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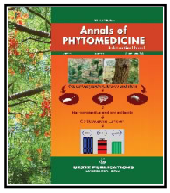

Original Article : Open Access

Characterization of phyto-synthesized silver nanoparticles using of *Nigella sativa* L. seed extract and evaluate antimicrobial efficacy against diabetic foot ulcer bacterial isolates

Asha Arora, Shweta Chhajed◆ and Priyansh Jain

Department of Botany, Mohan Lal Sukhadiya University, Udaipur-313001, Rajasthan, India

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Abstract

One of the major consequences of diabetic mellitus is diabetic foot ulcer (DFU) and due to their susceptibility to infection, are the main cause of hospitalization and lower limb amputation. Silver nanoparticles (AgNPs) antimicrobial efficacy has been very well studied in various reports; therefore, use of silver nanoparticles in biomedical field is a new trend. Synthesis of silver nanoparticles using plants become a promising substitution to the other methods of nanoparticle synthesis because it is cost effective and nontoxic; herein in this study, we represent the antimicrobial efficacy of plant synthesized silver nanoparticles against DFU bacterial isolates. Nanoparticles were synthesized using *Nigella sativa* L. seed extract. Characterization of AgNPs was carried out by UV-Vis. spectroscopy, SEM (scanning electron microscopy) with EDX (energy dispersive X-ray analysis), TEM (transmission electron microscopy), XRD (X-ray differaction), FTIR (fourier transform infrared spectroscopy). The AgNPs were assayed for antimicrobial activity against five DFU isolates, *i.e.*, *Streptococcus aureus, Pseudomonas aeruginosa, Bacillus subtilis, Klebsiella pneumoniae* and *Escherichia coli*.

The biocidal property of synthesized nanoparticles was determined by well diffusion method by measuring inhibition zone and minimum inhibitory concentration was evaluated by broth dilution method. Synthesized silver nanoparticles showed effective antimicrobial efficacy against all selective diabetic foot ulcer bacterial isolates.

1. Introduction

Over production of glucose and low utilization by the tissues make fundamental basis of hyperglycemic conditions (Shirwaikar *et al*., 2005). Diabetes is the third main cause of death in the world, due to its high mortality, prevalence and morbidity (WHO, 1999). It also claimed that over the next three decades (2000-2030), the number of diabetic patients will be increased more than three fold, by 171 million in 2000 to 365 million in 2030 (Shen *et al*., 2017). The term diabetic foot ulcer refers to "A non-healing or poorly healing full-thickness wound below the ankle with diabetes critical in the natural history of the diabetic foot" (Boulton *et al*., 2005). Diabetic individuals are 25 times more likely than non-diabetic patients to lose a limb, with diabetes accounting for up to 70% of all leg amputations (Bakker *et al*., 2005). Complications of diabetic foot ulcer include foot ulceration, neurodisorders, peripheral vascular disease, and microbial infection with or without osteomyelitis, all of which may lead to gangrene and limb amputation (Khanolkar *et al*., 2008). The most prevalent isolates of diabetic foot infections were *E. coli* (20.3 %), *S. aureus* (16.2 %), *K. pneumoniae* (17.4 %), and *P. aeruginosa* (12.6 %). Clinicians have reverted to silver dressings with variable levels of silver due to the advent of a high number of antibiotic-resistant bacteria and constraints in the use of antibiotics (Gemmell *et al*., 2006). AgNPs are considered as safe to use as antimicrobials since they may suppress bacterial growth at concentrations much below to their cytotoxic limits (Mondal *et al*., 2011). Because microbes are becoming more resistant to antibiotics, scientists are investigating the biocidal properties of various extracts of medicinal plants from all over the world as a result, new and interesting biologically active plant compounds with significant potential in herbal therapy have been discovered (Duraisami *et al*., 2021). The tribal communities in all over the world, have the traditional knowledge to use various plants to treat different types of diseases (Husain, 2021; Naikodi *et al*., 2021). Silver nanoparticles made from plant extracts are found to be nontoxic to human, as well as highly effective against bacteria, pathogens, and viruses at low concentrations (Qais *et al*., 2019). *N. sativa* (black cumin) is an annual herb belonging to Ranunculaceae family and has been used in north Africa, Asia and middle east for treatment of various ailments (Yimer *et al*., 2019). *N. sativa* oil is used for joint pain and stiffness (Houghton *et al*., 1995). Therefore, this study concentrates on the green synthesis of AgNPs using extract of *N. sativa* seeds. Nanoparticles were characterized and further, subjected to *in vitro* antimicrobial analysis.

