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Studies on Bacterial Synthesis of Silver Nanoparticles and its Synergistic Antibacterial effect with antibiotics against Selected MDR Enteric Bacteria

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ABSTRACT

In the present study, the extracellular synthesis of silver nanoparticles was done using two different bacterial strains viz. Bacillus flexus and Bacillus pseudomycoides. The silver nanoparticles were confirmed by in color test and characterized by UV-visible spectroscopy and the λmax observed at 430 nm and 410 nm confirmed to the synthesis of AgNPs. FTIR analysis confirmed the presence of elemental silver and reveals the dual function of the biological molecule responsible for the reduction and stabilization of AgNPs in the aqueous medium. The XRD showed that silver nanoparticles produced crystalline in nature with size ranges from 30 to 70 nm. The SEM showed that produced silver nanoparticles were spherical, pseudo-spherical in shape with traces of agglomeration. Further through investigation of antibiotic sensitivity/resistant pattern expressed that out of eighteen virulent enteric bacterial isolates, three isolates showed MAR index equal to 1, which indicated the presence of multiple drug resistance (MDR). MIC values of AgNPs against MDR isolate E7 and K3 were established to be 80 µg/ml whereas, for isolate Sa1 the MIC value was 70 µg/ml. The synergistic effect of antibiotics in conjugation with biologically synthesized AgNPs encourages the susceptibility amongst the tested bacterial cultures; viz. Salmonella followed by *Klebsiella* and *E. coli*.

Key-words: Antibacterial activity, Biosynthesis, Multidrug-resistant (MDR), Silver nanoparticles, Synergistic activity

INTRODUCTION

Silver nanoparticles are having great interest today due to its different properties such as good conductivity, chemical stability, catalytic and antibacterial activity. Nanotechnology provides a good platform to modify metal in the form of nanoparticles. An important area of research in nanotechnology is the biosynthesis and Characterization of nanoparticles such as nanosilver. It was reported that highly stable silver nanoparticles (40 nm) could be synthesized by bioreduction of aqueous silver ions with a culture supernatant of some nonpathogenic and pathogenic Bacteria [1].

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Silver nanoparticles (AgNPs) have emerged as an arch product from the field of therapeutic nanotechnology. Resistance in human pathogens is a big challenge in pharmaceutical and biomedicine. The present study is focused on antibiotic-resistant enteric bacteria as these represent the most immediate urgent global concern [2,3]. Enteric diseases are among the most common causes of morbidity and mortality in low-income nations, strangely affecting children under the age of five [4]. The Silver-Nanoparticles against Multidrug-resistant enteric human pathogens have received minor attention by means of published citations. Hence, the biosynthesis of silver nanoparticles from bacteria with special reference to Potentiation of antibiotic activity against Multidrug-resistant enteric human pathogen has investigated.

MATERIALS AND METHODS

Synthesis of silver nitrate reductase enzyme- The silver nanoparticles were synthesized from two different silver-resistant bacterial isolates viz. *B. flexus*, *B.*

pseudomycoides [5]. Intended for the biosynthesis of silver nanoparticle, the bacterial cell-free extract was prepared by separately inoculating the bacterial isolates in 100 ml LB broth followed by shaking incubation at 220 rpm for 24 hours. The cell free extract was separated by ultracentrifugation at 20,000 rpm for 10 minutes and used as a crude source of reductase enzyme for the extracellular synthesis of nanoparticles.

Biosynthesis of silver nanoparticles- In a typical biosynthesis production scheme of silver nanoparticles, 2 ml of reductase enzyme was mixed separately with 100 ml of 1 mM aqueous solutions of filtered sterilized $AgNO_3$, in 250 ml conical flasks and the reaction mixture was further incubated on incubator shaker at 150 rpm (Remi make) at 37˚C up to 72 hours and allow for reduction. The set without $AgNO_3$ was maintained as Control. The work was done adopting the method suggested by Das *et al*. [5] with slight modifications.

Purification of silver nanoparticles- The silver nanoparticles were purified by three successive ultra centrifugations at 20,000 rpm for 15 minutes at 40˚C the supernatant clear suspension was redispersed in sterile deionized water to remove the residual biological molecules. The process was repeated thrice for complete removal of redundant residual entities from the silver nanoparticles. The purified solution was then dried to form the powder using hot air oven at 60˚C for overnight [6].

