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Photobiological effect of eugenol-derived 3-benzoylcoumarin associated with led lights against MDR microorganisms

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Abstract

The problem of antibiotic resistance by bacteria threatens human health. Therefore, studies in this area seek alternatives to circumvent it. The study with coumarins and eugenol has already proven that these classes of compounds act against bacteria. In this same aspect, exposure to LED also shows a bactericidal effect. Seeking a possible enhancement of this effect, the present work studied coumarins derived from eugenol in association with LED to investigate the bactericidal effect. Four compounds were tested. For this, minimum inhibitory concentrations (MICs) and modulation with three antibiotics against *Escherichia coli* and *Staphylococcus aureus* bacteria were determined. To test the behavior of the activity against exposure to LED, the plates were exposed for 20 min to blue light, 415 nm and then incubated at 37°C for 24 h. For control, duplicates were made, and one of them did not undergo this exposure. C1 exhibited better activity against *S. aureus*, as synergism prevailed under the conditions tested. C3 and C4 were promising against *E. coli* as they showed synergism in

Abbreviations: °C, degrees celsius; ATCC, American Type Culture Collection; BHI, brain heart infusion; C1, Compound 1; C2, Compound 2; C3, Compound 3; μg/mL, microgram per milliliter; μL, microliter; C4, Compound 4; CFU/mL, colony forming unit per milliliter; DMSO, dimethylsulfoxide; DNA, deoxyribonucleic acid; h, hours; LED, light-emitting diode; LMBM, Laboratory of Microbiology and Molecular Biology; LQFar, Pharmaceutical Chemistry Research Laboratory; MDF, medium density fiberboard; MIC, minimum inhibitory concentration; nm, nanometer; ROS, reactive oxygen species; UNIFAL, Federal University of Alfenas; URCA, Regional University of Cariri.

association with the three antibiotics both with and without LED exposure. Thus, the compounds showed bactericidal activity, and LED was shown to enhance synergism.

KEYWORDS
antibiotic resistance, bactericidal activity, coumarins, phototherapy

1 | INTRODUCTION

Stagnation in the discovery of new antibiotics, coupled with the development of antimicrobial resistance by bacteria, threaten human health. *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Escherichia coli*, *Klebsiella pneumoniae*, *Enterococcus faecalis*, and *Acinetobacter baumannii* are pathogens often cited as resistant to a wide variety of antibiotics [1–3].

Furthermore, it is necessary to highlight the high prevalence of *Helicobacter pylori* in the population, as the treatment of infection by this microorganism requires the use of a combination of antibiotics that can contribute to the increase in antimicrobial resistance. This infection could be prevented and improved with lifestyle and diet changes associated with the use of certain known beneficial foods and probiotic bacteria [4].

Another study reports more specifically the effect of using probiotic bacteria as an outlet for the treatment of diseases caused by bacteria that have already shown immunomodulatory effects, improving the immune system's response to bacteria, reducing the use of antibiotics, and may also represent an outlet for antimicrobial resistance [5].

Thus, research is looking for new alternatives to the use of antibiotics to overcome such resistance. Coumarins have already had their bacterial activity previously studied, and their mechanism of action was mainly attributed to their ability to bind to the B subunit of the bacterial DNA gyrase and inhibit DNA supercoiling by blocking the ATPase activity [6,7].

Furthermore, the use of therapeutic association can enhance activities already found in these compounds. Thus, the use of photodynamic antimicrobial chemotherapy associated with compounds with known antibacterial activity is suggested. This type of therapy is based on the activation of photosensitizers, leading to the formation of reactive oxygen species that cause bacterial death. Some classes of molecules showed potentiated bactericidal effects against multiresistant gram-positive and gram-negative bacteria when irradiated with visible light [8,9].

In this type of therapy, light-emitting diode (LED) is already used for various applications, such as anti-inflammatory and healing effects, depending on the wavelength used. It also has bactericidal activity, commonly used in the treatment of acne and dental biofilms, with blue light (407–420 nm) being the most indicated for its bactericidal effect [10]. That said, this study aimed to verify the antibacterial and modulatory activity of 3-benzoylcoumarins associated with blue LED light.

