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Green Synthesis of Silver Nanoparticles from Leaf Extract of *Calotropis Gigantea* and Evaluation of Their *in vitro* Antibacterial Properties Against Multidrug Resistant Bacteria

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Abstract

In recent years, green synthesis approaches for nanoparticles preparations are gaining a lot of attention in various fields including medicine, drug delivery, pharmaceuticals, and agriculture. Their preparation is relatively simple, inexpensive and represents eco-friendly properties. Due to the unprescribed and excess use of antibiotics has led the development of multidrug resistant (MDR) bacteria. Several studies are evidenced that green nanoparticles have potential bactericidal activity. In this study we have prepared silver nanoparticles from *Calotropis gigantea* leaf extract (Cg-AgNPs) and studied their *in vitro* antimicrobial activity against MDR Gram negative bacterial pathogens of septic wound infections such as *Escherichia coli, Klebsiella pneumoniae and Pseudomonas aeruginosa*. The particles characterization was made by using Dynamic Light Scattering experiment (DLS) and Scanning electron Microscope (SEM). The results of the present study revealed that the size of the Cg-AgNPs ranged between 30-70 nm with spherical shapes and showed a moderate antibacterial activity against MDR bacteria.

Keywords
Antibacterial effect, *Calotropis gigantea* (*C. gigantea*), green synthesis, *in vitro*, nanoparticles.

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INTRODUCTION

Nanotechnology is an emerging field of science catering a wide range of applications in various fronts like biomedical, pharmaceutical, food industry, agriculture, drug, and vaccine delivery. In order to treat microbial infections, widespread and excess use of antibiotics has led to development of multidrug resistant microorganisms. Infections caused by MDR organisms are associated with prolonged hospitalization, increased mortality, and inflated healthcare costs [1]. Hence, their therapy has become a global challenge. However, to address such problems, extensive research work in nanotechnology provided novel therapeutic approaches which are replacing excess use of antibiotics for the treatment of multidrug resistance bacteria [2]. It was found that metal nanoparticles provide the most effective results with their unique mode of action [3]. Nanoparticles are the smallest particles with size range from 1 to 100 nm shows exceptional properties due to large surface area to volume ratio. However, preparations of metal nanoparticles through conventional methods (physical and chemical methods) associated with few drawbacks including use of toxic precursor chemicals, solvents and generation of toxic by-products [2]. Recently, these limitations have overcome through green synthesis approaches, where the biological (green) materials like plant extracts, microbial extracts and algal extracts are being used as reducing agents. Among many nanoparticles (gold, silver, platinum, palladium, titanium, iron, aluminimum and copper), silver nanoparticles (AgNPs) have gained much attention due to their unique antimicrobial properties [4]. During synthesis of AgNPs by green approach, the biological extracts reduce silver salts (Ag^{+}) to metallic silver (Ag^{0}) and also act as capping agent. The *in-situ* capping of plant extracts prevents the agglomeration of the nanoparticles, reduces toxicity and improves antimicrobial action [5, 6, 7, 8, 9]. Several studies have reported that plant extracts have antimicrobial properties, and hence, a synergistic effect of silver nanoparticles and capped biomolecules may be found [4]. In a recent report, the green synthesized silver nanoparticles from Catharanthus *roseus* and *Azadirachta indica* extracts have shown antimicrobial effect against multidrug resistant bacteria and effective healing wounds in BALB/c mice [10]. Biosynthesized silver nanoparticles from C. *gigantea* have effectively inhibited the growth of *Vibrio alginolyticus*, isolated from wild *Artemia franciscana* cysts [11].

In this study we have used *Calotropis gigantea* (*C. gigantea*) leaf extract for prepared silver nanoparticles (Cg-AgNPs). Studies demonstrated that *C. gigantea plant* extracts have antimicrobial effects [12, 13]. *C. gigantea* (crown flower) is a large shrub belonging to family *Asclepiadeae*, widely distributed throughout the Indian subcontinent, southern China, Southeast Asia. In its Latex and plant parts including flowers, roots, bark and leaves have identified certain medicinal values and, hence widely used in ayurvedic medicine. The present study is undertaken to evaluate (*in vitro*) antimicrobial efficacy of Cg-AgNPs against multidrug resistant Gram-negative bacteria like *Escherichia coli* (*E. coli), Klebsiella pneumoniae (K. pneumoniae) and* *Pseudomonas aeruginosa* (*P. aeruginosa*) of septic wound infections.

MATERIALS AND METHODS

Preparation of *C. gigantea* leaf extract

The fresh leaves of *C. gigantea* were collected from Devuni kadapa (YSR District, Andhra Pradesh) and thoroughly washed under running tap water and followed by sterile distilled water and air dried under shade for 4 to 6 days and grinded into a fine powder. 10 grams of powder was dissolved in 100 ml of distilled water and filtered through the whatmann filter paper, and the filtrate was centrifuged at 8,000 rpm for 20 minutes. The obtained supernatant was used for preparation of silver nanopartilces.

