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Research Article

OPTIMIZATION OF PHYTO-SYNTHESIZED SILVER NANOPARTICLES AND THEIR ANTIMICROBIAL ACTIVITY AGAINST HUMAN PATHOGENIC BACTERIA

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ABSTRACT

The physicochemical and biological properties of metals change as the particles are reduced to nanoscale. This ability increases the application of nanoparticles in commercial and medical industries. Keeping in view this importance, we have reported a fast, convenient and extracellular method for the synthesis of silver nanoparticles by reducing silver nitrate with the help of *Polyalthialongifolia* aqueous leaf extract. The nanoparticles were analyzed by using UV- Visible Spectrophotometer and were optimized for different parameters including time, silver nitrate concentration, silver nitrate to leaf extract concentration ratio, temperature and pH. The nanoparticles optimized at 1mM silver nitrate concentration, 15 ml leaf extract concentration, 24 hours of reaction time, 30°C temperature and acidic pH (5) were collected and assayed for antibacterial activity against four different human pathogenic bacteria i.e. *E.coli*, *Klebsiellapneumoniae*, *Bacillus subtilis* and *Pseudomonas aeruginosa*. The bactericidal property of nanoparticles was analyzed by Broth dilution method as well as Agar well diffusion method and the synthesized nanoparticles were found to be efficient against human pathogenic bacteria.

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INTRODUCTION

Nanotechnology is an important and rapidly growing field of modern research which involves designing of minute particles known as nanoparticles, whose size ranges up to few 100 nm (Sun et al, 2014). As they possess higher surface area to volume ratio than their natural atomic form (Nadhman et al, 2014), metal nanoparticles have received more attention since last few years because of their remarkable properties and wide range of applications in catalysis (Paul et al, 2014), plasmonics (Khlebtsov and Dykman, 2010), optoelectronics (Muruganandam et al, 2014), biological sensors (Venkatesan and Santhanalakshmi, 2014), water treatment (Con and Loan 2011) and pharmaceutical applications (Ravichandran 2009; Roychoudhury et al, 2016). Among the metal nanoparticles, silver nanoparticles are considered to be the most potent antibacterial agents. Their antibacterial activity has been explored greatly over the last decade (Niraimathi et al, 2013).

Synthesis of nanoparticles can be done by various physical, chemical and biological methods (Anandini et al.2013). In view of the toxic nature and hazardous waste generated during the synthetic chemical- and radiation-based pathways, nanoparticle synthesis using biological materials has gained

considerable importance (Kannan et al. 2013). Biological synthesis of nanoparticles through enzymes (Willner et al, 2006), bacteria (Nair and Pradeep 2002), fungus (Shankar et al, 2003), algae (Li et al, 2011; Vieira et al, 2016), and higher plants (Mittal et al 2013; Rauwelet et al 2014) have proven to be advantageous over physical and chemical methods because of its environment friendly nature (Rai et al, 2012; Sharma et al, 2016). Plant extract as the means of nanoparticle synthesis is very attractive and is a big topic of research in present days as it offers an eco-friendly, clean, nontoxic, and inexpensive method and produce nanoparticles with different shape, size, and morphology (Sudhakar et al, 2015; Choudhary et al, 2016; Govindarajan et al, 2016). Plant extract-based nanoparticle synthesis is advantageous than any other biological entity, since it does not require multistep or complex procedures such as microorganism isolation, identification, growth optimization, culture preparation, and maintenance. Furthermore, plant-based synthesis is inexpensive, faster than using microorganisms, and easy to scale up for large-scale nanoparticle production (Li et al, 2011; Mittal et al, 2013; Iravani, 2011).

Our study aims at synthesis and optimization of silver nanoparticles using leaf extract of *Polyalthialongifolia*, which

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is a member of Annonaceae family and is native plant of India, Bangladesh and Sri-Lanka (Katkar et al., 2010). The plant is used as an antipyretic agent in indigenous systems of medicine. Pharmacologic studies on the leaves and bark of this plant display effective antimicrobial activity, cytotoxic function and hypotensive effects (Faizi et al., 2008). The investigation also aims to evaluate antibacterial activity of these synthesized silver nanoparticles against different human pathogenic bacterial strains.

