ISSN: 0975-8585

Research Journal of Pharmaceutical, Biological and Chemical Sciences

Phytogenic Silver Nanoparticle Synthesis with Potential Antibacterial Activity and Dye Degrading Ability

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ABSTRACT

In light of the growing use of nanoparticles in various fields, their synthesis method plays a crucial role. Existing physical and chemical methods poses certain disadvantages in terms of toxic chemicals and harsh conditions employed. Biological means of synthesis is offering safe alternative in terms of its environment suitability. The main aim of this study is to fabricate silver nanoparticles using the pods of *Phaseolus vulgaris* under benign conditions. The antibacterial activity of the phytonanoparticles is evaluated against the pathogenic strains *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853 and *Staphylococcus aureus* ATCC 25923, *Salmonella typhi* ATCC 6539 and *Klebsiella pneumoniae* NCIM 2883. Their potential to degrade the dyes Congo red and Mordant black 17 was also checked. Plant mediated silver nanoparticles of size in the range of 20 – 80 nm were successfully synthesized. They were found to possess significant antibacterial activity and the MIC was determined to be 1.08µg/ml. On the other hand, the silver nanoparticles were able to decolorize Congo red dye up to 50% while Mordant Black 17 was minimally decolorized which may be due to their complex structure.

Keywords: Plants; Phytonanoparticles; SEM; TEM; Antibacterial activity; Dye degradation.

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ISSN: 0975-8585

INTRODUCTION

Nanobiotechnology was born as a hybrid discipline, a combination of biotechnology and nanoscience. In recent years, nanoparticles with sizes typically below 100 nm have been applied in several fields of bioscience and biomedicine, with an increasing number of commercial applications. Some of the physical properties exhibited by nanomaterials are due to large surface atom, large surface energy and spatial confinement and reduced imperfections. Currently there are several methods for the production of nanoparticles like chemical and physical methods. But there are evidences regarding the harmfulness of these methods to the environment. [1] This is leading to a growing awareness for the need to develop clean, nontoxic and environmentally friendly procedures for synthesis and assembly of nanoparticles that are best suited to environment. Reinvestigating the manufacturing process and adopting a much benign route to synthesis is the order of the day. Nature itself provides the answer in terms of the diverse species it houses from plants to microbes. [2, 3, 4] There are certain evidences that authenticate the use of microbes ranging from fungi to bacteria [5] and plants in the synthesis of nanoparticles. Exploiting plants in the synthesis of nanoparticles has an edge over microbes as this eliminates the elaborate procedure of maintaining cell cultures. [1] It can also be suitably scaled up for large-scale synthesis of nanoparticles.

The first report of plants synthesizing nanoparticles appeared in 2002 when it was shown that gold nanoparticles, ranging in size from 2 to 20 nm, could form inside alfalfa seedlings. [6] Subsequently it was shown that alfalfa also could form silver nanoparticles (AgNPs) when exposed to a silver rich solid medium. [7] Several plants have been successfully used later on for efficient and rapid extracellular synthesis of silver and gold nanoparticles. Leaf extracts of geranium (*Pelargonium graveolens*), [8] lemongrass (*Cymbopogon flexuosus*), [9] *Cinnamommum campho,* [10] neem (*Azadirachta indica*), [11] *Aloe vera,* [12] tamarind (*Tamarindus indica*) [13] and fruit extract of *Emblica officinalis* [14] have shown potential in reducing Au(III) ions to form gold nanoparticles Au(0) and silver nitrate to form silver nanoparticles Ag(0). This is followed by several other investigations employing different plants namely papaya, [15] soap nuts, [16] *Spinacia oleracea* and *Lactuca* sativa [17] and medicinal plants [18] to mention a few. Attempts to synthesize nanoparticles internally was also successful when alfalfa (*Medicago sativa*) [6], C*hilopsis linearis* [19] and *Sesbania* seedlings [20] showed synthesis of gold nanoparticles inside living plant parts.