Characterization of silver nanoparticles- The dried powder of silver nanoparticles was then mixed with 10 ml of deionized water and kept on a sonicator to prevent aggregation of molecules and further Characterized by UV-Visible spectroscopic analysis; FT-IR analysis; XRD analysis, and SEM analysis.

Antibacterial activity of Silver Nanoparticles against MDR Enteric bacteria

Isolation and Identification of Enteric Human Pathogens- The isolation of pathogen was done for three consecutive years on selective as well as differential enteric media. Frequently reported enteric human pathogens viz. *E. coli*, *Klebsiella* sp., *Salmonella* sp., and *Shigella* species were isolated from urine, stool and sewage samples respectively [7]. All the isolates were further screened for the virulence by India ink degradation. The obtained virulence strains were identified by the conventional method. The multidrug resistance strains were screened adopting antibiotic susceptibility test [8]. The assays were implemented in triplicate and expressed in terms of central tendency. The S/R blueprints of the isolates were determined by comparing the values of inhibition zones with "Disc diffusion supplemental table"[9] MAR (Multiple antibiotic resistance) indexes were calculated by standard formula [10]. The isolate showing MAR indexes equal to 1 was selected for further analysis.

MAR Index =

$$\frac{\text{Number of antibiotics to which isolates showed resistance}}{\text{Total number of antibiotics tested}}$$

Independent and Synergistic Antibacterial activity of Silver Nanoparticles- Standard stock solutions of different concentration (100 µg/ml to 10 µg/ml) of obtained silver nanoparticles were prepared. The control was used as autoclaved deionized water. The suspensions were sonicated for 20 minutes to avoid deposition of AgNPs and use for disc impregnate. The AgNPs impregnated discs were placed aseptically on MH agar plates speeded with test pathogens and incubated at 37˚C for 16 to 18 hours. Post incubation, the zone of inhibition was measured and MIC of AgNPs was determined. Assays were implemented in triplicate and expressed in terms of central tendency.

For determining synergistic effects, each standard antibiotic disc was impregnated with respective MIC of AgNPs against test MDR bacteria viz., *E. coli* (E7), *Klebsiella* (K3) and *Salmonella* (Sa1). The impregnated discs were placed aseptically on MH agar plates speeded with test pathogens and incubated at 37˚C for 16 to 18 hours. Further, the zone of inhibition was measured as mm diameter. The assays were implemented in triplicate and expressed in terms of central tendency. Both the readings obtained were then compared and expressed in terms of fold area increase in antibacterial activity, by using the formula [11].

$$\text{Increase in fold area} = (b^2 - a^2) / a^2$$

Where, a= Zone of inhibition (mm) obtained by antibiotic alone

b= Zone of inhibition (mm) obtained by antibiotic combination with AgNPs

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RESULTS

Biosynthesis of Silver Nanoparticles- The isolates *B. flexus* and *B. pseudomycoides* showed the reduction of Ag+ ions, since, visualizing the change in color from colorless to dark brown. The results revealed the possible use of the bacterial strains for rapid synthesis of Silver nanoparticles hence conceivably to be used in the biosynthesis process for large-scale production.

Characterization of synthesized Silver Nanoparticles- The purified dried AgNPs powder samples viz. AK1 and AK2 were characterized by means of UV-Visible spectrum graphically represented in Fig. 1. During, which two strong peaks were observed at 430 nm and 410 nm which confirmed the synthesis of AgNPs [8].

