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RESEARCH ARTICLE

OPTIMIZATION OF GREEN SYNTHESIZED SILVER NANOPARTICLES USING Azadirachta indica (NEEM) FOR THEIR INVESTIGATION OF ANTIBACTERIAL ACTIVITY

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Abstract

The present study demonstrates an economical and eco-friendly method for the synthesis of silver nanoparticles (AgNPs) using the Azadirachta indica (neem). The process involves reduction of Ag^+ ions to Ag°nanoparticles.The synthesis of AgNPs was confirmed and the products characterized by UV-visible spectroscopy and X-ray diffraction analysis. The average sizes of AgNPs were found to be in a range of 5 to 9 nm in controlled environment. The formation of smaller size silver nanoparticles was seen at higher concentration of extract (25gm/100ml), silver nitrate (4mM) and higher temperature (70°C to 80°C). The antibacterial activity of resulting silver nanoparticles was found to be effective against Staphylococcus aureus ATCC 25923 and Escherichia coli ATCC 25922, Klebsiella pneumoniae ATCC 700603. However synthesized silver nanoparticles were observed less effective against Pseudomonas aeruginosaATCC 27853. The resulting silver nanoparticles showed a synergism of neem extractand silver nanoparticles on bactericidal effectand process of synthesis creates new opportunities in process development for the synthesis of safe and eco-friendly AgNPs.

Introduction:-

Nanoparticles are defined as a nanoscale particle of size ranging from 1 to 100 nm[3]. NPs are well-known to exhibit a strong antimicrobial activity against various microorganisms such as bacteria, viruses, and fungi due to its smaller in size and large surface area[2]. Among the metallic nanoparticles, silver nanoparticles (AgNPs) have gained increasingly attention due to its unique physical, biological and chemical properties [4].

It was concluded that silver has strong antimicrobial property and also shows toxicity to cells[2]. Silver ions interacts with macromolecules like proteins and deoxyribonucleic acid (DNA) and interferes with the formation and mechanism of proteins thus disrupting the cell metabolism resulting in inhibition of bacterial cell and cell wall growth which leads to cell death. The term is also defined as oligodynamic effect [15].

Silver containing materials has been used in different medical procedures, for example, to reduce infection in burn treatment and arthroplasty as well as to prevent bacteria colonization on prostheses, catheters, vascular grafts, dental materials, stainless steel materials and human skin [7]. Silver containing materials are also used in various industries like water treatment and for elimination of microorganisms on textile fabrics. Recent studies also have shown that

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silver nanoparticles also have cytoprotective activity in HIV infected cells. Due to all these wide range of applications different synthesis technique are made for synthesis of silver nanoparticle [13].

Green synthesis of AgNPs employing either biological microorganisms or plant extracts has emerged as a simple and alternative to chemical synthesis. Plant extracts mediated synthesis of AgNPs can be advantageous compared with other biological processes as it does not require the process of maintaining the cell cultures and aseptic environments [8].Terpenoids and flavanones are phytochemicals which act as reducing and capping agent to form AgNPs[14].

In recent years, antimicrobial resistance has emerged as one of the main public health problems. Silver nanoparticles have proved to be a likely candidate for antimicrobial agent since their large surface to volume ratio ensures a broadrange of attack on bacterial surface. In this study four bacteria were taken to investigate antibacterial activity of silver nanoparticles against pathogenic bacteria such as Escherichia coli, Staphylococcus aureus K. pneumoniaeand P. aeruginosa.

Materials and Methods:-

Collection of plant material

Healthy leaves of neem were collected from Kalanki, Kathmandu.

Preparation of leaf extract (reducing agent)

Fine pieces of neem leaves were boiled for 20 minutes and filtered [14].

Synthesis and optimization of silver nanoparticles

Synthesis and optimization process was done according to [10] with slight changes.

Effect of neemand Concentration

20ml of 1mM $AgNO_3$ was added to each 25 mL of 10gm/100ml neem extract adjusting pH at10 using 0.1M NaOH solution and placed in a shaking water-bath at temperature of 80°C for 20 minutes. The above procedure was repeated for plant extract concentrations of 15gm/100ml, 20g/100ml, and 25g/100ml.

Effect of Variation of Concentration of $AgNO_3$

20ml of 1mM $AgNO_3$ was added to 25 ml of 10g/100ml neem extract adjusting pH to10 using 0.1M NaOH solution and placed in a shaking water-bath at temperature of 80°C for 20 minutes. The above procedure was repeated for $AgNO_3$concentration values of 2mM, 3mM and 4mM.

Effect of Variation of temperature

20 ml of 1mM $AgNO_3$was added to 25 mL of 10g/100ml neem extract adjusting pHat 10 using 0.1M NaOH solution. Then placed in a shaking water-bath at temperature of 40°C for 20 minutes. The above procedure was repeated for temperature values of 50°C, 60°C and 70°C.

Recovery of nanoparticles

Thesolutions were centrifuged at 5000rpm for 30 minutes to recovered and washed. Then the nanoparticles were dried at 60°C according to [12].

UV-spectrophotometer

UV-Visspectrophotometer in the wavelength range of 250– 700 nm to obtain the UV-Visible spectra of the sample. The distilled water used as a blank reference [10].

