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ORIGINAL ARTICLE

Efficacy of green synthesis of Silver nanoparticles from Tulsi (*Ocimum sanctum*) leaf aqueous extract and its antibacterial effect on clinical multidrug-resistant *Staphylococcus aureus* in West Bengal

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Abstract

Rapid augmentation in the prevalence of multidrug-resistant (MDR) *Staphylococcus aureus* is a worldwide threat. Advising newer antibiotics may fail to reduce the chances of the emergence of newer drug-resistant *Staphylococcus aureus*. Very little shreds of evidence can be found to treat clinical MDR *Staphylococcus aureus* with biogenic silver nanoparticles (AgNPs) in West Bengal. To prepare AgNPs biogenically using aqueous tulsi leaf extract (TLE) and also to assess its antibacterial effect upon clinical MDR *Staphylococcus aureus*, biogenic synthesis of the AgNPs using aqueous TLE was done, characterized those with UV-Vis Spectrophotometer, dynamic light scattering, field emission scanning electron microscopy, Fourier transform infrared spectroscopy, and evaluated the antibacterial activity against the clinical MDR *Staphylococcus aureus*. ANOVA followed by LSD post hoc test was used to test the differences between the OD (optical density) of different experimental sets. The biosynthesized AgNPs were spherical, monodispersed, and of smaller size (9-23 nm) with the involvement of eugenol, quercetin, and oleanolic acid present in the *tulsi* leaf. A significant change in OD was observed in AgNPs (prepared using TLE) treated broth compared to only *tulsi* leaf extract treated culture. There was a significant similarity between the efficacies of AgNPs and clindamycin ($P < 0.05$). Our findings propose that AgNPs synthesized using TLE were fast and efficient to ameliorate the bacterial growth, which may be used as a potent antibacterial agent for the treatment of clinical MDR *Staphylococcus aureus* infection in near future.

Keywords: *Ag Nanoparticles; Biogenic; Clindamycin; MDR; MRSA; Staphylococcus aureus; Tulsi.*

How to cite this article

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INTRODUCTION

From the origin of the concept of nanoparticles in 1954 by eminent scientist Paul Ehlrich [1] to the 21st century, there is an immense change in the craze of using nanoparticles in research work has been observed. Biologically prepared nanoparticles have the potential to lead us to find solutions to a wide range of issues that are being encountered nowadays. Chemically reduced silver nanoparticles have an adverse effect on human health as well as it gives low yield and requires high energy [2]. As an alternative, biogenic silver nanoparticles emerged as a good antibacterial, as well as an antifungal, and anticancer agent. Apart from this, the literature suggests the use of AgNPs (Silver nanoparticles) in different sectors like clothing [3], water treatment/purification

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[4], medical devices [5], etc. Silver nanoparticles can be prepared from various sources like neem, tulsi, aloe vera, etc [1]. A review published recently in 2020 [1], from our laboratory, has observed that very less evidence can be found about the antibacterial outcomes of green silver nanoparticles upon clinical multidrug-resistant (MDR) *Staphylococcus aureus* in the last decade in India, especially in West Bengal.

Staphylococcus aureus is a common human pathogen having commensal relations with the human body [6]. The emergence of multidrug-resistant *Staphylococcus aureus* especially MRSA methicillin-resistant *Staphylococcus aureus*) & VRSA (vancomycin resistant *Staphylococcus aureus*) is the global concern at this time. The prevalence of MRSA in West Bengal is 11% to 56%, as reported earlier in our study [7]. The mortality of the infections caused due to MRSA is approximately 31-63% for bacteremia [8-9], 24-57% for infective endocarditis [10-11], 10% for pneumonia [12], and 21-29% for common MRSA infections [13-14]. *Staphylococcus aureus* is raising questions about the use of vancomycin in the challenging treatment of MRSA infection, as the bacteria are becoming resistant to vancomycin itself, emerging as VRSA [15]. *Staphylococcus aureus* can adapt itself in the hospital environment and can make humans their host [16]. The enormity of this problem demands an effective treatment for MDR (multidrug-resistant) *Staphylococcus aureus* infection, which is emerging as MRSA and VRSA, reducing the efficiency of methicillin and vancomycin.

Shambat *et al.,* published an article in 2012 described the highly diverse group of STs (Sequence Types) and CC (Clonal Complex) for *Staphylococcus aureus*. As per sequence typing is concerned, they found different variety of *Staphylococcus aureus* like ST22, ST772, ST8, ST672, ST291, ST30, ST121, ST1208, ST199, ST45. Whereas they have also identified 12 various CC for *Staphylococcus aureus* [17]. For any bacterial population, phylogenetic lineage is not random at all the evolution of any bacterial population majorly depends upon the use of different antibiotics as well as horizontal transfer of genes [6, 18].

Though there is a lack in the knowledge of the mode of action of the nanoparticles targeting bacteria to kill [19-20]. Literature suggests that the nanoparticles can target different biomolecules of the bacteria to eliminate the same [19]. Nanoparticles, due to their special physical characteristics (smaller size & surface to volume ratio) are efficiently able to penetrate the cell wall of the bacteria, produce ROS (reactive oxygen species), and also to disturb the transcription and translation process inside the cell [21]. It has been seen that nanoparticles have bactericidal effect upon a vast range of bacterial species as well as other eukaryotic cells or viruses [22-23]. Nanoparticles have also shown effect against MDR bacteria including MRSA and VRSA [24]. According to literature there are three major mechanisms followed by nanoparticles to act as antibacterial agent, which includes making bacterial membrane porous, altering the structure of DNA and proteins & by releasing silver ions later on interacting with the cellular components [25].

