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Antibacterial Activity of Synthesized Silver Nanoparticles from *Tinospora cordifolia* against Multi Drug Resistant Strains of *Pseudomonas aeruginosa* Isolated from Burn Patients

Khushboo Singh[1], Manju Panghal[1], Sangeeta Kadyan[1], Uma Chaudhary[2] and Jaya Parkash Yadav[1]*

[1]*Department of Genetics, M.D. University, Rohtak-124001, Haryana, India*
[2]*Department of Microbiology, Post Graduate Institute of Medical Sciences Rohtak-124001, Haryana, India*

Abstract

In the present study, antibacterial activity of silver nanoparticles synthesized from stem of *Tinospora cordifolia* were analysed against multidrug-resistant strains of *Pseudomonas aeruginosa* isolated from burn patients. As *Pseudomonas aeruginosa* is a scourge of hospital burn units and its emergence as multidrug-resistant strains is a major problem in the control of nosocomial infections. Therefore, we tried to establish a combination of medicinal values of *Tinospora cordifolia* and nanotechnology possibly with the field of medicine for the development of antibacterial agents against these MDR strains.The synthesized silver nanoparticles were characterized by UV-visible spectroscopy, Energy Dispersive Spectroscopy and Fourier Transform Infrared Spectroscopy. Transmission Electron Microscopy and X-Ray Diffraction have revealed the size of silver nanoparticles 9 ± 36 nm and 12.49 nm respectively. Further antibacterial activity of silver nanoparticles prepared from *Tinospora cordifolia* against multidrug resistant strains was determined by agar well diffusion assay and Minimum Inhibitory Concentration (MIC) was estimated by qualitative experimentation by resazurin based micro broth dilution method. All experiments were done in triplicate. The silver nanoparticles of stem of *Tinospora cordifolia* showed the zone of inhibition ranges from 10 ± 0.58 to 21 ± 0.25mm. The MIC of AgNPs from stem extract was found to be 6.25 to 200 µg/ml against *Pseudomonas aeruginosa* strains. Silver nanoparticles from *Tinospora cordifolia* possess very good antibacterial activity which makes them a potent source of antibacterial agent.

Keywords: *Tinospora cordifolia*; Silver nanoparticles; Burn patients; MDR; Antibacterial

Introduction

Pseudomonas aeruginosa is an opportunistic pathogen capable of causing nosocomial infections. Patients with burns are at extraordinary risk of acquiring *P. aeruginosa* infection of the burn wound with subsequent septicaemia and death because burn injury disrupts both the normal skin barrier and many of the systemic host defence mechanisms, which makes skin susceptible to microbial colonization resulting in development of burn wound sepsis [1]. Also *P.aeruginosa* is a scourge of hospital burn units [2]. This is also justified by various studies [3,4]. *P. aeruginosa* is naturally resistant to a significant number of antimicrobials and this resistance of *P. aeruginosa* to commonly used therapeutic agents has increased in recent years [5]. In case of burn patients, the burned skin remains vulnerable to invasive microbial infections of all kinds until complete epithelial repair has occurred. Microbial drug resistant is emerged as a major problem in health care industry as microbes involve in the change of their metabolism and genetic structure to acquire resistant against the drugs used in the treatment of infectious disease. MDR can be defined as resistance to at least four classes of antibiotics used during treatment of these infections. Emergence of MDR strains is often may due to selective pressure of antimicrobial therapy [5].

These drug resistant pathogens are more pathogenic with high mortality rate than that of wild strain. To overcome microbial drug resistant, scientists are looking forward for the development of alternative and novel drugs. Nanotechnology is expected to open some new aspects to fight and prevent diseases using atomic scale tailoring of materials [6]. Nanotechnology is currently employed as a tool to explore the darkest avenues of medical sciences because silver nanoparticles have been well known for its strong inhibitory and bactericidal effects and can effectively used for the treatment of various infectious diseases [7]. Therefore antimicrobial silver is now used extensively to combat organisms in wounds and burns. It works because pathogens cannot mutate to avoid the antimicrobial effect of silver. The importance of silver ions has been also found in the treatment of burn wound by various researchers who studied the antimicrobial properties of silver nanoparticles against virulent pathogens. The effect of the nanoparticles was found to be significantly more pronounced on MDR strains [6].

