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A NOVEL APPROACH FOR STUDYING THE COMBINED ANTIMICROBIAL EFFECTS OF SILVER NANOPARTICLES AND ANTIBIOTICS THROUGH AGAR OVER LAYER METHOD AND DISK DIFFUSION METHOD

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In the present investigation, the combinatorial inhibitory effects of silver nanoparticles impregnated with four broad spectrum antibiotics namely amoxicillin, chloremphenicol, erythromycin and rifamycin against four major pathogens was analysed. For studying the antimicrobial activity, a technique based analysis was performed by disk diffusion method and by agar over layer method (Bioautography). It was observed that silver nanoparticles (10 µg) showed potential zone of inhibition in similar with the effects of antibiotics (10 µg). Rather, when given together (0.5 µg each), an enhanced antibacterial activity depicting the inhibition zone (2 - 4 mm increase in diameter) was reported against certain pathogenic strains, *Bacillus cereus* (40 mm), *Bacillus subtilis* (28 mm), *Klebsiella pneumoniea* (24 mm) *and Vibrio cholerae* (20 mm). From this experimental analysis, rifamycin (antibiotic) offering maximum inhibitory effect, was separated by Thin Layer Chromatography (TLC). Bioautography (agar over layer method), a novel approach for studying the antimicrobial bahaviour of silver nanoparticles and silver nanoparticles impregnated with antibiotics was established. Two zones representing two reference points (black zone at loading point and red zone at separation point)) formed by agar over layer method indicates the zone of inhibtion produced by the silver nanoparticle and antibiotics respectively.

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Keyword: Antibacterial activity, Silver nanoparticles (AgNps), Antibiotics, Thin layer chromatography (TLC), Bioautography, Agar over layer method

1. Introduction

Since most of the antibiotics seem to be less sensitive to many organisms, researchers started finding a new potential antimicrobial agent [1]. With this respect, silver and silver-based compounds are having strong bacteriocidal and fungicidal activity [2, 3, 4].

Nanoparticles having larger surface area to volume ratio tend to pose higher antimicrobial activity [5]. Also, Silver has a lower propensity to stimulate microbial resistance than many other antimicrobial agents [1, 6]. Based on these properties, Ag-NPs have been used in wide range of applications such as to prevent infection, in (burn and traumatic) wound dressings, diabetic ulcers, coating of catheters, dental works, scaffold, and medical devices [7, 8].

Green synthesis, a novel green chemical route for producing nanoparticles from plant extracts are having more advantageous than the method of using microbes for nanoparticle synthesis [9]. Tea extract (*Camellia sinensis*) are effective reducing agents, because of their high polyphenol content are used in nanoparticle (Ag–NPs) synthesis.

The objective of the present study was to evaluate the antimicrobial activity of the synthesized silver nanoparticles and compared with the inhibitory effects produced by the

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antibiotics. Also, a detailed study on the combined effects of nanoparticles impregnated along with major broad spectrum antibiotics was also analysed. Besides, technique based analysis on antimicrobial behavior was evaluated by disk diffusion method (Microbiological analysis) and by bioautography through TLC (Analytical technique).

2. Materials and methods

2.1 Synthesis and Characterization of silver nanoparticles

The silver nanoparticles were synthesized by novel green chemical route [9]. The nanoparticles were characterized by X-RD analysis, (X-ray diffraction method) to predict the size of the nanoparticle. SEM analysis (Scanning Electron Microscopy) was performed for studying the surface morphology.

2.2 Test organisms used for the analysis

Clinical isolates of *Bacillus cereus, Bacillus subtilis, Klebsiella pneumoniea, Vibrio cholerae* were obtained from KMCH, Coimbatore (India). A loop of single colony of each test strain was inoculated in Mueller Hinton liquid medium (broth) and incubated in a temperature controlled shaker (120 rpm) at 30 °C overnight. Antibiotics used for the analysis (amoxicillin, chloremphenicol, erythromycin and rifamycin) were purchased from Sigma Aldrich, Bangalore, India and Merck Limited, Mumbai, India.

