Effect of colloidal silver against Ciprofloxacin persisters of *Pseudomonas aeruginosa* PAO1

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Abstract

Pseudomonas aeruginosa causes chronic multidrug-resistant infections, often linked to antibiotic-tolerant persister cells in biofilms. The treatment of such infections poses chemotherapeutic limitations. This study evaluates the efficacy of colloidal silver (SilverSol® technology) against Ciprofloxacin-tolerant persisters of P. aeruginosa PAO1 in planktonic and biofilm populations. Dose-kill assays showed persister formation at 20×MIC of ciprofloxacin in planktonic cells and 50×MIC in biofilms. Colloidal silver exhibited a MIC of 3ppm for planktonic cultures and 4 ppm for biofilm inhibition.

Complete eradication of Ciprofloxacin persisters occurred using colloidal silver at 5×MIC in planktonic cells and 3×MIC in biofilms within 1 hour. Total biofilm eradication was possible at 4 ppm using TTC viability assays. Checkerboard assay revealed an additive effect of ciprofloxacin and colloidal silver (FIC index: 0.65). FESEM imaging and Allison-Sutherland staining confirmed the absence of extracellular matrix, demonstrating biofilm inhibition by the colloidal silver. Hence, these findings highlight colloidal silver as a promising therapeutic strategy against persistent infections caused by ESKAPE pathogens such as P. aeruginosa PAO1.

Keywords: *P. aeruginosa* PAO1, Colloidal silver, ESKAPE pathogen, Persister cells.

Introduction

Antibiotic resistance is a growing global health crisis, often referred to as a silent pandemic[26]. *Pseudomonas aeruginosa* is a major pathogen that enhances the burden of antimicrobial resistance and poses a serious health threat. *P. aeruginosa* is one of the ESKAPE pathogens (*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa* and *Enterobacter* spp.) which is associated with severe primary, secondary and nosocomial infections[15]. These infections are often recurrent and multidrug resistant[7,38]. The virulence of *P. aeruginosa* is due to the formation of biofilms and production of siderophores and extracellular enzymes like elastases and proteases. It also produces exotoxins, hemolysins, pyocyanins and type III secretion system effectors[7,36-37]. These factors, in addition to drug resistance, add to the severity of *P. aeruginosa* infections. Under antibiotic stress, *P. aeruginosa* forms a subpopulation of tolerant cells known as persisters[19]. These are phenotypic variants that survive antibiotic exposure without the generation of genetic resistance[23,25,28]. The prevalence of persisters is significantly higher in biofilms compared to planktonic populations[27,34]. Exopolysaccharides present in biofilms restrict antibiotic penetration, contributing to persistent infections[10,13]. Ciprofloxacin, a widely used fluoroquinolone, is the drug of choice for *P. aeruginosa* infections due to its biofilm permeability[30,31]. However, emerging reports indicate increasing ciprofloxacin tolerance in *P. aeruginosa*[11,45]. Persister-mediated drug tolerance leads to recurrent infections, necessitating novel therapeutic strategies[27,34,50] .The known methods for eliminating persisters include electrochemical scaffold potentiation of antibiotics with sugars[2,6,52], use of organic acids[47], metal nanoparticles[16, 35] and combination drug therapy[22,51].

Metal nanoparticles have been proven to be efficient against ESKAPE pathogens[35] with silver nanoparticles being particularly effective against *P. aeruginosa*[14,20,29]. Colloidal silver has been reported to be effective as an antibacterial agent[4,24,49]. Colloidal nanosilver refers to a suspension of engineered silver nanoparticles in a liquid medium, typically water. These nanoparticles range from 1 to 100nm in size. Colloidal nanosilver particles exhibit unique physical, chemical and optical properties that differ from bulk silver. Due to their small size and high surface-area-to-volume ratio, colloidal nanosilver particles release silver ions (Ag^+) more efficiently, enhancing their antibacterial and antifungal properties[18].

The enhanced stability and controlled ion release of colloidal nanosilver make it a preferred choice over traditional colloidal silver in various applications. Colloidal silver comprises of silver in two distinct forms, metallic silver particles and silver ions. The total amount of silver that is reported as the silver concentration (in parts per million) is a total of the silver contained in the particles and the silver contained as ions. Colloidal silver particles get attracted to the surrounding water molecules present in biomolecules. This makes it more stable and bioavailable than other forms of silver. Ionic silver can accept only one electron and carries a positive electrostatic charge, making it more reactive but less stable than colloidal silver particles[44].

SilverSol® is produced from pure silver (Ag 99.99%) by electrolysis with a particle size of 5-50nm as compared to common colloidal silver which is 8nm-10micron. SilverSol® is manufactured using a patented technology that uniquely harnesses antimicrobial activity of silver. SilverSol® exhibits properties such as efficacy at extremely low concentrations, prolonged stability and absence of

adverse side effects[44]. Previous studies showed colloidal silver produced with SilverSol® to be effective against ampicillin persisters of *E. coli*[9].

P. aeruginosa PAO1 is a virulent strain with an ability to form biofilms, produce pyocyanin and form persisters to ciprofloxacin. This study investigated the potential of colloidal silver as an antimicrobial agent that could contribute to alternative strategies for addressing antibiotic tolerance and persistent *P. aeruginosa* infections. The study determined the efficacy of colloidal silver against ciprofloxacin-induced persister cells of *P. aeruginosa* PAO1, both in planktonic form (log phase) and biofilm populations.

Material and Methods

Bacterial strains and growth medium: *Pseudomonas aeruginosa* PAO1 MTCC 741 was obtained from MTCC, Chandigarh, India. Luria Bertani (LB) broth and Luria Bertani (LB) agar were used as growth media. Log phase culture was prepared by inoculating 500µl of a saline suspension (OD_{530} - 0.4) of overnight grown culture of *P. aeruginosa* PAO1 MTCC 741 into 100ml of sterile LB broth and incubating for 3.5 hours at 37°C at 220 rpm. Log phase was achieved at OD_{530} - 0.5 as determined from growth curve experiments.

Antibiotics and Colloidal silver solution: Ciprofloxacin hydrochloride monohydrate was obtained from HiMedia laboratory, India. Stock solutions of ciprofloxacin (10mg/ml) were prepared in sterile distilled water and stored at -20°C. The stock solution was freshly diluted before each experiment with sterile distilled water or sterile LB broth. Colloidal silver solution was sourced from Viridis Biopharma Pvt. Ltd, Mumbai. It was diluted in sterile distilled water or sterile LB broth.

Minimum Inhibitory Concentration (MIC) of Ciprofloxacin: MIC of Ciprofloxacin for *Pseudomonas aeruginosa* PAO1 was determined by the broth dilution method according to Clinical and Laboratory Standards Institute (CLSI) Standards[12]. The concentration range used was 1-10µg/ml of ciprofloxacin. Presence of growth was measured as optical density at 530nm after 24 hours.

***Pseudomonas aeruginosa* PAO1 persisters to Ciprofloxacin:** The presence of *Pseudomonas aeruginosa* PAO1 persisters to ciprofloxacin was determined by dose dependent and time dependent kill curve.

Dose dependent kill curve for *Pseudomonas aeruginosa* PAO1: To determine the antibiotic concentrations that result in the survival of only drug-tolerant persister cells, killing curves were performed. Dose-dependent study was done using different concentrations (10×, 20×, 40× MIC) of ciprofloxacin against *Pseudomonas aeruginosa* PAO1. Respective antibiotic concentrations were added to 10ml of log phase culture and further incubated at 37°C at 220rpm for one hour. The medium was separated by centrifugation at 8000 rpm for 10 minutes and the cell pellets were washed thrice with sterile saline and then suspended in 10ml sterile saline. Cell counts were determined in triplicate by drop plate technique on LB agar plates using 10µl of the dilutions[32,34].

Time dependent kill curve for *Pseudomonas aeruginosa* PAO1: The antibiotic concentration determined by the dose dependent kill curve was added to 10ml of log phase culture and incubated at 37°C at 220 rpm for time intervals of 1, 2 and 3 hours. After the designated incubation time, samples were centrifuged at 8000rpm for 10 minutes. The cell pellets were washed thrice with sterile saline and then suspended in 10ml sterile saline. Cell counts were determined in triplicate by drop plate technique on LB agar plates using 10µl of the dilutions to determine the number of surviving persister cells.

Persister cells from biofilm: Biofilms were formed on coverslips by immersing them in 24 hour old LB broth culture of *P. aeruginosa* PAO1 diluted 1:100 with fresh LB and incubating at 37˚C for 24 hours under static conditions. After biofilm formation, the coverslips were rinsed twice with sterile saline to remove loosely attached/ non-adhering cells. The coverslips were then transferred to fresh tubes with 10ml of sterile saline and vortexed thoroughly at high speed to completely disrupt the biofilms and release the bound sessile cells. The sessile cells thus recovered, were centrifuged and resuspended in 10ml of LB broth. The sessile cell suspensions were then used for obtaining persister cells.

Dose dependent kill curve for persisters in sessile population: To obtain persister cells, the sessile cells suspensions as obtained above in 10 ml LB broth were treated with 10×, 20×, 40×, 50×MIC of ciprofloxacin and incubated at 37°C, 220 rpm for one hour. The culture suspensions were centrifuged at 8000 rpm for 10 minutes and rinsed with saline twice to remove traces of antibiotic. Cell pellets were resuspended in saline and enumerated by drop plate technique on LB agar.

Time dependent kill curve for persisters in sessile population: Persister cells in the sessile population were observed at 150µg/ml (50× MIC) of ciprofloxacin. Hence, for time dependent kill curve experiment, sessile cells in 10 ml of LB broth were treated with 150µg/ml of ciprofloxacin and incubated at 37°C at 220 rpm for time intervals of 1, 2 and 3 hours and processed as mentioned above. The cell pellets thus obtained were resuspended in saline and enumerated by drop plate technique on LB agar plates.

MIC of colloidal silver for planktonic and sessile population: MIC of colloidal silver for the planktonic and sessile populations of *P. aeruginosa* PAO1 was determined using the broth dilution method according to CLSI standards[12]. Colloidal silver was used at a concentration

range of 1-10ppm. Growth was measured as optical density at 530nm after 24 hours.

Effect of colloidal silver on the formation of *P. aeruginosa* PAO1 persister cells to Ciprofloxacin: Colloidal silver at MIC concentration of 3ppm was added to the log phase culture in three ways:

i) Addition of colloidal silver at log phase followed by addition of 60µg/ml ciprofloxacin after 1 hour incubation
ii) 60µg/ml of ciprofloxacin was added to culture at log phase followed by addition of colloidal silver after 1 hour incubation
iii) Colloidal silver and 60µg/ml of ciprofloxacin were added simultaneously at log phase. Persister population was determined after each treatment.

