was tabulated as shown in Table 1 and 2. The synthesized AgNPs displayed efficient antibacterial activity against both Gram-negative and Gram-positive bacteria. The silver nanoparticles synthesized by *Withania coagulans* extract showed the maximum zone of inhibition around 19.9 mm for *Bacillus Subtilis*, which were followed by *Bacillus cereus* (18.8mm), *Escherichia coli* (18.6mm), *Staphylococcus aureus* (12.6mm) and *Pseudomonas aeruginosa* (12.2mm) at a synthesized nano particle concentration of 1000µg/ml. The two fungi studied high zone of inhibition was observed for *A niger* (15.1mm). On the other hand, the negative control (distilled water) did not exhibit any zone of inhibition and the crude extract shows very less zone of inhibition.

S No	Name of the Organism	Zone inhibition observed for concentration (µg/ml) of AgNPs					
		1000	500	250	100	10	1
1	*Bacillus Subtilis*	19.9	15.7	11.6	8.5	5.6	---
2	*Escherichia coli*	18.6	15.1	10.9	8.3	5.1	---
3	*Pseudomonas aeruginosa*	12.2	9.1	7.6	5.1	2.9	---
4	*Staphylococcus aureus*	12.6	9.5	8.1	5.3	3.2	---
5	*Bacillus cereus*	18.8	15.5	11.3	8.2	5.4	---

Table 1: Anti Bacterial zone inhibition activity results of synthesized silver nano particles

S No	Name of the Organism	Zone inhibition observed for concentration (µg/ml) of AgNPs					
		1000	500	250	100	10	1
1	*Aspergillus niger*	15.1	12.7	9.6	6.4	3.2	---
2	*Rhizopus oryzae*	12.5	9.4	6.7	4.1	---	---

Table 2: Anti Fungal zone inhibition activity results of synthesized silver nano particles

