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RESEARCH ARTICLE

Glutathione's potential to attenuate quorum sensing induced biofilm formation in *Klebsiella pneumoniae* and *Serratia marcescens*

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ABSTRACT

Rapid advancements not only facilitate human adaptation but also trigger environmental adjustments. While pivotal discoveries like Penicillin revolutionized medicine, the subsequent overuse of antibiotics led to diminishing efficacy due to antibiotic resistance. Addressing biofilm formation as a major contributor to antimicrobial resistance and recognizing quorum sensing as a key factor in biofilm formation, there is a need for new strategies. Glutathione, a natural antioxidant, has shown promising potential as an effective antimicrobial agent and a reliable component for cellular defence in the immune system. This study explores the capability of Glutathione to mitigate quorum sensing-induced biofilm formation in *Klebsiella pneumoniae* and *Serratia marcescens*. The results demonstrated that glutathione induced ROS-mediated cell death in these bacteria. Glutathione exhibited a maximum inhibition of approximately 85% in biofilm formation for both *K. pneumoniae* and *S. marcescens*. It also effectively disrupted preformed biofilms by degrading the eDNA of the EPS layer of matured biofilms. Interestingly, glutathione attenuated the quorum sensing pathway, as evidenced by reduced production of virulence factors, thereby mitigating QS-induced biofilm formation in both bacteria. This work lays the groundwork for further exploration in developing glutathione as a novel antibiotic to combat antibiotic resistance.

Keywords: Glutathione; antibiotic resistance; biofilms; quorum sensing; *Serratia marcescens*; *Klebsiella pneumoniae*

1. Introduction

In the pursuit of a disease-free world, the daunting challenge of antibiotic resistance poses a significant obstacle, morphing into a global menace that disrupts healthcare landscapes worldwide. This resistance not only precipitates a surge in the global death toll, impacting millions annually, but it also casts a formidable shadow over developed nations like Australia. Beyond its grim human toll, antimicrobial resistance exacts a substantial economic toll, with projections hinting at a potential global cost surpassing a trillion by 2050. Within this complex scenario, bacterial biofilms emerge as pivotal contributors to antimicrobial resistance (AMR), forming resilient colonies that defy conventional antimicrobial treatments and evade host immune responses[1].

Bacterial biofilms, intricate communities of microorganisms encased in an extracellular matrix, are pivotal players in the complex landscape of antibiotic resistance (AMR). Unlike their free-floating counterparts, bacteria within biofilms exhibit heightened resistance to antimicrobials, presenting a formidable challenge in

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both clinical and environmental settings. These resilient structures not only shield bacteria from the host immune system but also create a conducive environment for genetic exchange, promoting the spread of resistance traits among bacterial populations[2]. The development of antibiotic resistance within biofilms is a multifaceted process. The closed and compact architecture of biofilms provides a protective shield, making it challenging for conventional antibiotics to penetrate and effectively target bacterial cells. Moreover, the slowed growth rate of bacteria within biofilms, coupled with the presence of persister cells, contributes to their inherent resistance. Persister cells, resistant to many antibiotics due to antitoxin systems, act as a reservoir for resistance traits, enabling the survival of the entire biofilm colony. Both *K. Pneumoniae* and *S. marcescens* belong to ESKAPE pathogens and are known to form firm biofilms. They are showing resistance to almost all available antibiotics and there is a need to develop new antibiotics that can target their biofilms.

As biofilms continue to pose a significant threat, novel strategies are imperative to tackle the escalating menace of AMR. Glutathione, a tripeptide with diverse cellular functions, emerges as a potential protagonist in this battle. Its multifaceted role extends beyond being a crucial antioxidant, encompassing functions such as cell cycle regulation, growth, and the maintenance of redox homeostasis[1,3,4].

The synthesis and metabolism of glutathione involve energy-dependent processes, orchestrated by enzymes like γ-glutamylcysteine synthetase (γ-GCS) and GSH synthetase. Maintaining a delicate balance between reduced glutathione (GSH) and oxidized glutathione disulphide (GSSG) is vital, as it serves as a sensitive indicator of oxidative stress within cells. Disruptions in this balance, often influenced by enzymes like glutathione reductase, can tip the scale towards oxidative stress, influencing the biofilm formation process[5]. Glutathione's potential as an antimicrobial agent is not only confined to its direct bactericidal effects but also extends to its ability to interfere with biofilm formation. By disrupting the oxidative balance within bacterial cells, glutathione can impede the intricate processes involved in biofilm development. Its anti-biofilm and anti-quorum sensing properties position it as a promising candidate in the pursuit of innovative strategies to combat antibiotic resistance.