This article has an objective to rapidly prepare AgNPs of smaller size by using the tulsi leaf extract and evaluate its effect upon the clinical MDR *Staphylococcus aureus* growth isolated from West Bengal.

MATERIALS AND METHODS

Ethical approval

The collection of clinical isolates was priorly approved and permitted by Institutional ethical committee of University of Calcutta, Kolkata and patients' consent were also taken from the concerned donor institutions.

Staphylococcus aureus identification

Gram staining followed by basic biochemical tests like catalase, coagulase and mannitol fermentation tests were used to identify clinical *Staphylococcus aureus*, collected from different hospitals and private laboratories in West-Bengal [26].

Identification of MDR S. aureus strain

Multidrug resistance of the tested *S. aureus* was assessed by disc agar diffusion test (DAD) [27] as per CLSI guidelines [28].

Aqueous plant leaf extract (PLE) preparation

Tulsi (*Ocimum sanctum*) leaves were bought from the local market of Kolkata, Sukea street area. 25 gram leaves were washed twice and air dried in the laboratory at room temperature. Then the leaves were cut into small pieces and boiled in 100 mL ddH_2O (double distilled water) at 70-80 °C for 10 minutes and filtered with whatmann□s

number 1 filter paper. Then the filtrate was kept at 4 °C for further use [29].

$AgNO_3$ solution

1mM $AgNO_3$ (silver nitrate) solution was prepared by solubilising 17 mg $AgNO_3$ ddH_2O at room temperature and making the volume 100 mL in a volumetric flask. Flask was kept covered with aluminium foil to avoid any light induced reaction with the silver nitrate present in the solution [29].

Preparation of silver nanoparticles (AgNPs)

Light induced reduction method was followed for the rapid preparation of biogenic silver nanoparticles. 1ml of 5% & 7% PLE was mixed with 9 mL 1 mM $AgNO_3$ solution in separate test tubes. 1 mL ddH_2O was mixed with 9 mL 1 mM silver nitrate solution as control. All the tubes were allowed to stand for the reactions under sunlight at room temperature [29] and the colour change was observed.

Purifying, concentrating and quantifying AgNPs

AgNPs prepared using 7% PLE was taken for further purification and characterization. 2 mL solution was centrifuged at 15000 rpm for 15 minutes, then the pellets were dispersed in 1 mL ddH_2O and again centrifuged for another 15 minutes at the same speed and the pellets were dispersed in 1 mL ddH_2O, labelled as filtered AgNP colloid (F – AgNPs), and then studied under UV-Vis spectrophotometer. The F – AgNPs were lyophilised for the quantification of the nanoparticles present in the solution [30]. According to the literature, there is always a need of proper method to quantify different kind of nanoparticle based upon size and capping agents [31], the lyophilisation method was adopted for this study. Another quantification method was also evaluated for the comparison, described by Teruya *et al.* [32].

Characterization of AgNPs

Colour change

Change in colour of the solution was observed for 24 hours at various time intervals [33-35] (figure - 2).

UV-Vis spectrophotometric analysis

All the crude solutions were analysed and scanned for absorbance at various time intervals from 300 to 700 nm wavelength of light using UV-Vis spectrophotometer (Systronics, UV-VIS Spectrophotometer 117, India) with 10mm path length. The F – AgNPs were also studied using the same at 260-600 nm wavelength [34, 36].

Dynamic light scattering (DLS)

To analyse the monodispersity and particle size, AgNPs prepared using 7% PLE was examined under DLS instrument [37].

FESEM

Field emission scanning electron microscopy (FESEM) was used to analyse the size and shape of the prepared AgNPs. The F – AgNPs were drop casted on FTO (Fluorine doped tin oxide) coated glass slides and then observed under FESEM for imaging [33, 36, 38].

FTIR

Fourier transform infrared spectroscopy (FTIR) was done with the lyophilized AgNPs and PLE, to assess the functional groups involved in the preparation of green AgNPs. The spectra were recorded between 400 and 4000 cm^{-1} wavenumbers and analysed by subtracting the spectra of KBr as the lyophilized powders were examined by mixing with pure KBr.

Antibacterial activity:

MIC test

Minimum inhibitory concentration (MIC) of the biologically synthesized AgNPs was calculated using Broth Microdilution Method.

Bacterial growth curve

Growth curve of the tested MDR *S. aureus* was plotted with proper observation of the strain with 10% (2ml AgNP solution and 18 ml broth) of F – AgNPs (10.3 µg/mL) and the antibiotic clindamycin as positive control, as the strain was sensitive against clindamycin [39]. The inoculum added was 100 µL and that had a density of 0.5 McFarland (Figure - 1 & Table - 1).

Effect of PLE and silver nitrate upon bacteria

The antibacterial activity of Clindamycin (CLI) (0.5 µg/mL), Azithromycin (AZM) (8µg/mL), PLE (1 mL in 10 mL broth), silver nitrate (1 mL in 10 mL broth) and AgNPs (10.3µg/mL) were also tested using the same concentration (as in growth curve) of inoculum in the broth medium after incubating for 32 hours.