Preparation of green silver nanoparticles

For green synthesis of AgNPs, 10 ml of 3 mM silver nitrate ($AgNO_3$) solution was added into 20 ml of *C. gigantea* leaf extract and incubated at room temperature with continuous shaking (150 rpm) under dark condition for 24 hours. The solution colour was changed to dark brown color indicating the formation of silver nanoparticles. Then the solution was centrifuged at 10,000 rpm for 20 minutes at 4^{0}C and the supernatant was separated carefully without disturbing the pellet. Following which the pellet was washed thrice with distilled water at 10,000 rpm for 20 minutes at 4^{0}C for washout of any coordinated plant or silver compounds. Finally, the pellet (Cg-AgNPs) dissolved in 3 ml of sterile de-ionized water.

Characterization of Cg-AgNPs

Dynamic Light Scattering (DLS) was used for the measurement of mean particle size of prepared silver nanoparticles by Zeta sizer S-90 (Malvern, UK). For DLS analysis, Cg-AgNPs were appropriately diluted with distilled water and were performed at a scattering angle of 90^{0} at room temperature [14, 15, 16, 17]. The morphological structure of dried Cg-AgNPs Sample was studied under Scanning Electron Microscope (SEM). Briefly, green synthesized nanoparticles were mounted on aluminium metal stubs using conductive double-sided carbon adhesive tape and over it coated with gold-palladium using a sputter coater. After that the sample was placed in SEM chamber and images were recorded in high vacuum mode at a voltage of 20kV using JEOL JSM-IT500 scanning electron microscope [16, 18].

Antimicrobial activity of Cg-AgNPs

To determine the antibacterial activity of Cg- AgNPs against MDR bacteria viz. *E. coli, K. pneumoniae* and *P. aeruginosa* of septic wound infections (gifted by Department of Microbiology, Yogi Vemana University, Kadapa) followed agar disc diffusion method. The loop full of bacterial culture was aseptically spread on to the nutrient agar plates and over on it placed whatmann number 1 filter paper discs (~10 mm). Later each sample [Cg-AgNPs, plant extract and antibiotics (Norfloxacin and Tetracycline as positive control)] of 10 µl loaded on to the discs and incubated at 37^{0}C. After 24 hours, zone of inhibition around the disc was measured by using vernier caliper.

RESULTS AND DISCUSSION

Green-synthesized nanoparticles have many applications in medicine, pharmaceuticals, and agriculture. Their preparation is relatively simple, cost effective and free of harsh chemicals, and hence, they are nontoxic, eco-friendly, and safe for humans. Green-synthesized AgNPs have been used in biomedicine, agriculture, food industry, cosmetics and textile industry as a novel drug carrier, plant disease protector, preservative as well as shelf-life extension agent and coloring agent, respectively. In the present work, color of plant leaf extract of *C. gigantea* has turned into yellowish brown with silver nitrate solution (Fig 1). It was due to reduction of silver ions into silver particles. Synthesis of AgNPs was visibly characterized with the formation of yellowish-brown color due to the excitation of surface plasmon resonance for AgNPs [19]. Plant extracts contain many biocompounds (alkaloids, phenolic compounds, terpenoids, enzymes, co-enzymes, proteins, and sugars etc.), which have potential to reduce metal salts from positive oxidation state to zero oxidation state [2]. Hence, the bio compounds of *C. gigantea* has reduced the silver nitrate salt (Ag^{+}) to silver nanoparticles (Ag^{0}). However, the particle size remain depends on the nature of the bio compounds of the plant extract. If the plant extract possessed strong reductants, which promotes quick reaction rate and favors the formation of smaller nanoparticles [2]. In this study, Cg-AgNPs characterization was made by using the SEM and DLS. The particles morphology of Cg-AgNPs under SEM at 500 nm was identified as dispersed spherical shapes with various sizes range from 30 to 70 nm (Fig.2A). Similarly, in DLS study, intense peak was observed between 90 nm to 140 nm with 80 percent of particle intensity (Fig.2B). In an *in vitro* biomedical study AgNPs were synthesized through a greener route using aqueous latex extract of *C.gigantea* and their morphological features were characterized by Field emission scanning electron microscopic (Fe-SEM) and Transmission electron microscope (TEM) reveals that nanoparticles are spherical in shape with the size range between 5 and 30 nm [20]. The precise mechanism for bactericidal action of biosynthesized AgNPs was not well understood, however, if these nanoparticles change the membrane integrity of bacteria by accumulating on their membranes which leads to death, irrespective of their drug-resistance nature [21]. In this study we have studied the *in vitro* bactericidal effect of Cg-AgNPs against three MDR Gram negative bacteria (*K. pneumoniae, P. aeruginosa and E. coli)* isolated from septic wound infections. It was noticed that the zone of inhibition with strains of *K. pneumoniae, P. aeruginosa* and *E. coli* are 9 mm,6.5 mm and 11.5 mm respectively (Table 1). These results demonstrating that nanoparticles have a moderate growth inhibition effect against these bacteria. Among three bacteria, *E. coli* is highly susceptible to Cg-AgNPs followed by *K. pneumoniae, P. aeruginosa* Whereas the aqueous leaf extract (*C. gigantea*) alone not showed any inhibition zone against three bacteria in plate diffusion method. The reason might be the less concentration of the sample which unable to exhibit antibacterial properties. Rajkuberan et al, [20] reported that green synthesized AgNPs from latex aqueous extract of *C. gigantea* have shown remarkable antimicrobial activity against different bacterial pathogens such as *Bacillus cereus*, *Enterococci* sp, *Shigella* sp, *Pseudomonas aeruginosa*, *K. pneumoniae, Staphylococcus aureus* and *E. coli* when compared to the crude latex aqueous extract alone.