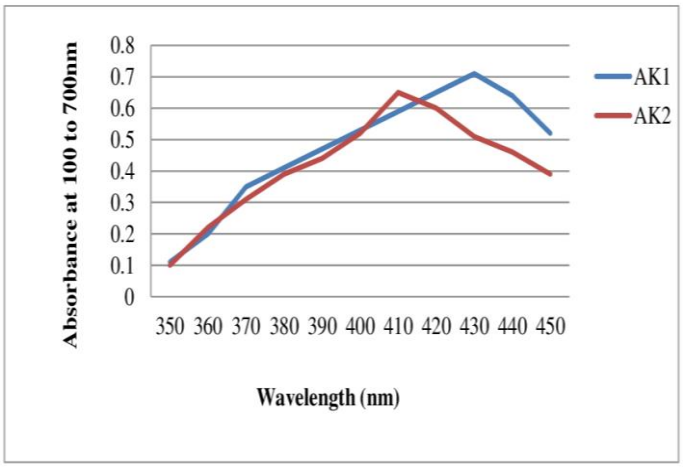
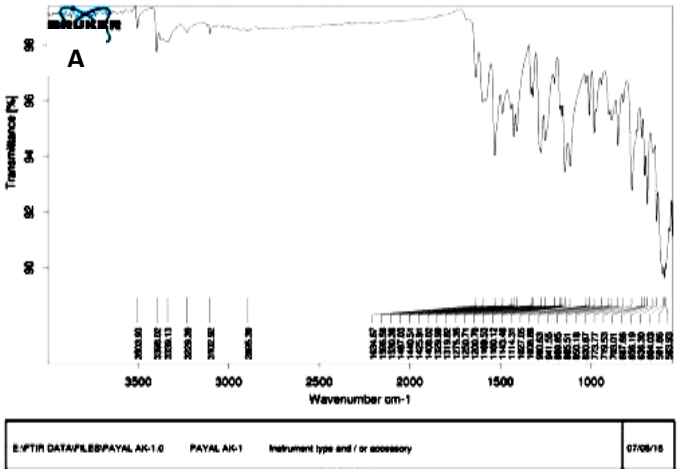


Fig. 1: UV-Visible absorbance spectra of synthesized silver nanoparticles

The results of FTIR for two AgNPs samples (viz. AK1 and AK2) were represented in Fig. 2, the bands obtained at 591.86 $cm^{-1}$and 577.46 cm^{-1}. Hence the FTIR analysis confirms the presence of elemental silver, [12].



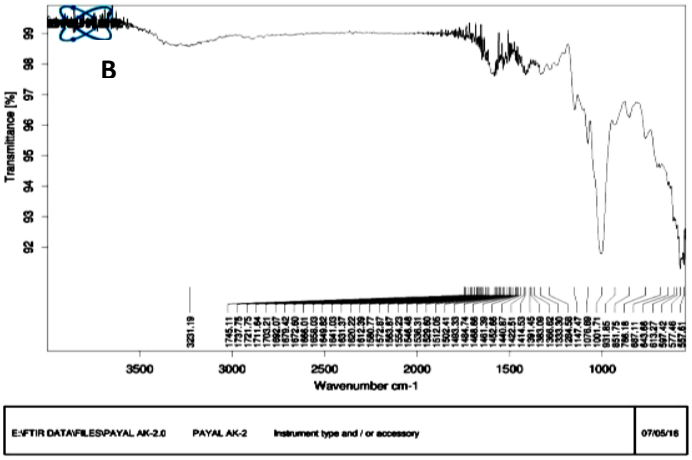


Fig. 2: FTIR Spectrum of Silver nanoparticles synthesized from *B. flexus and B. pseudomycoides*

The XRD pattern obtained for two AgNPs samples (viz. AK1 and AK2) were represented in Fig. 3. Comparisons of XRD spectrum with the standard powder diffraction card of Joint Committee on Powder Diffraction Standards (JCPDS), silver file No. 04-0783, confirms that the silver nanoparticles found in the present study were in the form of nano-crystals as evident from the peak at 2θ values 111, 200, 220, 311 respectively for silver and are in accordance with calculated particle size calculated. In Table 1, we are also observed that all the samples contain different sizes of silver nanoparticle with size ranges from 30 to 70 nm.

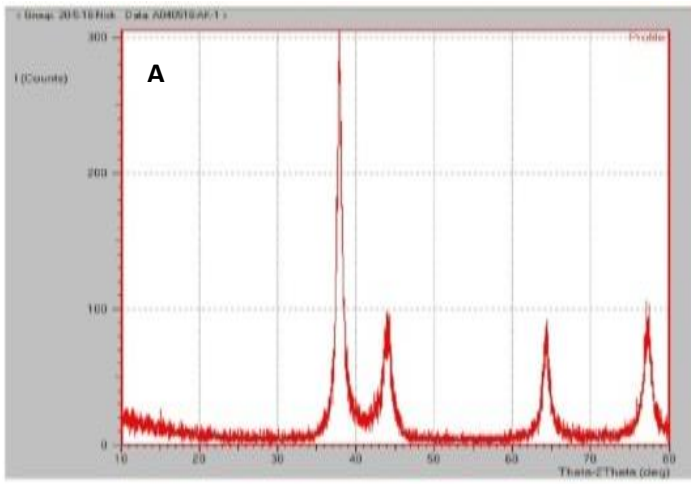


Fig. 3 (a): XRD Spectrum of silver nanoparticles synthesized from *B. flexus* sp.