XRD (X-ray diffraction)

The dried powdered of silver nanoparticles were used to determine crystalline structure of silver and the XRDwas done at NAST, Khumaltar, Nepal using D2 phazer machine of Bruker Company with Cu-Kα (λ=1.54056 Å) radiation. The diffraction pattern was recorded from diffraction angle range of 10º to 90º at a 2θ pattern. The average crystalline size was calculated from the width of the XRD peaks using the Scherrer formula:$D = K\lambda / \beta\cos\theta$ Where,

λ is the X-ray wavelength in nanometer (nm)β is the peak width of the diffraction peak profile at half maximum height resulting from small crystallite size in radians θ is the angle of incidence of X-ray and K is a constant related to crystallite shape and taken as 0.9[12].

Assessment of Antimicrobial activity silver nanoparticle

Antimicrobial activity of synthesized silver nanoparticle was tested by agar well diffusion method.Bacteria including Klebsiella pneumoniae ATCC 700603, Escherichia coli ATCC 25922, Pseudomonas aeruginosa ATCC 27853 and Staphylococcus aureus ATCC 25923 were used as hosts to test the antimicrobial activity. Antibiotics such as Streptomycin 10mg/disc, Ciprofloxacin 30mcg/disc and Gentamycin 10mcg/disc were used as positive control [15].

Results: -

UV-Spectrophotometric analysis

A distinct color change from pale yellow to brown was observed after addition of aqueous neemextract to silver nitrate solution.

***Azadirachta indica* (Neem)extractconcentrationvariation in Neem-mediated synthesized AgNPs**

At 10gm/100ml of neem extract peak absorbance was 0.640 at 426nm wavelength. Followed by 15gm/100ml of neem extract concentration, the peak absorbance was 0.447 at 415nm wavelength. Then at 20gm/100ml extract peak absorbance was 0.577 at 431nm. Then at 25gm/100ml extract peak absorbance was 1.343 at 442nm wavelength as shown in figure 1.

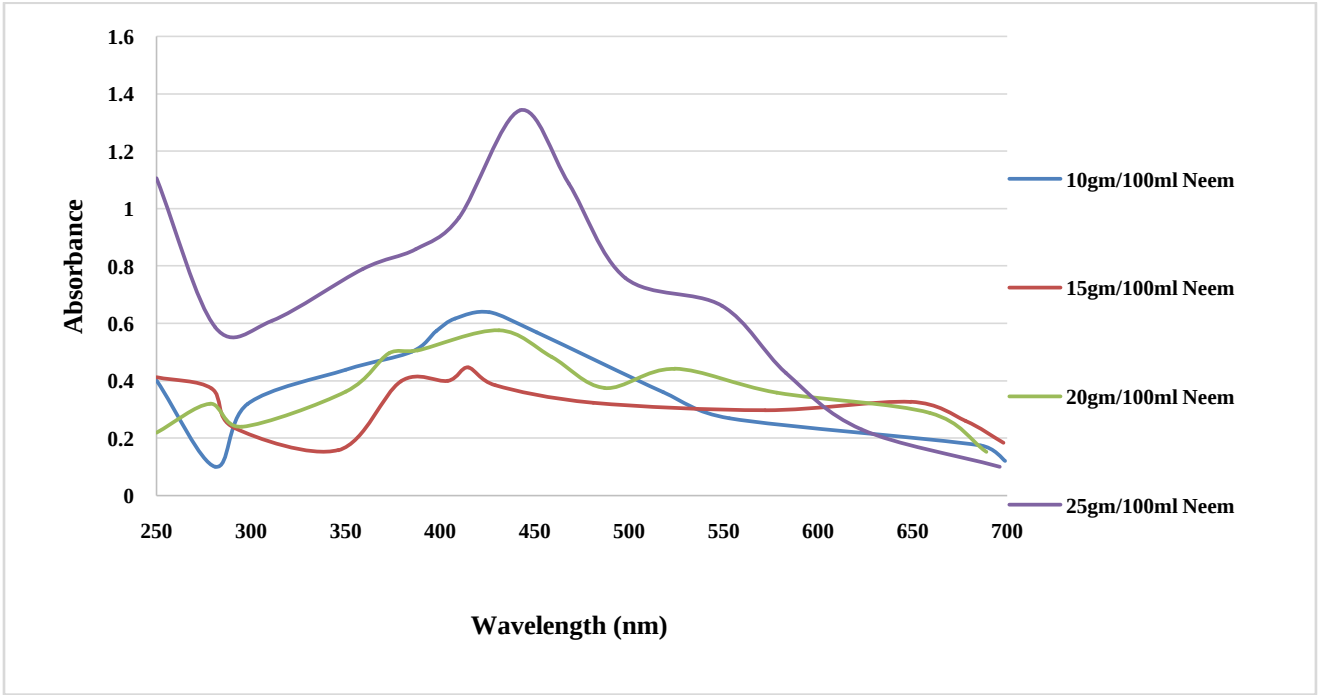


Fig. 1:-UV–vis absorbance spectra of silver nanoparticles synthesized using Neem under different concentrations of neem.

$AgNO_3$ variation in neem-mediated synthesized AgNPs

At 1mM of $AgNO_3$ peak absorbance was 0.589 at 419nm wavelength. Followed by 2mM of $AgNO_3$ concentration, the peak absorbance was 0.859 at 428nm wavelength. Then at 3mM of $AgNO_3$ peak absorbance was 1.270 at 414nm. Then at 4mM of $AgNO_3$ extract peak absorbance was 1.984 at 436nm wavelength as shown in figure 2.