2.3 Disk Diffusion method

2.3.1 Antimicrobial activity of silver nanoparticles impregnated with antibiotics (combined effects)

Four major pathogens *(B.cereus, B.subtilis, K.pneumoniea, V.chlorae)* were taken for analysis. Four plates for each organism in such a way 16 plates containing solidified agar media were prepared and swabbed with respective microbial inoculum. Four disks in each plate were fixed at equal distance. The first disk was loaded with 10 µl of distillled water which ser(Fig. 1)ves as control. The second disk was impregnated with 10 µg of silver nanoparticle. The third disk was impregnated with 10 µg of different reference antibiotics (namely amoxicillin, chloremphenicol, erythromycin and rifamycin) and the fourth disk was loaded with 10 µg of nanoparticle and reference antibiotics (0.5 µg each) respectively. All these 16 plates were kept at 37 ^{o}C for incubation overnight. After 24 hrs of incubation the zone of inhibition were measured and compared.

2.4 Thin layer chromatography

It is used to separate the individual compounds formulated in the antibiotics (crude). The separation of the compound also depends on the type of the solvent used. The antibiotic representing maximum zone of inhibition (rifamycin) based on disk diffusion method was used for TLC analysis. A sample of 10 mg/ml concentration of antibiotics in methanol was prepared. From this solution, 4µl of the sample prepared was taken and spotted on the silica coated TLC plates. It was then kept in slanting position with the solvent to run under capillary pressure. Here methanol and chloroform in the ratio of 3:7 was used as a solvent. The spots were then identified both in the UV light, far light and in the iodine chamber [10].

The R_f values were calculated. R_f value is defined as the distance travelled by the solute to the distance travelled by the solvent.

2.5 Bioautography – Agar over layer method

The developed TLC plates were kept in the sterile petriplates. Then the nutrient agar prepared was poured over the thin layer which was further spreaded over the entire petridish. 24 hours cultures of *(B.subtilis, B.cereus, V.cholerae and K.pneumoniea)* were swabbed on it. The plates were then incubated at 37 º C for 24 hours. Zone of inhibition obtained at varying separation point were observed.

Similarly chromatogram was developed by loading 1µg concentration of nanoparticle impregnated with rifamycin (antibiotic). The characteristic zone of inhibition thus obtained at the separation point by agar over layer method was investigated [11]. The results were discussed.

3. Results and discussions

3.1 X-RD analysis of silver nanoparticles

Based upon the characteristic peak obtained (2θ value taken from the graph Fig.1), the particle size of the crystal was calculated using the formula,

$$D = 0.9\lambda / \beta \cos\theta$$

Where, D represents the average size of the particle, λ corresponds to wave length of copper Kα line (1.5406 A˚), β represents full width at half maximum of peak, θ corresponds to diffraction angle .The size of the particle (D) was calculated as 5.3 nm.

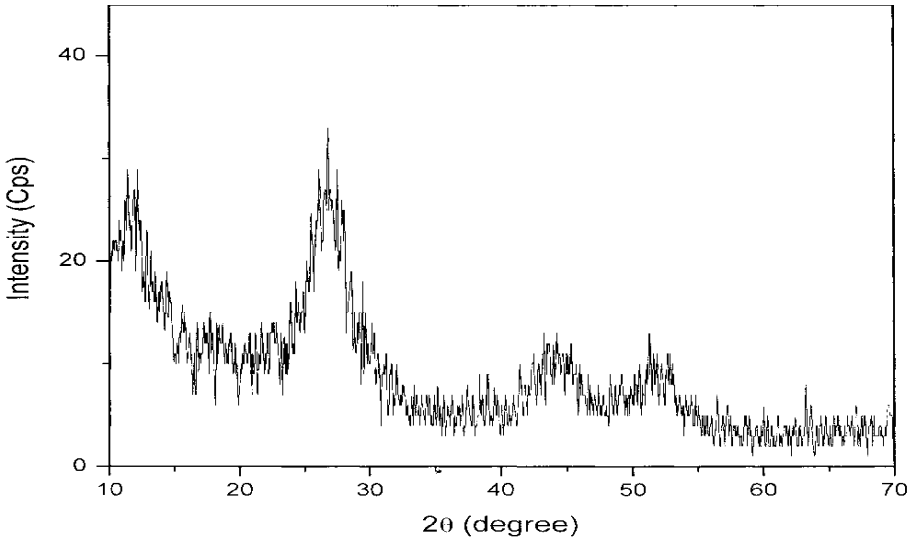

Fig. 1. X-RD pattern of silver nanoparticles.