Corresponding author: Ms. Shweta Chhajed

Department of Botany, Mohan Lal Sukhadiya University, Udaipur-313001, Rajasthan, India.

E-mail: Shweta09chhajed@gmail.com.

Tel.: +91-9784643265

2. Materials and Methods

2.1 Collection and identification of plant sample

Seeds of *N. sativa* were procured from a local shop at Udaipur, Rajasthan and stored at room temperature. For the validation, herbarium specimen was prepared and submitted at University of Rajasthan, Jaipur, India and the Voucher No. is RUBL211517. The dry seeds were finely grounded and stored in a sterile container for extraction.

2.2 Preparation of aqueous extract (Marsali *et al.*, 2018)

10 g dry powder of seeds of *N. sativa* was added in 50 ml of deionized water, then stirred for 20-30 min at 60°C. The seed extract was then cooled. The extract was then filtered using Whatman No. 3 filter paper. The process is followed by vacuum filtration using filter paper with 0.2 µm pore size. The filtrate thus obtained was stored at 4°C temperature for further use.

2.3 Green synthesis of silver nanoparticles (Mohideen, 2021)

AgNPs were synthesized by adding 5 ml of seed extract of *N. sativa* to 95 ml of 1 mM silver nitrate solution at room temperature for 24 h. The colour was changed from pale yellow to brown with time intervals, indicated the synthesis of AgNPs. The silver nanoparticles precipitates were purified by centrifuge at 12,000 rpm for 20-30 min. The pellets were dried using hot air oven at 50°C. The dried powder was stored in sterile dark bottle.

2.4 Characterization of green silver nanoparticles

The phyto-synthesized AgNPs were monitored by UV-vis spectrophotometry (Chand *et al.*, 2021). Nanoparticles in the size range of 2-100 nm provide a characteristic peak in the visible region at 200-800 nm. The crystalline structure of AgNPs, was observed by X- ray diffraction analytic technique and to calculate the size of the particles Debye-scherrer equation was applied:

$$t = \frac{K\lambda}{\beta \cos \theta}$$

where,

β = is the line broadening at half the maximum intensity, radian

λ = X-ray wavelength (1.5405)

θ = observed peak angle

τ = is the mean size of the ordered (crystalline) domains

K = crystalline shape factor (0.9)

The structure and chemical composition of the synthesized silver nanoparticles were characterized by scanning electron microscopy with EDX. EDX technique is used to determine elemental composition of AgNPs. All elements present in AgNPs have different atomic structure producing sharp peaks in X- ray spectrum (Yao *et al.*, 2007), TEM provide information on size and morphology (Williams, 2009), and FTIR technique is used to identify chemical compounds and substituent group. FTIR is used to determine the functional group and composition of AgNPs (Vasantharaj *et al.*, 2018).

2.5 Antimicrobial assay

2.5.1 Preparation of stock solution of silver nanoparticles

A stock solution of 1000 ppm was prepared by dissolving 100 mg of synthesized silver nanoparticles in 100 ml of deionized water. From this stock solution, 10 ml of 250 ppm, 500 ppm and 750 ppm solutions were prepared.

2.5.2 Preparation of test bacterial strain

Bacterial isolates (*S. aureus*, *P. aeruginosa*, *K. pneumonia*, *B. subtilis* and *E. coli*) were obtained from IMTech. Chandigarh, were used for evaluating antimicrobial activity (McCracken and Cowsan *et al.*, 1983). One inoculation loop of pure culture of bacterial strain was inoculated into 5 ml of nutrient agar solution and then incubated at 37°C for 24 h in the incubator. Test bacterial strain was prepared by inserting one inoculation loop of cultured bacteria into 5 ml of 0.19 % NaCl solution.

2.5.3 Antimicrobial assay (Magaldi *et al.*, 2004)

Evaluation of antimicrobial activity of seed extract was carried out by agar well method. 20 ml of muller hinton agar (MHA) was molted and poured into sterile-petri plates and then allowed to set at room temperature for 20-30 min. 100 µl of standardized bacterial inoculum were swabbed uniformly to the solidified MHA plates using sterile L shape spreader under aseptic conditions and allowed the plates to dry for 5 min. Further, 6 mm diameter wells were punched aseptically with a cork borer on agar plates. Distilled water was used as negative control, whereas antibiotic streptomycin was used as positive control. Thereafter, 20 µl of plant extract of *N. sativa*, $AgNO_3$ and AgNPs with 250 ppm, 500 ppm and 750 ppm concentration were transferred in respective wells. The plates were labelled and then incubated upside down at 37°C for 24 h. The zones of inhibitions were measured (Sethumathi *et al.*, 2021).