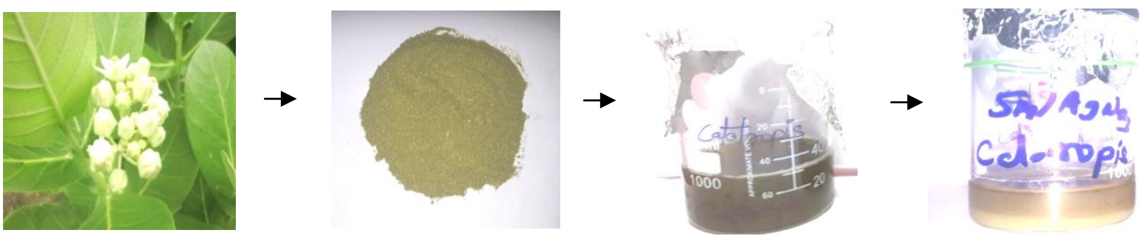
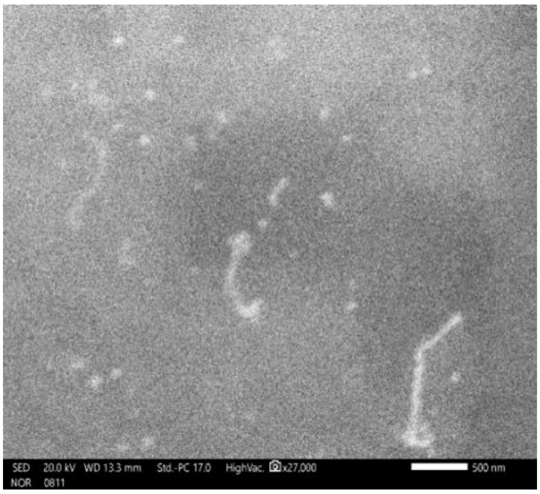
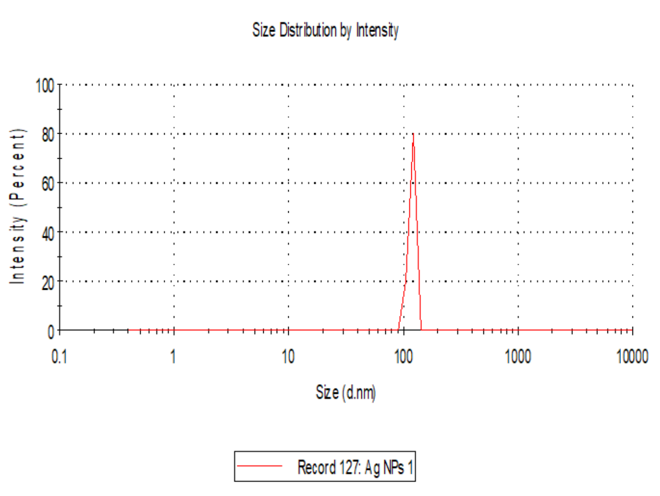


Fig.1 Green preparation of AgNPs from *C. gigantiea* leaf extract (Cg-AgNPs)





A **B**

Fig.2 Characterization ofCg- AgNPs: A) SEM image of Cg-AgNPs B) Size distribution of nanoparticles by DLS.

Table. 1 Antimicrobial activity of Cg-AgNPs against *K. pneumoniae, P. aeruginosa* and *E. coli.*

S.No.	*Strain*	Zone of Inhibition			
		AgNPs of C. gigantea	***C. gigantea Plant leaf extracts***	***Antibiotics***	
				Neroflaxine	***Tetracycline***
1	*K. pneumoniae*	9 mm	N.D	N.D	7 mm
2	*P.aeruginosa*	6.5 mm	N.D	23.5 mm	N.D
3	*E. coli*	11.5 mm	N.D	30 mm	N.D

ND: Zone of inhibition not detected

CONCLUSION

Silver nanoparticles were prepared from the leaf extract of *C. gigantea* (Cg-AgNPs) and they were in spherical shape with size range between 30-70 nm under SEM. Cg-AgNPs were tested *in vitro* against multidrug resistance bacteria (*E. coli, K. pneumonia and P. aeruginosa*) and noticed a moderate antibacterial activity. .

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