Synergistic action of Ciprofloxacin and colloidal silver against persister cells of *P. aeruginosa* PAO1: Synergistic action was determined using the checkerboard assay in microtitre plates[5]. Persister cells to ciprofloxacin were obtained as per the earlier mentioned protocol and used as inoculum for the checkerboard assay. Stock solutions of both ciprofloxacin and colloidal silver were prepared in LB medium. The reaction volume in each well in the microtiter plate was set as 150µl of ciprofloxacin at the specified concentration in LB medium + 150µl of colloidal silver at the specified concentration in LB medium and 20µl of persister cell suspension ($3x10^5$ cfu/ml). The concentrations of ciprofloxacin used were 0 - 3µg/ml while that of colloidal silver were 0 - 5ppm.

After incubation at 37°C for 24 hours, the growth was determined by measuring optical density at 530nm and further confirmed by addition of 10µl of triphenyl tetrazolium chloride (TTC) solution (1%w/v) to each well. Conversion of colourless TTC solution to red formazan indicated growth. The formazan intensity was measured at 490nm. According to the CLSI guidelines, synergy between drugs is determined by calculating the fractional inhibitory concentration (ΣFIC) index. The FIC index is calculated as the sum of the FIC values for each tested drug where the FIC for each drug is obtained by dividing its inhibitory concentration in combination by its MIC when used alone. In the given study, ΣFIC = FIC Ciprofloxacin + FIC colloidal silver combination is considered synergistic when the ΣFIC is = 0.5; additive, when the ΣFIC is >0.5 to < 2 and is antagonistic when the ΣFIC is = 2.

Effect of colloidal silver on biofilm formation by *P. aeruginosa* PAO1: 150µl of 24 hour old *P. aeruginosa* PAO1 broth culture adjusted to 0.5 OD_{530} was added to 150µl of LB broth containing different concentrations of colloidal silver (1-10ppm) in a 96 well microtiter plate to achieve a density of 10^5cfu/ml. Plates were incubated at 37˚C for 24 hours after which the culture was removed and the wells were washed twice with saline. Crystal violet assay was performed to estimate the amount of biofilm formed in comparison with the untreated control. Crystal violet (0.1% w/v) was added to the air-dried wells and incubated for 15 minutes at room temperature. The wells were then rinsed with distilled water gently and allowed to dry completely. Dye bound to the biofilm was solubilized in 70% ethanol and absorbance was measured at 600nm using microplate reader (Varioskan™ LUX multimode microplate reader, Thermofisher). Percent biofilm inhibition was determined using the formula:

$$\text{Percentage inhibition} = \frac{A_{600nm}\text{ without colloidal silver} - A_{600nm}\text{ with colloidal silver}}{A_{600nm}\text{ without colloidal silver}} \times 100$$

Effect of colloidal silver on the viability of sessile population of *P. aeruginosa* PAO1: *P. aeruginosa* PAO1 was inoculated in LB broth containing colloidal silver in microtiter plates as above. After incubation at 37˚C for 24 hours, the planktonic cells were removed and plates were rinsed twice with saline. The plates were dried for 15 minutes. To determine the inhibitory action of colloidal silver, 50µl of 1.2%w/v TTC dye solution (Triphenyl tetrazolium chloride) was added to wells and incubated in the dark for 4 hours at 37˚C. The intensity of formazan was quantified by measuring absorbance at 490 nm. Percent viability was determined using the formula:

$$\text{Percentage viability} = \frac{A_{490nm}\text{ with colloidal silver}}{A_{490nm}\text{ without colloidal silver}} \times 100$$

Microscopic analysis of silver treated biofilms of *P. aeruginosa* PAO1

Allison Sutherland staining: Biofilms were allowed to form on sterile glass coverslips (18mm x 18mm) in LB broth containing 4ppm of colloidal silver (concentration inhibiting biofilm formation) along with untreated control at 37˚C for 24 hours. The biofilms formed were stained by the modified Allison and Sutherland method. The biofilms were fixed with 10mM cetylpyridinium chloride for 30 minutes, rinsed with saline and air dried. The coverslips were stained for 15 minutes with a 2:1 mixture of saturated aqueous congo red solution and 10% (v/v) tween 80 and rinsed in distilled water. Lastly, the coverslips were stained with 10% (v/v) Ziehl carbol fuchsin for 6 minutes, rinsed in distilled water and air dried overnight. Ziehl carbol fuschin stains bacterial cell whereas congo red stains the biofilm orange/pink.

Field Emission Scanning Electron Microscopy (FESEM) analysis: Biofilms were formed on coverslips as above**.** The coverslips were rinsed with saline and processed for FESEM. The cells were fixed with 2% glutaraldehyde at 4˚C for 2 hours followed by rinsing two times with sterile distilled water. Dehydration was done sequentially with graded concentrations of ethanol viz 20%, 40%, 60%, 80% and 90% for 15 minutes with each concentration and then

twice with absolute ethanol. Coverslips were dried completely, mounted on copper stubs with carbon tape followed by coating with iridium and observed under FESEM (JSM 7600 F, SAIF- IIT Bombay).

Tolerance of *P. aeruginosa* PAO1 to colloidal silver: 10ml of log phase cultures in LB broth (2 $\times 10^{14}$ cfu/ml) of *P. aeruginosa* PAO1 was treated with colloidal silver at a concentration range of 3 – 21 ppm at intervals of 3ppm and 10ml of sessile cell suspensions (10^9 cfu/ml) in LB broth obtained from biofilms and were treated with colloidal silver at a concentration range of 4–20 ppm at intervals of 4ppm. The treated cultures were incubated at 37ºC at 250 rpm for one hour. After incubation, the cell pellets obtained by centrifugation were rinsed with sterile saline thrice to remove traces of colloidal silver. Cell pellets were resuspended in sterile saline and enumeration was done on LB agar plates by drop plate technique.

Effect of colloidal silver on *P. aeruginosa* PAO1 persisters to Ciprofloxacin: *P. aeruginosa* PAO1 persisters to ciprofloxacin in both planktonic and sessile population were inoculated in LB medium containing colloidal silver. The concentrations of colloidal silver used were 3 – 21ppm for planktonic persisters and 4 – 20ppm for persisters from the sessile population. Persister populations were exposed to colloidal silver for 1 hour. Enumeration of cell number was done in triplicate by drop plate method.

Statistical analysis: All experiments were performed as three independent experiments, each in duplicate. Graphs were prepared using Microsoft Excel 2016. Error bars in the graphs express standard error of the mean ± (SEM). For the effect of colloidal silver on persisters, mean values were compared within and between groups using One-way Analysis of Variance (ANOVA) followed by Bonferroni's test using SPSS software (file version 1.0.0.118). Results were considered statistically significant at P values less than 0.05.

Results

Minimum Inhibitory Concentration (MIC) of Ciprofloxacin: The MIC of ciprofloxacin for *P. aeruginosa* PAO1 MTCC 741 was determined to be 3µg/ml by broth dilution method.

***Pseudomonas aeruginosa* PAO1 persisters to Ciprofloxacin**

Dose dependent kill curve for *P. aeruginosa* PAO1: Dose dependent study was done using 10×, 20×, 40× MIC concentration of ciprofloxacin against log phase population of *P. aeruginosa* PAO1. Fig. 1 depicts the survival of *P. aeruginosa* PAO1 population on exposure to different concentrations of ciprofloxacin for 1 hour. Increase in ciprofloxacin concentration to 60µg/ml resulted in the rapid killing of majority of the population followed by a plateau consisting of drug-tolerant persister cells that have survived even with further increase in drug concentration (Fig. 2). Concentration of ciprofloxacin for obtaining persister population was selected as 60µg/ml as with further increase in drug concentration. No significant decrease in surviving population count was observed. Hence, 60µg/ml of ciprofloxacin was used for the isolation of persister cells from the log phase population of *P. aeruginosa* PAO1 throughout the study.

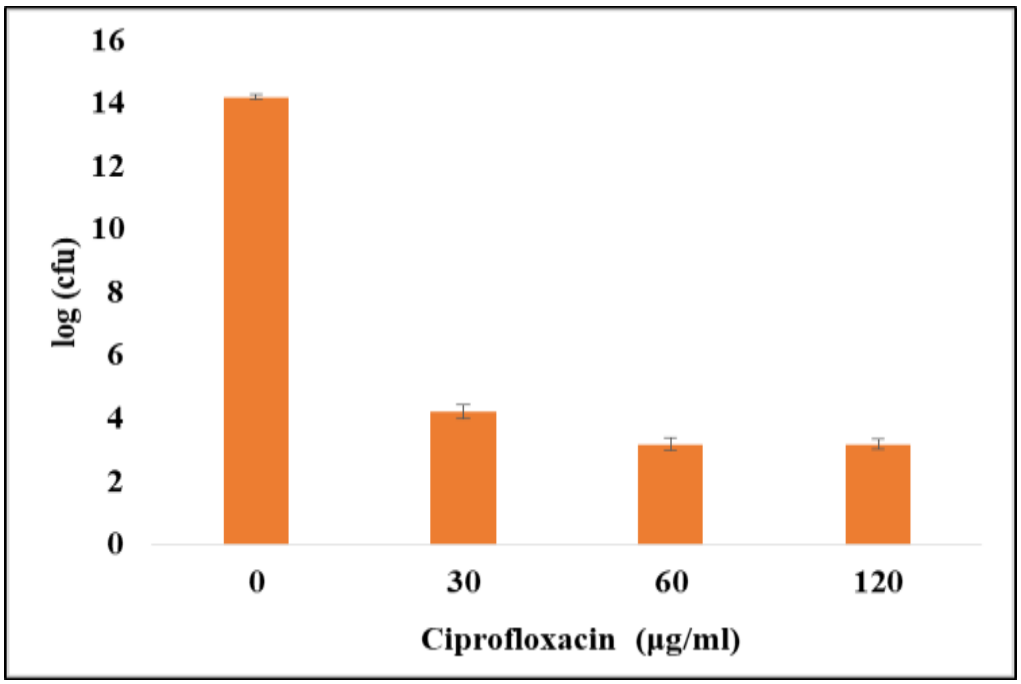

Fig. 1: Dose dependent killing for *P. aeruginosa* PAO1. Log phase culture was treated with different concentrations of Ciprofloxacin for 1 hour. Cell numbers are plotted as log CFU/ml. The values are average of three independent experiments. Error bars indicate ± SEM

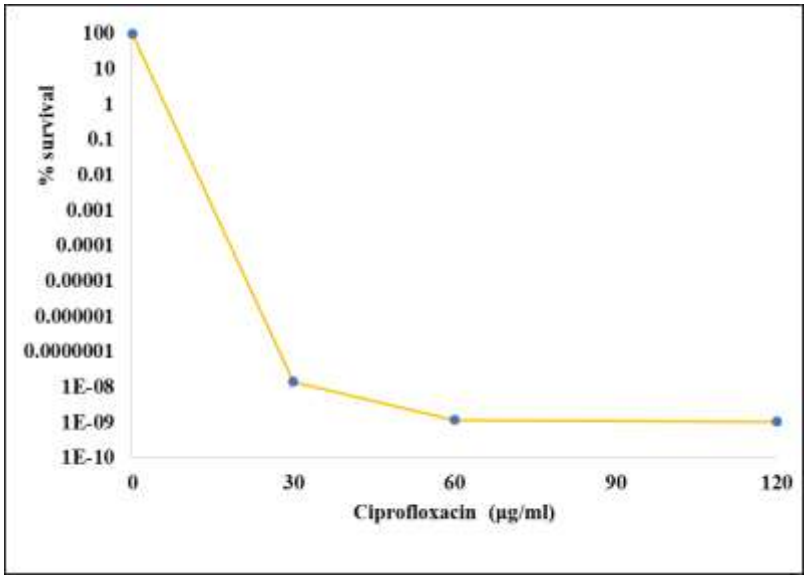

Fig. 2: Percentage survival for dose dependent kill curve for *P. aeruginosa* PAO1. Control sample (0µg/ml) was taken prior to antibiotic treatment