In this study a legume (*Phaseolus vulgaris* - French beans pods) a less effective metal accumulator [21] which are commonly available was selected for the phytogenic synthesis of silver nanoparticles. Periodic monitoring of the pH was carried out to comprehend the condition of the medium during the reduction process. These nanoparticles were checked for their medical and environmental applications. Investigations were carried out to evaluate the antibacterial activity of the silver nanoparticles against certain pathogenic strains. A preliminary study was also conducted to find out the potential of nanoparticles as dye degraders.

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MATERIALS AND METHODS

Silver nitrate was obtained from Sigma–Aldrich Chemicals. All glasswares have been washed with lavolene and distilled water and dried in oven before use. Fresh pods of beans have been collected from local markets, Chennai, India.

Preparation of the Extracts and Synthesis of Silver Nanoparticles

The pods of *Phaseolus vulgaris* were washed several times with ultrapure water to remove the dust. The extracts used for the synthesis was prepared from 20 g of thoroughly washed mass in a 500mL Erlenmayer flask and boiled in 250 mL ultrapure water for 20 min. 60 mL aqueous solution of 1 mM of silver nitrate was reduced using 2.5 mL of the extract at room temperature resulting in reddish brown color after the bioreduction.

Characterization Techniques

Synthesis of AgNPs by reducing respective metal ion solution with leaves extract may be easily observed by UV–vis spectroscopy. The absorption spectra of the synthesized nanoparticles were measured using a Perkin-Elmer Lamda-45 spectrophotometer in 300–1000nm range. Detailed analysis of the morphology, size and distribution of the nanoparticles was documented by various instrumental analyses like Scanning Electron Microscopy using Hitachi S-4500 SEM machine and Transmission Electron Microscopy using a TEM, JEM- 1200EX, JEOL Ltd., Japan. The possible phytochemicals involved in the synthesis and stabilization of nanoparticles was identified by performing FTIR analysis.

Antimicrobial Studies

The potential of silver nanoparticles as effective antimicrobial agents is well known. Antibacterial activity of the AgNPs synthesized was evaluated against *Escherichia coli* ATCC 25922*, Pseudomonas aeruginosa* ATCC 27853 and *Staphylococcus aureus* ATCC 25923, *Salmonella typhi* ATCC 6539 and *Klebsiella pneumoniae* NCIM 2883 by agar well diffusion method. [22] The minimum inhibitory concentration was determined by standard broth dilution method. Bacterial strains were grown overnight on MHA plates at 37°C before being used. MIC was determined using the initial bacterial inoculums of 2×108 CFU/ml and the time and temperature of incubation being 24 h at 37°C, respectively with different concentrations of the AgNPs (0.36, 0.72, 1.08, 1.44, 1.8, 3.6 µg in 10, 20, 30, 40, 50 and 100 µl respectively). The MIC is the lowest concentration of silver nanoparticles that completely visually inhibits 99% growth of the microorganisms.

Study on Dye Degradation

The synthesized silver nanoparticles were checked for their capability to degrade two dyes namely Mordant black 17 and Congo red. Decolourization of the dyes (1 ppm) was carried out with two concentrations (10 µl and 25 µl) of the AgNPs to study the possibility of dye

ISSN: 0975-8585

degradation which is reported as percentage reduction of the dye and calculated using the formula

% Decolorisation =A - B/A * 100

Where A is initial absorbance and B is the final absorbance.

RESULTS

Biogenic silver nanoparticle synthesis is on the rise especially plant mediated. The pods of *Phaseolus vulgaris* (French beans) have been used to successfully achieve the silver nanoparticle synthesis. The potential of phytochemicals as synthesizers of silver nanoparticles is reflected in the results obtained. The aqueous extract of *Phaseolus vulgaris* pods when challenged with AgNO3 showed a rapid change in the colour of the solution from pale white to reddish brown indicating the formation of silver nanoparticles (Figure 1). [23]

Figure 1. Formation of Silver Nanoparticles by the Aqueous Extracts of *Phaseolus vulgaris* Indicated by the Colour Change from Pale White to Brown.