2 | MATERIALS AND METHODS

2.1 | Obtaining compounds

The substances used were kindly provided by the Pharmaceutical Chemistry Research Laboratory (LQFar), from the Faculty of Pharmaceutical Sciences at Federal University of Alfenas (UNIFAL), Minas Gerais, Brazil. For this study, four substances were tested, namely: compound 1 (C1) to 3-benzoyl-8-methoxy-2H-chromen-2-one; compound 2 (C2) to 8-methoxy-3-(4-nitrobenzoyl)-2H-chromen-2-one; compound 3 (C3) to 8-methoxy-2-oxo-2H-chromen-3-carboxylic acid; and compound 4 (C4) to 3-(4-aminobenzoyl)-8-methoxy-2H-chromen-2-one.

2.2 | Microorganisms

Bacteria from standard strains (*E. coli* ATCC 25922 and *S. aureus* ATCC 25923) and multiresistant strains (*S. aureus* 10 and *E. coli* 06) supplied by the Laboratory of Microbiology and Molecular Biology (LMBM) of the Regional University of Cariri (URCA) were used.

2.3 | Minimum inhibitory concentration (MIC)

The determination of MIC was performed using the broth microdilution technique using 96-well sterilized plates with serial dilutions 1:1 [11]. Microbial cultures kept in refrigerated stock agar were subcultured in brain heart infusion (BHI) broth and incubated at 37°C for 24 h. After this period, the inoculum was standardized by preparing a suspension in BHI, whose turbidity is similar to the 0.5 tube of the McFarland scale (1×108 CFU/ml). This suspension was diluted 100× in BHI medium, which corresponds approximately to a suspension containing 1×106 CFU/ml, from which

100 μl was removed and placed in each well with 900 μl of BHI, filling the plate column and microdilution with 100 μl of the product to be tested until the penultimate well.

Test solutions were prepared using 10 mg of the products solubilized in 9265 μl of distilled water and 500 μl of dimethylsulfoxide (DMSO), obtaining an initial concentration of 1024 μg/ml. The final concentrations of the samples in the culture medium were 512, 256, 128, 64, 32, 16, and 8 μg/ml.

The tests were performed in triplicate on the plate columns and on plates in duplicate, since one of them was exposed to LED for 20 min. Plates were incubated at 35 ± 2°C for 24 h. After this period, the plates were developed with a specific dye, resazurin. This solution was prepared in sterile distilled water at a concentration of 0.01% (w/v). After the incubation period, 20 μg/mL of the indicator solution was added to the plates in each well, and after 1 h at room temperature, the test reading was performed. This is determined by the coloration of the culture medium, which is considered positive for wells that do not show microbial growth, that is, those with a red color remain blue and negative [12].

The positive control of the test was carried out with the culture media containing the inoculum. The MIC was defined as the lowest concentration capable of completely inhibiting microbial growth in microdilution wells as detected macroscopically.

After determining the MIC, the products were modulated with antibiotics. For this procedure, 1.5 ml Eppendorf®-type tubes are used, of which 150 μl are bacterial inoculum, product volume in sub-inhibitory concentration (MIC/8), and the remainder of the volume mean BHI. Subsequently, microdilution is performed with antibiotics, diluting in water or DMSO (depending on the antibiotic solubility) and using 100 μl to microdilution in the wells until the penultimate cavity. In this plate, a column is also made containing antibiotic, medium, and inoculum that will be used as modulation control.

2.4 | Evaluation of photoactive effect with led exposure

To carry out these tests, the same methodologies were initially used for the test to assess the antibacterial activity by microdilution. Then the plates were subdivided into two groups, first was subjected to blue LED light for 20 min each plate and the second group was not subjected to LED lights. To expose the plates, three devices from the New Estética® brand were used, using a medium density fiberboard (MDF) box covered internally with laminated paper and with three holes in the lid, as an apparatus to isolate each plate from the external light [13]. Plates were incubated at 35 ± 2°C for 24 h.

2.5 | Statistical analysis

The central data and standard deviations were obtained according to the methodology of Freitas et al. [14] on microbiological analysis in microdilution plates. Data were analyzed using the statistical program GraphPad Prisma 5.0 through a two-way ANOVA test. Afterwards, a post hoc Bonferroni test was performed (where $P < 0.05$ was considered significant and $P > 0.05$ nonsignificant).

