

## Coumarin (2H-1-Benzopyran-2-one) act against planktonic and biofilm forms of *Staphylococcus aureus*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa*

Laísa Vilar Cordeiro<sup>1\*</sup>, Helivaldo Diógenes da Silva Souza<sup>2</sup>, Giulian César da Silva Sá<sup>3</sup>, Aleson Pereira Sousa<sup>1</sup>, Pedro Thiago Ramalho de Figueiredo<sup>1</sup>, Maria das Neves Silva Neta<sup>1</sup>, José Maria Barbosa Filho<sup>1</sup>, Edeltrudes de Oliveira Lima<sup>1</sup>

<sup>1</sup> Department of Pharmaceutical Science, Health Sciences Center, Federal University of Paraíba, 58033-455, João Pessoa, Brazil.

<sup>2</sup> Biochemistry Department, Biosciences Center, Federal University of Rio Grande do Norte, Natal, Brazil.

<sup>3</sup> Chemistry Department, Exact and Natural Sciences Center, Federal University of Paraíba, João Pessoa, Brazil.

\* Corresponding author e-mail: [laisavilar@gmail.com](mailto:laisavilar@gmail.com)

Received: February 2, 2021

Corrected: March 21, 2021

Accepted: March 29, 2021

### SUMMARY

**Introduction:** biofilm-related infections caused by *Staphylococcus aureus*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* are difficult to treat and few effective pharmacological options are currently available for this purpose. In this context, coumarin (2H-1-Benzopyran-2-one) has been reported to have antibacterial and antibiofilm activity, but this potential remains poorly understood. **Aim:** to investigate the action of coumarin on planktonic and biofilm forms of *S. aureus*, *K. pneumoniae* and *P. aeruginosa*. **Results:** a minimum inhibitory concentration (MIC) of coumarin ranging from 256 to 1024 µg/mL was observed, with a remarkable ability to inhibit the formation of biofilms and to act on mature biofilms in concentrations close to MIC. **Conclusion:** coumarin has strong activity against planktonic and biofilm forms on the three species of great relevance in the clinical scenario. These results are interesting to enable a pharmacological alternative for the treatment of these infections.

**Keywords:** Coumarin, antibacterial activity, biofilms, 2H-1-Benzopyran-2-one.

## RESUMEN

La cumarina (2H-1-Benzopiran-2-ona) actúa contra las formas planctónicas y de biopelícula de *Staphylococcus aureus*, *Klebsiella pneumoniae* y *Pseudomonas aeruginosa*

**Introducción:** las infecciones relacionadas con la biopelícula causadas por *Staphylococcus aureus*, *Klebsiella pneumoniae* y *Pseudomonas aeruginosa* son difíciles de tratar y actualmente existen pocas opciones farmacológicas eficaces para este propósito. En este contexto, se ha informado que la cumarina (2H-1-Benzopiran-2-ona) tiene actividad antibacteriana y antibiofilm, pero este potencial sigue siendo poco conocido. **Objetivo:** investigar la acción de la cumarina sobre formas planctónicas y de biopelículas de *S. aureus*, *K. pneumoniae* y *P. aeruginosa*. **Resultados:** se observó una concentración inhibitoria mínima (CMI) de cumarina en el rango de 256 a 1024 µg/mL, con una notable capacidad para inhibir la formación de biofilms y actuar sobre biofilms maduros en concentraciones cercanas a la CMI. **Conclusión:** la cumarina tiene una fuerte actividad contra las formas planctónicas y biofilm sobre las tres especies de gran relevancia en el escenario clínico. Estos resultados son interesantes para habilitar una alternativa farmacológica para el tratamiento de estas infecciones.

*Palabras clave:* Cumarina, actividad antibacteriana, biopelículas, 2H-1-Benzopiran-2-ona.

## RESUMO

A cumarina (2H-1-benzopiran-2-ona) age contra as formas planctônicas e de biofilme de *Staphylococcus aureus*, *Klebsiella pneumoniae* e *Pseudomonas aeruginosa*

**Introdução:** as infecções relacionadas ao biofilme causadas por *Staphylococcus aureus*, *Klebsiella pneumoniae* e *Pseudomonas aeruginosa* são difíceis de tratar e poucas opções farmacológicas eficazes estão disponíveis atualmente para esse propósito. Nesse contexto, foi relatado que a cumarina (2H-1-benzopirano-2-ona) tem atividade antibacteriana e antibiofilme, mas esse potencial permanece pouco conhecido. **Objetivo:** investigar a ação da cumarina sobre as formas planctônicas e de biofilme de *S. aureus*, *K. pneumoniae* e *P. aeruginosa*. **Resultados:** observou-se uma concentração inibitória mínima (CIM) de cumarina variando de 256 a 1024 µg/mL, com notável capacidade de inibir a formação de biofilmes e de atuar sobre biofilmes maduros em concentrações próximas à CIM. **Conclusão:** a cumarina possui forte

