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Antibiotics-Silver Nanoparticles (AgNPs) Blend – A Novel Route to Overcome Drug Resistance

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Currently, antimicrobial resistance is becoming one of the major threats affecting the socio-economic status of one's country. The evolution of such drug-resistant bacterial species stems from multitude of factors and the push for combating life threatening diseases by way of novel antimicrobials with biomedical significance would be the major breakthrough in materials research. Nanobiotechnological formulation has developed different methods for synthesizing various metal nanoparticles of biomedical significance and ecological considerations. In this study, silver nanoparticles were synthesized extracellularly using *Bacillus subtilis* A1 and exposed to bacterial strains resistant to antibiotics such as Amoxicillin, Tetracycline and Vancomycin. The toxic nature of silver nanoparticles inhibited bacterial growth in a dose dependent manner (10μg, 15μg and 20μg/mL). AgNPs at concentration 5 μg/mL when combined with standard antibiotics (MIC dose) enhanced the susceptibility pattern as observed from the zone of inhibition toward drug resistant *Staphylococcus aureus and Proteus mirabilis*. To understand the principle, Amoxicillin was used along with AgNPs to extend its biological half life. The spectral analysis of amoxicillin-AgNPs blend revealed the possible interaction accounting for enhanced biocidal activity. This combinatorial antimicrobial efficacy induced by AgNPs in Amoxicillin-AgNPs blend might help formulate biomaterials possessing broad spectrum pharmacognostics to overcome drug resistance.

Key words: Silver nanoparticles, Drug resistance, Amoxicillin, Vancomycin, Tetracycline.

Antibiotic resistance or drug resistance is the biggest challenge faced by pharmaceutical and biomedical sectors at global level. There are several mechanisms accounting for multidrug resistance *viz*. inactivation of drug, alteration of target, alternate metabolic pathway and efflux of drug[1]. The emergence and re-emergence of such multidrug resistant strains has made the so-called *elixir* (drug) ineffective posing a major threat at global level with significant increase in mortality, morbidity and cost of treatment[2]. The irrational use of antibiotics not only influences healthcare industry but also the environment, since their exposure through human and veterinary wastes. The persistence of resistance in the environment is promoted by horizontal gene transfer where the resistant genes get distributed to other species of susceptible bacteria[3]. Therefore, development, modification or hunt for novel antimicrobial compounds having biocidal potential against multidrug resistant strains[4] is a priority area of research. The use of silver in medical applications dates back to 1700 where silver nitrate was used to treat venereal diseases, burns, ophthalmia neonatorum[5] etc despite its electrochemical applications. As prevalence of several antibiotic resistant pathogenic bacteria are on the rise, silver nanoparticles are the new hope to control them. This study elucidates the bactericidal potential of silver nanoparticles (AgNPs) and antibiotic-AgNPs blend against MDR bacteria in overcoming drug resistance.

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MATERIALS AND METHODS

Silver nitrate (99.9% pure, AR grade) was procured from HiMedia, India. The standard antibiotic discs such as Amoxicillin, Vancomycin and Tetracycline were supplied by HiMedia India. The bacterial pathogens used in the experiment: *Staphylococcus aureus*, *Enterococcus faecalis*, *Klebsiella pneumoniae* and *Proteus mirabilis* and their respective resistant strains were obtained from Sharp Clinical Laboratory, Chennai and were maintained in NA slants at 4ºC.

Synthesis and Characterization of silver nanoparticles (AgNPs)

Silver nanoparticles were synthesized extracellularly using the bacterium, *Bacillus subtilis* A1 exposed to 1mM $AgNO_3$. Characterization studies on silver nanoparticles were performed to determine the size, shape and nature of nanoparticles as reported in our previous study[6].

