Rapid biogenic fabrication of silver nanoparticles using *Ziziphus nummularia* under optimised conditions and evaluation of their antimicrobial synergy

V. Kumar[a,b,*], S. S. Lakhawat[b], S. Kumar[b], A. A. Chaudhary[c], H. A. Rudayni[c], M. Al-Zahrani[c], U. K. Gilhotra[d]
[a]*Research Scholar, Faculty of Pharmacy, Rajasthan University of Health Sciences, Jaipur 302033, Rajasthan, India*
[b]*Amity Institute of Biotechnology, Amity University Rajasthan, Jaipur 303002, Rajasthan India*
[c]*Department of Biology, College of Science, Imam Mohammad Ibn Saud Islamic University (IMSUI), Riyadh, Saudi Arabia*
[d]*Department of Pharmacology, G.D.Memorial College of Pharmacy, Jodhpur 342005, Rajasthan, India*

Nanotechnology deals with the Nanoparticles having a size of 1-100 nm in one dimension used significantly concerning medical chemistry, atomic physics, and all other known fields. These particles can be prepared easily by different physical, chemical and biological approaches. But the biological approach is the most emerging approach of preparation because this method is easier than the other methods, eco-friendly and less time consuming. In this study green synthesis of silver nanoparticles (AgNPs) of *Ziziphus nummularia (ZN)* was done using the magnetic stirrer method. These AgNPs were characterized by UV-vis Spectroscopy, FTIR and SEM technique and the optimization was done by varying the root extract volume, silver nitrate concentrations, pH and temperature. It was observed that ZN extract can reduce the silver ions in to AgNPs with in 30 min of reaction time. The surface plasmon resonance peak observed near 430 nm, confirmed the reduction of Ag^+ to Ag^0. Maximum absorbance peak 430 nm was observed with 2mM $AgNO_3$ solution with standard condition of 9pH and 70°C temp. at magnetic stirrer followed by 42 hrs incubation during the optimization of stable AgNPs. SEM images clearly shows that synthesized AgNPs are in spherical shape with size range 20-50 nm. FTIR clearly indicating the presence of capping and reducing agents around the AgNPs synthesised from crude extract of *ZN*. The biosynthesized AgNPs exhibited significant antimicrobial activity against pathogenic *Staphylococcus aureus*, *Enterococcus faecalis* (a Gram-positive bacterium), *Pseudomonas aeruginosa* and *Escherichia coli* (a gram negative bacterium) and *Aspergillus niger* (plant pathogenic fungal strain), *Candida albicans* (human pathogenic fungal strain). This novel approach of bionanotechnology can be taken up by the researcher in near future to develop the effective bionaomedicine, biopesticides, nanofood composites etc.

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Keywords: Silver nanoparticles, AgNPs; *Ziziphus nummularia*, Antimicrobial, Bacteria

1. Introduction

Nanotechnology is a modern science, engineering, and technology based converging technique to produce nanoscale particles, which is about 1 to 100 nanometers. Physicist Richard Feynman is the father of nanotechnology. In recent years many scientist have made their efforts to produce a specific desired nanoparticles in the field of drug delivery, catalysis, molecular imaging, biosensors, DNA sequencing and electrical devices. [1–3] Nanoparticles have very good impact in all fields of modern sciences including physics, chemistry, biology, electronics, biotechnology, biomedical and medicine. Nanoparticles have very specific features based on their shape, size, charge, scattering, optoelectronic, magnetic, and mechanical, which differs from bulk.[4,5]

* Corresponding author: vikramyadav05@gmail.com
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For the last five years, Green synthesis is given importance over the conventional methods. The abundance of raw materials makes green synthesis a cost-effective process. Nearly no harmful chemicals are involved in green synthesis where physical and chemical methods require high energy resources, huge amount of time, involves number of chemicals and are neither cost effective nor environmentally friendly.[6]

Thus, to overcome the drawbacks of Physical and chemical methods, Biological synthesis fills all the voids. Biogenic synthesis of nanoparticles is compatible with the principle of green chemistry. The resulting nanoparticles produced through biogenic synthesis are comparatively stable than that of physical and chemical methods. Further experiments and studies are under process to improvise the Green synthesis of nanoparticles. [7]

During the biogenic silver nanoparticle formation the bio reduction of $Ag^{+++}$to Ag^{o} occurs in presence of reducing and stabilizing agents which are naturally present in fungi, bacteria, yeast and plants [8]. Silver nanoparticles have the potential antimicrobial activity for the broad range of fungi, bacteria and virus [9] and they intensively used as a antimicrobial agents in various field of food storage, medicine, health and textile in a numeral ecological applications [10].

