

Bio-assisted synthesis of bi-metallic (Ag-Zn) nanoparticles by leaf extract of *Azadirachta indica* and its antimicrobial properties

Indranil Singh, Tooba Mazhar, Vikas Shrivastava*, Rajesh Singh Tomar

Amity Institute of Biotechnology, Amity University Madhya Pradesh, Gwalior, India, 474005

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Abstract

In the last decade, the bio-assisted synthesis of nanoparticles has been significantly exploited seeing the shortfall of chemical synthesis and urgent need to find the substitution. The chemical synthesis of nanoparticles is a rapid process, which might be appealing in various frontiers but has got some serious issues to take care of. In pursuit of finding a process that is cleaner, non-toxic, eco-friendly, low cost, and renewable, nature introduces us to the prospect of biosynthesis. In our study, we have successfully prepared bimetallic nanoparticles through a bio-assisted route. We have formed (Ag-Zn) silver doped zinc oxide nanoparticles with the help of an aqueous leaf extract of *Azadirachta indica*. Ag-Zn nanoparticles were further characterized by FTIR, P-XRD, SEM-EDX methods. The data obtained from X-ray diffraction has shown the peaks of silver doped zinc at 2θ value 38.1° thereby telling particle size is approx. 12.53 nm size as calculated by Scherrer's equation. FTIR analysis gave characteristic peaks of functional groups. SEM-EDX confirmed successful doping and grain size of the particle. The study has further characterized the anti-microbial activities of the Ag-Zn BMNPs (Bi-metallic nanoparticles) with the help of the Kirby-Bauer method showing maximum inhibition of *Streptococcus aureus* species. The result of the study can be advantageous to develop an understanding of the development of nano-based medicine.

Keywords: Antimicrobial; *Azadirachta Indica*; Bio-Assisted; Nanoparticles; Nanotechnology.

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INTRODUCTION

A massive amount of surge in nanomaterial research has been observed in the past few decades. Nanobiotechnology has been the central figure of emerging research in the field of nanomaterials. It is the field that has brought nanotechnology, material sciences, chemistry, physics, and biotechnology to a single interface. Of all the established processes of nanomaterial synthesis ranging from numerous physical and chemical processes, it is the biological process that has started making name for itself. Nanobiotechnology opting greener route has emerged as an eco-friendly, cheap, hygienic, process of nanomaterial synthesis. Several nanomaterial hence formed has resulted in an

increased anti-bacterial, anti-viral, anti-cancer potential [1]. Plant extract and other biological elements have proved to be more capable as efficient capping agents. They are also believed to assist as well as introduce some functional properties that could help us to target tumours, eradication of biofilm, can be used as anti-viral etc. [2-4].

The bio-assisted synthesis of nanoparticles has gained a lot of attention recently. It has been due to the emergence of this cheap and eco-friendly method [5]. Synthesis of silver nanoparticles using greener routes has been reported in the literature [6] but, work on bimetallic silver doped zinc oxide nanoparticles through the bio-assisted method is quite scanty.

This tendency of the ZnO has resulted in its

* Corresponding Author Email: vshrivastava@gwa.amity.edu

application in electronics, sensors, a biocide, detector, optoelectronics as well as photo-catalyst [7-9]. It is further preferred for its chemical stability, being biologically safe, and does have a low cost and toxicity [10]. Bio-assisted methods generally employ one of the following three methods to produce the nanoparticle. It includes plants extract, microorganisms, and enzymes for the reduction as well as the capping of the hence formed nanoparticle [11]. Zinc oxide nanoparticles were found to be effective against fungal phyto-pathogens but inhibition by particles was dependent upon the size of synthesized nanoparticles as well as their concentration used [12-14].