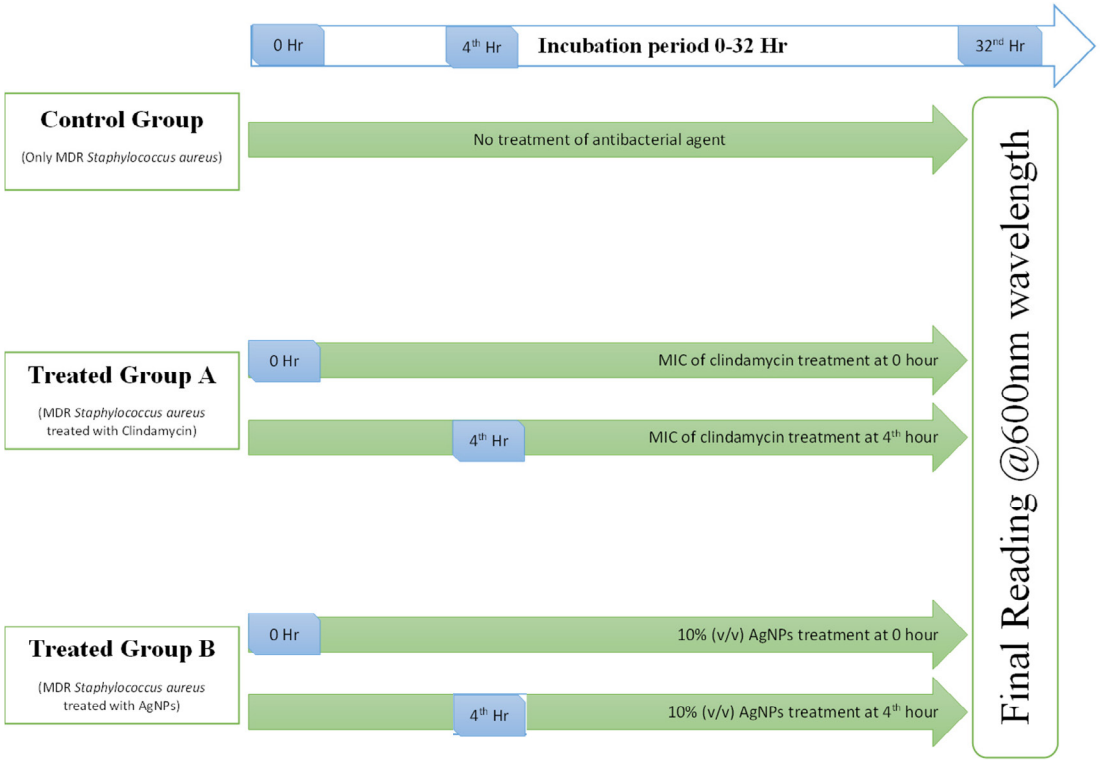

Fig. 1. Plan of assessment of antibacterial activity of biosynthesized silver nanoparticles (Final reading = OD) (10% v/v AgNPs stands for 10.3 µg/mL AgNPs).

Table 1. *MDR Staphylococcus aureus* growth observations with clindamycin and biosynthesized AgNPs.

MDR *Staphylococcus aureus* growth observations					
Experimental groups	Control	Clindamycin treatment at 0 hour	AgNP treatment at 0 hour	Clindamycin treatment at 4th hour	AgNP treatment at 4th hour
Study design	No antibacterial agent was added throughout the incubation period.	MIC (0.5µg/ml) of clindamycin was added at the time of inoculation.	10.3µg/ml biosynthesized AgNPs were mixed at the time of inoculation.	Bacteria were left to grow for 4 hours as control and then MIC (0.5µg/ml) of clindamycin was added to assess the effect.	After 4 hours of incubation as control, 10.3µg/ml AgNPs were added to check the antibacterial activity of the biogenically prepared silver nanoparticles.
Observation after 32 hours of incubation	The control group MDR *Staphylococcus aureus* attained the exponential phase, afterwards stationary.	As the MDR bacteria was sensitive against clindamycin, the graph was flat parallel to the baseline. No or little growth was observed.	A huge spike was seen at the time of introduction of inoculum and AgNPs due to the immiscible nature of the AgNPs with the broth media. With the progression of time, the absorbance was observed to be reduced. The final segment of the curve was found to be similar with the clindamycin.	Clindamycin was found to be efficient to kill the bacteria, which can be observed by the absorbance after 32 hours of incubation. At 4th hour, the graph was seemed to gain the exponential phase, which was gradually stopped by the treatment of MIC of clindamycin.	Till the 4th hour of incubation, the MDR bacteria was growing as the absorbance was increasing gradually. Addition of AgNPs again showed a sudden rise in the graph might be due to the immiscibility of the AgNPs solution with the media. At 32nd hour of incubation, the graph was found to be similar with the MIC.