The current study explores Glutathione's role in inhibiting bacterial biofilms, offering insights into its antibacterial, anti-biofilm, and anti-quorum sensing properties against *Klebsiella pneumoniae* and *Serratia marcescens*. This research aims to develop Glutathione as a novel therapeutic strategy against antibiotic resistance, contributing to the ongoing efforts to combat infectious diseases.

2. Materials and methods

2.1. Bacterial strains and their growth conditions

All the experiments were performed in a sterile environment inside an LAF (laminar airflow). Bacterial strains Klebsiella pneumoniae (MTCC 432) and Serratia marcescens (MTCC 2645) were cultured and maintained in Growth media 3 (Beef Extract, Yeast Extract, Peptone and NaCl). Bacteria were grown at 37 ℃, 120 rpm in an incubator shaker. All the chemicals required for the experiments were purchased from Sigma-Aldrich, USA.

2.2. Minimum Inhibitory Concentrations (MIC) and Minimum Bactericidal Concentration (MBC)

Minimum inhibitory concentration is defined as the lowest concentration of an antimicrobial agent which inhibits the visible growth of the organism. Microdilution method was used to check the effect of the minimum concentration of glutathione against the two strains Serratia marcescens and Klebsiella pneumoniae. A 10^6 CFU/mL of cells were treated with increasing concentrations of glutathione (0–20 mM/mL) for 24 h. Post incubation (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) assay was performed to

check the viability of the cells. The assay is based on the conversion of MTT by NADPH-dependent cellular oxidoreductase enzymes to formazan crystals which is usually determined by the cell's metabolic activity. Cellular viability was determined by plotting glutathione concentration versus the percentage cell viability graph. Minimum bactericidal concentration is the concentration at which the bacteria were killed after the drug is removed. In this, the treated/untreated cells were again incubated with fresh GM3 media post-glutathione treatment for another 24 h and the viability was confirmed using the MTT assay[6,7].

2.3. Growth kill assay

Growth kill assay or time-kill assay was carried out to evaluate the effect of glutathione on the growth of bacteria over time. A 10^8 CFU/mL of cells were treated with increasing concentrations of glutathione (0–20 mM/mL) for 48 h at 120 rpm, 37 ℃. Bacterial growth was measured spectrometrically every hour till 48 h, and the results were plotted for time on the x-axis and O.D. on the y-axis[8–10].

2.4. Reactive Oxygen Species (ROS) assay

The ability of glutathione to induce ROS was evaluated by quantifying intracellular ROS production. Bacterial cells were allowed to grow till they reached an O.D. of 0.5 and then treated with a fluorogenic dye along with different concentrations of glutathione to measure the ROS accumulation in the cell. In this assay DCFDA or fluorescent dye dichlorodihydrofluorescein diacetate (DCFDA) was used which was deacetylated by cellular esterase which were then oxidized by ROS to form a fluorescent compound which is measured spectrophotometrically with an excitation wavelength of 485 nm and emission wavelength of 535 nm every hour till 5 hours and the graph was plotted as glutathione concentration on the x-axis and DCF fluorescence intensity on the y-axis[10].

2.5. Biofilms inhibition assay

Crystal violet quantification assay was used to check the ability of glutathione to inhibit the formation of bacterial biofilms. The positively charged crystal violet binds to the negatively charged substances present on the EPS layer of the biofilms, hence resulting in staining of the biofilms. Bacterial cell cultures were grown till an O.D. of 0.5 and then treated with increasing concentrations of glutathione (0–20 mM/mL) for 24 h. Post-incubation, the biofilms were treated with 1% crystal violet and incubated in the dark for 30 min. After staining, the cells were washed twice with distilled water, air dried and absolute ethanol was added. After 10 min the absorbance was measured at 590 nm. Results were plotted by taking glutathione concentration on the x-axis and % biofilm inhibition on the Y-axis[6,11,12].

2.6. Biofilms eradication assay

Glutathione's ability to eradicate or distort pre-formed biofilms was evaluated by this assay. Bacterial cultures were treated with 15 mM of hydrogen peroxide to allow biofilm formation. Preformed biofilms were then treated then with increasing concentrations of glutathione (0–20 mM/mL) for 24 h. Post incubation, biofilms were washed, air dried, stained with 1% crystal violet and incubated in the dark for 30 min. After staining, the cells were washed twice with distilled water, air dried and absolute ethanol was added. After 10 min of incubation at room temperature, the absorbance was calculated at 590 nm. Results were plotted by taking glutathione concentration on the x-axis and % biofilm eradication on the y-axis[13].