Silver is a long-established anti-microbial agent which has a wide-ranging application [15-16]. It has been one of the potent anti-microbial used in the form of topical ointment over burns and wounds [17]. Interestingly, they have shown a large amount of inhibitory effect against microorganism but has failed to show a similar toxic effect on animal cells [18]. Post application of the silver ion has shown to function as stress inducers which cause detrimental effects by the condensation of the genetic material inside the cell. The other chains of events post application are DNA damage, cell detachment from the cell wall, shrinkage, dehydration, and formation of granules inside as well as outside of the cell [19-21]. Antifungal effects of silver have established significant results [22].

Past studies over the mechanism of the nanoparticle functioning have reported the disruption of the cell wall by anchoring of NPs followed by penetration leading to the disruption of the cell through disruption of the cell wall integrity, change in the permeability, uncontrolled transportation [23]. Some work has also argued about the reactive oxygen species (ROS) generation as a potential method acquired by nanoparticles for their anti-bacterial mechanism [24]. A study over silver nanoparticle coated endotracheal tubes which are used in ventilator-assisted breathing has been beneficial in reducing the no. of cases as well as onset time [25]. The shape of synthesized nanoparticles also affects their antibacterial action against several microbial species [26].

Today there are numerous options in form of the biological system that could eventually help us to synthesize nanoparticles. There are reported processes in the case of fungi [27], yeast [28],

diatoms [29], and bacteria [30], including algae and plants [31] which could assist in the conversion of inorganic metal into the desired size and shape of the nanoparticle. The reducing potential of the substrate has been interlinked to the different metabolites and proteins that are present in it.

Brassica juncea, *Medicago sativa*, *Iris pseudacorus* are a few plants of many that have been reported to accumulate the nanoparticles of the metal on which they have grown [32-34]. The heterogeneity in terms of shape which varies from hexagons, triangles, spheres, ellipsoids, cubes, nanorods, nanowires, in terms of size, and accumulation has driven research towards more effective and flexible synthesis with tailored size and shape of nanoparticles. Now days this has been achieved in-vitro from plant extracts of different plant parts that have been successfully reduced numerous metals salts as well as acids to count gold, silver, copper, iron, titanium, etc. [35-37]. Plants parts of *Pelargonium graveolen* [38], *Cymbopogon flexuosus* [39], *Azadirachta indica* [40], *Aloe barbadensis* [41], etc. are reported to possess the reducing potential. The FTIR spectroscopy of the hence formed nanoparticles has confirmed the role of sugars, flavonoids (luteolin, quercetin), polyphenols, terpenoids (eugenol), alkaloids, proteins, phenolic acids as important in the reduction of metal ions to nanoparticles [42-43].

In this study, we have taken *Azadirachta indica* leaves for their availability, rapid growth, and for them being widely cultivated throughout India. *Azadirachta indica* has widely been used for its various applications in countering medical conditions and has various versatile applications in the field of pharmaceuticals. It has established the potential to act as an anticancer, antibacterial, and antioxidant agent [44].

Our study reports, a comparatively simpler biological synthesis for bi-metallic (Ag-ZnO) nanoparticles by the leaf extract of *A. indica*, wherein, plant extract has played the dual role of both reducing as well as of capping agent. The nanoparticles were characterized using SEM-EDX, FTIR and P-XRD techniques. This process is eco-friendly, cheap, and a one-pot synthesis at room temperature hence it could be easily used for the scale-up.

MATERIALS AND METHODS

Sample Collection

Azadirachta indica leaves were collected from Amity University Madhya Pradesh, Gwalior, India.

The leaves hence collected were stored in the air-tight zipper bags made up of polyethene which was then kept under shade. The collected leaves were then transferred to the beaker where they were washed twice by the tap water followed by three-time washing done with the help of distilled water. Care must be taken that leaves were properly washed with almost no dust particle being attached to the leaves. They were then weighed and then left at room temperature for a few days to allow leaves to get dried completely. Alternatively, one can sun dry or oven-dry the leaves at the temperature of 60 °C for 24 hr [45].