3.2 Scanning Electron Microscopy (SEM) Analysis

The SEM image represents the formation of silver nanoparticles was depicted in Fig. 2. Also the grains have aggregated to form nanoclusters.

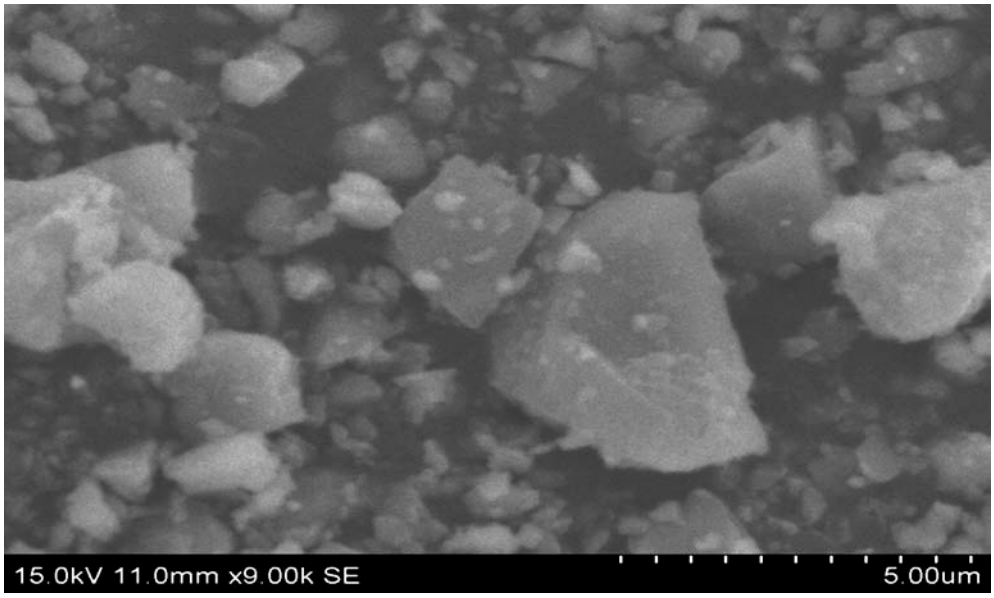

Fig. 2. SEM image of silver nanoparticles.

3.3 Combined Effect

3.3.1 Comparative analysis of antimicrobial effects among nanoparticles, antibiotics and nanoparticles impregnated with antibiotics

The comparative analysis was focused on the sensitivity of the microorganisms towards silver nanoparticles, antibiotics and their combined effects with reference to the control. A minimum of 2 to 4 mm increase in the diameter of zone of inhibition was observed when nanoparticle and the antibiotics are give together. Table 1 shows that the antimicrobial potency of nanoparticle and the four broad spectrum antibiotics against four major pathogens.

B. subtilis offered the maximum zone of inhibition, almost 24-28 mm diameter for all the four antibiotics (Fig.3). *B. cereus* posed very high inhibitory effect in which the zones overlapped against amoxicillin (even for 1µg final concentration, inferring antibacterial activity beyond maximum) and the rest showed a characteristic antimicrobial activity (36-40 mm).This combined effect was depicted in (Fig. 4). *K.pneumoniea* offered a typical (combined) zone of inhibition of 24-27 mm diameter (Fig.5). With respect to *V.cholerae,* amoxicillin and erythromycin offered no antibacterial property, only the silver nanoparticles pose a selective antibacterial effect. But, chloremphenicol and rifamycin showed a unique zone of inhibition around 29 mm and 20 mm respectively (Fig.6). The zone of inhibition values are tabulated (Table.1). Similar type of work was previously established for nanoparticles combined with flucanazole (antibiotic) against fungal species [12].The result obtained with the use of silver nanoparticles was found to be comparable with previous work established.

