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Green synthesis of stable silver nanoparticles using *Euphorbia milii* extract and study of its antimicrobial activity against *Escherichia coli*

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Abstract
Single step green synthesis of stable silver nanoparticles from silver nitrate solution has been successfully carried out with the help of natural reducing agent from the extract of *Euphorbia milii* plant by simple heating at 90^0 C. The nanoparticles were characterized using UV-visible spectroscopy and transmission electron microscopy. The diameters of the almost spherical nanoparticles were found in the range of 6-50 nm with average particle size of nearly 23 nm. Zone inhibition test for the study of antimicrobial activity of silver nanoparticles was conducted against the strain of *E.coli* pathogen and significant inhibition of the bacterial strain was observed.

Keywords: Silver nanoparticles; *Euphorbia milii*; Antimicrobial; *E. coli*.

1. Introduction
Metal Nanoparticles find widespread applications in the fields such as photonics [1-3], micro-electronics [4-6], catalysis [7-10], medicine [11, 12] *etc*. and hence proliferated research works on them are being witnessed in recent times. On account of special property of the nanoparticles with high surface area to volume ratio, they fit into multifaceted applications. Silver nanoparticles (AgNP) amongst the metallic nanoparticles owe its importance mainly due to its potent antimicrobial activity against varieties of pathogenic microorganisms [13-17]. In general, different physical and chemical processes are employed to synthesize AgNPs [18-24]. However, these processes involve use of high pressure, energy, temperature and toxic chemicals that compelled the scientific community to look for 'green synthesis' processes. Plant extracts with broad varieties of phytochemicals aiding reduction of silver ions, give an alternate way of green process of AgNP synthesis [25, 26]. The water soluble phytochemicals like flavones, terpenoids, organic acids, quinones etc. are responsible for efficient reduction of silver ions (Ag^+) to neutral silver atoms (Ag^0) that nucleate in small clusters forming nanoparticles [25]. The major advantage of using plant extracts for AgNP synthesis is that they are easily available, safe and nontoxic in most cases. There are significant numbers of reports in the literature on synthesis of AgNPs using extract of different plants [27-31].
In our study, the reducing agent comes from extracts of *Euphorbia milii*, which is a spiny ornamental plant of *Euphorbiacea* family, popularly known as *Crown of Thorns* or *Christ Plant*. *Euphorbia milii* plays a role in folk medicine. *Euphorbia milii* latex has been used as herbal medicine and proved to be a potent plant molluscicide [32]. Phytochemical studies of methanolic extract of *Euphorbia milli* revealed the presence of β-sitosterol, cycloartenol, β-amyrin acetate, lupeol, euphol and flavonoids [33, 34]. These polyphenolic compounds with numerous –OH groups facilitates the reduction of silver ion (Ag^+) thereby giving neutral silver atom (Ag^0). R.A. de Matos *et al.* reported the synthesis of AgNPs and significant particle size reduction by irradiation of light from Xenon lamp onto silver nitrate and *Euphorbia milii* latex solution followed by application of ultrashort laser pulses [35]. However, no report on synthesis of AgNPs using *Euphorbia milii* extract by simple means of reaction at optimized temperature without sophisticated physical processes, has been found.
In this paper, the results of green synthesis of AgNPs by heating using the extract from the plant of *Euphorbia milii* as reducing agent in aqueous silver nitrate solution have been reported. The resulting nanoparticles were characterized by transmission electron microscope (TEM) and ultraviolet-visible (UV) spectroscopy. The resulting nanoparticles were also tested for its antimicrobial activity against the pathogen strain of *Escherichia Coli* (*E. coli*).

2. Materials and methods

2.1 Materials

Silver nitrate (99%, Merck), soluble starch (Merck) analytical grade reagents were purchased and used as received. Eosin methylene blue (EMB) agar (Sigma-Aldrich) was also used without further purification. Double distilled water was used in preparation of all the solutions. Antimicrobial activity of AgNPs was tested against *Escherichia coli,* (MTCC code 1687). Amoxicillin 500 gm capsule (Rizmox 500mg, Ritz Pharma, India) was also collected from local medical store for use in negative control plate.