Extraction Method

The leaf extract of *Azadirachta indica* was prepared with 20g of dry leaves which were taken and then grounded in a mortar pestle. The dry leaf powder was then dissolved in 50% ethanol and left overnight at room temperature. The next day extract was filtered first with muslin cloth and then with Whatman filter paper. The extract was collected in a bottle and stored at 4 °C until further use. The extract was light green.

Phytochemical Analysis of ethanolic extract

The plant extract was screened using HPLC to check the presence of secondary metabolites such as tannins, alkaloids, glycosides, terpenoids, saponins, flavonoids, phenols, steroids, catechins, coumarins, quinones and xanthoproteins [46-47].

Synthesis of Bi-metallic (Ag-Zn) nanoparticles

The stock solution was prepared by dissolving 1mM Zinc acetate (Merck, Mumbai, India) and volume was then made up to 100 mL. In a separate flask, a stock solution of 0.1 mM of Silver nitrate (AgNO_3 ; Merck, Mumbai, India) was prepared and then the volume was made up to 100mL. Pour both hence formed solutions into a 250 mL beaker with simultaneous added 5 mL ethanolic neem leaf extract in a drop-wise manner. The beaker was kept over a magnetic stirrer at room temperature. The solutions were stirred for 6-8 hrs similarly at 250 rpm [48-49].

Test microorganism

The test microorganisms were taken from The Institute of Microbial Technology, Chandigarh, India. The antibacterial assay was performed over *Salmonella enterica typhimurium* (MTCC No. 98), *Streptococcus pyogenes* (MTCC No. 1926),

Staphylococcus aureus (MTCC No. 9760), *Proteus mirabilis* (MTCC No. 3310), *Streptococcus aureus* (MTCC No. 1926), *Staphylococcus saprophyticus subsp. saprophyticus* (MTCC No. 6155), *Escherichia coli* (MTCC No. 40), *Pseudomonas aeruginosa* (MTCC No. 424), *Listeria monocytogenes* (MTCC No. 657), *Bacillus cereus* (MTCC No. 430), and *Salmonella enterica ser. paratyphi*. (MTCC No. 735).

Characterization of Bi-metallic Nanoparticle (Ag-Zn)

The nanoparticles that have been formed through the above-mentioned process were then subjected to characterization, done at Sophisticated Analytical Instrument Facility (SAIF) of Sophisticated Test and Instrumentation Centre (STIC), Cochi, Kerala. Techniques include Fourier-transform infrared spectroscopy (by Thermo Nicolet Avtar 370 4000 cm^{-1} to 400 cm^{-1} , USA), Scanning Electron Microscope-Energy Dispersive X-Ray Analysis (by Jeol 6390LA/ OXFORD XMX N, Jeol USA Inc.) and X-ray Diffractometer (Powder Method) (by Bruker D8 Advance Twin- Twin, US) that provided in-depth analysis of particle morphology, size and bond association.

Antibacterial Assay

The complete antibacterial analysis was based on the Kirby Bauer well diffusion method [50] where we have taken Mueller Hinton Agar to act as the medium of growth. The wells formed were of diameter 5 mm. The microorganism that has been taken under consideration was first grown in the sterilized nutrient broth at 37°C in an incubator. For the characterization of antibacterial activity of the fabricated Ag-Zn nanoparticles following steps have been taken under consideration. The 100 microliters of the overnight grown culture have been spread over the Mueller Hinton Agar plate. It is followed by the creation of wells. Wells were then loaded with the 100 μl of nanoparticles of the desired conc. to analyse its anti-bacterial potential. The same procedure has been repeated in the entire test microorganism mentioned above. Then, the so formed plates were incubated overnight at 37°C to check the effect of nanoparticles on the growth of bacteria.

RESULT AND DISCUSSION

Phytochemical Analysis of ethanolic neem leaf extract:

Phytochemical screening was done to evaluate

the presence of different phytomolecules in *Azadirachta indica* plant extracts (Table 1). The key below Table 1 signifies the quantification of variability in presence of different phytomolecules. The negative sign indicates absence, while a single positive sign signifies the weak presence of phytomolecules. An increase in the number of these positive signs indicates the abundance of these phytomolecules in the extract. The result illustrated in Table 1 depicts the presence of saponins, flavonoids, alkaloids, tannins, etc. and it has been shown by us and others, that these phytomolecules have the potential of driving photo-reduction of compounds [51].