2.5.4 Minimum inhibitory concentration (MIC) evaluation (Wiegand *et al.*, 2008)

Antimicrobial activity of the nanoparticles synthesized was determined by broth dilution method. From the silver nanoparticles stock solution of 1000 ppm, two-fold serial dilution was done to prepare 500 ppm, 250 ppm, 125 ppm, 62.5 ppm, 31.25 ppm, 15.62 ppm, 7.81 ppm, 3.90 ppm, 1.95 ppm, 0.97 ppm, 0.48 ppm, 0.24 ppm solutions. Then, 100 µl of microbial suspension equaling 0.5 McFarland (1.5 × 106 CFU) was added to each tube. The tubes were kept for 24 h in an incubator with shaker at 37°C. Control contained only inoculated muller hinton broth. The MIC was noted by the visual turbidity and optical density (OD) was taken of the tubes both before and after incubation. After the MIC determination of the AgNPs, aliquots of 50 µl from all tubes which showed no visible bacterial growth were seeded in MHA plates, which are not supplemented with AgNPs, were incubated for 24 h at 37ºC. The MBC was observed for presence or absence of bacterial growth in agar plates For AgNPs.

3. Results

3.1 Green synthesis of AgNPs

Addition of plant extract to silver nitrate solution results change in colour from yellow to brown within no time because silver salts reduce to silver ions by the reducing agents present in seed extract of *N. sativa*. The silver nanoparticles were centrifuged, collected and dried for further analysis.

3.2 Characterization of AgNPs

After the initial identification, the biosynthesis of silver nanoparticles was confirmed by UV-visible spectroscopy, as this is one of the most basic techniques for structural characterization of nano-molecules. The absorption spectrum of AgNP's, showed a different but proximate single band absorption peak at 419 nm. This confirms the synthesis of silver nanoparticles (Figure 1).

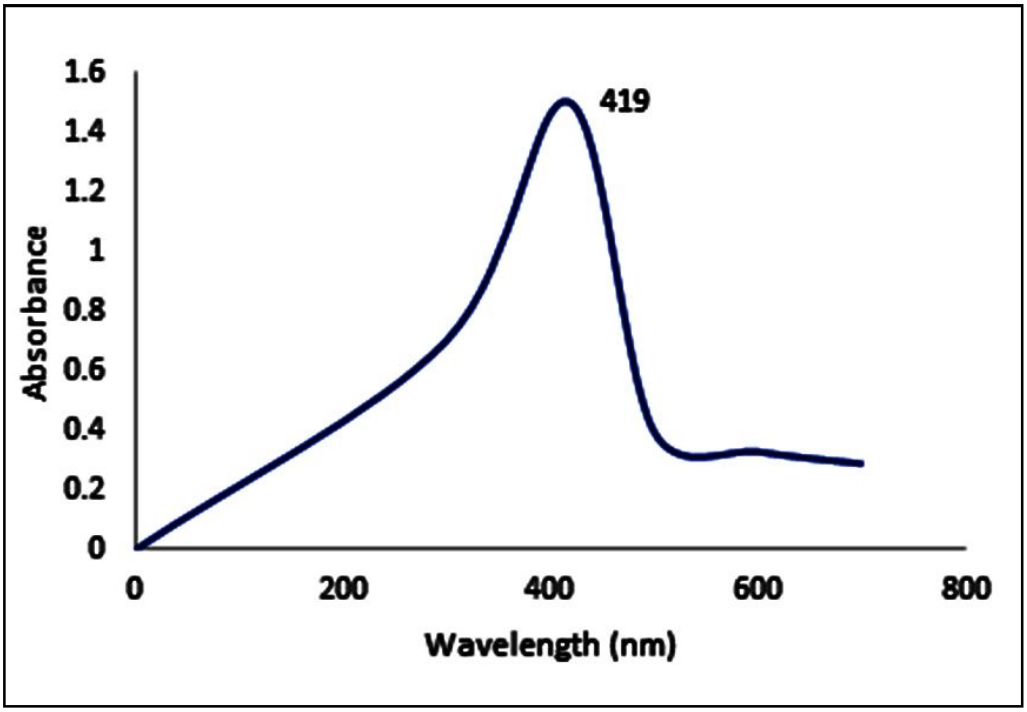


Figure 1: UV-Vis spectrophotometry of silver nanoparticles synthesized using *N. sativa*.