2.2 Experimental

2.2.1 *Preparation of Euphorbia milii* plant *extract*

Fresh stems of *Euphorbia milli* were collected from NERIST, Nirjuli campus, and shaved off thorns and skin followed by removing central hard portion of the plant that do not contain latex. The delaminated fleshes of the plant were thoroughly washed with distilled water and chopped into very small pieces up to the weight of 15 grams. The chopped pieces were put into 50 ml of de-ionized water and subjected to centrifuge for 30 min. at room temperature at a speed of 2000 rpm. The supernatant was separated and filtered with filter paper with the help of vacuum filter. The filtered solution was collected to use for the reduction of silver ions (Ag^+) to AgNPs (Ag^0).

2.2.2 *Synthesis of AgNPs*

2.5 ml of *Euphorbia milii* extract was added to 1 ml of aqueous $AgNO_3$ solution (0.1 M) in a conical flask (Cap. 100 ml) followed by addition of 50 ml aqueous 2% soluble starch solution. Shaking or swirling of the solution mixture is strictly avoided during the addition of each ingredient. The slightly greenish mixture solution was placed in preset hot air oven at 90 ^{0}C. The solution was kept in oven for 2.5 h considering the color changed to yellowish. At the interval of every 30 minutes, a small amount of the solution was taken out using dropper and its UV-visible spectrum was recorded to verify the absorption maxima.

2.3 Microbiological Experimentation

E. coli (MTCC code 1687) was selected as indicators of faecal contamination of water. EMB agar was used as the growing medium. Bacteria were grown aerobically in EMB agar at 37 ^{0}C for 24 h. The cultures were centrifuged and the cells were washed and suspended in distilled water.

2.3.1 Zone of Inhibition Test

For zone of inhibition test, culture media was prepared by adding 3.6 grams of EMB agar in 100 ml of distilled water and autoclaved at 15 psi for 15 minutes followed by cooling of the media to 45 ^{o}C-50 ^{o}C. The media was poured in three Petri discs and allowed to solidify in a laminar flow table for about 24 hours. After solidification, three bores were made on each media using a borer and *E. coli* strain was spread on the media using spreader. Of three plates, first plate was left blank without adding any inhibitor. This plate is called positive control plate. In the second plate, AgNP sample was poured into the bores, which is the sample plate. In the third plate, an antibiotic amoxicillin solution was poured into the bores. Amoxicillin solution is prepared by adding 1 mg of amoxicillin powder in 1 ml of ultra pure water in a centrifuge vial. This plate is called negative control plate. All the three plates were then incubated at 37 ^{0}C for 24 h in bacteriological incubator [36].

2.4 Characterization

UV-visible spectra were recorded with Shimadzu UV-2550 spectrophotometer in the range of 350 – 600 nm. The analyses were performed at room temperature using quartz cuvettes (1 cm optical length with the blank considering corresponding *Euphorbia milii* extract.

JEM-2100, 200 kV, Jeol model of Transmission Electron Microscope was used to take the microghaphs of synthesized nanoparticles to study its size and morphology. The samples were prepared by putting few drops of dispersed solution on carbon coated copper grids and the excess liquid was allowed to slowly evaporate prior to imaging of the sample.

3. Results and discussion

3.1 Synthesis of Silver Nanaoparticles

Stable dispersion of AgNPs was attempted to synthesize with 1 ml of 0.1M $AgNO_3$, 2.5 ml *Euphorbia milii* extract and 50 ml of 0.2% soluble starch at 90^{o}C allowing the reaction time to be 2 h and 2.5 h as optimized. The progress in reaction was observed by viewing color change from slight greenish to yellowish (Figure 1) during the course of reaction. *Euphorbia milii* extract was used as reducing agent to promote the reduction of silver ions to neutral silver atoms. Use of natural reducing agent, soluble starch as natural stabilizer, and universal solvent i.e. water to prepare the stable colloidal AgNP dispersion are the reason for claiming this process to be green synthesis process. Soluble starch acts as stabilizer which inhibits the aggregation of AgNPs. Aggregation can be inhibited by the thick electrical double layers that form around metal nanoparticles in low ionic strength suspensions [37]. In our experimental work, silver colloidal dispersion found the most stable for the allowed reaction time of 2 h and 2.5 h. However, excess time of heating of reaction mixture may lead to increase in tendency of agglomeration of the already formed nanoparticles and thereby losing the stability. Attempts were also made to prepare AgNP using *Euphorbia milii* extract to reduce Ag^+ ions to neutral AgNPs with stirring but the color change obtained to yellowish is soon converted to dark solution indicating aggregation of silver particles.

