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RESEARCH ARTICLE

CLAUSENA DENTATA LEAF EXTRACT MEDIATED SILVER NANOPARTICLE SYNTHESIS AND ITS ANTIBACTERIAL ACTIVITY AGAINST MULTI-DRUG RESISTANT *ESCHERICHIA COLI*

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ABSTRACT

Community-acquired infections, particularly respiratory tract and urinary tract infections are caused by *Escherichia coli* represents a significant burden for most health care systems in the globe. The excessive use of commercial antibiotics leads to raising resistance in microbes and cause severe side effects. There is an urgent need to require alternate antibiotics to overcome this problem. The current study was deals with the isolation and identification of multi-drug resistant *E. coli* from clinical samples. In addition, we perform green synthesis of silver nanoparticles using *Clausena dentata* leaf extract and its antibacterial potential. The biosynthesized AgNPs were characterized by SEM-EDAX, XRD, UV–Vis and FT-IR spectroscopy techniques. UV-Vis spectroscopy of AgNPs showed maximum absorption at 430-460 nm. XRD and SEM analysis revealed that AgNPs are face-centered, a cubic structure being spherical in shape with an average particle size of 40-50 nm. The antibacterial activity result highlights biosynthesized silver nanoparticles reported more potent activity against multi-drug resistant *E. coli* compared to control.

Key words: *Clausena dentate,* Nanoparticles; MDR *E. coli,* Spectral & SEM analysis.

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INTRODUCTION

Escherichia coli is one of the leading organism cause several diseases such as urinary tract infections (UTI), neonatal meningitis and community-acquired infections, including respiratory tract infections (RTIs), respectively. Worldwide, due to indiscriminate use of antibiotics, the prevalence of drug-resistant pathogens (e.g. extended-spectrum beta -lactamase (ESBL)-producing *E. coli*) are common and create life threatening issues. The consumption of least amount of antibiotics is possible without harm public health and also reduces the risk of microbial resistance (Tinelli *et al*., 2012). In recent years, the synthesis of silver nanoparticles (AgNPs) using green technology is evolving into important and new branch of nanotechnology (Armendariz, 2002). Nanoparticles exhibit new or improved properties based on their size, distribution and morphology. The ancient times silver has act as disinfecting agent and used in several applications from traditional medicines to culinary items. It has been reported that silver nanoparticles (SNPs) are non-toxic to humans and most effective against microorganisms at low concentrations and lack of any side effects (Kathiresan, 2009). Nanoparticles can be synthesized by physical, chemical and biological methods.

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During the past few years, the biological approach has gained more widespread interests among the scientists (Rao, 2001; Kulkarni, 2007) due to its simple experimental procedure and eco-friendliness (Agnihotri *et al*., 2009). The nanoparticles are reported as having several medical applications (Xu, 2006) such as treatment of cancer (Farokhzad *et al*., 2006) and kill human bacterial pathogens (Sondi, 2004; Stoimenov *et al*., 2002; Morones *et al*., 2005). The antimicrobial potential of plant mediated AgNPs has been done using various plants like, *Acalypha indica* (Krishnaraj, 2009), *Pelargonium graveolens* (Shankar, 2003), *Parthenium hysterophorus* (Parashar, 2009), *Aloe barbadensis* (Chandran, 2006) and *Gliricidia sepium* (Rajesh *et al*., 2009) against various pathogens including urinary tract infections causing bacterial pathogens (Ravikumar *et al*., 2010). Considering the above cited informations, the present study has chosen *Clausena dentata* is a shrubby medicinal plant belonging to the Rutaceae family and widely distributed in South India. The people of Tamil Nadu use this plant for medicinal and nutritional purposes (Chokeprasert *et al*., 2007; Zafar, 1994). Xanthyletin, imperatorin, dentain, nordentatin, sabinene, biofloratriene, borneol compounds were isolated from different parts of the plant (Rajkumar, 2010). The pharmacological aspects of the *C. dentata* have been reported as anticancer, antimicrobial, antioxidant, antidiabetic, antiinflamatory, larvicidal and immunomodulatory properties (Arbab *et al*., 2012).

Hence, the present study was designed to isolat multi-drug resistant *E. coli* from clinical samples and test its antibacterial potential using *Clausena dentata* leaves mediated AgNPs.

