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Research Article
Antibacterial Potential of *Cippadessa baccifera* Leaf Extract Mediated AgNPs Against Multi-drug Resistant Bacterial Isolates

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Abstract

Background and Objectives: Multi-drug resistant bacteria (MDRB) have become a widespread serious problem in tropical and subtropical countries. The present study was aimed to investigate the isolation, identification, antibacterial resistance/sensitivity pattern of MDRB (*S. aureus*, *E. coli* and *S. pneumoniae*) from clinical samples and green synthesis of AgNPs for their control. **Materials and Methods:** The routine isolation and identification steps were followed for MDRB from samples. The antibacterial resistant/sensitivity pattern of isolated bacteria were tested against commercial antibiotics. In addition, *Cippadessa baccifera* plant mediated silver nano-particles were synthesized and tested against the target bacteria. The green synthesized silver nano-particles were characterized by using UV-Vis, XRD, EDX, FTIR and SEM analysis. **Results:** About 136 bacterial isolates (*S. aureus* (65), *E. coli* (60) and *S. pneumoniae* (11)) were identified based on their colony morphology and microscopic observation. A total of 121 bacterial isolates were confirmed as resistant against selected antibiotics like penicillin, methicillin, ampicillin and amoxicillin clanonic acid. Plant mediated AgNPs exhibited strong antibacterial activity against target bacteria with better growth inhibition zones i.e., *E. coli* (35±0.9 mm), *S. aureus* (33.6±0.4 mm) and *S. pneumoniae* (23.3±0.5 mm) than other extracts. UV-Vis spectroscopy results of green AgNPs showed maximum absorption peak at 370 nm. XRD and SEM analysis revealed that AgNPs were face-centered, cubic structure being spherical in shape with an average particle size of 45 nm. FTIR result of nanoparticles showed capped with C-X, -CH_3, N=O, C=N, C=O functional groups of plant compounds. The particle size of AgNPs was found to be poly-dispersed nature. **Conclusion:** The outcome of results suggested that, plant mediated AgNPs express better antibacterial effect against multi drug resistant bacteria (MDRB) and it can be used as a potential source for various biomedical applications.

Key words: Multi-drug resistant bacteria, antibiotic resistance pattern, silver nanoparticles, antibacterial activity, *Cippadessa baccifera*, microscopy and spectral study

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Multi-drug resistant bacteria (MDRB) are leading micro-organisms that can be cause severe health problems in human as well as animals. In recent years, the expanding bacterial resistance to antibiotics has become a growing concern worldwide[1]. Intensive care physicians consider antibiotic-resistant bacteria having a significant or major problem in the treatment of patients[2]. The emergence of multi-drug resistant strains complicated with the treatment of infectious diseases in immunocompromised patients. Increasing bacterial resistance is prompting a resurgence in research of the antimicrobial role of herbs against resistant strains[3,4]. A vast number of medicinal plants have been recognized as valuable resources of natural antimicrobial compounds[5]. Medicinal plant extracts offer considerable potential for the development of new agents which are effective against infections[6]. A survey of earlier literature suggests that leaf extracts from various plants namely *Azadirachta indica*, *Aloe vera*, *Bryophyllum* sp., *Cyperus* sp., *Hydrilla* sp., *Gliricidia sepium* and *Rosa rugosa* reported as potential antibacterial agents[7-11]. The development of reliable green process for the synthesis of silver nano-particles is an important aspect of current nanotechnology research.

Green mediated nanoparticles synthesis is an emerging highlight of the intersection of nanotechnology, it have increased attention due to growing need to develop environmentally benign technologies in material synthesis[12]. A great deal of effort has been put into the green synthesis of inorganic material, especially metal nano-particle using micro-organisms and plants[13]. Nanosilver has many important applications viz. antimicrobial agent, applied in textiles, home water purification systems, medical devices, cosmetics, electronics and household appliances[14]. Besides, silver nano-particles exhibit strong optical features making the nano-particles suitable for biological sensing and imaging[15]. Due to their high conductivity, silver nano-particles are applied in conductive inks, adhesives and pastes for a range of electronic devices[16]. Silver nano-particles are synthesized by reduction and oxidation in solutions[17,18], thermal decomposition of silver compounds[19], microwave assisted synthesis[20] and biological reduction method[21]. The green synthesis of nano-particles offers one step, eco-friendly and stable in nature. *Cippadessa baccifera* (Meliaceae) plant are available in western and eastern Ghats of Tamil Nadu, India. The plant leaf and fruits used as folklore medicine for treat piles problem, diabetes, diarrhoea and headache[22]. *Cippadessa baccifera* plant crude extract having better antimicrobial activities against *Staphylococcus aureus, Pseudomonas aeruginosa*, *Salmonella typhi*, *Shigella flexneri* and *Vibrio cholera*[23]. This study also enlighten the application of natural sources (along with nanotechnology) especially from plants offer potent antimitotic effect against target MDRB. The present work was designed to perform the isolation of MDRB bacteria, synthesis of silver nanoparticles with *C. baccifera* plant leaf extract and evaluate its antibacterial activity against isolated multidrug resistance bacteria.

