European Journal of Pharmaceutical Sciences 158 (2021) 105695

Contents lists available at ScienceDirect

European Journal of Pharmaceutical Sciences

journal homepage: www.elsevier.com/locate/ejps

# Aminophenyl chalcones potentiating antibiotic activity and inhibiting bacterial efflux pump

Marina Micaele Rodrigues Siqueira [a], Paulo de Tarso Cavalcante Freire [a,c], Beatriz Gonçalves Cruz [a], Thiago Sampaio de Freitas [a], Paulo Nogueira Bandeira [b], Hélcio Silva dos Santos [a,b], Carlos Emidío Sampaio Nogueira [a,d], Alexandre Magno Rodrigues Teixeira [a,d], Raimundo Luiz Silva Pereira [a], Jayze da Cunha Xavier [a], Fábia Ferreira Campina [a], Cristina Rodrigues dos Santos Barbosa [a], José Bezerra de Araújo Neto [a], Maria Milene Costa da Silva [a], José Pinto Siqueira-Júnior [e], Henrique Douglas Melo Coutinho [a,*]

[a] Department of Biological Chemistry, Regional University of Cariri, Crato, CE, Brazil
[b] Science and Technology Centre - Course of Chemistry, State University Vale do Acaraú, Sobral, CE, Brazil
[c] Department of Physics, Federal University of Ceará, Fortaleza, CE, Brazil
[d] Department of Physics, Regional University of Cariri, Juazeiro do Norte, CE, Brazil
[e] Department of Molecular Biology, Federal University of Paraíba, João Pessoa, PB, Brazil

ARTICLE INFO

*Keywords:*
Antimicrobial activity
Antibiotic potentiating activity
Efflux pump
Docking

ABSTRACT

Chalcones and their derivatives are substances of great interest for medicinal chemistry due to their antibacterial activities. As the bacterial resistance to clinically available antibiotics has become a worldwide public health problem, it is essential to search for compounds capable of reverting the bacterial resistance. As a possibility, the chalcone class could be an interesting answer to this problem. The chalcones (2*E*)-1-(4′-aminophenyl)-3-(phenyl)-prop-2-en-1-one (APCHAL), and (2*E*)-1-(4′-aminophenyl)-3-(4-chlorophenyl)-prop-2-en-1-one (ACLOPHENYL) were synthesized by the Claisen-Schmidt condensation and characterized by $^{1}H$ and $^{13}C$ nuclear magnetic resonance (NMR), Fourier-transform infrared (FT-IR), and mass spectrometry (MS), In addition, microbiological tests were performed to investigate the antibacterial activity, modulatory potential, and efflux pump inhibition against *Staphylococcus aureus* (*S. aureus*) multi-resistant strains. Regarding the *S. aureus* Gram-positive model, the APCHAL presented synergism with gentamicin and antagonism with penicillin. APCHAL reduced the Minimum inhibitory concentration (MIC) of gentamicin by almost 70%. When comparing the effects of the antibiotic modifying activity of ACLOPHENYL and APCHAL, a loss of synergism is noted with gentamicin due to the addition of a chlorine to the substance structure. For *Escherichia coli* (*E. coli*) a total lack of effect, synergistic or antagonistic, was observed between ACLOPHENYL and the antibiotics. In the evaluation of inhibition of the efflux pump, both chalcones presented a synergistic effect with norfloxacin and ciprofloxacin against *S. aureus*, although the effect is much less pronounced with ACLOPHENYL. The effect of APCHAL is particularly notable against the K2068 (MepA overexpresser) strain, with synergistic effects with both ciprofloxacin and ethidium bromide. The docking results also show that both compounds bind to roughly the same region of the binding site of 1199B (NorA overexpresser), and that this region overlaps with the preferred binding region of norfloxacin. The APCHAL chalcone may contribute to the prevention or treatment of infectious diseases caused by multidrug-resistant *S. aureus*.

## 1. Introduction

Chalcones are used as precursors and intermediates in the synthesis of active compounds and in the biosynthesis of flavonoids. Their name stems from the Greek chalcos, meaning bronze. Chalcones have in their molecular structure an enone chain (Cβ=Cα-C=O) with a characteristic

* Corresponding author.

https://doi.org/10.1016/j.ejps.2020.105695
Received 9 July 2020; Received in revised form 2 December 2020; Accepted 22 December 2020
Available online 29 December 2020

α,β-unsaturated carbonyl (Mariño et al., 2016). This compound class has a yellow coloration; however, in an alkaline medium, it presents a red color. Chalcones are present in the pigmentation of flowers, making them attractive to birds and insects that contribute to the pollination process of plants (Katsori and Hadjipavlou-Litina, 2009).