MATERIALS AND METHOD

Leaf extract preparation

Leaves of *P.longifolia* were collected from university campus. They were first washed with tap water followed with deionized water and then wiped individually using tissue paper. 25g of leaves were cut into small pieces and were boiled in 150ml deionized water contained in a 250ml conical flask for 5 min. The extract was decanted using Whatmann filter paper 1 and the resulting filtrate was stored at 4°C (Geetha et al., 2013).

Synthesis of silver nanoparticles

Silver nanoparticles were prepared by adding 5 ml of freshly prepared leaf extract to 95ml of 1mM silver nitrate (Merck Life Sciences Pvt. Ltd.) solution (Neran et al., 2014) and incubated in dark conditions at room temperature for 24 hours. The formation of silver nanoparticles was confirmed by spectrophotometric analysis.

UV-Vis Characterization of Nanoparticles

The UV visible spectral analysis was done using Elico SL 159 UV-Vis spectrophotometer. The bio-reduction of Ag⁺ ions in solution were monitored using UV-visible spectrometer at a wavelength ranging from 300nm to 700nm. Spectrum between absorbance and wavelength was generated using deionized water as reference.

Optimization of Nanoparticles

The synthesized nanoparticles were optimized for different parameters such as time, concentration of silver nitrate, concentration of leaf extract, temperature and pH. The optimum parameters were recorded and further used for bulk preparations.

Time

The time was optimized by monitoring the absorbance of solution prepared by above mentioned process at a difference of 1 hour till 4 hours and then after 24 hours.

Temperature

The optimization of temperature was done by keeping the nanoparticle solution prepared by above mentioned process at 4°C (in refrigerator), Room temperature (30°C), 60°C (in oven) and 90°C (in oven) and was analysed through spectrophotometer after 24 hours.

pH

The pH optimization was done by keeping the nanoparticle solution prepared by above mentioned process at pH 3,5,7,9 and 11 with the help of 0.1N NaOH and 0.1 N HCl. Further the UV visible spectral analysis was done after 24 hours.

Concentration of Silver nitrate

The above mentioned procedure was used for fixation of silver nitrate concentration, where the reaction was monitored using different concentrations of silver nitrate (1mM, 2mM, 3mM, 4mM, 5mM) whose absorbance was further measured spectrophotometrically after 24 hours.

Concentration ratio of silver nitrate solution and leaf extract
The ratio of silver nitrate: leaf extract was varied (95ml+5ml, 85ml+15ml, 75ml+25ml, 65ml+35ml, 55ml+ 45ml respectively) in the above mentioned procedure, to observe the maximum synthesis of nanoparticles. The absorbance of resulting solution was measured spectrophotometrically.

Purification and collection of nanoparticles after optimizing different parameters the best parameters at which maximum nanoparticles were synthesized were taken further for the investigation of their antimicrobial assay. The nanoparticle solution of optimized parameters was centrifuged at 15000rpm for 15 min and the pellet obtained was washed thrice with deionized water and dried in oven (Neran et al., 2014).

Antimicrobial assay of synthesized nanoparticles
Antimicrobial assay of biosynthesized nanoparticles was performed against human pathogenic bacteria by two different methods (Christopher et al., 2015):

Broth Dilution Method

In a test tube containing 9 ml of NB, 1 ml AgNP solution and 100 µl inoculum were added. Nutrient broth solution inoculated with bacteria was taken as control. Each sample was inoculated in duplicates and all the tubes were incubated in shaker for 24 hours.

Agar Well Diffusion Method

Molten agar was poured in sterilized petriplate and allowed to solidify. It was subsequently seeded with the test organism (15µl) by spreading it on the surface with the help of a spreader. Then 10 mm wide well was bored in this agar plate and filled with 200 µl of the solution of AgNP. The plates were then incubated at 37°C and zone of inhibition was measured after 24 hours. Water was taken as control.