MATERIALS AND METHODS

Isolation, identification and antibitotic susceptibility test of MDRB: Totally 500 clinical samples (pus, blood, wound, urine and sputum) were collected (using sterile containers) from Government hospitals, Salem and Namakkal Districts, Tamil Nadu, India for isolation of target group of bacteria. This work was approved by the institutional human ethical committee (PU/IEC/HR/2014/008). The preliminary isolation of multi-drug resistant bacteria using the different medium viz., Mannitol Salt Agar, Eosin Methylene Blue Agar (EMB) and Blood Agar Medium. The multi-drug resistant bacteria was identified based on the morphological, biochemical and sugar fermentation test. The suspension of each confirmed MDRB strain compared with susecptible strains are *S. aureus* MTCC 96, *E. coli* ATCC 25922 and *S. pneumoniae* ATCC 41619. The isolation and identification of MDRB was confirmed as per the recommended guidelines[24]. Antibacterial susceptibility of isolates (*S. aureus*, *E. coli* and *S. pneumoniae*) was determined by using modified Kirby-Bauer disc diffusion method as recommended by CLSI[24]. The commercial antibiotics like methicillin (30 mg), penicillin (10 mg), ampicillin (10 mg), amoxicillin clanonic acid (30 mg), vancomycin (30 mg), erythromycin (15 mg), gentamicin (10 mg), streptomycin (10 mg), tetracycline (30 mg) and ciprofloxacin (10 mg) were used in this study. A sterile disc used as a negative control and the plates were incubated for 24 h at 37°C.

Collection and prepation of leaf extracts: The healthy and young leaves of *Cippadessa baccifera* (Fig. 1) were collected from Kalvarayan hills, (Latitude11°14'46-12°53'30', 77°32'52'-78053'05' longitude) Salem district, Tamil Nadu, India. The plant taxonomic identification was done by Dr. D. Natarajan, Assistant Professor, Department of Biotechnology, Periyar University, Salem. The voucher specimen was deposited in the research laboratory for future reference. The leaves were thoroughly washed with tap-water and shade-dried for

Fig. 1: *Cippadessa baccifera* plant

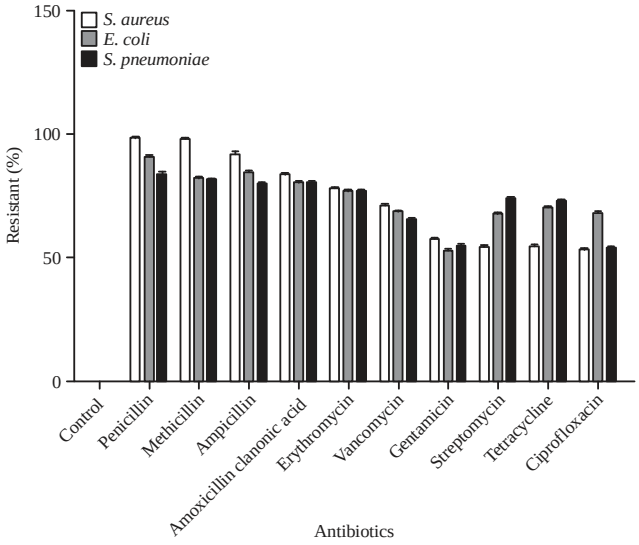

Fig. 2: Antibiotic resistant patterns

7-10 days. Dry leaves were ground in a grinder to make a fine powder. About 10 g of the powder was dissolved in 100 mL of deionised water and boiling for 20 min at 60°C. The filtrate was collected in 250 mL Erlenmeyer flask and stored at 4°C.