The synthesis of chalcone compounds is essential, given that the volume of material that can be extracted through natural processes is usually insignificant and inappropriate for use at an industrial scale. Among the methods of chalcones syntheses, the aldol condensation of Claisen-Schmidt stands out (Nandedkar et al., 2013). Recently, studies have shown different pharmacological activities for chalcones, such as antifungal (Gupta and Jain, 2015), antibacterial (Tran et al., 2012), anticancer (Bandeira et al., 2019; Bhat et al., 2005; Katsori and Hadjipavlou-Litina, 2009), antioxidant, and antidiabetic activity (Eichenberger et al., 2017).

Health care-associated infections (HCAI) are considered a major problem for public health, impacting on morbidity and mortality rates during the hospitalization period, as well as increasing diagnostic and therapeutic expenses (O'Neill, 2016; Oliveira et al., 2010). Antibiotic resistance is considered a world health disorder, which impacts the effectiveness of antibiotics for the treatment of infections (WHO, 2009). In this context, the use of a natural or synthetic substance to modulate bacterial resistance against an antibiotic has proven to be an alternative to drugs that are more effective in the treatment of infectious diseases (Rios et al., 1988).

Bacterial resistance to antibiotics can be internal or obtained through genetic transmission or mutation, modifying the mechanisms by which antibiotics become incapable of combating bacterial strains (Sun et al., 2014). An important mechanism of bacterial resistance is the presence of proteins from efflux pumps in their membranes. It is known that the efflux pump is one of the main causes of drug resistance (Webber and Piddock, 2003). The efflux pumps act by active transport, causing the extrusion of one or several types of antibiotics from the bacterial cytoplasm (Sharma et al., 2019). As examples of multi-drug resistance pumps (MDR pumps) are NorA and MepA, analyzed in this article. In this way, efflux pump inhibitors become an important therapeutic alternative in the treatment of infectious diseases.

Previous studies had reported that some compounds can inhibit efflux pumps, such as certain compounds derived from indole (Lepri et al., 2016). Derivatives of 2-arylquinoline (Felicetti et al., 2020) and boronic acid derivatives have the ability to inhibit NorA efflux pumps (Fontaine et al., 2014), while compounds derived from 2-phenylquinoline showed the ability to inhibit NorA and MepA efflux pumps (Felicetti et al., 2018). Previous studies had shown that chalcones can exhibit antibacterial properties via efflux pump inhibition (Dan and Dai, 2020; Holler et al., 2012; Thai et al., 2015). Holler et al. screened a library of 117 chalcones for efflux pump inhibition activity against NorA (Holler et al., 2012). In this study, twenty chalcones were shown to be inhibitors of the NorA efflux pump in everted membrane vesicles. It was also shown that two chalcones exhibited potential activity equal to the known efflux pump inhibitor reserpine. These results lead them to suggest that chalcones can be transformed into drugs for overcoming multi-drug resistance based on efflux transporters of microorganisms.

Thai et al. carried out a virtual screening and molecular docking in a series of forty-seven natural compounds, including chalcones, to investigate the novel *Staphylococcus aureus* NorA efflux pump inhibitors (Thai et al., 2015). They observed that seven of them were predicted as NorA inhibitors, among them the (2*E*)−1-(4′-aminophenyl)- 3-(4-chlorophenyl)-prop-2-en-1-one, which is one of the chalcones that will be investigated in this work.

An attempt to contain the bacterial resistance by efflux pump inhibition happens through the association of antibiotics with substances that are capable of inhibiting these pumps. In this study, we investigated the antimicrobial and antibiotic potentiating activity of the chalcones (2*E*)−1-(4′-aminophenyl)−3-(phenyl)-prop-2-en-1-one ($C_{15}H_{13}NO$, hereafter named APCHAL) and (2*E*)−1-(4′-aminophenyl)−3-(4-chlorophenyl)-prop-2-en-1-one ($C_{15}H_{12}NOCl$, hereafter named ACLOPHENYL). The evaluation of inhibition of the efflux pump for both compounds was also performed. Two strains of *S. aureus* were used: 1199B, which overexpresses the NorA gene and the multi-drug resistant (MDR) mutant strain K2068, which presents the MepA efflux pump.

## 2. Materials and methods

### 2.1. Synthesis of chalcones

The *p*-aminochalcones derivatives used in this study were synthesized via Claisen-Schmidt condensation conducted under basic conditions. A solution of *p*-aminoacetophenone (2 mmol) in ethanol (5 mL) was added to a solution of benzaldehyde (2 mmol) in ethanol (5 mL) containing 10 drops of 50% v/v sodium hydroxide, and the resulting mixture was stirred for 48 h. The mixture was filtered under vacuum, washed with cold water to pH 7.0, and analyzed by Thin-layer chromatography (TLC) (Scheme 1).