In the way of nanotechnology, many researchers demonstrated the green synthesis of silver nanoparticles including bacteria, actinomycetes, fungi and plants. The plant materials have been successfully used for silver nanoparticles synthesis, due to their potential medicinal property, huge availability, faster rate of synthesis [8,9]. *Tinospora cordifolia*, an important medicinal plant,is a diploid (2n=22), deciduous climbing shrub belonging to family Menispermaceae. In ancient traditional Ayurvedic system of India, it is a constituent of several remediation used for various treatment such as general debility, dyspepsia and urinary diseases [10]. So keeping in view the advantage of silver nanoparticles synthesized from medicinal plants, our study is based on the synthesis of silver nanoparticles from *T. cordifolia* and checked its antibacterial activity against MDR strains of *P. aeruginosa*.

***Corresponding author:** Jaya Parkash Yadav, Department of Genetics, M D University, Rohtak-124001, Haryana, India, Tel: 91-1262-393055; E-mail: yadav1964@rediffmail.com

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Material and Methods

Preparation of the plant extract

The stem of *Tinospora cordifolia* was collected locally from Botanical Garden, M.D. University, Rohtak, Haryana, India. It was thoroughly washed in distilled water, cut into fine pieces. 15 g of fresh plant material was boiled into 100 ml sterile distilled water and filtered through Whatman`s No.1 filter paper. The extract was stored at 4°C for further experiments.

Synthesis of silver nanoparticles from plant extract

The aqueous solution of 1 mM silver nitrate ($AgNO_3$) was prepared and used for the synthesis of silver nanoparticles. 15 ml of plantextract was added into 200 ml of aqueous solution of 1 mM silver nitrate for reduction into Ag^+ ions and kept for 15-20 minutes at 70-75°C. This aqueous extract acts as reducing and stabilizing agent for 1mM of $AgNO_3$. The prepared AgNPs were further characterized.

Characterization techniques

The techniques used for characterization were as follows:

UV-VIS spectroscopy: The Ag nanoparticles were characterized in a Shimadzu UV-VIS Spectrophotometer. The scanning range for the samples was 300-800 nm. The double distilled water used as a blank reference.

Fourier Transform Infra-red Spectroscopy (FTIR): To remove any free biomass residue or compound that is not the capping ligand of the nanoparticles, after complete reduction, silver nanoparticles were concentrated by repeated centrifugation (3 times) of the reaction mixture at 15,000 rpm for 20 min. The supernatant was replaced by distilled water each time. Thereafter, the purified suspension was freeze dried to obtain dried powder. Finally, the dried nanoparticles were analysed by ALPHA FT-IR Spectrometer (from Bruker, Germany) for the detection of different functional groups by showing peaks from the region of 4000 cm^{-1} to 500 cm^{-1}.

Transmission Electron Microscopy (TEM) and Energy Dispersive X-Ray Spectroscopy (EDX): The shape and size of AgNPs was determined by transmission electron microscopy. A drop (2 µl) of water that dissolved synthesized nanoparticles was placed on a copper grid. The images were obtained with a Tecnai, Twin 200 KV (FEI, Netherlands) at a bias voltage of 200 kV used to analyse samples. The composition of the silver nanoparticles was determined using the EDX coupled to the TEM.

X- Ray Diffraction (XRD): The X-ray diffraction data were obtained by X-Pert Pro Diffractometer using step scan technique and with Cu-Ka radiation (1.500 Å, 40 kV, 30 mA) in h–2h configuration. The metal nanoparticles were coated on to the glass substrate and after drying the sample was analysed by X-ray diffractometer. The crystallite domain size was calculated using the Debye-Scherrer formula.

Multi drug resistant *P. aeruginosa* from clinical isolates: Twenty*P. aeruginosa*isolates were obtained from the various samples of burn patients receiving in Microbiology Department of Post Graduate Institute of Medical Sciences, Rohtak. The purity and identity of each isolate was confirmed in laboratory by standard microbiological methods [11-13]. The sources of the clinical isolates were urine, wounds, blood, and body fluids of burn cases. The ATCC 27853strain of *P. Aeruginosa* was served as positive control.

The 10 most cost-effective antibiotics routinely used to treat *P. aeruginosa* infections were employed in the susceptibility test. The antibiotics included were amikacin, aztreonam, ceftizoxime, cefepime, gentamicin, imipenem, netilmicin, ofloxacin, piperacillin and tazobactum. For isolation of MDR strains, these antibiotics were used and susceptibility was checked by Kirby-Bauer disc method [14]. The strain which were resistant to 6 or 7 antibiotics was taken as MDR strain.

