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ANTIMICROBIAL AND ANTIBIOFILM ACTIVITY OF *AZADIRACHTA INDICA* SILVER NANOPARTICLES AGAINST ENTERIC PATHOGENS ISOLATED FROM EFFLUENT SAMPLES

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Keywords:

Biofilms, Silver Nanoparticles, *Azadirachta indica*, Effluent, Sewage

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ABSTRACT: The aim of this study was to evaluate the antimicrobial and antibiofilm activity of silver nanoparticles of *Azadirachta indica* (AISNPs) against the pathogens isolated from effluent. Green synthesis approach was opted for the synthesis of the synthesis of AISNPs by using the ethanolic extract of *Azadirachta indica* which was added to 1mM solution of $AgNO_3$. These synthesized nanoparticles were molecularly characterized by UV-Visible spectrophotometer, TEM, Zeta Sizer, Zeta Potential, XRD and FTIR respectively. Antimicrobial activity of the AISNPs was carried by well diffusion method on Muller Hinton Agar against biofilm-producing multi-drug resistant effluent pathogens, which were identified as *E. coli*, *P. aeruginosa*, *S. aureus* and *K. pneumoniae*. The zone of inhibition of AISNPs observed in case of *E. coli* was 16.08 mm, 18.65 mm against *S. aureus*, 10.46 mm against *P. aeruginosa* and 15.34 mm against *K. pneumoniae*. Antibiofilm activity of AISNPs demonstrated the decline of biofilm producing activity by 76% against *P. aeruginosa*, 74% against *S. aureus*, 60% against *K. pneumoniae* and 59% against *E. coli* respectively, indicating the strong antibiofilm properties of the AISNPs. It can be concluded from the present study that green synthesized AISNPs can be used as an alternate approach to combat the multiple drug-resistant biofilm producing pathogens isolated from effluent.

INTRODUCTION: Environment is a multi-factorial component, comprising of the natural resources like air, soil and water as essential ingredients and each of these units are directly associated with humans and all the living population found on this planet. Amongst these environmental components utilized by the humans, water is one of the most vital sources.

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These days the water is being polluted by various chemical and biological disposal units which can cause various types of clinical and pathological conditions in human beings [1]. Recently, it has been observed that the disposal of effluent wastes in water bodies has led to the emergence of various microbial population which can be highly pathogenic to the human beings [2].

Majority of these pathogens are found to be environmental habitants and hence are found in air, water and soil [3]. Pollution of these natural resources by chemicals and effluents acts as an inducer for the emergence of pathogens which can cause various diseases in human beings like Cholera, Typhoid, Gastroenteritis and Hepatitis A

etc. [4]. These diseases are caused due to the consumption of water contaminated by chemical and biological sources, including the disposal of effluents which promotes the accumulation of pathogenic bacteria like Coli forms [5, 6]. The significance of these environmental pathogens also increases as they can resist various drugs and antibiotics and hence are called as Multi-Drug Resistant or MDR pathogens [7]. Several factors can contribute to the development of MDR related activity bacteria and one of these phenomena is the Biofilm formation. Several pathogens like *Pseudomonas aeruginosa*, *Escherichia coli*, *Staphylococcus aureus etc*. has the ability to produce biofilms, which is a complex formed by the microorganisms, covered by an exopolysaccharide matrix [8] and imparts drug resistance like properties, thus enabling them to escape the line of treatment [9].

Mechanisms underlying the biofilm production involves the genetic as well as molecular parameters which leads to the spontaneous mutations in the microorganisms, thus, promoting the reduced susceptibility to antibiotics [10]. Moreover, the close accumulation of the microbes can mediate communication *via* quorum sensing and which helps them to coordinate their metabolic activity through which they can promote the formation of biofilm [11]. Biofilms consisting of drug resistant bacteria can transfer its genes horizontally and promote the drug resistance mechanisms among other communities [12] and has become a point of global concern as it has led to the emergence of drug resistance in pathogens [13].

Current strategies involved in the treatment of biofilms is complicated as the biofilms have enhanced resistance to antibiotics [11]. Thus, application of novel strategies in order to overcome this issue is the need of the hour. Application and integration of nanotechnology as an alternate to combat the biofilm strategy has been recently explored and have been found to be valuable in delimiting the biofilm population to a greater extent [14, 15]. In the present study, we synthesized silver-nanoparticles using the green synthesis mechanism by using the ethanolic extract of *Azadirachta indicaas* a reducing agent for the production of AgNP required for formation of silver nanoparticles. *Azadirachta indica*, also called as Indian lilac or Neem is one of the ancient medicinal plants reported in history of medicinal plants [16]. Possessing an immense resource of Flavonoids like Quercetin and limonoids which are known to possess anticancer properties [17] and are commonly found in leaves of the neem plants. Apart from this, the ethanolic extract of *A. indica* has also been known for the strong antimicrobial activities and has been used since ages in the form of tincture [18]. The toxicity profiling of *A. indica* has also revealed its extracts to be non-bio-accumulative in water or the environmental sources as well as no acute toxicity against the marine flora and fauna has been reported [19]. Based on these properties, this study focused on the application of the ethanolic extract of the *A. indica* for the green synthesis of the silver nanoparticles.

