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Comparison of seven structurally related coumarins on the inhibition of *Quorum sensing* of *Pseudomonas aeruginosa* and *Chromobacterium violaceum*

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ABSTRACT

Quorum sensing (QS) is a cell-to-cell signaling communication system that controls the virulence behavior of a broad spectrum of bacterial pathogens, participating also in the development of biofilms, responsible of the antibiotic ineffectiveness in many infections. Therefore, QS system is an attractive target for antimicrobial therapy. In this study, we compare the effect of seven structurally related coumarins against bacterial growth, biofilm formation and elastase activity of *Pseudomonas aeruginosa*. In addition, the anti-pathogenic capacity of the seven coumarins was evaluated on the wild type and the biosensor strain of *Chromobacterium violaceum*.

The comparative study of coumarins showed that molecules with hydroxyl groups on the aromatic ring displayed higher activity on the inhibition of biofilm formation of *P. aeruginosa* over coumarins with substituents in positions 3 and 4 or without the double 3,4-bond. These 3 or 4-hydroxylated positions caused a decrease in the anti-biofilm activity obtained for coumarin. However, the hydroxyl group in position 3 of the pyrone ring was important for the inhibition of *C. violaceum* QS and elastolytic activity of *P. aeruginosa*. The effects observed were active independently of any effect on growth. According to our results, coumarin and its hydroxylated derivatives represent an interesting group of compounds to use as anti-virulence agents against the human pathogen *P. aeruginosa*.

1. Introduction

Development of alternative therapies that can target the multidrug-resistant bacteria emerged in the last few decades because of the indiscriminate use of antibiotics needs immediate attention by the world public health systems [1]. An interesting approach is the inhibition of *Quorum sensing* (QS), which involves the production, release, and subsequent detection of chemical signaling molecules, called autoinducers, by a population-density-dependent cell-cell communication system affording bacteria the capacity for multicellular control of gene expression linked to virulence and pathogenesis. In general, these autoinducers include oligopeptides and *N*-acyl homoserine lactones (AHL) in gram-positive and -negative bacteria, respectively [2]. During infections, bacteria communicate and behave as a group for social interactions like a multi-cellular organism, which provide them significant benefits in host colonization, formation of biofilms, defense against competitors, and adaptation to changing environments. In this sense, the QS system is an attractive target for antimicrobial therapy [3] and can be blocked in different ways, including (i) inhibition of AHL molecule biosynthesis, (ii) degradation of AHL molecules by bacterial lactonases, and (iii) inactivation of AHL receptor protein [4].

Many natural compounds have been reported as anti-QS inhibitors [5], and some of the most promising anti-QS molecules found

Abbreviations: AHLs, *N*-acyl homoserine lactones; CFU, colony forming units; DMSO, dimethyl sulfoxide; HHL, *N*-hexanoyl homoserine lactone; QS, *Quorum sensing*; LB, Luria–Bertani; IC50, half inhibitory concentration.

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in plants are from the coumarin family, a large structurally diverse family of plant phenolic compounds characterized by their pharmacological properties [6]. Coumarin and its derivatives have been reported to have important biological activities like antitumour [7–9], anti-inflammatory [10,11], anticoagulant [12,13], antibacterial [14,15], among others. And in the last few years, coumarins were reported to inhibit biofilm and reduce virulence factors of human pathogenic bacteria such as *Escherichia coli* and *Pseudomonas aeruginosa* [16,17].

In this work, we selected structurally related coumarins to compare their ability to inhibit QS signaling on the AHL-based system of Gram-negative bacteria using a biosensor strain of *Chromobacterium violaceum*. Their anti-pathogenic capacity was also compared by inhibiting biofilm and elastase, an important virulence factor of *Pseudomonas aeruginosa*, a bacterial pathogen responsible for forming resistant biofilms during respiratory diseases.

2. Results and discussion

Coumarin belonging to plant phenolic compounds was identified as a potent anti-virulent compound against a broad spectrum of pathogens, and some coumarin derivatives have previously shown to possess promising pharmacological properties, with evidence of interference in the AI-1 and AI-2 QS systems, as well as hinder the formation of biofilm in *E. coli*, *Salmonella typhimurium* and *P. aeruginosa* [17,18]. Because different substitutions in specific sites significantly affect the biological activity of coumarins [19–21], the potency of seven structurally related coumarins in the inhibition of QS-mediated pathogenesis of *Chromobacterium violaceum* and *P. aeruginosa* was compared.