Observations

Silver nanoparticle synthesis

The time of addition of extract into the metal ion solution was considered as the start of the reaction. The formation of nanoparticles was checked preliminarily by the change of solution's colour from yellow to brown that took place within 20 minutes when *P.longifolia* extract was added to silver nitrate solution (Roy K et al., 2013).

Optimization of nanoparticles

Effect of Time

The magnitude of the yield of nanoparticles depends on the length of the reaction time, i.e., the duration of the interaction of the silver salt with the plant extract (Prathna et al., 2011). Increasing the time of reaction resulted in gradual increase in the absorbance spectrum with Surface Plasmon Resonance at 431.9 nm (Figure 8, Table 1) and increased colour intensity

with the duration of incubation (Figure 1).The maximum absorbance was observed after 24 hours(Umoren *et al*, 2014).



A Samples incubated after adding leaf extract



B Samples after 24 hours of incubation

Figure No 1 Effect of time on nanoparticle formation

Table No 1 Absorbance peaks and wavelengths at different time durations

Time	Absorbance	Wavelength(nm)
1 hour	0.325	428.7
2 hours	0.416	429.2
3 hours	0.517	424.8
4 hours	0.511	431.4
24 hours	1.162	431.9

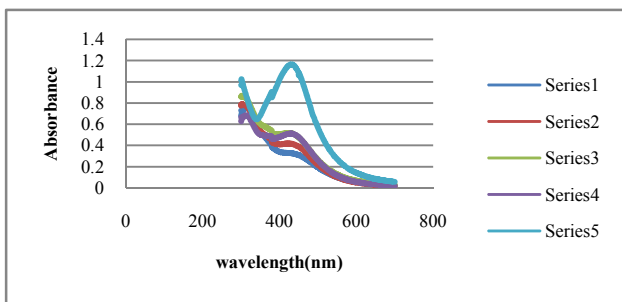


Figure No 8Time Optimization: Series 1 - 5 represents incubation time of 1 hr, 2hrs, 3 hrs, 4 hrs, 24 hrs respectively

Effect of silver nitrate concentration

Characteristic surface Plasmon absorption band was observed at 434 nm for the brown coloured silver nanoparticles synthesized (Figure 2) from the 1 mM silver nitrate. Though the absorbance increased by increasing concentration from 1mM to 5mM,(Figure 9, Table 2) the absorbance peak became unclear (Park *et al*, 2012) making 1mM as the optimum concentration for nanoparticle synthesis (Ahmad *et al*, 2013).



Figure No 2 Samples with 1mM, 2mM, 3mM, 4mM, 5mM AgNO₃ concentrations.(L-R)

Table no. 2 Absorbance peaks and wavelengths at different AgNO₃ concentrations.

Concentration	Absorbance	Wavelength(nm)
1mM	0.883	434.1
2mM	0.929	438.5
3mM	1.116	441.8
4mM	1.031	452.3
5mM	1.001	452.8

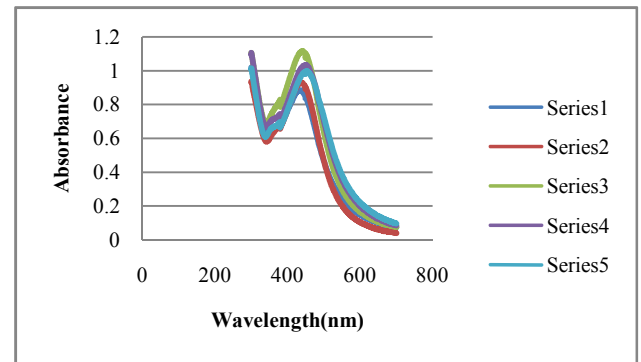


Figure No 9AgNO₃ concentration: Series 1 - 5 represents 1mM, 2mM, 3mM, 4mM, 5mM AgNO₃ solution respectively

Effect of Leaf Extract Concentration

Different concentrations ratios of extracts to AgNO₃ were obtained by changing the volume of the added extract solution to different volumes of 1mM AgNO₃ solution (Figure 3).As the concentration increased, absorbance peak was found to be decreased (Figure 10, Table 3).Maximum absorbance of 1.523 was recorded at 431.9 nm and 15 ml leaf extract concentration, making it optimum for nanoparticle synthesis.