Antibacterial Efficacy - Qualitative Analysis (Disc diffusion method)

The efficacy of the synthesized bio-nanoparticles (AgNPs) was determined by performing Antimicrobial Susceptibility test using Kirby Bauer's method[7]. Three hours broth culture of the test organisms was uniformly spread over MHA plate using a sterile swab (Hi Media, India) after adjusting the inoculum size to ~ 0.5 McFarland standard. Two sets of Antibiotics *viz.* Amoxicillin trihydrate (AMX, 10 mcg), Vancomycin (VA, 30 mcg) and Tetracycline (TE, 30 mcg) were placed over the lawn cultured MHA plate of which one set constituted antibiotic control. Antibiotic-AgNPs blend was prepared by diluting 5 mcg of AgNPs in 1 ml of sterile saline and adsorbed on to antibiotic discs using sterile micropipette and dried, the final concentration worked out to 15mcg (AMX-AgNPs), 35mcg (VA-AgNPs) and 35mcg (TE-AgNPs). Control was run using saline and aqueous silver nitrate. The plates were incubated at 37°C for 24 hours and the diameter of zone of inhibition was measured.

Preparation of Amoxicillin-AgNPs blend

Amoxicillin, a broad spectrum antibiotic was preferred due to its short biological half-life. In order to enhance the efficacy AgNPs was conjugated with antibiotic as described[8] and subjected to spectral analysis.

RESULTS AND DISCUSSION

Synthesis and Characterization

The typical Plasmon resonance of silver visualized from the presentation color to brown in 72h upon exposure confirmed the formation of silver nanoparticles with maximum absorbance in the range 420 – 450nm. The XRD pattern revealed zero-valent face centric cubic symmetry and crystalline nature of the nanoparticles formed with spherical in shape and average mean size of 30 – 60nm as evidenced from FESEM. The EDX spectrum showed strong signals from Ag, C, O and N[6].

Antibacterial Efficacy - Qualitative Analysis (Disc diffusion method)

The antibacterial activity of the synthesized silver nanoparticles was tested toward *S. aureus*, *Enterococcus faecalis*, *Klebsiella*

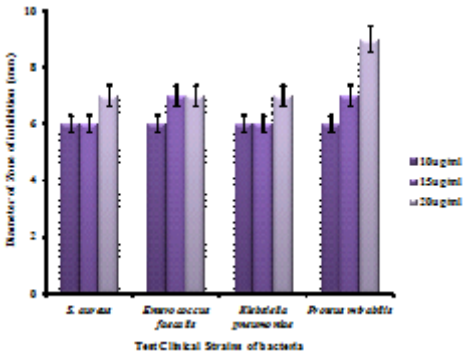

Fig 1. Antimicrobial Efficacy of silver nanoparticles (AgNPs) against pathogens. The data are presented as mean ± standard deviation (n=3)

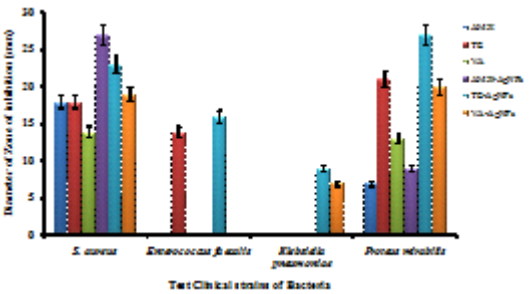

Fig 2. Comparative and synergistic analysis of antibiotics and Antibiotics-AgNPs blend against resistant strains. The data are presented as mean ± standard deviation (n=3)

pneumoniae and *Proteus mirabilis* by Disc diffusion method. The AgNPs at three different concentrations ie., 10 µg/ml, 15 µg/ml and 20 µg/ml showed significant inhibition zone when challenged against all the gram positive (*S. aureus* and *Enterococcus faecalis*) and gram negative pathogens (*Klebsiella pneumoniae* and *Proteus mirabilis*) whereas no inhibition zone was observed around the cell free extract as in Fig. 1. This clearly demonstrates that the antibacterial activity came from the silver nanoparticles producing an average zone size ranging 6 – 9mm.