Characterization of Biogenic Silver Nanoparticles

The preliminary detection of silver nanoparticles due to the reduction of Ag ions in the solution with the ingredients present in the aqueous extracts was observed by the UV-Vis spectroscopy. The UV-vis spectra of the AgNPs synthesized by *Phaseolus vulgaris* (Figure 2) displayed a characteristic absorption peaks at 435 nm, specific to silver nanoparticles with less absorbance that signifies smaller size of nanoparticles [24].

ISSN: 0975-8585

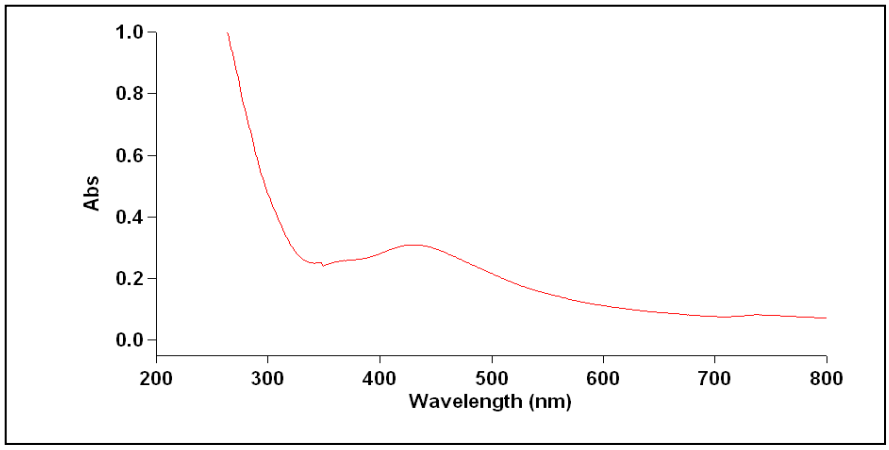

Figure 2. UV/Vis Spectra Recorded for AgNPs Synthesized Using *Phaseolus vulgaris* Showing the Characteristic Peak

Change in pH

Periodic monitoring of the pH was carried out at 30 min interval for 7 hrs to verify the change during the formation of nanoparticles (Table 1). The initial pH observed for the *Phaseolus vulgaris* was observed to be 5.6. It has been noticed that the bioreduction of silver was carried out in acidic pH as there was not much variation during the synthesis process. This result is supported by an earlier study by Ranjan et al. who reported at that lower pH, polydispersity of the nanoparticles were reflected in the UV- vis peaks. [25]

Table 1. Changes in pH of the Aqueous Extract of *Phaseolus vulgaris* During the Bioreduction

Sample	Hrs	pH
Plain extract	0	5.6
Extract challenged with $AgNO_3$	0.5	5.94
	1	5.97
	1.5	5.96
	2	5.96
	2.5	5.96
	3	5.98
	3.5	6.17
	4	6.21
	4.5	5.7
	5	5.65
	5.5	5.63
	6	5.63
	6.5	5.62
	7	5.62

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Instrumental Analyses

A further insight regarding the features of the silver nanoparticles was provided by the various instrumental analyses. The most popularly used microscopic techniques to comprehend the morphology, size and distribution of the silver nanoparticles are the Scanning electron microscopy (SEM) and Transmission electron microscopy (TEM).

SEM and TEM characterization

SEM analysis was done by preparing thin films of the sample 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 film on the SEM grid were allowed to dry by putting it under a mercury lamp for 5 min.

SEM micrographs of the silver nanoparticles synthesized by *Phaseolus vulgaris* (Figure 3) bared a relatively spherical shaped, dispersed silver nanoparticles. Comprehensible information was obtained by the TEM micrographs documented using the nanoparticle suspensions by placing the samples on the carbon coated copper grid making a thin film and removing the extra samples using the cone of a blotting paper. The TEM micrographs so obtained are given in Figure 4.