Figure No 3Samples with 5ml, 15ml, 25ml, 35ml and 45ml leaf extract concentration (L-R)

Table No. 3 Absorbance peaks and wavelengths at different concentrations of leaf extract.

Concentration	Absorbance	Wavelength(nm)
5 ml	0.988	427
15 ml	1.523	431.9
25 ml	1.206	434.1
35 ml	0.318	440.7
45 ml	0.785	445.1

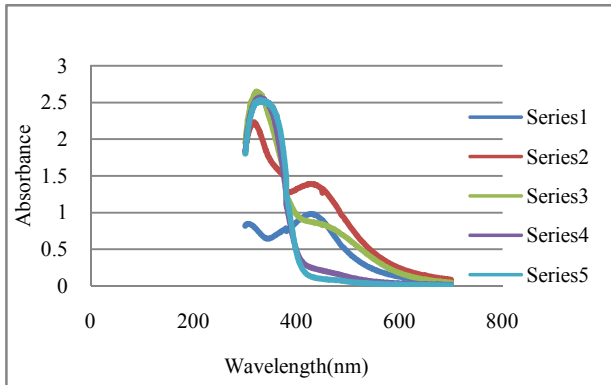


Figure No10 Leaf Extract Concentrations: Series 1 to 5 represents 5 ml, 15 ml, 25 ml, 35 ml, 45 ml respectively

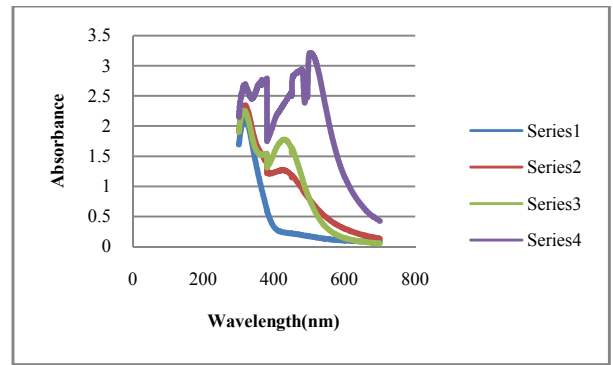


Figure NO 11 Temperature Optimization: Series 1 to 4 represents 4°C, 30°C, 60°C, 90°C respectively

Effect of pH

pH plays an important role in nanoparticle synthesis. The UV-Visible absorption spectra shows that the maximum absorption at pH 5 (Figure 12, Table 5). With increase in pH of the solution, the colour intensity was increased (Figure 5) and the absorbance was found to be decreased. At pH 5, absorbance 1.737 at 427.2 nm was recorded.



Figure No 5 Samples with pH 3, 5, 7, 9, 11(L-R)

Table No 5 Absorbance peaks and wavelengths at different pH.

pH	Absorbance	Wavelength(nm)
3	-	-
5	1.327	427.2
7	-	-
9	-	-
11	-	-

(- represents unclear peaks)

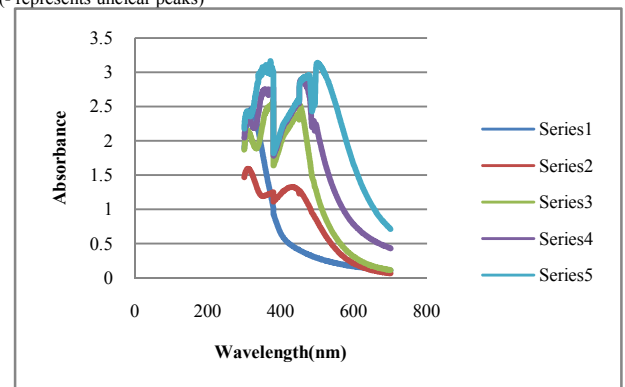


Figure No 12 pH Optimization: Series 1 to 5 represents pH 3, pH 5, pH 7, pH 9, pH 11 respectively

Effect of Temperature

The absorbance increases as the temperature of the solution is increased. But room temperature(30°C)was found to be optimal. Lower temperatures produce sharp peaks, representing uniform-sized AgNPs, while, higher temperature (40°C or above) produce broad peaks (Zhanget al,2013). But our observations were not in accordance with their studies. Though higher absorbance peak was observed at 60°C, but the nanoparticles formed were not stable and agglomeration was observed (Figure 4). Hence, 30°C was taken as optimum temperature with absorbance 1.264 at 425.4 nm (Figure 11, Table 4).