Components that are responsible for the reduction of silver ions were identified by FTIR analytic technique with spectrum range 500-4000 cm^{-1}. The FTIR spectrum of *N. sativa*-AgNPs (Figure 2), the most dominant bands/peaks at 604.08, 1032.46, 1405.21, 1652.32, 2960.58, 3443.26 cm^{-1} which represent C-H stretching of the alkanes, N-H vibrations of amides, C=C stretching of aromatic compounds and O-H stretching of phenols and alcohols. The peaks at 2960.58, 3443.26 cm^{-1} were responsible for Ag^{+} reduction.

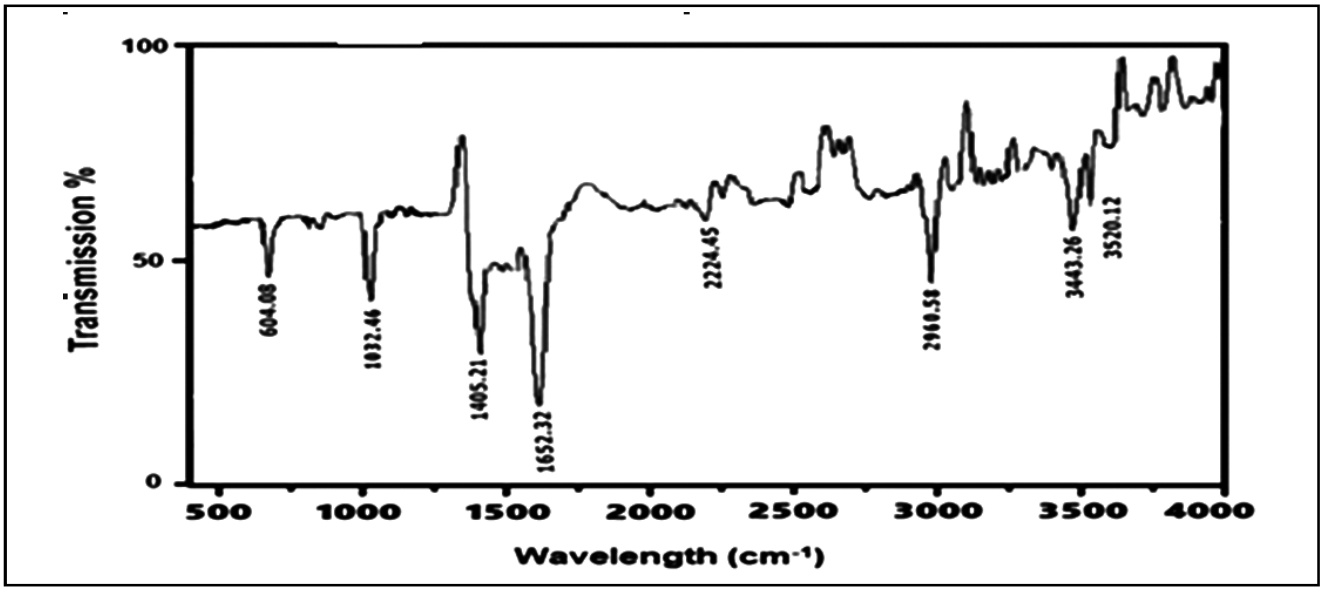


Figure 2: FTIR spectrum of synthesized AgNPs of *N. sativa* seed extract.

XRD spectrum of *N. sativa*-AgNPs (Figure 3) revealed that the dominant peaks were obtained at 29.68°, 35.12°, 39.15°, 46.78°, 78.2° which can be indexed to (111), (200), (220), (240), (311) crystalline planes of AgNPs, respectively, which inferred for faced-centered cubic structures of silver nanoparticles.

Scanning electron microscpe images of silver nanoparticles synthesized seed extract of *N. sativa* reveal spherical shape and their size to be less than 80 nm in *N. sativa* (Figure 5a). The EDX spectra of all synthesized silver nanoparticles showed high intensity and significant absorption peak for Ag^+ at 3 keV, (Figure 4) which is well known absorption signal for Ag^+. EDX analysis reveals silver was 68.8% and oxygen 18.6% (Table 2). *N. sativa* AgNPs, were found to be dispersed, spherical in shape with average size ranging between 10 and 20 nm (Figure 5b).