Synthesis and characterization of silver nanoparticles: The plant aqueous extract (10 mL) was added to 90 mL of 1 mM $AgNO_3$ solution and incubated for 30 min. The change of solution from yellow to grey colour indicates the formation of AgNPs. The reduction of pure Ag+ions was monitored by measuring the UV-Vis spectrum of the reaction medium. The UV-vis spectroscopic analysis was done with ranges from 300-700 nm. The synthesis of silver nanoparticles were subjected to lyophilization and powdered for XRD analysis. The diffracted intensities were recorded 10-80° at 2θ. The size of synthesized silver nano-particles was confirmed by observing under (SEM) scanning electron microscope. EDX analysis was conducted to confirm the elemental compositions of the sample. The identified biomolecules present in the *C. baccifera* AgNPs were analyzed using FTIR spectroscopy recorded in the range of 3500-5000 cm^{-1}. The various modes of vibration were identified and assigned to determine the different functional groups present in the *C. baccifera* AgNPs. The zeta potentials of sample was suspended in aqueous solution was measured using a Malvern instruments nano-zetasizer based on the electrophoretic light scattering method that measures the migration rate of dispersed particles under the influence of an electric field. After five series of measurement, the mean value and standard deviation of the zeta potential was obtained.

Antibacterial activity of green synthesized AgNPs: The antibacterial activity of plant mediated AgNPs was evaluated against MDRB, using the agar well diffusion method[25]. The overnight bacterial cultures (MDRB) were spread over the freshly prepared Muller-Hinton agar plates. The different concentrations of green synthesized AgNPs solution (25, 50 and 75 µL) was poured into the corresponding well using a micropipette. As control, 25 µL of *C. baccifera* $AgNO_3$ without treatment of plant extract solution was poured into control well. The plates were incubated at 37°C for 24 h and the zone of growth inhibition was measured and the experiments were done in triplicate for calculation of standard deviation (Mean±SD).

RESULTS

Isolation, identification and antibiotics resistant pattern of MDRB: Out of 500 clinical samples, 136 bacterial isolates (*S. aureus* (65*)*, *E. coli* (60) and *S. pneumoniae* (11)) were identified based on their colony morphology, microscopic observations and biochemical tests (Table 1). The antibiotic sensitivity/resistant pattern of isolates resulted *S. aureus* express maximum percentage of resistant against most of the tested commercial antibiotics followed by *E. coli* and *S. pneumoniae* (Fig. 2).

Synthesis and characterization of plant mediated AgNPs: The colour change was observed in the *C. baccifera* leaf extract, incubated with $AgNO_3$ and leaf extracts without $AgNO_3$ did not show any change in colour (Fig. 3a). The