Figure No 4 Samples incubated at 4°C, 30°C, 60°C, 90°C (L-R)

Table no 4 Absorbance peaks and wavelengths at different Temperatures

Temperature	Absorbance	Wavelength(nm)
4°C	-	-
30°C	1.264	425.4
60°C	1.775	428.1
90°C	-	-

(- represents unclear peaks)

Purification and collection of nanoparticles

Centrifugation of the solution resulted in brown coloured pellet which was collected and washed thrice with deionized water. The pellet was then dried in hot air oven, powdered and collected in clean vial.

Antimicrobial assay of synthesized nanoparticles

Nanoparticles were assayed for their antibacterial properties by two different methods and following observations were recorded:

Broth Dilution Method

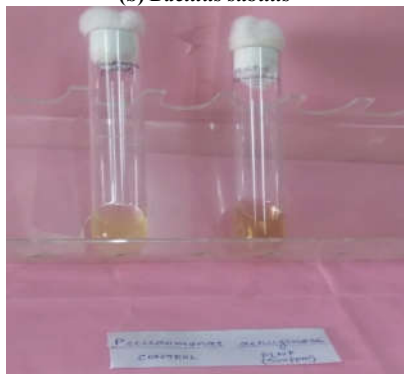
Absorbance of the samples was measured at 600 nm using UV-Vis spectrophotometer. No growth was observed in tubes containing 500 ppm nanoparticles as the solution was clear and not turbid (Figure 6, Table 6).



(a) *E. Coli*



(b) *Bacillus subtilis*



(c) *Pseudomonas aeruginosa*



(d) *Klebsiella pneumoniae*

Figure No 6 Broth Dilution Method
Table No 6 Antibacterial Activity by Both Dilution Method

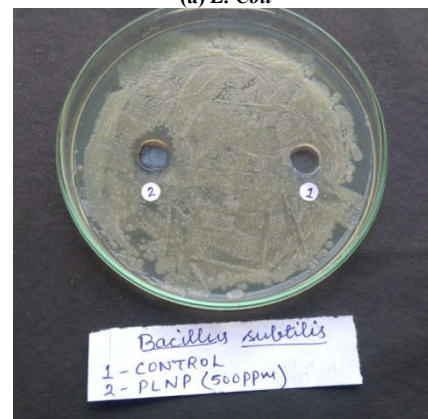
S.No.	Bacteria	Sample	Absorbance	Transmittance (%)
1.	<i>E.coli</i>	Control	1.427	3.7
		Test	0.085	82.2
2.	<i>Bacillus subtilis</i>	Control	0.942	11.4
		Test	0.068	85.5
3.	<i>Pseudomonas aeruginosa</i>	Control	1.053	8.8
		Test	0.077	83.6
4.	<i>Klebsiella pneumonia</i>	Control	1.059	3
		Test	0.063	85.5

Agar Well Diffusion Method

After 24 hours of incubation, zone of inhibition was measured against all four bacteria (Figure 7, Table 7).