The nanoparticles so produced were screened for the distribution and surface characteristics via UV-Visible Spectrophotometry, XRD, FTIR, TEM and Zeta Potential and antimicrobial activity. In order to check the activity against biofilm producing multi drug resistant oral pathogens, the AISNPs were tested against the MDR Pathogens isolated from the effluent sample. This paper is an attempt to implicate a novel approach of ethanolic extract based synthesis of the AISNPs as an alternate strategy to eliminate the effluent pathogens and promote the reduction of biofilm formation.

MATERIALS AND METHODS:

Isolation and Identification of Effluent Pathogens: There were 20 effluent sample that were collected from the drainage unit of various industrial sites located in Greater Noida and Ghaziabad region which comprised of 10 samples from Greater Noida and 10 samples from Ghaziabad. Isolation of bacteria from the effluent water sample was done by the serial dilution technique as previously described by Saha and Santra (2014) [20]. 1 ml of the effluent water sample was added to 10 ml of the sterile distilled water and vortexed for 1 minute. The prepared sample was used as a stock from which the serial dilutions were performed. 1 ml of the stock sample was transferred with the help of a sterile pipette containing 9 ml of sterile distilled water, making the stock dilution as 10^{-1}. In the similar way dilutions were prepared from 10^{-1} to 10^{-7}. 100µl from each dilution was transferred into Nutrient

Agarmedia, labelled with the dilution and coded as GN and GZB respectively, corresponding to the dilution numbers mentioned on them *i.e.,* 10^{-1} to 10^{-7}. 100 µl of samples from each dilution were inoculated on the plates and spread with the help of a glass spreader followed by incubation at 37°C for 24 hours. The isolated colonies obtained after the incubation, were further used for the morphological and biochemical identification of the pathogens.

Morphological and Biochemical Ientification of the Isolated Effluent Water Pathogens: The isolated pathogens were morphologically identified based on their cultural characteristic and gram staining morphology. The bacterial isolates obtained were subjected to biochemical tests IMViC, Catalase, Oxidase, TSI Agar Slant metabolic analysis, Urea hydrolysis and Nitrate reduction. Also, the isolated pathogens were also streaked on various selective and differential media like MacConkey Agar, Eosin Methylene Blue Agar and Mannitol Salt Agar for their identification.

Antibiotic Sensitivity Testing of Effluent Pathogens: Antibiotic sensitivity activity was performed as per the CLSI guidelines [21]. The isolated effluent pathogens were grown in the Brain Heart Infusion Broth and incubated at 37°C for 18 hours. The young cultures thus obtained were then swabbed on Muller Hinton Agar with the help of a sterile cotton swab followed by the incorporation of antibiotic discs for Gram-positive and Gram-negative bacteria respectively. The plates were then incubated at 37°C for 24 hours after which it was observed for the zone of inhibition next day.

Synthesis of Silver Nanoparticles: Protocol opted for the synthesis of silver nanoparticles was in accordance with the study carried by Priyadarshini *et al.*, [22]. 0.017 gm of silver nitrate was dissolved in 100 ml of distilled water in order to prepare 1mM $AgNO_3$solution.90 ml of the prepared solution was mixed with 10 ml of the ethanolic extract of *Azadirachta indica*. The flask containing the reaction mixture was then immediately wrapped in Aluminum foil in order to prevent the photoactivation of the silver nanoparticles and it was incubated at the room temperature for 24 hours. Post incubation the color of the reaction mixture changed from pale yellow to brownish grey, indicating the production of silver nanoparticles (Silver nanoparticles). The obtained silver nanoparticles were centrifuged at 10,000 rpm for 15 minutes to separate the supernatant and pellet. The supernatant was discarded and the pellet collected was washed thrice with sterile distilled water and was recentrifuged again at 12,000 rpm for 20 minutes. This process was repeated thrice and the pellet obtained was oven dried at 65°C for 10 hours. The dried powder was collected and labelled as AISNPs (*Azadirachta indica* Silver Nanoparticles) and stored in a universal container and stored at 4°C for the characterization and antimicrobial assessment.

Characterization of Silver Nanoparticles: The synthesized silver nanoparticles were scanned from 200-800 nm for detecting the Surface Plasmon Response parameter of the synthesized AISNPs on UV-Visible Spectrophotometer (Shimadzu UV-1800). The crystallinity of the nanoparticles was screened by XRD by using the Pan-Analytical (Netherlands) X' Pert PRO X-ray diffractometer and Cu-Kα radiation (λ=1.54059Å) with an angular range of 20-80˚at 40kV and 30 mA. The powdered nanoparticle was mixed with KBr in a ratio of 1:100 and analyzed for the presence for various functional groups within the range of 500-4000 cm-1 through the FTIR. Size and surface morphology of the synthesized silver nanoparticles was done by dropping the sonicated nanoparticles on copper grids, which were dried and examined under the Transmission electron microscope (JEOL-JEM-1400) at 120kV accelerating voltage. The AISNPs were also analyzed for their hydrodynamic particle diameter through Dynamic Light Scattering and Zeta Potential analysis activity was proceeded through the Zeta Sizer instrument (Malvern, UK).