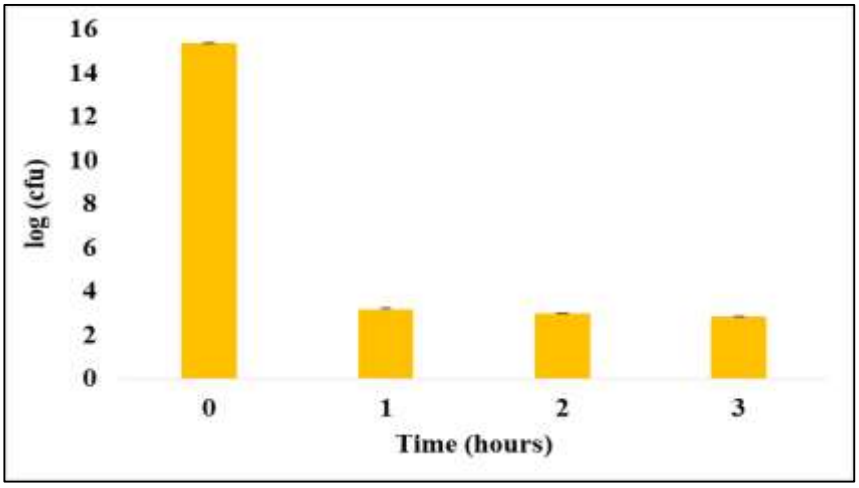

Fig. 3: Time dependent killing of *P. aeruginosa* PAO1 at 60µg/ml Ciprofloxacin. Cell numbers are plotted as log CFU/ml. The values are average of three independent experiments. Error bars indicate ± SEM

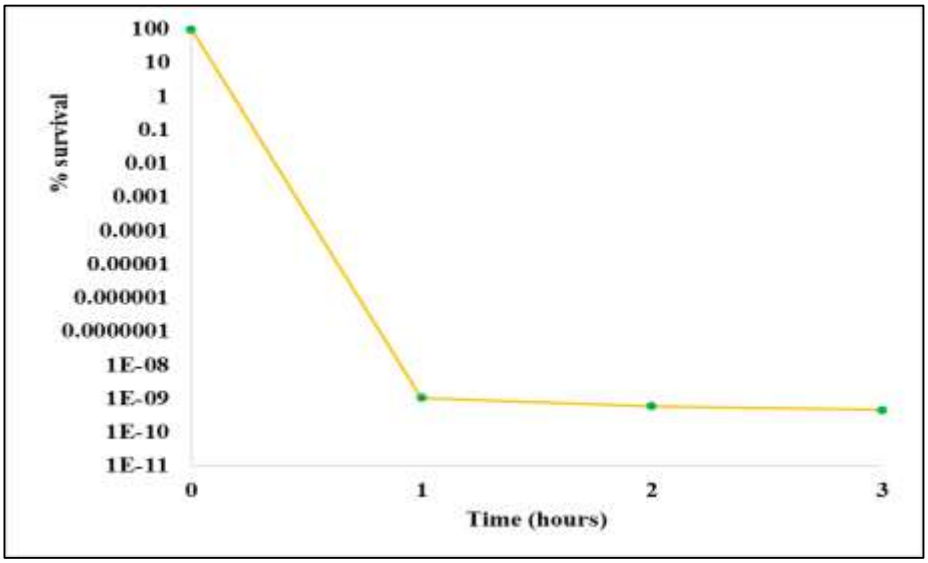

Fig. 4: Percentage survival of *P. aeruginosa* PAO1 for time dependent kill curve at 60µg/ml Ciprofloxacin. Control sample (0 hour) was taken prior to antibiotic treatment

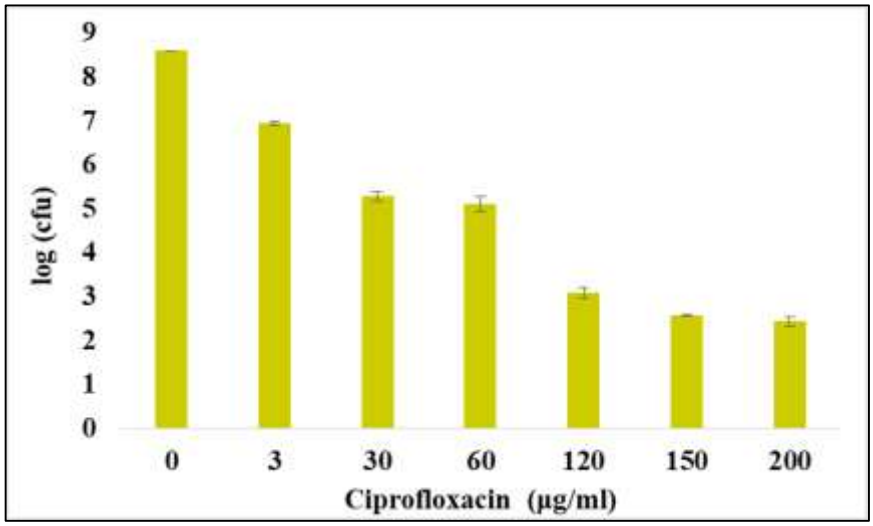

Fig. 5: Dose dependent killing for sessile population of *P. aeruginosa* PAO1. Cells from the sessile population were treated with different concentrations of Ciprofloxacin. Cell numbers are plotted as log CFU/ml. The values are average of three independent experiments. Error bars indicate ± SE

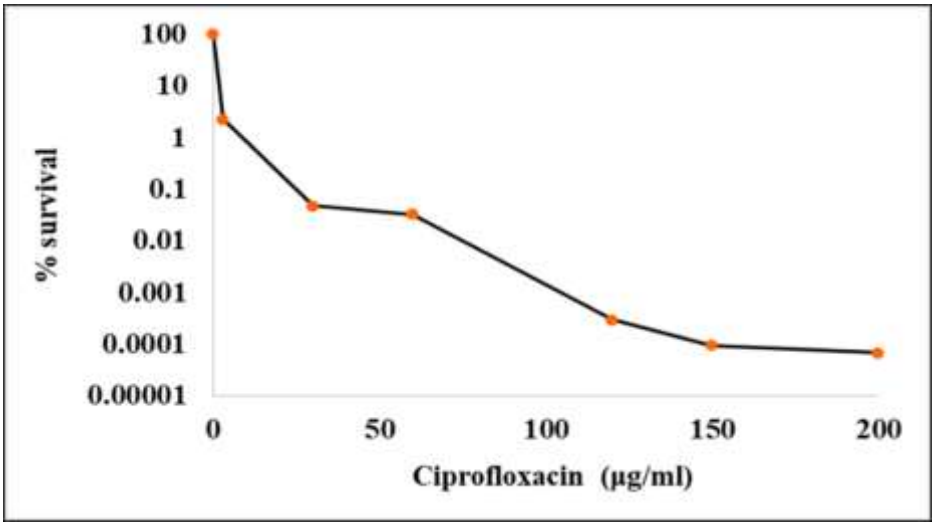

Fig. 6: Percentage survival for dose dependent kill curve for sessile population of *P. aeruginosa* PAO1. Control sample (0µg/ml) was taken prior to antibiotic treatment

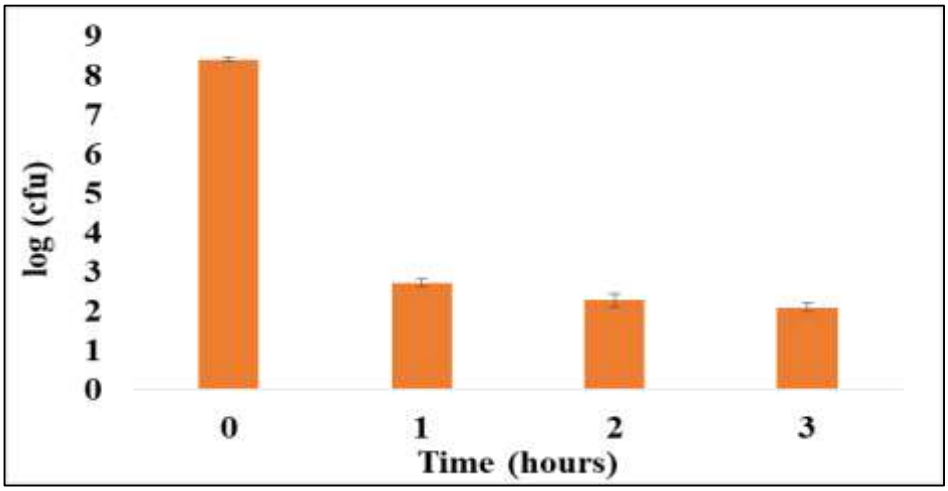

Fig. 7: Time dependent killing at 150µg/ml Ciprofloxacin for sessile population of *P. aeruginosa* PAO1. Sessile population was treated with 150µg/ml of Ciprofloxacin and incubated for different time intervals. Cell numbers are plotted as log CFU/ml. The values are average of three independent experiments. Error bars indicate ± SEM

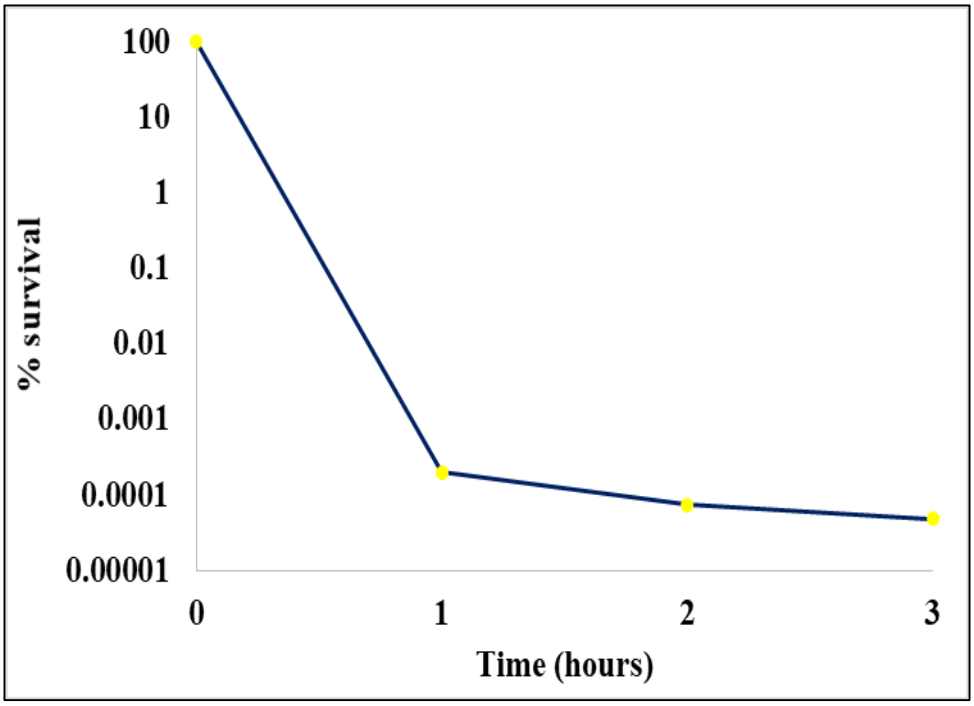

Fig. 8: Percentage survival for time dependent kill curve at 150µg/ml Ciprofloxacin for sessile population of *P. aeruginosa* PAO1. Control sample (0 hr) was taken before antibiotic treatment