2.1. Structurally related coumarins inhibit C. violaceum QS systems

The QS mechanism in *C. violaceum* ATCC 12472, directly controlled by the CviI/CviR system (LuxI/LuxR homologues), is correlated with the production of the purple pigment violacein in response to threshold concentrations of the autoinducer *N*-hexanoyl homoserine lactone (HHL) [22]. The parallel use of *C. violaceum* Tn5-mutant CV026, which violacein production depends on auto-inducer exogenous addition [23], allows simultaneous discrimination of compounds that act as quenchers of the AHL signal from those that inhibit the AHL synthesis [24]. Anti-QS compounds inhibit production of violacein in both cases making these strains excellent for screening [25] (see Fig. 1).

In the *C. violaceum* CV026 bioassay, the presence of turbid halos on a violet background indicated QS inhibition without affecting microbial growth. At 50 μg/disk, coumarins were able to inhibit QS system-associated pigment production (Fig. 2) indicating that they might have a modulated effect on the interaction of the HHL with the CviR receptor or are able to degrade the signaling molecule before it reaches the bacteria (quorum quenchers) [25]. Antagonist molecules that bind in place of the natural AHL ligand induce a CviR conformation that prevents DNA binding to the transcription factor that activates gene expression and induces *C. violaceum* virulence [25].

The order of violacein inhibition for coumarins was 6,7-dihydroxycumarin ≫ 7-hydroxycoumarin > 3-hydroxycoumarin > 6-hydroxycoumarin > coumarin. Dihydrocoumarin and 4-hydroxycoumarin at the concentrations tested could not inhibit violacein production mediated by QS.

Based on the qualitative bioassay results, we tested the possibility of QS inhibitory activity of coumarins at level of the AHL synthesis by quantifying the production of violacein in the *C. violaceum* ATCC 12472. AHL synthesis in the wild type strain showed to be altered in presence of coumarins in a dose-dependent manner and the potencies displayed (IC50) were in the range of 4.5 to 8.5 μg/mL for the most active coumarins: 3-hydroxycoumarin > 7-hydroxycoumarin > coumarin > 6,7-dihydroxycoumarin > 6-hydroxycoumarin; whereas the IC50 for 4-hydroxycoumarin and dihydrocoumarin were up to 70 μg/mL (Fig. 3). Cell viability was not affected after coumarin treatments (Table 1), therefore, the inhibition in the production of violacein was due to a blockage in cellular communication rather than to inhibition in cell growth.

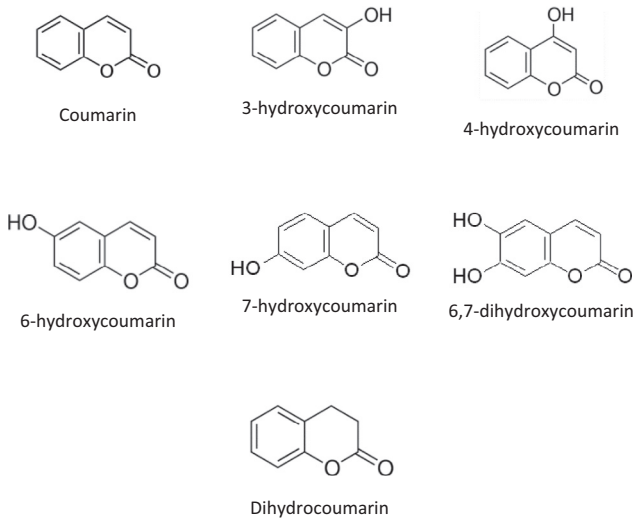

Fig. 1. Coumarin and coumarin hydroxylated derivatives.

2.2. Structurally related coumarins inhibit P. Aeruginosa QS system

On the other hand, *P. aeruginosa*, an opportunistic pathogen implicated in many difficult-to-treat infections that often result in significant morbidity and mortality, play a prominent role in hospital infections [26]. *P. aeruginosa* has known QS systems, LasI/R and RhlI/R, to control several genes involved in biofilm formation and virulence factor production such as, LasB elastase, LasA protease, pyocyanin, rhamnolipids, and exotoxins [27].