Some characteristics of (2*E*)−1-(4′-aminophenyl)−3-(phenyl)-prop-2-en-1-one (**APCHAL)** are given as follows. Yellow solid (Yield: 25.60%), m.p. 109.3 – 109.9 °C; FT-IR (KBr, $\nu_{cm}^{-1}$): 3522, 3434, 1623, 1578, 1554. $^{1}$H NMR ($CD_3OD$, 300 MHz) δ: 7.40 – 7.42 (m, H-3/H-5, H-4), 7.93 (d, H-2′/H-6′, $J = 8.7$ Hz), 6.73 (d, H-3′/H-5′, $J = 8.7$ Hz), 7.69 – 7.72 (m, H-2/H-6, H-α, H-β). $^{13}$C NMR ($CD_3OD$, 75 MHz) δ: C-1 136.8, C-2/C-6 129.6 C-3/C-5 130.1, C-4 131.4, C-1′128.2, C-2′/C-6′ 132.6, C-3′/C-5′ 115.1, C-4′ 154.8, C-α 123.3, C-β 144.4, C═O 190.3. MS (EI) m/z ($M^{+\cdot}$ 223), calcd for $C_{15}H_{13}NO$/223 (Bandeira et al., 2019; Santiago et al., 2018).

Some characteristics of (2*E*)−1-(4′-aminophenyl)−3-(4-clorophenyl)-prop-2-en-1-one (**ACLOPHENYL**) are given as follows. Yellow solid (Yield: 53.59%), m.p. 162,9 - 163,3 °C; FT-IR (KBr, $\nu_{cm}^{-1}$): 3555, 3350, 1621, 1570, 1550, 1490. $^{1}$H NMR ($CD_3OD$, 300 MHz): δ 7.91 (d, H-2/H-6, $J = 8.73$ Hz), 7.69 (d, H-3/H-5, $J = 8.79$ Hz), 7.42 (d, H-2′/H-6′, $J = 8.46$ Hz), 6.68 (d, H-3′/H-5′, *J* Hz), 7.70 (d, H-α, $J = 14.28$ Hz), 7.74 (d, H-β, $J = 15.60$ Hz). $^{13}$C NMR ($CD_3OD$, 75 MHz): δ C-1 135.6, C-2/C-6 132.7, C-3/C-5 130.9, C-4 137.5, C-1′ 127.6, C-2′/C-6′ 130.8, C-3′/C-5′ 114.6, C-4′ 155.8, C-α 124.3, C-β 142.9, C═O 189.9. MS (EI) m/z ($M^{+\cdot}$ 257.5), calcd for: $C_{15}H_{12}NOCl$/257.5 (Bandeira et al., 2019).

### 2.2. Microorganisms

In the studies related to the modification of antibiotic activity, the standard bacterial strains used were *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 25923, while the resistant strains were *E. coli* 06 and *Staphylococcus aureus* 10. The *E. coli* 06 strain is resistant to several β-lactams such as cephalothin, cephalexin, and ceftriaxone, while the *Staphylococcus aureus* 10 strain is resistant to β-lactams, fluoroquinolones, and macrolides.

In the efflux pump tests, the bacterial strains of *Staphylococcus aureus* 1199B (which overexpresses the NorA gene encoding the NorA efflux protein) and *Staphylococcus aureus* K2068 (which presents the MepA efflux pump) were used. All the bacterial strains were cultivated in heart infusion agar (HIA) during 24 h. Brain heart infusion (BHI) broth was the medium used at the time of the test.

### 2.3. Antimicrobial activity and effect

#### 2.3.1. Drugs

For the evaluation of the antibiotic potentiating activity of the APCHAL and ACLOPHENYL chalcones, two antibiotics were used: the aminoglycoside antibiotic gentamicin, the β-lactam antibiotic penicillin.

In tests with efflux pumps, the antibiotics chosen act as specific substrates for each one of the bacterial strains: norfloxacin for SA 1199B, and ciprofloxacin for SA K2068.

The solutions were prepared on the basis of recommendations established in CLSI (CLSI, 2008) and diluted in sterile water to reach a

concentration of 1024 μg/mL. In addition to antibiotics, ethidium bromide was used to check for efflux pumps.

For the tests, a solution prepared from the APCHAL and ACLOPHENYL compounds were used at a concentration of 10 mg/mL, dissolved in 0,5 mL DMSO (dimethyl sulfoxide), and further diluted with distilled water to a concentration of 1024 μg/mL.

#### 2.3.2. Antibacterial activity

MIC (minimum inhibitory concentration) was determined using broth microdilution tests (Coutinho et al., 2008) with 96-well plates. The bacteria were suspended in BHI broth at a concentration of $10^5$ CFU/mL. The final concentration of the APCHAL and ACLOPHENYL samples ranged from 512 to 8 μg/mL. MICs were evidenced using a colorimetric method with resazurin blue, and their values correspond to the lowest concentrations capable of inhibiting bacterial growth.