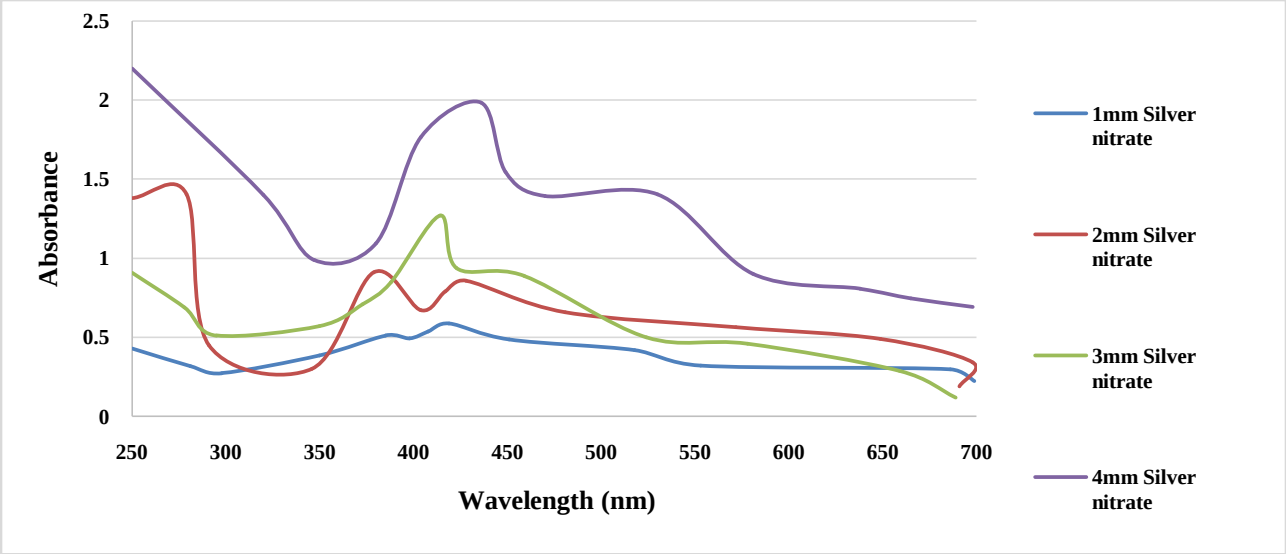


Fig. 1:-UV–vis absorbance spectra of silver nanoparticles synthesized using Neem under different concentrations of $AgNO_3$

Temperature variation in neem-mediated synthesized silver nanoparticle

At 40°C peak absorbance was 0.401 at 420nm wavelength. Followed by 50°C the peak absorbance was 0.479 at 419nm wavelength. Then at 60°C peak absorbance was 0.777 at 423nm. Then at 70°C of $AgNO_3$ extract peak absorbance was 0.943 at 422nm wavelength as shown in figure 3.

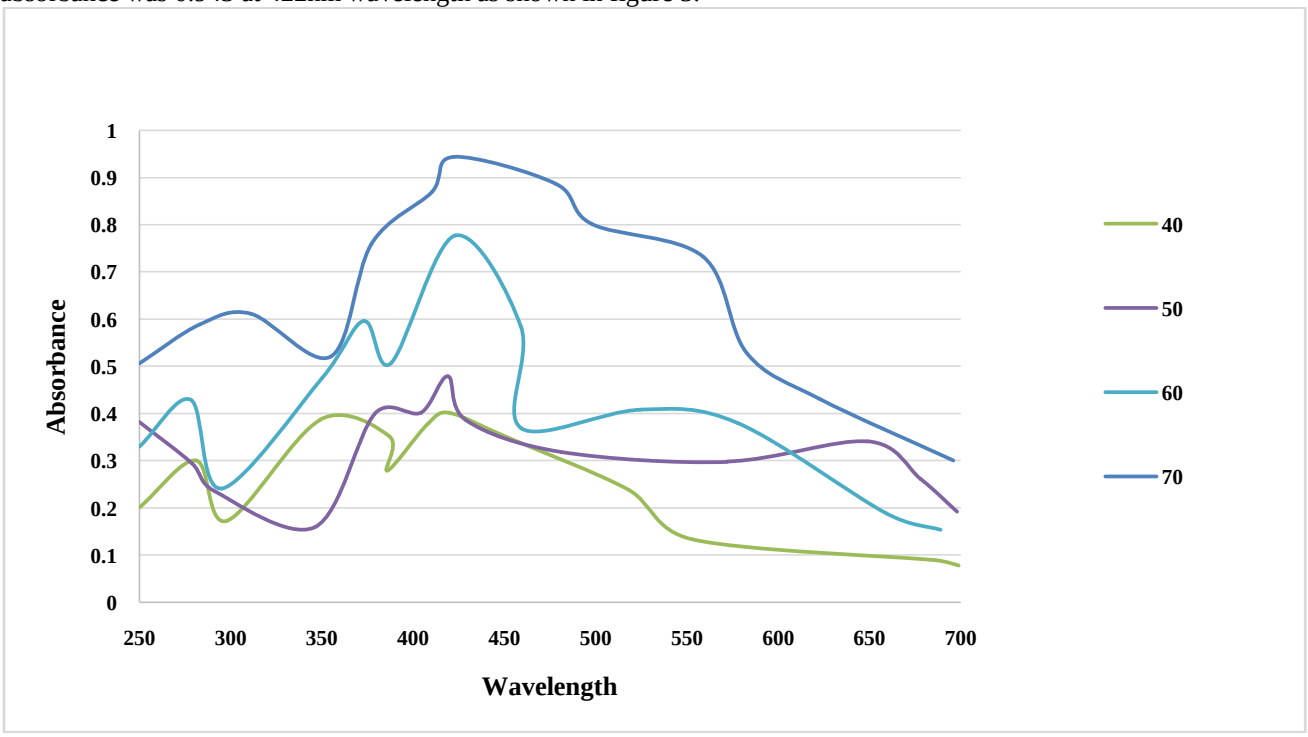


Fig. 3:-UV–vis absorbance spectra of silver nanoparticles synthesized using Neem under different temperature.