HPLC Results of Neem leaf extract

According to results obtained from HPLC (High-performance Liquid Chromatography) quantification of ellagic acid, eugenol, gallic acid, and quercetin were obtained using standard graphs. The peak in the chromatogram (Fig. 1) was identified based on the retention time of standards injected separately and by the addition of standard solutions.

Synthesis of Nanoparticle and the visible changes

The biosynthesis of a nanoparticle is a three-step synthesis process. It usually begins with the reduction of the metal ions and simultaneous nucleation [52]. This is followed by coalescing of the nanoparticle, generally termed as the growth

phase. The final step in the biosynthesis involves termination providing the final identical shape to the nanoparticles. In simpler terms, metal salts dissolved in the distilled water soon turns out to be divided into the ionic form post addition of plant extract through bio-reduction. The possibility is that the functional group present in the extract must be responsible for these conversions of metal ions into its zero-valent state, which then undergoes the growth phase to form the nanoparticle.

P-XRD Results

Powder X-Ray Diffraction is used for the study of crystalline size and the structural properties of formed bimetallic nanoparticles. The XRD is done with the 2θ range of 10° to 80° and the result is summarised in Fig. 2. The peaks were found to be sharp as well as narrow which indicates the wurtzite hexagonal phase of the nanoparticle [48]. The strongest peaks were detected at 2θ values 31.86° , 34.41° , 36.18° , 38.15° , 44.33° , 47.38° , 56.70° , 62.69° , 77.22° corresponding to dhkl values (100), (002), (101), (111), (200), (102), (110), (220), (202) respectively [53]. Crystal lattice peaks (100), (002) and (101) corresponds to JCPDS file no. 89-1397 and (103) indicates the presence of silver corresponding to JCPDS file no. 89-3722 [54]. Further, we have used the Scherrer equation to determine the mean crystalline size of the particle from the line broadening XRD measurement using

Table 1. Showing presence of phytochemicals in *neem* leaf extract.

Sr. No.	Phytochemicals in ethanol extract	Presence or absence
1.	Tannins	++
2.	Alkaloids	+++
3.	Glycosides	++
4.	Saponins	+++
5.	Terpenoids	+++
6.	Flavonoids	+
7.	Phenols	++
8.	Steroids	+++
9.	Catechins	---
10.	Coumarins	++
11.	Quinones	+++
12.	Xanthoprotein	---

(+ Present; - Absent)