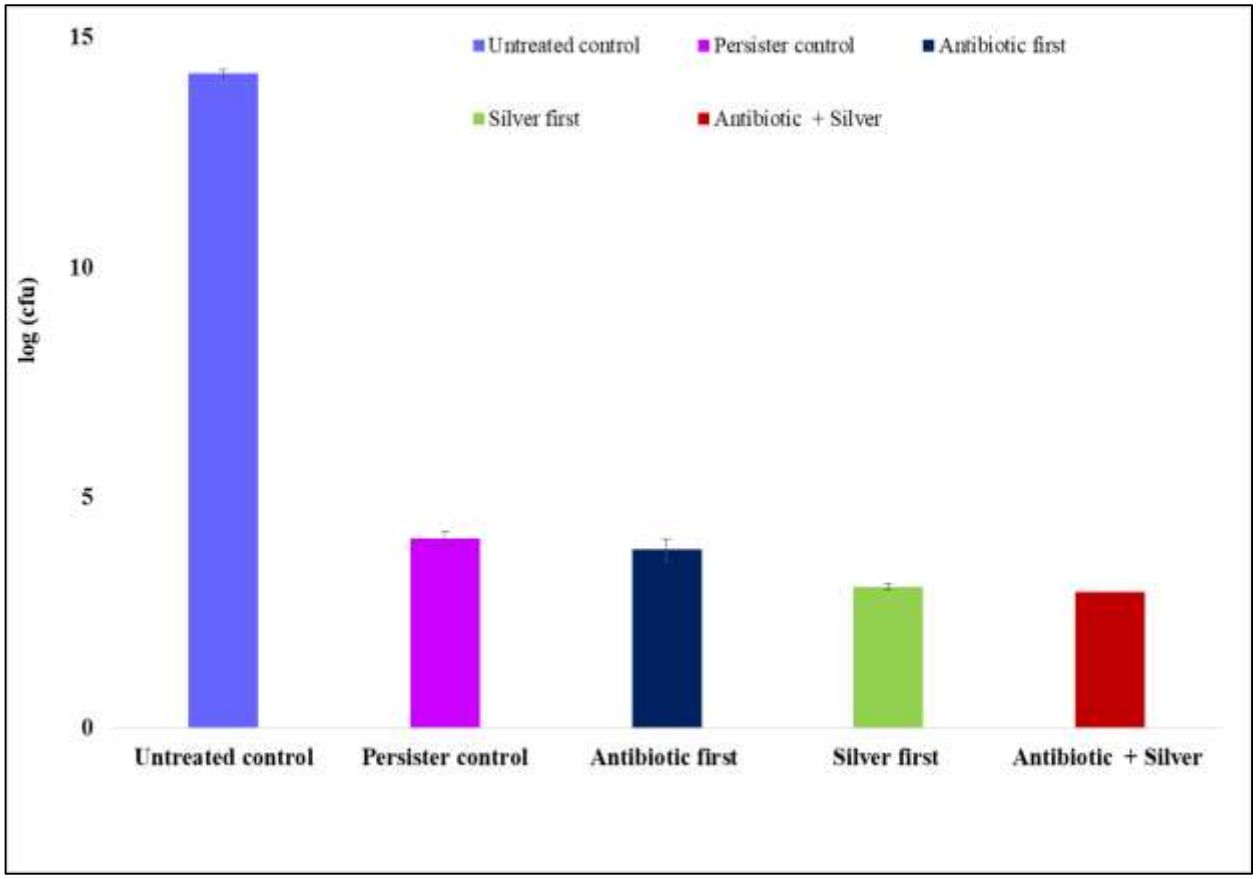

Fig. 9: Effect of colloidal silver on the formation of *P. aeruginosa* PAO1 persisters to Ciprofloxacin. Untreated control indicates log phase culture not treated with antibiotic or silver. Persister control indicates log phase culture treated with 60µg/ml Ciprofloxacin for 1 hour. Antibiotic first indicates the addition of 60µg/ml Ciprofloxacin at log phase followed by 3ppm colloidal silver. Silver first indicates addition of 3ppm colloidal silver at log phase followed by 60µg/ml Ciprofloxacin. Antibiotic + silver indicates addition of both simultaneously at log phase. Cell numbers are plotted as log CFU/ml. The values are average of three independent experiments. Error bars indicates ± SEM

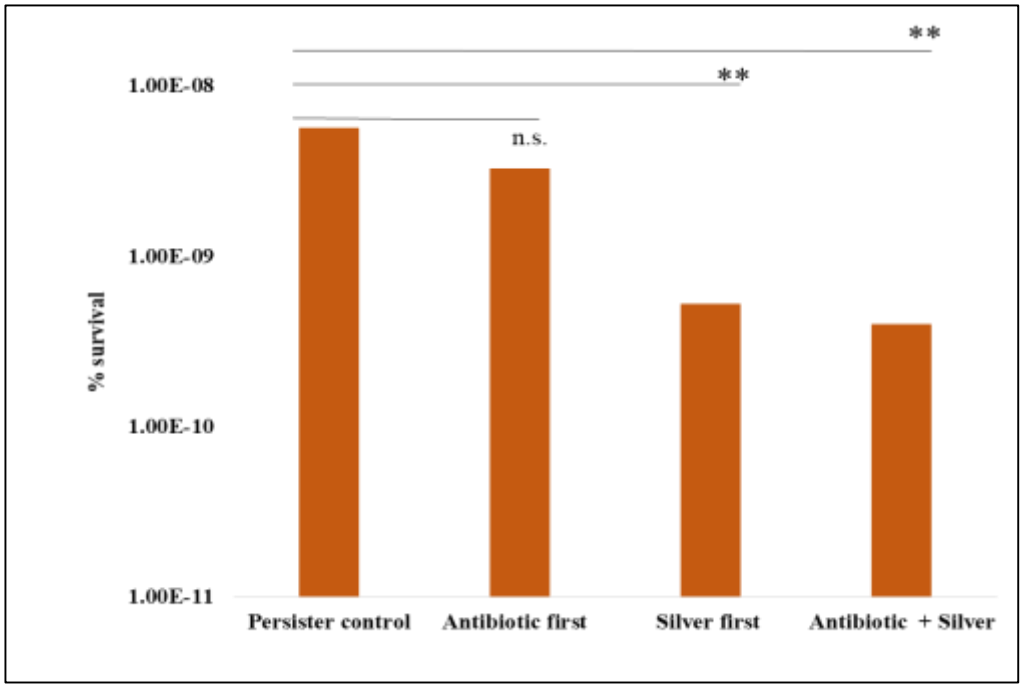

Fig. 10: Effect of colloidal silver on persister formation in *P. aeruginosa* PAO1 (Percentage survival). For treatment methods- Antibiotic first, Silver first and Antibiotic + silver, the percentage survival was calculated in comparison to persister control. Persister control indicates persister cells not exposed to colloidal silver. The values are average of three independent experiments. n.s. indicates not significant, *indicates P<0.05 and ** indicates P< 0.01 compared to persister control for each treatment method respectively. Error bars indicate ± SEM

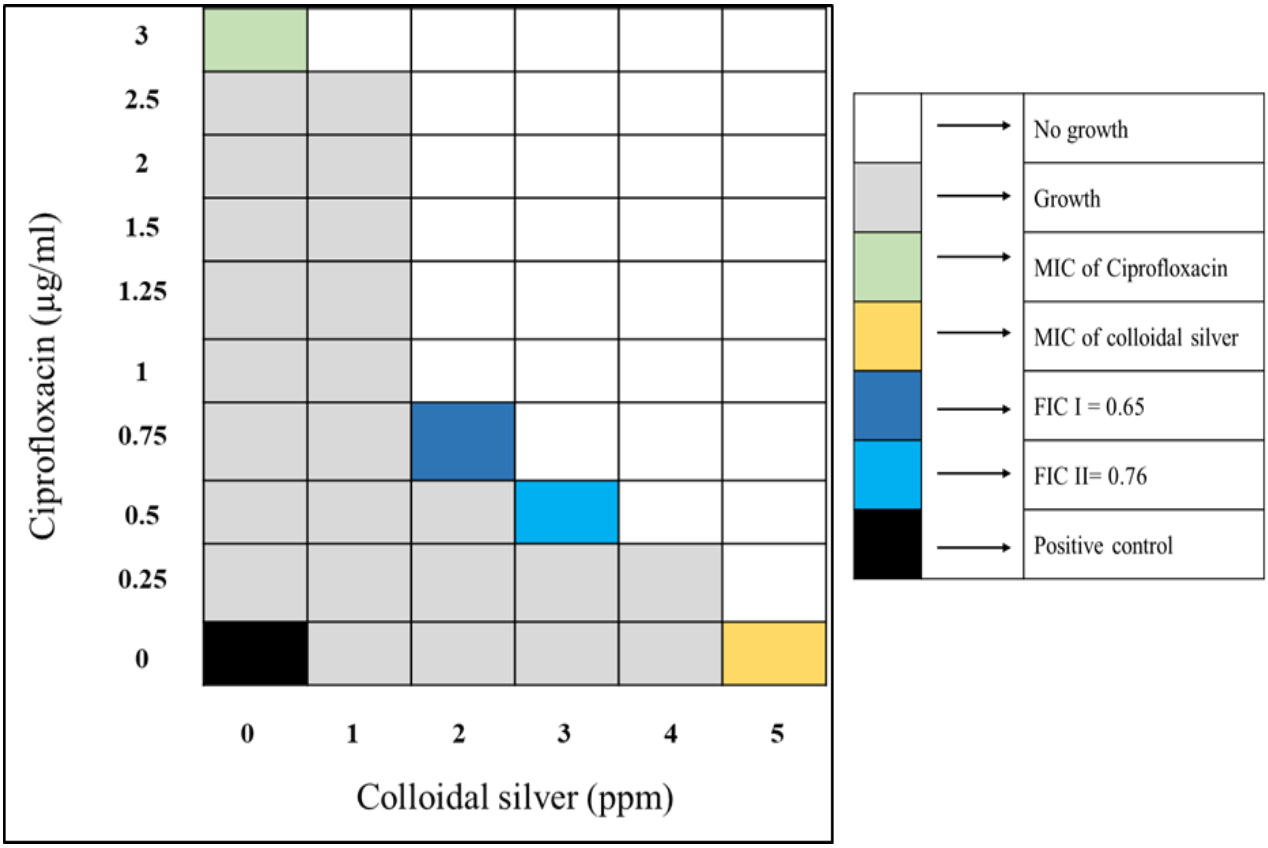

Fig. 11: Checkerboard assay with Ciprofloxacin and colloidal silver for persisters of *P. aeruginosa* PAO1. Concentrations used for Ciprofloxacin and colloidal silver were 0.25-3µg/ml and 1-5ppm respectively. Positive control indicates maximum growth and viability of persister cells. Fractional inhibitory concentration (FIC) was calculated for two combinations I & II where growth was inhibited at a concentration lower than the MIC of Ciprofloxacin and colloidal silver.