Table 1: XRD data of biosynthesized AgNPs of *N. sativa*

S. No.	Diffraction angle (2θ)	Crystal orientation	Intensity	FWHM (2θ angle)	β cos θ	Crystal size	Crystal shape
1.	29.68°	(111)	64.39	0.41	0.006904	47 nm	Face-centered
2.	35.12°	(200)	100	0.36	0.008234	33 nm	cubic (FCC)
3.	39.15°	(220)	31.06	1.05	0.002356	17 nm	structured
4.	46.78°	(240)	56.82	0.91	0.002389	23 nm	lattice
5.	78.20°	(311)	15.15	1.02	0.007678	15 nm	

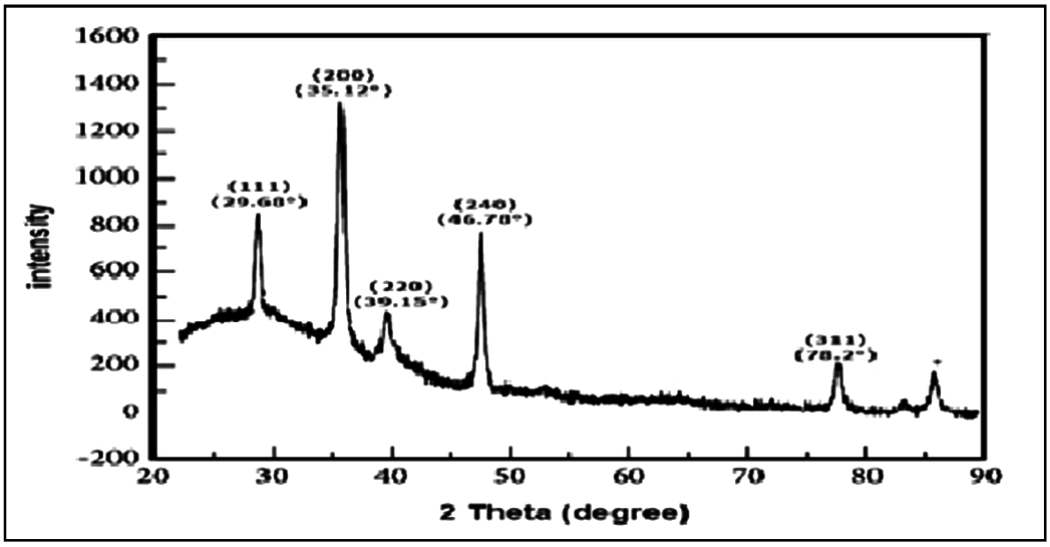


Figure 3: XRD spectrum of synthesized AgNPs of *N. sativa* seed extract.

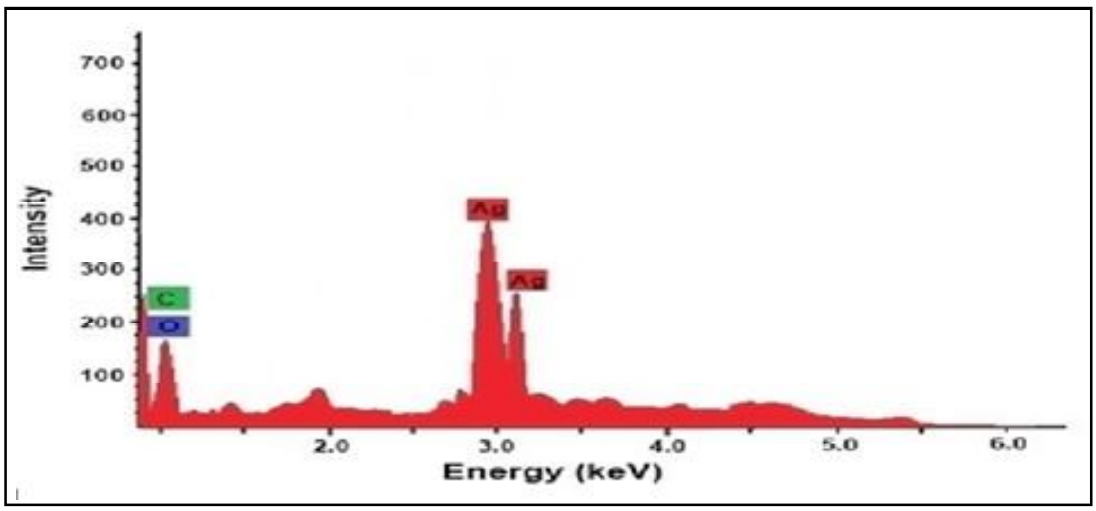


Figure 4: EDX spectrum of AgNPs synthesized from seed extract of *N. sativa*.