+++ Strongly present; ++ Present

+Weakly present; - Absent

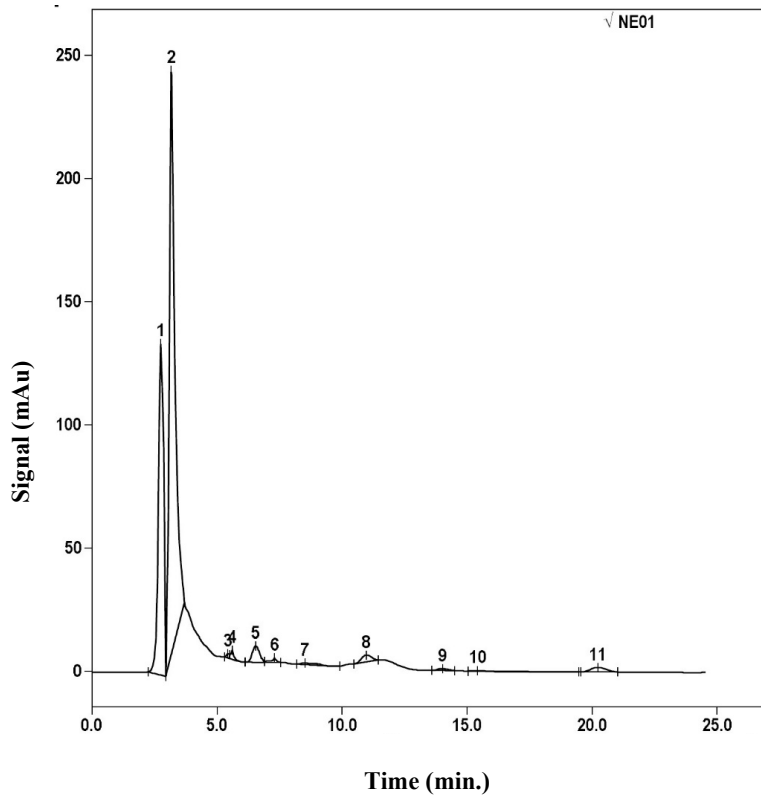


Fig. 1. HPLC Graph showing the presence of different phytochemicals.

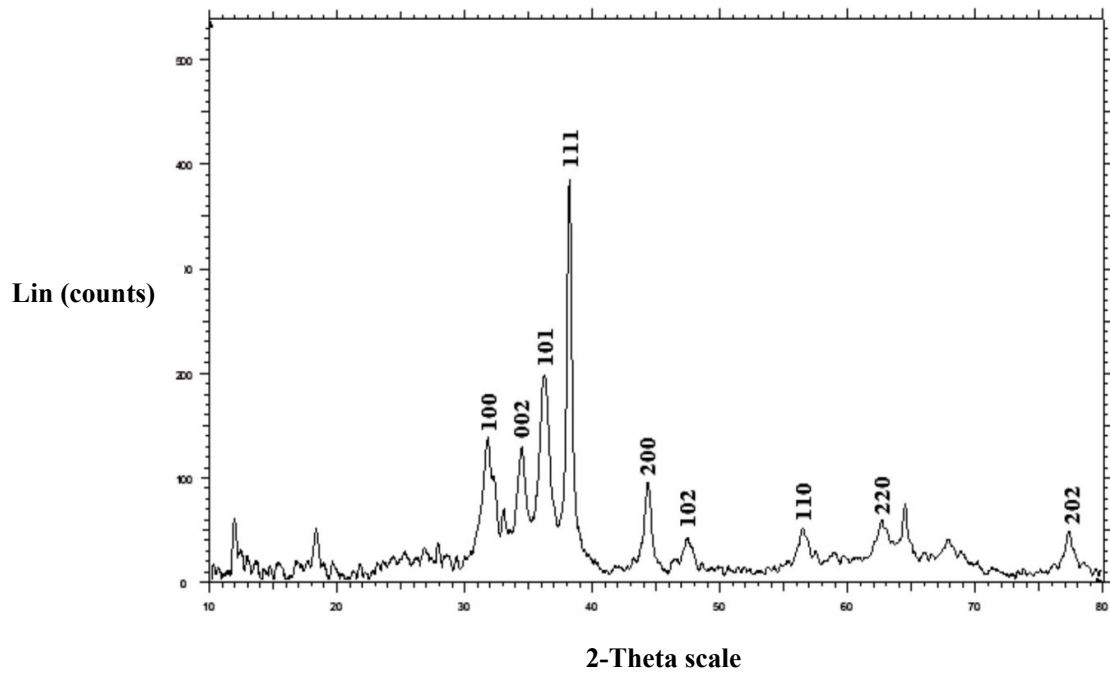


Fig. 2. P-XRD Graph showing different peaks of Bimetallic Ag doped Zinc Oxide Nanoparticle.

the following equation:

$$D = 0.89\lambda / (\beta \cos\theta)$$

Where λ is the wavelength of radiation and can be calculated through $2d \sin\theta$. β stands for the FWHM i.e., full-width half-maximum of the Ag-Zn nanoparticles and the diffraction angle is denoted with the help of θ . The resulted size of the crystallite based on the above-stated formula was calculated to be 12.53 nm.