Antibiotic-AgNPs blend vs antibiotics

In the second line of susceptibility testing, the result on combinatorial antibacterial activity of AMX-AgNPs, TE-AgNPs and VA-AgNPs blend toward the resistant strains: *S. aureus, Enterococcus faecalis, Klebsiella pneumoniae* and *Proteus mirabilis* showed significant increase in inhibition zone than the antibiotic control. Resistant strain of *S. aureus* showed maximum susceptibility to Antibiotics-AgNPs blend at all concentrations with a difference of 9mm, 7mm and 5mm from that of antibiotic control. Similarly *Proteus mirabilis* showed a significant difference in 2mm, 6mm and 7mm compared to antibiotic control. However, *Enterococcus faecalis* and *Klebsiella pneumoniae* that doesn't respond to the antibiotics were inhibited by antibiotic-AgNPs blend proving the additive effect induced by AgNPs as observed from Fig. 2.

Antibacterial Efficacy and mechanism

The antimicrobial activity was performed using three antibiotics namely moderate spectrum β-lactam amoxicillin, glycopeptide vancomycin and broad spectrum polyketide tetracycline possessing specific mechanism of action. The antibiotic-AgNPs blend (1:1) with a final concentration of 15mcg, 35mcg and 35mcg showed a significant growth inhibition. Notable synergistic efficacy was observed between AMX-AgNPs, TE-AgNPs and VA-AgNPs against gram positive (*S. aureus*) supported by 1.5 x, 1.3 x and 1.4 x increase in zone diameter compared to antibiotic control. Similarly, *P. mirabilis* showed a maximum of 1.6 x fold increase in zone diameter to VA-AgNPs blend compared to 1.3 x fold increase observed among other combinations and antibiotic control. Similar study[9] on the synergistic effect between AgNPs and chloramphenicol / vancomycin against *P. aeruginosa* showed a 4.9 x, 4.2 x increase in diameter and 11.8 fold increase in case of streptomycin-AgNPs. The development of manifold increase in the zone size might probably be due to the involvement of varied phytochemical constituents of therapeutic importance present in addition to the reduced silver ions. Lara *et al.* 2010 [10] reported that commercial silver nanoparticles could bring biocidal action against multidrug resistant pathogens such as *P. aeruginosa, E. coli* and *Streptococcus pyogenes*. It is noteworthy that the size and shape of the nanoparticles had accounted for biocidal action[11]. Absence of such an effect against all the test organisms relies probably on another mechanism. Spectral evidence showed complete encapsulation of the drug (AMX) by AgNPs as shown in Fig. 3 and XRD analysis with reduced peak intensity of final formulation indicated the reduction in crystallinity of drug entrapped by AgNPs (data not shown) accounting for biological activity[12].

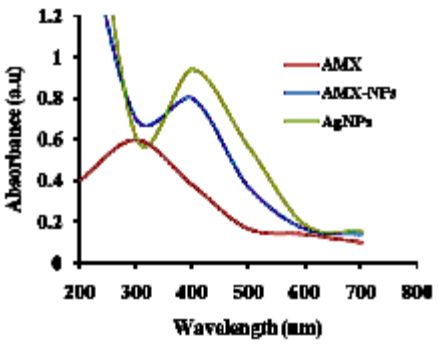

Fig. 3. Spectral analysis of Antibiotic (Amoxicillin) and Antibiotic-AgNPs blend

The presence of –S– group in amoxicillin may also be the limiting factor for interaction of AgNPs in blend formulation. As a result, these tiny non-specific NPs in the blend gain easy access into the resistant bacterium followed by the release of drug which then exhibits its mechanism of action paralyzing the cell[13] and it may be anticipated that the same trend follows for other antibiotics in blend formation demanding future research studies. Moreover, the electrostatic interaction of silver ions toward bacterial cell[14], membrane damage[15] with respect to the broad target range determines bactericidal potential. Hence, it can be assumed that silver nanoparticles are broad spectrum agents

whose performance is not obstructed by antibiotic resistant mechanisms[16]. This formulation would serve as a lead for unresolved mysteries of drug resistance in lieu of increased dose of antimicrobials and the fate of AgNPs in bioprocess in future study.

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