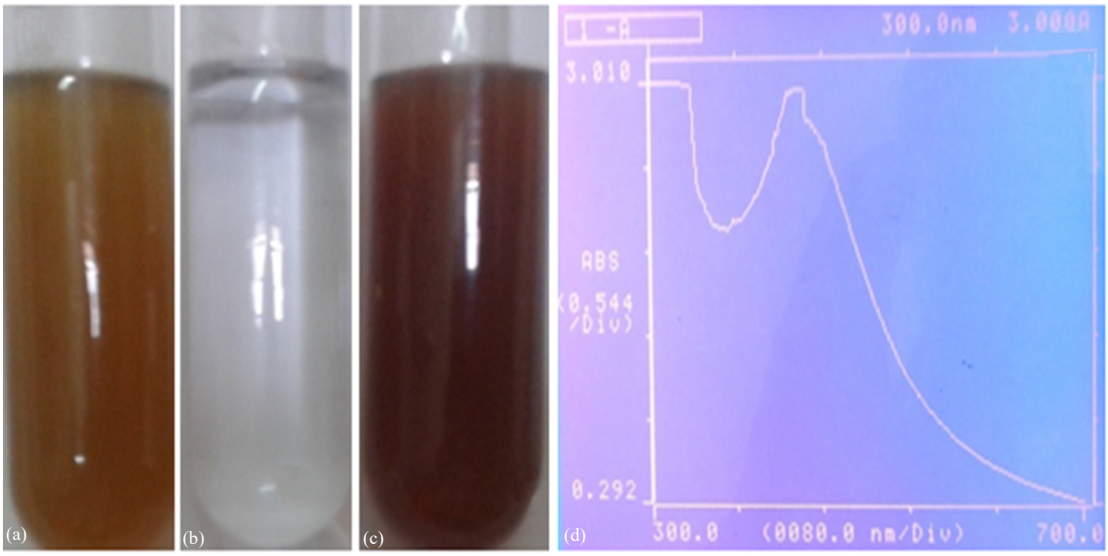

Fig. 3(a-d): Biosynthesis of nano-particles from *C. baccifera,* (a) *C. baccifera* leaf extract, (b) $AgNO_3$, (c) *C. baccifera* mediated $AgNO_3$ and (d) UV-Vis spectrum of AgNPs from *C. baccifera*

Table 1: Identification of multidrug resistant bacteria

Characterization	*S. aureus*	*E. coli*	*S. pneumoniae*
No. of bacterial isolates from 500 clinical samples	65	60	11
Morphological character			
Colony colour	Golden yellow color, bluish-green color	Dark blue-black colony	Green and Moist colony
Microscopic characters			
Gram staining	Gram-positive (cocci, grape like arrangement)	Gram-negative (rod shape)	Gram-positive dipole Cocci shape
Motility	Non-motile	Non-motile	Non-motile
Spore staining	Non-spore forming	Non-spore forming -, positive +, negative for production	Non-spore forming
Biochemical characters			
Catalase	+	+	-
Coagulase	+	+	-
Hemolysis	α hemolysis	No-hemolysis	β-hemolysis
Methyl red	+	+	-
Indole	+	+	-
Oxidase	+	-	-
Voges, p-test	-	-	-
Sugar fermentation test			
Urease test	-	-	-
Citrate utilization	-	+	-
Lactose	-	+	+
Mannitol	+	-	-
Sucrose	-	+	-

-: Positive for production, +: Negative for production

solution colour change was observed in absorption spectrum and the wavelength range from 300-700 nm. The better result was revealed a peak at 370 nm (Fig. 3b). The UV-vis spectroscopic results are similar to the surface Plasmon vibrations with characteristic peaks of the silver nano-particles prepared by chemical reduction.

X-ray diffraction pattern of the biosynthesized silver nano-structure produced by the *C. baccifera* leaf was further demonstrated and confirmed by the characteristic peaks observed in the XRD image (Fig. 4). The XRD pattern shows four intense peaks (27.94, 32.32, 38.56 and 46.36) from the whole spectrum of 2θ value ranged from 20-70°C. The SEM