Preparation of Bacterial Inoculum: The microbes obtained from the effluent water were grown in Brain heart infusion broth or BHIB and incubated at 37°C for incubation for 24 hours prior to the screening of antimicrobial and biofilm production assay.

Screening of Antimicrobial Activities of Synthesized Silver Nanoparticles: The bacteria were grown in BHIB overnight to attain the CFU of ~10^6 per ml. 100 µl of the bacterial culture was spread on Muller Hinton Agar plates and five agar wells (8 mm) were punched with the help of

sterilized microtips. The wells were loaded with 20, 30, 40 and 50 µlof the AISNPs, having a concentration of 50 µg/ml and the plates were incubated at 37°C for 24 hours. The results were compared by replicating the same experiment except for the addition of Streptomycin Sulfate, with the same concentration and volume as that of AISNPs.

MIC Evaluation of Antimicrobial Activities of AISNPs: For the estimation of MIC, the standard CLSI guidelines were opted to analyze the antimicrobial potential of AISNPs by the turbidimetric method for estimating the growth or presence of microorganisms in broth [21]. Two-fold dilutions of AISNPs were made in the concentrations ranging from 0.1 mg/ml to 0.003 mg/ml while the Bacterial concentration was adjusted to 10^6 CFU/ml. The control had only the inoculated broth and was incubated along with the test samples at 37°C for incubation for 24 hours. Presence of turbidity was noted pre and post incubation in order to determine the MIC value.

Screening of Antibiofilm Activities: For antibiofilm activity of AISNPs on bacterial cells Crystal Violet estimation method was used with slight modifications [23]. The bacterial cultures were diluted in a ratio of 1:100 and incubated at 37ºC for 24 hours for the formation of biofilm. The planktonic cells were removed by washing twice with sterile normal saline. After the establishment of biofilms, AISNPs in the concentration of 100, 50 and 25 µg/ml were added to each well. The plates were incubated further for 24 hours at 37ºC followed by rewash with sterile normal saline. Post washing, 1% (W/V) Crystal Violet was added to each well for 20 minutes followed by the addition of 95% ethanol (200µl/well). The absorbance was measured using the ELISA plate Reader (ERBA, TransAsia) at OD 600.

RESULT AND DISCUSSION:

Isolation and Identification of Effluent Pathogens: Plates with isolated colonies of bacteria were selected further used for the morphological and biochemical characterization. Morphological identification of the isolates obtained on the higher dilution plates revealed the presence of Gram-negative bacilli in 9 out of 10 samples (90%) while 1 bacterial population was that of Gram-positive cocci (10%) in effluent samples from the Greater Noida, while the samples from Ghaziabad had 10 out of 10 population of Gram-negative bacilli (100%). From the characterization of GN and GZB sample it was observed that 5 out of 10 samples from Greater Noida effluent samples were *E. coli* (50%) while 3 out 10 samples were of *Pseudomonas* spp. (30%) and 2 sample comprised of *Staphylococcus aureus* (20%).

In Ghaziabad effluent samples 3 out of 10 samples were of *P. aeruginosa* (30%), 2 out of 10 samples were of *Klebsiella* spp. (20%). and 5 samples of *E. coli* (50%) were observed, the detailed report of which is described in **Table 1** along with the biochemical test performed on these isolates. Both of the tests, morphological and biochemical characterization of the isolated strains provided the idea of the expected microorganisms that could be present in effluent samples and elucidate their role in the spread of disease and other conditions.

TABLE 1: MORPHOLOGICAL AND BIOCHEMICAL CHARACTERIZATION OF THE BACTERIA ISOLATED FROM EFFLUENT SAMPLES OF GREATER NOIDA (GN1-10) AND GHAZIABAD (GZB1-10). IT WAS OBSERVED THAT APART FROM SAMPLE GN10, WHICH WAS A GPC (GRAM POSITIVE COCCI), THE OTHERS WERE GNB (GRAM NEGATIVE BACILLI)

Sample no.	Gram Reaction	Indole	Methyl Red	Voges Prausker	Citrate Utilisation	Catalase	Oxidase	Urease	Nitrate Reductase
GN1	GNB	-	-	-	+	+	+	-	+
GN2	GNB	+	+	-	-	+	-	-	+
GN3	GNB	-	+	+	+	+	-	+	+
GN4	GNB	+	+	-	-	+	-	+	+
GN5	GNB	+	+	-	-	+	-	+	+
GN6	GNB	+	+	-	-	+	-	+	+
GN7	GNB	-	-	-	+	+	+	+	+
GN8	GNB	-	-	-	+	+	+	+	+
GN9	GNB	+	+	-	-	+	-	+	+
GN10	GPC	+	+	-	-	+	-	-	+