X-ray diffraction

X-ray diffraction of neem-mediated AgNPs at neem concentration variation

The average size of the silver nanoparticle synthesized from neem extract at the concentration of 10gm/100ml of extract was 7.08, Likewise at 15gm/100ml was 5.39, at 20gm/100ml was 4.66 and at 25gm/100ml was 4.71 as shown in table 2.

Table 1:-XRD particle size evaluation of neem-mediated Ag-NPs atneemconcentration variation using Scherrer equation.

S.N.	FWHM	β	2θ	θ	radian	Crystallite size (D)	average	total average
10gm/100ml	0.73	0.01274	32.26	16.13	0.28152	11.3285	7.0839	5.4657
	1.52	0.02653	38.17	19.09	0.3331	5.53049		
	1.18	0.02059	44.02	22.01	0.38415	7.26169		
	1.99	0.03473	64.49	32.25	0.56278	4.72006		
	1.55	0.02705	77.65	38.83	0.67762	6.57885		
15gm/100ml	1.22	0.02129	32.26	16.13	0.28152	6.77856	5.396	
	2.62	0.04573	38.3	19.15	0.33423	3.20979		
	1.83	0.03194	44.35	22.18	0.38703	4.68788		
	1.81	0.03159	64.51	32.26	0.56296	5.19003		
	1.43	0.02496	77.31	38.66	0.67466	7.11397		
20gm/100ml	0.97	0.01693	32.27	16.14	0.28161	8.52582	4.6635	
	2.49	0.04346	38.13	19.07	0.33275	3.37564		
	2.4	0.04189	44.19	22.1	0.38563	3.57248		
	3.7	0.06458	64.49	32.25	0.56278	2.53863		
	1.92	0.03351	77.48	38.74	0.67614	5.30472		
25gm/100ml	1.82	0.03176	32.26	16.13	0.28152	4.54387	4.7194	
	2.71	0.0473	38.33	19.17	0.33449	3.10348		
	1.84	0.03211	44.34	22.17	0.38694	4.66224		
	1.83	0.03194	64.35	32.18	0.56156	5.12879		
	1.65	0.0288	77.15	38.58	0.67326	6.15856		

The total average size of crystallite is 5.4657nm.

3.1.1 X-ray diffraction of neem-mediated AgNPs at $AgNO_3$ concentration variation

The average size of the silver nanoparticle synthesized from neem extract at the concentration of 1mM of $AgNO_3$ was 6.09, Likewise at 2mM was 6.55, at 3mM was 6.46 and at 4mM was 5.55 as shown in table 2.

Table 2:-XRD particle size evaluation of neem-mediated Ag-NPs at $AgNO_3$ concentration variation using Scherrer equation.

Concentration		FWHM	β	2θ	θ	radian	Crystallite size (D)	average	total average
1mM		1.21	0.021118	32.26	16.13	0.281522	6.83458	6.0998	6.1674
		0.9	0.015708	38.11	19.06	0.332572	9.3387		
		1.47	0.025656	44.01	22.01	0.38406	5.82891		
		1.84	0.032114	64.32	32.16	0.561298	5.10008		
		2.98	0.052011	76.59	38.3	0.668374	3.39674		
2mM		1.4	0.024435	31.9	15.95	0.27838	5.9017	6.55247	
		1.31	0.022864	37.86	18.93	0.330391	6.41108		
		1.17	0.02042	44.01	22.01	0.38406	7.3235		

		1.41	0.024609	64.31	32.16	0.561211	6.65506		
		1.57	0.027402	77.12	38.56	0.672999	6.47103		
3mM		1.22	0.021293	32.26	16.13	0.281522	6.77856	6.46042	
		1.62	0.028274	38.31	19.16	0.334318	5.1913		
		1.97	0.034383	44.01	22.01	0.38406	4.34949		
		1.25	0.021817	64.5	32.25	0.562869	7.51475		
		1.2	0.020944	77.15	38.58	0.673261	8.46803		
4mM		1.08	0.01885	32.27	16.14	0.281609	7.65745	5.55704	
		2.06	0.035954	38.32	19.16	0.334405	4.0826		
		1.52	0.026529	43.85	21.93	0.382663	5.63399		
		1.57	0.027402	64.83	32.42	0.565748	5.994		
		2.3	0.040143	77.12	38.56	0.672999	4.41718		

The total average size of crystallite is 6.1674nm.

X-ray diffraction of neem-mediated AgNPs at temperature variation

The average size of the silver nanoparticle synthesized from neem extract at temperature of 40°C was 12.36, Likewise at 50°C was 9.73, at 60°C was 7.30 and 70°C was 7.13 as shown in table 3.

Table 6:-XRD particle size evaluation of neem-mediated Ag-NPs at temperature variation using Scherrer equation.