In view of the importance of the nanoparticles, this study was designed to green synthesis of silver nanoparticles using the *ZN* and the characterization along with optimization was also done using the different techniques i.e. UV, SEM and FTIR and finally evaluated the antimicrobial activity of biogenic silver nanoparticles (AgNPs) using human pathogen such as *Staphylococcus aureus, Enterococcus faecalis* (a Gram-positive bacterium), *Pseudomonas aeruginosa* and *Escherichia coli* (a gram negative bacterium) and *Aspergillus niger (plant pathogenic fungal strain), Candida albicans* (human pathogenic fungal strain). The biosynthesized AgNPs exhibited significant antimicrobial activity against all the selected pathogenic bacterial and fungal strain.

2. Materials and methods

2.1. Chemicals, reagents, culture strains and plant sample

The chemicals and reagents used in this study were either analytical or microbiological grade and were obtained from the Amity Institute of Biotechnology and Amity Institute of Microbial Technology Laboratory of Amity University Rajasthan. The bacterial strains used in the experiments were procured from Microbial Culture Collection Centre (MTCC), Institute of Microbial Technology (IMTECH), Chandigarh, and Amity Institute of Microbial Technology, Amity University Rajasthan, Jaipur. Fresh roots of *ZN* were collected from the nearby field of Amity University Rajasthan and the plant sample was authenticated from the CSIR-National Institute of Science Communication and Information Resources, Ministry of Science and Technology, Govt. of India with reference number NISCAIR/RHMD/Consult/2020/3645-46.

2.2. Preparation of crude extract

Sun dried fresh root sample was grinded to form homogenous powder and then 25gm root sample added to soxhlet apparatus and run the process with azeotropic mixture of 70% ethanol in distilled water for 48 hrs with temperature 60±5ºc. Dried biomass was collected using the vaccum evaporator and stored at 4°C temperature for further study.

2.3. Synthesis of AgNPs

First, Stock solution of 10% crude extract was prepared using the 10gm of dried biomass extract in 100 ml distilled water. Silver nanoparticles were synthesized by adding 100 mL of a 2 millimolar (mM) aqueous solution of silver nitrate into 50 mL of 10% *Ananas comosus* peels extract taken into beaker and pH level 9 was adjusted using 1M NaOH solution. Then the final solution was heated for 15 min at 70±5ºC and stirred using magnetic stirrer simultaneously in dark area. The color of the solution was started changing from light brown to dark brown within 15 min indicating the formation of nanoparticles and further, no change in color was observed and incubated the sample for 72 hrs at room temperature. The separation of silver nanoparticles from the dispersion was carried out by centrifugation at 10,000 rpm for 15 min after that AgNPs were

washed 4 times with distilled water and acetone to remove water soluble impurities and then nanoparticles were lyophilized and stored in dry bottles for further study [11].

2.4. Characterization of silver nanoparticles

Characterization of Ag-NPs was done using standard characterization techniques like UV-Visible (UV-Vis) spectroscopy, SEM, FTIR. UV-visible absorption measurements were carried out on high-quality monochromator based UV/VIS spectrophotometer (Thermo Scientific Multiskan GO) at 350–600 nm wavelength. The UV peaks in the range of 400 to 450 confirm the formation of AgNPs. FTIR (Thermo Scientific Nicolet iS5) was done for the qualitative study of the nanoparticles i.e. to study the functional groups present in the nanoparticles. The FTIR spectrum was collected at a spatial resolution of 4 cm^{-1} in the transmission mode, between 4000-600 cm^{-1}. Also, SEM (Nova NanoSEM™) was done to analyze the exact shape and size of the synthesized nanoparticles [11].