GZB-1	GNB	-	-	-	+	+	+	-	+
GZB-2	GNB	-	-	+	+	+	-	+	+
GZB-3	GNB	-	-	+	+	+	-	+	+
GZB-4	GNB	+	+	-	-	+	-	-	+
GZB-5	GNB	+	+	-	-	+	-	-	+
GZB-6	GNB	+	+	-	-	+	-	-	+
GZB-7	GNB	+	+	-	-	+	-	-	+
GZB-8	GNB	+	+	-	-	+	-	-	+
GZB-9	GNB	-	-	-	+	+	+	-	+
GZB-10	GNB	-	-	-	+	+	+	-	+

As evident from the previous studies conducted on the effluent water sample [6], it was concluded that most of the effluent pathogens that are associated belong to the family of Gram negative bacteria and comprised of multiple genera of microorganisms that can cause multiple infections [24]. Based on the biochemical characterizations, the isolated pathogens were identified as *E. coli, P. aeruginosa, K. pneumoniae* and *S. aureus*.

Antibiotic Sensitivity of Isolated Effluent Pathogens: Assessment of the antimicrobial activities was carried out by the Kirby -Bauer method against the antibiotic discs comprising of antibiotics Ampicillin (20 mcg), Cotrimoxazole (25 mcg), Tazobactam (110 mcg), Chloramphenicol (30 mcg), Ciprofloxacin (5 mcg), Ceftriaxone (30 mcg), Tetracycline (30 mcg), Gentamicin (10 mcg), Amikacin(30 mcg) and Levofloxacin (5 mcg), was used against the gram-negative isolates, while antibiotics such as Ampicillin (20mcg), Cotrimoxazole (25mcg), Cephalexin (30mcg), Tetracyclin (30mcg), Ciprofloxacin (5mcg), Levofloxacin (5mcg), Linezolid (30mcg), Cloxacilin (5mcg), Roxithromycin (15mcg), Lincomycin (2mcg) and Gentamicin (10mcg) were used against Gram positive isolates. The details of these antibiotic sensitivity tests are given in **Table 2**. On the basis of the antibiotic sensitive activities, it was observed that the isolated effluent pathogen *E. coli* was absolutely resistant to all the category of antibiotics present in the multi-disc and for the some of the antibiotics, for which the zone of inhibition was seen, fell under the category of multi drug resistant or MDR, when compared to the standard data of CLSI guidelines.

Other group of microorganisms like *Klebsiella* spp. was also found to be resistant to Ampicillin, Cotrimoxazole, Cefotaxime, Ciprofloxacin, Ceftriaxone, Tetracycline, Ofloxacin and Levofloxacin, while it was found to be sensitive for Tazobactam and Chloramphenicol. Based on the resistant pattern of the *Klebsiella* spp. it was also categorized as an MDR strain. *Pseudomonas aeruginosa* was found to be sensitive against Cotrimoxazole, Chloramphenicol, Ciprofloxacin, Ceftriaxone, Ofloxacin, Amikacin and Levofloxacin. Antibiotics for which the resistance was noted were Ampicillin, Cefotaxime and Gentamicin. All the above mentioned strains on the basis of the CLSI guidelines fell under the category of MDRs and similar type of MDR bacterial strains have been isolated from effluent samples in studies conducted previously [6].

TABLE 2: ANTIBIOTIC SENSITIVITY TEST OF MICROBES ISOLATED FROM EFFLUENT. ALL THE ISOLATED PATHOGENS *P. AERUGINOSA, K. PNEUMONIAE* AND *E. COLI* WERE FOUND TO BE RESISTANT AGAINST MULTIPLE ANTIBIOTICS AND HENCE WERE DESIGNATED AS MDR STRAINS

Microorganism	Antibiotic	Symbol	Zone of Inhibition (mm)	Category
P. aeruginosa	Ampicillin	AS	0	Resistant
	Cotrimoxazole	BA	35	Sensitive
	Cefotaxime	CF	0	Resistant
	Tazobactam	TZP	19	Intermediate
	Chloramphenicol	CH	30	Sensitive
	Ciprofloxacin	CP	32	Sensitive
	Ceftriaxone	CR	34	Sensitive
	Tetracycline	TE	13	Intermediate
	Ofloxacin	OF	32	Sensitive
	Gentamicin	GM	10	Resistant
	Amikacin	AK	29	Sensitive