Temperature	FWHM	β	2θ	θ	radian	Crystallite Size (D)	average	Total average
40°C	0.71	0.012392	32.04	16.02	0.279602	11.64122	12.36962	9.13555
	1.16	0.020246	37.89	18.95	0.330653	7.240752		
	0.21	0.003665	44.05	22.03	0.384409	40.80811		
	2.58	0.045029	54.67	27.34	0.477086	3.466232		
	1.98	0.034558	64.28	32.14	0.560949	4.738428		
	1.6	0.027925	76.51	38.26	0.667676	6.322953		
50°C	0.55	0.009599	31.94	15.97	0.278729	15.024	9.73525	
	0.68	0.011868	37.95	18.98	0.331176	12.35409		
	1.22	0.021293	44.13	22.07	0.385107	7.026333		
	1.36	0.023736	64.32	32.16	0.561298	6.900107		
	1.38	0.024086	77.31	38.66	0.674657	7.371718		
60°C	0.91	0.015882	32.12	16.06	0.2803	9.084534	7.301765	
	0.76	0.013265	37.93	18.97	0.331002	11.053		
	1.69	0.029496	44.05	22.03	0.384409	5.07083		
	1.75	0.030543	54.7	27.35	0.477348	5.110908		
	2.13	0.037176	64.48	32.24	0.562694	4.409578		
	1.12	0.019548	77.29	38.65	0.674482	9.081742		
70°C	0.84	0.014661	32.27	16.14	0.281609	9.845297	7.135564	
	1	0.017453	38.13	19.07	0.332747	8.405333		
	1.26	0.021991	44.37	22.19	0.387201	6.80907		
	1.29	0.022515	54.7	27.35	0.477348	6.933403		

	1.64	0.028623	64.49	32.25	0.562781	5.727389		
	2	0.034907	77.49	38.75	0.676228	5.092891		

The total average size of crystallite is 9.13555nm.

Antibacterial Activity

Antibacterial activity of neem-mediated AgNPs against bacterial pathogens at neem concentration variation

The AgNPs of 3mM and 4mM concentration of neem extract showed highest and almost same amount of antibacterial activity against S.aureus, E.coli, K.pneumoniae and P.aeruginosa. Beside that there was very small or no zone of inhibition was observed by 1mM and 2mM $AgNO_3$ concentration against P.aeruginosa as shown in figure 4.

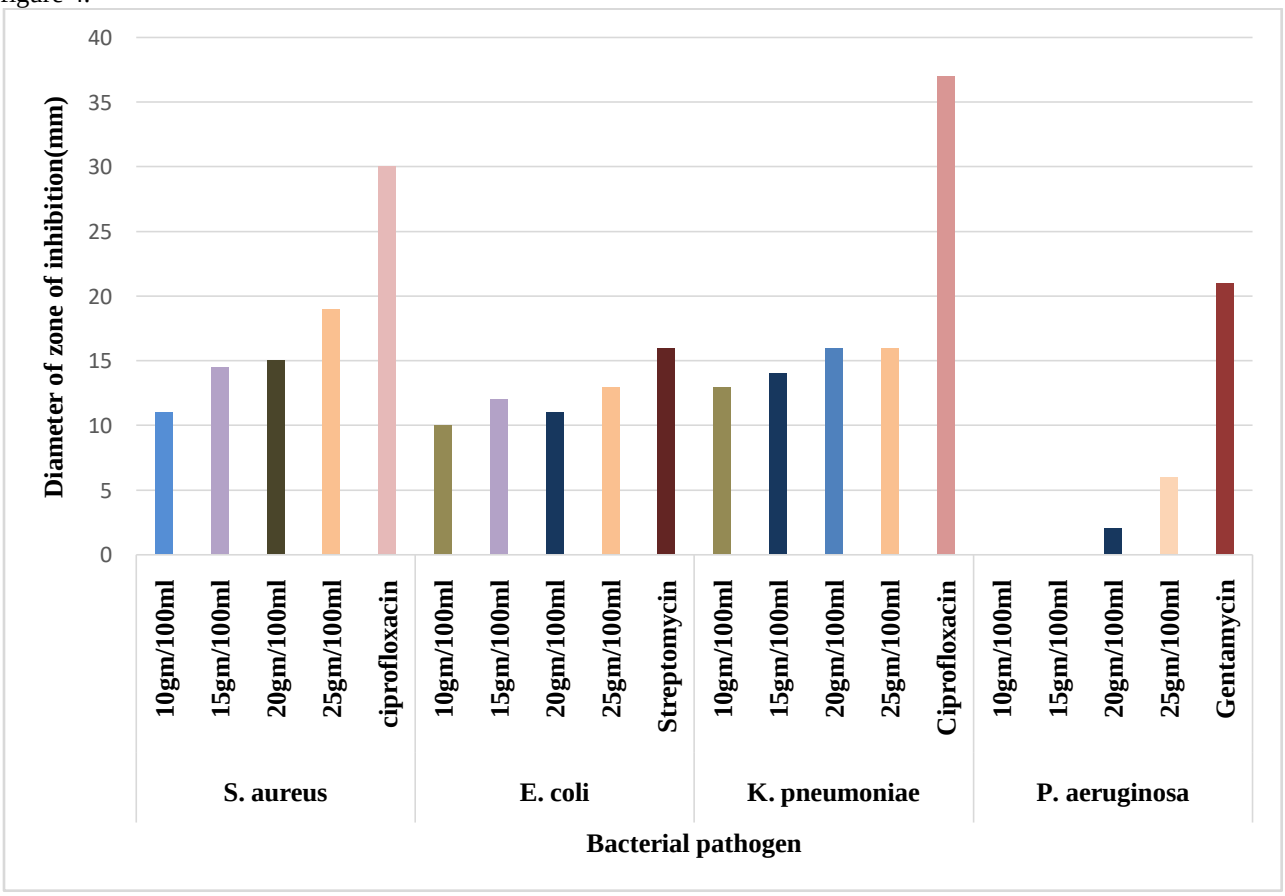


Fig. 4:- Zone of inhibition shown by neem-mediated AgNPs against *S. aureus, E. coli, K. pneumoniae and P. aeruginosa* at different neem concentration.