Table 2: EDX of AgNPs synthesized from seed extract of *N. sativa*

S. No.	Element	Wt. %	At. %
1.	O K	18.68	20.21
2.	C K	12.51	37.79
3.	Ag L	68.81	28.33

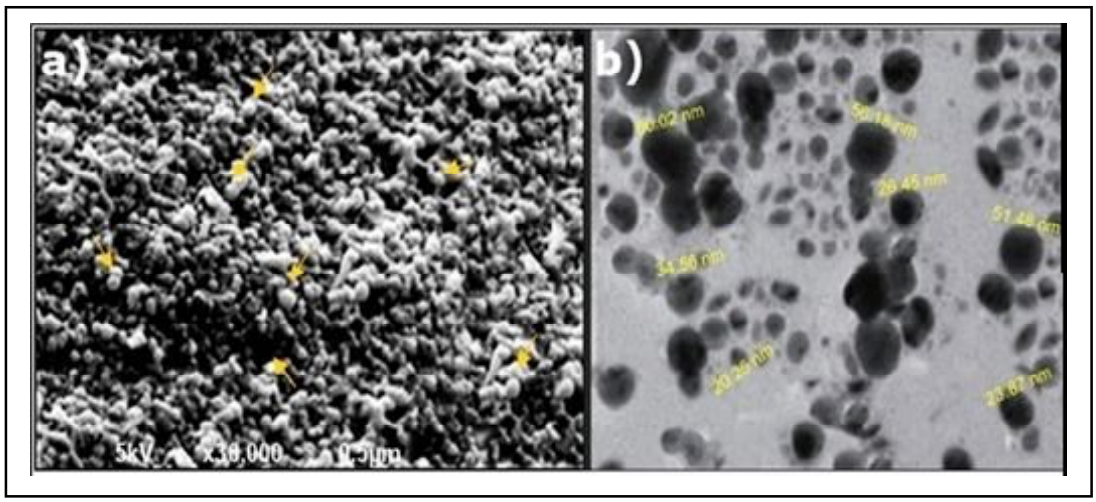


Figure 5: (a) SEM image and (b) TEM image of AgNPs synthesized using seed extract of *N.* sativa.