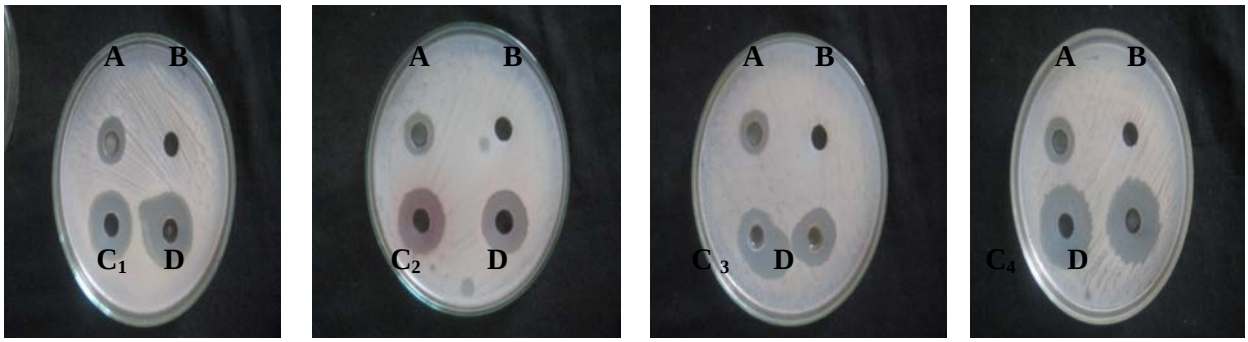

Fig. 3. Zone of inhibition against Bacillus subtillis.

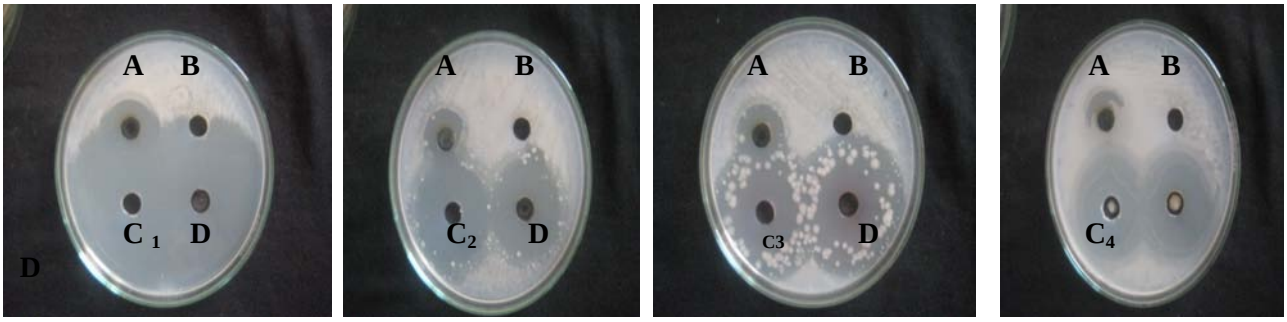

Fig. 4. Zone of inhibition against Bacillus cereus.

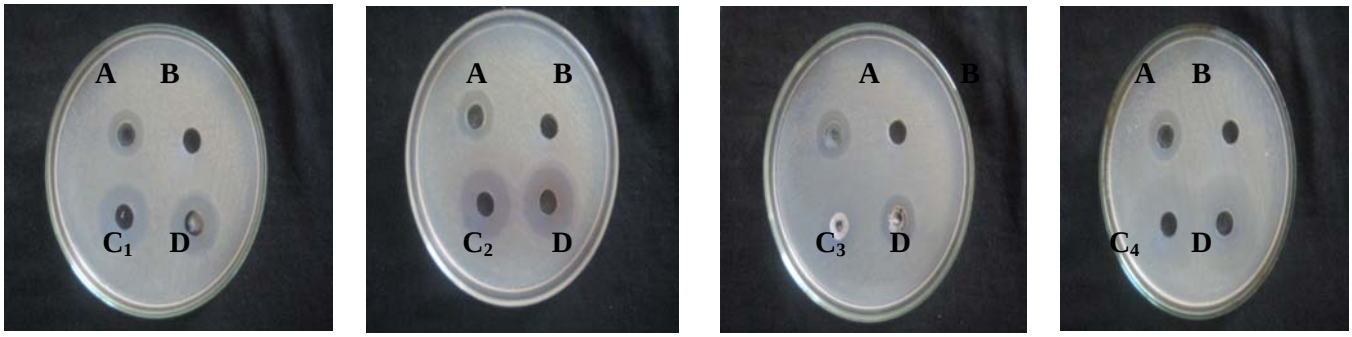

Fig. 5. Zone of inhibition against Klebsiella pneumoniae.