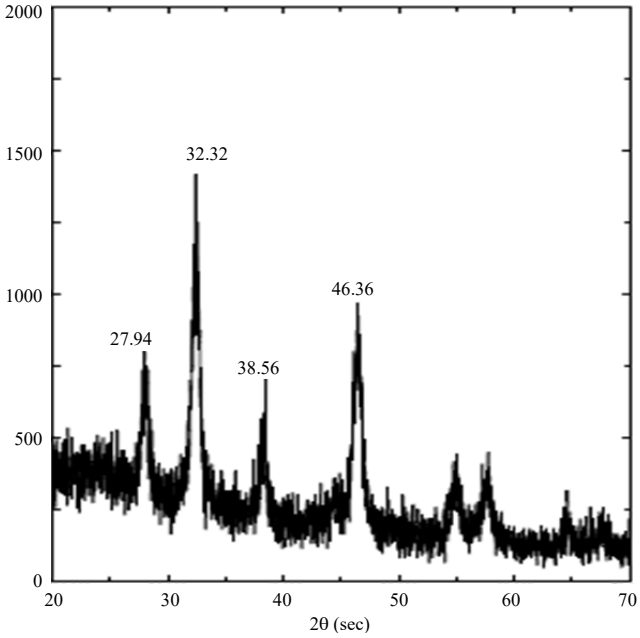

Fig. 4: XRD analysis of AgNPs

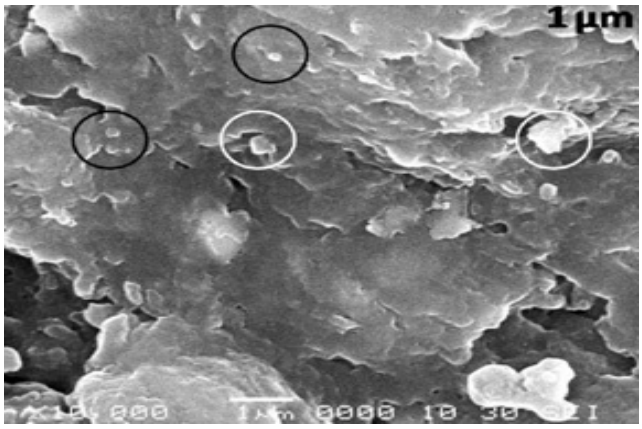

Fig. 5: Scanning electron microscopy analysis of *C. baccifera* AgNPs

analysis result reflected the understanding of topology and size of the AgNPs, showed the synthesis of higher density polydispersed irregular shape with some smaller particle (black circles) of spherical sizes. The present study observed AgNPs of various sizes ranged from 44-64 nm. Most of the nano-particles were aggregated and only few of them were scattered in nature (Fig. 5). The EDX spectrum of synthesized silver nano-particles (Fig. 6) clearly exhibited the absence of elemental nitrogen and oxygen peaks and the presence of elemental silver metal.

FTIR measurement of the *C. baccifera* mediated silver nano-particles were carried out to identify the possible biomolecules responsible for the reduction of Ag^+ ions and capping of the bioreduced silver nano-particles synthesized by the leaf extract. The major peaks in the FTIR spectrum of silver nanoparticles (Fig. 7) observed the peaks at 1339.61, 1419.79, 1518.96, 1647.37, 1771.73 and 3447.36 cm^{-1} reflects the cyclic functional groups of C-X, -CH_3, N=O, C=N, C=O and H bonds, respectively. Zeta potential measurement for the obtained nano-particles, found to be -0.0616 mV with a peak area of 100% intensity (Fig. 8).

Antibacterial study of green synthesized AgNPs: The antibacterial effects of three different concentrations (25, 50 and 75 µL) of plant mediated AgNPs were assessed on the basis of zone of growth inhibition (Table 2 and Fig. 9). $AgNO_3$ compared with AgNPs exhibited strong antibacterial activity on selected and the highly resistant MDRB isolates and maximum zone of inhibition was found in *E. coli* (35±0.9 mm), followed by *S. aureus* (33.6±0.4 mm) and *S. pneumoniae* (23.3±0.5 mm). The *C. baccifera* leaf extract alone produce least inhibitory effect against the organism tested (10±0.8 mm).

DISCUSSION

The *S. aureus* colonies identified as red to yellow colour formation due to mannitol fermentation. *E. coli* colonies show a characteristic green metallic sheen, indicate that lactose fermentation. *S. pneumoniae* colonies characteristically produce a clear zone of alpha haemolysis. Out of 136 isolates, 121 showing multi-drug resistance bacterial isolates (*S. aureus*, *E. coli* and *S. pneumoniae*) against various commercial antibiotics like penicillin (99, 90 and 87%), methicillin (98, 86 and 85%), ampicillin (97, 87 and 80%), amoxicillin clanonic acid (85, 80 and 80%) and erythromycin (77, 70 and 71%), respectively.

The reduction of silver during exposure to plant extracts could be attributed by a color change. The silver nano-particles expressed a dark yellowish-brown color in the aqueous solution due to the surface Plasmon resonance phenomenon[26]. Significant color change was observed after 2-3 days, indicates slow reduction of the $AgNO_3$ by the aqueous leaf extract[27]. The present study observed the rapid biosynthesis (90% reduction of the metal ions within 30 min) of stable silver nano-particles with 10 g of *C. baccifera* leaf and 1 mM aqueous $AgNO_3$. The dissimilarity in the rates of bio-reduction may be due to the differences in the activities of the molecules present in the *C. baccifera* aqueous leaf extract[28,29]. The XRD, EDX and SEM analysis of green synthesized AgNPs shown the characteristic feature of the