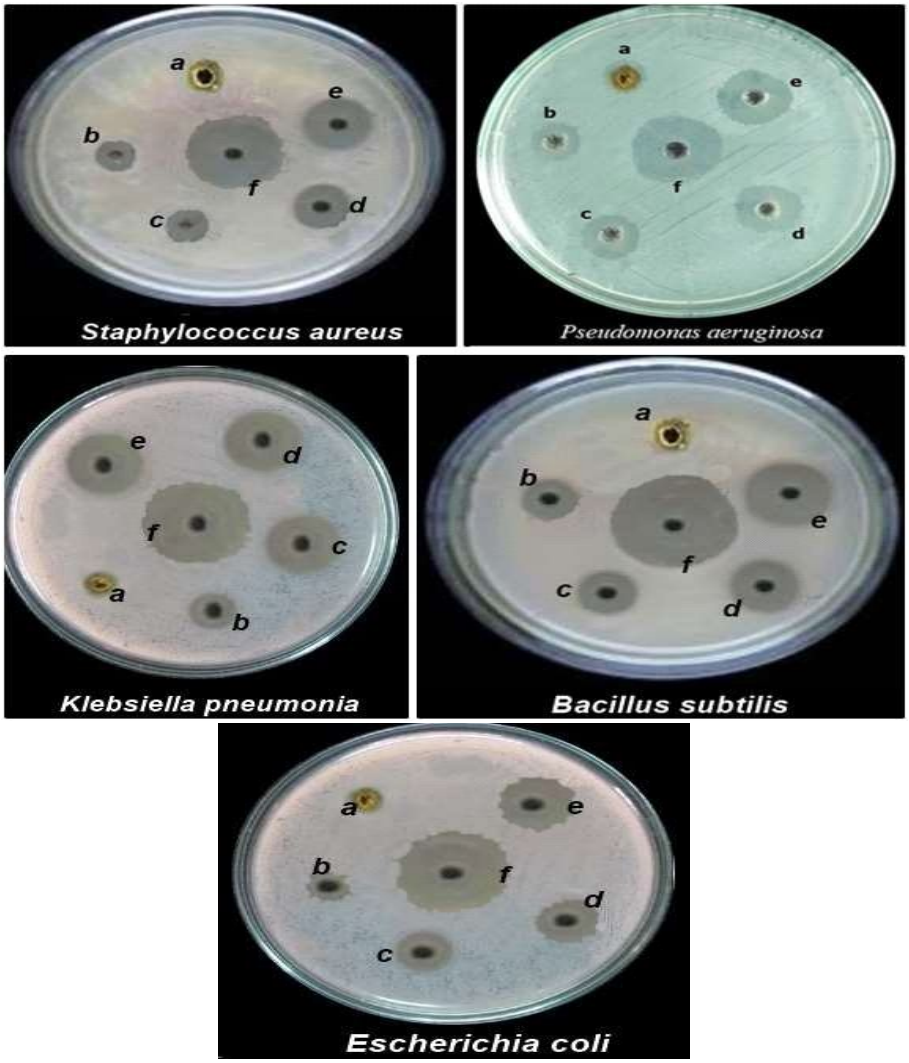
3.3 Antimicrobial activity

The antimicrobial efficacy of the silver nanoparticles synthesized from seed extract of *N. sativa* showed desired results against all bacterial isolates at high concentration of AgNPs mentioned in table 3 (Figure 6). The efficacy of seed extract was not that effective against all the bacteria isolates, as compare to the AgNPs. For seed extract, the highest inhibition zone was observed against *B. subtilis* (9.78 ± 0.14) and the least inhibition zone was observed against *S. aureus* (7.28 ± 0.08), whereas zone of inhibition observed against *P. aeruginosa* was (9.21 ± 0.41), *K. pneumonia was* (8.81 ± 0.43) and *E.coli* was (8.82 ± 0.45). As for the $AgNO_3$, the highest inhibition zone was observed against *P. aeruginosa* (13.38 ± 0.64), followed by *S. aureus* and *B. subtilis* (12.46 ± 0.48), *K. pneumoniae* (12.29 ± 0.56) and the minimum zone of inhibition was found against *E. coli* (11.56 ± 0.49). At the 250 ppm concentration of silver nanoparticles, the highest zone of inhibition was found against *P. aeruginosa* (16.32 ± 0.63), followed by *B. subtilis* (15.75 ± 0.69), *S. aureus* (15.15 ± 0.40), and the least zone of inhibition was found against *E. coli* (14.27 ± 0.72). At the 500 ppm concentration of silver nanoparticles, the highest zone of inhibition was found against *B. subtilis* (19.13 ± 0.43), followed by *P. aeruginosa* (18.68 ± 0.87) and *E. coli* (17.33 ± 0.66), and the minimum zone of inhibition was found against *S. aureus* (17.19 ± 0.64) and *K. pneumoniae* (16.51 ± 0.17). The synthesized silver nanoparticles from the seed extract of *N. sativa* showed significant antimicrobial activity, as bacterial species of DFU were susceptible against higher concentration of AgNPs. At the 750 ppm concentration of silver nanoparticles, the highest zone of inhibition was found against *P. aeruginosa* (20.45 ± 0.59), followed by *B. subtilis* (19.71 ± 0.66) and *E. coli* (19.57 ± 0.25), and the minimum zone of inhibition was found against *S. aureus* (19.18 ± 0.43) and *K. pneumoniae* (18.62 ± 0.34).

Table 3: Zone of inhibition of seed extract, $AgNO_3$ and synthesized silver nanoparticles from *N. sativa*

	Zone of inhibition (mm)					
Bacterial strains	**Plant extract (20 µl)**	**$AgNO_3$ (20 µl)**	**AgNps (250 ppm)**	**AgNps (500 ppm)**	**AgNps (750 ppm)**	**Streptomycin**
S. aureus	7.28 ± 0.08***	12.46 ± 0.48	15.15 ± 0.40**	17.19 ± 0.64**	19.18 ± 0.43	23.47 ± 0.53
P. aeruginosa	9.21 ± 0.41	13.38 ± 0.64**	16.32 ± 0.63	18.68 ± 0.87***	20.45 ± 0.59**	29.44 ± 0.49**
K. pneumonia	8.81 ± 0.43**	12.29 ± 0.56	14.53 ± 0.45***	16.51 ± 0.17***	18.62 ± 0.34	28.22 ± 0.35
B. subtilis	9.78 ± 0.14***	12.82 ± 0.13***	15.75 ± 0.69***	19.13 ± 0.43	19.71 ± 0.66	31.67 ± 0.13***
E. coli	8.82 ± 0.45	11.56 ± 0.49	14.29 ± 0.72	17.33 ± 0.66	19.57 ± 0.25***	29.09 ± 0.77

(*mean ± SE); n = 3; *** $p< 0.001$; ** $p< 0.01$; * $p< 0.05$



(*a = Plant extract, b = $AgNO_3$, c = AgNP 250 ppm, d = AgNP 500 ppm, e = AgNP 750 ppm, f = Streptomycin)

Figure 6: Zone of inhibition of aqueous leaves extract and silver nanoparticles synthesized from *N. sativa*.

