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Investigation of the Role of Vitamin C In Enhancing the Activity of Antibacterial Agents and Biofilm Formation

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Abstract: Resistance to antibiotics is rapidly spreading across the globe, posing a new health-care challenge for all countries. Bacterial biofilms are three-dimensional formations made up of cells encased in a matrix of polymeric, making cells resistant to the drugs and the immune system. As a result, new tactics for inhibiting the production of the EPS matrix may lead to more efficient use of already available antibiotics. The mechanism of vitamins C effect on boosting the effectiveness of several anti-bacterial drugs was investigated in this study, the target isolates were obtained from University of Mosul/ Biology department/ bacterial culture collections and evaluated qualitatively and quantitatively. The isolates involved: *Staphylococcus aureus*, *Escherichia coli*, *Klebsiella sp.*, *Serratia marcescens* and *Pseudomonas aeruginosa*. Antibiotic sensitivity tests and biofilm producing assay results revealed that the majority of the isolates were resistant to a range of antibiotics and had a large capacity for biofilm formation when grown on a cover glass surface. Vitamin C is an antioxidant, a scavenger of active metabolites. The Minimum Inhibitory Concentration (MIC) of Vitamin C against selected isolates had been determined, and all further experiments used concentrations below the MIC. Our results showed that Vit.C pre-treatment enhance the bactericidal effect of antibiotic and increases bacterial susceptibility to antibiotics. Using light microscopy, experiments of sub inhibitory doses of Vitamin C revealed good suppression of selected isolates biofilm development on the cover glass surface. Vitamin C can be utilized as an antibiotic adjuvant in combination with antibiotic and has effective biofilm inhibition, caused by multidrug-resistant bacteria, according to evidence.

Keywords: Vitamin C, Antibacterial agents and biofilm formation.

Introduction

Vitamin C has previously been reported for its antibacterial properties towards Mycobacterium TB, the germs that cause tuberculosis in humans, since the 1930s. (Boissevain et. al., 1937). In a 1933 in vivo investigation, Feeding TB sputum to vitamin C-deficient guinea pigs resulted in intestinal tuberculosis, while providing a same guinea pigs vitamin C-rich tomato juice did not produce sickness (McConkey et. al., 1933). Vitamin C, an antioxidant, and N-acetyl cysteine (NAC), an active metabolite scavenger, both prevent hepatotoxicity. Vitamin C's antimicrobial properties were previously assumed to be linked to its pH-lowering properties. In contrast, one study suggests that

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vitamin C has potent antimicrobial activity toward group A hemolytic streptococci, even in pH-neutral conditions. (Mehmeti et. al., 2013). Microdilution experiments were employed in other studies to look into the antimicrobial properties of vitamin C against a variety of microbial (opportunistic) illnesses (Holloway et. al., 2011). Vitamin C dosages of 0.31 mg/mL were found to be effective in reducing the growth of *Pseudomonas aeruginosa* in vitro. Notably, co-administration of vitamin C can significantly boost the antibacterial activity of other drugs like epigallocatechin gallate, such as against multi-drug resistant bacteria. (Hatano et al. 2008), this was likewise true for vitamin C combined with deferoxamine in the treatment of Gram-positive cocci., such as *Staphylococcus epidermidis* and *Staphylococcus aureus*, as well as against Gram negative rods, including, *P. mirabilis*, *K. pneumoniae* and *E. coli*. Vitamin C mixed with quercetin had a synergistic antibacterial effects, and vitamin C combined with extracts such as white tea and pomegranate rind extracts had a synergistic bactericidal activity. (Chen et. al., 2018).

Method

Selection of the Isolates

The isolates were obtained from University of Mosul/ Biology department/ bacterial culture collections, the isolates involved: *Staphylococcus aureus*, *Escherichia coli*, *Klebsiella* sp., *Serratia marcescens* and *Pseudomonas aeruginosa*

Morphological Characterisation

In order to confirm the purity and the diagnosis, the morphological and cultural characteristics were conducted for all the pure isolates on prepared medium NA and BHI (Difco). The morphological characteristic include shape, Gram staining. Growth patterns were checked using NA, BHI and Mannitol Salt Agar (MSA) media after incubation at 37 °C for 24 hours.

