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Protamine Sulfate Enhances Biofilm Inhibitory Activity of Curcumin against Pseudomonas Aeruginosa Biofilm Grown on Ex Vivo Porcine Skin Model

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Abstract

A bacterial biofilm is one of the most difficult structures to eradicate and is involved in the enhancement of the virulence and resistance of bacteria. Several strategies have been used to fight bacterial virulence and biofilm formation through targeting the quorum sensing (QS) mechanism of communication. Curcumin is a natural component extract from Turmeric and has antimicrobial and quorum sensing inhibitory (QSI) effects on many microbes and their biofilm. This study aimed to evaluate the effect of curcumin-protamine sulfate combination against the biofilm formation by *P. aeruginosa* (ATCC 27853). Neither the curcumin nor the protamine sulfate exhibited antibiofilm activity when tested against the *P .aeruginosa* biofilm alone. However, when curcumin was combined with protamine sulfate at different concentrations, a significant reduction in biofilm formation was detected. The highest inhibition percentage was detected against 48 hour biofilm when the biofilm was treated with 62.5 µg/ml curcumin with 62.5 µg/ml protamine sulfate using the 96-well plate method. The porcine *ex-vivo* model was used to confirm the previous result. The highest inhibition of biofilm was 95% when the porcine skin was treated with 500 µg/ml of curcumin with 500 µg/ml protamine sulfate. The obtained results from this study highly suggest that the combination of curcumin with protamine sulfate improves the effect against the biofilm, and ensures that the effect is due to the prevention of biofilm formation mainly through interruption of QS rather than killing of the bacteria.

Keywords: Biofilm; curcumin; protamine sulfate; Quorum sensing inhibition; *Pseudomonas aeruginosa* .

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1. Introduction

Pseudomonas aeruginosa is one of the most common opportunistic human pathogens that is a major problem in burn and wound infections [1–4]. Eradication is complicated by its ability to form antibiotic resistant biofilms [5–10]. New biofilm inhibitory strategies are in demand as antibiotic resistance comprises an emerging problem [11,12]. Curcumin is a lipophilic polyphenol found in the rhizome of Curcuma longa [13,14]. Curcumin has antibacterial and antibiofilm activity against *Pseudomonas aeruginosa* through quorum sensing inhibition [15–18]. Its effect on *Pseudomonas aeruginosa* have been confirmed by many studies [16,19–25]. As curcumin is considered a safe therapeutic option we investigated the enhancement of curcumin activity by protamine sulfate [26]. Protamine sulfate, a cationic peptide, has demonstrated antimicrobial activity against *Pseudomonas aeruginosa* [27]. It enhanced the activity of antibiotics and nonantibiotics against *Pseudomonas aeruginosa* [28–32]. In this work we are investigating the effect of combining curcumin with protamine sulfate against *Pseudomonas aeruginosa* biofilm grown in ex vivo porcine skin explant model that mimics wound conditions. This novel combination will provide a new strategy to combat *Pseudomonas aeruginosa* biofilm utilizing nonantibiotic measures.

2. Materials

2.1

Curcumin was purchased from (abcr, Deutschland). Protamine sulfate was purchased from (Sigma-Aldrich, USA). Porcine skin explants were collected freshly from local slaughtering markets in Jordan and kept in freezer at -20 ̊C for preservation

2.2

Bacterial Strain and Culture Conditions

2.3

Pseudomonas aeruginosa ATCC 27853 was purchased from American Type Culture Collection preserved at -20 ºCin 30 % Glycerol, until needed.

3. Methodology

3.1 Minimum Inhibitory concentration (MIC) and Minimum biocidal Concentration (MBC)

MIC was determined using the method described Wedajo and co-authors [33]. Briefly, *Pseudomonas aeruginosa* was cultured in nutrient broth at 37°C overnight and bacterial suspension was adjusted to 1.5×10^8 CFU/mL. Five hundred µL of nutrient broth were added into each well of Twenty-four well plates. Afterwards, 500 µL of each solution (20 mg/mL curcumin and 10 mg/mL protamine sulfate) were added to the first well in column one. Serial dilution was performed along the remaining wells except wells 23 and 24, which were used

as controls. One hundred microliters of the bacterial suspension (1.5×10^8 CFU/mL) were added to each well before the plates were incubated for 18 hours at 37°C. The MIC was determined as the lowest concentration with no bacterial growth. MBC was tested by culturing 100 µl of the bacterial suspension in wells with no growth on nutrient agar plates. Plates were incubated overnight at 37°C. MBC was determined as the lowest concentration where no viable bacterial count was detected.