Table 4: MIC of synthesized AgNPs from seed extract of *N. sativa*

Bacterial strains	MIC of plant extract (ppm)	MIC of AgNPs (ppm)
S. aureus	125	0.98
P. aeruginosa	62.50	1.95
K. pneumonia	31.2	515.62
B. subtilis	1251	5.62
E. coli	125	0.97

MIC was the lowest concentration of biocidal agent which can inhibits 99.9% microbial growth. The MIC for seed extract was observed 125 ppm for *S. aureus*, 62.50 ppm for *P. aeruginosa*, 31.25 ppm for *K. pneumoniae*, 125 ppm for *B. subtilis* and 125 ppm for *E. coli* mentioned in Table 4.The MIC for AgNPs synthesized using seed extract of *N. sativa* was observed 0.98 ppm for *S. aureus*, 1.95 ppm for *P. aeruginosa*, 15.62 ppm for *K. pneumoniae*, 15.62 ppm for *B. subtilis* and 0.97 ppm for *E. coli*.

4. Discussion

Since last few decades, AgNPs were reported as the most effective biocidal agents because of their sturdy antimicrobial efficacy against bacterial isolates (Loo *et al.*, 2018). Therefore, this study concentrates on the green synthesis of AgNPs using *N. sativa* seed extract as a reducing agent. The synthesis of AgNPs was initiated by addition of $AgNO_3$ to plant extract. The intensity of brown color increases with incubation time indicated the formation of AgNPs (Thirumurugan *et al.*, 2010). The biosynthesized AgNPs were monitored by UV-Vis spectrum in the range of 200-700 nm and the sharp peak was obtained at 419 nm corresponding to the plasmon (Kalyani *et al.*, 2019). FTIR

spectroscopy investigated various functional groups such as amines, aromatic compounds, nitrogen and phenol, which act as a reducing agent and posses binding affinity to silver ions. These compounds are responsible for the formation of AgNPs (Li *et al*., 2014; Kumar *et al.*, 2015). The size of AgNPs was calculated by Debye scherer's formula (Ajitha *et al*., 2015).

Results of the XRD analysis of *N. sativa* silver nanoparticles were revealed the face centered cubic structure of AgNPs (Ali *et al*., 2019). High intensity and significant absorption peaks were obtained in EDX spectra for silver ions at 3 keV $^{+}$, as previously reported in some studies (Kaviya *et al.*, 2011). The surface quantification revealed 68.8 % with 18.68 % of Ag-O species in *N. sativa*. In SEM analysis, the obtained micrographs revealed that the nanoparticles were dispersed, spherical shaped with less than 80 nm size.TEM results revealed the widespread distribution of AgNPs in the range of 10-20 nm, as previous studies reported (Almatroudi *et al*., 2020; Huang *et al*., 2007). Many studies reports suggested that AgNPs have strong antimicrobial efficacy (Sharma *et al*., 2014). In this study, the aqueous extract of *N. sativa* and $AgNO_3$ showed less antimicrobial activity as compared to synthesized AgNPs. AgNPs at higher concentration show effective antimicrobial activity for all bacterial isolates (Sanchooli *et al*., 2018). *N. sativa* seed extract showed higher MIC values as compared to silver nanoparticles. Since MICs reflect the inhibitory status of antimicrobial agent/AgNPs, it is possible that if the AgNPs is removed, the microbe will grow again, so to confirm the inhibitory capacity MBC test was done (Wypij *et al*., 2018; Panpaliya *et al*., 2019). Several reports suggested that lower MIC values had stronger antimicrobial activity (Ahmad *et al*., 2017; Navarro Garcia *et al*., 2006). MIC value of AgNPs possess higher antimicrobial efficacy as compared to *N. sativa* seed extract.

5. Conclusion

Green synthesis of AgNPs using *N. sativa* seed extract was found to be a convenient and safe method. The colour changed from pale yellow to dark brown indicated the formation of AgNPs and a sharp absorbance peak was observed at 419 nm. The X-ray diffraction analysis revealed the structure and chemical property of green synthesized AgNPs. Size and morphology were determined by SEM and TEM. The AgNPs chemical composition was revealed by EDX. FTIR spectroscopy investigated the presence of bioactive functional groups and exhibited vibrational peaks of compounds which are present in the seed extract. The synthesized AgNPs of the seed extract showed effective antimicrobial efficacy at higher concentrations against bacterial isolates of DFU.

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Authors contribution statement

Dr. Asha Arora designed and conceptualized this research work, Shweta Chhajed collected the sample and analyzed. Dr. Priyansh Jain calculated the data and interpreted all the data. All authors contributed in revising and editing the manuscript.

Conflict of interest

The authors declare no conflicts of interest relevant to this article.

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