MATERIALS AND METHODS

Sample collection: Totally 500 clinical samples (urine, blood, wound, sputum and pus) were collected (using sterile containers) from the clinical laboratories and hospitals, in and around Salem and Namakkal Districts, Tamil Nadu, India. The collected samples were carefully brought out in research laboratory and stored for further process.

Isolation and identification of *E. coli:* The preliminary isolation and identification of *E. coli* was done using EMB (Eosin methylene Blue Agar media). The colonies show a characteristic green metallic sheen due to the rapid fermentation of lactose. The identification of *E. coli* isolates based on morphological, microscopic and biochemical, sugar fermentation test as per the standard guidelines of Clinical and Laboratory Standards Institute (CLSI) (Wayne, 2012).

Antibiotics susceptibility/resistant test: Antibiotic susceptibility/resistant test of all isolates were done against several antibiotics (viz Penicillin 10 mg, vancomycin 30 mg, erythromycin 15mg, gentamicin 10 mg, amoxicillin-clavulanic acid 30mg, ampicillin 10 mg, tetracycline 30, and ciprofloxacin10 mg) as per the modified method of Kirby-Bauer disc diffusion method on Muller-Hinton agar as recommended by Clinical and Laboratory Standard Institute (CLSI). The plates were incubated an overnight at 37^0C. The *E. coli* ATCC 25922 was used as control strain.

Plant collection: The fresh and healthy leaves of *Clausena dentata* were collected from Kalrayan hills, (Latitude$11^o14'46$ -$12^0 53'30'$ North $77^0 32'52'$-$78053'05'$East longitude) Villupuram district, Tamil Nadu, India. The taxonomic identification of plant was done by Dr. D. Natarajan, Assistant Professor, Department of Biotechnology, Periyar University, Salem. The voucher specimen was deposited in the research laboratory for further reference.

Preparation of the leaf extract: Young leaves of *C. dentata* (Fig. 1) were washed thoroughly with tap water followed by distilled water and air dried on a paper towel for 4-6 days. Dry leaves were ground in a tissue grinder to make a fine powder. Ten grams of the powder were dissolved in 100 ml sterile deionised water and heated for one hour at 80°C. The obtained extract was filtered through What man No.1 filter paper. The filtrate was collected in 250ml Erlenmeyer flash and stored at 4°C for further studies (Verastegui *et al*., 1996).

Green synthesis of AgNPs by aqueous extract of *C. dentate:* The silver nanoparticles were prepared by treating 90 ml of 1 mM silver nitrate with 10 ml of plant leaf extract and incubated at room temperature. The colour change of the solution was checked periodically and resulted the yellowish-brown color indicates the formation of silver nanoparticles. It showed that aqueous silver ions could be reduced by the plant extract. Further, the synthesized silver nanoparticles were used for antimicrobial studies (Sathishkumar *et al*., 2009). The particles were isolated by centrifuging 20 ml of suspension in de-ionized water containing Ag nanoparticles for 15 min at 10,000 rpm. The pellets were collected and dried in an oven at 40^oC to remove excess amount of water.

Characterization of silver nanoparticles

UV -visible Spectroscopy: UV- visible spectral analysis of for nanoparticles synthesised from *C. dentata* leaves were carried out by measuring the optical density (OD) using scanning spectrophotometer. Measurements were performed (between 200 and 800 nm) with a resolution of 1nm and scanning speed of 300nm/min. The reduction of Ag+ ions was monitored by measuring the UV-Vis spectrum of 1ml aliquots of samples and 2ml de-ionized water in quartz cell as indicated earlier (Wiley *et al*., 2006) Silver nitrate (1mM) was used as a blank.

X-ray diffraction XRD: The powdered or dried AgNPs were coated on XRD grid and the spectra were recorded Rich seifert p 300 instrument operated at a voltage of 40 KV and a current of 30 mA with Cu Kα radiation. X- ray diffraction (XRD) pattern analysis of the sample was done to observe the nature of the nanoparticles. The biosynthesized AgNPs using leaf extract was lyophilized into a powder.

FTIR analyses: For FTIR analysis, the AgNPs solution was centrifuged at 12,000 rpm for 30min. The pellet was washed three times with 25ml of de-ionized water to get rid of the free proteins or enzymes that are not capping the AgNPs (Kanipandian *et al*., 2014). The particles were dried and ground with KBr and analyzed on a Perkin Elmer infrared spectroscopy (from 3500 to500 cm-1).