3.2 Minimum biofilm inhibitory concentration (MBIC)

MBIC activity of curcumin, protamine sulfate, and curcumin-protamine sulfate combinations were tested according to the method described by Chen and co-authors with slight modifications [34]. Fifty µL of each solution (20 mg/mL Curcumin, 1 mg/mL Protamine Sulfate, or combination of Curcumin and protamine sulfate) were added to the first column of 96-well plates and serially diluted using nutrient broth. Combinations were done by mixing 25 µL of each curcumin and Protamine sulfate as follows: 0.125 mg/ml curcumin + 0.125 mg/ml protamine sulfate, 0.125 mg/ml curcumin + 62.5 µg/ml protamine sulfate, 0.125 mg/ml curcumin + 31.25 µg/ml protamine sulfate, 62.5 µg/ml curcumin + 62.5 µg/ml protamine sulfate, and 62.5 µg/ml curcumin + 31.25 µg/ml protamine sulfate). One hundred µl of *Pseudomonas aeruginosa* bacterial suspension (1.5×10^8 CFU/mL) was added to each well and allowed to grow and form biofilm for 48 hr or 72 hr at 37 °C. At the end of the incubation period, the plates were washed with running tap water three times to remove any planktonic cells before they were air dried at room temperature for 15 minutes. Two hundred µl of 1% crystal violet solution was added to each well and the plate was incubated at room temperature for 15 minutes. Crystal violet was washed away and the wells were allowed to dry. Two hundred µl of absolute ethanol were added to dissolve the stain. The optical density was measured at 600 nm and the antibiofilm activity was calculated using the following equation:

$I\% = ((\text{control } OD_{600} - \text{test } OD_{600}) / \text{control } OD_{600}) \times 100\%$

Where:

I% - is the biofilm inhibition percentage

OD_{600} - is the absorbance at 600 nm

Control is the untreated wells

3.3 Ex vivo porcine wound model

Pseudomonas aeruginosa biofilm was grown on *ex vivo* wound bed according to the method described by Phillips and co-authors with some modifications [35]. Explants were prepared by cutting frozen porcine skin with round cutter to obtain 12 mm diameter explants approximately 3-4 mm thick. A high-speed drill (Louxor, China) with round cutter was utilized to form a wound bed in the center of each explant that is 3 mm in diameter and 1.5 mm in depth. Expalnts were washed three times with normal saline and sterilized by chlorine gas for 45 minutes. Chlorine gas was generated by mixing 40 mL acetic acid with 20 mL commercial grade bleach

(Chlorox®) in a covered plastic reaction chamber. Each explant was washed three times with normal saline and aseptically placed in in 24-well plates having soft nutrient agar (0.5% agar). The wound bed was inoculated with 10 µl (*ca.* 6×10^8 CFU/ml) of bacterial suspension that was cultured overnight in nutrient broth. Explants were prepared according to the following groups: curcumin group treated with 100 µL of 500 µg/ml curcumin; protamine sulfate group treated with 100 µL of 500 µg/ml protamine sulfate; three treatment groups treated with 100 µL of 500 µg/ml curcumin combined with different protamine sulfate concentrations (125 µg/ml, 250 µg/ml, and 500 µg/ml), control group that did not receive any treatment, and a vehicle group that was treated with vehicle without any treatment. Explants were cultured for 48h at 37 ºC. Then, each explant was washed three times with sterile PBS, and aseptically placed into 15 mL test tube containing 5 ml of cold sterile PBS with 5 µl/L Tween 80. To liberate the bacteria from the biofilm, explants were sonicated for 30 seconds and vortexed for another 30 seconds. The bacterial suspension was serially diluted then plated on nutrient agar plates and incubated overnight at 37ºC to determine the bacterial load (CFU/ml). Bacterial Load (CFU/mL) was log transformed to determine log cycle reduction.

3.4 Statistical Analysis

Bacterial Load (CFU/mL) was log transformed and calculated as average of three trials ± SD. Analysis of variance was conducted and differences between groups were tested for significance by one-way ANOVA using SPSS. Differences at $P< 0.05$ were considered statistically significant.

4. Results

4.1 Minimum Inhibitory concentration (MIC) and Minimum biocidal Concentration (MBC)

Curcumin MIC was 625 µg/ml, and MBC value 10 mg/ml. Protamine sulfate MIC of was 156 µg/ml, while MBC was 312 µg/ml.

4.2 Minimum biofilm inhibitory concentration (MBIC)

This method was not useful for curcumin MBIC evaluation as it depends on UV absorbance. The concentrations 20 mg/ml - 0.125 mg/ml were not successfully tested using this essay because of the yellow color of curcumin which affected the reading of the spectrophotometer and so the antibiofilm activity was difficult to measure. The rest of the tested concentrations of curcumin did not show any biofilm inhibitory activity. The concentrations 1 mg/ml - 0.125 mg/ml were also difficult to determine because protamine sulfate at these concentrations make a layer that adheres to the wells either in treated or in blank wells. The concentrations 62.6 µg/ml -31.25 µg/ml did not show any activity. Combination of curcumin with protamine sulfate inhibited biofilm formation (Figure1).