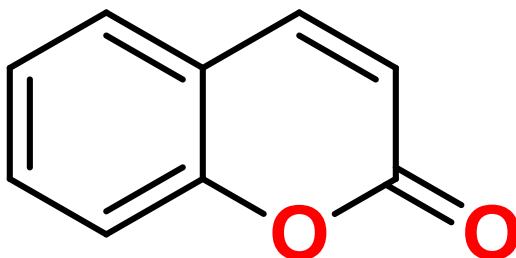
atividade contra as formas planctônicas e de biofilme sobre as três espécies de grande relevância no cenário clínico. Esses resultados são interessantes para possibilitar uma alternativa farmacológica para o tratamento dessas infecções.

*Palavras-chave:* Cumarina, atividade antibacteriana, biofilmes, 2H-1-benzopirano-2-ona.

## INTRODUCTION

Bacteria exhibit two modes of growth. Those that live free in a liquid medium, where they are dispersed, are called planktonic bacteria. When these planktonic bacteria are attached to a surface, they are called sessile cells. In the sessile state, these bacteria differ from their planktonic condition, because living in structured communities they begin to acquire some survival advantages [1]. Bacterial biofilms are formed when planktonic cells adhere to an inert surface and undergo several changes that culminate in the formation of an aggregate complex composed of sessile cells wrapped in an exopolymeric matrix. These complex structures are extremely resistant to the action of the immune system and antibiotics, being closely related to persistent infections. The most important biofilm-forming bacteria include *Staphylococcus aureus*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa*, leading to severe clinical complications often with lethal outcomes [2, 3].

Coumarin (2H-1-Benzopyran-2-one, figure 1) is a natural product found in several plant species, which is related to a wide range of biological activities [4]. Among the applicabilities of coumarin, its antibacterial and antibiofilm potential has been shown [5-8], although its action is not completely clear. Based on the need for new therapeutic alternatives to treat infections caused by species producing biofilms, this study aimed to investigate the action of coumarin on planktonic and biofilm forms of *S. aureus*, *K. pneumoniae* and *P. aeruginosa*.



**Figure 1.** Structure of 2H-1-benzopyran-2-one.

## EXPERIMENTAL

### Substances

The Coumarin (2H-1-Benzopyran-2-one) was obtained from Merck/Sigma-Aldrich® (Purity  $\geq 98\%$ ). The substance was properly weighed and solubilized in dimethyl sulfoxide (DMSO) at 5 % and Tween-80 at 2 %, to obtain emulsions in the concentrations necessary for use in the tests.

### Strains

In this study, 15 bacterial strains were used: *Staphylococcus aureus* (ATCC-13150, LM-116, LM-182, LM-297, LM-356), *Klebsiella pneumoniae* (ATCC-700603, LM-143, LM-166, LM-176, LM-260), *Pseudomonas aeruginosa* (ATCC-15442, LM-163, LM-230, LM-286, LM-362). Except for the American Type Culture Collection strains (ATCC-13150, ATCC-700603, ATCC-15442), all other strains come from clinical isolates, which are part of the MICOTECA of the Laboratory for Research on Antibacterial and Antifungal Activities at the Federal University of Paraíba, Brazil. For use in the assays, bacterial suspensions were prepared in 0.9 % saline, from fresh cultures, and adjusted to the McFarland standard 0.5 scale.

### Minimum Inhibitory Concentration (MIC)

Coumarin activity on planktonic cells was determined by the minimum inhibitory concentration (MIC) of the substance on the different strains. The analysis was carried out using the broth microdilution technique in a 96-well plate to obtain different concentrations of the substances [9]. (CLSI 2015). Initially, 100  $\mu\text{L}$  of double strength Brain Heart Infusion (BHI) broth (Sigma-Aldrich/ Merck®) was added to the wells, and 100  $\mu\text{L}$  of the substance emulsion was subsequently added. Serial microdilutions were performed in which a 100  $\mu\text{L}$  aliquot from a well containing more concentrated medium was transferred to the next well with less concentrated medium, producing final coumarin concentrations ranging from 2048 to 4  $\mu\text{g}/\text{mL}$ . Finally, 10  $\mu\text{L}$  of bacterial inoculum was added to the wells such that each column contained a different strain. At the same time, sterility, cell viability and interference controls of vehicles used in the preparation of emulsions of substances (DMSO and Tween-80) were also performed. The test was performed in triplicate.