SEM EDX analyses: Scanning Electron Microscope (SEM) analysis of the sample was performed using SIGMA model, CASLZEISS operated at an accelerating voltage of 10kv. The sample was prepared on a carbon coated copper grid by simply dropping a very small amount of the samples on the grid and using blotting paper, the excess solution was removed. Then, the film was allowed to dry under a mercury lamp for 5min (Ramalingam, 2013). EDX analysis of the samples was performed using the oxford instrument Thermo EDX attached with SEM.

Particle Size and Zeta Potential Analysis: The synthesized AgNPs solution from the plant sample was analyzed by DLS particle size analyzer (ZETA Seizers Nanoseries Malvern instrument Nano ZS) to determine the distributional size of nanoparticles. The clear disposable cell was rinsed with ethanol and de-ionized water was used as the dispersant. A sample was placed in clear disposable zeta cell. Twelve scans were performed at 25°C.

Antibacterial activity of silver nanoparticles against MDR *E. coli:* The green synthesized silver nanoparticles from *C. dentata* leaves were tested for the antibacterial activity against multidrug resistant *E. coli* by standard well diffusion method (Saravanan, 2011). The pure culture of *E. coli* was grown in nutrient broth at 37° C for 18-24 hours. Wells were made on the Mueller- Hinton agar plates using a gel puncture and the plates were inoculated by swabbing the bacterial pathogens to create a confluent lawn of bacterial growth. The different concentrations of biosynthesized AgNPs solution (25, 50 and 75 µL) were poured into the corresponding well using a micropipette and negative control 75 µL of $AgNO_3$ and *C. dentata* $AgNO_3$ without treatment of plant extract. After an appropriate incubation time (at 37° C for 24 hours), the diameter of the growth inhibition zone of each well was measured. The experiment was done in triplicates**.**

Fig. 1. Habit of *Clausena dentate*

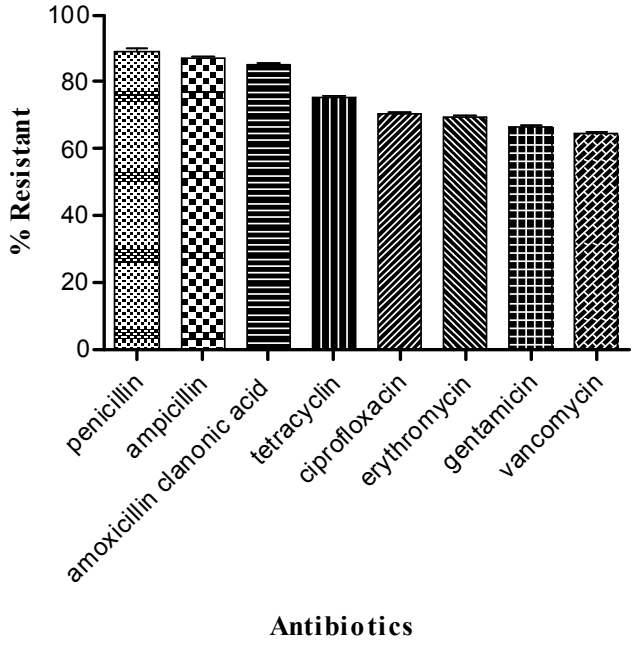

Fig. 2. Antibiotics resistant patterns of *C. dentata*

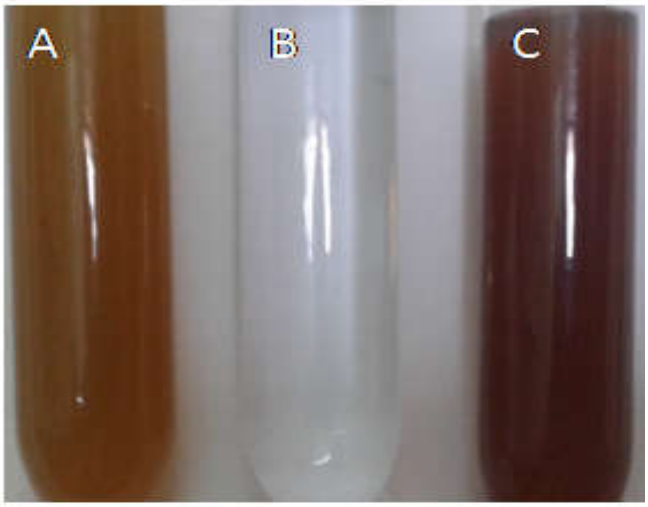

Fig. 3. Biosynthesis of nanoparticles (A) *C. dentata* leaf extract (B) $AgNO_3$ (C) $AgNO_3$ treated with *C. dentata* leaf extract.