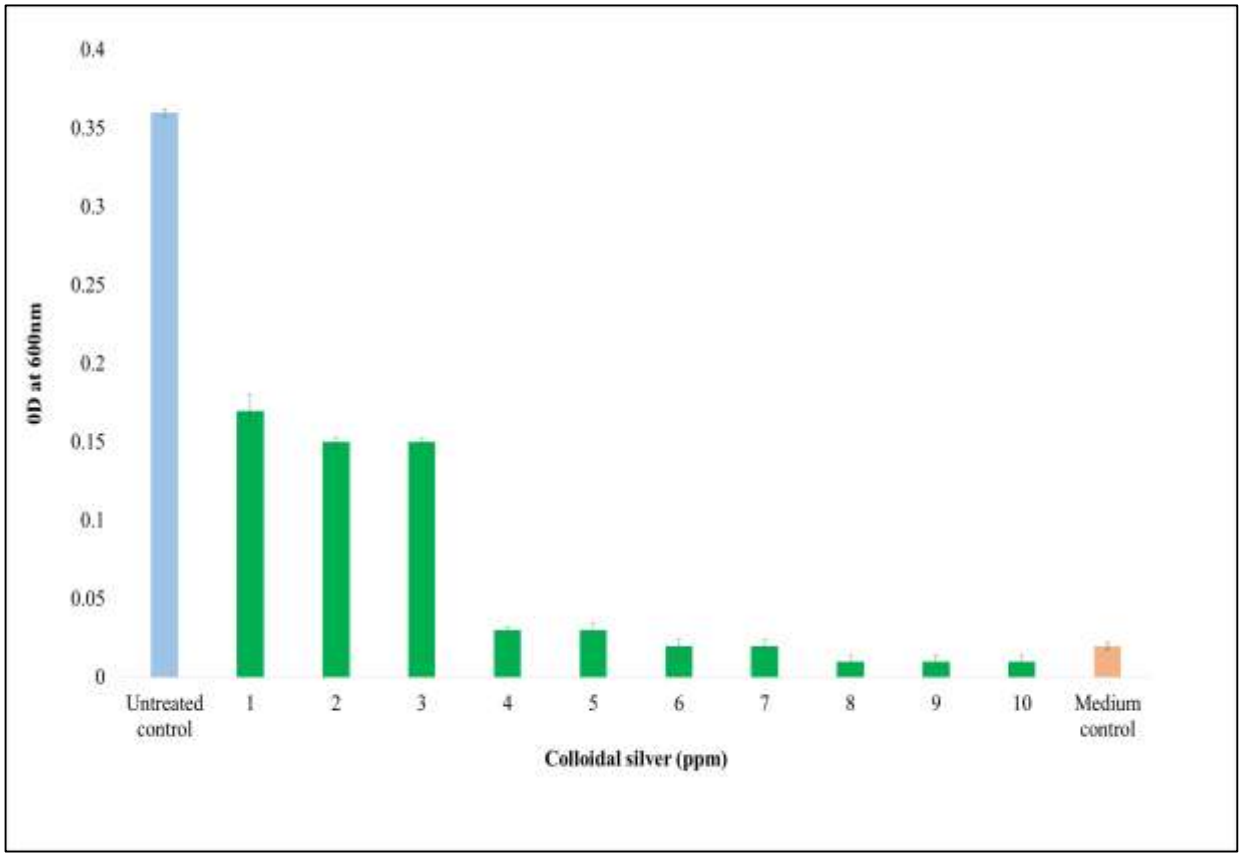

Fig. 12: Effect of colloidal silver on Biofilm formation/synthesis in *P. aeruginosa* PAO1 (Crystal Violet assay). Untreated control indicates maximum biofilm formation in the absence of colloidal silver. Medium control indicates absorbance of uninoculated medium without bacterial cells at 600nm. Error bars indicate ± SEM

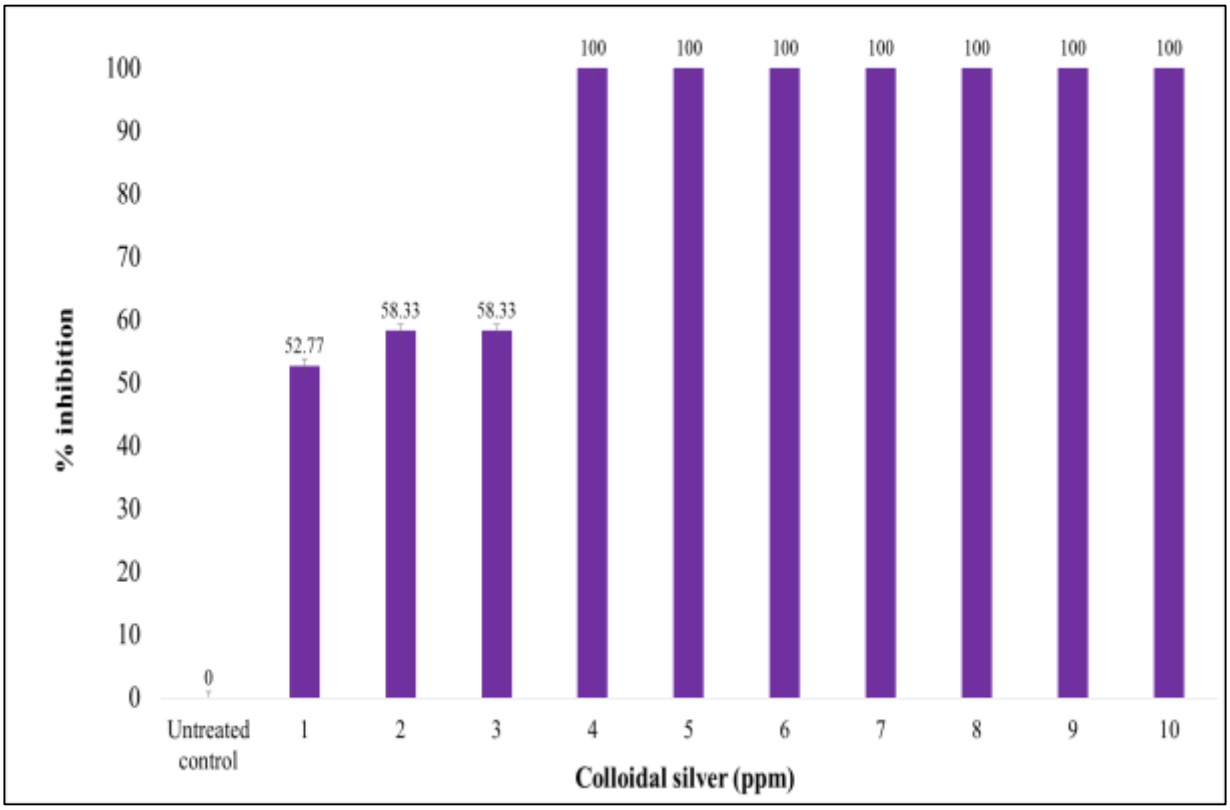

Fig. 13: Effect of colloidal silver on Biofilm formation/synthesis in *P. aeruginosa* PAO1 (percentage inhibition). Untreated control indicates maximum biofilm formation in the absence of colloidal silver. Error bars indicate ± SEM

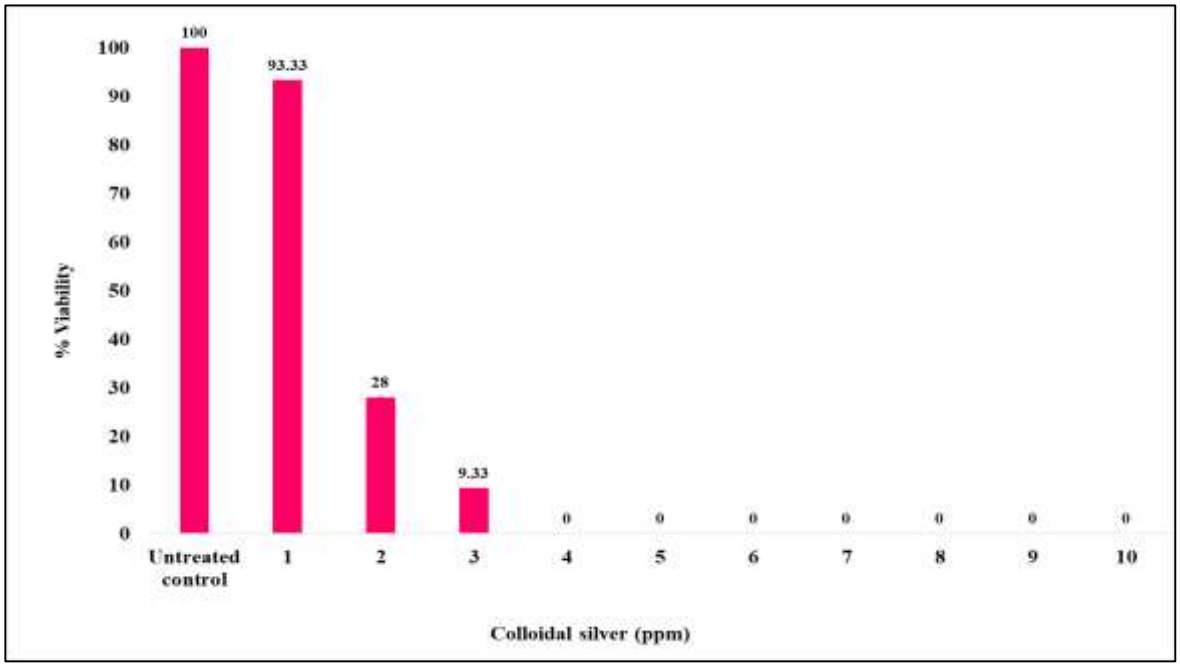

Fig. 14: Effect of colloidal silver on the viability of sessile population in biofilms of *P. aeruginosa* PAO1 (TTC assay). Untreated control indicates biofilm formation in the absence of colloidal silver and the maximum viability of the sessile population. Error bars indicate ± SEM

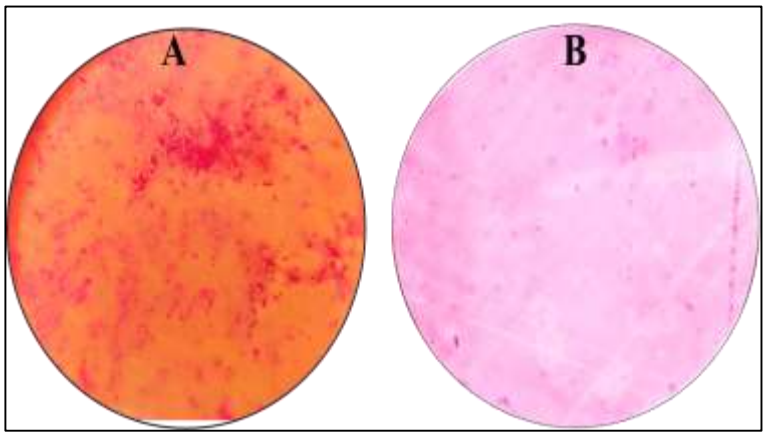

Fig. 15: Allison Sutherland staining of *P. aeruginosa* PAO1 Biofilms. A) Biofilm formation in the absence of colloidal silver. B) Biofilm formation in the presence of 4ppm colloidal silver.