DNase Activity Assay

DNA extraction: boiling method

Colonies of overnight growth for the selected isolates were used. Briefly, the bacterial pellets were suspended in 200 µl of TE buffer Tris-HCl (10 mM): EDTA (1 mM) and subjected to 15 min of boiling. Immediately after boiling, the tubes were placed in an ice for 15 min and then centrifuged for 5 minutes at 14,000 rpm at room temperature. The supernatant containing DNA (100 µl) was transferred to another clean tube and stored at -20°C.

DNase Tube test

With each isolate, colonies of *Staphylococcus aureus*, *Escherichia coli*, *Klebsiella* sp., *Serratia marcescens*, and *Pseudomonas aeruginosa* were taken from the pure culture and 0.1 g of DNA was added to 200 l BHI broth in an Eppendorf tube. The DNA and bacteria-containing tubes were incubated at 37°C with shake (150 rpm). Following 24 hours, 20 µl of media was removed and spun for 1 minute at 10,000 g. The supernatants were processed in a 0.9 percent gel electrophoresis device containing EtBr and observed with an Ultraviolet trans illuminator (Gerceker et.al., 2009).

Antibiotic Sensitivity Assay

The isolates were screened for antibiotic sensitivity using disk diffusion (Kirby Bauer's) method on Mueller–Hinton agar (Merck, Germany). The assays to establish the susceptibility of drugs were conducted according to the Clinical and Laboratory Standards Institute (CLSI) (Bayer et. al., 1966). Tetracycline, Ciprofloxacin, Flucloxacillin, Streptomycin, and Ampicillin were among the antibiotics utilized.

Determined Of Minimal Inhibitory Concentration (MIC) Of Sodium Ascorbate Against *S. Aureus* and *Klebsiella* Sp.

A broth dilution assay against chosen isolates was used to identify the minimum dosage of sodium ascorbate that suppressed growth of bacteria (CLSI, 2006). Two-fold serial dilutions of sodium ascorbate (0, 100, 50, 25, 12.5, 6.25, 3.15 mg/ml) were performed in 1 ml LB broth. 0.1 ml of cells were added to each concentration of ascorbate dilutions and incubated at 37°C for 24 hours. The lowest concentration that stopped observable growth of bacteria was referred to as the (MIC). The MIC for *S. aureus* and *Klebsiella* sp. was 12.5 mg/ mL, 25 mg/mL respectively. Depending on pre- treatment cells for the selected isolates in the presence of sub-inhibitory concentrations 6.25 mg/mL of sodium ascorbate, determination of the antibiotic susceptibility and biofilm forming for the selected isolates were done.

Assessment of Antibiotic Susceptibility of *S. Aureus* and *Klebsiella* Sp.

Antibiotic susceptibility of *S. aureus* and *Klebsiella* sp. were assessed Briefly, inoculation of the 0.5 ml (1×10^8) CFU/ml of overnight cultures of isolates were done using 5 ml LB broth which contained sub-inhibitory concentrations of sodium ascorbate at 6.25 mg/ml. They were then incubated at 37°C for 16–18 h. The isolates were tested for antibiotic sensitivity using disk diffusion (Kirby Bauer's) technique on Mueller–Hinton agar using the antibiotic that were resistance to it. The results were compared with the untreated cultures. The experiments were done in triplicate and the mean values were utilised.

Biofilm Forming Assay

The study of biofilm using optical microscopy was carried out according to procedures of (Martin-Cereceda et. al., 2001) with little modification. Briefly, in 24-well MTP, 1% of overnight isolate colonies were introduced to 1 ml of fresh growth medium with a 1cm glass cover and cultured for 24 hours. The cover glasses were removed carefully following incubation and rinsed with deionized water to eliminate the planktonic cells. The assumed biofilms on the cover glass were dyed with a 0.4 percent crystal violet solution and then examined under a light microscope.

Effect of Sub Inhibitory Concentrations of Sodium Ascorbate on Biofilm Forming of *S. Aureus* and *Klebsiella* Sp.