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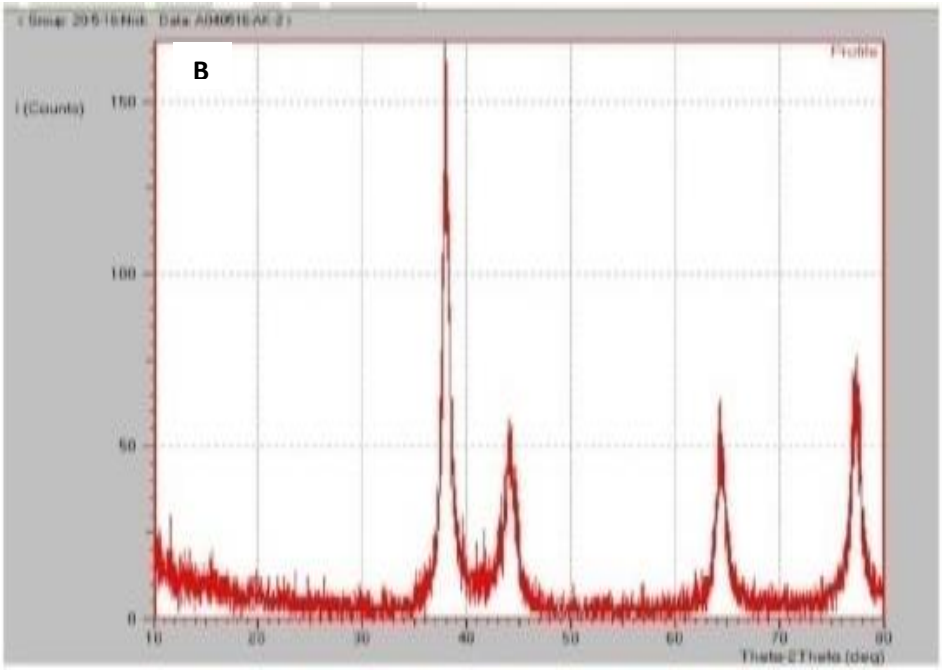

Fig. 3 (b): XRD Spectrum of silver nanoparticles synthesized from *B. pseudomycoides* sp.

Table 1: Peak indexing from d-spacing and particle size of synthesized silver nanopowder

2θ	θ	D	$1000/d^2$	$(1000/d^2)/60.62$	Hkl	FWHM(β)	β cos θ	Particle (D) size (nm)
				Sample AK1				
37.91	18.955	2.371	177.904	2.934	111	0.0041	0.00407	34
43.98	21.99	2.056	236.574	3.902	200	0.0048	0.00479	29
64.21	32.105	1.449	476.417	7.859	220	0.0038	0.00292	47
77.20	38.6	1.234	657.030	10.838	311	0.0034	0.00210	66
				Sample AK2				
38	19	2.366	178.667	2.947	111	0.0041	0.00405	34
44.15	22.075	2.049	238.208	3.929	200	0.0048	0.00478	29
64.36	32.18	1.446	478.468	7.892	220	0.0038	0.00274	50
77.28	38.64	1.233	657.894	10.852	311	0.0034	0.00200	69

In the present study, Fig. 4 shows representative SEM images recorded at high magnifications of biosynthesized silver nanoparticles, it was observed that the produced silver nanoparticles were scattered as well as in aggregated of varying sizes.