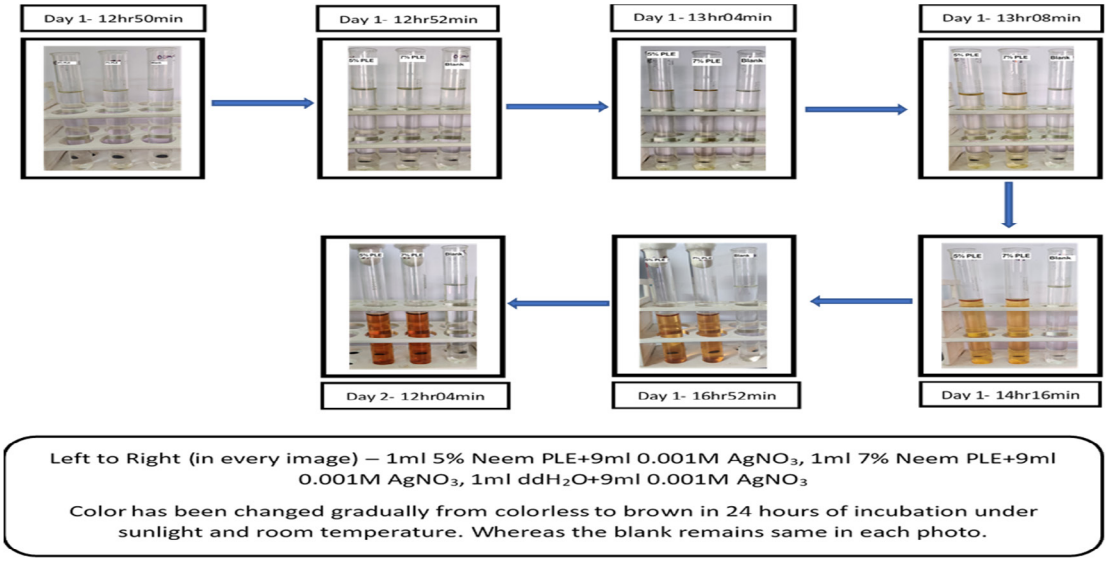

Fig. 2. Gradual change in colour of the solutions as evidence of production of biogenic silver nanoparticles.

Statistical analysis

ANOVA followed by LSD post hoc test was used to observe the difference in the optical density between the control, NP treated and clindamycin treated groups at 95% confidence level ($P < 0.05$) (Table - 2).

RESULTS AND DISCUSSION

Clinical MDR Staphylococcus aureus identification

Multidrug resistant bacteria can be defined as the bacteria resistant against any agent of at least three or more classes of antibiotics [40]. The bacteria sample used in the study were found to be resistant against the following categories of antibiotics: Penicillin (amoxycillin), Macrolide (azithromycin, clarithromycin), Quinolones and Fluoroquinolones (levofloxacin) and Penicillin combination (amoxycillin with clavulanic acid). Thus, the bacteria used were MDR isolate of *Staphylococcus aureus*, as they were also tested positive for catalase, coagulase test and 7.5% mannitol salt agar growth test.

Preparation and Characterization of Silver Nanoparticles

Biological preparation of nanoparticles is attracting the focus of researchers due to its unique characters and properties. The phytochemicals like eugenol, β-caryophyllene, β-elemene, cyclopropylidene, linalool, xanthones, flavonoids, etc [29, 41] are present in the PLE supposedly helps reducing the silver ions of silver nitrate to the silver atom, which gets capped by the phytochemicals. It is also supposed that, a number of atoms start aggregating to form nanometre scale particles, which will be stabilized by the same or different phytochemicals present in the PLE to stop further aggregation to limit the aggregation in nanometre scale resulting in the production of nanoparticles [41]. Diagrammatic representation of silver nanoparticle synthesis is shown in the figure – 3.

Colour change

The gradual change in colour from (colourless to brown) with time is shown in the figure-2, which suggests the biogenic preparation of AgNPs using the biological source.

UV-Vis Spectrophotometer analysis

After purification of the mixture, the sample was analysed in UV-Vis spectrophotometer for the characterization of the biogenic silver nanoparticles (Figure - 4 & 5). The peak was observed at 460 nm wavelength. After analysing the absorbance at various intervals, it was found that the nanoparticle preparation starts just after the incubation started. Though the solution left for approximately 24 hours for better efficacy (Figure – 4). On the other hand, 5% and 7% PLE was used to prepare the AgNPs, AgNPs produced using 7%

Table 2. LSD post-hoc test result, no significant difference between the OD of AgNP @ 32 Hr and Clindamycin @32 Hr.

Multiple Comparisons

Dependent Variable: Result at 600nm wavelength

LSD

(I) Groups involving the study	(J) Groups involving the study	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Control @ 0Hr	Control @ 32Hr	-.209333*	.001139	.000	-.21181	-.20685
	Clindamycin treated @ 0Hr	-.002667*	.001139	.037	-.00515	-.00019
	Clindamycin treated @ 32 Hr	-.022667*	.001139	.000	-.02515	-.02019
	AgNP treated @ 0Hr	-.051667*	.001139	.000	-.05415	-.04919
	AgNP treated @ 32 Hr	-.020667*	.001139	.000	-.02315	-.01819
Control @ 32Hr	Control @ 0Hr	.209333*	.001139	.000	.20685	.21181
	Clindamycin treated @ 0Hr	.206667*	.001139	.000	.20419	.20915
	Clindamycin treated @ 32 Hr	.186667*	.001139	.000	.18419	.18915
	AgNP treated @ 0Hr	.157667*	.001139	.000	.15519	.16015
	AgNP treated @ 32 Hr	.188667*	.001139	.000	.18619	.19115
Clindamycin treated @ 0Hr	Control @ 0Hr	.002667*	.001139	.037	.00019	.00515
	Control @ 32Hr	-.206667*	.001139	.000	-.20915	-.20419
	Clindamycin treated @ 32 Hr	-.020000*	.001139	.000	-.02248	-.01752
	AgNP treated @ 0Hr	-.049000*	.001139	.000	-.05148	-.04652
	AgNP treated @ 32 Hr	-.018000*	.001139	.000	-.02048	-.01552
Clindamycin treated @ 32 Hr	Control @ 0Hr	.022667*	.001139	.000	.02019	.02515
	Control @ 32Hr	-.186667*	.001139	.000	-.18915	-.18419
	Clindamycin treated @ 0Hr	.020000*	.001139	.000	.01752	.02248
	AgNP treated @ 0Hr	-.029000*	.001139	.000	-.03148	-.02652
	AgNP treated @ 32 Hr	.002000	.001139	.104	-.00048	.00448
AgNP treated @ 0Hr	Control @ 0Hr	.051667*	.001139	.000	.04919	.05415
	Control @ 32Hr	-.157667*	.001139	.000	-.16015	-.15519
	Clindamycin treated @ 0Hr	.049000*	.001139	.000	.04652	.05148
	Clindamycin treated @ 32 Hr	.029000*	.001139	.000	.02652	.03148
	AgNP treated @ 32 Hr	.031000*	.001139	.000	.02852	.03348
AgNP treated @ 32 Hr	Control @ 0Hr	.020667*	.001139	.000	.01819	.02315
	Control @ 32Hr	-.188667*	.001139	.000	-.19115	-.18619
	Clindamycin treated @ 0Hr	.018000*	.001139	.000	.01552	.02048
	Clindamycin treated @ 32 Hr	-.002000	.001139	.104	-.00448	.00048
	AgNP treated @ 0Hr	-.031000*	.001139	.000	-.03348	-.02852

