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Research Article

Curcumin and Colistin are Synergistic in Inhibiting the Growth and Biofilm Formation of *Pseudomonas aeruginosa* Isolated from Environmental Sample

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Abstract

Colistin is commonly known as a last-resort antibiotic used to treat fatal infections caused by gram-negative bacteria. Increasing drug resistance and the absence of alternative drugs in the market has led to the indiscriminate application of colistin, resulting in low to moderate resistance against this antibiotic in different pathogens. Another important fact is; the transmission of antibiotic-resistance genes from the hospital to the community setting causes an outspread of resistance against this crucial antibiotic. To address the problem in a different way, we have used well-known phytochemical curcumin and applied it either separately or in combination with colistin to observe the antimicrobial effect against opportunist pathogen *Pseudomonas aeruginosa* isolated from environmental samples. Applying both antimicrobial agents separately under sub-inhibitory concentration causes an increase in biofilm formation, which is abrogated when the concentration increases to an inhibitory level. This effect persists for up to 48 hours and the loss of biofilm is noticed after that due to dispersal from the matrix.The gradual loss of swarming motility was observed in response to increasing concentration of both antimicrobials. Moreover, the combinatorial application exhibited the synergistic inhibition of *Pseudomonas aeruginosa*, vindicating the enhancement of the inhibitory effect in presence of both antimicrobials at a concentration well below the minimum inhibitory concentration, which has an important pharmacological consequence in the context of drug delivery. Our result, therefore, indicates the enhancement of the therapeutic efficacy ofcolistin in eradicating infection when combined with a conventional bioactive compound exploring the possibility of an alternative strategy against antimicrobial resistance.

Keywords: Colistin; Curcumin; Antibiotic resistance; Biofilm; Motility; *Pseudomonas aeruginosa*

Introduction

One of the most significant global public health challenges is antibiotic resistance due to the indiscriminate usage of different antibiotics in the hospital sector and beyond. Clinically important pathogens are getting increasingly more resistant to traditional antibiotics, and the availability of novel antibiotics is rare. The lack of novel antibiotics to treat infections caused by the multi-drug resistance "ESKAPE" group (*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa,* and *Enterobacter* species) therefore necessitate the revaluation of previously abandoned antibiotics [1]. Colistin belongs to the polymyxin family of antibiotics, which are cationic cyclic lipo- decapeptides that arise from the genomes of the *Paenibacillus* species. Despite showing neurotoxicity and nephrotoxicity, it has been widely regarded as the last resort antibiotic against gram-negative bacteria. However, the extensive usage of this antibiotic has led to resistance against this antibiotic which is very alarming for the future perspective.

The primary mechanisms of colistin resistance in gram-negative bacteria are structural alterations of bacterial lipopolysaccharide [2]. Bacterial resistance to Colistin can also disrupt membrane integrity by displacing Mg^{2+} and Ca^{2+} cations from the outer membrane leading to cell lysis [3]. The newly discovered horizontally transferable plasmid-mediated mobile colistinresistance gene (*Mcr*) is extremely concerning because it has been found in more than 20 nations, including Europe, Asia, South America, North America, and Africa, India [4,5]. Mcr genes (*Mcr*1 to *Mcr*10) with several variations have been found in isolates from people, animals, and the environment in more than 60 nations on six of the seven continents, with Antarctica being the exception [6,7]. Public health is severely impacted by the contamination of soil, aquatic systems, plants, and wildlife caused by the release of antimicrobials, antimicrobial-resistant bacteria, and antibiotic-resistant genes into the environment by both people and animals (via ejection or through anthropogenic actions) [8]. Recently, it has been discovered that environmental colistin resistance is directly connected to sewer water releasedfrom hospital and pharmaceutical industries in North and West India [9]. This poses a severe threat to public health; therefore, it is vital to research bacterial culture from clinical and environmental samples.

P. aeruginosa frequently exhibits colistin resistance like other gram negative bacteria such as *Escherichia coli* [10]. Infections caused by *P. aeruginosa* are tremendously hard to treat with available antibiotics due to their underlying resistance mechanisms and low outer membrane permeability. The resistance mechanism can be either intrinsic (efflux systems, outer membrane permeability, etc.) or acquired (chromosomal mutations, mobile resistance genes, and adaptive resistance) [11]. Biofilm formation as an adaptive mechanism is considered a critical virulence factor enhancingthe survival of antibiotic exposure and initiating chronic infections [12]. The ability of *P. aeruginosa* to form dense and persistent biofilms is responsible for over 60% of human infections. Biofilms comprise polysaccharides (Pel, Psl, and alginate) and extracellular DNA. *P. aeruginosa* enters the quorum-sensing mode in response to changes in cell density and environmental cues or stresses such as antibiotics, starvation, pH, heat, etc. [13]. Motility is a critical element in the production of *P. aeruginosa* biofilms. Early phases of biofilm formation are aided by swarming movement. Few studies have demonstrated the

link between biofilm formation, swarming motility, and quorum sensing [14].

Antimicrobial treatments of *P. aeruginosa* infections are challenging, primarily due to biofilm production. Therapeutic management of *P. aeruginosa* infections poses unique challenges for the clinical use of conventional antimicrobials. Recent studies have reported several novel non-antibiotic therapeutic approaches and targets for alternative or complementary treatments to traditional antimicrobials and antibiotics that are highly effective in combating infections caused by resistant *P. aeruginosa* by facilitating biofilm dispersal, improving drug penetration, and therefore maximizing efficacy against the bacteria and their established biofilms [13]. These approaches include iron chelation, phage therapy, biofilm remediation strategies, nanoparticles, antimicrobial peptides, and natural agents like curcumin [7,11].

In the present study, we analyzed how different colistin concentrations affect biofilm production and motility in a resistant strain of *P. aeruginosa* isolated from the environmental soil sample of the hospital in the Kolkata region. We also studied the effect of curcumin on *Pseudomonas aeruginosa* motility and biofilm formation. Moreover, the combined impact of colistin and curcumin on *P. aeruginosa* vindicates the utility of combinatorial therapy of different biomolecules on the opportunist pathogen. This combined application of colistin and curcumin can be used as alternative therapeutic approach against *pseudomonas* infections.

Material and Methods

P. aeruginosa was isolated from the soil of Kolkata, West Bengal. Luria Bertani (LB) broth and agar medium was procured from Himedia. Colistin and curcumin were purchased from SRL Chemicals.