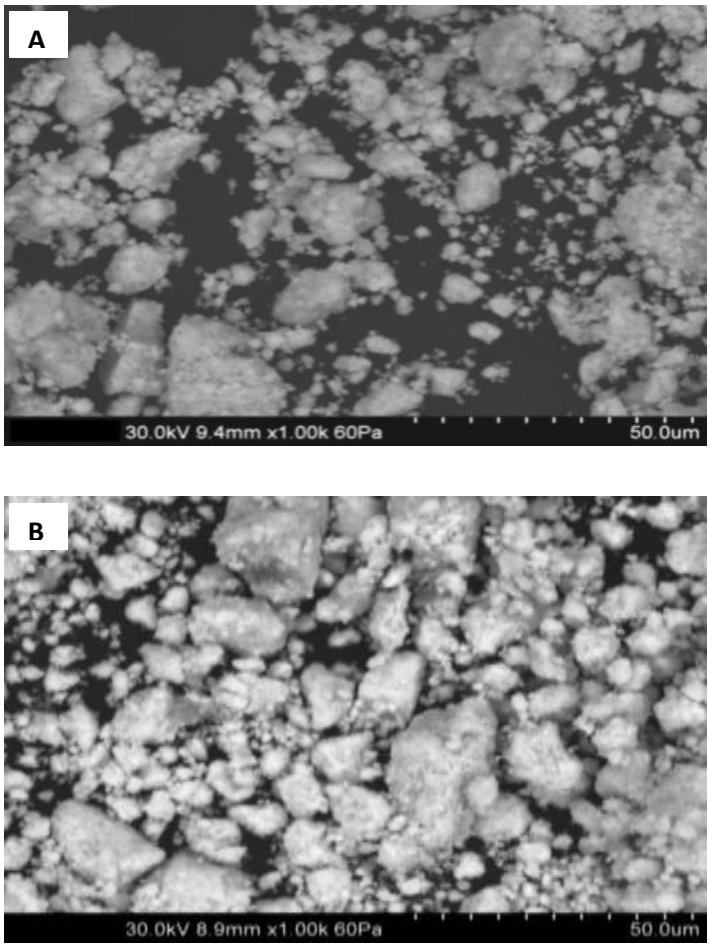

Fig. 4: SEM images of Silver Nanoparticles synthesized from *B. flexus and B. pseudomycoides*

Antibacterial activity of Silver Nanoparticles against MDR Enteric bacteria

Isolation and Identification of Enteric Human Pathogens- In favor of the antibacterial study of AgNPs against enteric human pathogens viz, *E. coli, Klebsiella, Salmonella* and *Shigella* species. All the isolates were further confirmed by screening the virulence adopting India ink degradation. The obtained virulence strains were identified and labeled as E1 to E10 for *E. coli* as well as K1 to K4 for Klebsiella, Sa1, Sa2 for Salmonella, Sh1, Sh2 for Shigella species respectively. The findings on antimicrobial susceptibility testing are graphically presented in Fig. 5 from the figure, maximum isolates (88%) among tested pathogens showed resistance to Gentamycin, Co-trimoxazole and Tetracyclin followed by 83% isolates showed resistance to Nitrofurantoin and Ceftriaxone. Whereas, in the case of Azithromycin and Chloramphenicol, the (72%) isolates among the test pathogens showed at par resistance against both the antibiotics.

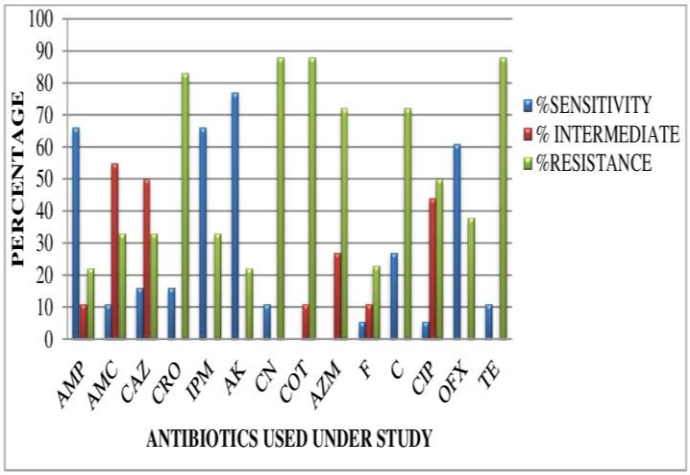

Fig 5: Antibiotic Sensitivity/Resistant Pattern of the isolated human enteric pathogens

The resistance was exhibited by only 22 - 50% of isolates under study against Ampicillin, Amoxicillin-clavulanate, Ceftazidime, Imipenem, Amikacin, Ciprofloxacin, and Ofloxacin. The results on MAR index of test isolates are graphically presented in Fig. 6. From the Fig. 6, it was established that out of eighteen isolates, isolates E7, K3 and Sa1 showed the MAR index equal to one, which indicates the presence of multiple drug resistance (MDR) in these isolates and their origin from a high-risk source of contamination where antibiotics are often used [13]. Hence, only E7, K3, and Sa1 isolates were used for further investigations.