	Levofloxacin	LE	30	Sensitive
Microorganism	**Antibiotic**	**Symbol**	**Zone of Inhibition (mm)**	**Category**
K. pneumoniae	Ampicillin	AS	0	Resistant
	Cotrimoxazole	BA	0	Resistant
	Cefotaxime	CF	0	Resistant
	Tazobactam	TZP	17	Sensitive
	Chloramphenicol	CH	17	Sensitive
	Ciprofloxacin	CP	0	Resistant
	Ceftriaxone	CR	0	Resistant
	Tetracycline	TE	0	Resistant
	Ofloxacin	OF	0	Resistant
	Gentamicin	GM	16	Intermediate
	Amikacin	AK	16	Intermediate
	Levofloxacin	LE	0	Resistant
Microorganism	**Antibiotic**	**Symbol**	**Zone of Inhibition (mm)**	**Category**
E. coli	Ampicillin	AS	0	Resistant
	Cotrimoxazole	BA	0	Resistant
	Cefotaxime	CF	0	Resistant
	Tazobactam	TZP	17	Resistant
	Chloramphenicol	CH	17	Resistant
	Ciprofloxacin	CP	0	Resistant
	Ceftriaxone	CR	0	Resistant
	Tetracycline	TE	0	Resistant
	Ofloxacin	OF	0	Resistant
	Gentamicin	GM	16	Resistant
	Amikacin	AK	16	Resistant
	Levofloxacin	LE	0	Resistant

Synthesis of Silver Nanoparticles: Nanoparticle formation was detected by the development of dark brown coloration of the solution, which was the resulted due to the reduction of 1mM $AgNO_3$ after the addition of ethanolic extract of *Azadirachta indica*, indicating the bio-reduction of Ag^+ to Ag^0, indicating the effect of surface plasmon resonance as shown in **Fig. 1**. On the other hand, the medium devoid of extract potion of *Azadirachta indica* displayed no color change.

FIG. 1: ETHANOLIC EXTRACT OF *AZADIRACHTA INDICA* AND SYNTHESIS OF AISNPS

Characterization of Silver Nanoparticles:

UV-Visible Spectrophotometry: The surface plasmon resonance activity of AISNPs was noticed with the addition of ethanolic extracts of *Azadirachta indica* to 1mM of AgNO3solution and the color change from pale yellow solution to brownish brown was noticed, which was similar to earlier studies [25, 26]. The surface plasmon resonance was observed at the spectrum of 430 nm and was detected by the UV Visible spectroscopy as shown in **Fig. 2**. One of the key parameters that lead to the change in color of the silver nitrate solution to silver nanoparticles is due to the ability of the ethanolic extracts to act as a bio-reductant and promote the conversion of the silver nitrate to silver nanoparticles [25, 27]. The peak intensity on UV Visible spectrophotometer, thus obtained indicated the formation of silver nanoparticles.

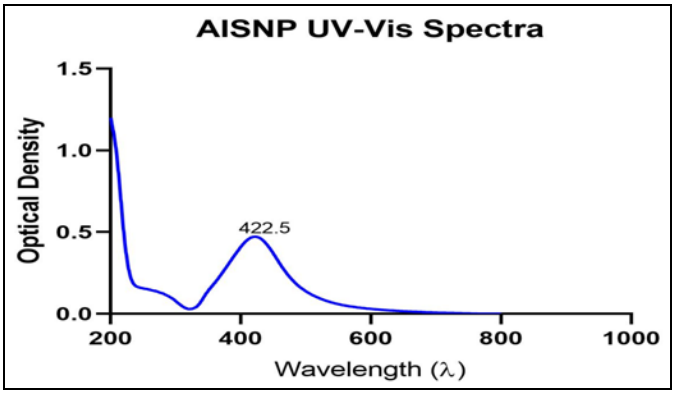

FIG. 2: AISNP UV-VISIBLE SPECTROSCOPY GRAPH; PEAK WAS OBSERVED AT 422.5 NM INDICATING THE SURFACE PLASMON RESONANCE OF THE SYNTHESIZED NANO-PARTICLES

XRD: Data of the various angular diffraction obtained after the analysis of the powdered AISNPs on XRD was then analyzed by using the software X' Pert High score Plus (Version 3.0, P Analytical, Netherlands). The result thus obtained was used for the calculation of the crystalline nature of the nanoparticles and also the various parameters like peaks, Full Wavelength Half Maxima (FWHM) and Miller's indices for the estimation of crystallinity of AISNPs. The diffraction peaks were received at 2θ of 38.1° (111), 44.2 ° (200), 64.5° (220) and 77.4° (311) crystallographic planes, respectively as shown in **Fig. 3**. The outcome of the XRD was found in accordance with the standard JCPD No. 00-004-0783, as referred in earlier studies [28, 29]. Analysis of the crystal from the XRD crystallography revealed the Face Centered Cubic shape of the AISNPs and was similar to the studies described earlier [30, 31]. The crystallite size of the AISNPs was found to be 9.6 nm and was calculated by the Debye-Scherrer equation:

$$D = K\lambda / \beta \cos \theta$$

Where, D is the crystallite size of AISNPs, K is Scherrer constant which is 0.9, λ is the X-ray Wavelength, θ is the Bragg diffraction angle, and β is the full-width at the half maximum of the diffraction peak corresponding to the plane [32, 33].