Antibacterial assay of silver nanoparticles

Preparation of test samples: 4 Test samples of the AgNPs were prepared in DMSO (Dimethyl Sulfoxide). The concentration ranges from 12.5-200 µg/ml i.e. 12.5, 25, 50, 100, and 200 µg/ml.

Antimicrobial bioassay: The antimicrobial activities were determined by modified agar well diffusion assay [15]. Under aseptic conditions, in to the Bio safety chamber, 20 ml of MHA medium was dispensed in to pre-sterilized petridishes. Once the media solidifies it was then inoculated with micro-organism suspended in peptone water. The media was then punched with 6mm diameter hole and filled with different dilutions (varying from 2.5 to 20 µl) of AgNPs extract from stock of 20 mg/ml. Streptomycin discs for bacteria (10 µg/disc) were used as positive controls and DMSO was used as a negative control. Finally, the petridishes were incubated for 24 hours at 37°C. The diameter of zone of inhibition as indicated by clear area which was devoid of growth of microbes was measured. Each experiment was done in triplicate.

Minimum inhibitory concentration method (MIC)

This method is based on a micro broth dilution method in 96 multi-well microtitre plates with slight modifications [16]. Qualitative experimentation by resazurin indication solution prepared by dissolving a 270 mg tablet in 40 ml of sterile distilled water. Indicator resazurin of purple color reduced in the presence of living bacteria. Color change from purple to pink or to colorless. In the absence of living bacteria the color of the indicator were remain purple. The lowest conc. at which color change occurred was taken as MIC.

Result and Discussion

Synthesis of AgNPs

The green synthesis of silver nanoparticles through plant extracts were carried out. On mixing the plant extract of *T. cordifolia* with silver nitrate solution (1mM), a change in the colour from pale yellow to dark brown was observed (Figure 1). Similar results were also reported by many researchers [17-19]. The brown colour confirms that it was due to the reduction of Ag^+ which indicates the formation of Ag nanoparticles.

Characterization of Ag Nanoparticles

UV–VIS spectral analysis: In our results peak specific for the synthesis of silver nanoparticles was obtained at 420-425 nm by UV-Visible spectroscope in the form of a sharp peak (Figure 2), which was specific for the synthesis of AgNPs. It is well known that colloidal silver nanoparticles exhibit absorption at the wavelength from 390 to 420 nm due to Mie scattering [20]. Hence, the band at 420-430 nm can be attributed to the property of Mie scattering. This may not include the protecting agent, because the Mie scattering responds only to the silver metal [21].

FTIR analysis: The aim of IR spectroscopic analysis is to determine chemical functional groups in the sample. The amide linkages between amino acid residues in polypeptides and proteins give rise to well-known signatures in the infra-red region of the electromagnetic

Figure 1: Picture shows the colour change (a) Before (b) After the reduction of Ag+ into AgNPs of *T. cordifolia*.

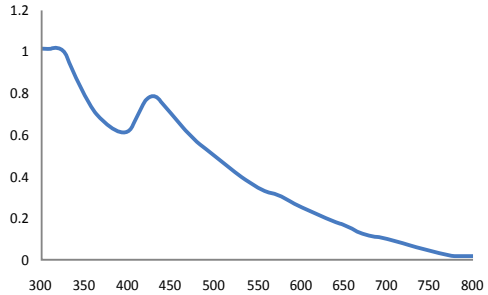

Figure 2: UV-VIS absorption spectra of AgNPs of *T. cordifolia* at a range of 300-800 nm.

Figure 3: FTIR spectra of vacuum dried powder of synthesized AgNPs of *T. cordifolia* from stem.

spectrum.

Different functional groups absorb characteristic frequencies of IR radiation. Thus, IR spectroscope is an important and popular tool for structural elucidation and compound identification. Our observation confirms the presence of such compounds in the sample which coat covering the silver nanoparticles known as capping agents. FTIR analysis of AgNPs from stem of *T. cordifolia* has been shown in Figure 3. FTIR showed the presence of bands due to O-H stretching due to (3,285cm^{-1}) vibration of the alcoholic compounds, aldehydic C–H stretch (2,916 and 2,849 cm^{-1}), C-O stretch (1,643cm^{-1}) arises from carbonyl group, N-O, C-C (1,361 and 1,337 cm^{-1}) and C-O stretch (dialkyl) (1,149 cm^{-1}), C-N (1,077 cm^{-1}), C-H (761 cm^{-1}). Similar kind of results has been showed by many researchers too [18,19] in FTIR analysis of AgNPs of *T. cordifolia*. This study gives the evidence of formation and stabilization of silver nanoparticles in the aqueous medium by using biological molecules.