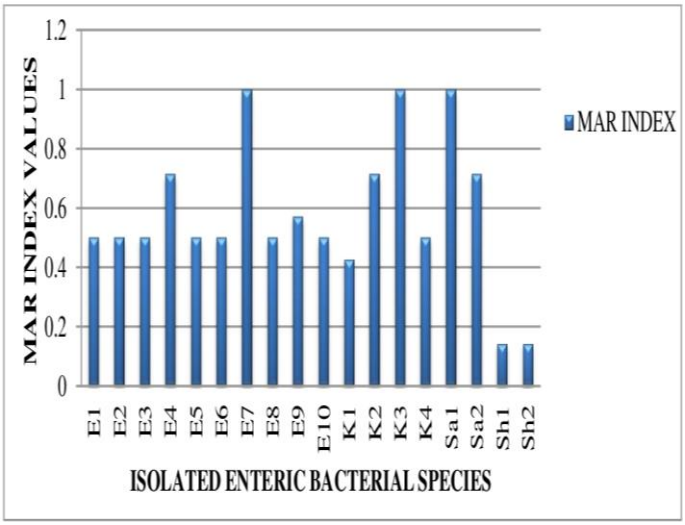

Fig 6: MAR Index of the isolated human enteric pathogens

Independent and synergistic antibacterial activity of silver nanoparticles- The individual antibacterial activity of AgNPs against test pathogens viz. E7, K3, and Sa1 are depicted in Fig. 7, MIC values for isolate E7 and K3 were

recorded to be 80 µg/ml whereas, for isolate Sa1 MIC value was 70 µg/ml. The results obtained on combined antibacterial activity are depicted in Fig. 8, from the results; it was observed that in the case of study on antibacterial activity of antibiotics alone all the selected MDR isolates exhibited resistance(R). In case of study on antibacterial activity of AgNPs alone, mild bactericidal activities were observed in terms of zone of inhibition ranging from 10–11 mm.

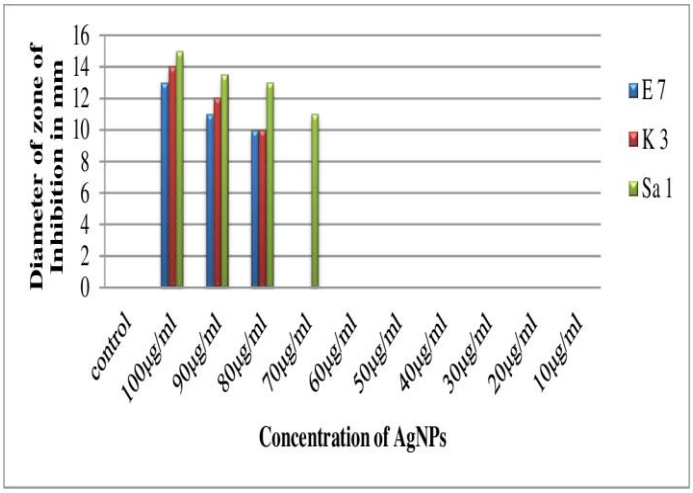

Fig 7: MIC values of AgNPs against test pathogens

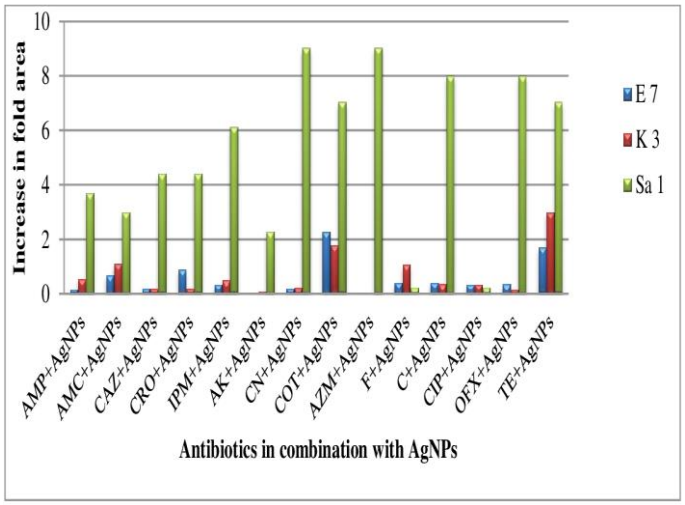

Fig 8: Increase in Fold Area of antibiotics in combination with AgNPS

Notes: In the absence of bacterial growth inhibition zones (NI), the disc's diameter (6mm) was used to calculate the fold increases [11]. Increase in fold area=(b^2-a^2)/a^2. (R)-Resistance, (S)-Sensitive, (I)-Intermediate