As shown in Fig. 4B, coumarins significantly reduced the biofilm formation of *P. aeruginosa*, without affecting the cell growth (Fig. 4A). The most potent compounds capable of inhibiting in 63, 61, 53, 46, and 38% the biofilm formation were 6,7-dihydroxicoumarin, 7-hydroxycoumarin, coumarin, 6-hydroxycoumarin, and 3-hydroxycoumarin at 100 μg/mL, respectively. On the other hand, 4-hydroxycoumarin and dihydrocoumarin at the same concentration only inhibited biofilm formation in 20 and 18% respectively.

The identification of compounds with the ability of inhibiting biofilm without cytotoxicity to the bacterial cell is the major importance to reduce the risk of drug resistance [28].

Similar results were shown for coumarin, 7-hydroxycoumarin and 6,7-dihydroxycoumarin in the inhibition of biofilm and virulence of the enterohemorrhagic *Escherichia coli* O157:H7, responsible for the most common hemorrhagic colitis [29,16].

Against *P. aeruginosa* ATCC 27853 the position of the hydroxyl group also affected the biofilm activity in a similar way that Lee and collaborators [16] reported on *E. coli* O157:H7. Biofilm inhibition was strongly diminished in presence of the 4-hydroxylated coumarin, and enhanced when the hydroxyl groups were in position 6 or 7. Hydroxylation in position 3 could not surpass the inhibition caused by coumarin, and dihydrocoumarin only inhibited in an 18% the biofilm formation, indicating the requirement of hydro-