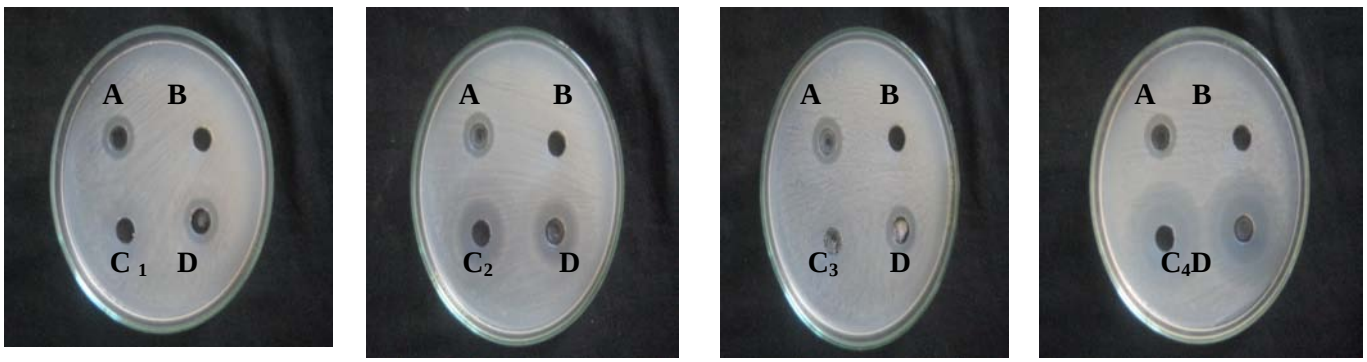

Fig. 6. Zone of inhibition against Vibrio cholerae.
A - Nanoparticles ; B - Controls ; C_1 - Amoxicillin ; C_2- Chloremphenicol; C_3- Erythromycin ; C_4 – Rifamycin ; D - Nanoparticles + Antibiotics

Table 1. Zone of inhibition produced by AgNps, antibiotics and AgNps with antibiotics.

Organism used	Antibiotics used	Control (mm)	Ag Nps (mm)	Antibiotic (mm)	AgNps + Antibiotic (±1mm)
Bacillus subtilis	Amoxicillin	-	15	22	24
	Chloramphenicol	-	16	27	28
	Erythromycin	-	14	17	18
	Rifamycin	-	14	22	23
Bacillus cereus	Amoxicillin	-	*	*	*
	Chloramphenicol	-	20	36	40
	Erythromycin	-	19	35	36
	Rifamycin	-	20	40	43
Vibrio cholerae	Amoxicillin	-	14	-	14
	Chloramphenicol	-	13	27	29
	Erythromycin	-	15	-	15
	Rifamycin	-	14	20	20
Klebsiella pneumoniae	Amoxicillin	-	15	20	18
	Chloramphenicol	-	17	25	27
	Erythromycin	-	16	12	16
	Rifamycin	-	16	23	24

3.4 Detection of separated compounds of antibiotics by TLC

The chromatogram was developed in methanol/chloroform (3:7 v/v) solvent system and the spots were identified by subjecting the chromatogram to UV light (Fig. 7) or iodine vapours (Fig. 8). Three characteristic bands inferring three significant derivatives of rifamycin were separated by TLC. The R_f values were calculated and the compounds were identified by comparing with reference standard [13].

$$R_f \ value = \frac{Distance \ \ moved \ \ by \ \ the \ \ solute}{Distance \ \ moved \ \ by \ \ the \ \ solvent}$$

$$R_{f_1} = 1.7/3.7 = 0.459 \ , \ R_{f_2} = 2.8/3.7 = 0.756 \ , \ R_{f3} = 3.2/3.7 = 0.864$$

The first derivative was found to be 3-aminorifamycin S, second derivative was found to be 3-ethylamino rifamycin S (R'-octyl group) and the third derivative was found to be 3-aminorifamycin (R-Phenethyl, R'-Allyl)

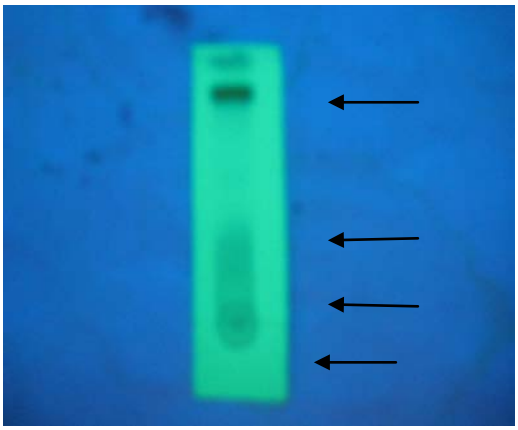

Fig. 7. Detection by U-V light.