3 | RESULTS

3-Benzoyl-8-methoxy-2H-chromen-2-one (C1), 8-methoxy-3-(4-nitrobenzoyl)-2H-chromen-2-one (C2), 8-methoxy-2-oxo acid-2H-chromen-3-carboxylic acid (C3), and 3-(4-aminobenzoyl)-8-methoxy-2H-chromen-2-one (C4) (Figure 1) had their activities tested against *S. aureus* and *E. coli* bacteria, standard and multiresistant strains, modulated with the antibiotics norfloxacin, gentamicin, and ampicillin, also used as modulation control. The MIC of these compounds, for the standard and multiresistant strains of the tested bacteria, was greater than or equal to 1024 μg/ml in all tests with and without exposure to LED; the only exception was coumarin C3, which exhibited a MIC of 512 with LED exposure, for multiresistant *E. coli* strain (Table 1).

In the modulation tests for *S. aureus* 10 (multiresistant strain) when modulated with norfloxacin, 1,3-benzoyl-8-methoxy-2H-chromen-2-one (C1) showed synergism reducing MIC when compared to the antibiotic alone, but it has not been shown to significantly interfere in the MICs of the antibiotics gentamicin and ampicillin. When associated with LED, the behavior of the antibiotics gentamicin and ampicillin was altered, showing antagonism, both when isolated (control) and associated with the compound. There was synergism only with norfloxacin, which alone when

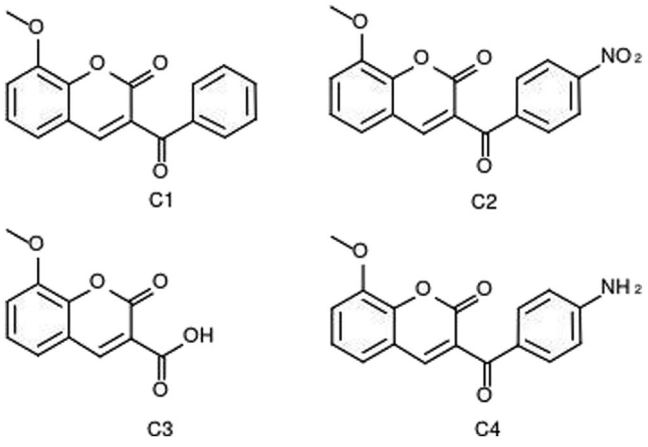

FIGURE 1 Structures of eugenol-derived coumarins

TABLE 1 Minimum inhibitory concentration (MIC) in microgram per milliliter of eugenol-derived coumarins

Treatment	E.C. ATCC 25922	S.A. ATCC 25923	E.C. 06	S.A. 10
C1	1024	1024	1024	1024
C1 + blue LED	1024	1024	1024	1024
C2	1024	1024	1024	1024
C2 + blue LED	1024	1024	1024	1024
C3	1024	1024	1024	1024
C3 + blue LED	1024	1024	512	1024
C4	1024	1024	1024	1024
C4 + blue LED	1024	1024	1024	1024

Note: MIC values were expressed in microgram per milliliter.
Abbreviations: C1, 3-benzoyl-8-methoxy-2H-chromen-2-one; C2, 8-methoxy-3-(4-nitrobenzoyl)-2H-chromen-2-one; C3, 8-methoxy-2-oxo-2H-chromen-3-carboxylic acid; C4, 3-(4-aminobenzoyl)-8-methoxy-2H-chromen-2-one; E.C., *E. coli*; S.A., *S. aureus*.