2.7. Quantification of Extracellular Polysaccharide (EPS)

To test the effect of glutathione on distorting the protective EPS layer of the matured biofilms, this assay was performed. As described above, biofilms were preformed as mentioned above and then treated with increasing concentrations of glutathione (0–20 mM/mL) for 24 h. Further, the cells were treated with 10%

TCA and equal volumes of acetone were added and incubated at 4 ℃ for 24 h. The tubes were centrifuged at 10000 rpm for 10 min at 25 ℃. The weight of the pellet was compared with before and after drug treatment and the difference in weight was used to calculate the percentage of EPS[14].

2.8. Quantification of Extracellular DNA (eDNA)

eDNA was extracted from the standard protocol as mentioned in Vishwakarma and Vavilala 2020. Preformed biofilms were treated with increasing concentrations of glutathione (0–20 mM/mL) and incubated at 37 ℃ for 24 h. Further, the biofilms were kept at 4 ℃ for 1 h, then 1 µL of 0.5 M EDTA was added and centrifuged. The pellet was further dissolved in 50 mM of Tris-HCl of pH 8. e-DNA was extracted by treating the pellet with equal volumes of phenol:chloroform:isoamyl alcohol (25:24:1) and centrifuged. A 400 µL of supernatant was then treated with chloroform: isoamyl alcohol (24:1) in equal volumes and centrifuged. The aqueous phase obtained was then treated with 3 volumes of chilled ethanol and 1/10th volume of sodium acetate of pH 5.2 and the mixture was stored at −20 ℃ overnight. eDNA was pelleted by centrifuging at 18,000 x g at 4 ℃ for 20 min. The pellet was then treated with 10 µL of TE buffer. Purified e-DNA was quantified spectrophotometrically by using the absorbance ratio of A260/ A28[7].

2.9. Quantification of QS induced-bacterial virulence factors production

2.9.1. Lipase activity

The cultures were treated with increasing concentrations of glutathione to determine the lipase activity in the cells. Post incubation, cells were centrifuged at 10,000 x g for 10 min at 4 ℃. The pellets were resuspended in 500 µL of Tris- EDTA buffer of pH 8.0. The resulting cell suspension was sonicated for 30 seconds to lyse the cells. Cell supernatant was used to assay the lipase activity. p-Nitrophenyl Palmitate (p-NPP) was used as a substrate for this assay. 100 µL of supernatant was added to 900 µL of substrate mixture which included two solutions. Solution A contains 3 mg of p-NPP in 1 mL of isopropanol and Solution B contains 10 mg of gum arabicum, 40 µL of Triton X-100 in 9 mL of 50 mM of Tris HCl buffer of pH 8.0. Lipase activity was quantified spectrophotometrically at 410 nm following 20 min of incubation at 60 ℃ in a water bath[15].

2.9.2. Urease activity

Glutathione-treated/untreated bacterial cultures were centrifuged at 5000 rpm for 5 min at room temperature. A 100 µL of bacterial supernatant was then incubated with 50 µL of urea for 3 h at 37 ℃. After incubation, 10 µL of Nessler's reagent was added to the wells and incubated for 5 min. The amount of urease activity was quantified spectrophotometrically at 530 nm[16].

2.9.3. Protease activity

Bacterial protease activity was determined using azocasein as a substrate. The culture supernatant was treated with equal volumes of 0.3% azocasein in 0.05 M Tris-HCl and 0.5 mM calcium chloride. The mixture was then incubated at 37 ℃ for 30 min and after which 600 µL of 10% Trichloroacetic acid (TCA) was added to the mixture to terminate the reaction. The mixture was incubated at −20 ℃ for 20 min. The mixture was then subjected to centrifugation at 8000 rpm for 15 min. To the supernatant 200 µL of sodium hydroxide was added and the protease activity was measured spectrophotometrically at 400 nm[17].

3. Results

3.1. Antibacterial studies

The MIC_{50} of glutathione against *Serratia marcescens* was found to be 6 mM/mL and it is 4 mM/mL for *Klebsiella pneumonia*e respectively. Glutathione showed a bacteriostatic effect for both organisms. Thus, Glutathione showed a promising potential as a good antimicrobial agent to kill bacterial cells.

3.2. Growth kill curves

The growth of the bacteria in the presence of glutathione was assessed over a period of 0 h–48 h respectively. It was observed that bacterial growth was decreased with an increased concentration of glutathione (**Figure 1**). Glutathione significantly impacted the exponential growth of both bacteria. At higher concentrations of glutathione, a decline in the bacterial growth curve as compared to untreated controls was observed, which indicates that glutathione inhibits bacterial growth over a time period in a dose-dependent manner in both these bacteria.