Some sub inhibitory concentrations of sodium ascorbate showed no effect on the growth rate, but still were able to significantly inhibit biofilm formation. These concentrations were used for microscopic observation. The microscopical study of the biofilm was conducted using (Musthafa et. al., 2010) methods with minor modifications. Briefly, both overnight cultures that were untreated and treated with sub inhibitory concentrations of sodium ascorbate were mixed with 1 mL of fresh growth medium that had a cover glass of 1 cm and then incubated for 24 h. The cover glasses were removed carefully after incubation and were then rinsed with distilled water in order to remove the planktonic cells. A solution of 0.4% crystal violet was used to stain the presumed biofilms that adhered to the cover glasses before being studied under a light.

Results and Discussion

The diagnosis and purification of bacterial isolates obtained from Biology department/ bacterial culture collections were confirmed based on gram stain and biochemical tests, the results were identical to what was stated in the approved diagnostic systems (Gary, 2017). A DNase tubes testing was carried out for DNase activity for the growing culture of *S. aureus*, *Klebsiella* sp., *E. coli*, *Serratia marcescens* and *P. aeruginosa* (Figure 1). Only *S. aureus* and *Serratia marcescens* are positive in this test and showed degrade DNA. In a tube containing *Klebsiella* sp., *E. coli* and *P. aeruginosa* no DNA damage was observed.

Each of the isolates was subjected to screening for antibiotic susceptibility and the results were compared with the standard protocols produced by CLSI.

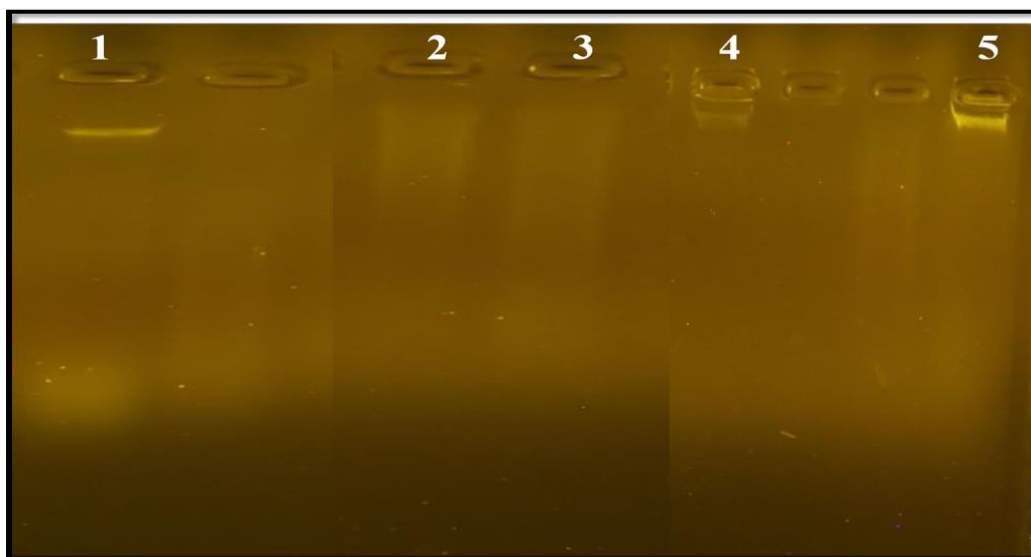


Figure 1. DNase activity of 1: *Escherichia coli*, 2: *Staphylococcus aureus*, 3: *Serratia marcescens*, 4: *Klebsiella sp.* and 5: *Pseudomonas aeruginosa* using the DNase tube test. The dashed band shows the degraded DNA and the vertical band shows the intact DNA.

The results showed that *Pseudomonas aeruginosa*, *Serratia marcescens*, were sensitive to Cip. and resistance for the TE., S, F and APX, *E. coli* was sensitive to Cip. and F, while *Klebsiella sp.* and *S. aureus* were resistance to all antibiotic used, therefor *Klebsiella sp.* and *S. aureus* were chosen for study the effect of sodium ascorbate assays (Table 1) (Figure 2).