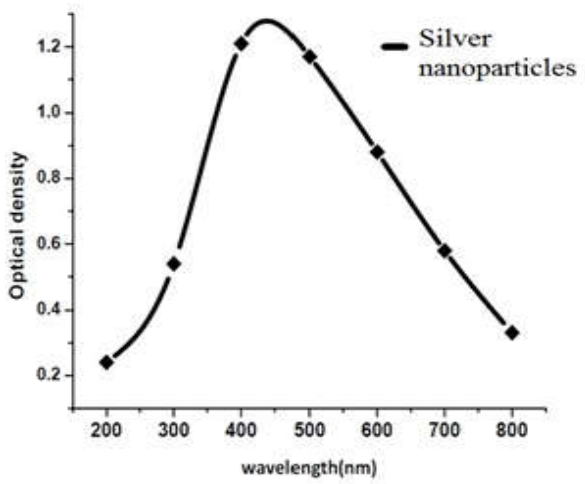

Fig. 4 UV- vis spectrum of nanoparticles from *C. dentate*

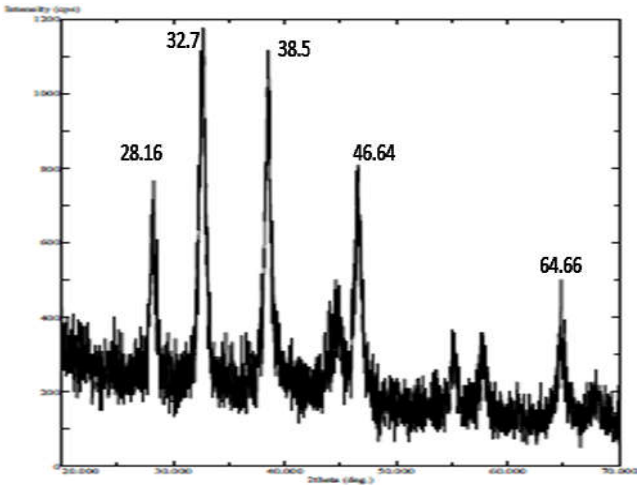

Fig. 5. XRD pattern of nanoparticles synthesised from *C. dentata* leaf extract

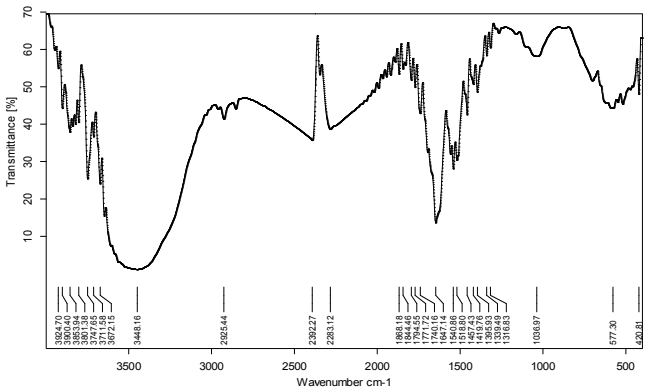

Fig. 6. FTIR spectrum of *C. dentata* leaf extract

RESULTS

Isolation and identification of *E. coli* strains: A total of 500 clinical samples was used for isolation of *E. coli* using different selective medias and perform biochemical reactions. A total of 85 *E.coli* individuals was isolated from all samples. The urine samples have maximum percentage of *E. coli* isolates followed by other samples (Table 1). Based on the morphology and biochemical characterization (green metallic sheen, shown lactose fermentation, microscopic, biochemical and sugar fermentation tests) of all *E. coli* isolates shown positive results (Table 2).