*. The mean difference is significant at the 0.05 level.

PLE was found to be more prominent (Figure - 5).

Dynamic Light Scattering (DLS) Analysis

As per the data taken from DLS, the size of the biogenic silver nanoparticles was found to be between 10-100 nm. The average size of the AgNPs was 51.9 nm (Figure- 6). The results observed in our laboratory seem similar with some articles published

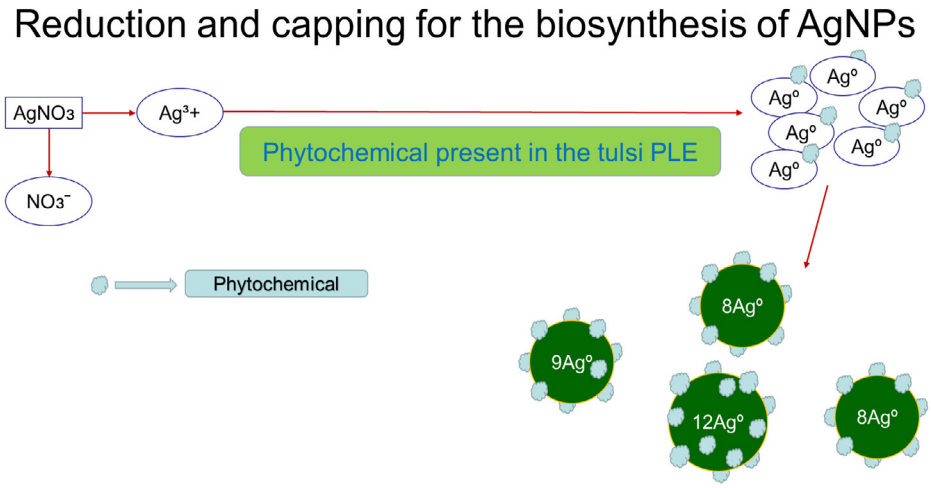

Fig. 3. Bio-reduction and stabilization for the production of AgNPs.

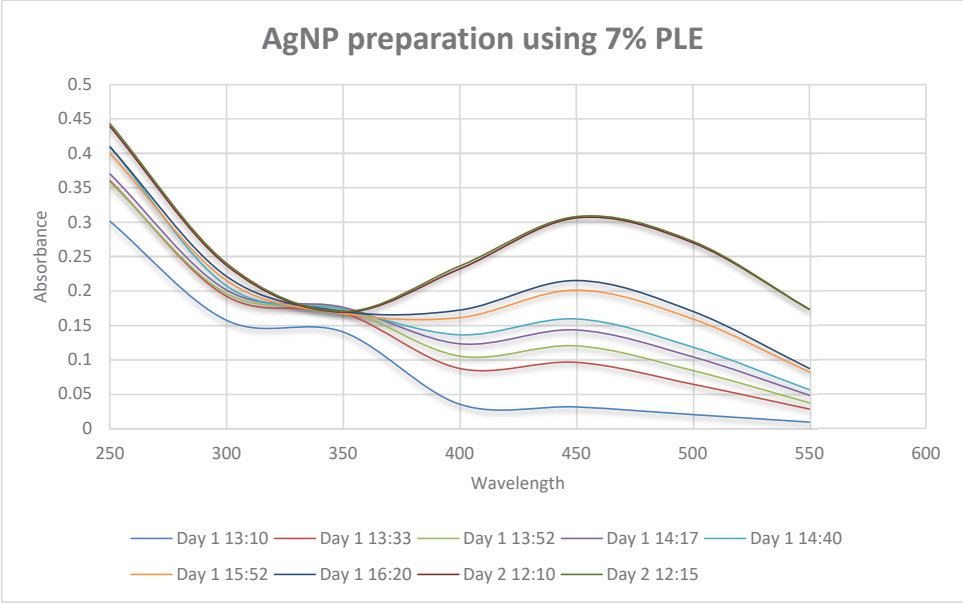

Fig. 4. UV-Vis absorbance at various time interval.

earlier. AgNPs prepared from fungal source recorded the average size to be 53.7 nm as per DLS results [42].