Bacterial isolates identification

Environmental bacterial isolates were collected and isolated from the different soil samples of an urban hospital in Kolkata, West Bengal. The stock culture was maintained at -80˚C in 30 % v/v frozen glycerol solution. Gram-negative bacteria were differentiated using the gram-staining method. They were streaked on nutrient agar media, and *P. aeruginosa* strain was chosen for further study due to its importance. The strain was phenotypically identified throughstreaking on a nutrient agar plate and Cetrimide agar, a selective media. Alternatively, they were reconfirmed by doing 16s rRNA sequence analysis. 100% query coverage and 99.08% identity were observed with *P. aeruginosa* strain DSM 50071 Accession no. NR_117678.1. Large, opaque, and non-mucoid colonies with a slightly rough edge appeared on the plate after incubating the plates for 16–24h. The production of pyocyanin and *2-aminoacetophenone* by *P. aeruginosa* colonies gave a greenish-blue appearance and grape-like odour respectively. The identity was reassessed by performing the VITEK experiment using the VITEK 2 machine.

MIC determination of colistin

The minimum inhibitory concentration (MIC) of colistin and curcumin was determined using the tube macro-dilution method as described earlier [15]. Briefly, a solid culture of *P. aeruginosa* grown on the Luria agar plate was used as inoculum for the MIC, and increasing concentrations of colistin and curcumin (as shown in figure S1/S2) were added in the 2 ml Luria Bertani medium. This was kept at 37℃ overnight, and the growth profile was checked by measuring absorbance at 600 nm optical density (OD). The concentration where growth was completely inhibited indicates the minimum inhibitory concentration of colistin and curcumin.

Motility

Swimming, Swarming, and Twitching motilities were examined through a plate-based method described in previous studies with some modifications [16]. Different agar concentrations were used in the LB medium to evaluate each form of motility. The swarm medium contained 0.5% (wt/vol) agar, whereas LB broth was supplemented with 0.3% and 1.0% (wt/vol) agar for the swim and twitch media. The agar solidifies after 3–4 h on the bench top, due to its low viscosity, a single colony of *P. aeruginosa* isolates was inoculated inside the surface of swarming agar plates (0.5% LB agar) and incubated at 37℃ for 24h. Inoculum should not come in contact with the basal petri plate surface, twitch motility may occur and interfere with the interpretation of the radial growth. The effect of colistin and curcumin on *P. aeruginosa* swarming motility was carried out as per the above-described protocol. *Staphylococcus aureus* strain was taken as a negative control as it is a non-motile bacterium. The different inhibitory and sub-inhibitory concentration of colistin and curcumin was incorporated in the 0.5% LB agar media. A single colony of *P. aeruginosa* colony was inoculated in the presence and absence (control) of different concentrations. Each experiment was carried out at least three times. All resulting colonies were analyzed by measuring the surface coverage on agar plates after 24 and 48 hours of incubation at 37℃.

Biofilm formation assay

Biofilm formation assays were performed following a previously described method with few modifications [17]. Crystal violet assay was done to determine biofilm formation quantitatively. *P. aeruginosa* is inoculated in the LB media and incubated at 37℃ for 24, 48, and 72 hrs to analyze biofilm formation. The sessile isolates forming biofilms on the walls of glass test tubes are stained with crystal violet for 15 mins. After planktonic cells are discharged by rinsing twice with the phosphate-buffered saline (PBS), the crystal violet-stained test tube is rinsed twice with PBS to release the excess stain. After air drying of the test tube,

visible film lined on the wall, and the bottom of the tube indicated biofilm production. The dye incorporated by the adherent cells was solubilized in 130µl of absolute ethanol. The colored alcoholic solution was transferred to 96 well microplate to take absorbance reading at 640 nm and assess the biofilm formation in Victor Nivo-Multimode Plate Reader. The different inhibitory and sub-inhibitory concentration of colistin and curcumin was incorporated for biofilm assay in 24, 42, and 72 hrs to assess the effect of colistin and curcumin on biofilm formation. The assays were performed in triplicates, and analysis was done by GraphPad Prism software.

Evaluation of viable planktonic cells

The viable cell forming unit method was used to determine the quantity of viable planktonic (suspended) bacteria. 100 µl of biofilm solutions were taken from different sets, serially diluted to 10^{-6} in PBS, and spread plate on a nutrient agar plate to determine viable planktonic bacteriaand expressed as CFU/mL as described previously with some modifications [18]. Single colonies were counted, and graphical analysis was done in GraphPad prism.

Haemolysis

Typically, ethylenediaminetetraacetic acid stabilized human blood samples were freshly obtained from healthy adult volunteers [19]. In the beginning, 5mL of blood sample was added to 10ml of 50 mM PBS, and then red blood cells (RBCs) were isolated from serum by centrifugation at 2000rpm for 10 min; the RBCs were further washed three times using 50mM PBS. The purified RBCs were then diluted to 5% hematocrit value with PBS. The overnight culture of *P. aeruginosa* was centrifuged at 3000 rpm for 3 mins, washed with PBS three times,and finally suspended in 2ml of PBS. A 100µL of RBC suspension was dispensed into an Eppendorf tube containing 100µL of bacterial suspension in PBS. Sub-inhibitory and inhibitory concentrations, i.e., 100 µg/ml and 300 µg/ml of curcumin, were chosen for the hemolysis test. After 1h of incubation at 37°C, the mixture was centrifuged at 1500 rpm for 10 min, and 20µL of the supernatant was added to 80µL of PBS in a fresh 96-well plate. Absorbance was measured at 414 nm OD using the microplate reader mentioned earlier. 0.1% Triton X-100 (v/v) was used as a positive control (100% hemolysis), whereas PBS as negative control (0% hemolysis). The percentage of hemolysis was calculated using the following equation.

% hemolysis = (OD of sample−OD of PBS)/ (OD of positive control−OD of PBS) × 100

Scanning electron microscopy (SEM) observation

The bacterial culture was prepared the same way as the bactericidal analysis performed [20]. The *P. aeruginosa* was grown to a mid-exponential phase, washed thrice with 10 mM PBS, and diluted in the same buffer to OD_{600} = 1.0 (cell number 10^9). The concentration of the colistin and curcumin were kept at 8 µg/ml and 100 µg/ml, 300 µg/ml, respectively. After twohours of incubation at 37℃, cells were removed by centrifuging at 6000 rpm for 10 minutes. The cells were washed thrice with 10 mM PBS (pH 7.5) to eliminate all kinds of salts associated with them. 2.5% of glutaraldehyde was used to fix the bacterial cell at 4℃ overnight. The next day, fixed cells were again collected by centrifugation at 6000 rpm for 10 minutes and washed with 10 mM PBS (pH 7.5) to wash out the excess fixative. SEM analysis was performed on Quanta FEG 250 operating at an accelerating voltage of 20 kV. Silicon wafers were used to prepare SEM specimens. For each sample, a droplet of bacterial suspensions was cast on a silicon wafer and dried overnight in a vacuum desiccator. The samples were gold coated before inserting into the microscope.