MIC is defined as the lowest concentration capable of causing complete inhibition of bacterial growth after 24 h at  $35 \pm 2^\circ\text{C}$ . The antibacterial activity of coumarin was interpreted and considered as active or inactive, according to the criteria already described in the scientific literature: up to 600  $\mu\text{g}/\text{mL}$ , the substance is considered to have

high activity, between 600 and 1500  $\mu\text{g}/\text{mL}$  with moderate activity; and MIC above 1500  $\mu\text{g}/\text{mL}$  characterizes the product with low activity or inactive against the tested strains [10, 11].

### Effect of coumarin on bacterial biofilms

The evaluation of coumarin activity on biofilm forms was performed using *S. aureus*, *K. pneumoniae* and *P. aeruginosa* strains. First, the potential of coumarin to inhibit biofilm formation was verified. For this, the microdilution plates were incubated statically with Brain Heart Infusion (BHI) broth containing different concentrations of coumarin in the presence of the bacterial inoculum (final concentration of  $10^7$  CFU/mL). Negative controls were carried out containing only culture broth and inoculum. After 24 h of incubation at  $35 \pm 2$  °C, the contents of the wells were discarded and washed three times with sterile distilled water, to remove planktonic cells, reserving them for drying at room temperature. After drying, the 1 % violet crystal solutions were transferred and left to stand for 40 min. The dye was discarded and its excess on the well walls was removed by washing with distilled water. Thus, the wells received absolute ethanol and, after 30 min at rest, the plate was read in a microplate spectrophotometer (Thermo Scientific Multiskan GO<sup>®</sup>/ Waltham, Massachusetts, EUA) at 590 nm [12]. All analyzes were performed in quintuplicate.

Then, coumarin action on mature biofilms was verified. That is, the action on preformed biofilms. The plates were previously incubated with BHI broth and inoculum (final concentration  $10^7$  CFU/mL) for 24 h at  $35 \pm 2$  °C, to form the biofilms in the wells. Then, the contents of the wells were removed and a new culture broth containing different concentrations of coumarin was added. The plates were incubated statically under the same conditions of time and temperature. Finally, the same process described above was performed for evaluation on a microplate spectrophotometer (Thermo Scientific Multiskan GO<sup>®</sup>/ Waltham, Massachusetts, EUA) [13].

Statistical significance was determined by the paired test using the t test and the results were considered statistically significant when  $p < 0.05$  for rejection of the null hypothesis. For this plot, the GraphPad Prism software (version 6.0 for Windows, San DIEGO, CA-USA) was used.

## RESULTS AND DISCUSSION

Coumarin activity on planktonic cells was assessed by determining the minimum inhibitory concentration (MIC). The MIC range obtained was 256 to 1024  $\mu\text{g}/\text{mL}$  (table 1).

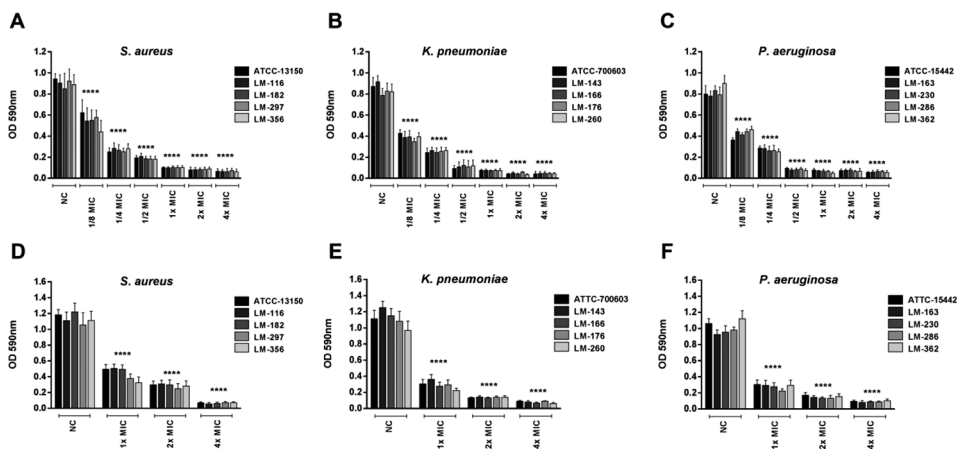
**Table 1.** Minimum inhibitory concentration (MIC) of 2H-1-Benzopyran-2-one against bacterial strains.