FTIR Analysis

The FT-IR (Fourier Transform Infrared) analysis (Fig. 3) of bio-assisted nanoparticle have shown absorption band at 1653.20; 1608.93 and 1379.68. The absorption peak in Fig. 3 is seen at 1653.20 representing the C=C stretching alkanes. While that of 1608.93 represents the C=C stretching-conjugated alkenes. On the same line, 1379.68 represents the O-H blending phenol. The mentioned absorption peak shows vibration, stretching, as well as bending characteristics of the various functional group that is being found in the sample. These peaks could refer to the C-H stretch of phenols, the N-H bond of primary amines, the O-H stretch of Phenols and the CH-CH bending

found in the various aromatic rings. These findings suggest that there has been efficient capping of the bi-metallic nanomaterial hence formed.

SEM Image

SEM makes use of a high energy beam of electrons to produce a range of signals on the specimen surface. These signals give an idea about the surface morphology of nanoparticles. Under SEM silver doped zinc oxide showed irregular-sized nanoparticles that were oriented randomly in the range of 113 nm-250 nm as seen in Fig. 4.

EDX analysis

The EDX spectrum, (Fig. 5) further clarifies and proves our claim of formation of bi-metallic nanoparticles through the bio-assisted method as well as the efficient doping of the silver nanoparticles along with the zinc nanoparticles. The EDX spectrum at the same time also had shown only the peaks of zinc, oxygen, silver and some minute amount of carbon and sodium. This is the result of the secondary metabolites that have been taken as leaf extract to cap and stabilize the nanoparticles. The atomic and the weight per cent value of the element could be found in table 2.

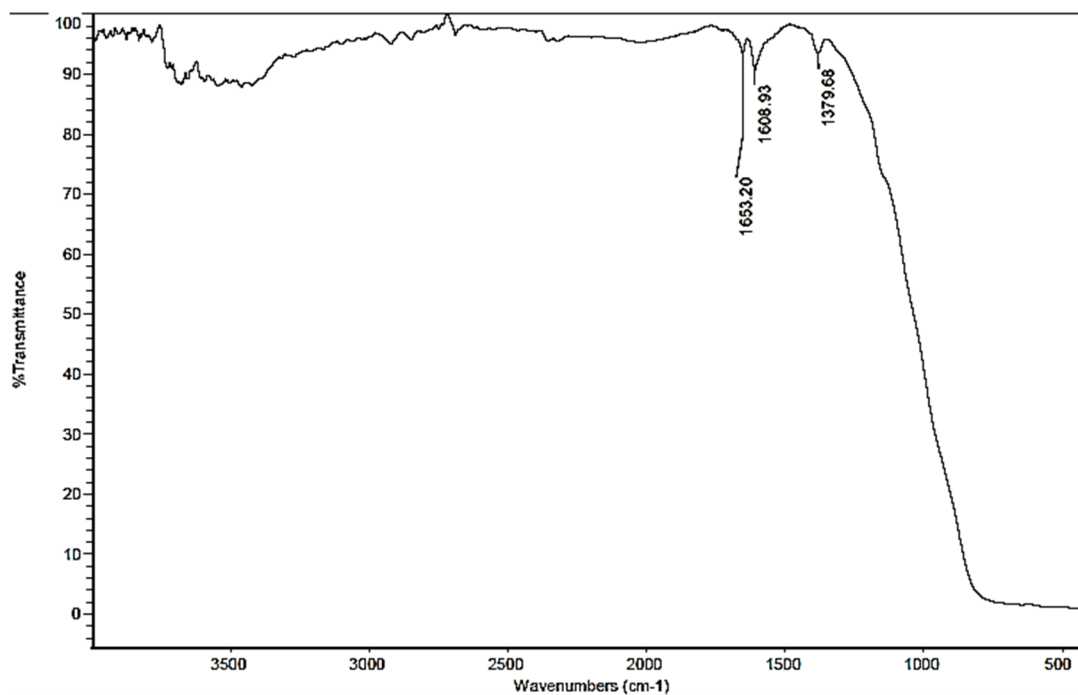


Fig. 3. FTIR graph showing vibrational frequencies of functional groups.