FESEM imaging

Before going for the FE-SEM, the sample (F - AgNPs) was concentrated by centrifugation at 15000 rpm for 15 minutes. As per the images obtained from FE-SEM imaging, round, spherical silver nanoparticles can be observed from 9-23 nm size. Images were taken at 30 kV and about 250000 X magnification (Figure – 7a, b, c, d). It has been seen that smaller the size of the nanoparticle, more will be the efficacy [25]. Another report published in 2019 the measured size of AgNPs between 11-90 nm [43]. AgNPs produced from neem has a size range of 9-56 nm as per SEM results [44].

FTIR analysis

FTIR analysis revealed that the tulsi leaf extract showed sharp peaks at 746 cm^{-1}, 856 cm^{-}

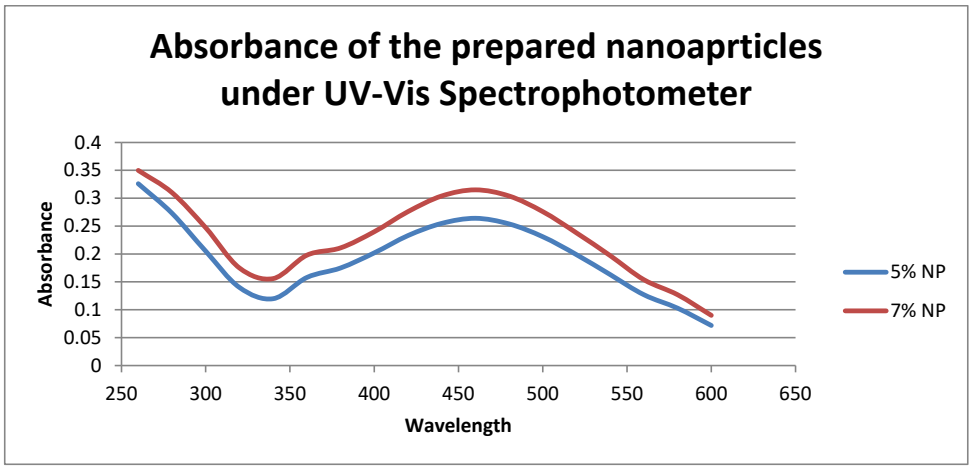

Fig. 5. UV-Vis spectrophotometer analysis of the prepared nanoparticles.

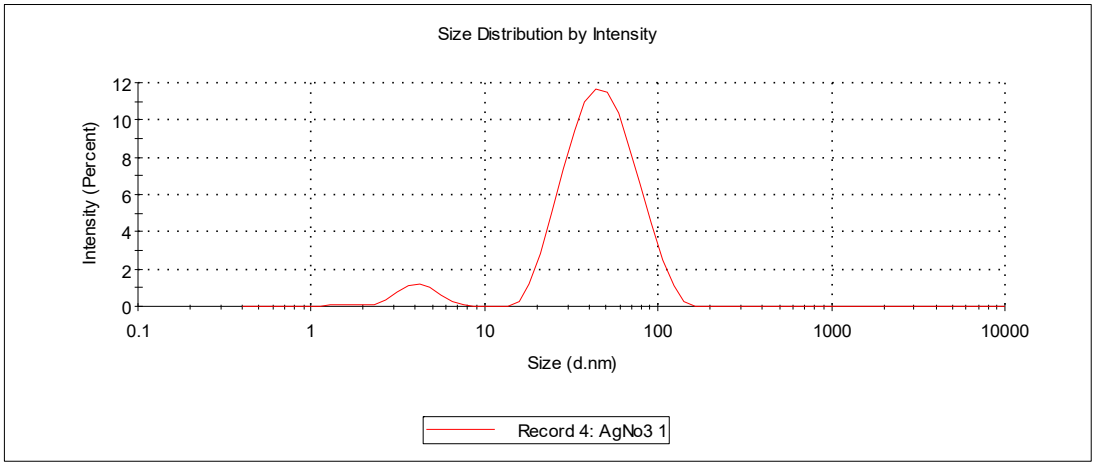

Fig. 6. Size distribution of the prepared silver nanoparticles analysed under DLS.

^{1}, 1018 cm^{-1}, 1276 cm^{-1}, 1483 cm^{-1}, 1747 cm^{-1}, 2364 cm^{-1}. Whereas the peaks of low intensity were observed at 725 cm^{-1}, 873 cm^{-1}, 1018 cm^{-1}, 1300 cm^{-1}, 1467 cm^{-1}, 1724 cm^{-1}, 2372 cm^{-1} in case of AgNPs prepared using the tulsi PLE (Figure - 8). The change in peak intensity suggests the involvement of the respective functional groups in the preparation of tulsi PLE mediated AgNPs [45]. The peaks were evidence of presence of eugenol, quercetin and oleanolic acid having C-H, C=C, C=O functional groups respectively. Whereas the IR peaks observed between 720 and 1250 were also observed as signature peaks for eugenol corresponding to C=C region [46]. Literature also suggests the appearance of IR peaks at 1012, 1080, 1614, 1654 cm^{-1} as evidence of the presence of C-C, C=C stretches present in eugenol, linalools and terpenes present in the tulsi PLE.

AgNPs quantification

The biogenically synthesised AgNPs yield was found to be approximately 97µg/mL in the solution. The quantification method which involved lyophilization provided us 103µg/mL after filtration of the primary mixture (1 mL 7% PLE & 9 mL silver nitrate).

Antibacterial activity

The antibacterial activity of green AgNPs is also supported by literature [47], and the biogenically prepared AgNPs were found to be potent to inhibit the growth of MDR *Staphylococcus aureus*.