Table 1. Results of the screening for antibiotic susceptibility assay

Bacteria	Cip	TE	S	F	APX
<i>Pseudomonas aeruginosa</i>	37mm Susceptible	7mm resistant	18mm susceptible	8mm resistant	9mm resistant
<i>Escherichia coli</i>	22mm Susceptible	7mm resistant	10mm resistant	17mm susceptible	8mm resistant
<i>Serratia marcescens</i>	30mm Susceptible	6mm resistant	18mm resistant	13mm resistant	7mm resistant
<i>Klebsiella sp.</i>	6mm resistant	6mm resistant	8mm resistant	7mm resistant	6mm resistant
<i>Staphylococcus aureus</i>	11mm resistant	6mm resistant	10mm resistant	8mm resistant	6mm resistant

Multidrug-resistant bacteria have been a cause of concern, and efforts to find new antibiotics have had mixed results. (Puzari et. al., 2018). As a result, a novel adjuvant to fight infectious disease is required. According to prior research, vitamin C is one such possible adjuvant. The effect of vitamin C sub-MIC concentrations on the development and biofilm generation of certain isolates was investigated in this study. In terms of isolates susceptibility profiling (Figure 2), resistant to a broad range of beta- and non-beta-lactam antibiotic had the largest percentage, and the majority of isolates were multi-drug resistant, which is consistent with prior researches (Guggenbichler et. al., 2011, Mansour et. al., 2009). Nearly half of biofilm producers are resistant to at least three different antibiotic families at the same time, that could be related to the biofilm matrix (Alves et al. 2014), and the physiological properties of the microbes that make it allow for antibiotic resistance. The antibacterial agent's delayed penetration, a shift in the microbial growth rate, or other physiological modifications linked to the biofilm's formation are generally the mechanisms of resistance (Donlan et. al., 2002). Enterococci, one of the commensal organisms in the genital tracts and intestines, are becoming key pathogenicity sources in hospitals. Because of their own regular resistance to commonly used antibiotics, as well as their ability to obtain additional resistance to other kinds, either through plasmid and transposon elements, as in glycopeptide and aminoglycoside compounds, or via mutations, as in penicillin, they are receiving more attention. (Cohen, 1992). This development is linked to the

dissemination of resistance-bearing plasmid and transposon vectors across a variety of *Enterococcus faecalis* and *Enterococcus faecium* strains, as well as a rise in vancomycin use in medical centres.

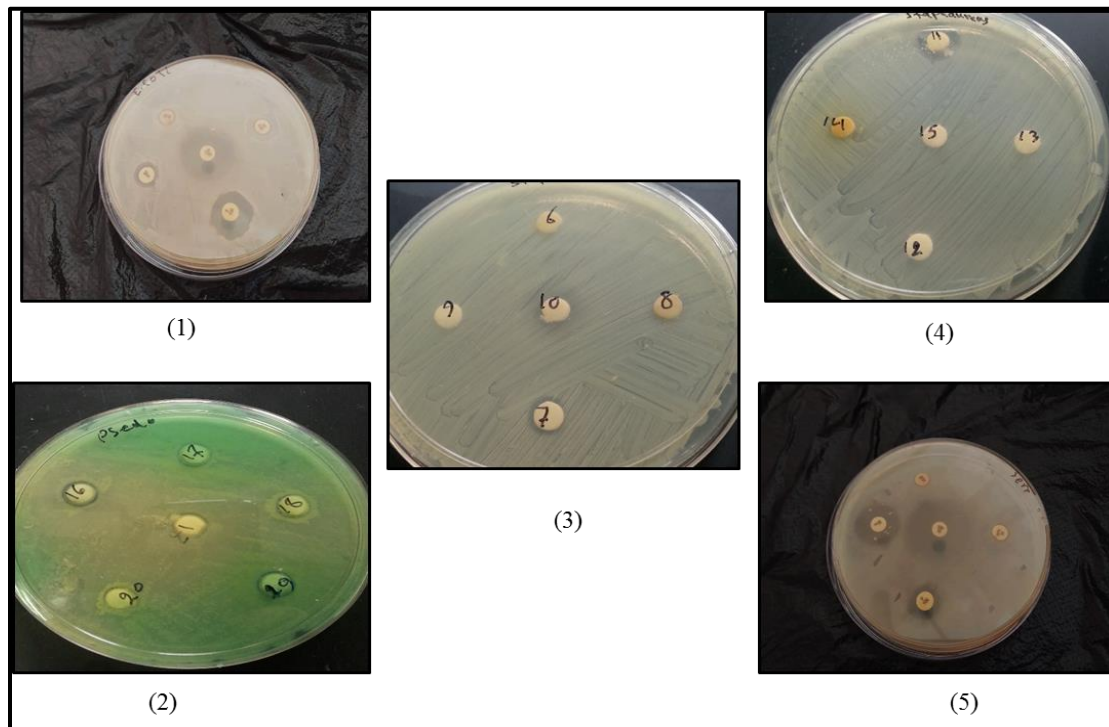


Figure 2. Sscreening for antibiotic susceptibility assay, 1- *Escherichia coli*, 2- *Pseudomonas aeruginosa*, 3- *Klebsiella* sp. 4- *Staphylococcus aureus* and 5- *Serratia marcescens*.