Fig 1: Change of colours of the reaction mixture before and after the reaction.

3.2 UV-Visible Spectra Analysis

The UV-Vis spectrophotometer was used for determination of AgNPs formation measuring optical absorbance spectra. Different sized nanoparticles will absorb different wavelengths of light. A large variance in particle size will result in a wider absorbance peak in UV-Vis spectrum. The unique optical properties of metal nanoparticles originate from the collective oscillations of conduction electrons, which, when excited by electromagnetic (EM) radiation, are termed surface plasmon polariton resonances (SPPR). The electric field of the incident electromagnetic radiation displaces the particle's electrons from equilibrium and, in turn, produces a restoring force that results in oscillatory motion of the electrons with a characteristic frequency, that is, the SPPR frequency [37]. The characteristic surface plasmon resonance vibration peak generally recorded in UV -Vis spectrum for AgNPs is around 420 nm [23, 27, 37]. The change in colour while synthesizing the AgNPs to yellow or brownish is due to this surface Plasmon resonance phenomenon.

Significant absorbance maxima peak at 425 nm corresponding to surface plasmon resonance vibration have been observed for two samples prepared using the extract at 90 ^{0}C for 2 h and 2.5 h time duration (Figure 2). This observation may be considered as strong evidence in support of formation of AgNPs. The case was confirmed also by recording UV-Vis spectra for 0.1 M $AgNO_3$- 2% starch solution and *Euphorbia milii* extract-2% starch solution, where no characteristic peak around 420 nm was observed. Hence, it may be summarized that *Euphorbia milii* extract is acting as reducing agent to form stable AgNPs from silver ions. Gradual development of surface plasmon resonance peak in the range 425-435 nm was also monitored by recording the absorption spectra at regular 30 min. interval of time as depicted in Figure 2. At the initial time, no peak around 420 nm was visible whereas after 2 h and 2.5 h of reaction time absorbance peak at 425 nm was recorded. The stability of AgNP dispersion was verified by change of color on storing and recording surface plasmon resonance peak after certain interval of time. Agglomerations of nanoparticle will either red shift the absorption peak or disappearance of characteristic peak around 420 nm.

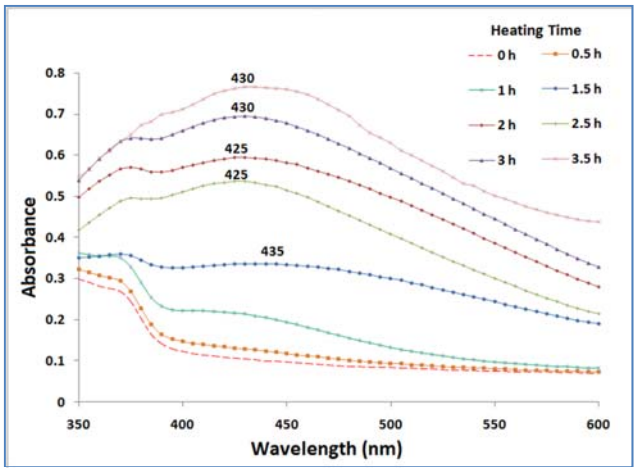


Fig 2: UV-visible spectra of the reaction mixture with respect to heating time.