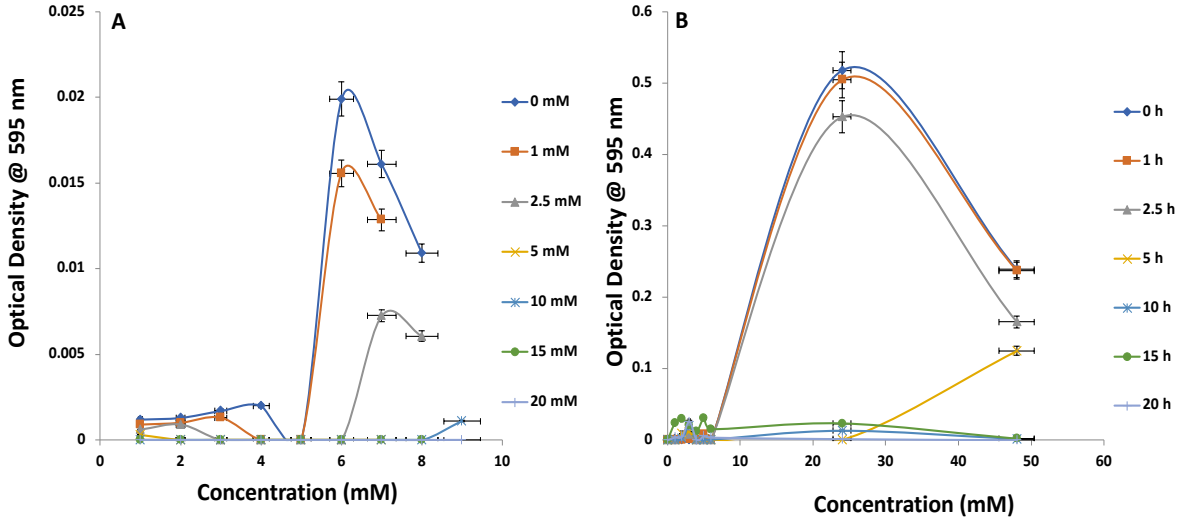

Figure 1. Time Kill assay showing the effect of glutathione on the growth of bacteria over time. **(A)** *S. marcescens* **(B)** *K. pneumoniae*.

3.3. Reactive Oxygen Species (ROS) assay

In order to understand the mechanistic insights into glutathione's ability to kill bacteria, intracellular ROS was quantified. It was observed that glutathione-treated bacterial cells showed increased amounts of ROS production in a dose-dependent manner as compared with the untreated control over a period of 5 h. At 15 mM/mL concentration of glutathione there was significantly higher intracellular ROS accumulation was observed as compared to untreated control (**Figure 2**). This result indicates that glutathione induces ROS production, that alters the redox environment of the bacteria leading to bacterial cell death.

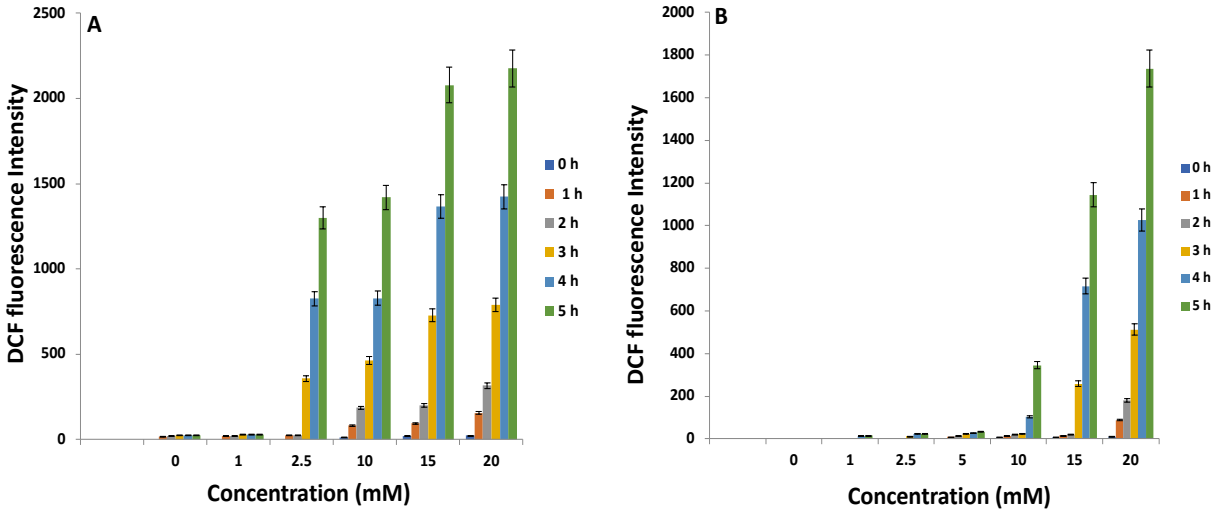

Figure 2. Quantification of intracellular ROS production post glutathione treatment **(A)** *S. marcescens* **(B)** *K. pneumoniae*.