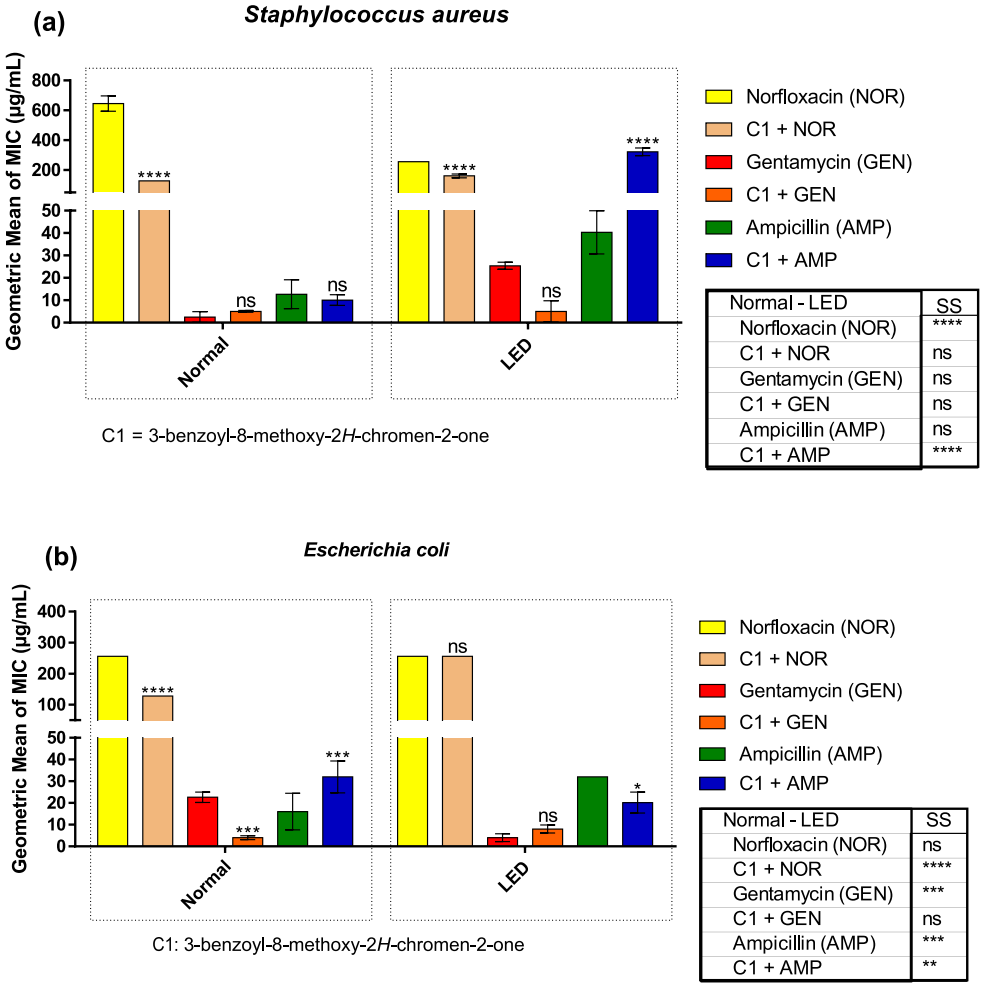

FIGURE 2 Geometric mean minimum inhibitory concentration (MIC) of the modulation of the compound 1,3-benzoyl-8-methoxy-2H-chromen-2-one against *Staphylococcus aureus* and *Escherichia coli* with and without exposure to blue LED. $^{**}P < 0.01$, $^{***}P < 0.001$, and $^{****}P < 0.0001$ indicate significant difference between the groups.

exposed to LED presented a reduction in MIC, responding differently when associated with C1, in which there was no change in MIC (Figure 2a).

The compound 1,3-benzoyl-8-methoxy-2H-chromen-2-one was also tested for the multiresistant strain of *E. coli*. In these tests, C1 showed synergism with norfloxacin, but when associated with LED, there was no significant difference to the control, but it is noted that this was due to the reduction in the MIC of the antibiotic. Concerning gentamicin, there was also synergism without exposure to LED, and when exposed to it, there was also a reduction in the MIC of the antibiotic and, because of that, there seems to be antagonism. Comparing the modulation of the compound to antibiotics with and without exposure to LED, it is noted that there was a synergistic response with ampicillin in the situation where there was exposure to LED (Figure 2b).