3.3 Transmission Electron Microscopy (TEM)

The morphology of the AgNPs was studied by transmission electron microscopy (TEM). TEM micrographs (Figure 3 and Figure 4) were obtained for the two samples of AgNP dispersion (reaction time 2 h and 2.5 h, respectively) prepared using *Euphorbia milii* extract as reducing agent. TEM analysis clearly showed almost spherical well dispersed AgNPs with size ranging from 6 to 50 nm. It was observed in TEM images that the nanoparticles obtained after 2.5 h of reaction time are more uniform in dispersion and spherical than that obtained after 2 h.

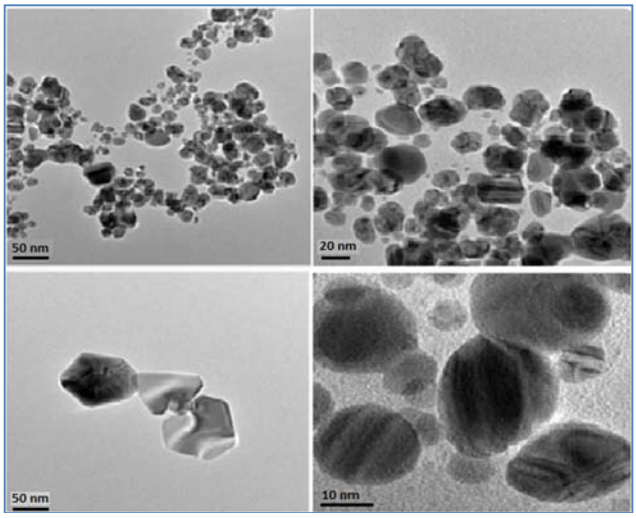


Fig 3: TEM images of AgNPs after the reaction time of 2 h.

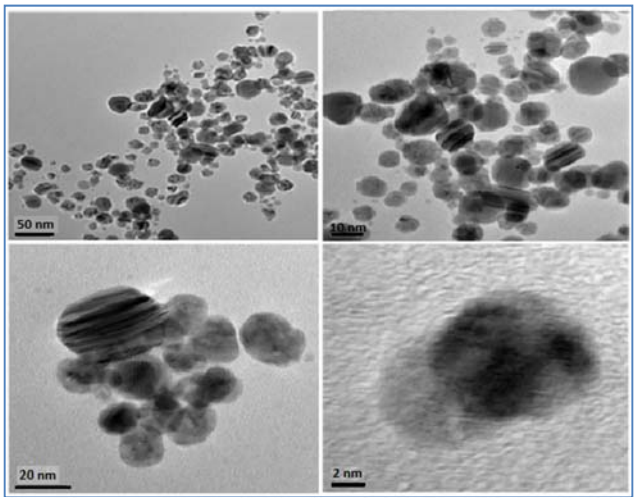
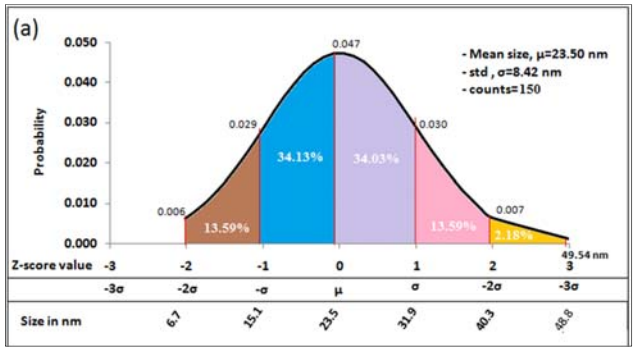


Fig 4: TEM images of AgNPs after the reaction time of 2.5 h.

Normal size distribution curve for the dispersed nanoparticles based on TEM images have been drawn for the samples with reaction time 2 h and 2.5 h respectively (Figure 5) considering the particle count to be 150. The sizes for the nanoparticles ranges 5.5-48 nm for 2.5 h reaction time sample and 6-50 nm for 2 h reaction time samples with average size 22.8 nm and 23.5 nm, respectively.



