ADHERENCE REDUCTION OF *PSEUDOMONAS AERUGINOSA* UFPEDA 416 UNDER BLUE LED LIGHT IRRADIATION AND CURCUMIN EXPOSURE

REDUÇÃO DA ADESÃO DE *PSEUDOMONAS AERUGINOSA* UFPEDA 416 SOB IRRADIAÇÃO DE LUZ DE LED AZUL E EXPOSIÇÃO À CURCUMINA

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**ABSTRACT:** This study assessed the adhesion of *Pseudomonas aeruginosa* UFPEDA 416 (ATCC 27853) exposed to curcumin-mediated Photodynamic Antimicrobial Chemotherapy (PACT). Initially, the Minimum Inhibitory Concentration of curcumin was determined (0.63 μg/mL). After, *P. aeruginosa* UFPEDA 416 was exposed to the MIC solution of curcumin and then irradiated with a high-power blue LED light at 480 nm for 20, 40, 60 and 120 minutes (28.03 mW/cm²). At each interval, 100 μL aliquots were transferred to microtubes containing Müller-Hinton broth and incubated at 30ºC for 24 h. The biofilm formed was quantified by the crystal violet method. The adherence rate was calculated using the difference in the absorbance of the treatment, compared to the control. There was a reduction in adhesion capacity from 40.8% in the first 20 minutes to approximately 11% after 60 minutes. The reduction in the adhesion rate accompanied the decrease in planktonic cells, determined from the measurement of the absorbance of the supernatant at 620 nm. The results indicated action by bacteriostasis.

**KEYWORDS:** Biofilms, Photodynamic Antimicrobial Chemotherapy, Natural Photosensitizer.

**RESUMO:** Este estudo avaliou a adesão de *Pseudomonas aeruginosa* UFPEDA 416 (ATCC 27853) exposta à Quimioterapia Fotodinâmica Antimicrobiana (PACT) mediada por curcumina. Inicialmente, foi determinada a Concentração Inhibitória Mínima de curcumina (0,63 μg/mL). Em seguida, a bactéria foi exposta à solução CIM de curcumina e então irradiada com uma luz LED azul de alta potência a 480 nm por 20, 40, 60 e 120 minutos (28,03 mW/cm²). A cada intervalo, alíquotas de 100 μL foram transferidas para microtubos contendo caldo MH e incubados à 30ºC por 24 h. O biofilme formado foi quantificado pelo método do cristal violeta. A taxa
1. Introduction

*Pseudomonas aeruginosa* is a Gram-negative rod, widespread in the environment and sometimes classified as an opportunistic pathogen because it can cause a variety of infections in both immunocompetent and immunocompromised people [1]. The bacterium exhibits a variety of virulence factors, which include pigment synthesis and exopolysaccharide production [2]. As a result, the WHO recently listed this rod as one of the twelve priority bacterial pathogens [3].

*P. aeruginosa* is also a microbe that exhibits high resistance to a great number of conventional antibiotics and disinfectants. Depending on the nature of the genes involved, resistance occurs through intrinsic mechanisms, such the low permeability of its membrane and its ability to form a biofilm [4]. Resistance also occurs through extrinsic mechanisms, such as plasmids and horizontal gene exchanges [5].

Many strategies have been proposed to combat *P. aeruginosa* and targets may be either molecular [6] or cellular structures [7]. Photodynamic Antimicrobial Chemotherapy (PACT) is a technique that involves the production of Reactive Oxygen Species (ROS) from a reaction between light,
photosensitizing molecule, and oxygen [8]. Photosensitized molecule leaves the ground state at a specific light wavelength to produce singlet oxygen. This is highly reactive with the cellular constituents, leading to death by oxidative damage [9].

In terms of sustainable development, substitutes that are less harmful to the environment can be used as a light source and photosensitizer [10]. LED light is recognized as safe, non-thermal, non-invasive, and non-toxic to the handler, without reports of side effects [11]. In addition, bioactive and biodegradable natural substances are more attractive molecules since they can be applied to replace synthetic compounds with respect to environmental impacts and intoxication [12].

Curcumin is a pigment that varies from reddish-brown to yellow, depending on the pH. It is extracted from the root of Curcuma longa (L.), whose bioactive properties had been reported before the 1st century AD [13]. The application of curcumin in different clinical modalities has generated interest in recent years. The molecule exhibits a variety of properties, such as antitumor [14], antioxidant [15], neuroprotective [16], anti-inflammatory [17], and antimicrobial [18].