(a) *E. Coli*



(b) *Bacillus subtilis*



(c) *Pseudomonas aeruginosa*



(d) *Klebsiella pneumoniae*

Figure No 7 Agar Well Diffusion method

Table No 7 Antibacterial Activity of Nanoparticles by Agar Well Diffusion Method

S.No.	Bacteria	Inhibition Diameter (mm)	
		Control	TEST
1.	<i>E.coli</i>	-	12.5
2.	<i>Bacillus subtilis</i>	-	11
3.	<i>Pseudomonas aeruginosa</i>	-	11.5
4.	<i>Klebsiella pneumoniae</i>	-	12

RESULTS AND DISCUSSION

The present study reports the facile approach of biosynthesizing silver nanoparticles from silver nitrate using the aqueous extract of *P. longifolia*. The adopted method is well suited with green chemistry principles as the plant extract serves as a dual functional molecule as reductant and a stabilizing agent for the synthesis of silver nanoparticles. After adding leaf extract to the silver nitrate solution, reduction of Ag^+ ions took place may be due to presence of different phytochemicals present in leaf extract. Thus the solution turned brown from pale yellow indicating formation of silver nanoparticles.

The efficiency and the influence of various parameters in the biosynthesis of silver nanoparticles analysed include time, silver nitrate concentration, leaf extract concentration, temperature and pH. 24 hours was recorded as the optimum reaction time as at this time, the absorbance was found to be

maximum i.e. 1.173 at 431.9 nm wavelength and the synthesized nanoparticles were stable without any agglomeration.

Raising silver nitrate concentration increases the yield of nanoparticles; however, an increase beyond 10 mM can lead to deposition of silver nitrate on the silver nanoparticles and their lethality. Higher concentration of $AgNO_3$ also produces unclear surfaces as shown by studies of Park et al (2012). In our study, with increasing concentration, though absorbance increases, the stability of silver nanoparticles formed was found to be reduced. At higher concentrations, more agglomeration was observed. Thus, 1mM was taken as optimum concentration of $AgNO_3$.

The synthesis of nanoparticles is also affected by the concentration ratio of leaf extract to silver nitrate. As the concentration of leaf extract increases, colour intensity and absorbance of the solution is decreased because as the ratio of extract to silver nitrate solution increases the synthesis of polydispersed nanoparticles takes place which results in shift of absorbance peak and decrease in colour intensity of the solution.

The absorbance peak increases with increase in temperature. Studies of Zhang et.al (2013) showed that as the reaction temperature increases, peak becomes broader. Further increase in temperature leads to disturbed peaks. 30°C was recorded as the optimum temperature where stable nanoparticles were produce without any agglomeration.

pH plays an important role in nanoparticle synthesis. Studies showed that alkaline pH is more suitable for formation of nanoparticles. But for the first time, we observed acidic pH 5 as the optimum pH for stable nanoparticle synthesis with no agglomeration.

Further, the biosynthesized silver nanoparticles showed significant antibacterial action on tested pathogenic microorganisms. The nanoparticles were assayed for their antibacterial activity by two different methods. In Broth Dilution method, it was observed that absorbance of sample containing 500 ppm of nanoparticles was less than the control samples which contained bacteria only (control). Presence of nanoparticles inhibited growth of bacteria showing their antibacterial nature. In Agar Well Diffusion method, inhibition zones ranging from 11 mm to 14 mm diameter were found in all four samples. This indicates that synthesized nanoparticles contained some antibacterial activity due to which the growth of bacteria was inhibited around the wells. As a result it is observed that a fine tuning of process variables may give the end product with typical physical characteristics.

Thus, the present work can further be explored for the applications of biosynthesized nanoparticles in different fields of medicine and biotechnology.

CONCLUSION

The biosynthesis of silver nanoparticles using *P.longifolia* extract was found to be an easy and safe method. Following the addition of extract to the silver nitrate solution, silver nanoparticles began to form within 20 mins and the results were observed with a change in colour and increase in

absorbance peak as shown by the UV-Vis spectroscopy. The reduction of silver ions to silver nanoparticles was found to be most efficient at 1mM AgNO₃ solution and 15 ml leaf extract concentration at acidic pH 5 and 30°C temperature. When assayed for their antibacterial activity against different human pathogenic bacteria *E.coli*, *Bacillus subtilis*, *Pseudomonas aeruginosa* and *Klebsiella pneumoniae*, they inhibited the growth of bacteria thus making them potent antibacterial agents and can be explored for their used in medical purposes. This green synthesis method is rapid, less time consuming, environmentally safe and thus can be used in various biotechnological applications.

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