2.5. Optimization of silver nanoparticles synthesis

Different $AgNO_3$ concentrations varied between 0.5 mM to 3 mM were incubated for 2 days and UV-visible spectra recorded at regular time interval from 24 to 72 h. The effect of change in pH range 4 to 10 using 1M of NaOH and HCl on the rate of AgNPs synthesis recorded. AgNPs synthesis was also optimized with respect to change in temperature ranging from 20°C to 90°C used for synthesis of nanoparticles [12].

2.6. Antibacterial assay test by diffusion method

Antibacterial activity of bogenic silver nano particles of *ZN* extract was assessed against the various human pathogen strains such as *Staphylococcus aureus, Enterococcus faecalis (a Gram-positive bacterium), Pseudomonas aeruginosa and Escherichia coli (a gram negative bacterium) and Aspergillus niger (plant pathogenic fungal strain), Candida albicans (human pathogenic fungal strain)* by using the disc diffusion method.

In brief, overnight grown bacterial cultures were spread onto nutrient agar medium plates under the laminar flow and place the 10 µl of different concentration of AgNPs paper discs (from 100, 200 and 300 µg/ml concentration of AgNPs to achieve 1, 2 and 3 µg of AgNPs discs respectively), impregnated standard Streptomycin and Amphotericin -B discs (5 µg/disc) and water dipped control disc on nutrient agar plates of each microbes. The plates were incubated at 37°C for 24 h and inhibition zones were observed and this process was repeated in triplicate [13]. Mean and Standard Error of Mean (SEM) of the zone of inhibition were calculated using the simple excel.

3. Results and discussion

3.1. Characterization of silver nanoparticles

3.1.1. Color change

Color changes of the AgNPs solution from light brown to dark brown was observed when $AgNO_3$ was treated with *ZN* extract, which clearly indicates the formation of AgNPs. Color changes of the medium are due to the Surface Plasmon Resonance (SPR) phenomenon, in which chemical compound such as alkaloids, flavonoids, saponins, steroids present in plant extract acts as a reducing agent that helped in the reduction of Ag^{+3} to Ag^0 [12-14].

3.1.2. UV-Visible spectra of silver nanoparticles

The oscillation waves of electrons of AgNPs gives the SPR absorption band 400-440 nm [15]. The formation of biogenic AgNPs using the *ZN* extract was monitored at different time intervals in UV-vis spectroscopy and maximum absorbance peak band was observed at 430nm (Fig. 5,7,9), which is clearly indicated the SPR and the AgNPs size ranging from 2 to 100nm. Absorbance peak always depend upon the optimum concentration of $AgNO_3$, which was found to at the 1:2 ratio of *ZN* extract and 2mM of $AgNO_3$. Effect of the reaction time on the biogenic

AgNPs formation was also evaluated with UV-Visible spectrum and found that as the time increased the absorbance peak increases but after a certain time interval the absorbance peak remained constant, which shows the total bio reduction of silver nitrate to silver ions.

3.1.3. SEM of silver nanoparticles

The morphological characteristic phenomenon of AgNPs were also analyzed by using the SEM technique to confirm the shape and size. SEM micrograph of AgNPs of *ZN* extract shows that the nanoparticles are uniformly dispersed with spherical in shape and size ranging 20-50 nm (Fig.1) and there were no nanoparticles with simple extract sample (Fig. 2). Some of particles size were more than the desired size as a result of the proteins binding on the surface of the nanoparticles and the aggregation of AgNPs. However, under the optimization process, smaller size of AgNPs were synthesized with average size of 25 nm (Fig. 1). This reduction in size of AgNPs are reported to increased contact surface area of AgNPs with pathogenic bacteria and make it better bactericidal [16]. Plants are the good source of capping agent, which play very important role for the formation of stable AgNPs in uniform shape and size by effectively modifying the surfaces of nanoparticles [17]. Uniform shape and small size AgNPs are more susceptible to release the Ag+ ions [18].

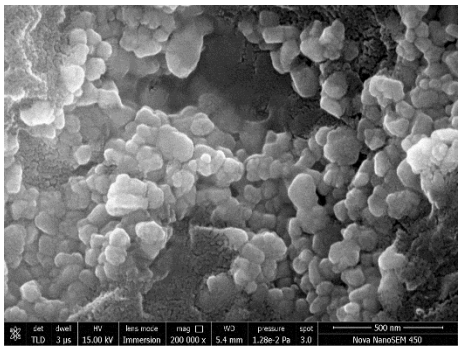

Fig. 1. SEM image of biogenic silver nanoparticles of Ziziphus nummularia extract.