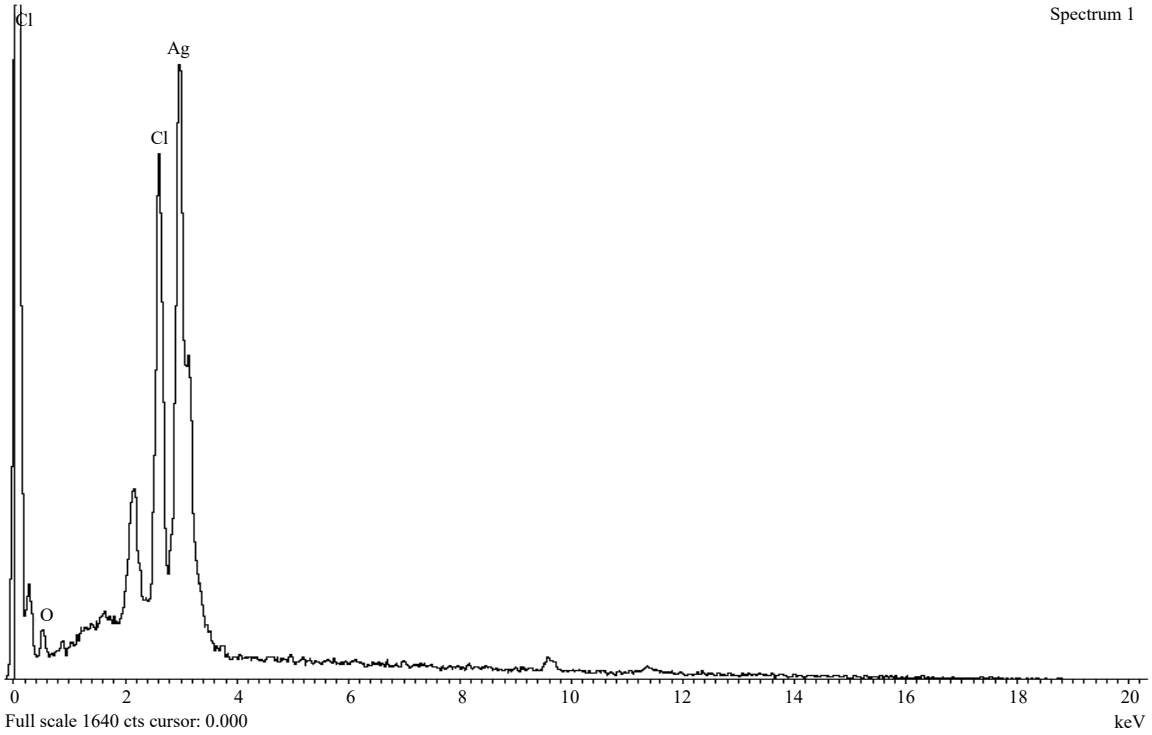

Fig. 6: EDX spectrum from *C. baccifera* AgNPs

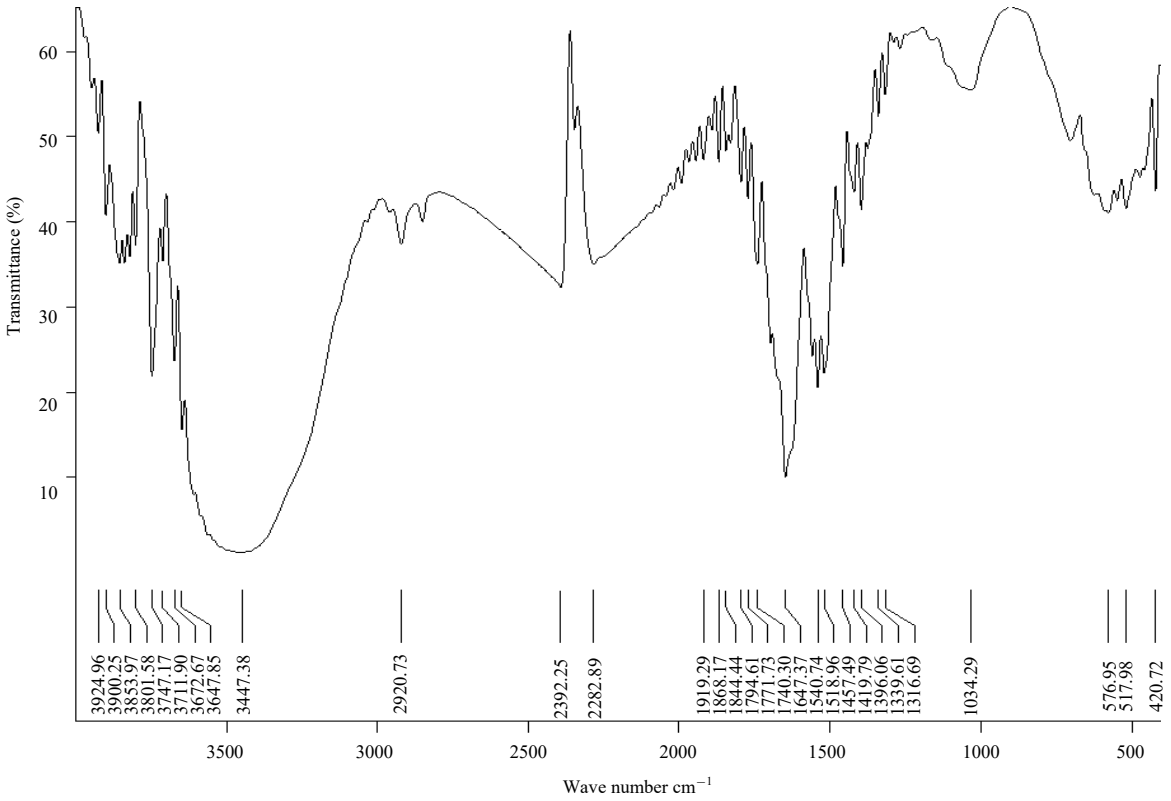

Fig. 7: FTIR spectrum of *C. baccifera* AgNPs

samples and FTIR spectrum reflect the cyclic functional groups of plant mediated AgNPs and these changes may be due to the polyols and phenols are mainly responsible for the reduction of Ag ions and oxidized to unsaturated carbonyl groups. The sharp signal peak of silver strongly confirmed the reduction of silver nitrate to silver nano-particles. The optical absorption band of the EDX peak in the range of 3-4 keV is typical for the absorption of metallic silver nanocrystallites[28,29]. The green synthesised silver nanoparticles have good antibacterial activity against clinically important multi-drug resistant pathogens. *E. coli* reported as strong antibacterial effect followed by other organisms. Another study supported