Antibacterial activity of neem-mediated AgNPs against bacterial pathogens at $AgNO_3$ concentration variation

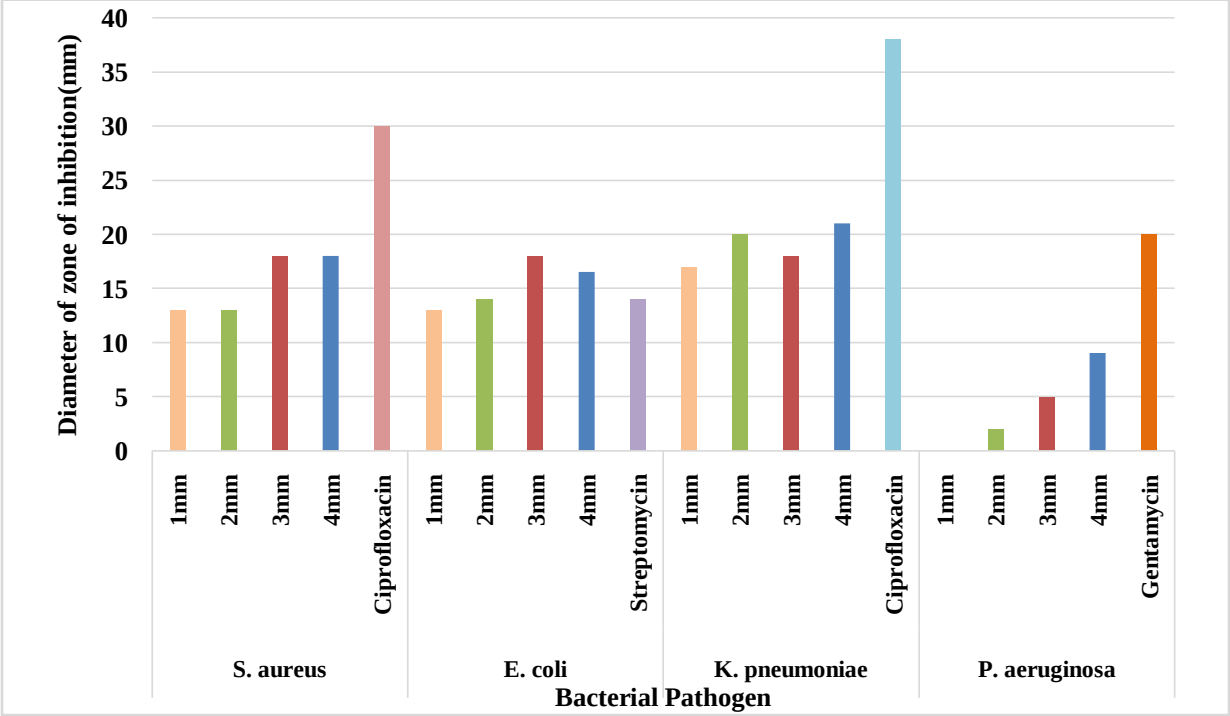


Fig. 5:- Zone of inhibition shown by neem-mediated AgNPs against *S. aureus, E. coli, K. pneumoniae and P. aeruginosa* at different $AgNO_3$ concentration.

Antibacterial activity of Neem-mediated AgNPs against bacterial pathogens at temperature variation

At 70°C synthesized AgNPs showed the larger zone of inhibition against S. aureus, E. coli with zone of inhibition 18mm, 12mm, 20mm respectively. The lowest inhibitory activity was seen at 40°C. However, no inhibitory activity was observed against P. aeruginosa as shown in figure 6.