The antimicrobial action of curcumin on P. aeruginosa occurs through different mechanisms, such as inhibition of quorum-sensing systems, alteration of membrane permeability and motility, downregulation of virulence factors and major disruption of biofilm architecture, as well as downregulation of genes for biofilm inactivation [19-20]. Curcumin absorbs blue light; in the spectrum between 400 and 500 nm. It can be used as an effective photosensitizer due to its property of forming singlet oxygen and excellent biocompatibility [21]. This present work aimed to evaluate the effect of curcumin, with respect to its Minimum Inhibitory Concentration (MIC) effect on biofilm formation in the strain Pseudomonas aeruginosa UFPEDA 416 exposed to blue LED light energy.
2. Material and Methods

2.1 Curcumin

Curcumin, 65% pure, of natural origin (Merk, 1386-5G, Batch: SHBN4217), was used. The standard solution was dissolved in 95% alcohol, obtaining an initial concentration at 20 μg/mL.

2.2 Determination of MIC and MBC

To determine the MIC of curcumin, the technique of microdilution in Müller-Hinton broth (MH) was used. A suspension *P. aeruginosa* UFPE-DA 416 (ATCC 27853) was prepared in a saline solution (NaCl 0.9%) with turbidity adjusted with tube n° 1 of the MacFarland scale [22]. Curcumin concentrations between 10.0 and 0.15 μg/mL were tested. Microplates were incubated at 36±1°C for 24 h. MIC was determined by visual analysis of turbidity, compared to control, as the lowest concentration of curcumin that inhibited growth [23]. The test was performed in triplicate.

MBC (Minimum Bactericidal Concentration) was determined by adding 10 μL of 0.1% resazurin solution to all wells. After 2 hours of incubation at room temperature (25°C), there was either the appearance of a pink color or absence of color, indicating viable cells. If the solution remained blue, this indicated the death of > 99.9% of the bacterial culture, in relation to the concentration of bacteria that was present in the wells at time zero [24]. Positive and negative controls were carried out with the same procedure as described for the MIC test.
2.3 *In vitro* biofilm formation assay

The tests were carried out in a box measuring 50 cm in height x 30 cm in width x 30 cm in length. In the upper part, a spotlight was installed coupled to a high frequency LED lamp (A-60, G-light), 3W, 100-240v, RGB light. At the bottom there was a base to support a 90 mm diameter Petri dish. The light source was 13 cm above the Petri dish. In the Petri dish, 10 mL of saline solution containing curcumin (0.63 mg/mL) and 1 mL of the inoculum, prepared as previously described, were dispensed. The light energy emitted by the LED light was calculated using the equation 1 [25]:

\[ P = 2\pi I_0 h^2 \times (1 - \frac{1}{1+\sqrt{(1+r/h)^2}}) \]  
(Eq. 1)

Where, \( I_0 \) – measured light intensity (\( \lambda \)), \( r \) – radius of the Petri dish, \( h \) – height of the light source at the center of the Petri dish. The system was irradiated for 20, 40, 60 and 120 minutes. At each interval, an aliquot of 100 \( \mu L \) was transferred to plastic microtubes containing 900 \( \mu L \) of MH broth and incubated for 48h at 29±1 °C. Afterwards, the contents of the microtubes were transferred to new previously sterilized tubes. The walls of the microtubes were washed 3 to 5 times with tap water in order to remove any remaining non-adherent cells, and after drying for 1 h, 1 mL of 1% crystal violet solution was added to the microtubes. Twenty minutes later, the dye was discarded and the excess of dye on the microtubes was removed with tap water. Then, 1 mL of ethanol 99% was added and after 30 minutes, and the absorbance at 590 nm (Quimis U2M) of the crystal violet-ethanol solution was measured. Test control was performed without addition of curcumin and light exposure [25].

The adherence percentage was calculated using the equation 2 [26]:

\[ \frac{[(ODC - ODT) \div ODC] \times 100}{1} \]  
(Eq. 2)
Where, ODC – mean optical density of the control; ODT – mean optical density of the treatment. Adherence of cells was classified as strong (≥ 80%), moderate (if > 40.01 and < 79.99%) or weak (if ≤ 40%) [27]. To determine the potential of cells to form biofilm, even under stress, the optical density of the medium, multiplied by three, served as the cutoff point. Any value above the cutoff point indicated the presence of viable cells [28].