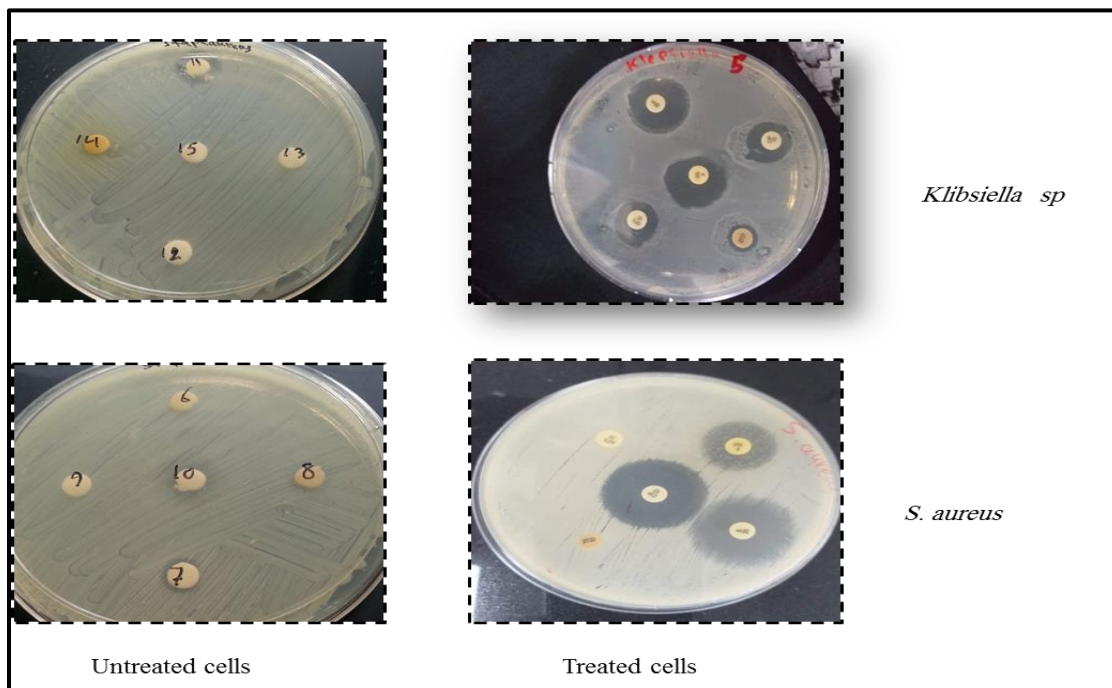


Figure 3. Antibiotic susceptibility test using disk diffusion method of selected bacteria showing the significant change of suseptibility after treated cells with sub inhibitory concentration of sodium ascorbate 6.25 mg/mL.

The survival of *S. aureus* and *Klribsiella* sp. in the presence of sub-MIC sodium ascorbate is vital to see if any of sodium ascorbate's effect are due to cell functioning changes rather than bactericidal or bacteriostatic actions. The MIC of sodium ascorbate for *S. aureus* and *Klribsiella* sp. were found to be 12.5 mg/ mL and 25 mg/mL using the broth dilution method, respectively. Sub inhibitory concentration of sodium ascorbate 6.25 mg/mL, where used for antibiotic susceptibility and biofilm forming assays for the selected isolates.

Antibiotic susceptibility assay demonstrated a significant change. The results of *Klribsiella* sp. treated cultures with sodium ascorbate showed the intermediate effect to TE, sensitive effect to S, F, AMP and Cip compared to untreated cultures. For the *S. aureus* treated cultures were intermediate effect to F and sensitive effect for only Cip and S compared to untreated cultures (Figure 3).