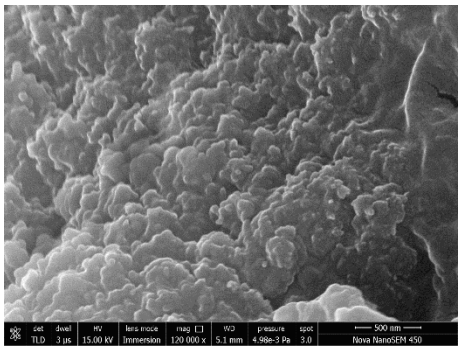

Fig. 2. SEM image of Ziziphus nummularia extract.

3.1.4. FTIR of silver nanoparticles

FT-IR spectroscopy was used to identify the surface and functional groups and its interaction with the AgNPs of *ZN*. The spectrum showed major absorption peaks at 1044.39, 1083.51, 1228.11, 1363.19, 1455.17, 1637.14, 1736.93, 2113.51 and 3320.54 cm^{-1}, which signify that the plant molecules act as capping agents that were bound on AgNPs (Fig. 3). The absorption peak at region 3320.54 cm^{-1} was the reason for -OH stretching of alcohol vibration. The absorption peak at 2113.51 cm^{-1} represented the C≡C stretching of alkyne. Hence, this spectral data confirmed the presence of proteins by the amine or amide I band at the region of 1637.14 and 1736.93 cm^{-1}. Also, the absorption band 1363.19 for the O-H bending of phenols and 1455.17

cm^{-1} for N-nitrosamines appended by AgNPs. The peaks at 1044.39, 1083.51 and 1228.11 cm^{-1} corresponding to the C-N stretching represents the presence of the amine group of proteins. AgNPs spectrum in Fig.3 shows that there was no free O-H group in spectra of silver nanoparticle as against 2905.58 and 2981.47 cm^{-1} in comparison to the spectrum of *ZN* extract (Fig.4), this suggested to result in binding of carboxylic acid group of reducing sugar to the silver. Hence, FTIR analysis confirmed the presence of capping and reducing agents around the AgNPs synthesised from crude extract of *ZN*. Furthermore, this result showed that phytochemical constituents like alkaloids, phenolic compounds, amino acids, carbohydrates and particularly tannins as a capping agents might protect the AgNPs from aggregation and thereby retain them for long term stability [17,18].

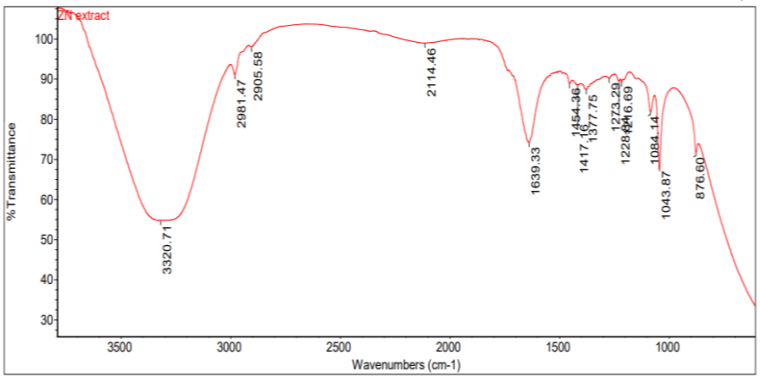

Fig. 3. FTIR analysis of Ziziphus nummularia extract.