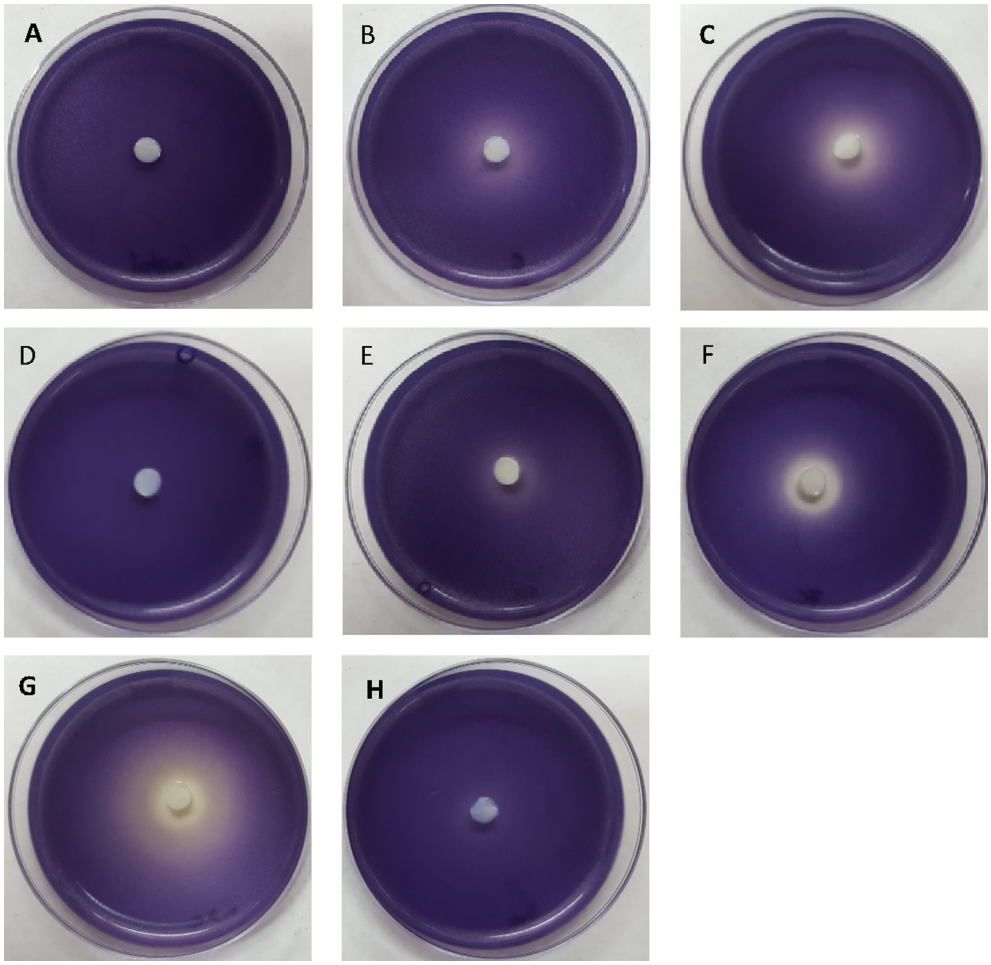

Fig. 2. Inhibition of violacein production in *C. violaceum* CV026 by structurally related coumarins in DMSO as control. A: Control, B: Coumarin, C: 3-hydroxycoumarin, D: 4-hydroxycoumarin, E: 6-hydroxycoumarin, F: 7-hydroxycoumarin, G: 6,7-dihydroxycoumarin, H: dihydrocoumarin.

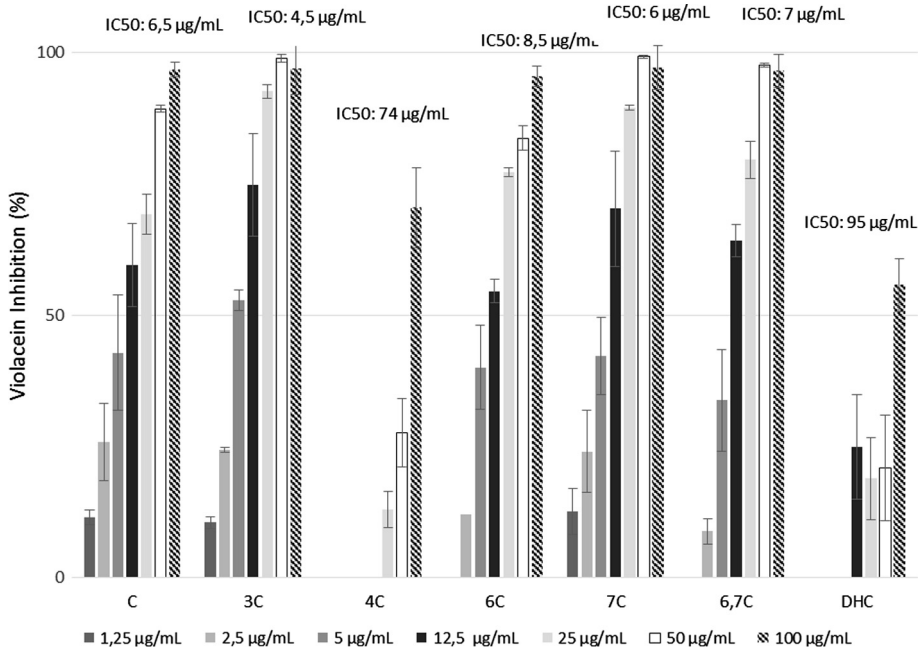

Fig. 3. Inhibition of violacein production in *C. violaceum* ATCC 12472 by different concentrations of structurally related coumarins. Potencies displayed as IC50 for each compound.

Table 1
Effect of structurally related coumarins on viability of *C. violaceum* ATCC 12472, as estimated by the standard plate count method after 24 h of incubation.

Coumarins 100 μg/mL	T0 CFU/mL	T24 CFU/mL
Control (DMSO 1%)	$1.13 \times 10^6 \pm 0.01$	$1.01 \times 10^8 \pm 0.02$
Coumarin	$1.32 \times 10^6 \pm 0.01$	$1.19 \times 10^8 \pm 0.01$
3-Hydroxycoumarin	$0.99 \times 10^6 \pm 0.03$	$1.44 \times 10^8 \pm 0.03$
4-Hydroxycoumarin	$1.21 \times 10^6 \pm 0.02$	$9.32 \times 10^7 \pm 0.04$
6-Hydroxycoumarin	$1.22 \times 10^6 \pm 0.01$	$8.06 \times 10^7 \pm 0.05$
7-Hydroxycoumarin	$1.17 \times 10^6 \pm 0.02$	$6.91 \times 10^8 \pm 0.02$
6,7-Dihydroxycoumarin	$1.25 \times 10^6 \pm 0.03$	$7.06 \times 10^7 \pm 0.05$
Dihydrocoumarin	$1.19 \times 10^6 \pm 0.04$	$6.48 \times 10^7 \pm 0.07$

Data are presented as mean ± standard deviation; $p < 0.05$, $n = 3$.

xyl groups in the benzene ring and the 3,4-double bond in the coumarin structure to obtain the anti-biofilm effect.