Table 1. Bacterial Isolates (*E. coli)* from different clinical samples

S. No	Clinical Sample	Number of target bacterial isolates	Percentage of isolates (%)
1	Urine	60	70.58
2	Blood	12	14.11
3	Wound	08	09.41
4	Sputum	03	03.52
5	Pus	02	02.35

Table 2. Morphological and biochemical characters of *E. coli* isolates

S. No	Morphological characters of *Escherichia coli* isolate	
1.	Colony shape	Mucoid colony
2.	Colony color	Dark blue-black colony
	Microscopic characters	
1.	Gram staining	Gram negative (Rod shape)
2.	Motility	Non-motile
3.	Spore staining	Non-spore former
	Biochemical tests	
1.	Catalase	+
2.	Indol	+
3.	Methyl red	+
4.	Voges, P test	–
5.	Urease test	–
6.	Citrate utilization	–
7.	Oxidise	–
	Acid from sugars	
1.	Glucose	+
2.	Lactose	+
3.	Manitol	+
4.	Sucrose	+

-, Positive for production, +, Negative for production

Table 3. Antibacterial activity of *C. dentata* mediated AgNPs on MDR *E. coli*

sample	(Diameter of growth inhibition zone in mm)			
	Conc. (µL)	*E. coli*	$AgNO_3$ ontrol	Crude extract
Plant mediated AgNPs	25	10 ± 0.4	9±0.0	10±0.0
	50	25 ± 0.8	9±0.2	10.1±0.6
	75	32 ± 0.4	9±0.4	10±0.9

Results were expressed as mean ± SD. n=3

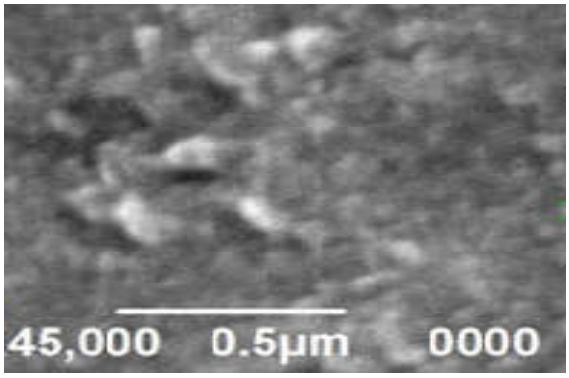

Fig. 7 Scanning Electron Microscopy analysis of nanoparticles from *C. dentata* leaf extract