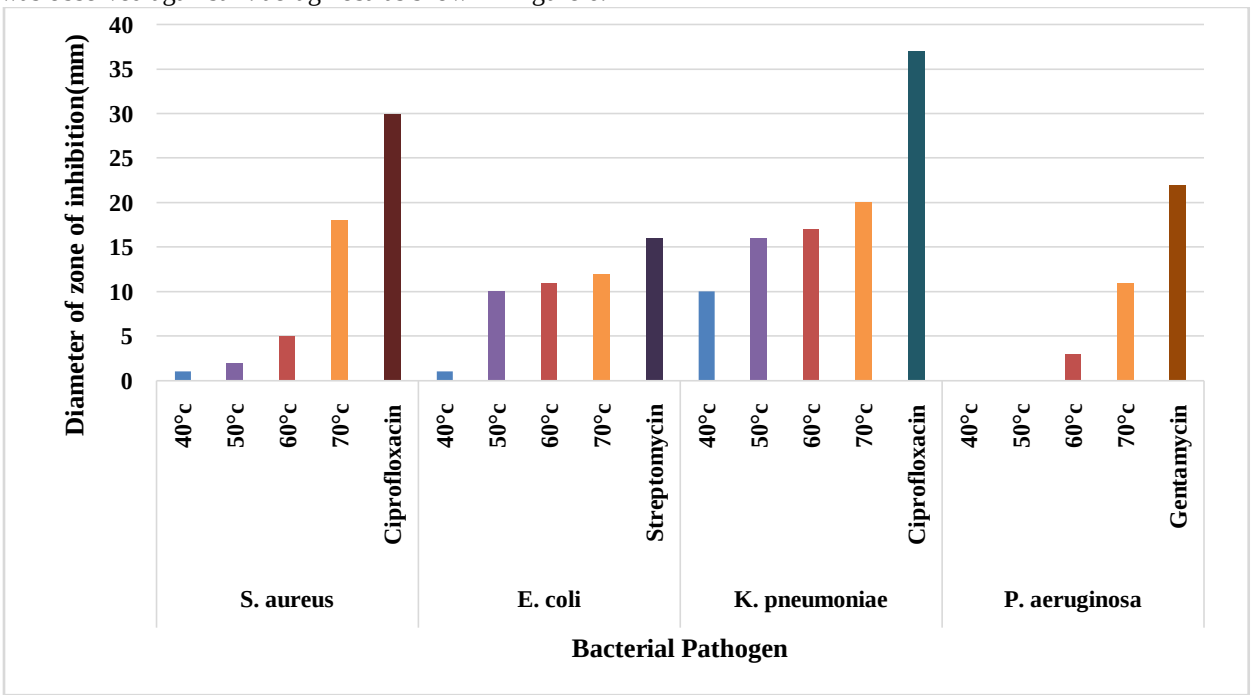
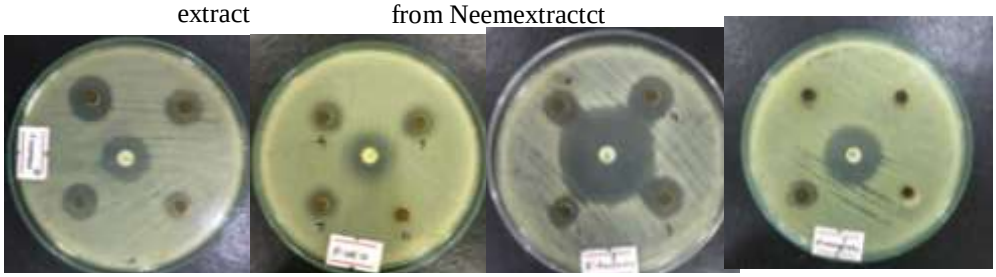


Fig. 6:- Zone of inhibition shown by neem-mediated AgNPs against *S. aureus, E. coli, K. pneumoniae and P. aeruginosa* at different temperature.

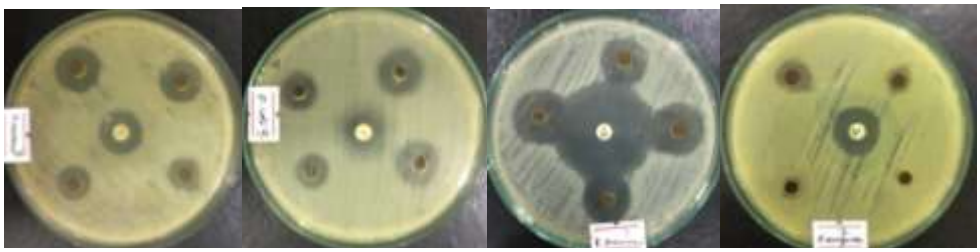



Neem

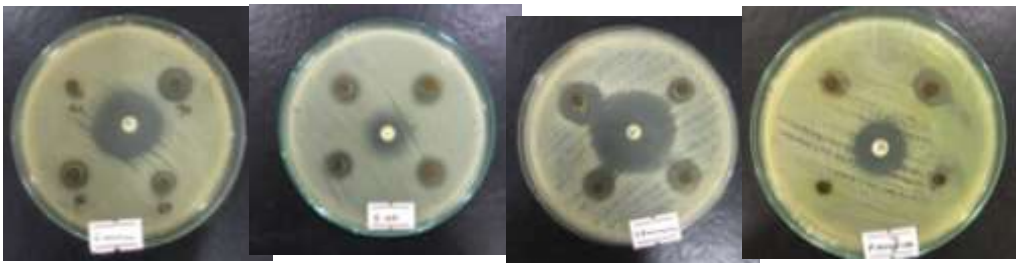
Preparing Neem extract | Synthesized AgNPs from Neemextractct | Synthesized AgNPs



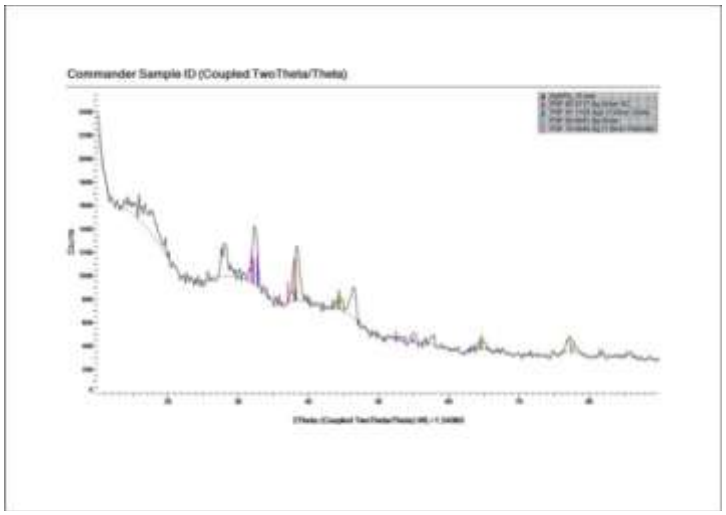
Antibacterial activity of neem-mediated AgNPs at neem extract concentration variation against bacterial pathogens: S. aureus,E. coli, K. pneumoniae, P. aeruginosarespectively