2.4 Determination of the planktonic cells

The test was carried out in triplicate, by optical determination of the supernatant collected in the in vitro biofilm formation assay. An aliquot of 500 μL from the supernatant was diluted in an equal volume of saline and the absorbance at 620 nm was measured [29]. The test control used growth without the addition of curcumin.

3. Results

3.1 MIC and MBC

The MIC of curcumin on P. aeruginosa UFPEDA 416 was 0.63 μg/mL, demonstrating only growth inhibitory activity without observation of MBC (Table 1). Thus, the action of curcumin on the strain was bacteriostatic.

<table>
<thead>
<tr>
<th>Curcumin (μg/mL)</th>
<th>MIC</th>
<th>MBC</th>
</tr>
</thead>
<tbody>
<tr>
<td>10.00</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>5.00</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>2.50</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>0.63</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>0.31</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>0.15</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Control</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

(+) formation of turbidity, indicating growth; (-) absence of growth.
3.2 *In vitro* biofilm formation assay

The LED light source emitted an energy of 28.03 mW/cm$^2$, disturbed the formation of the biofilm of *P. aeruginosa* UFPEDA 416, witnessed by the weak adherence (Table 2). Compared to the control, a reduction in adherence was observed to 40.8% after 20 minutes of exposure to the treatment, reaching approximately 11% from 60 minutes and staying the same up to 120 minutes.

The cutoff point was 2.129 (mean optical density = 0.710). This indicated that under all conditions, there were cells capable of adhering, although curcumin did exhibit activity.

Planktonic cell density was reduced by more than 90% within 20 minutes of exposure to blue LED light, followed by a cell regrowth over the next 120 minutes. It suggested that the treatment was more disruptive for the sessile cell state than for the planktonic cell state, confirming the bacteriostatic effect of the photosensitizer.

Table 2. Optical density of sessile and planktonic cells of *Pseudomonas aeruginosa* UFPEDA 416 and their percentages of adhesion (%adh) and reduction (%red) when exposed to 0.63 μg/mL of curcumin at different times of irradiation by blue LED light (λ = 480 nm)

<table>
<thead>
<tr>
<th>Time (minutes)</th>
<th>20</th>
<th>40</th>
<th>60</th>
<th>120</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Sessile</strong></td>
<td>0.741±0.074</td>
<td>0.870±0.091</td>
<td>1.109±0.069</td>
<td>1.107±0.003</td>
</tr>
<tr>
<td>% adh</td>
<td>40.8</td>
<td>30.4</td>
<td>11.1</td>
<td>11.4</td>
</tr>
<tr>
<td><strong>Planktonic</strong></td>
<td>0.117±0.027</td>
<td>0.870±0.091</td>
<td>1.109±0.069</td>
<td>1.107±0.462</td>
</tr>
<tr>
<td>% red</td>
<td>93.8</td>
<td>54.0</td>
<td>41.4</td>
<td>41.5</td>
</tr>
</tbody>
</table>

Control: sessile cells (1.250±0.012) and planktonic cells (1.893±0.020)

4. Discussion

One of the main mechanisms that helps *P. aeruginosa* to exhibit resistance is its ability to colonize a biofilm [30]. The bacterial lifestyle related to the formation of biofilms involves changes in physiological processes
resulting in a switch from propagation of planktonic cells throughout the environment to organization of complex colonies [31].

This work verified the effectiveness of PACT against *P. aeruginosa* UFPEDA 416, using curcumin as a photosensitizer and a high frequency LED lamp in the blue spectrum range as light energy. Light plays a crucial role in the formation and development of biofilms. It provides heat, serving not only as a source of energy, but also promotion of the adhesion process of cells [32]. Certain wavelengths significantly interfere with the development of initial biofilms (average wavelength from 470 nm). Other variables need to be considered, such as the time of exposure to light, the microbial physiologic status, and the nature of the photosensitizer [33].

The use of biodegradable naturally-occurring bioactive molecules is an attractive strategy to replace synthetic compounds [34]. Natural pigments are potential candidates in PACT [35]. Some studies have successfully used chlorophyll [36], indigo [37] and curcumin [38].

The antimicrobial activity of curcumin spans a broad spectrum for both Gram-positive and Gram-negative bacteria. The curcumin molecule acts on bacterial growth through reactions with oxygen, interfering with the viability of planktonic cells, as well as with the mechanisms of biofilm formation [19]. Preliminary data suggest that the antibiofilm activity of curcumin occurs via regulatory mechanisms of important operons responsible for cell adhesion to a given substrate [39].