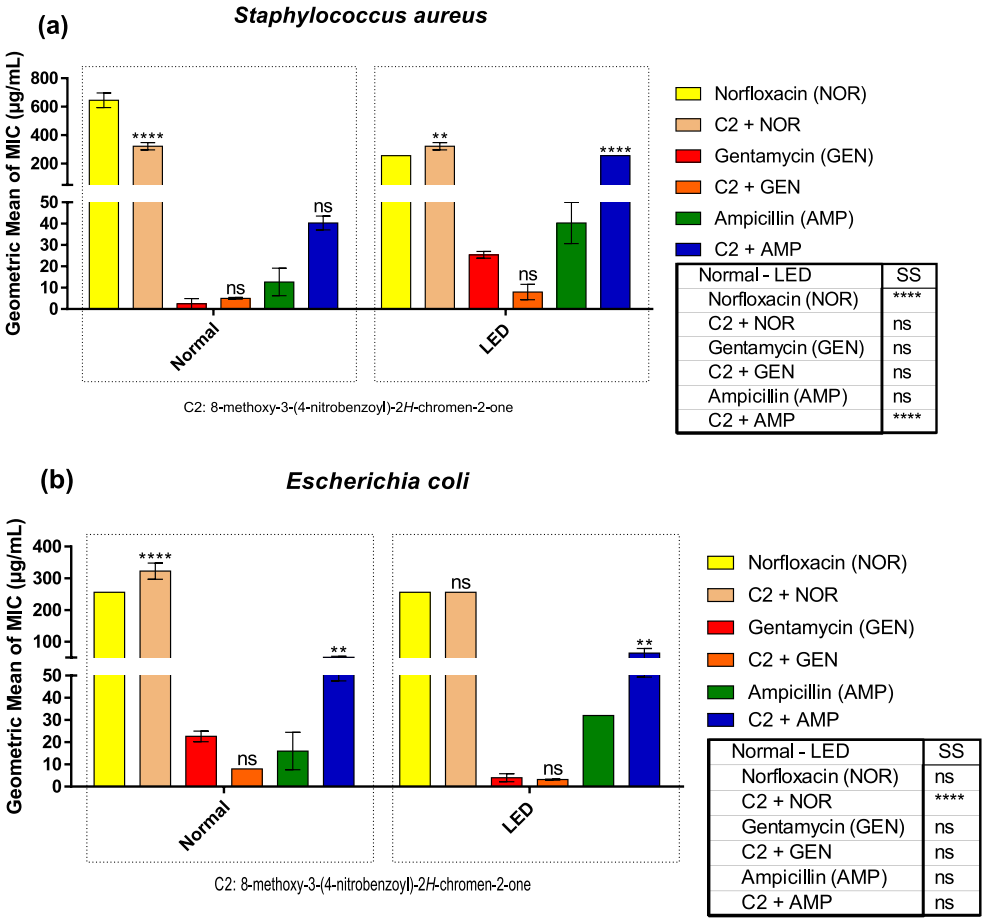

FIGURE 3 Geometric mean minimum inhibitory concentration (MIC) of the modulation of the compound 8-methoxy-3-(4-nitrobenzoyl)-2H-chromen-2-one against *Staphylococcus aureus* and *Escherichia coli* with and without exposure to blue LED. $^{**}P < 0.05$ and $^{****}P < 0.0001$ indicates significant difference between groups; ns, non significative.

For the compound 8-methoxy-3-(4-nitrobenzoyl)-2H-chromen-2-one against *S. aureus*, there was synergism in its modulation with norfloxacin. Exposure to LED did not interfere with this response. About gentamicin and ampicillin, there was antagonism (Figure 3a). In the tests of this compound for *E. coli*, the modulation response of the compound with norfloxacin showed antagonism, when associated with LED, the MIC was reduced, but without significant difference. In conjunction with gentamicin, the response was synergistic and antagonistic to ampicillin, both without and with LED exposure. It is also noteworthy that there was a decrease in gentamicin MIC with exposure to blue LED (Figure 3b).

8-Methoxy-2-oxo-2H-chromen-3-carboxylic acid showed synergistic activity to gentamicin and ampicillin for *S. aureus* when exposed to LED and indifferent to norfloxacin activity. It is also noted that in comparison with modulation without exposure to LED, the compound had not shown the same behavior, exhibiting antagonism to norfloxacin and ampicillin, and indifferent to gentamicin (Figure 4a). Regarding the tests with *E. coli*, this same compound showed synergism with the three antibiotics, without exposure to LED. In the plates that were exposed, an indifference in their response associated with norfloxacin and gentamicin was noted, continuing to exhibit synergism with ampicillin (Figure 4b).