Result

Determination of MIC of Colistin and Curcumin for the isolated strain

Determination of minimum inhibitory concentration (MIC) is essential as it indicates the efficiency of drugs. The MIC of colistin and curcumin were measured separately. In the case of colistin, MIC determination was evaluated gradually from 0.5 µg/ml to 10 µg/ml. LB broth without any antibiotic was kept as a negative control. Test tubes with 0.5, 1, 2, 4, and 5 µg/ml concentration showed visible growth, while no growth was seen in the tube having 6 µg/ml ofconcentration. Therefore, 6 µg/ml stands as MIC for colistin (S2 Figure A). We, therefore, chose 4 µg/ml as the sub-inhibitory concentration and 8 µg/ml as the inhibitory concentration in the preceding experiments.

MIC of curcumin was done similarly with appropriate concentration from 50µg/ml to 500µg/ml in MHB medium. As curcumin is a colored compound, the absorbance of each concentration of curcumin was noted and subtracted from the respective set where bacteria are growing (S2 Figure B). Results indicate that 200µg/ml to 250µg/ml concentration was found to be a threshold of the inhibition above which the growth of the organism reduced significantly. Thus, we considered 250 µg/ml as the MIC of curcumin, and anything above this concentration is selected as an inhibitory concentration.

Characterization of swarming type motility and inhibitory effect of colistin on swarming type of motility

Motility often influences biofilm formation in *P. aeruginosa.* The property of biofilm formation is inversely affected by swarming motility, whereas swimming motility increases initial attachment to surfaces during biofilm development [16]. To evaluate the relationship between biofilm formation and motility in our strain, we did various experiments with colistinto determine the possible correlation between these two phenomena. The swarming activity of *P. aeruginosa* in Luria agar is highest compared to swimming

and twitching types. (Figure 1A c). Motility decreases steadily in high colistin concentrations (Figure 1B h).

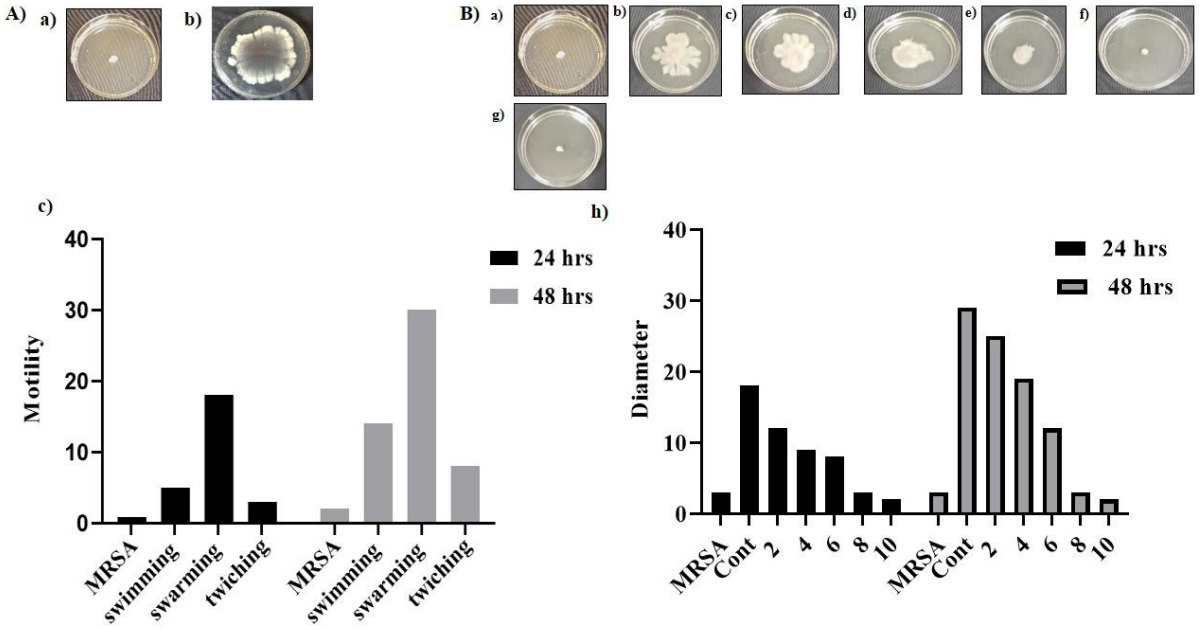

Figure 1: Motility analysis in presence and absence of colistin. A. Figure represents motile nature of *P. aeruginosa* on a motility plate containing 0.5% agar. Non-motile MRSA strain has been taken as a control. **B.** Swarming motility of *P. aeruginosa* on a plate containing 2 µg/ml, 4 µg/ml, 6 µg/ml, 8 µg/ml, and 10 µg/ml of Colistin. The picture depicts a gradual decrease in the diameter of motility as the concentration of antibiotic is increased. **C.** Graph represents comparison between different types of motility of *P. aeruginosa. Pseudomonas sp.* shows the highest swarming motility and lowest twitching motility whereas swimming has an intermediate effect. A relative enhancement of motility was observed compared at 48 h time point in compare to 24 h. **D.** Graphical representation of effect of Colistin on Swarming Motility of *P. aeruginosa. The motility decreases with increasing concentration of colistin.* There is a visible increase of motility in 48 h when compared to 24 h culture. 8 µg/ml and 10 µg/ml of colistin treated samples showed minimum swarming motility in both cases.

Quantitative analysis of Biofilm formation in the presence and absence of colistin

While *P. aeruginosa* strains possess complex, diversified genomes [21] and readily form biofilms [22]. It is unclear whether there are any variations in biofilm formation among clonally diverse clinical and environmental isolates of *P. aeruginosa*. The environmental isolate was tested for biofilm-forming ability using the Test Tube method using crystal violet staining. Absorbance at 640 nm was taken to determine the quantity of biofilm produced at 24, 48, and 72h. Graphical representation showing the quantitative analysis of Biofilm formation by *P. aeruginosa*. (Figure 2A). Without any effector, biofilm production increases at 48 hours and gradually decreases at 72h. After 48h of incubation, biofilm formation is highest and considered to be 100%. The extent of biofilm formation at other time points has been normalized against 48-hour results. After 48 hours, biofilm formation decreased, and the lowest amount of biofilm was seen at 72 hours. Dose-response experiments were conducted tostudy the effect of antibacterial agents such as colistin and curcumin. Results show that biofilmproduction increases in the presence of sub-inhibitory doses of both colistin and curcumin. Theamount of biofilm increases with 2 to 4µg/ml of colistin, which are sub-inhibitory concentrations (Figure 2B). However, biofilm production decreases in the presence of an inhibitory dose (8 µg/ml) due to better accumulation inside the matrix that can eventually kill the biofilm-producing cells. We also analyzed the viability of *P. aeruginosa* while producingbiofilm. As expected, severe loss of bacterial viability was seen when the biofilm was exposedto an inhibitory dose of colistin (S3 Figure A). As a result, lower number of viable planktonic cells were present with 2 µg/ml as the most of the cells are engaged in formation of biofilm leaving smaller number of planktonic cells available in the medium. The number of planktonic growth increases after 72 h as the biofilm dispersal takes place at this point of time.