3.4. Effect of glutathione in inhibiting biofilm formation assay

As glutathione showed promising antibacterial activity, its antibiofilm potential was then tested. It was observed that glutathione efficiently inhibited biofilm formation in both bacteria. In *Serratia marcescens* there is ~10%–85% biofilm inhibition was observed when treated with 1–20 mM glutathione (**Figure 3A**). In *K. pneumoniae* there was 10%–80% biofilm inhibition observed at 1–20 mM glutathione treatment (**Figure 3B**). This result showed that Glutathione has the capability to inhibit the bacterial ability to adhere to surface and initiate formation of biofilms.

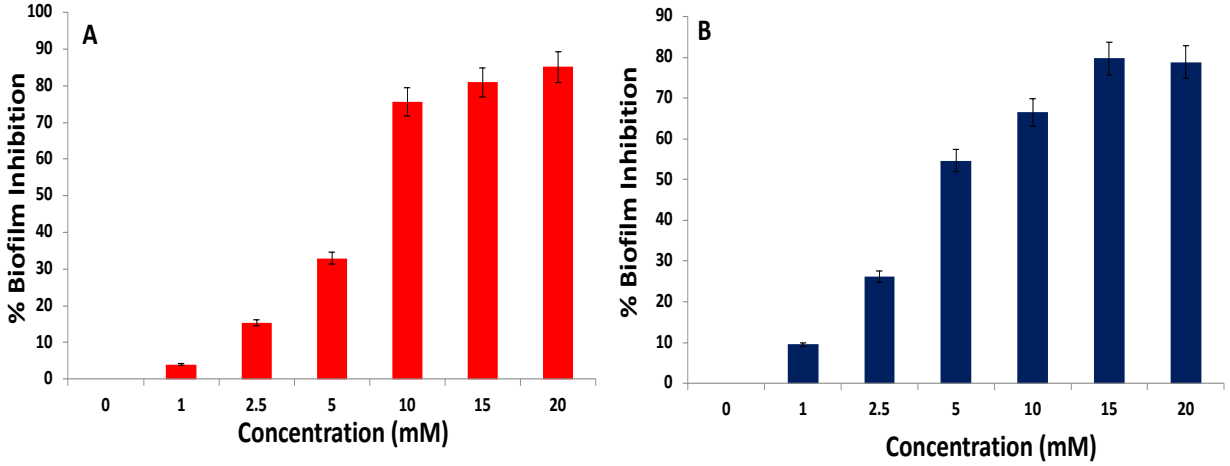

Figure 3. Effect of glutathione on bacterial biofilm Inhibition. **(A)** Biofilm inhibition in *S. marcescens* **(B)** Biofilm inhibition in *K. pneumoniae*.

3.5. Biofilms Eradication assay

To further check the ability of glutathione to eradicate the preformed biofilms, this assay was performed. It was observed that the preformed biofilms when treated with 1–20 mM glutathione showed 20%–60% biofilm eradication in *S. marcescens* (**Figure 4A**), while 10%–70% biofilms were eradicated in *K. pneumoniae* (**Figure 4B**). These results indicate that glutathione is capable of distorting existing biofilms and can offer a treatment for preexisting hard to treat diseases caused by these bacteria.

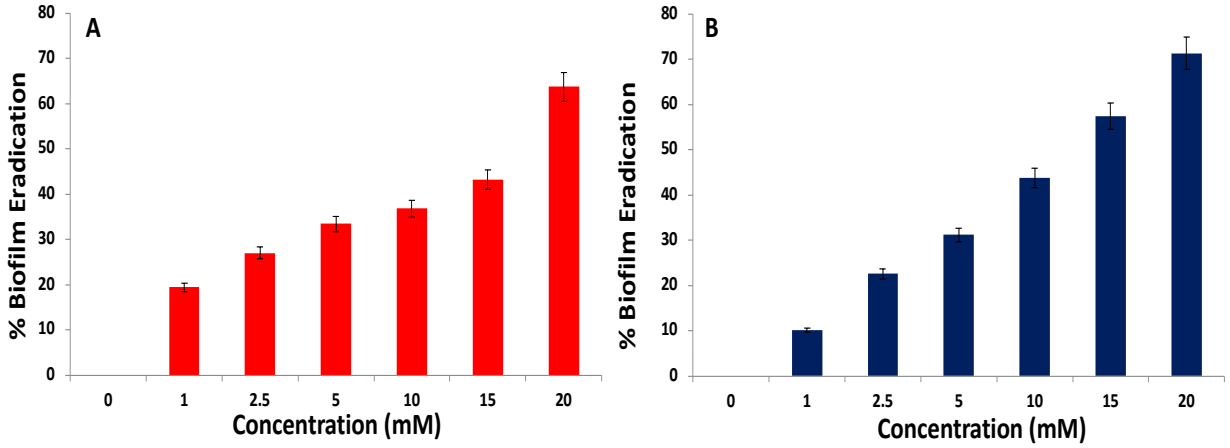

Figure 4. Glutathione's potential in eradicating pre-existing biofilms of **(A)** S. marcescens **(B)** *K. pneumoniae.*