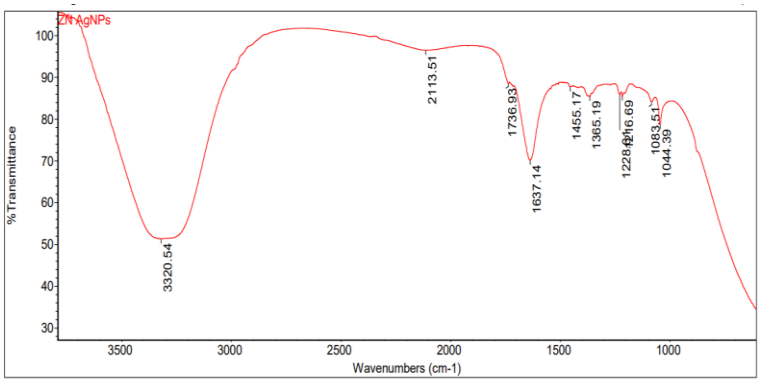

Fig. 4. FTIR analysis of biogenic AgNPs of Ziziphus nummularia extract.

3.2. Optimization of silver nanoparticles

3.2.1. Effect of concentration of $AgNO_3$ on AgNPs concentration

Different concentrations of $AgNO_3$ solution ranging from 0.5 mM to 3 mM were used for the synthesis of AgNPs using *ZN* crude extract and observed at regular interval from 24 to 72 h of incubation. At 0.5mM $AgNO_3$ concentration formation of AgNPs was found very low which increases up to 2mM $AgNO_3$ at 42 h incubation. Amount of AgNPs decreased after 72 h of incubation showing the complete reduction of Ag^+ at 48h. Maximum absorbance peak 430 nm was found with 2mM concentration (Fig.5,6) and further increase in $AgNO_3$ concentration led to decrease in the formation of AgNPs. This phenomenon can be expressed on the basis of enzyme substrate kinetics where the enzyme catalysing reduction site is saturated with the Ag ions and no more reduction site is available, which shows the total bio reduction of silver nitrate to silver ions; therefore, no further increase in biogenic AgNPs at higher concentration of $AgNO_3$ [12-14].

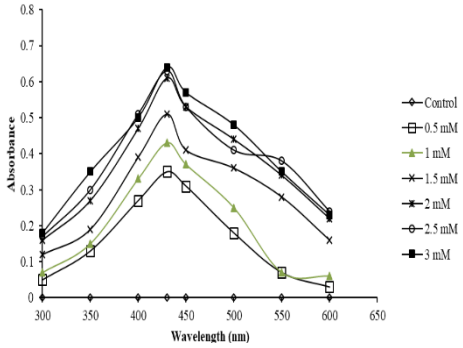

Fig. 5. UV–vis spectra of AgNPs after 48 h incubation with different conc. of $AgNO_3$.

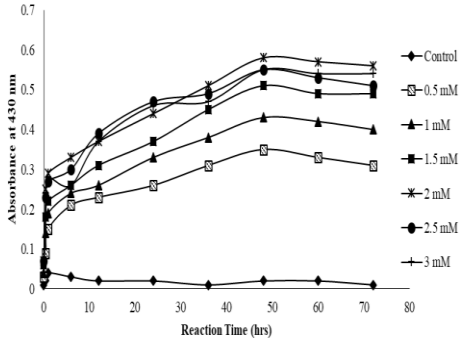

Fig. 6. Optimization of time dependent AgNPs synthesis at different conc. of $AgNO_3$.

3.2.2. Effect of pH on AgNPs concentration

To optimize the AgNPs synthesis the pH variation from 4 to 10 was studied using the 1M of NaOH and HCl. The initial pH of the extract sample was around 5.8. On incubation of different pH (4 to 10) extract sample with $AgNO_3$ for 72 h, the best yield of biogenic AgNPs was found with pH 9 maintained extract sample (Fig. 7,8). UV-vis spectra shows the maximum absorbance at 430 nm with pH 9 sample extract AgNPs at 48 hrs. This phenomenon may be due to amplified availability of OH^- at pH 9, which supports in complete reduction of Ag^+ into AgNPs by providing electrons [12-14]. However, further increase in pH up to 10 shows the reduction in AgNPs synthesis, it may be due to inactivation of enzyme responsible for AgNPs synthesis at higher pH level [13].

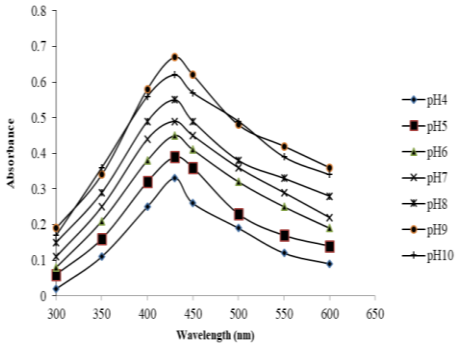

Fig. 7. UV–vis spectra of AgNPs after 48 h incubation at different pH.