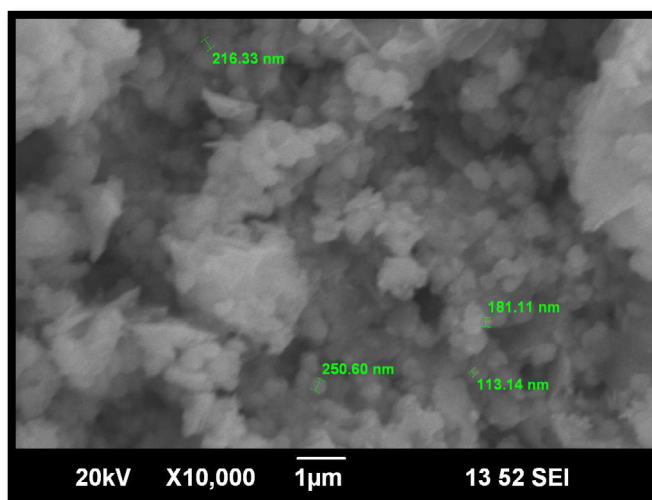


Fig. 4. Image showing surface morphology of Ag-doped Zinc oxide nanoparticle.

Table 2. Shows atomic and the weight per cent value of the element.

Element	Line Type	Wt.%	Atomic %
C	K-Series	10.02%	26.3%
O	K-Series	18.28%	36.01%
Na	K-Series	5.59%	7.67%
Ag	K-Series	56.35%	27.17%
Zn	L-Series	9.76%	2.85%

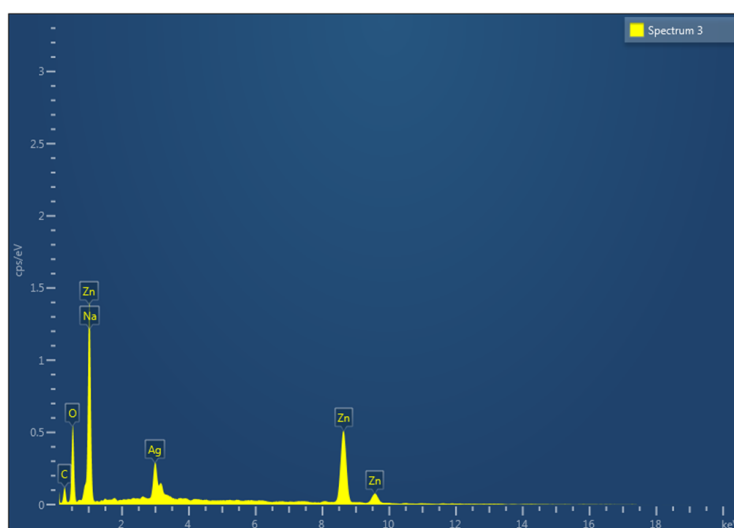


Fig. 5. Graph showing EDX pattern of synthesized bimetallic silver doped zinc oxide nanoparticles.

Anti-bacterial Activity

The anti-microbial activity of Zinc, as well as Silver, is a fact [55]. Silver showed anti-microbial activity against several pathogenic micro-

organisms at extremely low conc. [56]. Zinc has also proved to be toxic against a large number of gram-negative as well as gram-positive bacteria [57]. Whereas, the effects of doping of silver with

Table 3. Shows organisms along with their Zones of Inhibition at different nanoparticle concentration.