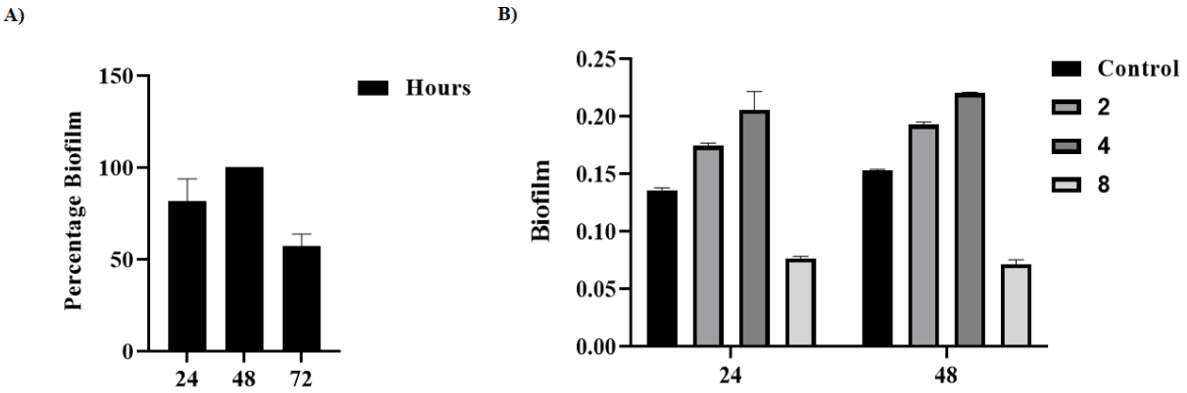

Figure 2: Biofilm formation in presence and absence of colistin. A. Graph represents the percentage formation of biofilm in different time points, i.e. 24h, 48h, and 72 hrs. Maximum biofilm was formed at 48 hour and considered as 100%. The amount of biofilm formation on other time points has been normalized against it. Reduction in biofilm formation was observed after 72 hrs. due to the dispersal process. **B.** Graph represents the effect of different concentrations of Colistin on biofilm formation at 24 h and 48 h. In the presence of a sub-inhibitory dose (2 µg/ml, 4 µg/ml), biofilm production is enhanced in comparison to control where no antibiotic is applied. In stark contrast, biofilm production drastically decreases at the inhibitory concentration (8 µg/ml). There is an overall enhancement of biofilm production at 48 h compared to the 24 h profile. Biofilm production is quantified by measuring the OD at 600 nm of the dissolved crystal violet. Data are expressed as mean ± standard deviation (STD).

Characterization of the inhibitory effect of curcumin on swarming type of motility

The effect of curcumin on the swarming motility of Pseudomonas was observed. The result implicates the decrease in motility in presence of gradual higher concentrations of curcumin (Figure 3A). Although the color of curcumin is an obstacle to growth measurement, we could estimate the motility of *P. aeruginosa* by subtracting the absorbance from the blank in each set. Loss of motility at inhibitory concentrations may be due to loss of survival or distortion of the structure of pilus or there is any effect at the genetic level is a matter of future investigation. Generally, bacterial responses to antimicrobials are concentration-dependent, and our result manifests the same trait.

Quantitative analysis of Biofilm formation in presence of curcumin

Results show that biofilm production increases in the presence of sub-inhibitory doses of curcumin such as 100 and 200 µg/ml (Figure 3B). The highest amount of biofilm formation was observed at 200 µg/ml of concentration. However, biofilm production decreases in the presence of an inhibitory dose of curcumin (300 µg/ml) which is correlated to the mortality of the bacteria under an inhibitory dose of the phytochemical. We also analyzed the viability of the bacterial cells during biofilm production of the *Pseudomonas* strains in the presence of predetermined sub-inhibitory and inhibitory doses of curcumin. As expected, severe loss of bacterial viability was seen when the biofilm was exposed to an inhibitory dose of the antibacterial substance (S3 Figure B). In presence of a sub-inhibitory dose (100 µg/ml), the highest viability was seen at 24 h. This result manifests low biofilm formation and more number of planktonic cells were observed under that condition. The number of live bacteria decreases drastically at 48 h in presence of sub-inhibitory curcumin which reinforce the inverse correlation between biofilm formation and presence of the planktonic cells in the medium.

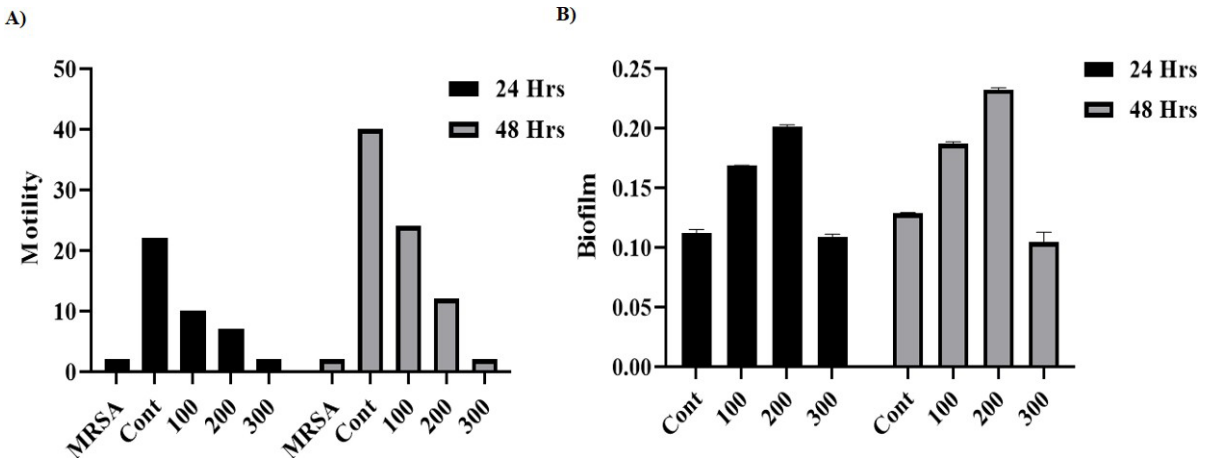

Figure 3: Motility analysis in presence of curcumin. A) Effect of different concentrations of Curcumin on swarming motility of *P. aeruginosa*. Higher concentrations of curcumin affect the swarming motility of P.aeruginosa. The graphical representation shows that in presence of 100 µg/ml, 200 µg/ml, and 300 µg/ml of curcumin, motility sharply decreased in 24 h culture. The overall motility was increased at 48 h but the relative motility followed the same trend as 24 h culture, i.e. motility gradually decreased in presence of an increasing concentration of colistin. Lowest motility was observed with the inhibitory concentration (300 µg/ml of colistin). **B) Biofilm formation analysis in presence of curcumin.** The graph represents the effect of Curcumin on biofilm formation over 48 h time point. In the presence of sub-inhibitory doses (100 µg/ml and 200 µg/ml) of Curcumin, biofilm production is enhanced. Biofilm production drastically decreases with inhibitory concentration (300 µg/ml). The relative profile of biofilm production remained same at both time points. Biofilm production is quantified by measuring the absorbance at 600 nm of dissolved crystal violet. Data are expressed as mean ± STD.