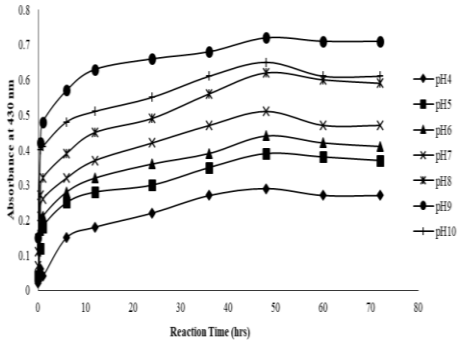

Fig. 8. Optimization of time dependent AgNPs synthesis at different pH.

3.2.3. Effect of Temperature on AgNPs concentration

During the AgNPs synthesis optimum level of temperature also play very important role for their optimization. The effect of temperature on AgNPs formation was observed at different temperature range from 20°C to 90°C at an interval of 10°C along with 2 mM $AgNO_3$, pH 9 and regular time interval for 72 hrs incubation (Fig. 9,10). At 70°C, color change of the sample from light brown to dark brown was observed within 15 min and formation of uniformly dispersed AgNPs were observed within 24 h and remains stable for longer time period indicated rapid and stabilized biosynthesis. However, further increase in temperature up to 90°C shows the reduction in AgNPs synthesis, it may be due to the thermal degradation of molecules which are involving in the formation of AgNPs formation. The results represents that the optimum higher level of temperature leads to an increase in the activation energy of the molecules and faster rate of Ag^+ ions reduction. As a result, there is a decrease in the size of synthesized AgNPs and, hence, uniformly dispersed small nanoparticles are formed without undergoing the AgNPs aggregation [19].

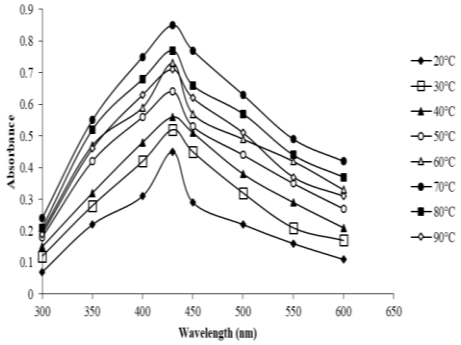

Fig. 9. UV–vis spectra of AgNPs after 48 h incubation at different temperature.

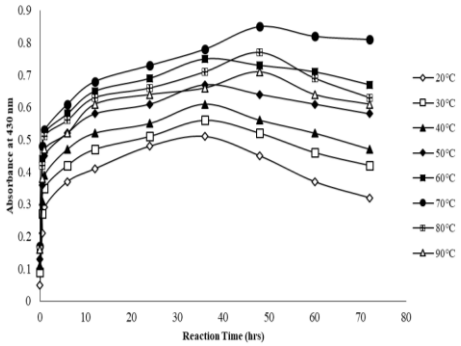

Fig. 10. Optimization of time dependent AgNPs synthesis at different temperature.