Time dependent kill curve for *P. aeruginosa* PAO1: Survival of log phase population of *P. aeruginosa* PAO1 on exposure to 60µg/ml of ciprofloxacin at different time intervals is presented in fig. 3. Untreated log phase culture was control. The time kill curve in fig. 4 shows a typical biphasic curve with rapid decrease in population at the end of 1 hour, followed by constant surviving population count even with further increase in the time of exposure to the antibiotic. As exposure time of one hour resulted in maximum persister cell number, persister population was obtained by exposing log phase culture of *P. aeruginosa* PAO1 to 60µg/ml of ciprofloxacin for 1 hour at 37°C and 220 rpm.

Isolation of persister cells from biofilms: Dose dependent study was done using 10×, 20×, 40×, 50× MIC of ciprofloxacin against sessile population of *P. aeruginosa* PAO1. Fig. 5 depicts the survival of cells from the sessile population of *P. aeruginosa* PAO1 at different concentrations of ciprofloxacin for 1 hour. Characteristic biphasic curve was observed at 150µg/ml (50×MIC) of ciprofloxacin followed by a plateau of drug-tolerant persister cells as shown in fig. 6. Survival of cells from the sessile population of *P. aeruginosa* PAO1 on exposure to 150µg/ml of ciprofloxacin at different time intervals is presented in fig. 7. Time kill curve (Fig. 8) showed a rapid decrease in population at the end of 1 hour followed by a constant persister cell number with further increase in incubation time. Hence, for the isolation of persister cells from sessile population, exposure to 150µg/ml of ciprofloxacin for 1 hour was used in the study.

Effect of colloidal silver on planktonic and biofilm population

Minimum Inhibitory Concentration (MIC) of colloidal silver: The MIC of colloidal silver for the planktonic population of *P. aeruginosa* PAO1 was 3ppm and for the sessile population in the biofilms, it was found to be 4ppm as determined by the broth dilution method.

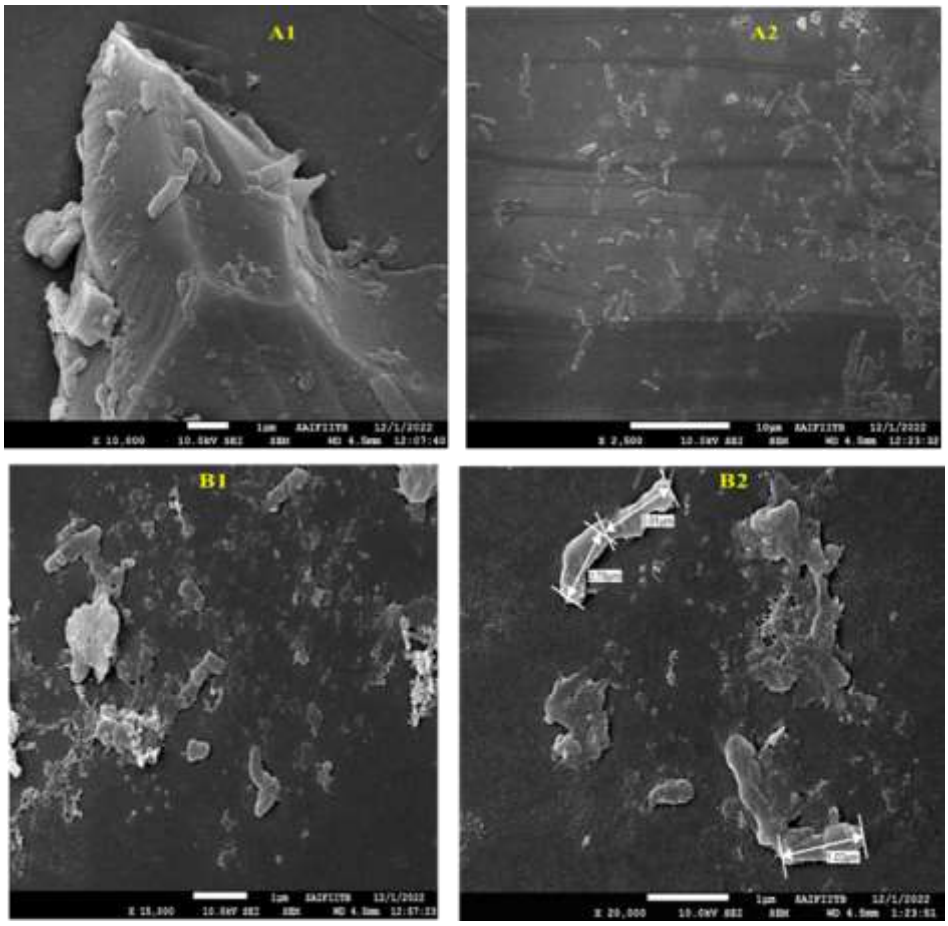

Fig. 16: FESEM imaging of *P. aeruginosa* PAO1 biofilms. A1, A2 - Untreated biofilm with extracellular matrix. B1, B2 - Reduced numbers of cells with disrupted extracellular matrix in the presence of 4ppm colloidal silver

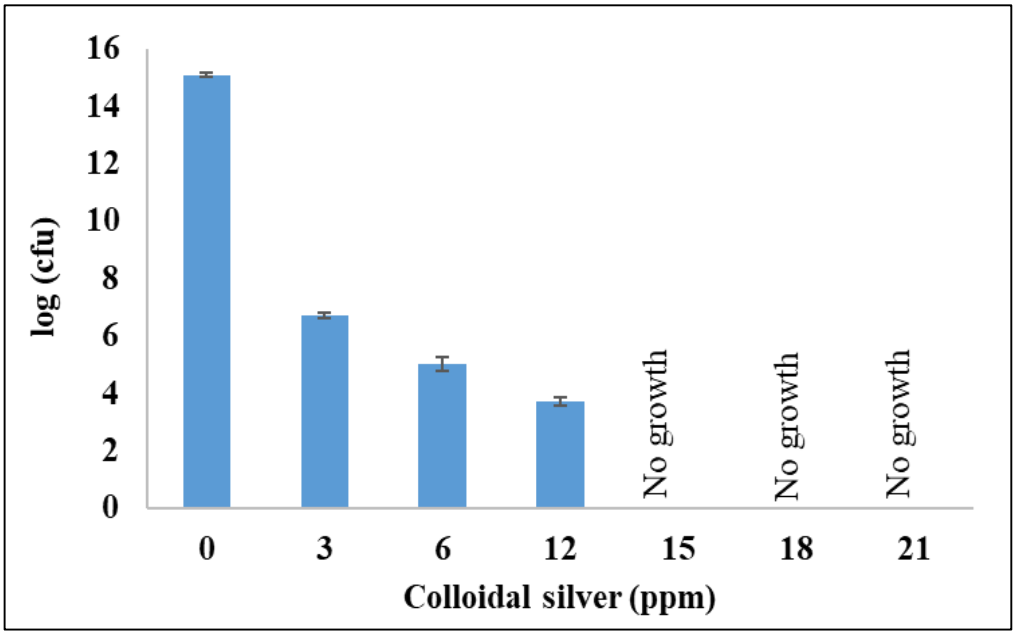

Fig. 17: Tolerance of log phase population of *P. aeruginosa* PAO1 to colloidal silver. Log phase culture was exposed to different concentrations of colloidal silver for 1 hour. Growth was inhibited at 15ppm and above. Cell numbers are plotted as log CFU/ml. The values are average of three independent experiments. Error bars indicate ± SEM

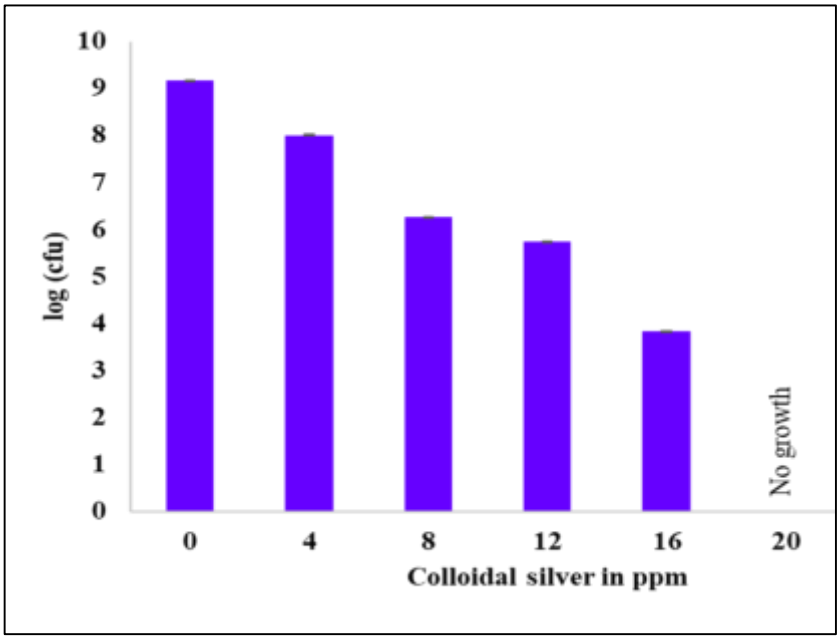

Fig. 18: Tolerance of sessile population of *P. aeruginosa* PAO1 to colloidal silver. Sessile cells from biofilms were exposed to different concentrations of colloidal silver for 1 hour. . Growth was inhibited at 20ppm. Cell numbers are plotted as log CFU/ml. The values are average of three independent experiments. Error bars indicate ± SEM