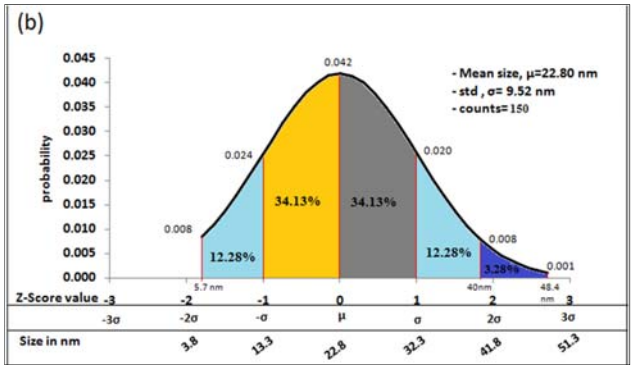


Fig 5: Normal distribution curve and average particle size of AgNPs (a) after the raction time of 2h, (b) after the reaction time of 2.5 h.

3.4 Microbiological Test Results

In this study, the application of AgNPs as an antimicrobial agent was investigated by growing *E.coli* on EMB agar plates supplemented with AgNPs, antibiotics and a blank as explained in experimental section. After 24 hours of incubation at 37 ^{0}C, it has been observed that the positive control plate is completely covered with *E.coli* colony. In the negative control plate, big rings around bores were observed. The area inside this ring shows inhibition zone of the antibiotic solution. In the sample plate, comparatively medium sized rings around the bores were observed. The observations are depicted in the Figure 6. The area inside this ring shows inhibition zone of the AgNPs sample to the growth of *E. coli.* However, the smaller rings around the bores of sample plate compared to negative control plate may be due to lesser concentration of AgNPs in the solution. If the concentration of the AgNPs in the sample is increased, bigger ring around the sample will form. The inhibition zone test against *E. coli* bacteria clearly depicted the antimicrobial effect of synthesized AgNPs against the bacterial strain. The mechanism of the inhibitory effects of AgNP on microorganisms is not completely clear, however, AgNPs interact with a wide range of molecular processes within microorganisms resulting in a range of effects from inhibition of growth, loss of infectivity to cell death which depends on shape. Therefore results in E. coli suggested AgNPs may damage the structure of bacterial cell membrane and depress the activity of some membranous enzymes, which cause *E. coli* bacteria to die eventually [15, 38].

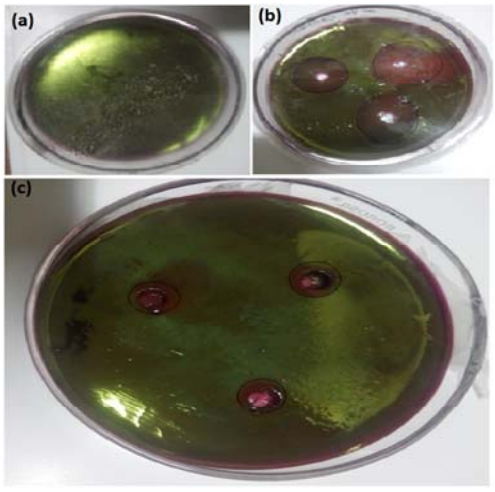


Fig 6: Zone of inhibition test result for *E. coli* (a) positive control plate (b) negative control plate, and (c) sample plate.

4. Conclusion

The synthesis of AgNPs using *Euphorbia milii* extract provides environmental friendly, simple and efficient route for synthesis of benign nanoparticles. The results are very promising since the extract promotes the formation of nanoparticles in a single step of simple heating and no harmful chemicals. The synthesized AgNPs were characterized using UV-Visible spectrophotometer and TEM. The corresponding surface plasmon resonance absorption peak has been found at 425 nm. The diameter of the AgNPs were found in the range of 6-50 nm with average particle size nearly 23 nm, as found in TEM experiment. The particles are generally observed to be spherical with distortion in a few cases. Antimicrobial activity against *E. coli* bacteria species was also confirmed conducting zone inhibition test. Precise applications of nanoparticles demands synthesis techniques capable of producing highly crystalline metal particles of many different sizes and narrow size distribution. With control over particle size and distribution, the particles can be incorporated into various matrices forming nanocomposites that exhibit desirable novel properties.

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