Unlike the results reported by Lee et al. [16] for *E. coli*, our results showed that the *P. aeruginosa* biofilm formation was significantly inhibited in the presence of the dihydroxylated coumarin in position 6 and 7, being the most potent anti-biofilm compound in our screening.

Elastolytic activity of *P. aeruginosa* is an important virulence factor under control of the QS system. More than 75% of clinical isolates of *P. aeruginosa* secrete elastase B (LasB), an elastolytic metalloproteinase that is encoded by the lasB gene. *In vitro* studies have demonstrated that LasB degrades several components in both the innate and adaptive immune systems [30]. In addition, LasB acts within the bacterial cell as a key regulator in the generation of the secreted polysaccharides that constitute the bacterial biofilm [31]. Inhibition of one of the main virulence factors (such as elastase activity) of bacteria turn them susceptible to the attack of the host immune system. Thus, LasB inhibition by natural products could be an important strategy for controlling *Pseudomonas* virulence in bacterial infections [32].

Hence, we determined the *P. aeruginosa* elastolytic activity in presence of the seven structurally related coumarins (Fig. 4C). Our results showed an inhibition of 52, 47, 45, 43, 37, and 33% of *P. aeruginosa* elastase B activity caused by 20 μg/mL of 7-hydroxycoumarin, coumarin, 3-hydroxycoumarin, 6-hydroxycoumarin, 4-hydroxycoumarin and 6,7-dihydroxycoumarin, respectively. At this concentration, dihydrocoumarin inhibited the enzyme activity in only 3%, although at 50 μg/mL caused 30% of inhibition. The other coumarins inhibited in 100% the elastolytic activity at 50 μg/mL.

3. Materials and methods

3.1. Bacterial strains and growth conditions

The bacterial strains used were *C. violaceum* ATCC 12472, *C. violaceum* 026 and *P. aeruginosa* ATCC 27853. Before experiments, both *C. violaceum* strains and *P. aeruginosa* were inoculated into broth and cultured 16 h with shaking in suitable Luria Bertani (LB) broth for each strain at 26 °C and 37 °C, respectively. Colony counting was carried out when necessary by serial decimal dilution in sterile distillated water and plating out on the optimum medium for each bacteria and incubation at the appropriate temperature for 24 h.

N-hexanoyl homoserine lactone (HHL) from Sigma-Aldrich (Argentine) was dissolved in absolute ethanol. Dihydrocoumarin, coumarin, 3-hydroxycoumarin, 4-hydroxycoumarin, 6-hidroxycoumarin, 7-hydroxycoumarin and 6,7-dihydroxycoumarin were purchased from Sigma- Aldrich (Argentine).

All assays were performed with coumarins at final concentrations of 1.25, 2.5, 5, 10, 12.5, 25, 50 and 100 μg/mL and dissolved in DMSO (1% final concentration).

3.2. Antibacterial assay

Antibacterial activity of coumarins was determined in the appropriate medium using a broth microdilution method in the 96-well polystyrene microtiter plates [33]. Overnight-grown bacteria diluted culture (OD560: 0.08 ± 0.02) in LB medium (10^6 CFU/mL) was mixed with coumarins and incubated statically at 37 °C for 18–24 h.

Inhibition of bacterial growth containing the coumarins was assessed by comparison with bacterial growth in the control wells with DMSO (1% final concentration).

3.3. Biosensor assay

Detection of QS inhibition by coumarins was carried out by a biosensor assay using *C. violaceum* CV026 reporter strain [23]. Sterile paper disk of 6 mm diameter were loaded with 2 μl of a 25 μg/μL coumarin solutions to obtain 50 μg per disk. The paper disks with coumarins at 50 μg were placed onto the surfaces of *C. violaceum* CV026 inoculated LB agar plates supplemented with 5 μg of HHL. DMSO was included as vehicle-only controls. After incubation at 27 °C for 18–24 h, inhibition of QS was detected by the presence of a zone of colorless but viable cells around the sample that is clearly distinguishable from the zone of growth inhibition.