3.6. Quantification of EPS

The important characteristic feature of matured biofilms is the presence of firm and thick EPS layer. To check if glutathione can disturb this protective EPS layer, this assay was performed. The results indicate that

glutathione could effectively damage the EPS layer formed in these organisms. In *S. marcescens* there was 50% reduced EPS production was observed with increased glutathione concentration, while in *K. pneumoniae* there was 40% EPS reduction (**Figure 5A,B**).

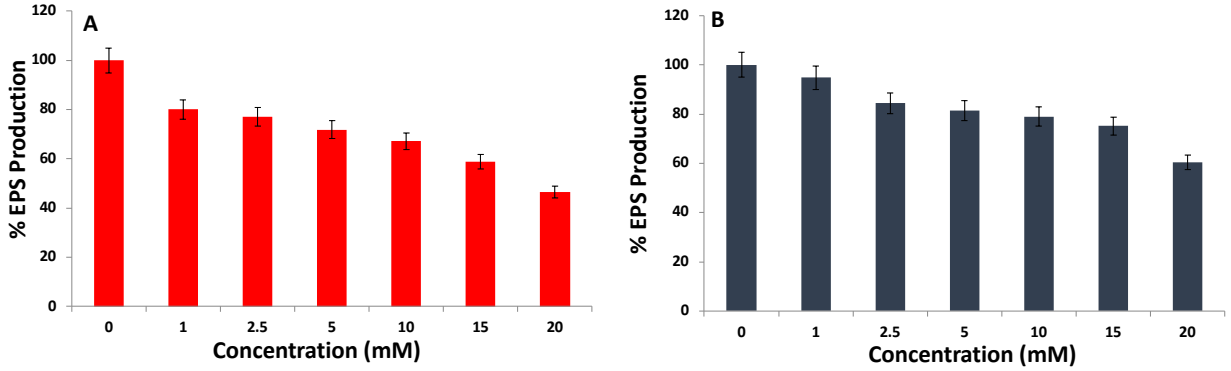

Figure 5. Quantification of the EPS layer of matured biofilms post glutathione treatment in **(A)** *S. marcescens* **(B)** *K. pneumoniae.*

3.7. eDNA quantification assay

eDNA was involved in the maturation and stabilization of biofilms, hence it was an important part of the biofilm structural integrity and maintenance. Bacterial preformed biofilms after treatment with glutathione, when the total eDNA was quantified, it was observed that there was a drastic reduction in the total e-DNA content in both bacteria. In *S. marcescens* there was a maximum of 2-fold decrease in total eDNA, while in *K. pneumoniae* there was a 4-fold decrease in eDNA content as compared to untreated controls (**Figure 6**). These results indicates that probably, glutathione must be interacting with the eDNA of the EPS layer, hence distorting it.

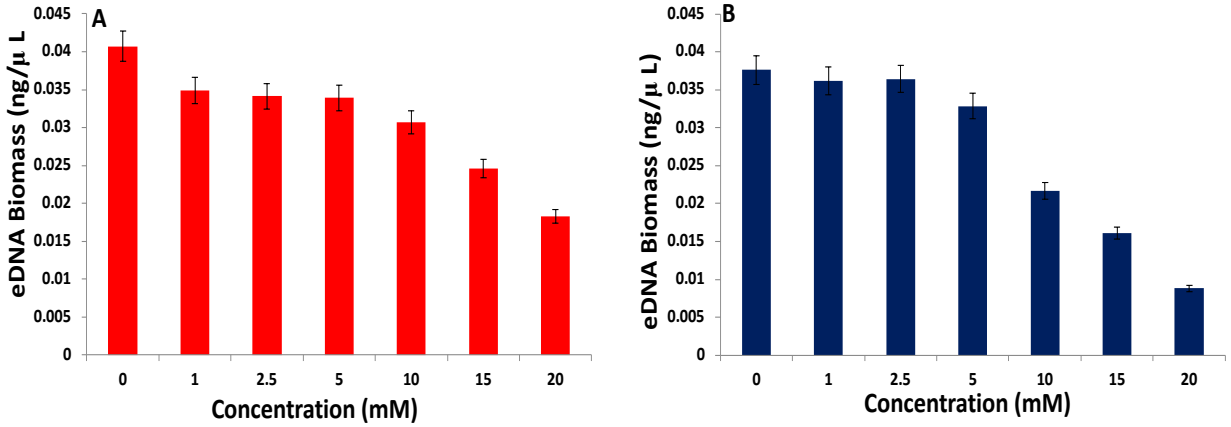

Figure 6. Quantification of total eDNA post Glutathione treatment in. **(A)** *S. marcescens* **(B)** *K. pneumoniae.*