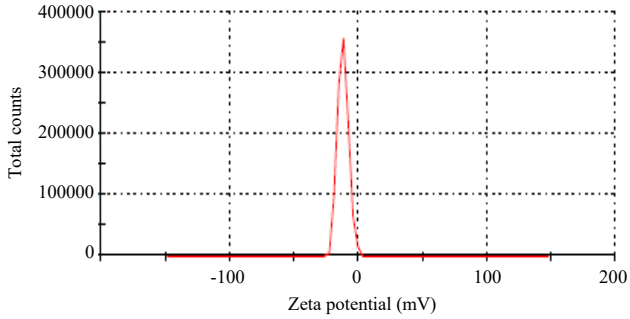

Fig. 8: Zeta potential distribution of AgNPs from *C. baccifera*

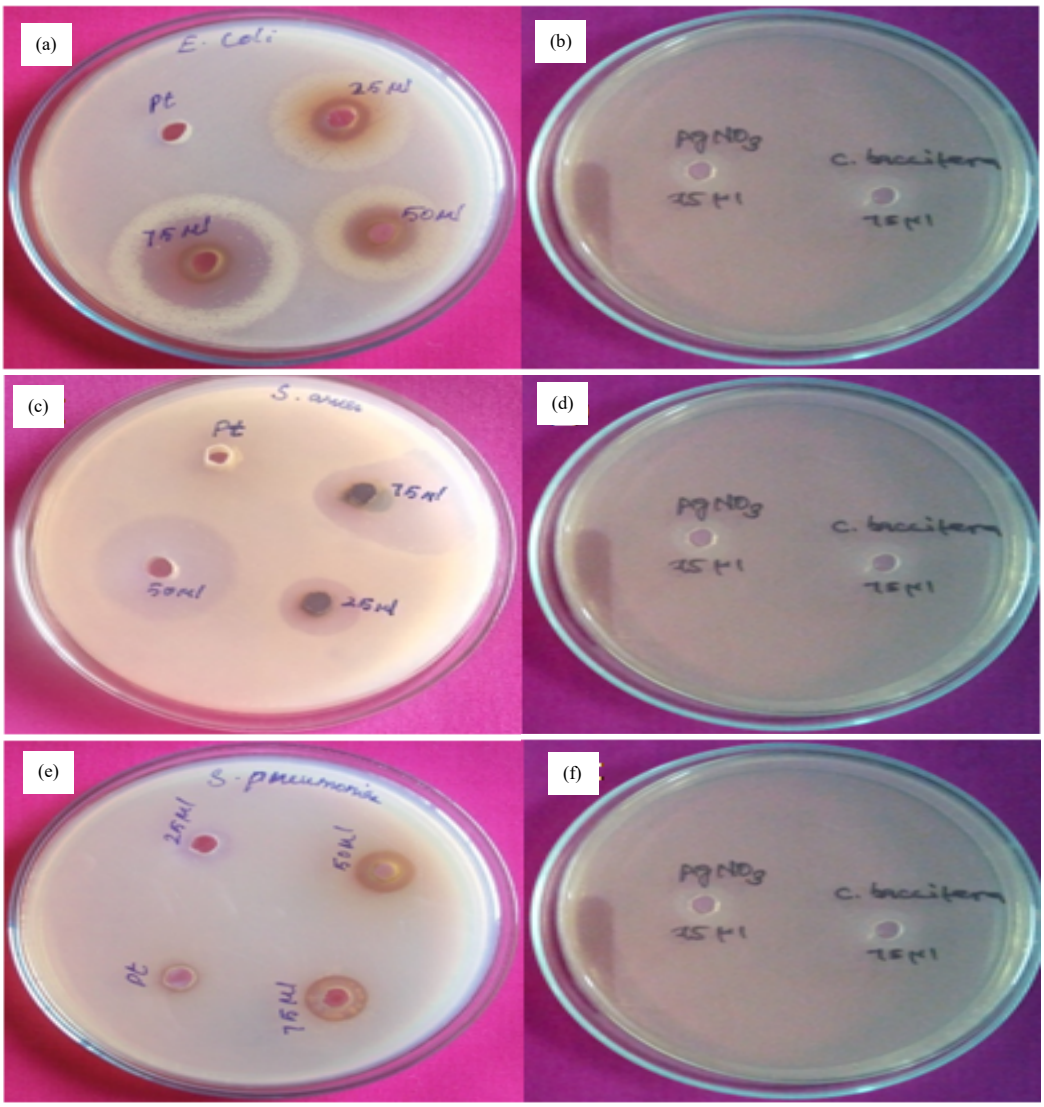

Fig. 9(a-f): Zone of inhibition of silver nanoparticles against MDRB (a) *E. coli*, (b) *E. coli* control on $AgNO_3$, aqueous extract, (c) *S. aureus*, (d) *S. aureus* control on $AgNO_3$, aqueous extract, (e) *S. pneumoniae* and (f) *S. pneumoniae* control on $AgNO_3$, aqueous extract

Table 2: Antibacterial activity of *C. baccifera* AgNPs on MDR bacteria

		Name of the micro-organisms (diameter of inhibition zone in mm)				
Name of the sample	Conc. (µL)	*E. coli*	*S. aureus*	*S. penumoniae*	$AgNO_3$ control	Crude extract
Plant mediated AgNPs	25	20±0.8	16.6±0.9	9.8±0.8	9±0.0	10.0±0.0
	50	28±0.8	26.3±0.4	15.9±0.8	9±0.2	10.1±0.6
	75	35±0.9	33.6±0.4	23.3±0.5	9±0.4	10.0±0.0

Results were expressed as Mean±SD, n = 3

the leaf extract of *Mentha piperita* (Lamiaceae) was very good bioreductant for the synthesis of nanoparticles which are active against clinically isolated human pathogens[30]. Similar kind of works have been carried out by several authors using orange peels, *Sesbania grandifolia* leaf extract and *Klebsiella pneumoniae* extra cellular metabolites[31-33]. This study also provide the baseline information and also open new avenues for isolating antibacterial lead molecules from plants for combating against MDRB.

CONCLUSION

The green synthesised silver nanoparticles using *C. baccifera* leaf extract, which is an economical, efficient and eco-friendly process. UV-Vis spectrophotometer, XRD, FTIR, SEM, EDX and particle size analyser tools have been used for confirm the reduction of silver nitrate to silver nano-particles. The antibacterial screening results showed that plant mediated AgNPs has been observed an efficient antibacterial effect against multi-drug resistant pathogenic bacteria (MDRB) and it can be used as a potential candidate for various biomedical applications in future.

SIGNIFICANCE STATEMENT

The present study was carried out the isolation and identification of MDRB from clinical samples and plant mediated AgNPs reported to have significant antibacterial effect against target bacteria. This study will helps the researchers towards the active mechanism study of plant AgNPs against microbes.

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