3.4. Quantification of violacein inhibition by structurally related coumarins

Based on the bioassay results, detailed investigation of inhibition of QS-controlled violacein production in *C. violaceum* ATCC 12472 and coumarins was carried out according to Choo et al. [34] with some modifications to be applicable to microtiter well plates. Briefly, 198 μL per well of overnight-grown *C. violaceum* ATCC 12472 diluted culture in LB medium to reach a concentration of 1 x 10^6 CFU/mL was added onto sterile 96-micro well plates. Two microliters of different concentrations of coumarins in DMSO were added to the well with the bacteria solution and incubated at 28 °C for 18–24 h. Solvent controls were prepared with DMSO without coumarins. After incubation, the violacein produced in each well was centrifuged at 10,000 rpm for 10 min to sediment the insoluble pigment and the supernatants were discarded. The violacein was dissolved in 200 μL DMSO and the cells separated by centrifuging (13,000 rpm, 10 min). Then supernatants were transferred to a new 96-wells plate and the OD was measured at 590 nm on a plate reader (Power Wave XS2, Biotek, Vermont, USA). The concentration of coumarins necessary to inhibit the 50% of violacein production (IC50) was calculated respect to control. The assay was run in triplicate wells and in three independent experiments. Bacterial viability in the presence of tested concentrations of coumarins in the culture broth was measured after serial dilution by a standard plate count method.

3.5. Effect of structurally related coumarins on biofilm formation regulated by QS in P. aeruginosa

Inhibition of biofilm formation was studied as described by O'Toole & Kolter [35] with some modifications. Briefly, in 96 well-microtiter polystyrene plate, 190 μL of overnight-grown *P. aeruginosa* diluted culture (OD560: 0.1 ± 0.02) in LB medium was mixed with 10 μl of different concentrations of coumarins in DMSO and incubated statically for 24 h. After incubation, the medium was carefully removed, washed thrice with PBS and the biofilm dried at room temperature. Biofilms were stained with 200 μL of an aqueous solution of crystal violet, (0.1% w/v) for 20 min, unbound stain was removed by two washes with water, dried and crystal violet bound to biofilm was solubilized with 200 μL