In this study, the soluble forms of vitamin C increases bacterial susceptibility to antibiotics, This observation is consistent with the results of (Helgadóttir et al., 2017; Vilchèze et. al., 2013), who stated that drugs that target bacterial growth might work synergistically with some other antibacterial drugs to reduce pathogens resistance.

A known ROS-generator is vitamin C, a natural food additive that can be used safely in medical care. Pretreatment of cells with vitamin C at various intervals with 48 hours of bacterial growth may improve antibacterial activity and eradicate specific bacterial species. (Vilchèze et al. 2013; Foti et. al., 2012).

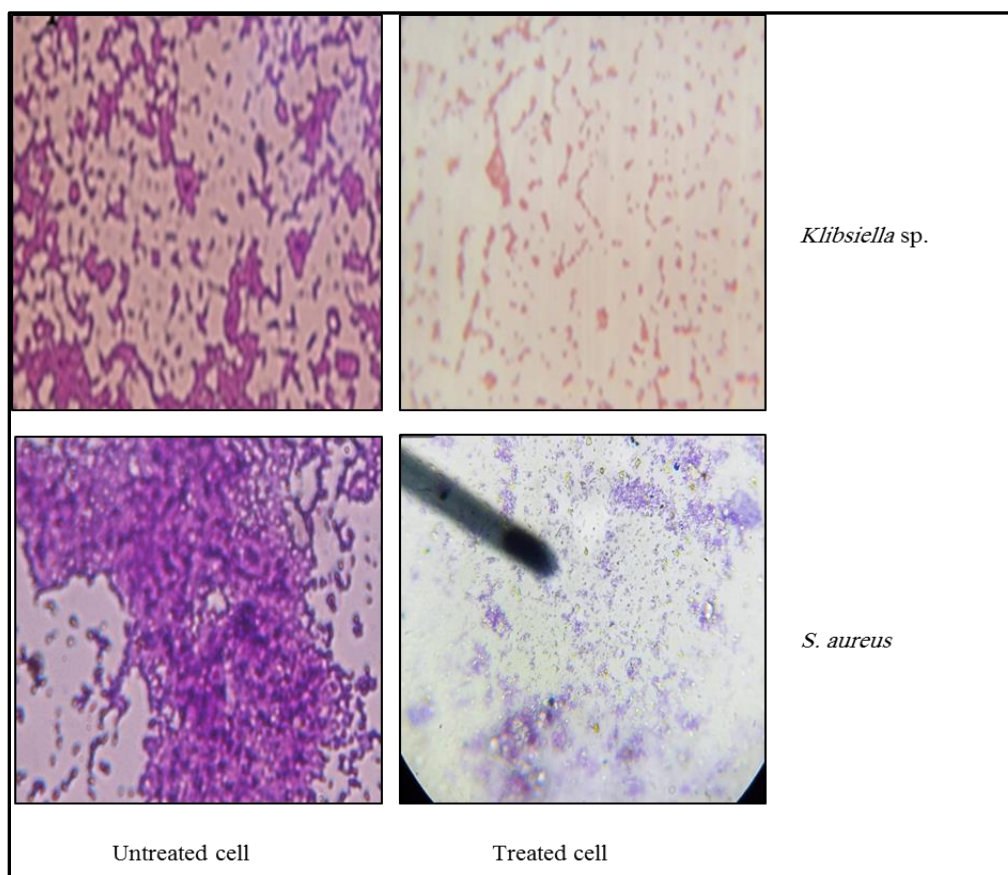


Figure 4. light microscopic images. Biofilm development of the *Klribsiella* sp. and *S. aureus* grown in the absence and presence of sub inhibitory concentration of sodium ascorbate 6.25 mg/mL showing inhibitory activity.

Vit C pre - treatment improves the bactericidal effect of the drug and behaves as just a lipocalin antimicrobials binding inhibitor, increasing the effective concentration of antibiotics around bacterial cells, indicating that Vit C can be used as an antimicrobials adjuvant in combination therapy to treat infections caused by multi drug resistance pathogens. Biofilms are up to 1,000 times more resistant to the antibiotics than bacteria in their planktonic form, and they cause 60% of human infections, with a higher risk of getting antibiotic resistance (Mai-Prochno et. al., 2015).

Interbacterial communication is essential for the production of biofilms by *S. aureus* and *Klasiella* sp. The influence of sodium ascorbate on the production of biofilms is depicted in (Figure 4). Biofilm was shown to be significantly reduced in overall when *S. aureus* and *Klasiella* sp. was grown with concentration of sub-MIC sodium ascorbate.