Antibacterial activity of neem-mediated AgNPs at $AgNO_3$ concentration variation against bacterial pathogens: *S. aureus, E. coli,K. pneumoniae,P. aeruginosa*respectively



Antibacterial activity of neem-mediated AgNPs at temperature variation against bacterial pathogens: *S. aureus, E. coli, K. pneumoniae, P. aeruginosa*respectively



XRD pattern of synthesized AgNPs from neem at 4mM $AgNO_3$ concentration.

Discussion:-

Bioactive compounds such as terpenoids and flavanone [14] present in Azadirachta indica act as excellent natural reducing, capping and stabilizing agent.The alkaline environment is very important as it provides hydroxide ions which results the increment of reduction capacity of silver ions [14]. Also, at greater pH than 7 agglomeration is discouraged due to all groups are deprotonated favoring the repulsion between the nanoparticles [3]. The color of solution was changed during the synthesis process from light yellow to dark brown which indicated the formation of silver nanoparticles. The color was changed because of surface plasmon resonance (SPR) and this band is ascribed to excitation of valence electrons [12]. The UV-Vis Spectrophotometer analysis was done at 250 to 700 nanometer wavelengths. X-ray diffraction was done for the further confirmation of colloidal suspension. The formation of silver nanoparticle increases with the concentration of plant extract. The reason may be related to abundant availability of reducing agent present in these plants same result was observed in [11]. Also, at higher concentration of $AgNO_3$, there is access amount of silver ions present and at higher temperature, the absorbance was maximum. Formation of smaller silver nanoparticles was occurred at high temperature by reducing the extent of aggregation of the nanoparticles due to increase in the rate of adsorption of silver nitrate and the viscosity of the coat-phase [11]. Bragg peaks representative of silver nanoparticles, unassigned peaks were also observed. That could be due to the presence of some bioorganic compounds/proteins in the leaf extract and crystallizes on the surface of the silver. The line broadening of the peaks was primarily due to small particle size as suggested by [1].

Antibacterial activity of silver nanoparticles was investigated through agar well diffusion method against *Staphylococcus aureus*, *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*. 500mg/ml concentration of AgNPs were added against pathogens According to [9], the positive antibacterial activity shown by silver nanoparticles is because AgNPs can attach to the negatively charged cell surface altering the physiochemical components of cell membrane [5] then easily invade the inner cell components and cause severe damage to the cells by interacting with sulfur and phosphorus- containing compounds, such as proteins and genetic materials, which leads to complete cell. Also, the formation of free radicals, the inactivation of proteins in the cell by silver ions and the production of reactive oxygen species (ROS) may be another reason [8]. The inhibitory activity of synthesized silver nanoparticles was quite satisfying against *Staphylococcus aureus*, *Escherichia coli*, *Klebsiella pneumoniae*and *Pseudomonas aeruginosa*.However,*Pseudomonas aeruginosa*was less susceptible to silver nanoparticles.Same result was reported by [6].

Conclusion:-

The study concluded theAzadirachta indicahas the ability to synthesize the silver nanoparticles. The surface plasmon resonance of synthesized silver nanoparticles were detected through UV spectroscopy method which was around 400nm to 450nm indicated the presence of silver nanoparticles. Through X-ray diffraction method the average size of silver nanoparticles was detected and confirmed which was 5-9nm. From the conducted research, silver nanoparticle synthesis at higher concentration variations of plant extract, silver nitrate including higher temperature at alkaline pH were found more effective to produce smaller size nanoparticles. The synthesized silver nanoparticles

exhibited antibacterial activity against Staphylococcus aureus, Escherichia coli, Klebsiella pneumoniae and Pseudomonas aeruginosa. It was proved that Pseudomonas aeruginosa was less susceptible to silver nanoparticles. The ability of bacterial inhibition by silver nanoparticles was significant objective of this study. It supported the evidence that silver nanoparticles seem to be alternative antibacterial agents and have the ability to overcome the bacterial resistance against antibiotics.

Data Availability
The data used to support the findings of this study are added in the article.

Abbreviations
AgNPs: Silver nanoparticles
NPs: Nanoparticles
$AgNO_3$:Silver nitrate
NaOH: Sodium hydroxide
UV-vis:Ultra violet- visible
ROS:Reactive oxygen species
XRD:X-ray diffraction
nm:Nanometer
DNA:deoxyribonucleic acid
HIV:Human immunodeficiency virus
NAST:Nepal Academy of Science and Technology
ATCC:The American Type Culture Collection

Authors Contribution
SP has contributed to the plan of the research work, sample collection, sample processing, data analysis, intellectual content design and result interpretation. HJR helped in collection of samples, interpreting result. AB supervised the research project Both the authors drafted the manuscript and agreed for its publication.

Competing interests
No competing interests

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Ethical approval and consent
Not applicable.

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