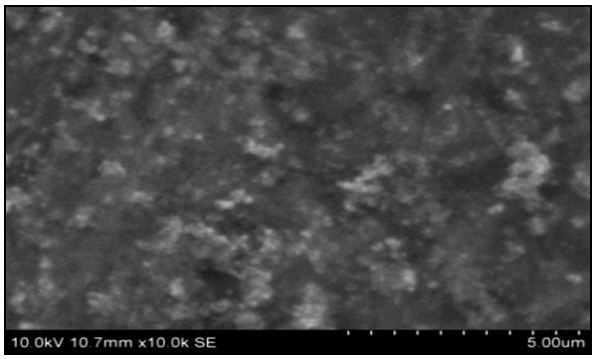

Figure 3. SEM Micrograph of the Silver Nanoparticles Synthesized by *Phaseolus vulgaris*

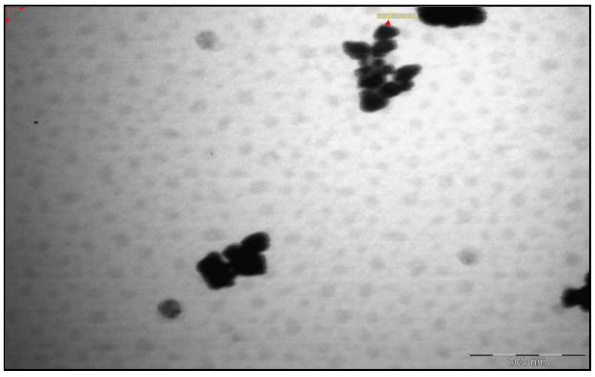

Figure 4. TEM Micrograph of the Silver Nanoparticles Synthesized by *Phaseolus vulgaris*

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The nanoparticles synthesized by *Phaseolus vulgaris* were found to be relatively spherically shaped with the size in the range of 30-50 nm. These AgNPs were found to be fairly clustered or grouped with a few of them observed to be dispersed.

FTIR Analysis

Currently, the mechanism behind the biological synthesis is not fully understood. But an attempt is made to possibly understand about the nature of phytocompounds that may be involved in the synthesis of nanoparticles. FTIR analysis was performed using FTIR Nicolet Avatar 660 (Nicolet, USA) to obtain the spectra for the plain aqueous extract and the extract with AgNPs (Figure 5).