3.3. Antimicrobial activity of AgNPs

Antimicrobial assay of biogenic AgNPs of *ZN* was studied against the various human pathogenic microbes such as *Staphylococcus aureus, Enterococcus faecalis (a Gram-positive bacterium), Pseudomonas aeruginosa and Escherichia coli (a gram negative bacterium) and Aspergillus niger (plant pathogenic fungal strain), Candida albicans (human pathogenic fungal strain)* by using the disc diffusion method and zone of inhibition was observed (Table 1). During this study we found that zone of inhibition was significantly increased with the increase in the concentration of AgNPs as shown in Fig.11, the satisfactory zone of inhibition was observed with 2 µg AgNPs with all selected pathogenic strains in comparison with standard Streptomycin and Amphotericin -B antibiotic. Zone of inhibition with 2 µg AgNPs concentrations against the *S. aureus, E. faecalis, P. aeruginosa, E. coli, A. niger, and C. albicans* was found to be 16.13±0.34, 14.77±0.32, 13.13±0.38, 16.07±0.20, 15.13±0.20 and 14.23±0.26 mm respectively. There was a satisfactory zone of inhibition with positive control standard Streptomycin (against bacterial strains) and Amphotericin –B (against fungal strains) treated discs in all these concerned microbial strains and there was no zone of inhibition found with negative control water treated discs in all these microbial plates. These results clearly represents that AgNPs of *ZN* were most effective against gram negative bacterium in comparison with gram positive bacterial strains and fungal strains. This might be due to the Ag^{+} from AgNPs can anchor to the negatively charged bacterial cell wall lead to perforation and results in cell lysis. Moreover, role of free radicals produced by AgNPs in contact with bacteria was also reported as demonstrated by electron spin resonance spectroscopy [20]. This excellent antimicrobial effects might be due to the attachment of AgNPs to the cell surface and disrupt the cell membrane by interacting with Sulphur and phosphorous containing components like DNA, protein [21].

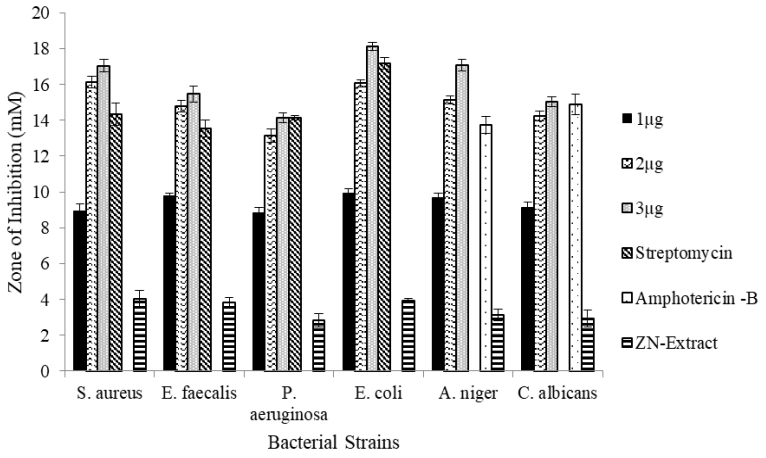

Fig. 11. Antimicrobial activity of AgNPs in relation with Zone of Inhibition.

Table 1. Antimicrobial activity of biogenic AgNPs of Ziziphus nummularia.

Microbial Strains	Antimicrobial activity of AgNPs of *Ziziphus nummularia* (Zone of Inhibition ± SEM)					
	1µg	2µg	3µg	Streptomycin	Amphotericin -B	ZN-Extract
S. aureus	8.93±0.38	16.13±0.34	17.03±0.35	14.33±0.60	NA	4.03±0.47
E. faecalis	9.77±0.18	14.77±0.32	15.47±0.44	13.53±0.48	NA	3.83±0.29
P. aeruginosa	8.83±0.30	13.13±0.38	14.13±0.29	14.13±0.12	NA	2.83±0.35
E. coli	9.93±0.26	16.07±0.20	18.13±0.23	17.17±0.32	NA	3.93±0.15
A. niger	9.67±0.27	15.13±0.20	17.07±0.34	NA	13.73±0.46	3.13±0.33
C. albicans	9.13±0.32	14.23±0.26	15.03±0.29	NA	14.87±0.58	2.93±0.49

3. Conclusion

This is the first report on biogenic formation of AgNPs using the *ZN* under the optimized condition. Optimization clearly confirmed that average monodispersed AgNPs were formed by mixing of *ZN* extract with 2mM $AgNO_3$ solution with standard condition of 9pH and 70°C temp. at magnetic stirrer followed by 42 hrs incubation. UV-vis spectroscopy based maximum absorbance peak band was observed at 430nm. Monodispersed spherical AgNPs of size 20-50 nm and presence of surface functional groups on AgNPs were characterized by using the SEM and FTIR techniques respectively. AgNPs of *ZN* exhibited excellent antibacterial and antifungal activity on pathogenic microbial strains. In near future researcher can explore the potential biomedical application of biogenic AgNPs of *ZN* by developing eco-friendly and cost-effective nanomedicine formulations.

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