The modulation of 3-(4-aminobenzoyl)-8-methoxy-2H-chromen-2-one against *S. aureus* showed synergism with norfloxacin when exposed to LED and antagonism and indifference to other antibiotics, as well as when without LED exposure (Figure 5a). For *E. coli*, there was synergism with the three antibiotics without exposure to LED, suggesting that the compound has a modulatory activity with an antibacterial response, but with exposure to LED, there was a reduction in the difference between the averages of the MICs, changing the response to ampicillin, for which he showed antagonism (Figure 5b).

4 | DISCUSSION

In a study, Vazquez-Rodriguez et al. [15] synthesized coumarin–chalcone hybrid compounds with different patterns of substitution, and performed, among other tests, the activity against human pathogenic bacteria (*E. coli*, *S. aureus*, and *P. aeruginosa*); such compounds were not active against human pathogenic bacteria. This is consistent with the current study regarding 3-benzoylcoumarins, which, in isolation, did

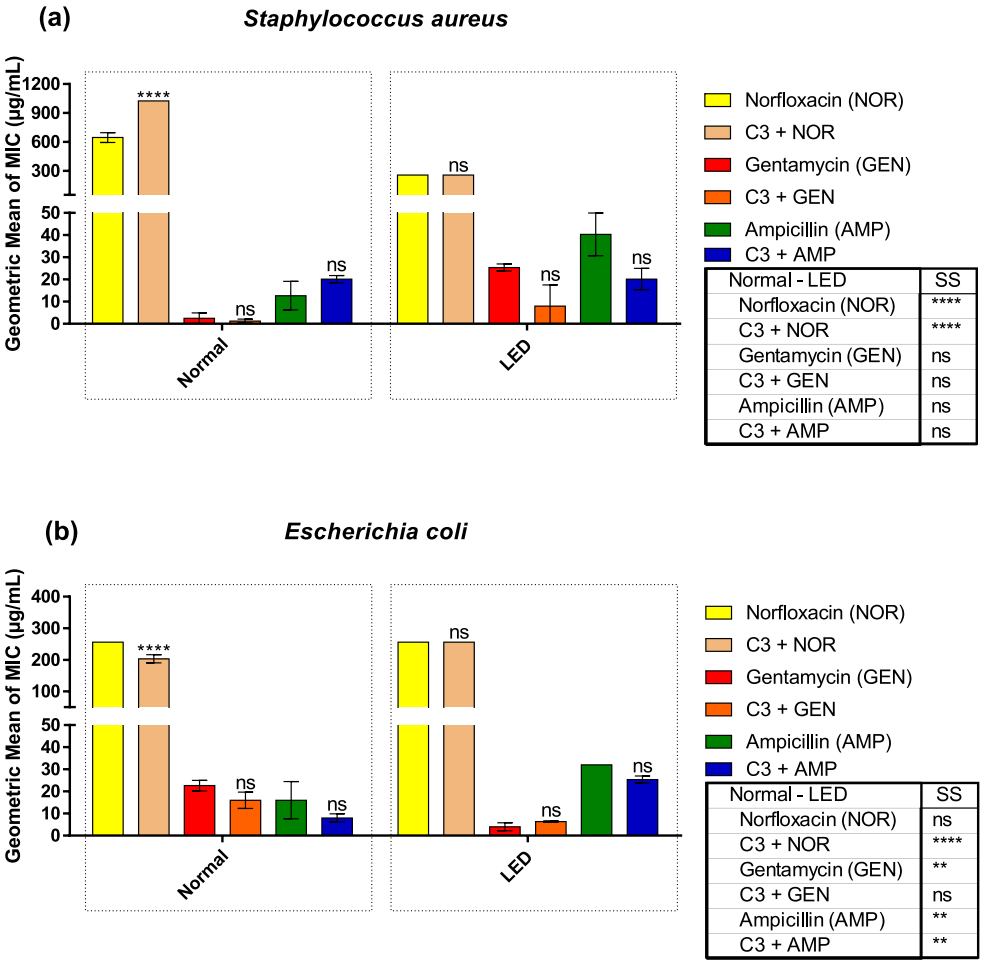

FIGURE 4 Geometric mean minimum inhibitory concentration (MIC) of the modulation of the compound 8-methoxy-2-oxo-2H-chromen-3-carboxylic acid against *Staphylococcus aureus* and *Escherichia coli* with and without exposure to blue LED. $^{**}P < 0.01$ and $^{****}P < 0.0001$ indicate significant difference between groups.

not show significant antibacterial activity, with MIC greater than 1024 µg/ml.