3.8. Potential of glutathione in inhibition of virulence factors production

Quorum sensing in bacteria is an important signalling pathway that produces various virulence factors, biofilm formation, luminescence etc. To check if glutathione has any potential role in attenuating the QS pathway in these bacteria, the various virulence factors were quantified post glutathione treatment. It was observed that glutathione treated bacterial cells showed dose-dependent reduction in all virulence factors production. In *S. marcescens* there was a maximum of 75% reduced urease activity (**Figure 7A**), 55% reduced lipase activity (**Figure 7B**) and 88% reduced protease activity in case of *S. marcescens* (**Figure 7C**). While in *K. pneumoniae*, there was 97% reduced urease production (**Figure 7D**), 95% lipase production (**Figure 7E**) and 10% protease production (**Figure 7F**) in a dose dependent manner. This result clearly indicates that

glutathione indeed targets the QS pathway of these bacteria and is preventing the biofilm formation, maturation and its associated pathogenicity.

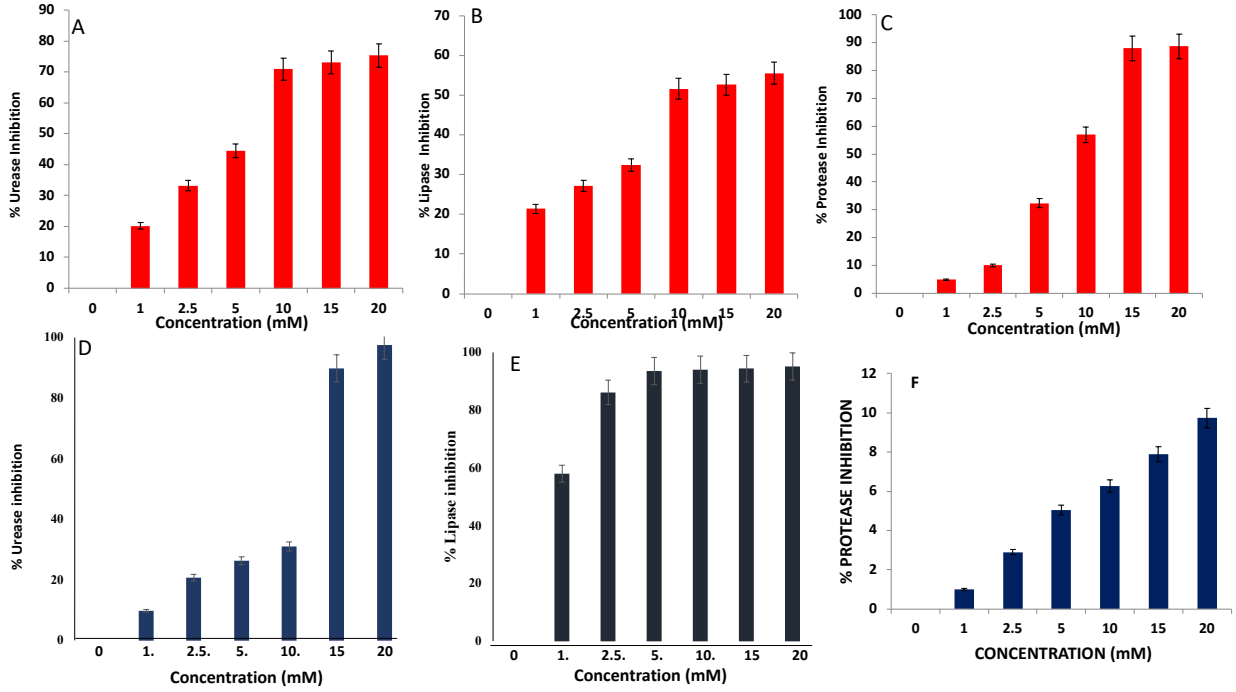

Figure 7. Effect of glutathione on quorum sensing induced virulence factors production **(A)** Urease inhibition in *S. marcescens* **(B)** Lipase inhibition in *S. marcescens* **(C)** Protease inhibition in *S. marcescens* **(D)** Urease inhibition in *K. Pneumoniae* **(E)** Lipase inhibition in *K. Pneumoniae* **(F)** Protease inhibition in *K. Pneumoniae*.