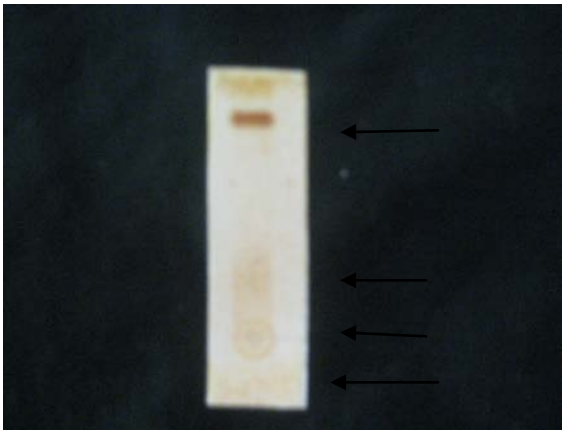

Fig. 8. Detection through iodine vapours.

3.5 Bioautography analysis of antibiotics and antibiotics with nanoparticles

A characteristic zone of inhibition (from the separation point on the thin layer to the agar on the petridish) diffused radially was observed. Rf value nearer to 0.859 indicates the antibacterial efficiency inferred by 3-amino rifamycin (R-phenethyl, R'-allyl). However, in combination with nanoparticle, the zone of inhibition was shared between nanoparticle and rifamycin at two different points. Zone at the point of application corresponds to the antibacterial property inferred by silver nanoparticles and zone at the separation point infers inhibitory effects of rifamycin derivatives.

In Fig.9 and Fig.10, rifamycin (derivative) showed a single broader characteristic zone (in red colour) against *B. cereus* and *B. subtillis* respectively. But, in Fig.9.1 and Fig.10.1, two characteriestic zones were observed, a black zone representing the nanoparticle, and a red zone representing rifamycin confers a little diminished inhibitory effect. A significant observation of this technique based analysis was perfectly on the zone produced at reference points. The zone will radially outwards from thin layer to the petriplate in circular motion, thereby inferring the antibacterial efficiency of the particles [14, 15]. Higher the diameter of the zone, higher the antimicrobial activity.

Similarly, in Fig. 11 and Fig. 12, against *K. pneumoniea* and *V. cholerae*, a single broader spherical shaped zone (in red colour) representing rifamycin was observed. Two zones (black zone for nanoparticle and red zone for rifamycin) at two different points posing equivalent inhibitory effect was observed in Fig. 11.1 and Fig. 12.1, respectively.

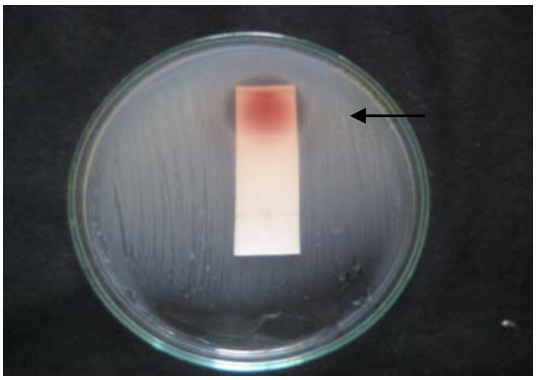

Fig. 9. Seperated rifamycin diffused over Bacillus cereus (red zone).

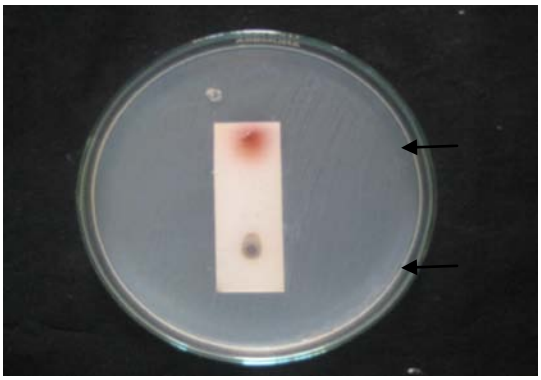

Fig. 9.1. Unseperated Ag Nps diffused over Bacillus cereus (black zone) Seperated rifamycin diffused over Bacillus cereus (red zone).