The evaluation of four natural coumarins, 5-geranyloxy-7-methoxycoumarin, artanin, isopimpinellin, and fellopterin, was tested for their antibacterial effects in vitro with an emphasis on their potential to restore the activity of conventional antibacterial agents against clinical strains of *S. aureus* resistant to methicillin. These coumarins showed a promising inhibition with MICs of 8 to 64 µg/ml. Furthermore, they also exhibited different degrees of synergism with eight conventional antibacterial agents and against 10 clinical strains of multidrug resistant *S. aureus* [16].

The compounds analyzed in this study had variable effects against *S. aureus* and did not always exhibit synergism with the antibiotics tested, even belonging to the same class as the aforementioned study. However, in some cases, it was possible to observe a potentiation of the antibacterial effect after exposure to LED. The coumarins C1 and C4 stood out to this characteristic, which differ only in the presence of the amino group.

In a similar work, the modulatory activity of the essential oil *Ocimum gratissimum* L. in association with blue, red, and yellow LED lights was tested. Exposure to LED lights increased the antibacterial effects against *E. coli*. The yellow and red lights caused bacterial growth inhibitions. For *S. aureus*, the study showed an improvement in the antibacterial effect by association with red light, while the blue and yellow lights showed an antagonistic effect [17]. This effect was similar to that observed in the present study regarding the behavior of C1 and C4 against *S. aureus*.

In a previous study, the essential oil of *Eugenia brasiliensis* showed synergism with amikacin and gentamicin against *E. coli*, enhanced by exposure to blue LED light [18]. This response was similar to that presented by compounds C2 and C3 for *E. coli*; however, in this study, there was no improvement in synergism by exposure to LED. Thus, LED activity may behave differently depending on the photosensitizer used.

Blue light is known to be more effective in killing bacteria because the production of reactive oxygen species is higher compared to other wavelengths; thus, blue light kills bacteria more than other lights of different colors [19].

The use of light with a length of 415 nm in this study corroborates the study by Lipovsky et al. [20], who observed a higher production and ROS than in 455 nm light, reducing the colony count of *S. aureus* and