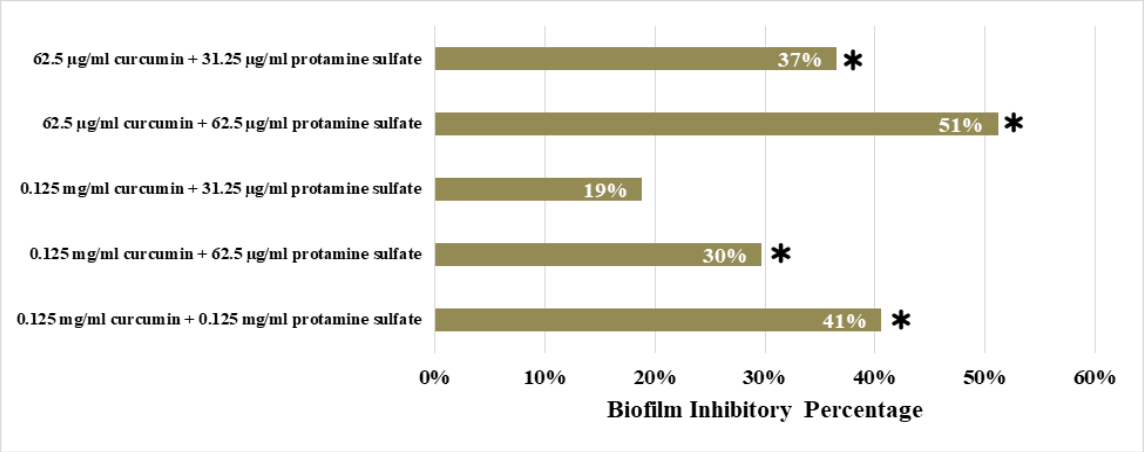

Figure 1: Biofilm inhibitory percentages of curcumin - protamine sulfate combinations on 48 hr Pseudomonas aeruginosa biofilm. ✱: significant difference p <0.05.

All tested concentrations of both curcumin and protamine sulfate did not show any biofilm inhibition activity when *Pseudomonas aeruginosa* was allowed to grow and form biofilm for 72 hr. While curcumin - protamine sulfate combination showed inhibtory activity (Figure 2).

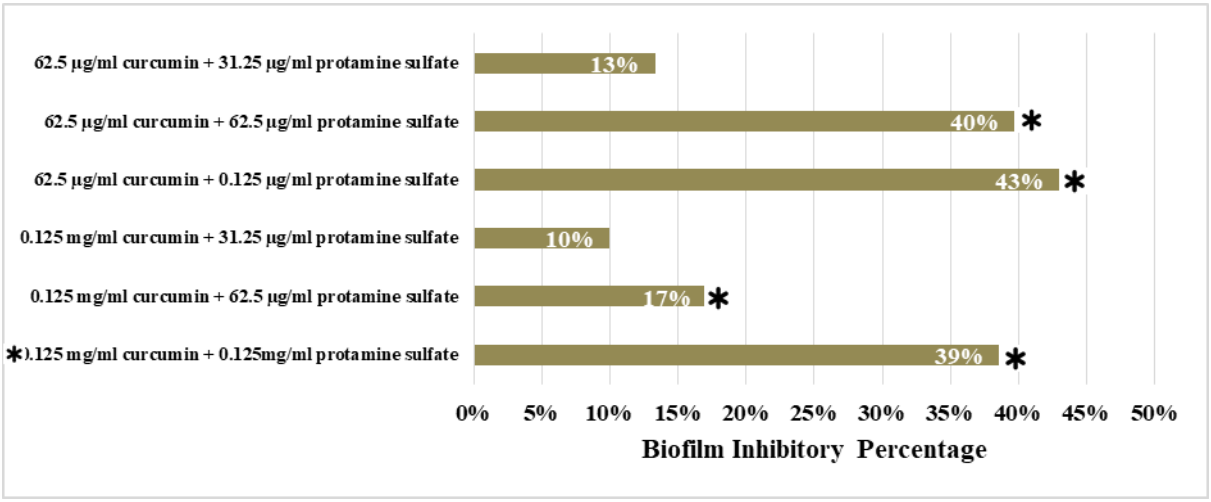

Figure 2: Biofilm inhibitory percentages of curcumin - protamine sulfate combinations on 72 hr Pseudomonas aeruginosa biofilm. ✱: significant difference p <0.05.