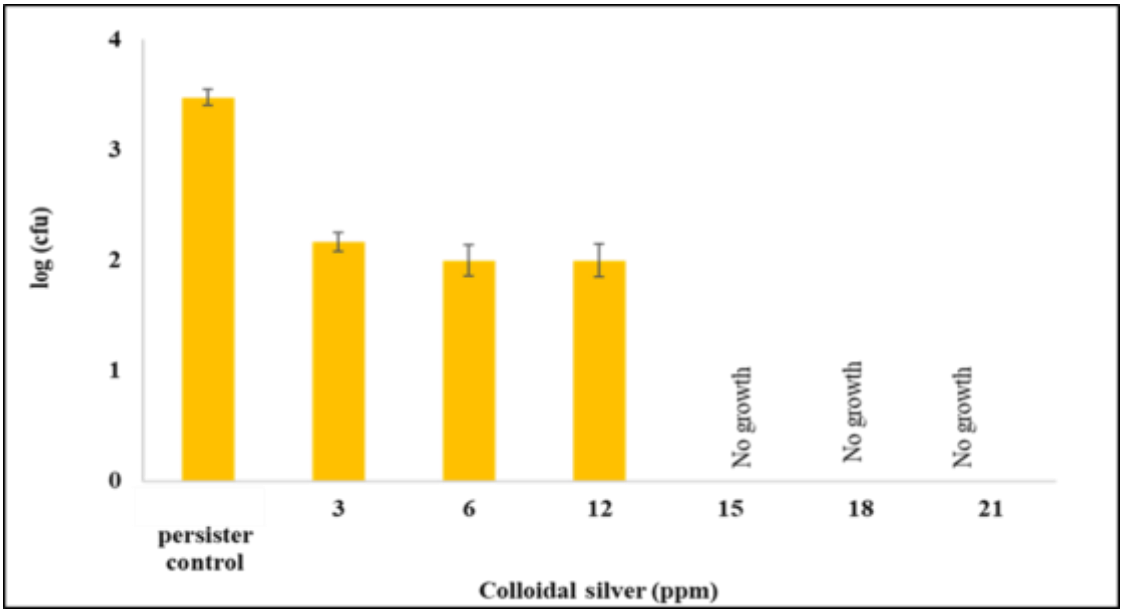

Fig. 19: Effect of colloidal silver on Ciprofloxacin persisters of *P. aeruginosa* PAO1. Persister control indicates persister cells unexposed to colloidal silver. Persister cells were exposed to 3-21ppm of colloidal silver for 1 hour. Cell numbers are plotted as log CFU/ml. Persister cells were inhibited at 15ppm and above. The values are average of three independent experiments. Error bars indicate ± SEM

Determination of the effect of colloidal silver on the formation of *P. aeruginosa* PAO1 persister cells to Ciprofloxacin: Colloidal silver was found to affect the formation of persister cells to ciprofloxacin in *P. aeruginosa* PAO1 as observed in fig. 9. There was a decrease in the number of persisters when silver was added prior to antibiotic addition or along with the antibiotic. Fig. 10 represents the percentage survival under the three different experimental conditions. The decrease in persisters was significantly higher when silver was added prior to the antibiotic as compared to persister control indicating that persister formation is inhibited by colloidal silver. There was no significant decrease in persisters when silver was added after the antibiotic addition.

Synergistic action of Ciprofloxacin and colloidal silver against persister cells of *P. aeruginosa* PAO1: The result of the microtitre plate checker board assay to determine the synergistic action of Ciprofloxacin and colloidal silver on persisters of *P. aeruginosa* PAO1 is represented in fig. 11. Ciprofloxacin and colloidal silver in combination inhibited the persisters at concentrations lower than their respective

MIC concentrations. The ∑FIC was calculated for the two combinations showing inhibition as indicated in the fig. 11.

$$\sum FIC = \frac{\text{Inhibitory concentration of Ciprofloxacin in combination}}{\text{MIC concentration of Ciprofloxacin}} + \frac{\text{Inhibitory concentration of Colloidal silver in combination}}{\text{MIC concentration of Colloidal silver}}$$

∑FIC I = (0.75/3 +2/5) = 0.65 ∑FIC II = (0.5/3+ 3/5) = 0.76

A combined action is considered additive, if the ∑FIC lies between 0.5 to 2. Hence, colloidal silver and ciprofloxacin were found to exert an additive effect on the persisters of *P. aeruginosa* PAO1. Colloidal silver significantly reduced the inhibitory concentration of ciprofloxacin.

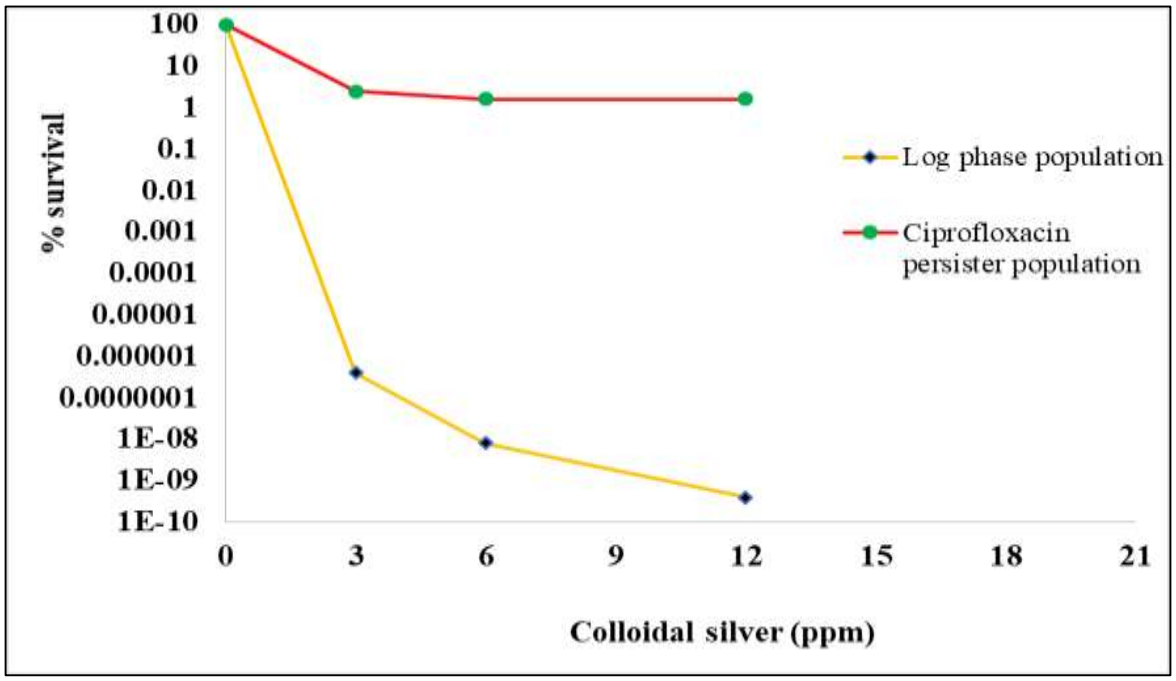

Fig. 20: Survival of log phase population and Ciprofloxacin persisters of *P. aeruginosa* PAO1 in the presence of colloidal silver. The population counts taken prior to the treatment with colloidal silver were considered as 100%.

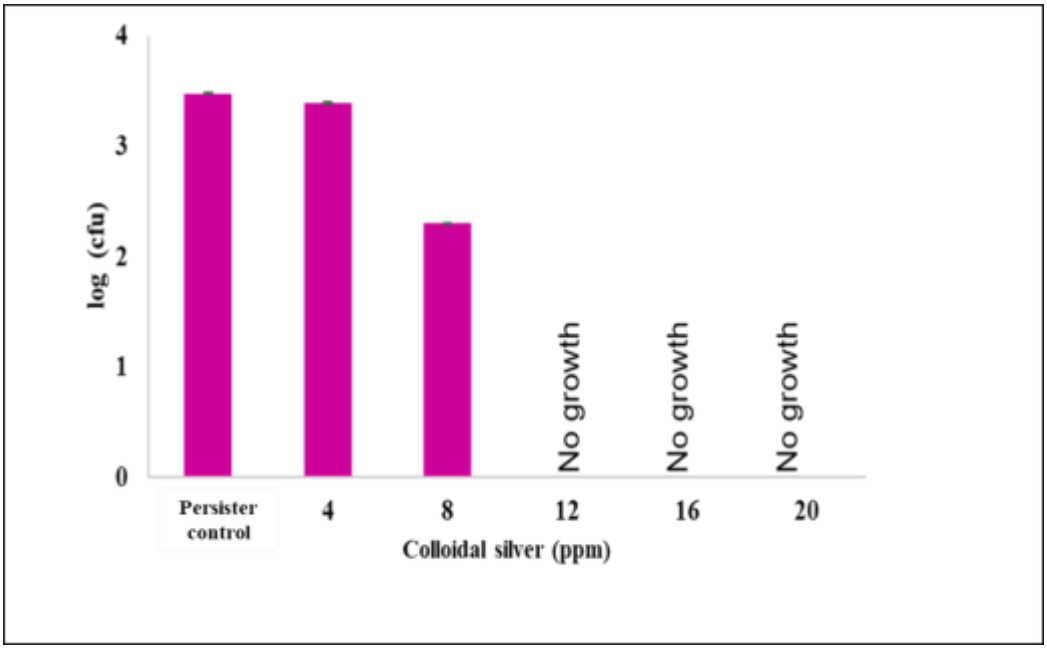

Fig. 21: Effect of colloidal silver on Ciprofloxacin persister cells from sessile population of biofilms of *P. aeruginosa* PAO1. Persister control indicates persister cells from sessile population unexposed to colloidal silver. Persister cells were exposed to 4-20ppm of colloidal silver. Cell numbers are plotted as log CFU/ml. Growth was inhibited at 12ppm and above. The values are average of three independent experiments. Error bars indicate ± SEM

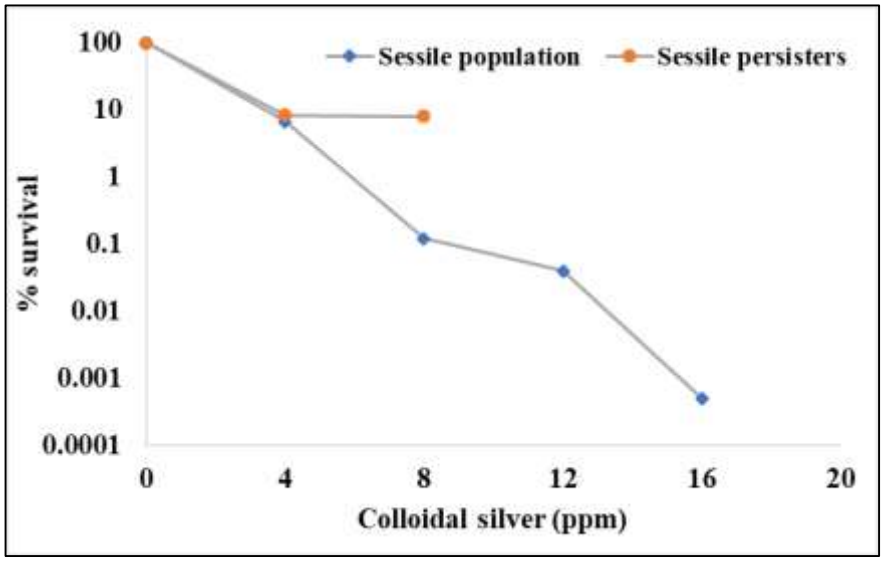

Fig. 22: Survival of sessile population and sessile persisters to Ciprofloxacin of *P. aeruginosa* PAO1 in the presence of colloidal silver. The population counts taken prior to the treatment with colloidal silver were considered as 100%

Effect of silver on Biofilm formation/synthesis: Inhibition of biofilm formation by colloidal silver was determined by crystal violet assay at the concentration range of 1 to 10ppm. It was observed that beyond 4ppm, the absorbance was either constant or was found to decrease. The lowest concentration of colloidal silver inhibiting biofilm formation was 4ppm since the absorbance was equal to that of medium control (Fig. 12). 100% inhibition of biofilm was observed at 4ppm concentration of colloidal silver (Fig. 13).