Strains	2H-1-Benzopyran-2-one	Controls	
	MIC	Microorganism	Culture broth
<i>S. aureus</i>			
ATCC-13150	512 µg/mL	+	-
LM-116	1024 µg/mL	+	-
LM-182	1024 µg/mL	+	-
LM-297	512 µg/mL	+	-
LM-356	512 µg/mL	+	-
<i>K. pneumoniae</i>			
ATCC-700603	256 µg/mL	+	-
LM-143	512 µg/mL	+	-
LM-166	256 µg/mL	+	-
LM-176	256 µg/mL	+	-
LM-260	512 µg/mL		
<i>P. aeruginosa</i>			
ATCC-15442	512 µg/mL	+	-
LM-163	512 µg/mL		
LM-230	256 µg/mL	+	-
LM-286	512 µg/mL	+	-
LM-362	256 µg/mL	+	-

(+): bacterial growth; (-): absence of bacterial growth.

The MIC<sub>50</sub> of coumarin was 512 µg/mL on the bacterial species used in this study, indicating moderate antibacterial activity, according to the classification criteria for substances of natural origin [10]. These values corroborate with other authors. Smyth *et al.* (2009) and Souza *et al.* (2005) observed coumarin action, with MIC ranging from 500 to 1000 µg/mL and better activity on Gram-negative strains, as well as what was identified in the present study (table 1). It is suggested that the antibacterial activity of coumarins and their synthetic derivatives is due to the inhibition of DNA gyrase preventing supercoiling, in addition to possibly also acting as photosensitizers for thymine dimers [14]. However, these mechanisms are not fully understood.

When evaluating the activity of coumarin on biofilm forms, a strong activity was noted in inhibiting the formation of biofilms by the three bacterial species studied (Figure 2A-C). The average biofilm inhibition was 89 % in MIC, 91 % and 93 % in 2x MIC and 4x MIC, respectively. Even at subinhibitory concentrations, coumarin was still able to significantly reduce the biofilm formation.



**Figure 2.** Effect of coumarin on biofilm forms. Inhibition of biofilm formation: **A.** *S. aureus*. **B.** *K. pneumoniae*. **C.** *P. aeruginosa*. Action on mature biofilms formed by **D.** *S. aureus*. **E.** *K. pneumoniae*. **F.** *P. aeruginosa*. Statistical analysis compared to the negative control (NC): \*\*\*\*  $p \leq 0.0001$ .

Biofilms are much more resistant to antibiotics and can tolerate concentrations 10 to 1000 higher than their respective planktonic forms. Several conventional antibacterial drugs are ineffective against biofilms, even in high concentrations [2]. Another major problem is that some drugs induce an increase in the formation of biofilms when they are in subinhibitory concentrations. During the infection treatment, part of the microorganism's population is exposed to suboptimal concentrations and low doses of these drugs can complicate these diseases [3]. Coumarin did not increase biofilm formation under these conditions, which makes it an interesting molecule to be investigated for this purpose.

Observing coumarin effect on mature biofilms, which, once established, are difficult to remove, there is a significant capacity to act on them, mainly in 4 x MIC (figure 2D-F), where there is an average reduction of 94 % compared to negative controls.

Lee *et al.* [7] and Zhang *et al.* [8] identified coumarin's strong potential to reduce *E. coli* and *P. aeruginosa* biofilms, respectively, also affecting other virulence factors, although the mechanism by which this occurs is not yet fully understood. In addition, other stu-

dies demonstrate that structural modifications based on the chemical skeleton of coumarin result in a great improvement in activity against planktonic and biofilm forms [15, 16].

## CONCLUSION

Based on this, it is concluded that coumarin has strong activity against planktonic and biofilm forms on the three species of great relevance in the clinical scenario. It is interesting that more studies investigate this potential of this natural product, to enable a pharmacological alternative for the treatment of these infections.

## ACKNOWLEDGEMENTS

This study was supported by Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (Capes).

## CONFLICT OF INTERESTS

No potential conflict of interest was reported by the authors.