4.3 Ex vivo porcine wound model

In accordance with the invitro results, neither curcumin alone nor protamine sulfate alone showed significant inhibitory activity against *Pseudomonas aeruginosa* biofilm grown on *ex vivo* porcine skin wound bed. However, the biofilm was susceptible to the combination of curcumin with protamine sulfate (Figure 3). The activity increased with increasing concentration of protamine sulfate. Maximum activity was attained by the combination of curcumin (500 µg/mL) and protamine sulfate (500 µg/mL) with approximately 1.3 log cycle reduction.

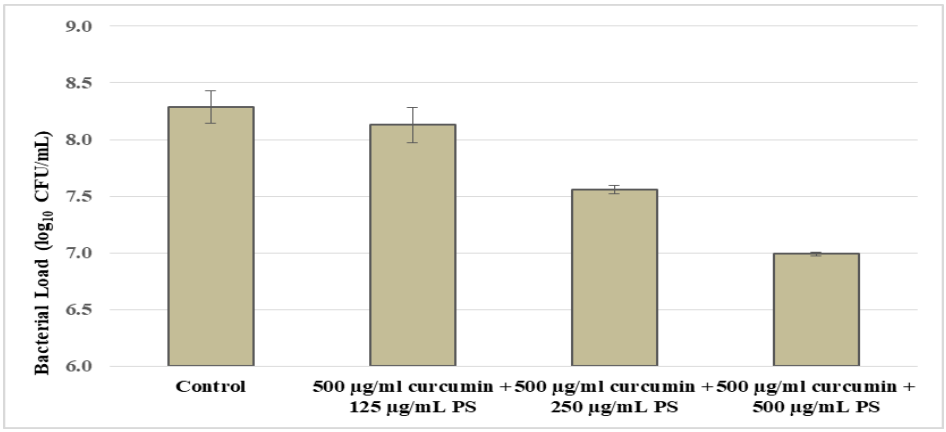

Figure 3: Effect of curcumin treatment (500 µg/ml) in combination with protamine sulfate (125 µg/ml, 250 µg/ml, and 500 µg/ml) on *Pseudomonas aeruginosa* biofilm grown on *ex vivo* skin explants. Results are means of n=3 ± SD (PS: protamine sulfate).

5. Discussion

Biofilm formation is one of the most common problems that increases virulence of bacteria and resistance towards antibiotics. As resistance to antibiotics is currently emerging, new treatment options to combat biofilms are needed. The aim of this work was to evaluate the effect of curcumin and protamine sulfate combination against *Pseudomonas aeruginosa* biofilm grown on *ex vivo* porcine skin explant model mimicking wound conditions. This model was utilized to simulate conditions in wounds as phenotypic characteristics of biofilms are determined mainly by growth conditions [35–37]. Minimum biofilm inhibitory concentrations of curcumin and protamine sulfate utilizing the conventional 96 well plate method were not obtained due to technical challenges. Curcumin has interfered with the UV absorbance that compromises the results. Protamine sulfate MBIC at concentrations 1 mg/ml - 0.125 mg/ml were also difficult to determine because it makes a layer that adheres to the wells either in treated or in blank wells. Moreover, lower concentrations 62.6 µg/ml -31.25 µg/ml did not show any activity. Combination of curcumin with protamine sulfate exhibited inhibitory activity against biofilm formation (Figure1). Due to these limitations, Viability count was adapted as a more accurate measure for assessing the antibiofilm activity. *Ex vivo* porcine skin explant model was utilized to simulate wound conditions. Sessile viability was employed to assess activity in this model as planktonic cells were washed not to exaggerate effect. Combination of curcumin and protamine sulfate showed significant inhibition of biofilm formation. Interestingly, curcumin and protamine sulfate didn't show any biofilm inhibitory effect separately. However, when curcumin was combined with protamine sulfate, the inhibition significantly improved when either 250 µg/mL or 500 µg/mL protamine sulfate were combined with curcumin (p value < 0.05). Sessile viability of *Pseudomonas aeruginosa* was reduced by 95% when 500 ug/mL protamine sulfate was combined with curcumin. Neither curcumin nor protamine sulfate alone had any antibiofilm activity on this biofilm wound model. The enhanced effect of curcumin-protamine sulfate combination may be attributed to the highly positively charged protamine sulfate that is mainly composed of L-arginine as bacterial cell wall is negatively charged. Previous results exhibited similar activity of protamine sulfate [28–32]. The activity of antibiotics and nonantibiotics against *Pseudomonas aeruginosa* was improved in accordance with the current results of

protamine sulfate / curcumin combination [28–32]. These findings could lead the way for combining other treatment options with protamine sulfate to enhance the antibiofilm activity. Results also lead us to suggest the potential benefit of this combination in wound dressings to protect against *Pseudomonas aeruginosa* biofilms. The enhanced activity with multiple treatments and different dosage forms with prolonged activity should also be considered.

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