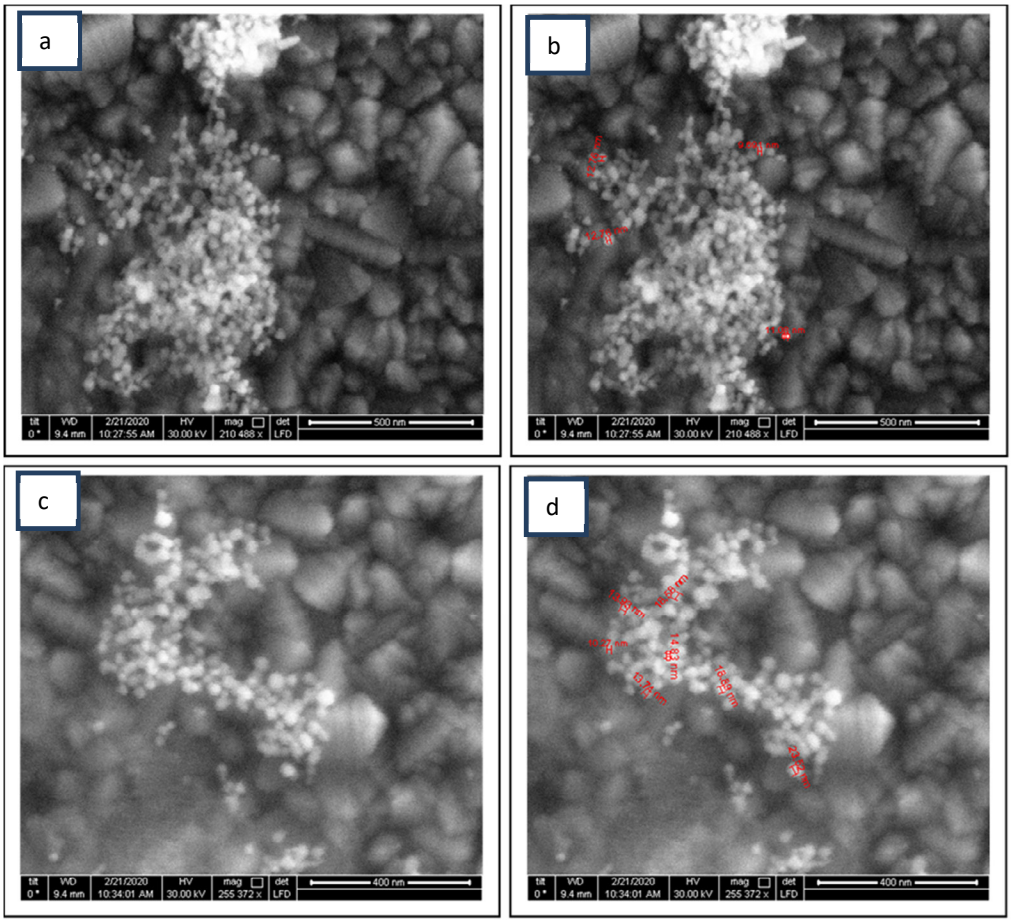

Fig. 7 (a, b, c, d). FE-SEM Images for the characterization of biologically prepared silver nanoparticles, showing spherical nature, size ranging between 9-23 nm.

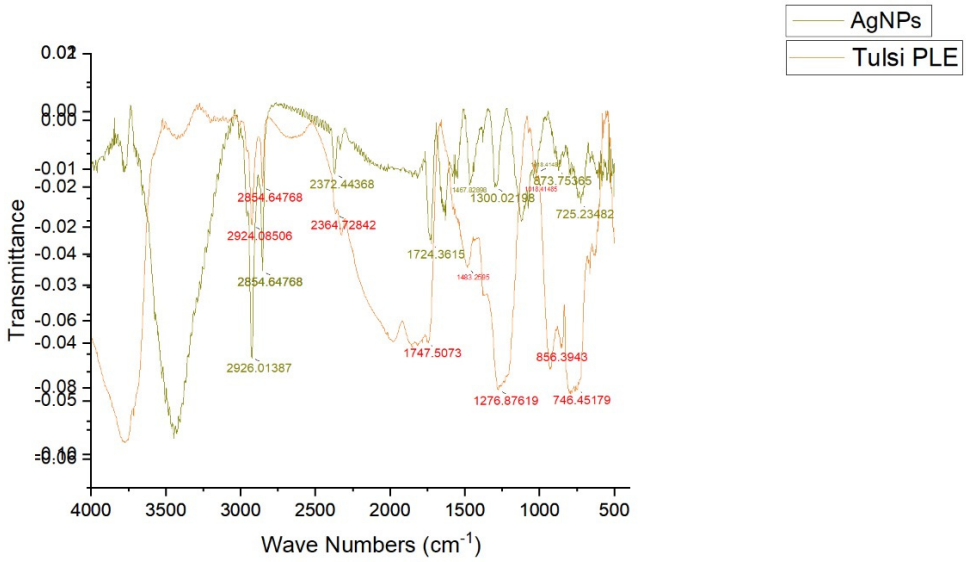

Fig. 8. FTIR spectra of tulsi PLE and AgNPs.