TEM analysis: TEM confirmed the development of silver nanostructures and gave clear image of silver nanoparticles. We observed that the shapewas spherical and sizeof silver nanoparticles was 36 ± 9 nm synthesized from *T. cordifolia* (Figure 4).

EDX analysis: EDX characterization has shown absorption of strong silver signal along with other elements, which may be originate from the biomolecules that are bound to the surface of nanosilver particles.From EDX spectra, shown in Figure 5, it is clear that silver nanoparticles reduced by *T. cordifolia.*

XRD analysis: The crystalline nature of the silver nanoparticles was carried out by XRD. The XRD pattern was ranging from five strong peaks were observed at 10.7, 11.15, 14.5, 7.03, 9.3, 11.2, 10.5, 23.8 and 14.3that corresponds to the planes, which are indexed to the face

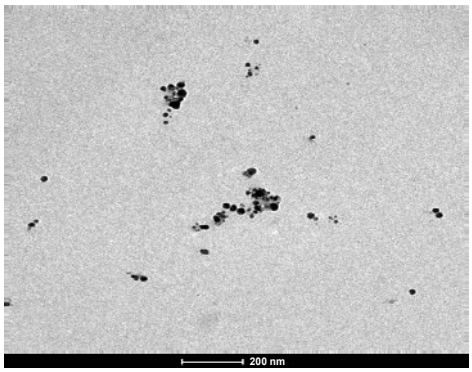

Figure 4: Image of transmission electron microscopic observation of synthesized silver Nanoparticles of *T. cordifolia* from stem.

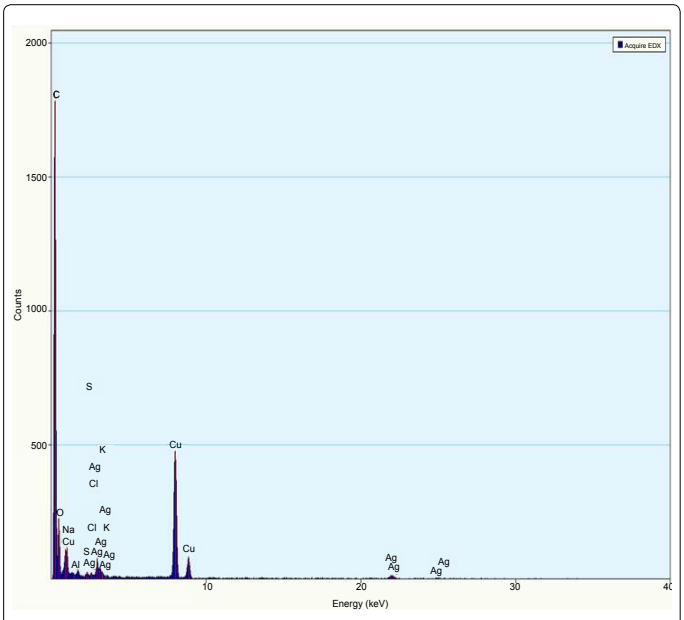

Figure 5: Image of EDX of synthesized silver Nanoparticles of *T. cordifolia* from stem.

S.No	2-theta(deg)	D (ang.)	FWHM (deg)	Int. I (cps deg)	Int. W(deg)	Size(nm)
1	28.006	3.183	0.796	189	0.949	10.7
2	32.237	2.774	0.774	387.58	0.929	11.1
3	38.191	2.354	0.601	856.65	0.979	14.5
4	44.347	2.040	1.273	220.52	1.541	7.03
5	46.209	1.962	0.963	211.25	1.113	9.3
6	54.771	1.674	0.829	65.73	1.108	11.2
7	57.517	1.601	0.893	73.6	1.270	10.5
8	64.578	1.441	0.411	175.65	0.766	23.8
9	77.352	1.232	0.738	209.36	1.083	14.3

Table1: Measurement of the size of AgNPs of *Tinospora cordifolia by* using Debye-Scherrer's equation.