Ag-ZnO Nanoparticles Antimicrobial activity						
Sr. No.	Organism strain code	Organism name	50 µg	100 µg	200 µg	400 µg
1	P.A. 424	<i>Pseudomonas aeruginosa</i>	0	14 mm	18 mm	20 mm
2	B.C.430	<i>Bacillus cereus</i>	0	12 mm	13 mm	14 mm
3	E.C.40	<i>Escherichia coli</i>	0	12 mm	13 mm	14 mm
4	SPT 735	<i>Salmonella enterica ser. paratyphi</i>	0	13 mm	15 mm	18 mm
5	L.M. 657	<i>Listeria monocytogenes</i>	0	11 mm	16 mm	18 mm
6	S.T. 98	<i>Salmonella enterica typhimurium</i>	0	14 mm	17 mm	22 mm
7	S.P. 1926	<i>Streptococcus aureus</i>	18 mm	-	22 mm	25 mm
8	S.A. 9760	<i>Staphylococcus aureus</i>	-	15 mm	17 mm	20 mm
9	P.M. 3310	<i>Proteus mirabilis</i>	0	0	0	0
10	S.S. 6155	<i>Staphylococcus saprophyticus subsp. saprophyticus</i>	0	0	0	0

zinc on microbial species as antimicrobial agent, were targeted in present study. The antibacterial activity of these particles has been checked against several bacteria and summarized in Table 3. Biosynthesized nanoparticles have exhibited a good zone of inhibition in almost all cases. It has to be kept in mind that for the antibacterial test we have taken only 100 µl of an aliquot in wells. In our findings, there was no significant inhibition at the 50 µg/100 µl in many of the bacteria. But there was a significant Zone of inhibition that could be seen in the case of *Streptococcus aureus* that shows 18mm after 24 hr. of incubation. This leads us to conclude that antimicrobial substances could have different sensitivity concerning different species and they may also vary on the sub-species level. The 100 µl of aliquot containing 100µg has shown a zone of inhibition from 11 mm in the case of *L. monocytogenes* to 15mm in *S. aureus*. While the concentration was increased to 200 µg of nanoparticles in 100 µl, it was found that there is a linear increase in the Zone of Inhibition that justifies the result of earlier concentration. Although Zone of Inhibition ranges from 14 mm-25 mm was achieved at the 400 µg of nanoparticles. It is to be noted here that 100 µg is sufficient in itself to irradiate the bacteria completely. It may be considered that 100 µg could be act as MBC (Minimum bactericidal concentration.). While MIC i.e. Minimum inhibitory concentration has been different for each of the bacteria but has in all cases lies between those of 0.5 mg/ml to 1 mg/ml. Hence the addition of 10% silver has increased the antibacterial activity of zinc nanoparticles as compared to the previous result that has been reported.

The antibacterial or antimicrobial activities of the nanoparticles are attributed to the size and

shape of nanoparticles. Another factor that also contributes to the activity of the nanoparticles is its capping agent, functional groups and also specific surface area [58]. With the increase in the concentration, there is an increase in the zone of inhibition due to an increase in the number of molecules like H₂O₂ which is responsible for the antimicrobial activity [59]. The leaf extracts also reported assisting the antimicrobial activity by the addition of the various phytochemicals [60]. It is also stated that smaller particle size directly correlates with the larger bandgap and subsequently made it impossible for the recombination of the excitons. Accumulation of these excitons results in the formation of the reactive oxygen species that prove to be detrimental to the survival of the bacteria [61].

CONCLUSION

Silver doped zinc oxide bimetallic nanoparticles were synthesized using ethanolic extract of dried *Azadirachta indica* leaves. To get an insight of properties of synthesized nanoparticles they were characterized using P-XRD, FTIR and SEM-EDX. Green synthesis has opted over physical and chemical as it is cheaper, easier to scale up, reliable and faster with no additional requirement of temperature maintenance. The Ag-Zn nanoparticle has shown excellent antibacterial and anti-microbial activity when tested against the set organisms especially *Streptococcus aureus*. Our study can pave way for treating many bacterial infections proving to be a boon in medical science.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR'S CONTRIBUTION

Experiment designs were conducted by VS and IS are. IS performed all the experiments. Interpretation of data was conducted by TM, VS, and IS. This project was supervised by RST, VS and the paper was written by IS and TM. RST and VS have proofread the paper. IS & TM are equal first authors.

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