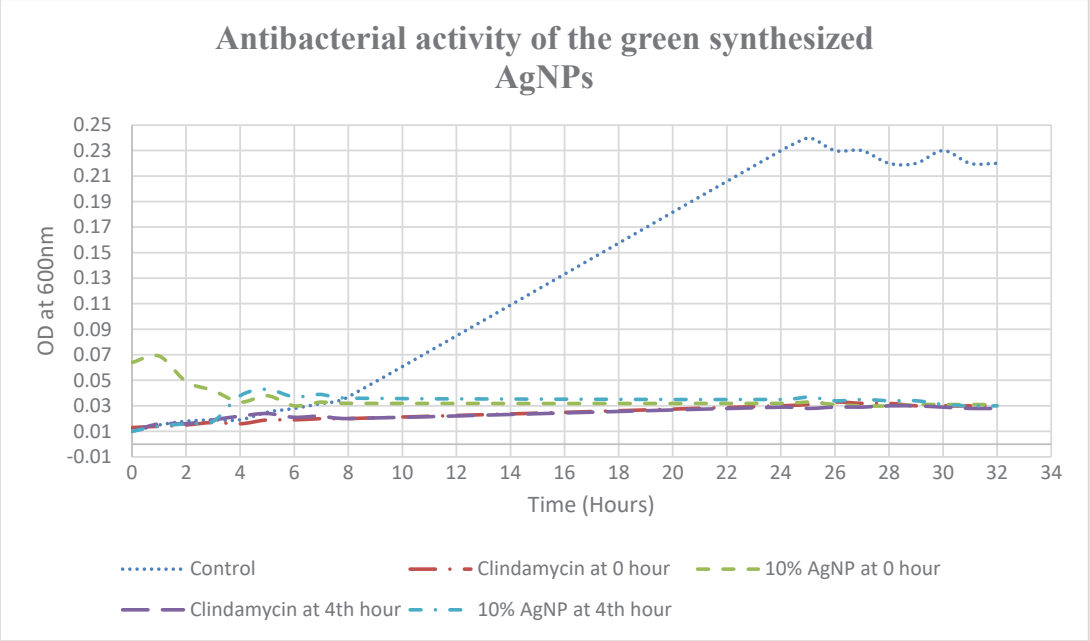

Fig. 9. Graphical representation of the bacterial growth curve (inoculum 100 µL of 0.5 McFarland dense primary culture).

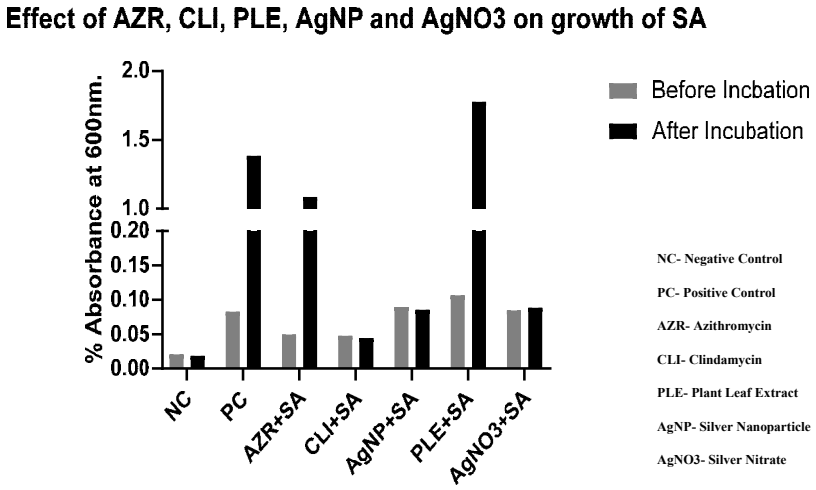

Fig. 10. Effect of PLE and $AgNO_3$ upon bacterial culture.

MIC test

Broth micro-dilution method revealed that the minimum inhibitory concentration of the prepared AgNPs (using 7% PLE) was 10% (v/v) (10.3 µg/mL) in the broth medium.

Bacterial Growth Curve

The efficiency of biogenically prepared silver nanoparticles were tested against clinical MDR *Staphylococcus aureus*. Growth of bacteria was analysed using UV-Vis spectrophotometer, and the OD (Optical Density) was taken at 600 nm wavelength [48] (Figure - 9).

Effect of PLE and silver nitrate upon bacteria

Despite of having antibacterial property, PLE was found to be less efficient to inhibit the growth of MDR *Staphylococcus aureus* than AgNPs and

$AgNO_3$ alone. The results are shown in Figure - 10.

Statistical analysis: One-way analysis of variance (ANOVA) followed by LSD post hoc test (Table - 2) was used to confirm the significant difference between the tested groups i.e., there was a significant difference between negative control (without antibiotic treatment) and positive control (clindamycin treatment), as well as there was a significant difference between negative control and AgNPs treated culture. Whereas, there was no significant difference between the positive control and AgNPs treated culture's OD at 95% confidence level ($P < 0.05$) after 32 hours of incubation [42].

CONCLUSION

PLE prepared from the tulsi plant was efficient to reduce the ionic Ag to Agº, due to the presence of biomolecules (eugenol, quercetin and oleanolic acid) present in the extract. The AgNPs were prepared within 20 minutes of incubation under sunlight at room temperature. The AgNPs have shown the SPR at approximately 450 nm wavelength and the size of the nanoparticles were 9-23 nm with a spherical morphology as well as the nanoparticles were efficient to act as antibacterial agent similar to clindamycin against MDR *Staphylococcus aureus*. As per the statistical analysis, the clindamycin and AgNPs treated group were not significantly different after 32 hours of incubation at the significance level of 0.05, confirming the efficacy of the biosynthesized AgNPs as potent as clindamycin against MDR *Staphylococcus aureus*. Thus, treating MDR bacterial infection may see a light of hope towards the use of biologically prepared silver nanoparticles.

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CONFLICTS OF INTEREST

The authors do not have any conflicts of interest.

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