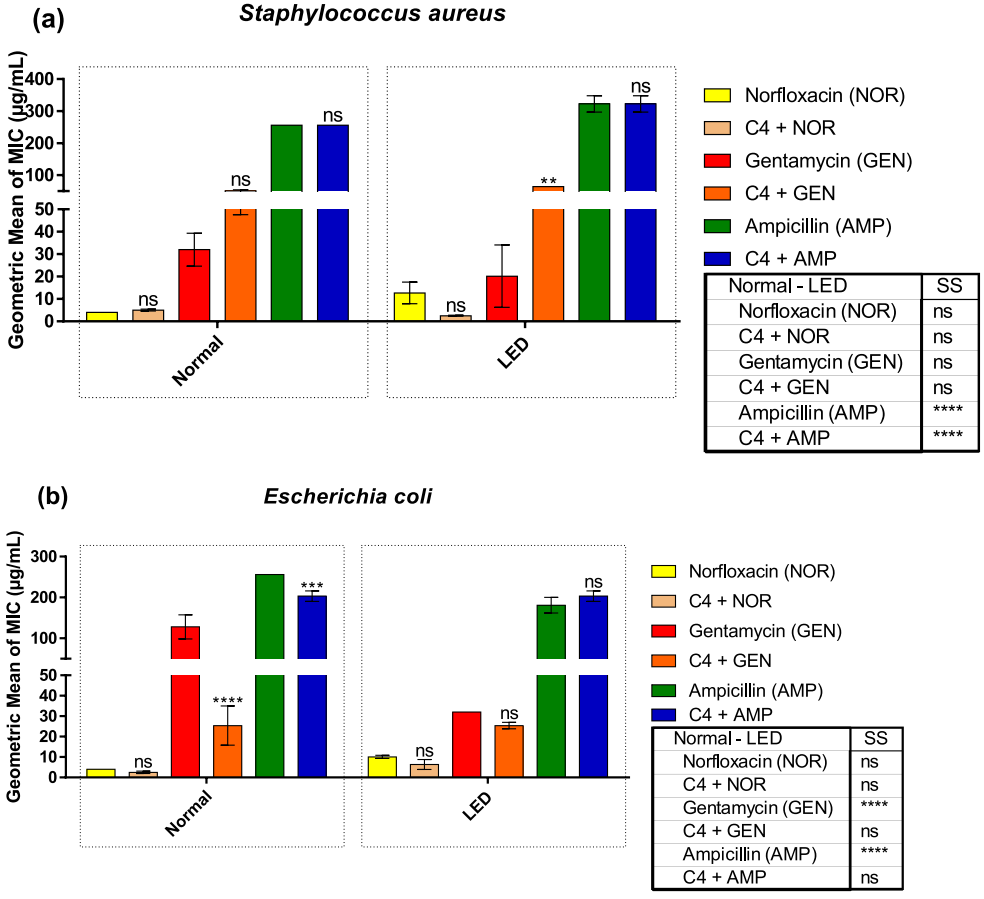

FIGURE 5 Geometric mean minimum inhibitory concentration (MIC) of the modulation of the compound 3-(4-aminobenzoyl)-8-methoxy-2H-chromen-2-one against *Staphylococcus aureus* and *Escherichia coli* with and without exposure to blue LED. $^{**}P < 0.05$, $^{***}P < 0.001$, and $^{****}P < 0.0001$ indicates significant difference between groups.

E. coli. In this study, it is also possible to observe different effects of the interaction of this light with the compounds and antibiotics used that may have interfered with the bactericidal activity of blue light, both potentiating (synergistic activity) and preventing (antagonistic activity) cell death.

In this sense, the action of the compounds and their activity when they interact with the LED need to be deepened, considering that these radicals may be formed, as occurs with photosensitizers; and there may be active on other mechanisms, such as barriers of bacterial permeability that governs susceptibility to antibiotics [21].

In a recently studied methodology, Oliveira et al. [22] suggest that the manual methodology of reading by resazurin, used in this study as a revealer of the results, should be reviewed, since there are limitations in the reading performed by the human eye. In this way, the variation in the results would be avoided, which can improve their interpretation and correlation with other studies of similar methodology.

5 | CONCLUSIONS

In the analysis of synergistic activity, coumarin C1 exhibited better results against *S. aureus*, both without and with LED exposure. With this coumarin, it was also possible to see a reversal of the antagonism caused by exposure to LED, when associated with gentamicin for this same class of bacteria, and this same behavior when modulated to ampicillin against *E. coli*.

Coumarins C3 and C4 exhibited promising bactericidal activity against *E. coli*, as there was synergism when associated with the three antibiotics, maintaining this behavior in association with LED in modulation with almost all antibiotics. And although they stood out for this class of bacteria, there was still synergism for *S. aureus* when C3 activity was modulated with gentamicin and C4 when it was associated with norfloxacin and exposed to LED.

In the association of C2 with antibiotics and with LED, the antagonism prevailed; thus, there was no evidence of a good bactericidal activity of this compound. Still, it is worth noting that LED was able to reverse the antagonism of this compound to *S. aureus* when modulated with gentamicin.

Thus, analyzing in general, the study showed a promising bactericidal response of the compounds tested both in modulation and in exposure to LED. Therefore, exposure to LED can give rise to a complementary therapy to the use of antibiotic therapy, improving the response of certain drugs already used traditionally and preventing the emergence of

resistance by new strains to other classes of antibiotics, as has recently been observed in clinical routine.

AUTHOR CONTRIBUTION

Conceptualization: Amanda Karine de Sousa and Henrique Douglas Melo Coutinho. *Methodology*: Janaina Esmeraldo Rocha, Thiago Sampaio de Freitas, Priscila Ramos Freitas, and Raimundo Luiz Silva Pereira. *Software*: Irwin Rose Alencar de Menezes. *Formal analysis*: Francisco Nascimento Pereira Júnior and Lucindo José Quintans Júnior. *Resources*: Guilherme Andrade Brancaglion, Daniela Carvalho de Paulo, and Diogo Teixeira Carvalho. *Writing—original draft preparation*: Amanda Karine de Sousa, and Lucindo José Quintans Júnior. *Supervision*: Lucindo José Quintans Júnior. *Project administration*: Henrique Douglas Melo Coutinho and All authors have read and agreed to the published version of the manuscript.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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