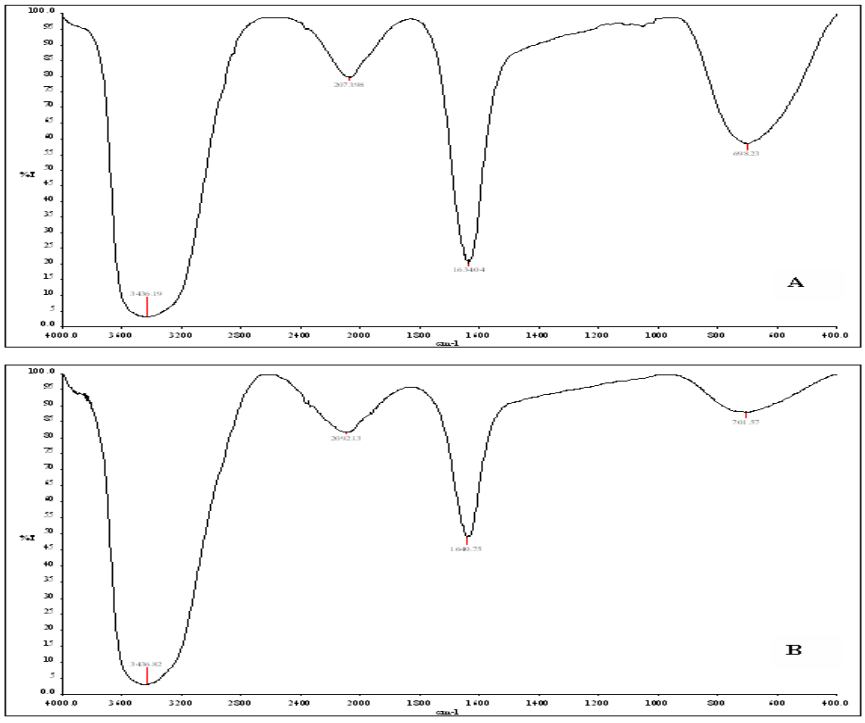

Figure 5. FTIR spectra A) Aqueous Extract of *Phaseolus vulgaris* B) Aqueous Extract of *Phaseolus vulgaris* Challenged with $AgNO_3$

The bands observed in the extracts of *Phaseolus vulgaris* before and after bioreduction showed similar peaks except for a variation in the size of two peaks (Figure 5A and 5B). The band at 3436.19 cm-1 and 3436.82 cm-1 and corresponds to O-H stretching H-bonded alcohols and phenols displaying the characteristic absorption of hydroxyl groups in the control as well as the sample. The peaks at 2073.98 cm-1 and 2092.13 cm-1 corresponds to aromatic C-O stretching bonds of polyol group. The band at 1634.04 cm-1 and 1640.75 cm-1 is assigned to C-H out of plane bending vibrations substituted ethylene systems CH=CH (cis). Visible variation in was observed at 698.23 cm-1 that showed a reduction in the peak area after bioreduction and this corresponded to C-H bend of alkynes.

ISSN: 0975-8585

Antibacterial Activity

The antibacterial activity of the phytonanoparticles was evaluated against a few pathogenic bacteria that were mentioned above. The AgNPs synthesized were displaying significant antibacterial activity that was observed by the zones of inhibitions produced. (Figure 6). The minimum inhibitory concentration of the silver nanoparticles was determined to be 0.72 µg for *Pseudomonas aueroginosa, Staphylococcus aureus* and *Salmonella typhi* and 1.08 µg for *Escherichia coli* and *Klebsiella pneumoniae*.

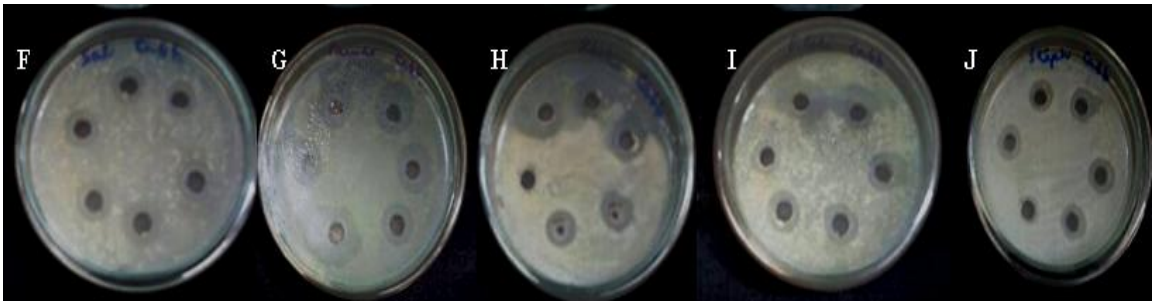

Figure 6. Antibacterial actIvity of the "Phytonanoparticles" Synthesized by *Phaseolus vulgaris* Observed through the Zones of Inhibition Against the Pathogens.
F- *Salmonella typhi*; G- *Pseudomonas aeruginosa*.
H- *Klebsiella pneumoniae;* I- *Escherichia coli;* J- *Staphylococcus aureus*

Dye Degradation

Dye degrading ability of AgNPs was studied with two dyes namely Mordant black 17 and Congo red. The silver nanoparticles of two concentrations (0.9 µg and 1.8 µg in 10 and 25 µl respectively) were used to degrade the dyes at 1 ppm. Significant decolourisation was observed for the two dyes which were initially observed by the color change (data not shown).