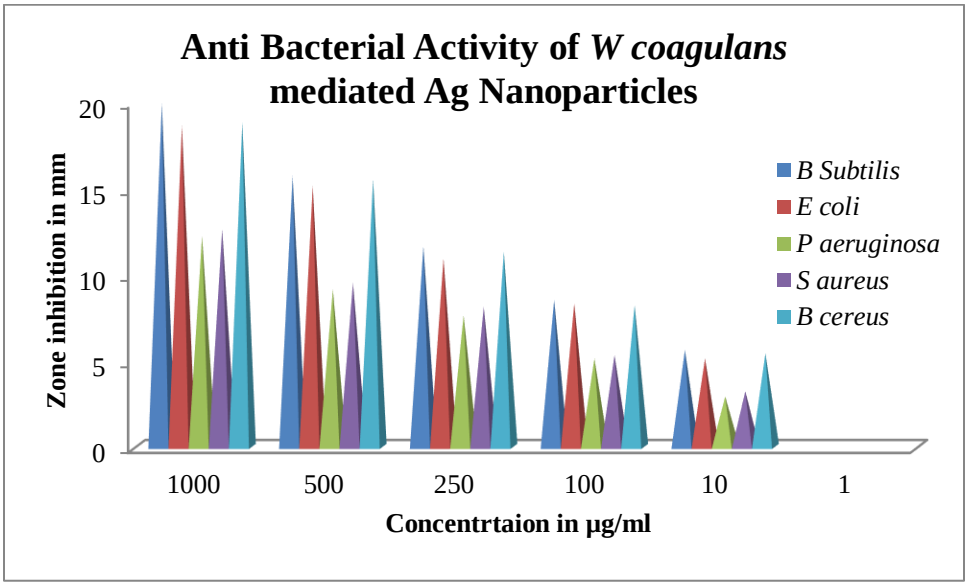


Figure 8: Comparative anti bacterial activity of synthesized AgNPs

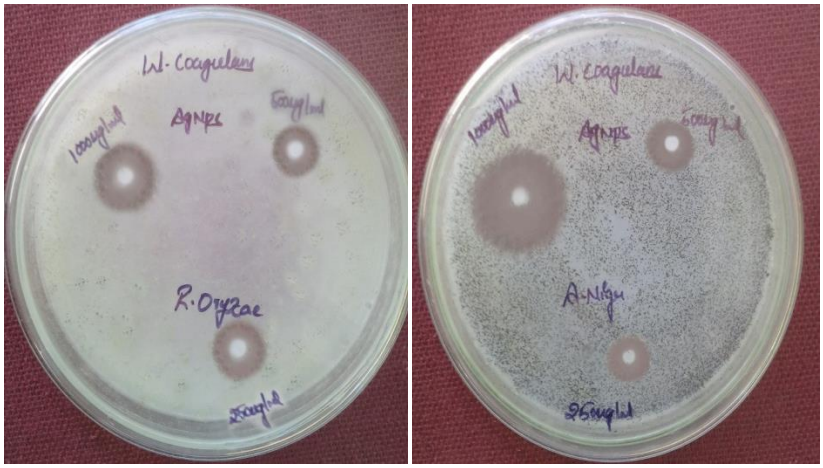


Figure 9: anti fungal activity results of synthesized silver nano partilces

3.2.2 Anti-Oxidant activity by DPPH method:

The antioxidant activity of the aqueous extract, Ascorbic acid and bio-conjugated AgNPs was evaluated using DPPH scavenging assay. The IC_{50} values of extract, standard and synthesized AgNPs are reported in Table 3. It can be inferred from the data that synthesized AgNPs displayed better antioxidant activity in comparison to crude extract. As can be seen from Table 3, there was a dose dependent increase in the percentage inhibition (% inhibition) of extracts and synthesized silver nanoparticles. DPPH free radical scavenging assay of the synthesized silver nanoparticle (AgNPs) when compared with the standard ascorbic acid showed promising activity. It was found that at a concentration of 50-100µg/mL, the bio-conjugated AgNPs exhibited better free radical scavenging activity with reference to drug ascorbic acid. These results suggest that at concentrations of 100µg/mL, the synthesized AgNPs may serve as potent radical scavengers. All these results suggest that intercalation of bio extracts with silver nanoparticles can serve as promising antiradical agents.

S No	Concentration in µg/ml	% DPPH inhibition		
		Ascorbic acid	Crude Extract	AgNPs
1	5	13.26203	0.427807	2.780749
2	10	21.39037	1.069519	9.625668
3	15	36.25668	4.919786	13.26203
4	20	45.34759	12.94118	24.49198
5	25	52.72727	18.18182	34.33155
6	30	60.53476	23.74332	40.85561
7	50	69.94652	43.85027	53.47594
8	100	86.63102	54.97326	67.59358
9	150	91.3369	65.88235	77.64706
10	200	93.79679	85.45455	90.58824
11	250	97.54011	89.83957	95.40107

Table 3: Results of anti oxidant activity (DPPH method) of synthesized silver nano particles

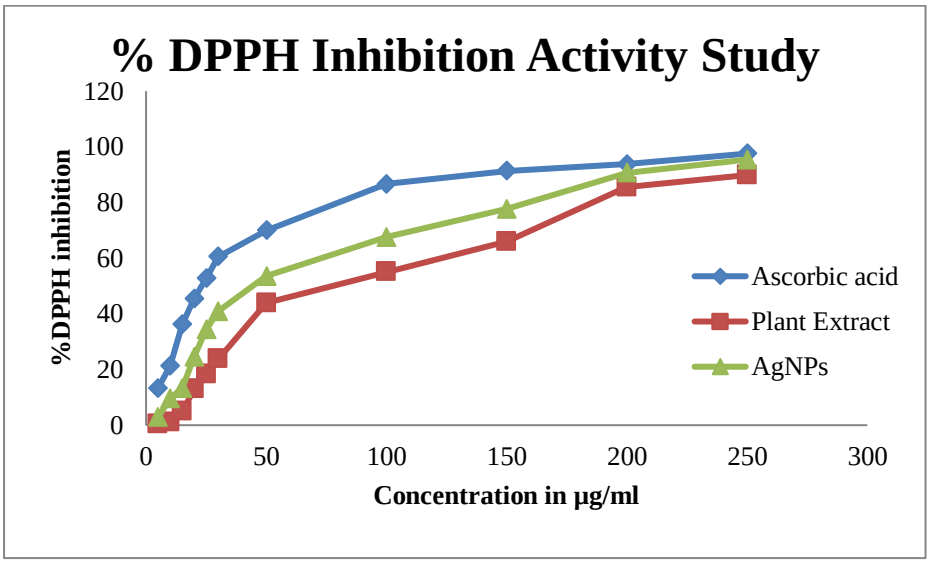


Figure 10: Graphical representation of anti oxidant activity (DPPH method)

4. Conclusion:

The bio-reduction of aqueous silver ions by the leaf extract of the *Withania coagulans* has been demonstrated. The reductions of the metal ions through leaf extract leading to the formation of synthesized nanoparticles are quite stable in solution. The control of shape and size of silver nanoparticles seems to be easy with the use of plant extracts. In the present study we found that Stem and Root extract can be good source for synthesis of silver nanoparticle. The synthetic methods based on naturally occurring biomaterials provide an alternative means for obtaining the nanoparticles. Use of plants in synthesis of nanoparticles is quite novel leading to truly 'green chemistry' route. This green chemistry approach towards the synthesis of nanoparticles has many advantages such as, process scaling up, economic viability and safe way to produce nanoparticles.

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