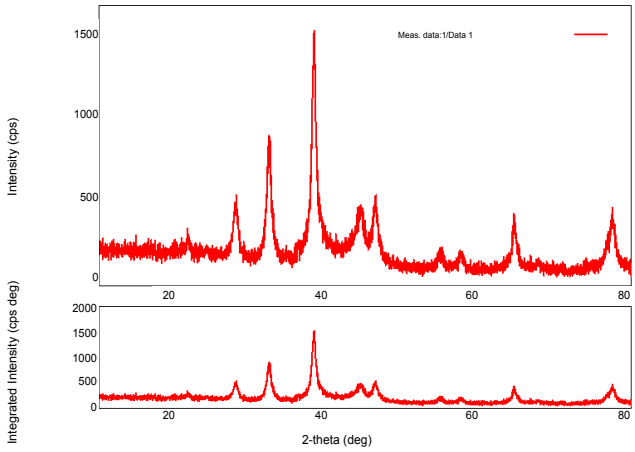

Figure 6: XRD diffraction pattern of synthesized AgNPs of *T. cordifolia*.

centred cubic structures of silver nanoparticles. The synthesis of silver nanoparticles with sharp bands of Bragg peaks, and this might be due to the stabilization of the synthesized nanoparticles by the various reducing agents of the *T.cordifolia*, and thus provides the crystallization nature of the silver nanoparticles [22]. The mean size of silver nanoparticles was calculated using the Debye-Scherrer's equation. An average size of the silver nanoparticles synthesized by *T. cordifolia* was 12.49 nm with size ranging from 9.3 nm and 23.8 nm (Table 1). XRD diffraction pattern of synthesized AgNPs of *T. cordifolia* was shown in Figure 6.

Antibacterial activity of AgNPs against MDR strains of *P.aeruginosa*

The inhibitory action of silver compounds and silver ions had been historically recognized and applied as a useful therapeutic agent for preventing wound infections. The inhibitory action of silver on bacterial cells is related to the strong interaction of silver with thiol groups present in key respiratory enzymes in bacteria [23]. Whereas, Nano crystalline silver shows the most effective inhibitory action with a rapid inhibition rate [24]. In the present study *T. cordifolia* was taken for synthesis of AgNPs because of its medicinal values. Various studies have been done by many researchers which confirm that *T. cordifolia* was found to be good antibacterial agentagainst pathogenic and non-pathogenic organisms [25-28]. The antibacterial effects of the bio-synthesized silver nanoparticles from *T. cordifolia* were also successfully investigated [19,29]. Also there are various reports which have been providing the evidences that silver nanoparticles were used as powerful tool against multidrug-resistant bacteria [30,31]. Kora and Arunachalam [32] showed that silver nanoparticles synthesized by UV photo-reduction method are showing promising antibacterial activity on *P. aeruginosa* at much lower concentrations. As these above reports suggests that both silver nanoparticles and *T. cordifolia* plant extract have shown good amount of antibacterial activity. Durairaj et al.[33] studied the antibacterial activity of purchased AgNPs (size 20-30nm) against 10 isolates of *P. aeruginosa* comprising of 5 MDR strains with an inhibition zone of 11 mm observed with10 µg dose of the nanoparticles. The nanoparticles exhibited MIC of50 µg/ml when added at the lag phase and the sub inhibitoryconcentration was measured as 100 µg/ml. In our experiment, when we compared the antibacterial activity of AgNPs and plant extract, it was found that silver AgNPs have shown more antibacterial activity than plant extracts. Our results clearly shows that the conventional plant extract showing some antibacterial activity but not much activity as AgNPs does against these MDR bacterial strains (Table 1), even taken in amount10 times more than AgNPs (Figure S1). It clearly indicates that these green AgNPs have shown considerable amount of activity than that of plant extract. Antibacterial activity of AgNPs significantly increases by more than 12-15% at very lower concentration i.e. almost 18 out of 20 strains have shown zone of inhibition comes at concentration of 4 mg/ml in case of plant extract whilein case of AgNPs 200 µg/ml is the highest concentration we have used. Therefore we further proceeds only with the results of antibacterial activity of AgNPs.