It has been observed in Table 2 that substantial decolourization was observed for Congo red by the AgNPs synthesized. There was a reduction of about 50 % of Congored within 24 hours of incubation by the AgNPs. But the reduction of Mordant black 17 was not very considerable which may be attributed to the azo bond and complexity in the structure of the dye. Similar results were reported by Nithya where the silver nanoparticles from *Pleurotus sajor caju* were able to bring about extensive degradation of Congored. [26]

Table 2. Percentage of Decolourisation of the Dyes with the AgNPs.

Ag-Nps	Conc. of AgNPs (µl)	% Decolourisation of Dye (1ppm)	
		Congo red	Mordant black
Phaseolus vulgaris mediated Ag-Nps	10	50	1
	25	55	5

ISSN: 0975-8585

DISCUSSION

Currently nanoparticles seem to play an indispensable role in drug delivery, diagnostics, imaging, sensing, gene delivery, artificial implants and tissue engineering. [26] In spite of their minute structure, their ability to trigger the chemical activity owing to their distinctive crystallographic nature that increases surface area makes their investigation supreme. Globally, more prominence is given to the biological syntheses techniques as these methods are nontoxic and ecofriendly. Exploring the ecosystem, which is an abode for various life forms ranging from microbes to plants, is currently in progress. In recent times, plant-mediated synthesis of nanoparticles is gaining importance due to its simplicity, rapidity and environment suitability. Ease of access to the plant material offers an inherent advantage in this method of synthesis.

It was earlier reported that smaller quantities of the plant material would suffice for the rapid synthesis of nanoparticles. [15] In the present study only 20 gms of the plant material which are effortlessly available were used to prepare the extracts for the bioreduction of silver that was primarily denoted by the color change as shown in Figure 1. It is well known that silver nanoparticles exhibit a yellowish-brown color in aqueous solution due to excitation of surface plasmon vibrations in silver nanoparticles. [1] The metal with free electrons (Ag) possesses plasmon resonances in the visible spectrum due to the combined vibration of these electrons in resonance with light wave [28] which gives rise to such intense colour i.e. golden yellowish colour. [29] The widely used technique to confirm the formation of nanoparticles is the UV- vis spectroscopy. The peak height of the spectra reflects the concentration of the nanoparticles. Broadening of peak signifies the dispersion of the silver nanoparticles. An increase in the absorbance capacity also showed that the size of the nanopartilces could be large. The smaller size of the *Phaseolus vulgaris*-AgNPs is well reproduced in the clear sharp peak observed in the spectra. (Figure 2)

The size and shape of the nanoparticles forms a basis for the wide variety of applications. The size of the plant mediated AgNPs were observed to be in the range of 25 – 55 nm with a spherical shape that were found to be dispersed. Nanoparticles have a large surface area compared with the total volume. The surface area to volume ratio is interesting because chemical reactions typically occurs on surfaces, so nanoparticles that have a high surface to energy ratio can be used in many interesting ways such as in catalysis. [28]

The ability of plants to act as chemical factories for synthesizing nanoparticles is mainly attributed to its various components like proteins [30] and polyphenols such as flavonoids. [31] The FTIR spectra of *Phaseolus vulgaris* (Figure 5A and B) that showed characteristic bands corresponding to certain phytochemicals like polyols reveals that these could have possibly been involved in the AgNP synthesis. The presence of alkaloids, cardiac glycoside, flavonoids, saponin, tannin and terpenoid has been earlier reported. [32] Similar results have been obtained earlier by Kavitha that disclosed that certain phytochemicals could be playing a major role in the overall reduction process. [33] The exact mechanism of the formation of these

nanoparticles in these biological media is still unknown. However, it was suggested that the electron released during Glycolysis (photosynthesis) for conversion of NAD to NADH would have led to the transformation of AgNO3 to form nanoparticles. Also, the release of an electron during the formation of ascorbate radicals from ascorbate could reduce the silver ions. [34]