Biofilm generation on medical equipment, in wounds, and in immunocompromised people is now recognized as a major cause of chronic infections (Bjarnsholt et. al., 2010). In immunocompromised patients, common pathogens such as *Escherichia coli* *Pseudomonas aeruginosa* and *Staphylococcus epidermidis*, which are often pathogenic, can cause dangerous long-term infections. Antibiotic overuse can lead to bacterial resistance, resulting in infections caused by antibiotic resistant bacteria, which is a serious increasing issue. (Flores-Encarnación et al., 2018). The global threat of resistance to antibiotics, according to WHO, is going to usher in a post-antibiotic period. (W. H. O., 2014). As a result, in addition to limiting antibiotic dosage, innovative techniques to bacterial infection treatment are urgently needed. Because conventional antibiotic therapies are ineffective in eradicating persistent biofilms, we sought out a combination therapeutic strategy to supplement them. The biofilm matrix's polysaccharides and proteins generate a hydrophobic covering that prevents antimicrobial compounds from penetrating and provides biofilm resistance (Roy et. al., 2018). Fighting bacterial infections by inhibiting EPS generation is a potential method. Antibiotics and other recognized antibacterial treatments are not powerful enough as to entirely eliminate biofilms. In this case, using a combination treatment strategy to achieve a significant bactericidal effect towards bacterial biofilms is indicated. Vitamin C, a vital nutritional supplement for human, has been shown to boost the antibacterial activity of a variety of antimicrobials. (Khameneh et. al., 2016). In most microbial cells, it is also known for producing reactive oxygen species (ROS). Because direct microscopic examination of biofilms following exposure to sodium ascorbate can provide significant data on the activity of sodium ascorbate on biofilms, light microscopy analysis was carried out (Manjunath Mandhira Doss, 2013).

In this study, microscopic analysis was carried out on sub-MIC concentrations that had no effect on the growth rates but inhibited biofilm formation significantly. Results analysis revealed that controls (untreated cells) had thick coating of biofilms while biofilms of treated isolates showed a visible reduction in the numbers of micro colonies. Moreover, the architecture of the biofilm was also deteriorated by sodium ascorbate, as observed with the Light microscopy analysis (Fig. 3). The same observations were also recorded when *P. mirabilis* and *S. marcescens* were administered with p-nitrophenyl glycerol (PNPG) and tannic acid (Jones et. al., 2009; Wei et. al., 2004).

Vit C pretreatment boosts the antimicrobial effect of cold plasma via decreasing survivability from 50 to percent in *P. aeruginosa* biofilm, 10% to 2% in *E. coli* biofilm, and 61 to 18 percent in *S. epidermidis* biofilm (Helgadóttir et al., 2017). Because lengthy CAP treatments are not realistic in clinical practice, we believe that pre-treating infect lesion with vit C before exposing them to CAP could be a valid idea for fast eradication of bacterial biofilms in a variety of applications.

According to our findings Vitamin C can significantly inhibit bacterial biofilms by suppressing EPS synthesis at concentration less than 25 mg/mL. Vitamin C's bactericidal activity towards mycobacteria was previously shown to be primarily connected with oxidative stress. (Pandit et al., 2017). Based on these data, Biofilms was prevented by vit C, which inhibits the quorum sensing and other stationary phase regulatory systems that underpin biofilm growth, resulting in reduced in polysaccharide production. Once EPS content is lowered at vit C concentrations, bacterial cells are totally exposed to the media, as a result, as proven in this research, they are more susceptible to antimicrobial medications or therapies, such as sodium Ascorbate oxidative. (Helgadóttir et al., 2017).

Conclusion

The synergistic effect of antibiotics with sub-MIC doses of Vit C salt was studied in this study. Furthermore, the effect of sub-MIC Vit C salt concentrations on biofilm architecture was explored in this study.

Recommendations

Based on our results, the study recommends using a Vit C- Antibiotic combination treatment strategy to achieve a significant bactericidal effect towards bacterial resistance and biofilms formation.

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Scientific Ethics Declaration

The author declares that the scientific ethical and legal responsibility of this article published in EPSTEM journal belongs to the author.

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