In the present study, the calculated crystallite size was found to be 9.6 nm was calculated by using the above equation, as shown in **Table 3**. The calculated crystallite value of thus obtained differed from the studies conducted previously [34, 35].

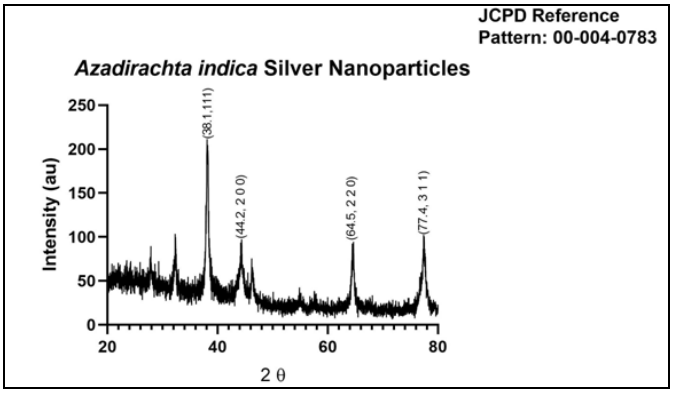

FIG. 3: XRD PEAKS OF NEEM LEAF SILVER NANOPARTICLES WITH MILLER'S INDICES PEAKS AT 38.1° (111), 44.2° (200), 64.5° (220) AND 77.4° (311). PRESENCE OF THESE PEAKS INDICATED THE CRYSTALLINE MORPHOLOGY OF AISNPS

TABLE 3: CALCULATION PARAMETERS OF CRYSTALLITE SIZE BY USING THE DEBYE-SCHERRER EQUATION

S. no.	2θ	θ	Radian	FWHM	D (nm)	h k l	Average Crystallite Size (nm)
1	27.84953	13.924765	0.243032997	1.0368	7.767679475	Unassigned	9.656623707
2	32.19725	16.098625	0.280974011	1.0368	7.689037355	Unassigned	
3	38.0952	19.0476	0.332443335	0.5184	15.12937719	111	
4	44.24185	22.120925	0.386082975	1.0368	7.413781751	200	
5	46.28915	23.144575	0.403949038	0.7776	9.811680838	Unassigned	
6	64.48912	32.24456	0.562773738	0.5184	13.53729892	220	
7	77.35802	38.67901	0.675076076	1.0368	6.247510427	311	

Dynamic Light Scattering and Zeta Potential: Hydrodynamic nanometer and ζ potential of AISNPs was analyzed on Malvern Zetasizer. AISNPs in the concentration of 100 µg/ml was dispersed in sterile double distilled water. The mean of ζ potential of the nanoparticles was found to be -19.7 mV and the size was 95.75 nm with the PDI value of 0.572, indicating the mono-dispersed solution of the synthesized nanoparticles as shown in **Fig. 4A** and **B** respectively [36].