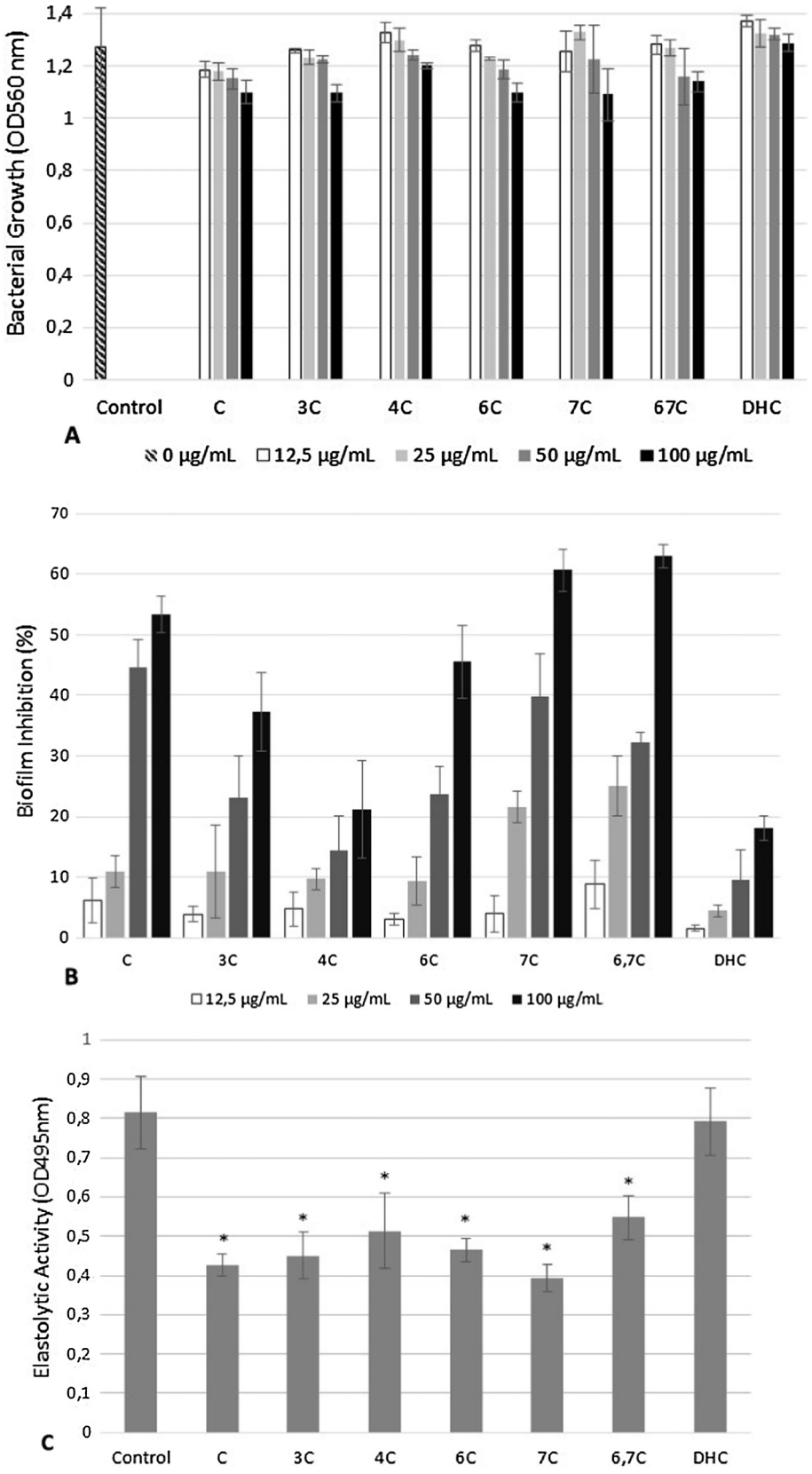

Fig. 4. A: *P. aeruginosa* growth and B: *P. aeruginosa* biofilm inhibition, in presence of 12.5, 25, 50 and 100 µg/mL of structurally related coumarins. C: Elastolytic activity of *P. aeruginosa* in presence of 20 µg/mL of structurally related coumarins.

absolute ethanol during 30 min at 37 °C with shaking. Absorbance at 540 nm of ethanol solutions of crystal violet was determined using a microtiter plate reader (Power Wave XS2. Biotek, Vermont, USA).

3.6. Elastase B activity assay

Elastolytic activity was determined using a modification of a previously reported method [36]. Elastin Congo red (100 μL) (Sigma) dissolved in Tris–HCl (pH 8.0) at a concentration of 5 mg/mL was mixed with 100 μL cell-free culture supernatant from *P. aeruginosa* ATCC 27853 grown, during 24 h, in LB media containing 20 μg/mL of coumarins. The reaction mixture (200 μL) was incubated at 37 °C for 24 h, and centrifuged at 13,000 rpm for 10 min to remove insoluble ECR. The absorbance (495 nm) of the supernatant is a measure of the elastase B activity.

3.7. Data analysis

All experiments were conducted in triplicates or quadruplicates at least thrice. ANOVA was used to analyze the differences between the treatments; and for potency (half inhibitory concentrations, IC50) calculations, data were fitted using a sigmoidal curve with variable slope, using GraphPad Prism 5. Data $p \leq 0.05$ was considered as significant unless otherwise specified.

4. Conclusion

In the present study, the anti-biofilm and anti-QS activity of a dihydrocoumarin (with a 3,4-unsaturated bond in the pyrone ring) and four hydroxycoumarins (two with the hydroxyl group on the aromatic ring of the molecule and two with the hydroxyl group replacing hydrogen of the pyrone ring) were compare with the anti-biofilm and anti-QS activity of coumarin, to determine how different substitutions in the coumarin skeleton inhibit biofilm and QS mechanisms of *C. violaceum* and *P. aeruginosa*. We can conclude that coumarins with hydroxyl groups on the aromatic ring display higher activity on the inhibition of biofilm formation of *P. aeruginosa* over coumarins with substituents in positions 3 and 4 or without the double 3,4-bond in the pyrone ring. However, the hydroxyl group in position 3 of the pyrone ring was important for the inhibition of *C. violaceum* QS as well as the elastolytic activity of *P. aeruginosa*.

In summary, coumarin and its hydroxylated derivatives represent an interesting group of compounds to use as anti-virulence agents against the human pathogen *P. aeruginosa*.

Conflicts of interest

The authors declare no conflict of interest.

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