Also if we use synthetic or purchased AgNPs then there may be a problem due to chemical agents used. So we synthesized AgNPs by plant extract of *T. cordifolia* which potentially eliminate the problem of chemical agents that may arise if we used any synthetic or chemically synthesized AgNPs, thus making nanoparticles biocompatible with the eco-friendly approach. The antibacterial efficacy of synthesized AgNPs enhances because the use of silver and *T. cordifolia*, as silver reduced in nano form which increases its surface area, thus make AgNPs more reactive and *T. cordifolia* enhances the therapeutic efficacy of AgNPs due to its good antibacterial efficacy. Therefore, in our study, AgNPs prepared by *T. cordifolia* were used for the development of antibacterial agents against MDR strains of *P. aeruginosa*.

We have checked the antibacterial activity of AgNPs by agar well diffusion method against twenty MDR strains of *P. aeruginosa* from burn patients. In our experiment, biosynthesized AgNPs showed excellent antibacterial activity against MDR strains of *P. aeruginosa* (Figure 7). Our results showed that AgNPs synthesized from *T. cordifolia* possess discrete antibacterial activity at different concentrations of 6.25-200 µg/mL (Table 2). The zone of inhibition ranges from 10 ± 0.58 to 21

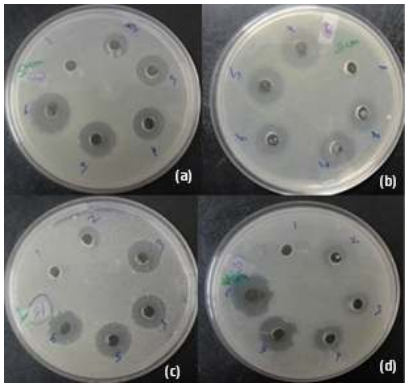

Figure 7: Showing antibacterial activity of AgNPs of *T. cordifolia* against MDR strains of *P. aeruginosa* from burn patients (a) MDR strain 2 (b) MDR strain 6 (c) MDR strain 15 (d) MDR strain 20.

P. aeruginosa MDR strains	Zone of Inhibition (in mm) at different AgNPs conc.					+ve control (Streptomycin)
	12.5 µg/ml	25 µg/ml	50 µg/ml	100 µg/ml	200 µg/ml	10 µg
1	-	-	11 ± 0.58	12 ± 2.30	17 ± 0.58	12 ± 0.58
2	10 ± 0.58	11 ± 1	15 ± 1	16 ± 0.58	19 ± 0.29	13 ± 0.14
3	10 ± 0.58	11 ± 0.46	11 ± 0.34	13 ± 0.17	14 ± 0.5	12 ± 1
4	-	-	-	-	10 ± 1	15 ± 0.77
5	-	10 ± 0.58	12 ± .06	13 ± 0.11	16 ± 0.29	14 ± 0.33
6	11 ± 0.55	13 ± 0.46	17 ± 0.34	18 ± 0.55	20 ± 0.58	12 ± 0.64
7	-	-	-	-	10 ± 0.46	15 ± 1
8	-	-	-	-	12 ± 0.5	16 ± 1
9	10 ± 0.58	10 ± 1	11 ± 0.34	12 ± 0.29	13 ± 0.58	11 ± 0.41
10	-	10 ± 0.55	11 ± 0.58	14 ± 0.50	15 ± 0.46	13 ± 0.28
11	-	-	-	-	15 ± 0.29	14 ± 1
12	-	10 ± 0.58	10 ± 1	13 ± 0.46	14 ± 0.58	13 ± 1
13	-	-	-	-	12 ± 0.58	15 ± 1
14	-	-	-	-	10 ± 1	16 ± 0.48
15	-	13 ± 1	17 ± 0.58	17 ± 0.33	18 ± 0.58	12 ± 0.33
16	-	13 ± 1	12 ± 0.58	11 ± 0.33	10 ± 0.58	12 ± 0.33
17	11 ± 0.46	13 ± 0.58	15 ± 0.58	16 ± 41	14 ± 0.52	11 ± 0.28
18	-	-	10 ± 0.58	11 ± 1	12 ± 0.58	11 ± 1
19	11 ± 0.46	13 ± 0.58	13 ± 0.58	14 ± 1	14 ± 0.52	12 ± 0.58
20	10 ± 1	11 ± 0.33	13 ± 0.58	17 ± 0.58	21 ± 0.25	10 ± 1

Table 2: Zone of inhibition of AgNPs of *T. cordifolia* at different concentration against 20 MDR strains of *Pseudomonas aeruginosa* isolated from burn patients.