Evaluation of the synergistic effect of different combinations of colistin and curcumin on *P. aeruginosa*

A combinatorial treatment was done with colistin and curcumin on *P. aeruginosa* to see whether colistin can augment its inhibitory effect in the presence of curcumin and vice versa. To perform this assay, we have chosen a sub-optimal concentration of one agent accompanied by the threshold concentration of the other from where it starts producing its inhibitory effect on the organism. For instance, 4 µg/ml is a threshold concentration of colistin, whereas 2 µg/ml is a concentration that doesn't hamper the growth of the bacteria at all. In the case of curcumin, similar low concentrations are 25 µg/ml and 50 µg/ml. If any functional synergism exists, a reduction of the minimum inhibitory concentration (MIC) of the threshold component should occur. Alternatively, we can see its effect on bacterial growth as well. Results showed that Colistin in combination with curcumin could effectively inhibit *P. aeruginosa* growth (Figure 4A). The 8 hrs long growth curve of *P. aeruginosa* in the presence of different combinations of curcumin and colistin shows functional synergism, where the separate application of curcumin (50 µg/ml) and Colistin (4 µg/ml) did not make any effect on the growth. Synergistic inhibition of growth was observed in different combinations such as (50 µg/ml curcumin + 4 µg/ml colistin,), (25 µg/ml curcumin + 4 µg/ml colistin), (25 µg/ml curcumin +2 µg/ml colistin), (50 µg/ml curcumin +2 µg/ml colistin) exhibiting 1.5 to 3-fold reduction in MIC values of colistin and 5 to 10-folds reduction of curcumin (Data not shown). To confirm the synergistic effects of colistin and curcumin on *P. aeruginosa,* the bactericidal assay was performed at 4 h and 8 h time points (Figure 4B). *P. aeruginosa* was treated with the same combination of antimicrobials (50 µg/ml curcumin + 4 µg/ml colistin), (50 µg/ml curcumin + 2µg/ml colistin), (50 µg/ml curcumin + 2 µg/ml colistin) and the result was manifested in terms of colony forming units (CFU) that indicates the presence of live bacteria in the culture. 50 µg/ml of Curcumin shows no effect on growth inhibition, while 4 µg/ml of Colistin exhibits a moderate effect. Almost no growth was observed when colistin and curcumin were combined, further validating our previous result. Therefore, it can be stated that Colistin shows a synergistic effect with curcumin inhibiting the growth of *P. aeruginosa.*

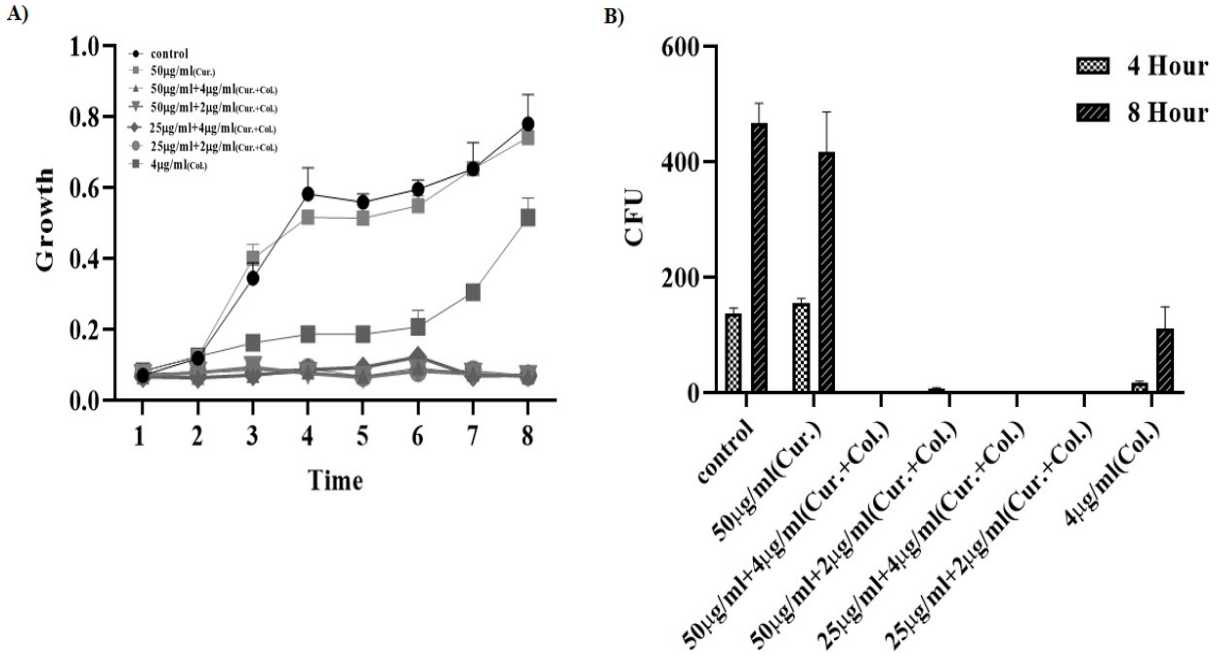

Figure 4: Synergistic effect of curcumin and colistin. A. Analysis of functional synergy between curcumin and colistin on the growth of *P. aeruginosa*. Sub-inhibitory concentrations of colistin, i.e., 4 µg/ml and 2 µg/ml, and similar concentrations of Curcumin i.e., 50 µg/ml and 25 µg/ml, were selected for the assay. LB-grown *P. aeruginosa* without antibiotic was used as a control. Synergistic effect of combinations of (50 µg/ml curcumin + 4 µg/ml colistin), (25 µg/ml curcumin + 4 µg/ml colistin), (25 µg/ml curcumin + 2µg/ml colistin), (50 µg/ml curcumin + 2 µg/ml colistin) was analyzed against 50 µg/ml of curcumin and 4 µg/ml of colistin. Cell viability was determined by measuring OD_{600} nm. The growth curve showed the synergistic effect of different combinations of curcumin and colistin. **B.** The presence of live bacterial cells was analyzed to confirm the synergistic effect of colistin and curcumin. Enumeration of the bacterial colony from 4 and 8 hours culture showed the highest CFU in control followed by the sample where 50 µg/ml of curcumin was applied. The effect was very negligible in presence of 4 µg/ml of colistin. Combinatorial application of both antimicrobials produces a drastic decrease in viable bacterial cell number.