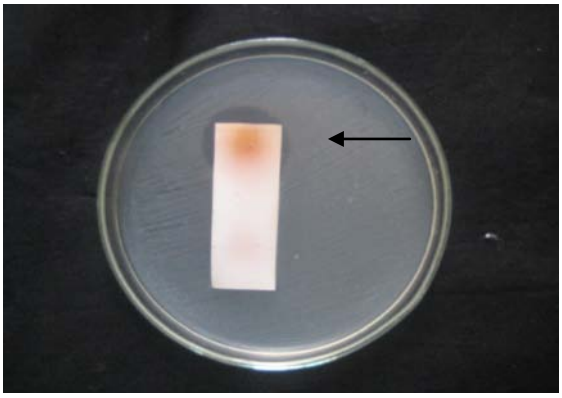

Fig.10. Seperated rifamycin diffused ov Bacillu subtilis (red zone)

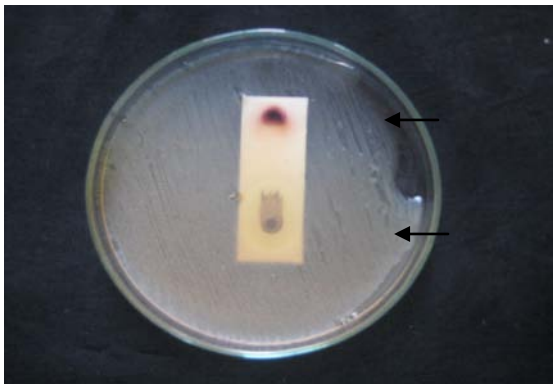

Fig.10.1. Unseperated Ag Nps diffused over Bacillus subtilis (black zone) Seperated rifamycin diffused over Bacillus subtilis (red zone).

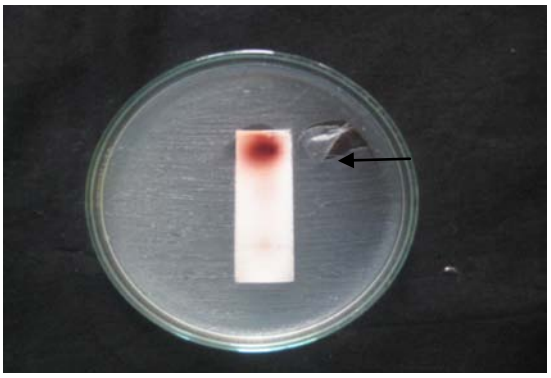

Fig.11. Seperated rifamycin diffused over Klebsiella pneumoniae (red zone).

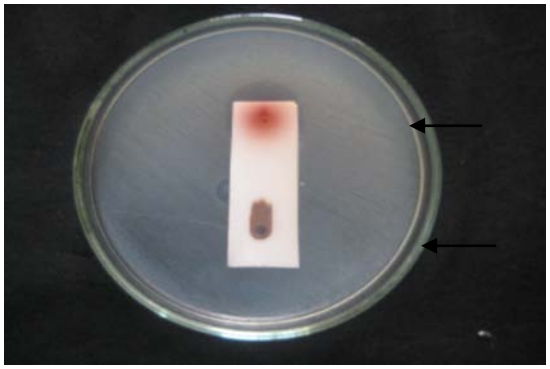

Fig.11.1 Unseperated Ag Nps diffused over Klebsiella pneumoniea (black zone) Seperated rifamycin diffused over Klebsiella pneumoniae (red zone).

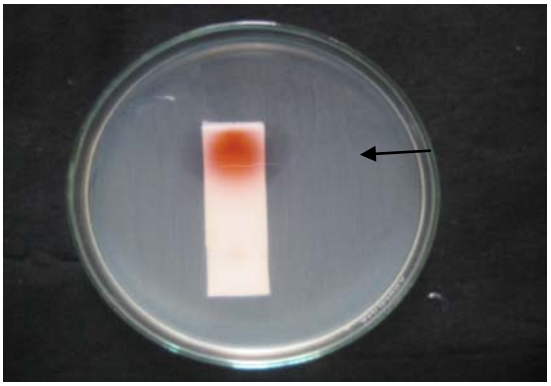

Fig.12. Seperated rifamycin diffused over Vibrio cholerae (red zone).