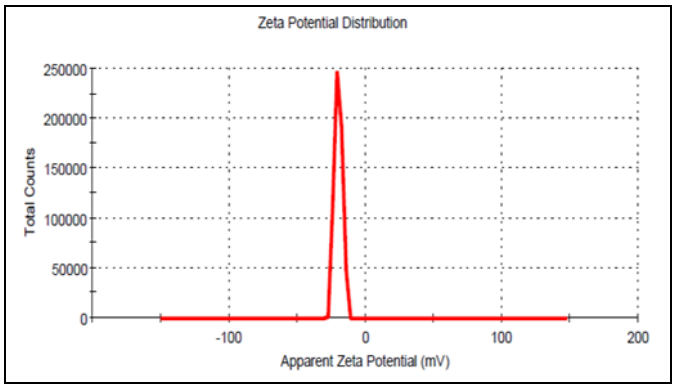

FIG. 4A: ZETA POTENTIAL OF AGNPS SYNTHESIZED BY *AZADIRACHTA INDICA*

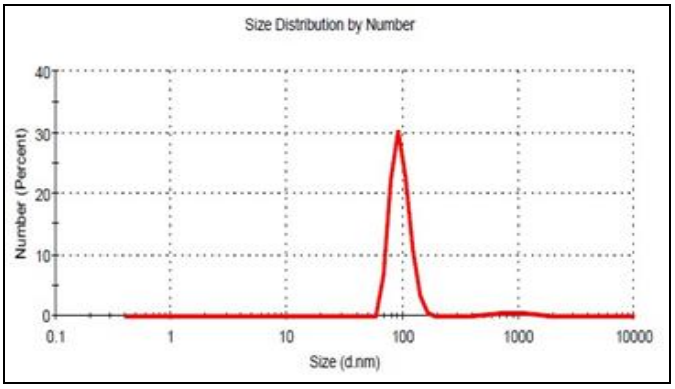

FIG. 4B: PARTICLE SIZE DISTRIBUTION OF AGNP SOLUTION SYNTHESIZED BY *AZADIRACHTA INDICA*

FTIR: In order to assess the presence of various biomolecules the AISNPs were analyzed through Fourier Transformed Infrared Spectroscopy. Peaks were observed at 3730.2, 3432.6, 2926.6, 1821.4, 1491.9, 1384.9, 1064.1 and 910.9 Wave numbers (cm^{-1}) as shown in **Fig. 5**. The trough peaks at 3432.6 displayed the presence of OH groups along with Carbohydrates, Proteins and Phenols, whereas the peaks at 2926.6 displayed the presence of CH and CH_2 aliphatic stretching. The band stretches at 1491.9, 1384.9, 1064.1 and 910.9 displayed the presence of weak C=C bonds, C-H bends of alkanes, strong C-F stretching and unsubstituted Alkenes respectively. The data thus obtained here is approximately close to the similar studies conducted previously [26] and depicts the presence of various biomolecules that promoted the interaction between silver ions and resulted in the bio-reduction of silver nitrate to silver nanoparticles.

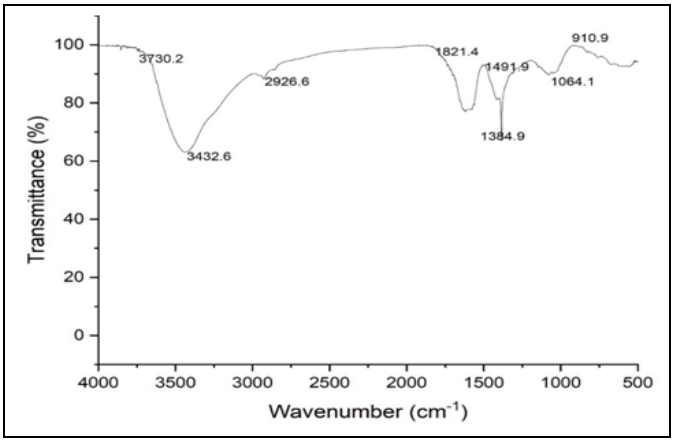

FIG. 5: FOURIER TRANSFORM INFRARED SPECTROSCOPY (FTIR) SPECTRUM OF AGNPS SYNTHESIZED BY REDUCTION OF AG+ IONS BY *AZADIRACHTA INDICA*

Transmission Electron Microscopy Analysis: TEM analysis of the AISNPs depicted the presence of the spherical silver nanoparticles and the mean diameter of the synthesized nanoparticles was found to be around 24.1 nm, which was calculated by the ImageJ software (Version 1.80, USA). Similar reports have been presented in some of the studies in which the green synthesized silver nanoparticles of *Azadirachta indica* was found between the range of 20-50 nm and possessed spherical shape, the result of which is as displayed in **Fig. 6**.

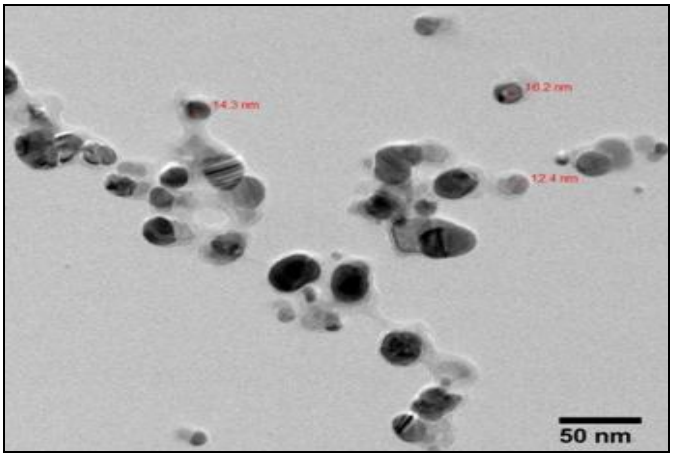

FIG. 6: TEM IMAGE OF SILVER NANOPARTICLES PRODUCED WITH *AZADIRACHTA INDICA*

Antimicrobial Activity of AISNPs: The Antimicrobial activity of AISNPs was assessed by the well diffusion method against multi-drug resistant effluent pathogens *E. coli, S. aureus*, *Klebsiella pneumoniae* and *P. aeruginosa*. The zones of each well containing 10-50 µl of AISNPs, having the concentration of 100µg/ml, was measured against each of the above-mentioned bacterial pathogens, the details of which is shown in **Fig. 7**. Amongst the tested bacteria, *S. aureus* was found to be most sensitive against the AISNPs. 50 µl of 100 µg/ml AISNPs produced an inhibition

zone of 16.40 mm against *S. aureus*, 15.39 mm against *P. aeruginosa*, 14.34 against E. coli and 12.65 mm against *K. pneumoniae*. Previously conducted studies to demonstrate the antimicrobial activities of the *Azadirachta indica* derived silver nanoparticles also supports this [22, 37, 38]. One of the reasons for the enhanced zone of inhibition displayed by the synthesized AISNPs is due to the large interactive surface area which promotes its antibacterial activity [22, 39]. MIC of the AISNPs was estimated via broth dilution technique and was tested against the test bacteria. The broth sample containing no turbidity of the test strains is depicted in **Table 4**. The calculated MIC values of AISNPs against *P. aeruginosa* was found to be 6.25 µg/ml whereas in case of *E. coliand K. pneumoniae* the value was found to be 12.5 µg/ml.