Curcumin reduces the hemolytic activity of *P. aeruginosa*

RBC hemolysis analysis was done in the presence of curcumin (Figure 5). 0.1% of Triton X100 (V/V) was taken as a positive control as it can lyse the red blood cells. In the absence of curcumin, *P. aeruginosa* lyse RBC to 70%. These opportunist gram-negative bacteria produce several hemolytic and other toxins that render toxicity to red blood cells (RBC). When curcumin was added at a sub-inhibitory concentration (100 µg/ml) and inhibitory concentration (300µg/ml) to *P. aeruginosa* culture, hemolysis decreased drastically, almost to the extent of 50%. At a concentration of 100µg/ml of curcumin, hemolysis was reduced to an extent of 35% in comparison to the value of positive control. Upon administration of inhibitory concentration (300µg/ml), the extent of hemolysis further decreased and ultimately came down to 20% of the positive control. Perhaps the loss of viability in the presence of an inhibitory dose of curcumincan reduce the hemolysis, or there might be direct genetic regulation of toxin genes that can influence the process.

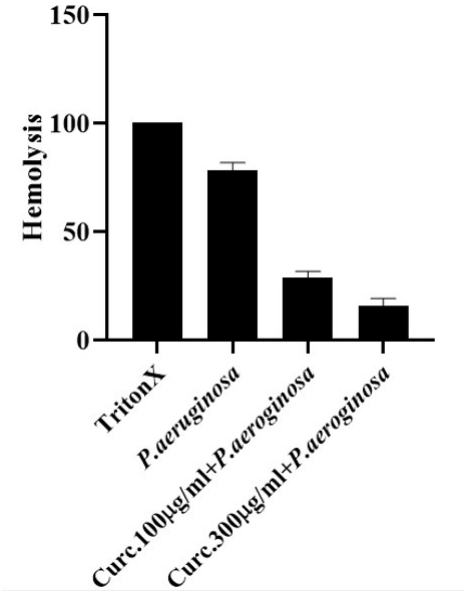

Figure 5: Hemolysis in presence of curcumin. RBC hemolysis analysis in the presence of curcumin. 0.1% of Triton X100 (V/V) completely lyse the RBC and is taken as a positive control. In absence of curcumin, *P. aeruginosa* lyse RBC in an extent of 70%. When Curcumin was added to the sample, the hemolysis property of *P. aeruginosa* decreased drastically decreases to an extent of almost 50%.

Phenotypic changes of *P. aeruginosa* observed under Scanning Electron Microscopy

Scanning electron microscope (SEM) analysis of *P. aeruginosa* was done in absence (Figure 6A) and presence of sub-inhibitory and inhibitory doses of curcumin and colistin. The methodology described the 24 h treatment of the antimicrobial agents in all cases. In presence of 100 µg/ml curcumin, some distortions in the cell shape were observed, such as some cells transformed into elongated shape due to the stress exerted by the antimicrobial compound (Figure 6B). In presence of 300 µg/ml concentration of curcumin which is very much lethal to the bacteria, a minimal number of cells are seen to be present under microscope vindicating its inhibitory effect on the bacteria. (Figure 6C). The small number of dead cells that are present under the microscopic view, clearly show the difference in morphology and shape in comparison to the active *P. aeruginosa* cells. In the presence of an inhibitory concentration of colistin (8 µg/ml), large clusters of cells were observed, implicating the loss of viability leading to aggregation of cellular mass. (Figure 6D).

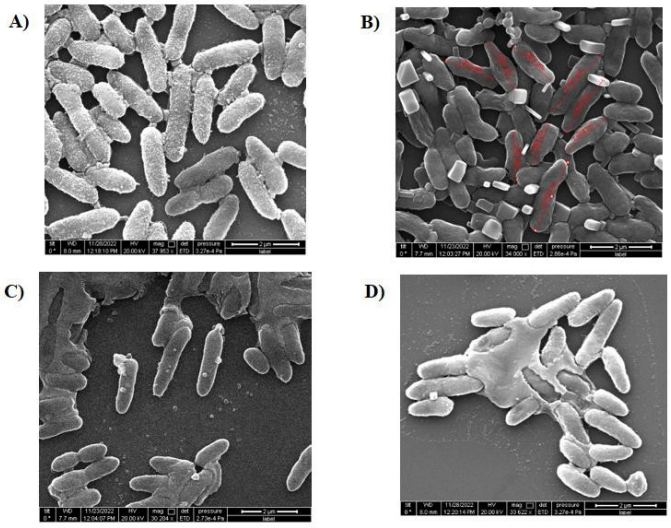

Figure 6: Scanning electron microscope (SEM) analysis. Scanning electron microscope analysis was done in presence of different curcumin concentrations. 2µm atomic resolution is displayed in all cases. A) *P. aeruginosa* cells were visible under scanning electron microscope without any antimicrobial. B) Distorted cells were observed in presence of 100 µg/ml of curcumin; some cells are shown to be more elongated than others. C) In presence of the inhibitory dose of curcumin (300 µg/ml), depleted bacterial cells were present under microscopic view D) Cells treated with the inhibitory dose of colistin (8 µg/ml) show aggravated clumping and aggregation due to complete loss of viability.

Discussion

P.aeruginosa is a primary soil bacterium gaining tremendous clinical importance nowadays due to its increasing antibiotic tolerance. The ability to produce biofilm facilitates the development of chronic infectious diseases and recalcitrant infection [23]. Biofilm production of *Pseudomonas* species is primarily driven by different genetic factors and environmental regulators based on where they reside. As biofilm is shown to be effective against antibiotic stress, other antibiotics initiate the process within a range of concentrations before it can finally kill the organism [24].

Various microorganisms have been reported to produce secondary metabolites that exert antibiofilm activity. Moreover, chemical substances isolated from plants prevent bacterial biofilm formation when applied in selective concentration. To observe the effect of colistin, which is one of the last resort antibiotics,

and phytochemical curcumin, which has significant antibacterial and anticancer effects, we found that both the substances promote biofilm formation within a range of concentration (subinhibitory concentration) and beyond that pointproduce a deleterious effect on the bacterium [25,26]. We noticed that *Pseudomonas* species have maximum biofilm in the presence of 4 µg/ml of colistin during the 48 h. Still, the amountgoes down as time progresses due to the dispersal of cells from the complex structure. The temporal expression of biofilm-producing genes regulates the amount of biofilm controlled byother environmental factors during different combinations [27]. Above 4 µg/ml concentration, the bacterium becomes sensitive towards colistin, and production of the biofilm decrease gradually, which causes incremental stress on the bacteria and causes a loss of viable numbers. The amount of biofilm almost diminishes at 8 µg/ml concentration during 72h (Figure 2) with concomitant reduction of viable cell number (S3 Figure). Interestingly after 48 hours, when *Pseudomonas aeruginosa* showed maximum biofilm formation in the absence of colistin, applying the sub-inhibitory dose (up to 4µg/ml) of the drug doesn't make any significant difference in terms of biofilm formation.