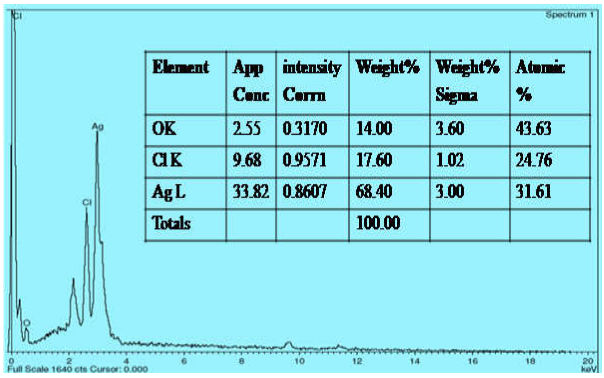

Fig. 8 EDX spectrum of AgNPS from *C. dentata* leaf extract

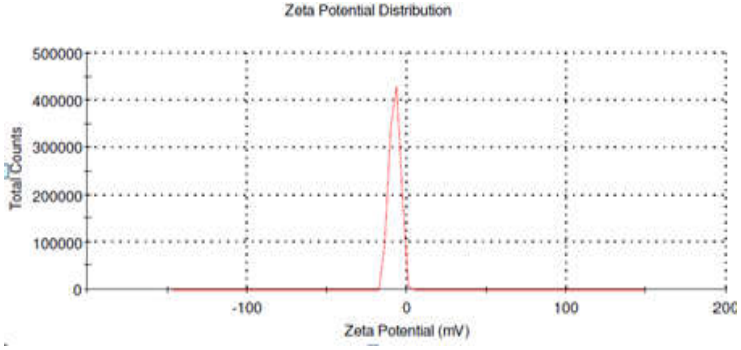

Fig. 9. Zeta potential distribution of bio-synthesized Ag NPs from *C. dentata*

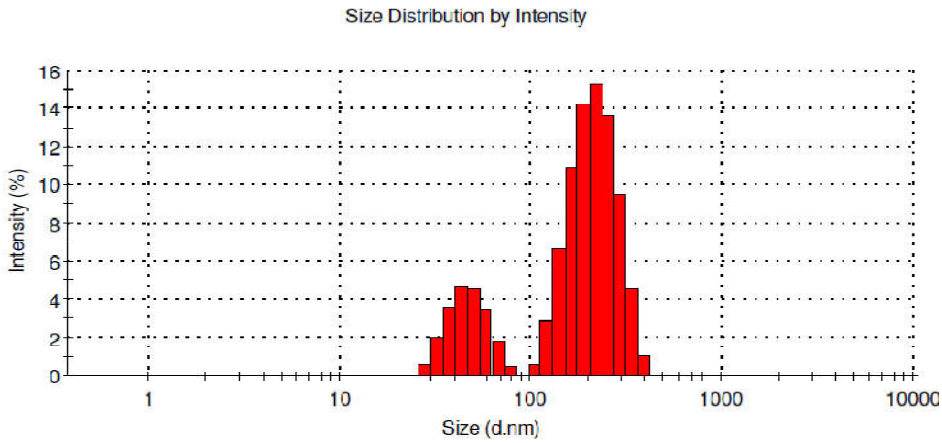

Fig. 10. Percentage intensity of particle size distribution of biosynthesized Ag NPs

Fig. 11. Antibacterial activity of plant extract against MDR *E. coli* (1) Treated with $AgNO_3$ (NPs 1) 25µl, (NPs 2) 50µl, (NPs 3) 75µl and (AB) Antibiotics less growth of inhibition (2) Plant extracts and $AgNO_3$ control (75µl)

Antibiotics susceptibility/resistant patterns of *E. coli* isolates: Antimicrobial susceptibility/resistant pattern of *E. coli* isolates were tested against the selected antibiotics and the results show high resistance was observed in penicillin (90%), ampicillin (85%), amoxicillin clanonic acid (82%) and tetracycline (70%) and ciprofloxacin (65%) respectively (Fig. 2).

Biosynthesis of silver nanoparticles: The biosynthesis of silver nanoparticles from *C. dentata* leaf extract has been observed by colour changes from green to yellowish-brown indicates the formation of silver nanoparticles. i.e. the reduction of the Ag+ ions (Fig. 3).

Characterisation of silver nanoparticles

UV- visible spectroscopy: The synthesis of silver nanoparticles were preliminary confirmed by the color change due to surface plasmon resonance of silver nanoparticles in the visible region. The absorbance intensity of the brown color increased steadily due to activity of reaction time. The maximum absorption was measured between the wavelength of 430 - 460 nm (Fig. 4) that which clearly indicates the formation of silver nanoparticles.

XRD analysis: The X-ray diffraction pattern of the biosynthesized silver nanostructure produced by the leaf extract was further demonstrated and confirmed by the characteristic peaks observed in the XRD image (Fig. 5).

The XRD pattern results observed a number of Bragg reflections with 2θ values of 28.16, 32.7, 38.5, 46.64 and 64.66 sets of lattice planes.

FTIR analysis: The characterization of plant extract and the silver nanoparticles were analyzed by FTIR (Fig. 6). The absorbance bands analysis in bio-reduction was observed in the region of 400-4000cm $^{-1}$ and peaks values are 1036.97, 1395.93, 1540.93, 1647.14, 1794.56 and 1740 cm-1. The observed peaks denotes that cyclic C-X, C-N, N=O, C=N, and C=O functional groups of the sample.

SEM analysis: The SEM analysis result show AgNPs were observed in the uniform size and the shape. The results of nanoparticles were spherical in shape with the size of 40-50nm. The surface deposited silver nanoparticles are clearly seen as the cubic crystalline silver.

EDX profile: The EDX profile of Ag nanoparticles showed strong signals for silver atoms (Fig. 8). The EDX pattern clearly shows that the Ag nanoparticles are crystalline in nature, which is developed by the reduction of silver ions using plant leaf extract. The EDX analysis confirmed the presence of silver nanoparticles (*C. dentata*) and shown strong signal energy peaks for silver atoms in the range 2–4 keV.

Zeta Potential measurement: A zeta potential measurement was used to determine the surface potential of the silver nanoparticles.

The obtained nanoparticles, zeta values were measured and found to be 7.39mV with a peak area of 100% intensity (Fig. 9).

Particle size analyser: Particle size analysis of plant-mediated $AgNO_3$ showed the presence of nanoparticles with polydispersity indices (PDI) value of 0.462 with intercept 0.799 **(**Fig. 10). The average particle size (z-average) was observed in 246.4nm.