4. Discussion

Bacteria acquire resistance to antibiotics through various strategies, with the formation of biofilms being a crucial one. Biofilms represent a diverse community of microorganisms displaying resistance to various antimicrobials, posing significant challenges in treatment. Effectively addressing pathogenic biofilms remains a persistent challenge, prompting intensified research efforts to develop novel antimicrobial classes capable of disrupting different stages of biofilm formation for more effective treatment. In the present study, we investigated the potential of glutathione, an antioxidant, to inhibit and eradicate the biofilms formed by *Serratia marcescens* and *Klebsiella pneumoniae* which are known to cause diverse nosocomial infections. Additionally, we assessed its potential to attenuate the quorum sensing pathway. Our results demonstrate that glutathione can effectively kill bacteria, showing strong antibacterial activity with MIC values of 6 mM/mL and 4 mM/mL for *Serratia marcescens* and *Klebsiella pneumoniae.* Earlier reports of exogenous glutathione effect on clinal isolates of *Acinetobacter bauminii* showed MIC values as high as 10 mM to 15 mM[18]. Also, it was reported that glutathione-stabilized silver nanoparticles showed effective antibacterial activity against *Campylobacter*[19]. Growth kill assay further validates the antibacterial potential of glutathione, which showed a significant reduction in the exponential growth of both *S. marcescens* and *K. pneumoniae* (**Figure 1**). Earlier reports also showed the antibacterial activity of 30 mM glutathione against a wide range of gram-positive and gram-negative bacteria like MRSA, MSSA, *E. coli*, *K. pneumoniae*, *S. pyogenes*, *Enterobacter* species etc.[1,20,21]. To get more insights into the mechanism of glutathione induced bacterial cell death intracellular ROS was quantified. Our results clearly showed that exogenous glutathione, indeed induced significantly higher mounts of RSO in these bacteria that lead to their death (**Figure 2**).

The anti-biofilm properties of glutathione against *S. marcescens* and *K. Pneumoniae* clearly showed that it efficiently inhibited the bacterial adhesion to surfaces, thereby prevented biofilm formation to as high as 80% in both the bacteria (**Figures 3** and **4**). Furthermore, glutathione efficiently eradicated preformed biofilms to around 65% and 70% in *Serratia marcescens* and *Klebsiella pneumoniae* respectively (**Figure 5**). Further to check if glutathione effects the EPS and its components, quantification of EPS and eDNA was performed. It was observed that glutathione treated bacterial biofilms of both the bacteria showed significantly lower EPS content (**Figure 6**) and drastic reduction in the overall eDNA content of the EPS layer (**Figure 7**). Earlier literature also showed that glutathione at higher concentration has efficient biofilm inhibition and biofilm disruption ability[1,22–30].

It is known that quorum sensing signalling pathway is a process by which bacteria communicate and alter the gene expression, this enhances its expression on host cells, and it also controls the production of various virulence factors and helps them survive in the environment[31]. Quorum sensing is also considered as one of the pivotal mechanisms in formation and maturation of biofilms. Controlling these virulence factors can serve as an effective mechanism to prevent and eliminate biofilms. Virulence factors such as urease, lipase, protease and prodigiosin play a major role in causing infections[32,33]. Thus, quorum sensing pathway becomes a potential target to attenuate biofilms formation and can cause or initiate infections[34]. Our results showed a significant inhibition of Urease, Lipase, Protease production in both the bacteria tested (**Figure 7**). This result indicates that glutathione is targeting the QS pathway, thereby mitigating biofilm formation, maturation and also its associated pathogenicity.

In conclusion, our findings indicate that glutathione hinders bacterial growth by altering the redox environment, leading to dose and time-dependent bacterial death. Moreover, it suppresses the quorum sensing pathway, hindering biofilm formation. Glutathione effectively degrades the extracellular DNA (eDNA) component of mature bacterial biofilms, destabilizing and disrupting the biofilm structure. The results also highlight the capability of glutathione to reduce the production of quorum sensing-induced virulence factors, showcasing its potential in targeting the quorum sensing pathway. Further validation could help glutathione develop as an antimicrobial agent in combination with antibiotics to combat infections associated with biofilms.

Author contributions

Performed all the experiments, did the investigation, data curation, validated the data and written the draft of the manuscript, AP; conceptualized the idea, executed the work, acquired resources to perform the experiments, validated all the results, supervised the project and edited the manuscript, SLV. All authors have read and agreed to the published version of the manuscript.

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Conflict of interest

The authors declare no conflict of interest.

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