Expectedly, the number of planktonic cells increases during the 72 h time point in the presence of a sub-inhibitory dose but is destroyed by an inhibitory dose (8 µg/ml) which also inhibits the production of biofilm. The result, therefore, interprets that the subinhibitory dose of colistin favors the growth of sessile cells over free-living planktonic cells; however, the effect is completely lost in the presence of a higher concentration of the drug (Figure 7). A similar phenomenon was seen with phytochemical curcumin. Curcumin increases the membrane permeability of the bacteria and induces different mechanosensitive channel protein that diffuses the stress produced by osmosis and other processes (Wray et al., 2021) [28]. After coming into the cytosol, curcumin inhibits the polymerization of filamenting temperature-sensitive mutant Z protofilaments and disturbs the guanosine triphosphatase activity in the cytoskeleton of *Escherichia coli* [5,29]. Therefore, this chemical agent works as an antimicrobial agent and promotes the action of other antibiotics when combined. *P. aeruginosa* strives to alleviate the stress caused by curcumin within a sub-inhibitory dosage (up to 200 µg/ml). The amount of biofilm production modestly increases at 48h compared to 24h. In presence of 300 µg/ml of curcumin, the biofilm production significantly decreases in both cases (Figure 3).

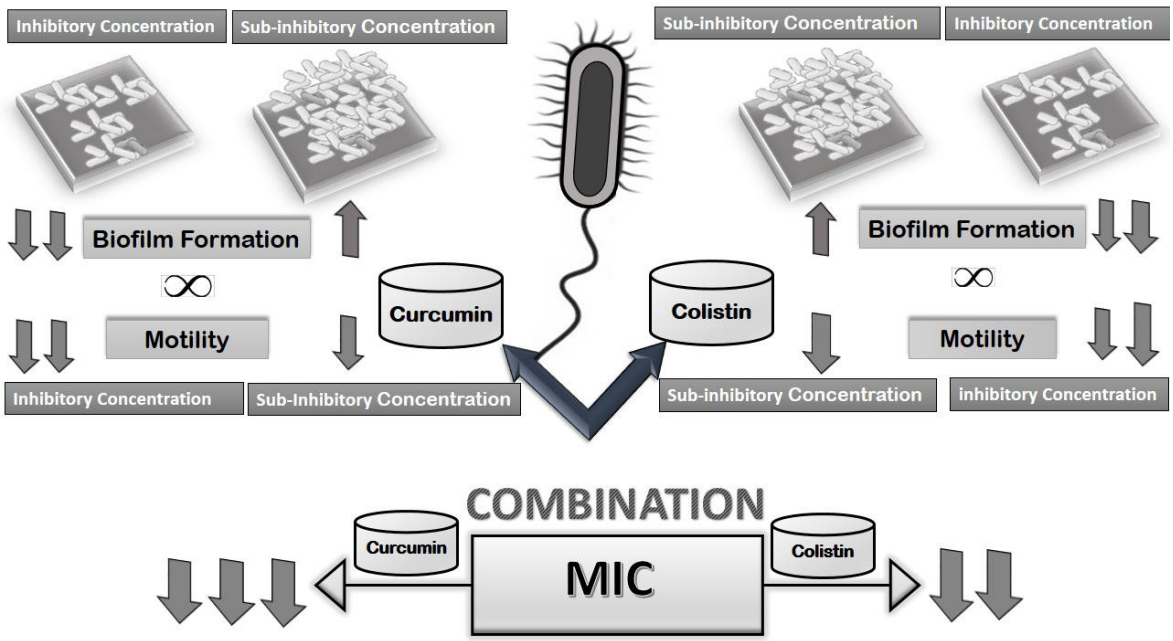

Figure 7: Model representation of the research findings of our study. Biofilm formation is promoted in presence of sub-inhibitory concentration of curcumin and colistin. When treated with inhibitory concentration, biofilm formation is decreased drastically. In context of motility, as the concentration of colistin and curcumin increases, we observe a gradual decrease in motility. While comparing motility and biofilm formation at sub-inhibitory concentrations, we saw an inverse correlation between these two phenotypic properties of *Pseudomonas* species. A combination of colistin and curcumin, when used on the *Pseudomonas aeruginosa,* showed a synergistic effect in inhibiting bacterial growth. The result was manifested with reduction in MIC and viable bacterial number.

When we checked the viability of the planktonic cells with the inhibitory dosage, irrespective of the time frame, a drastic loss of planktonic cells of *P .aeruginosa* was seen that correlates to the analysis done with colistin. To see the effect of colistin and curcumin when manifested together, we found these two agents work synergistically to kill the opportunistic pathogen, and the minimum inhibitory concentration (MIC) decreases 1.5 to 10 folds from their threshold value separately (Figure 7). That implies the coordinated role of two agents in destroying the bacteria.

Motility is an essential factor influenced by environmental conditions and controlled by several genetic factors helping different bacteria colonize on a particular surface. Apart from specific genes expressed during flagellar motion, chemical agents like colistin and curcumin seem to control the process based on the different concentrations they have been used. Curcumin inhibits motility either by binding to the flagella directly or interfering with the quorum sensing pathway that helps bacteria to gain resistance against environmental assaults and reduces pathogen activity during infection of the host cell [30-32]. In contrast, colistin doesn't directly affect the flagellar motility of different bacteria; however, it disrupts the outer membrane's organization and destroys the lipopolysaccharide layer, thereby reducing the motility by damaging the cell integrity [33]. As a consequence of the effect, we can notice that the motility of *Pseudomonas aeruginosa* decreases with a sub-inhibitor concentration of curcumin. In contrast, motility was almost lost in an inhibitory amount (Figure 7). Colistin also appears to be very effective in reducing bacterial motility as gradually increasing concentrations of this antibiotic impair the motile nature of the bacterium. Phenotypic alternation was observed in different concentrations of colistin and curcumin. Excess colistin shows loss of cell structure with aggregation of dead cells under scanning electron microscopy (SEM). As polymyxin class of antibiotics affects the cell wall structure by forming pores that help enter other antimicrobials inside the cell. Consequently, we could see the visible clumping of dead *Pseudomonas aeruginosa* throughout the microscopic field. Alternatively, curcumin imposes stress and causes cell death by inhibiting different cellular pathways necessary for cell survival. In higher concentrations, visible loss of cells was seen by scanning electron microscopy.