FIG. 7: ANTIMICROBIAL ACTIVITY OF AISNPS AGAINST EFFLUENT ISOLATED PATHOGENS ON MHA PLATE BY WELL DIFFUSION METHOD. N= AISNPs, Ec= *E. coli*, Kp= *Klebsiella pneumoniae*, Pa= *Pseudomonas aeruginosa* and Sa= *Staphylococcus aureus*, PC= Positive Control (50µg/ml).

TABLE 4: MIC VALUES OF AISNPS AGAINST THE EFFLUENT ISOLATES

Microorganisms	MIC of Streptomycin Sulfate (µg/ml)	MIC of AISNP (µg/ml)
E. coli	25	12.5
S. aureus	6.25	6.25
K. pneumoniae	25	25
P. aeruginosa	12.5	12.5

Antibiofilm Activity: Antibiofilm activity of AISNPs was evaluated by measuring biofilm growth of *S. aureus, E. coli* and *P. aeruginosa*, with crystal violet microtiter plate assay. The % reduction of the biofilm formation was calculated by the formula [40]:

Biofilm reduction %= (Optical Density of Test) / (Optical Density of Blank) × 100

Where the Test sample was the broth inoculated with microorganism and AISNPs and Blank is the broth sample inoculated with the bacteria but devoid of nanoparticles.

On the analysis, it was found that the AISNPs had a greater anti-biofilm reduction potential for *Pseudomonas aeruginosa* (76%), where the inhibition % was found to be approximately, followed by *S. aureus* (74%) and *E. coli* by (55%) as shown in **Fig. 8.**

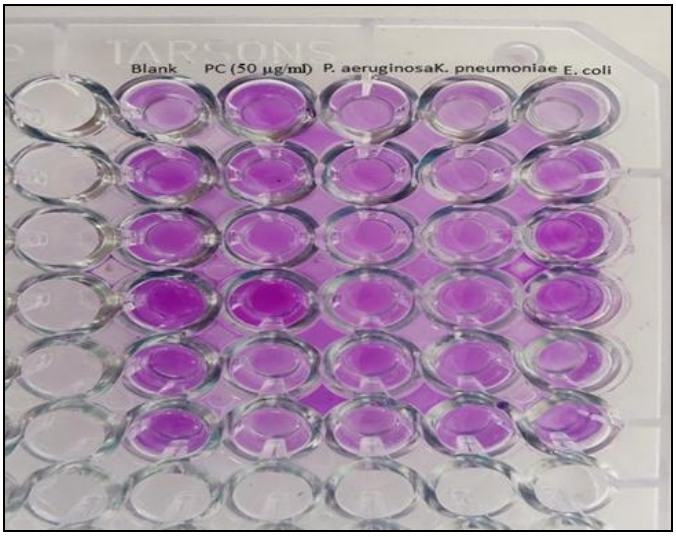

FIG. 8:

CONCLUSION: Water is one of the key and essential natural resource which directly impacts the health of an individual. Thus, it is an utmost requirement to apply mandatory regulatory parameters in order to maintain the potability of the water for the safe consumption. Presence of undesirable microorganisms like *E. coli, P. aeruginosa* and *K. pneumoniae* impacts the quality of water and makes it contaminated and unsafe for consumption and its application in various other processes [41]. In the present study, an attempt has been made to utilize the ethanolic extract derived

silver nanoparticles of *Azadirachta indica* and was tested for its antimicrobial and antibiofilm activities against the pathogens extracted from the effluent water samples, which were collected from the regions of Greater Noida and Ghaziabad. Based on the morphological and biochemical characterization, these pathogens were identified as *E. coli*, *P. aeruginosa* and *K. pneumoniae*. The results obtained in this study indicates the formation of spherical silver nanoparticles with the diameter of 24.1nm and possessed strong antimicrobial and antibiofilm related activities against the pathogens like *E. coli*, *P. aeruginosa* and *K. pneumoniae* which were extracted from the effluent waste. The AISNPs also displayed a strong potential as an antibiofilm agent and reduced the probability of biofilm formation by the micro-organisms by 76%, Hence, by considering the properties displayed by the AISNPs it can be concluded that these nanoparticles can be implicated as a novel strategy to minimize the presence and prevalence of the MDR strains.

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CONFLICTS OF INTEREST: None

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