Effect of colloidal silver on the viability of sessile population of *P. aeruginosa* PAO1: The viability of the sessile population of *P. aeruginosa* PAO1 was determined by TTC assay. Absorbance was measured at 490nm and percentage viability was calculated. No significant formazan was detected at 4ppm (Fig. 14). Hence it was confirmed that 4ppm colloidal silver inhibits the sessile population in the biofilms of *P. aeruginosa* PAO1.

Microscopic analysis of silver treated biofilms of *P. aeruginosa* PAO1

Allison Sutherland staining: Biofilms formed on sterile glass coverslips in LB broth containing 4ppm of colloidal silver (concentration inhibiting biofilm formation) and untreated control was stained after 24 hours. Dense exopolysaccharide (EPS) stained orange red embedded with dark pink short rods were observed in the untreated control (Fig. 15A) whereas absence of EPS with negligible short rods was observed on coverslips incubated with 4ppm concentration of colloidal silver (Fig.15B).

Field Emission Scanning Electron Microscopy (FESEM) analysis: FESEM images of untreated *P. aeruginosa* PAO1 biofilms showed higher number of cells along with intact extracellular matrix. Reduced cell numbers, disruption of cells as well as disruption of extracellular matrix were observed in the presence of 4ppm colloidal silver in comparison to untreated control biofilm (Fig. 16). This further confirms that biofilm formation was inhibited by 4ppm of colloidal silver.

Tolerance of *P. aeruginosa* PAO1 to colloidal silver: As colloidal silver was being used in the study to inhibit the formation of *P. aeruginosa* PAO1 persisters to ciprofloxacin, it was imperative to study the tolerance of *P. aeruginosa* to the colloidal silver in order to determine whether persisters to colloidal silver may be formed. Tolerance to colloidal silver of the log phase population of *P. aeruginosa* PAO1 was found to be 12 ppm and total killing was seen at 15ppm (Fig. 17). Sessile population from biofilms of *P. aeruginosa* PAO1 was found to tolerate 16ppm of colloidal silver and no growth was seen at 20ppm (Fig. 18). The tolerance to colloidal silver was just four times the MIC namely 12ppm (4 x 3ppm) for log phase culture and 16ppm (4 x 4ppm) for sessile population. This indicates that treatment with silver does not bring about persister formation to colloidal silver.

Effect of colloidal silver on *P. aeruginosa* PAO1 persisters to Ciprofloxacin: Ciprofloxacin persister population of *P. aeruginosa* PAO1 was found to tolerate 12ppm colloidal silver and was completely inhibited at 15ppm (Fig. 19). At 12ppm, a nine log reduction was seen in the log phase population while only a two log reduction was observed in persister population of *P. aeruginosa* PAO1 (Fig. 20). However, for the persister population from sessile cells of the biofilms, tolerance was found to be at 8ppm of colloidal silver (Fig. 21). At 12ppm, the sessile persister population was completely inhibited whereas the sessile population from biofilms required a MIC of 20ppm of colloidal silver (Fig. 22). At 12ppm, four log reduction was observed for sessile population. This indicates that sessile population of biofilm being sturdy and non-responsive does not get affected by antimicrobials easily. However, the persister population being stressed is more susceptible to colloidal silver.

Discussion

Pseudomonas aeruginosa is the most commonly reported ESKAPE pathogen, responsible for 13.2% to 22.6% of nosocomial infections and known to cause recurrent infections[40]. It poses a major therapeutic challenge for clinicians due to its drug resistance with the highest resistance reported (23.4%) to the commonly administered antibiotics meropenem and ciprofloxacin[43]. Antibiotic tolerance precedes drug resistance and antibiotic-tolerant persister cells play a crucial role in the recurrence of *P. aeruginosa* infections. These antibiotic-tolerant persister cells form under stress and exhibit a typical biphasic killing curve[8,17,28,48].

The killing curve of ciprofloxacin in this study exhibited two distinct phases: an initial rapid killing of the non-persister population, followed by the steady survival of persisters at 60µg/ml in the planktonic population and at 150µg/ml in the sessile population of *P. aeruginosa* PAO1 after one hour of incubation in the presence of the antibiotic (Fig. 2 and 8). These findings align with those of Mlynarcik and Kolar[33], confirming the presence of persister cells. Silver has been used medicinally for wound healing and disinfection since ancient times[1]. The antibacterial effect of silver nanoparticles (AgNP) depends on its size and form with highest activity obtained between 1–10nm.

Colloidal nanosilver, a suspension of AgNPs, offers enhanced antimicrobial properties due to its high surface-area-to-volume ratio and controlled silver ion release, making it a preferred choice over traditional colloidal silver. Previous studies have reported the antimicrobial activity of AgNPs against *P. aeruginosa.* Salomoni et al[42] showed that 10nm AgNPs were effective at 5ppm, while Al-Momani et al[3] reported a MIC of 7.5 ppm for *P. aeruginosa* ATCC 27853. Saeki et al[41] reported a MIC of 62.5 µM (~6.74 ppm) for AgNPs against *P. aeruginosa* PAO1. Platania et al[39] determined the MIC of colloidal silver solutions with different ratios of AgNPs and silver ions. MIC values obtained were 12.5ppm for 25% AgNPs/75% silver ions, 16.5ppm for 50% AgNPs/50% silver ions and 12.5 ppm for 100% AgNPs.

The colloidal silver solution used in this study contains silver nanoparticles having particle size of 5-50nm and elemental (zero-valent) metallic silver coated with silver oxide. The MIC of colloidal silver was 3ppm against planktonic population and 4ppm inhibited the formation of biofilms. As compared to previous studies[18,39,42], colloidal silver of SilverSol® technology exhibits better antimicrobial action against *P. aeruginosa* PAO1 at much lower MIC values.

Colloidal silver also affected the persister formation to ciprofloxacin in *P. aeruginosa* PAO1 (Fig. 9). The number of persisters decreased when silver was added either prior to the antibiotic or when added simultaneously with the antibiotic. The decrease in persisters was significantly higher ($P < 0.01$) when silver was added prior to the antibiotic as compared to persister control indicating that persister formation is inhibited by colloidal silver. There was no significant decrease in ciprofloxacin persisters when silver was added after the antibiotic addition. Hence, prior exposure to colloidal silver may modulate the physiological state of the cells or the extracellular environment, potentially inhibiting the mechanisms that promote persistence.

Previous studies[39] on the antimicrobial effect of colloidal silver have not reported any effect on antibiotic persisters of *P. aeruginosa* PAO1. The synergistic effect of colloidal silver and ciprofloxacin against persisters was studied using the checkerboard assay. The FIC index was 0.65, indicating an additive effect on ciprofloxacin persisters. Previous studies have reported a synergistic interaction (FIC < 0.5) between AgNPs and ciprofloxacin against *P. aeruginosa* isolates[41]. Since persisters are antibiotic tolerant, an additive effect with a higher FIC was observed. Persisters are known to be more prevalent in biofilms and exhibit higher tolerance to antimicrobial agents[21,28,34].

Our findings confirm that *P. aeruginosa* PAO1 biofilms harbor a significantly higher number of persister cells compared to planktonic populations. Specifically, 0.0001% of biofilm-associated cells survived the exposure to 150µg/ml of ciprofloxacin (Fig. 6) whereas only 1.00×10^{-9} of planktonic cells persisted at 60µg/ml (Fig. 2), indicating a fourfold increase in persister formation within biofilms. Colloidal silver inhibited biofilm formation in *P. aeruginosa* PAO1 completely at 4ppm as determined by the crystal violet assay and no viable biofilm population was detected by the TTC assay at 4ppm (Fig. 13 and 14). This was further confirmed by Allison Sutherland staining of *P. aeruginosa* PAO1 biofilms on coverslips.

Negligible short rods without EPS were observed in the presence of 4ppm colloidal silver (Fig. 15). FESEM images of untreated *P. aeruginosa* PAO1 biofilm showed a higher number of cells along with intact extracellular matrix. Decreased cell numbers, disruption of cells as well as extracellular matrix were observed in the presence of 4ppm colloidal silver as compared to untreated control biofilm (Fig. 16). This confirmed that colloidal silver effectively inhibits biofilm formation at 4ppm.

Colloidal silver significantly decreased the survival of planktonic, sessile and ciprofloxacin persister populations of *P. aeruginosa* PAO1. A nine-log reduction was observed in the log-phase population at 12ppm, whereas only a two-log reduction was noted for ciprofloxacin persisters at the same concentration. Ciprofloxacin persisters in biofilm-associated sessile populations were completely inhibited at 12ppm, while the overall sessile population required 20ppm for complete inhibition (Fig. 20 and 22).

These findings suggest that the sessile biofilm population is more resilient to antimicrobials whereas the stressed persister population is more susceptible to colloidal silver.

Conclusion

This study highlights the potent antimicrobial activity of colloidal silver against *P. aeruginosa PAO1*, particularly its effectiveness in reducing antibiotic-tolerant persisters and inhibiting biofilm formation. Colloidal silver demonstrated significant bactericidal action at lower MIC values compared to previously reported inhibition by silver nanoparticles[3,39,41,42], indicating its enhanced efficacy.

Furthermore, it effectively reduced persister formation to ciprofloxacin, with a stronger impact when administered prior to antibiotic exposure. The additive effect in combination with ciprofloxacin observed against persisters suggests its potential as an adjunct antimicrobial strategy. Notably, persisters within biofilms exhibited higher tolerance to antimicrobials, yet were more susceptible to colloidal silver than the overall biofilm population.

Given the increasing threat posed by multidrug-resistant infections, further studies need to be undertaken to evaluate the efficacy of colloidal silver against other ESKAPE pathogens, which are known for their high antibiotic resistance and their role in nosocomial infections. Additionally, testing a broader range of clinical isolates will help to confirm the consistency of these findings across different strains. These studies will provide deeper insights into the clinical relevance of colloidal silver as an alternative therapy for persistent bacterial infections.

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