Toxin production is one of the critical features of manifesting virulence. At the onset of the early stationary phase, when bacteria start producing toxins, a cellular regulatory circuit such as quorum sensing comes into play to control various physiological responses such as the production of toxin [32]. Curcumin was shown to affect toxin production, and when *Pseudomonas aeruginosa* was treated with an increasing concentration of this agent, the hemolytic effect of this organism decreased drastically (Figure 4). The hemolytic property of *Pseudomonas aeruginosa* is regulated by secretory protein phospholipase C, which might be indirectly controlled by curcumin, or the loss of survival of the *P. aeruginosa* by curcumin may also cause inhibition of hemolysis. The future experiment can determine the exact molecular mechanism of the incident. Increasing cases of antibiotic resistance found in multidrug resistance (MDR) organisms necessitate alternative therapy instead of using a single drug for the treatment process. Our combined therapy of colistin and curcumin has been demonstrated as an efficient system of inhibiting the growth and biofilm formation of the opportunist pathogen *Pseudomonas aeruginosa*.

As colistin is already established as a potential drug for treating gram-negative bacteria, it can be used with other antibiotics to get the best possible effect. Colistin and other drugs can be used by conjugating with a nanocarrier that produces a selective advantage of penetration inside the bacterial cell. Therefore, the implication of combined therapy is very significant and can be implemented in such cases where a single drug is not enough to get rid of the infection. Our article provides an initial idea of the combined therapy of different antimicrobials that may be more efficacious in treating nosocomial infections.

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Author's contribution

AB designed the experiments. BM, SB, AHK, and SM performed the experiments. AB, BM, PB, AG, and SC analyzed the data. AB, AD, BM, PB and ARM proofread the manuscript.

Compliance with ethical standards

This article contains no studies with human participants performed by any authors.

Conflict of interest

The authors declare that they have no conflicts of interest.

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Supplementary Figures

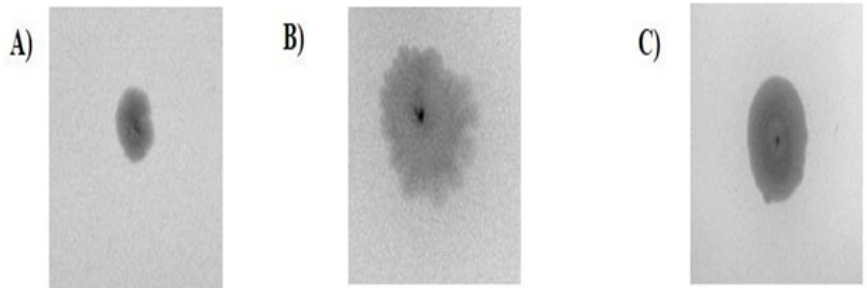

Figure S1: Motility analysis of *P. aeruginosa* isolated from the soil sample. A) MRSA strain motility assay on LB agar medium for negative control **B)** 1% agar plates for twitching motility **C)** 0.3% agar plates for swimming Plates were incubated at 37 °C for 24h.

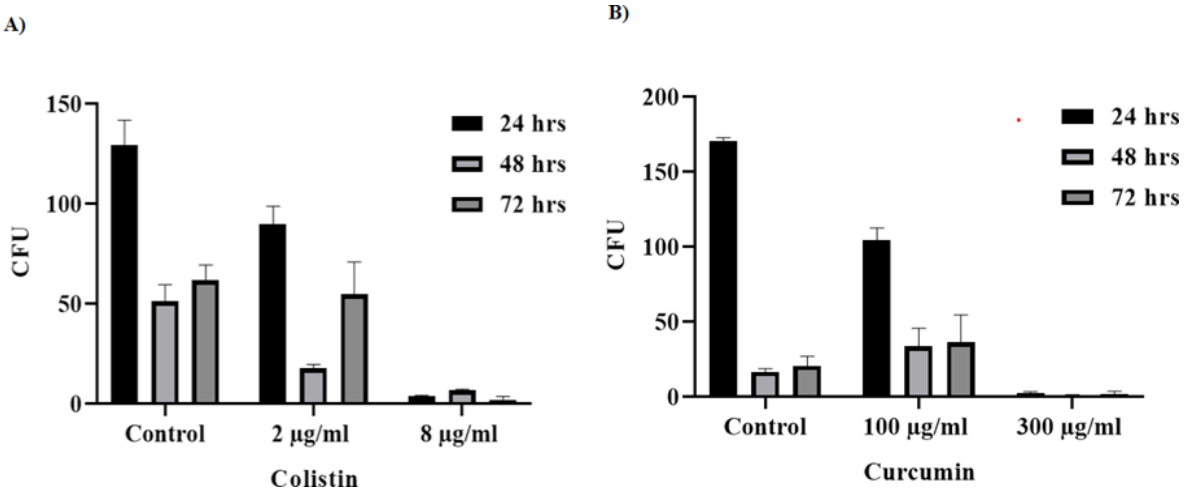

Figure S2: MIC against colistin and curcumin. A) MIC result of Colistin against the Pseudomonas strain. The concentration taken was 0.5–10 µg/ml. Growth decreased from 3 µg/ml and stopped at 6 µg/ml. Minimal growth was seen at 4, 4.5, 5, and 5.5 µg/ml concentrations. MIC was determined to be 6 µg/ml. **B)** MIC of curcumin against the Pseudomonas strain. Bacterial growth was measured in presence of 10 µg/ml – 400 µg/ml of curcumin. Growth decreased drastically from 150 µg/ml and stopped at 250 µg/ml. Therefore the MIC of curcumin falls in between 200-250 µg/ml. Concentration above that is termed as inhibitory concentration.

A)

Colistin concentration (µg/ml)	Growth profile
0	++++
0.5	++++
1	+++
1.5	+++
2	+++
2.5	+++
3	++
3.5	++
4	+
4.5	+
5	+
5.5	+
6	-
6.5	-
7	-
7.5	-
8	-
8.5	-
9	-
9.5	-
10	-

B)

Curcumin concentration (µg/ml)	Growth profile
0	++++
10	++++
50	+++
100	++
150	++
200	+
250	-
300	-
350	-
400	-

Figure S3: Viable cell count of planktonic growth in biofilm formation. A) Graphical representation of viable cell number of planktonic cells present during biofilm formation in presence of Colistin. An inhibitory concentration of 8 µg/ml and a sub-inhibitory concentration of 2 µg/ml were selected for the experiment. CFU counting was done using 10^{-7} dilutions. Within sub-inhibitory concentration, more number of viable planktonic cells were seen at 24 h. Since more biofilm formation takes place during 48 h, fewer planktonic cells were observed. Planktonic cells were increased at 72 h due to the dispersal process. **B)** Graphical representation of the number of viable planktonic cells during treatment of curcumin. An inhibitory concentration of 300 µg/ml and a subinhibitory concentration of 100 µg/ml were taken for the observation. Viable planktonic cells remained maximum during 24 h and gradually decreases after that. In presence of sub-inhibitory concentration (100 µg/ml) of curcumin, more biofilm is produced, resulting in fewer planktonic cells. Application of inhibitory dose (300 µg/ml) significantly reduce the viable cell number.