Antibacterial activity of green synthesised $AgNO_3$ using *C. dentata* leaf extract: The antibacterial effects of plant-mediated AgNPs at three different concentrations (25, 50 and 75 µL) were assessed on the basis of growth inhibition zone (Table 3, Fig. 11). The AgNPs exhibited potent antibacterial activity against multi-drug resistant *E.coli* and produced the maximum zone of growth inhibition (32 ± 0.4 mm in diameter) compared with $AgNO_3$. The *C. dentata* leaf extract alone produce least inhibition zone against the organism tested (10 ± 0.8 mm). This study clearly indicates that green synthesised silver nanoparticles have better antibacterial activity against the tested *E. coli.*

DISCUSSION

Urinary tract infection is one of the common disease of human beings caused by *E.coli* and need of medical attention (Den Heijer *et al*., 2010). The extensive use antibiotics to treat against infection have led to an increasing resistance in *E. coli.* (Johnson *et al*., 2008). The outcome of this study, we found *E. coli* show high resistance against to Penicillin, Ampicillin, amoxicillin clanonic acid, tetracycline and ciprofloxacin antibiotics, respectively. The synthesis of silver nanoparticles using leaves extract of *C. dentata* confirmed by observing its colour changes. Silver nanoparticles produce dark yellowish-brown color aqueous solution due to the surface Plasmon resonance phenomenon.

The synthesised nanoparticles were primarily characterized by Uv-Vis spectroscopy, reduction of silver ions in the aqueous solution from of the silver complex during the reaction, indicates the formation of silver nanoparticles (Shankar, 2004). Absorption spectra of silver nanoparticles formed in the reaction media have a strong absorbance peak (at 430 to 460 nm) indicates the particles are polydispersed. The results were supported by several researchers, who made the characterization of plant mediated nanoparticles using X-ray diffraction to confirm the crystalline nature of the particles **(**Huang *et al*., 2007; Udayasoorian *et al*., 2011). Results of XRD spectrum of present study reflects the silver particles formed as evidenced by the peak at 2 theta values of 28.16, 32.7, 38.5, 46.64 and 64.66 respectively. The X- ray diffraction results clearly indicate that the silver nanoparticles formed by the reduction of Ag ions by the *C. dentata* leaf extract and shown crystalline in nature. The silver nanoparticles were formed cubical with uniform shape. Xu and Kall (Xu, 2002) explained the shape of metal nanoparticles of the sample can be considerably changes based on their optical and electronic properties. The FTIR analysis result show the peaks corresponding to the presence of cyclic C-X, C-N, N=O, C=N, and C=O functional groups. The SEM image analysis of sample reports the spherical shape of the silver nanoparticles (ranging from 40–50 nm). Energy dispersive spectrometry (EDS) micro-analysis of AgNPs was performed by measuring the energy and intensity distribution of X-ray signals. Results of EDS spectra, clearly shown the silver nanoparticles reduced by *C. dentata* with strong signal energy peaks for silver atoms in the range of 2–4 keV. Study of particle size, distribution and zeta potential values of silver nanoparticles are important because of its association with other characterization of particles, such as saturation solubility and dissolution velocity, physical stability, or even biological performances (Soni *et al*., 2012). Present result showed the zeta values of nanoparticles from samples were found to be -7.39mV with a peak area of 100% intensity, using photon correlation spectroscopy. The size of the colloidal silver nanoparticles, their granulometric distribution has been recorded based on the particles number and their occupied volume (Sivaraman *et al*., 2013). The average particle size (z-average) has shown in 246.4 nm. The green synthesised silver nanoparticles have been demonstrated for antibacterial properties against target bacteria *E. coli* shown better inhibitory effect than crude plant extracts, because the nanoparticles are closely attached with the microbial cell and the activity begin size dependent (Carvalho, 1991). Another study supports the leaf extract of *Mentha piperita* (lamiaceae) is a very good bioreductant for the nanoparticles synthesis and active against clinically isolated human pathogen *E. coli* (Mubarak *et al*., 2011). The present study highlights that the antibacterial effect of *C. dentata* mediated AgNPs solution clearly indicates that silver nanoparticles have good antibacterial activity against multi-drug resistant *E. coli* (with the highest zone of growth inhibition 32 ± 0.4mm) than other extracts.

Conclusion

This study deals with isolation, identification and multi-drug resistant pattern of *E. coli* against commercially available antibiotics. Silver nanoparticles were synthesized by *C. dentata* leaves extract. The spectroscopic characterization of AgNPs (UV-visible, XRD, FTIR, SEM and zeta potential) supports the stability of the biosynthesized nanoparticles (average size of 40-50nm). The green synthesized nanoparticle from *C. dentata* found to be better and more effective against multi-drug resistant *E. coli*.

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