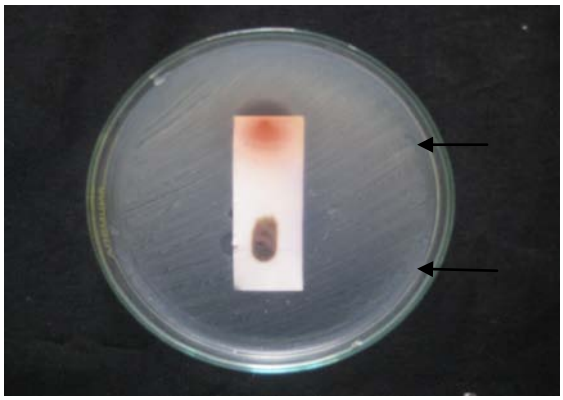

Fig.12.1. Unseperated Ag Nps diffused over Vibrio cholerae (red zone) Seperated rifamycin diffused over Vibrio cholerae (red zone).

4. Conclusions

From the above discussion, it was concluded that silver nanoparticles impregnated with antibiotics exhibit profoundly stipulated inhibitory effects (antimicrobial activity) when given together. This enhancement in the combined effect were preferably due to the difference in the mechanism of inhibition followed by nanoparticles and antibiotics. Though, the antibiotics (amoxicillin and erythromycin) failed to pose inhibitory effect over *V.cholerae*, the silver

nanoparticles pose specific antimicrobial activity (14-15 mm). Hence, nanoparticles can be used as a potential antimicrobial agent equivalent to the antibiotics against microbial infections. Based upon the TLC results, it was confirmed that 3-aminorifamycin (R-Phenethyl, R'-Allyl), a derivative of rifamycin, was responsible for antimicrobial activity of the antibiotic used. These results were confirmed by agar over layer method. Bioautography, a novel approach (differing from well diffusion method) was tried to determine the antimicrobial activity through TLC separation of silver nanoparticles and antibiotics. These results inferred the zone of inhibition of antibiotic produced at the separation point (red zone) and unseperated silver nanoparticles at the loading point (black zone). As being a metal nanoparticle, silver could not be separated over silica coated TLC plates.

References

[1] J. S. Kim, E. Kuk, K. N. Yu, J. H. Kim, et al., Nanomedicine: NBM **3**, 95 (2007).
[2] R. M. Slawson, M. I. Van Dyke, H. Lee, J. T. Trevors, Plasmid, **27**, 72 (1992).
[3] G. J. Zhao, S. E. Stevens, Biometals, **11**, 27 (1998).
[4] J. A. Spadaro, T. J. Berger, S. D. Barranco, et al., Microb. Agents Chemother. **6,** 637 (1974).
[5] C. N. Lok, C. M. Ho, R. Chen, et al., J. Biol. Inorg. Chem. **12 ,** 527 (2007).
[6] S. Silver, L. T. Phung, G. Silver, J. Ind. Microbiol. Biotechnol. **33**, 627 (2007).
[7] M. Rai, A. Yadav, A. Gade, Biotechnol. Adv. **27,** 76 (2006).
[8] V. Thomas, M. M. Yallapu, B. Sreedhar, et al., J. Colloid Interface Sci. **315**, 389 (2007).
[9] S. S. K. Kamal**,** P. K. Sahoo, J. Vimala, et al., Acta. Chim. Slov, **57,** 808 (2010).
[10] S. A. Nabi, M. A. Khan, S. N. Khowaja, et al., Acta. Chromatogr. **16**, 164 (2006).
[11] B. Pandey, P. Ghimire, V. P. Agrawal, Proceedings from the Great Himalayas: Climate, Health, Ecology, Management and Conservation, Kathmandu, January 12-15, (2004).
[12] M. Gajbhiye, J. Kesharwani, A. Ingle, et al., Nanomedicine: NBM **5**, 382, (2009).
[13] United States Patent, Bruzzese et al., 4, 372, 961 Feb 8, 1983.
[14] L. Rohalison, M. Hamburger, K. Hosttetman, et al., Phytochem. Anal. **2**, 199 (1991).
[15] O. B. Shittu, F. V. Alofe, G. O. Onawunmi, et al., Afr. J. Biomed. Res. **9** ,57 (2006).