Studies conducted by researchers in the recent past revealed that metal oxide nanoparticle formulations possessed significant antibacterial activity [35] and nanoparticles based antimicrobial formulations could be effective bactericidal materials. [36, 37] Utilizing silver in treatment of various infections has been in existence for a long time. The revelation that silver and its compounds are non-toxic to humans in lower concentrations and lethal to microorganisms [38] made it suitable for numerous applications ranging from household products to medical devices. The present study also reports significant activity of phytonanoparticles against the selected pathogenic strains and the minimum amount of AgNPs required was less to bring about the inhibition of the growth of the strains. Though the exact mechanism of inhibition is still not deciphered, three possible types of antimicrobial mechanisms could be stated. Firstly, plasmolysis followed by separation of cytoplasm from bacterial cell wall as was observed in Gram negative bacteria and Gram positive bacteria. Secondly, inhibition of cell wall synthesis and lastly induction of metabolic disturbances in pathogenic bacteria. [39]

Azo dyes characterized by the presence of one or more azo groups (N=N) in the molecule, are poorly biodegradable because of their structure [40] and represent a potential important class of organic pollutants. Mordant Black 17, an azo dye exhibits higher toxic effect which may be due to the metallic ions in them that are used as mordants. [41] Congo red, a secondary diazo dye, is the sodium salt of 3, 3'-([1, 1'-biphenyl]-4, 4'-diyl) bis (4-aminonaphthalene-1-sulfonic acid) and has a strong, though apparently non-covalent affinity to cellulose fibers. However, the use of congo red in the cellulose industries has long been abandoned, primarily because of its tendency to change color when touched by sweaty fingers, to run, and because of its toxicity. It may act as a potential mutagen for somatic cell lines, bacteria and yeast. Congo red is a benzidine-based dye and benzidine has been classified by IARC as Group 1 carcinogen. It is a recalcitrant and a known carcinogen. [42]

Microorganisms are frequently the sole means, biological or nonbiological, of converting synthetic chemicals to inorganic products. They harbor an amazing physiological versatility and catabolic potential for the breakdown of an enormous number of organic molecules. [43] Of late the potential of silver nanoparticles in degrading the dyes is being evaluated. The results obtained in this study reveal significant reduction (50 %) of Congo red [26] while there was very minimal reduction of Mordant Black. An earlier study has shown that silver-deposited anatase-titania nanotubes are more effective in enhancing the kinetics of the dye-removal via surface-adsorption and photocatalytic degradation mechanisms relative to the palladium-deposited anatase-titania nanotubes, which has been attributed to the differences in the surface-chemistry of anatase-titania nanotubes induced by the respective metal-deposition. [44]

ISSN: 0975-8585

CONCLUSION

Biological synthesis of silver nanopartilces is attracting attention as they do not involve the harsh conditions that are required in the chemical and physical synthesis methods. Phytofabrication of silver nanoparticles seems to be a better option than that of microbes as the latter needs appropriate conditions for survival. The present investigation reports the formation of silver nanoparticles with the size in the range of 20-60 nm using the most commonly available plant *Phaseolus vulgaris*. These AgNPs were found to be relatively spherical and showed little dispersion. Further these phytonanoparticles were found to possess significant antibacterial activity against some pathogenic strains used. These AgNPs when tested for their ability to decolourise the dyes like Mordant Black 17 and Congo red showed considerable decolourisation of the latter than the former which may be because of its structural complexity. Plants or their extracts can be efficiently used in the synthesis of silver nanoparticles as a greener route. Control over the shape and size of nanoparticles seems to be very easy with the use of plants. Though the mechanism of nanoparticle synthesis by plants is not yet fully understood the participation of phenols, proteins and reducing agents in their synthesis seems to be the most suitable answer. Achievement of such a green synthesis of nanoparticles, contributes to a raise in the efficiency of synthetic procedures using environmentally benign natural resources. Furthermore the low cost of the method as well as its simplicity and efficiency offers an alternative to chemical procedures.

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