However, in case of the combined activity of Antibiotics along with AgNPs; in case of isolate E7, the maximum increase i.e. (2.3) in fold area inhibition was recorded due to Cotrimoxazole-AgNPs combination followed by Tetracyclin-AgNPs combination (1.7). The remaining combinations showed the increase in fold area inhibition in the range of 0.1 to 0.9. However, in the case of Amikacin-AgNPs and Azithromycin-AgNPs conjugates, no change in fold area inhibition was observed. Similarly, in case of Isolate K3 maximum increase in fold area inhibition (3) was observed in Tetracyclin-AgNPs combination followed by Cotrimoxazole-AgNPs (1.8), Amoxicillin-clavulanate-AgNPs (1.13) and Nitrofurantoin-AgNPs (1.08) combinations respectively. The remaining combinations showed the increase in the fold inhibition in the range of (0.5) to (0.1) and Azithromycin-AgNPs showed no change in increase fold area inhibition. Isolate Sa1 showed the maximum increase in fold area inhibition (9.03) with Azithromycin-AgNPs combination followed by Gentamycin-AgNPs (9.02). Chloramphenicol-AgNPs and Ofloxacin-AgNPs combination showed at par increase in fold area inhibition of (8). Cotrimoxazole-AgNPs and Tetracyclin-AgNPs combination showed at par results (7.03), all of the remaining combinations showed an increase in inhibition fold area inhibition greater than (1) except Nitrofurantoin-AgNPs and Ciprofloxacin-AgNPs combination, which showed the increase in fold area inhibition of (0.21). Hence, a Maximum synergistic antibacterial activity of Cotrimoxazole-AgNPs combination was observed against isolate E7, Tetracyclin-AgNPs combination against K3 and Azithromycin- AgNPs combination against Sa1.

DISCUSSION

The AgNPs were synthesized by using two bacterial strains viz. *B. flexus* and *B. pseudomycoides*, characterization by UV-Visible spectrometry and FTIR revealed the presence of AgNPs in Synthesized samples. The overall result of XRD explained that silver nanoparticles found in the present study were in the form of nano-crystals with varying sizes [14] ; the scanning images showed the agglomeration which may be due to the fact that silver nanoparticles have the tendency to agglomerate due to their high surface energy and high surface tension of the ultrafine nanoparticles [15]. The research findings on antibiotic susceptibility testing of enteric human pathogens reported the persistence of antibiotic resistance in enteric human pathogens [16-19]. The consistency and overuse of antibiotics as well as resistant gene transfer from animals to man via Food chain might be the reason for resistance traits in

pathogens. Our findings on the Minimum Inhibitory Concentration of AgNPs reported the lethal effect of silver nanoparticles against different pathogens with MIC values in the range of the 50 to 75 µg/ml [20,21]. The results enlightened that the synergistic effect of antibiotics in conjugation with biologically synthesized AgNPs, increased the susceptibility among the tested MDR enteric bacteria in the following sequence; viz. *Salmonella* species followed by *Klebsiella* species and *E. coli* species respectively. These results are in line with the findings of Birla *et al.* [11] who mentioned increasing efficacies percentage of different antibiotics when used in combination with AgNPs against *P. aeruginosa, S. aureus* and *E. coli*. In a similar study, the antimicrobial activities of biologically synthesized AgNPs were assessed with commercially available antibiotics against G- and G+ bacteria [22].

CONCLUSIONS

The present study even though is very preamble; the studies enlighten the Potentiating of antibiotics activity due to presence of silver nanoparticles and provide helpful insights to the development of novel antimicrobial agents in combination with silver nanoparticle. This synergistic antibacterial effect may be considered as beneficial for the management of multiple drug resistance enteric pathogens however; more elaborate experimental shreds of evidence will be needed.

The focus may also be given towards the Toxicity studies of silver nanoparticles on the human pathogen in relation to human physiology which may open a door for a new combinational range of antibacterial agents using nanoparticles.

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CONTRIBUTION OF AUTHORS

The conception or design of the work, Data collection, Data analysis and interpretation for the work was done by Payal Agrawal. Whereas, Drafting of the article, Critical revision of the article for important intellectual content was done by Dr. Nikhilesh Kulkarni.

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