## REFERENCES

1. K.P. Rumbaugh, K. Sauer, Biofilm dispersion, *Nature Reviews Microbiology*, **18**, 571-586 (2020).
2. S. Srivastava, A. Bhargava, Biofilms and human health, *Biotechnology Letters*, **38**(1), 1-22 (2016).
3. M.R.M. Ranieri, C.B. Whitchurch, L.L. Burrows, Mechanisms of biofilm stimulation by subinhibitory concentrations of antimicrobials, *Current Opinion in Microbiology*, **45**, 164-169 (2018).
4. A. Stefanachi, F. Leonetti, L. Pisani, M. Catto, A. Carotti, Coumarin: A natural, privileged and versatile scaffold for bioactive compounds, *Molecules*, **23**(2), 250 (2018).



5. T. Smyth, V.N. Ramachandran, W.F. Smyth, A study of the antimicrobial activity of selected naturally occurring and synthetic coumarins, *International Journal of Antimicrobial Agents*, **33**(5), 421-426 (2009).
6. S.M. Souza, F. Monache, A. Smânia, Antibacterial activity of coumarins, *Zeitschrift für Naturforschung C*, **60**(9-10), 693-700 (2005).
7. J.-H. Lee, Y.-G. Kim, H.S. Cho, S.Y. Ryu, M.H. Cho, J. Lee, Coumarins reduce biofilm formation and the virulence of *Escherichia coli* O157:H7, *Phytomedicine*, **21**(8-9), 1037-1042 (2014).
8. Y. Zhang, A. Sass, H. V. Acker, J. Wille, B. Verhasselt, F.V. Nieuwerburgh, V. Kae-ver, A. Crabbé, T. Coenye, Coumarin reduces virulence and biofilm formation in *Pseudomonas aeruginosa* by affecting quorum sensing, type III secretion and c-di-GMP levels, *Frontiers in Microbiology*, **9**, 1952 (2018).
9. Clinical Laboratory Standards Institute (CLSI), *Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically. Approved standard M07-A10*, 2015.
10. A. Sartoratto, M.L.A. Machado, C. Delarmelina, M.G. Figueira, T.C.M. Duarte, G.L.V. Rehder, Composition and antimicrobial activity of essential oils from aromatic plants used in Brazil, *Brazilian Journal of Microbiology*, **35**, 275-280 (2004).
11. P.J. Houghton, M.J. Howes, C.C. Lee, G. Steventon, Uses and abuses of *in vitro* tests in ethnopharmacology: visualizing an elephant, *Journal of Ethnopharmacology*, **110**(3), 391-400 (2007).
12. D. Balasubramanian, L. Schneper, M. Merighi, R. Smith, G. Narasimhan, S. Lory, K. Mathee, The regulatory repertoire of *Pseudomonas aeruginosa* AmpC  $\beta$ -lactamase regulator AmpR includes virulence genes, *PLoS One*, **7**(3), e34067 (2012).
13. S.K. Rajasekharan, S. Ramesh, A.S. Satish, J. Lee, Antibiofilm and anti- $\beta$ -lactamase activities of burdock root extract and chlorogenic acid against *Klebsiella pneumoniae*, *Journal of Microbiology and Biotechnology*, **27**(3), 542-551 (2017).
14. S. Garg, J. Gupta, S. Sharma, D. Sahu, An insight into the therapeutic applications of coumarin compounds and their mechanisms of action, *European Journal of Pharmaceutical Sciences*, **152**, 105424 (2020).

15. M. Matos, S. Vazquez-Rodriguez, L. Santana, E. Uriarte, C. Fuentes-Edfuf, Y. Santos, A. Munoz-Crego, Looking for new targets: simple coumarins as antibacterial agentes, *Medicinal Chemistry*, **8**(6), 1140-1145 (2012).
16. T. Das, M.C. Das, A. Das, S. Bhowmik, P. Sandhu, Y. Akhter, S. Bhattacharjee, Modulation of *S. aureus* and *P. aeruginosa* biofilm: an in vitro study with new coumarin derivatives, *World Journal of Microbiology and Biotechnology*, **34**(11), 170 (2018).

### HOW TO CITE THIS ARTICLE

L. Vilar-Cordeiro, H.D. da Silva-Souza, G.C. da Silva-Sá, A. Pereira-Sousa, P.T. Ramalho de Figueiredo, M.d.N. Silva-Neta, J.M. Barbosa Filho, E. de Oliveira-Lima, Coumarin (2H-1-Benzopyran-2-one) act against planktonic and biofilm forms of *Staphylococcus aureus*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa*, *Rev. Colomb. Cienc. Quim. Farm.*, **50**(3), 685-694 (2021).