Reported studies of curcumin-mediated PACT using blue LED light have obtained conflicting results, especially with *P. aeruginosa*. Mahdizade-Ari et al. (2019) [40] observed a significant reduction in the number of viable cells with 80 μg/mL of curcumin under 445 nm (600 mW/cm²). On the other hand, Penha et al. (2017) [21] did not find activity of 75 μM curcumin at 470 nm (417 J/cm² for 30 minutes) against *P. aeruginosa ATCC 27853* (UFPEDA 416). This also was the only non-susceptible strain among those tested. Araújo et al. (2012) [41] when testing 0.015 μg/mL curcumin at 450 nm (67
mW/cm²), observed that Gram-negative bacteria were less susceptible to curcumin-mediated PACT. The authors justified the fact that these bacteria have a complex outer membrane with two lipid layers that act as a physical and functional barrier between cells and the environment. They are less sensitive than Gram-positive bacteria, whose walls are many times thicker and exhibit greater porosity and permeability.

On the other hand, relevant susceptibility has been achieved with other Gram-negative species, such as *Escherichia coli* [42], *Aeromonas hydrophila* ATCC 7966 [21], *Actinobacillus actinomycetemcomitans* (0.5 µg/mL of curcumin associated with chlorhexidine, 420-480 nm, 400 mW/cm², 5 minutes) [43], and *Vibrio parahaemolyticus* (10 µM, 470 nm, 3.6 J/cm² for 1 minute), which reduced bacteria by 6.5 log units [44].

Pre-treatment of curcumin, i.e., pre-exposure to blue LED light before contact with cells, enhances its activity as a photoinactivator, making it efficient against Gram-negative rods. Pre-exposure time ranged from 1 to 5 minutes at wavelengths between 455 to 470 nm, but the curcumin concentration was very variable in these studies [45-47]. Pre-exposure to the photosensitizer, however, was not used in our study.

Our results showed a progressive reduction in the adhered cell community, as well as the density of planktonic cells. The first 20 minutes of irradiation with blue LED light led to the greatest decrease in both quantifications. Reduced adherence, however, cannot be fully attributed to curcumin-mediated PACT. For this reason, quantification of planktonic cells was important. The percentage of reduction in planktonic cells increased by almost 50% and stabilized after 40 minutes of treatment, while the percentage of adherence also reduced.

In *P. aeruginosa*, weak adhesions are common [48]. According to Norat et al. (2022) [49], this particularity is associated with the evolution of the species, which activates detachment and migrates to colonize new substrates when it is exposed to environmental stress or seeks to avoid competition for
nutrients. In addition, as the test was carried out on the MIC of curcumin, it was possible to observe the bacteriostatic action of the molecules onto cells that may have migrated, followed by detachment, since were confined in the microtubes. Although this scenario is possible, the disturbance in the cells did not affect the ability to form biofilm. By switching this lifestyle, *P. aeruginosa* can resist inhibitory concentrations of antimicrobial compounds from 100 to 1000 times, compared to its planktonic phenotype [50].

Still related to resilience, *P. aeruginosa* exhibits the ability to express different virulence factors, which allow its flexibility and favors its response against different environmental stressors [51]. In this sense, curcumin-mediated PACT may become limited to intrinsically resistant microbes, such as *P. aeruginosa* since they express DNA repair capacity when irradiated [52].

Additionally, the stage of biofilm maturity is an important variable in terms of the effectiveness of curcumin in inhibiting their formation and stability. This may explain the large variation in results between studies with different bacterial species [45], as well as with different isolates of the same species [53]. On the other hand, although different concentrations of curcumin have been tested, efficacy is achieved at very low levels [54], which confers security and advantage over other natural and synthetic molecules.

**5. Conclusions**

Curcumin-mediated PACT on *P. aeruginosa* UFPEDA 416 produced a bacteriostatic action in the strain, especially in its planktonic form. The most promising exposure time was twenty minutes. Because genetic variability among specimens of *P. aeruginosa* can result in strains that are more resistant or less resistant, we suggest that future studies do not test curcumin just in its minimum inhibitory concentration form. Thus, a biocidal
effect can be achieved by curcumin 2xMIC and above, taking advantage of the safety of the molecule for human use.

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References