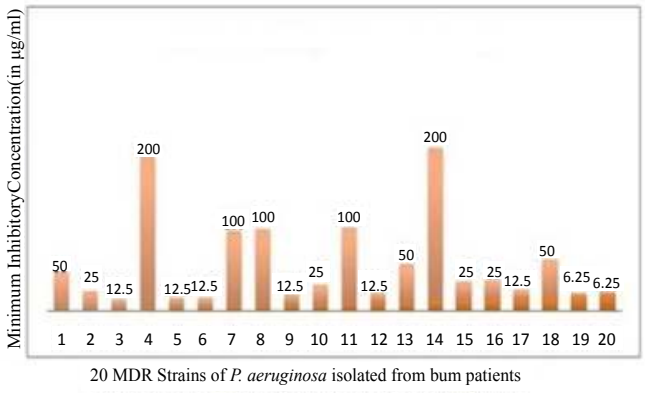

Figure 8: Minimum Inhibitory Concentration (MIC) of AgNPs of *T. cordifolia* against twenty MDR Strains of *P. aeruginosa* from burn patients.

± 0.25 mm. The highest zone of inhibition was 21 ± 0.25mm, found against MDR strain 20. Further MIC was calculated by resazurin based microtitre well plate method. The MIC of AgNPs from stem of *T.cordifolia* varies 6.25-100 µg/ml have been shown in Figure 8, was found to be strongly inhibitory against MDR strains. MDR strain 19 and 20 showed the lowest value of MIC 6.25 µg/mL while MDRstrain 4 and 14 showed the highest value MIC 200 µg/mL. The antibacterial activity of AgNPs have depicted that as we increases the conc. of AgNPs the antibacterial activity against MDR strains of *P. aeruginosa* increased parallely. The study revealed that high antibacterial activity was found against tested strains of *P. aeruginosa* at very low concentration of AgNPs (in µg/ml). Our resultsalso correlates with the work previously done by some researchers which studied the antibacterial effect of silver nanoparticles against Multi Drug Resistant strains of *P. aeruginosa* [6,33]. Afreen et al. [6] synthesized AgNPs from a fungus, *Rhizopussto lonifer* and checked its efficacy against two MDR strains isolated from burn cases from hospitals at Gulbarga region, Karnataka, India. Whereas, our study emphasises thatuse of plant extract reduces the cost of micro-organism isolation and also reducing the complicated process of maintaining the cell culture over nanoparticles synthesis by microorganisms. Durairaj et al. [33]studied the antibacterial activity of purchased AgNPs (size 20-30 nm) against 10 isolates of *P. aeruginosa* comprising of 5 MDR strains have shown significant antibacterial effect.

Moreover, when antibacterial activity of AgNPs synthesized from that plant extract was compared with the plant extract alone, it clearly shows that the conventional plant extract showing not much activity as AgNPs does against these MDR bacterial strains, even taken in amount10 times more than AgNPs (Figure S1). It clearly indicates that these green AgNPs have shown considerable amount of activity than that of plant extract. Antibacterial activity of AgNPs significantly increases by more than 12-15% i.e. zone of inhibition comes at concentration of mg/ml in case of plant extract and µg/ml in case of AgNPs. Therefore we further proceeds only with the results of antibacterial activity of AgNPs.Our method of synthesis of AgNPs was a simple, cost effective and eco-friendly method. The synthesized AgNPs of *T. cordifolia* from

Figure s1: Showing comparison of antibacterial activity of AgNPs of *T. cordifolia* with conventional plant extracts of *T. cordifolia.*

stem showed remarkable antibacterial activity against all the strains which makes them a potent source of antibacterial agent.

Conclusions

From the present study we conclude that even at very small concentration (in µg/ml) AgNPs from stem of *T. cordifolia* possess very good antibacterial activity which makes them a potent source of antibacterial agent against MDR strains of *P. aeruginosa*. Also, green synthesis of AgNPs can potentially eliminate the problem of chemical agents that may have adverse effects, thus making nanoparticles more compatible with the eco-friendly approach. Moreover the synthesized AgNPs enhance the therapeutic efficacy and strengthen the medicinal values of *T. cordifolia.* Hence, our results are promising and prove to be an important step in this direction as it decreases the burden of multidrug resistance in burn patients.

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