#### 2.3.3. Modulation

To evaluate the APCHAL substance as a microbial resistance modulator, the MIC of antimicrobials (gentamycin, and penicillin) was determined in the presence of the compound in a sub-inhibitory concentration (MIC/8), based on the methodology by Coutinho et al. (2008). Microorganisms at the $10^5$ CFU/mL concentration were suspended in the BHI medium in 96-well microdilution plates. The plates were incubated at 37 °C for 24 h, after which time readings were performed using resazurin.

#### 2.3.4. Efflux pump

The evaluation of inhibition of the efflux pump was performed according to Tintino et al. (2016). Two strains of *S. aureus* were used: *S. aureus* 1199B (SA-1199B), which overexpresses the NorA gene encoding the NorA efflux protein, and the multi-drug resistant (MDR) mutant strain *S. aureus* (SA-K2068) which presents the MepA efflux pump. The strains were maintained on blood agar base slants, and, before use, the cells were grown overnight at 37 °C in Heart Infusion Agar slants. The chlorpromazine, carbonyl cyanide m-chlorophenylhydrazone (CCCP), and ethidium bromide were obtained from Sigma Aldrich Co. Ltd. Norfloxacin and ciprofloxacin were the antibiotics used in NorA and MepA efflux inhibition, respectively. The antibiotics and the compounds under study were first dissolved in dimethyl sulfoxide (DMSO). Subsequently, the compounds dissolved in DMSO underwent a new dilution, this time in sterile water. Chlorpromazine and ethidium bromide were dissolved in sterile water and CCCP in methanol/water (1:1, v/v). All the compounds were stored at −20 °C at a final concentration of 1024 μg/mL. To evaluate the inhibition capacity of the efflux pumps, the MICs of the strain-specific antibiotics, as well as the ethidium bromide used as control, were compared with the MICs of their associations with standard inhibitors.

The test solutions used in the aforementioned controls were prepared in Eppendorf microtubes containing medium and inoculum. The test solutions used for comparison were added to the chalcones and standard inhibitors in quantities that correspond to MIC/8 (subinhibitory concentration). Then 100μL of Eppendorf`s content were transferred to 96-well microtiter tray with two-fold serial dilution by adding 100μL of antibiotics and ethidium bromide with final concentration ranging from 0.5 to 512 μg/mL. The trays were incubated at 37 °C for 24 h, and bacterial growth was revealed by staining with resazurin.

### 2.4. Octanol-water partition coefficient calculation

The Octanol-water partition coefficient was calculated using Molinspiration molecular property calculation services (Molinspiration, 2020).

### 2.5. Docking studies

NorA sequence of *S. aureus* 1199B strain was retrieved from the Universal Protein Resource database (Uniprot, Entry Q03325). The web-based SWISS-MODEL service was used to build a homology model of NorA (Waterhouse et al., 2018). The HHblits-based automated mode was used, resulting in 50 different templates with the best one based on the structure of *E. coli* YajR transporter (PDB-ID: 3wdo).

The MepA model was generated by retrieving the protein sequence for the NCTC 8325 strain from the Uniprot database. The same procedure as before was utilized. The template of the multi-drug and toxic compound extrusion (MATE) transporter of the Bacillus halodurans (PDB-ID: 5c6n) was chosen for the homology model.

Both models were then uploaded to the Molecular Dynamics on Web (MDWeb) (Hospital et al., 2012) service, where they were solvated and submitted to a 5 ps molecular dynamics run in canonical ensemble (NVT), followed by a 0.5 ns production run in isothermal–isobaric (NPT) ensemble using the AMBER99 force field. The root mean square deviation (RMSD) of both models reached a plateau after approximately 0.4 ns.

The grid box for the docking procedure was defined as a 70 Å x 70 Å x 70 Å box around the geometrical center of the protein model. Partial Gasteiger charges were added to ligand atoms, non-polar hydrogen atoms were merged, and rotational bonds were determined. Docking studies were carried out through the Lamarckian genetic algorithm in Autodock 4.0 (Morris et al., 2009), and all parameters were kept at their default values. The ten best results were chosen by the lowest binding energy (kcal/mol).

### 2.6. Data analysis

The tests were performed in triplicates, and the results were expressed as the geometric mean. A two-way NOVA with Tukey's post-hoc Bonferroni test statistical analysis was applied to verify the antibiotic potentiating activity of the APCHAL using the program GraphPadPrism 6.02 [30][30], where $p < 0.05$ was considered significant.

## 3. Results and discussion

### 3.1. Structural determination

The $^{13}C$ NMR spectra of the APCHAL and ACLOPHENYL chalcones presented characteristic signals of ketone (C═O) conjugated to the α, β-unsaturated system with values of $\delta_C$ 190.3 and 189.9 ppm, respectively. They also revealed signs for Cα with values of $\delta_C$ 123.3 and 124.3 ppm and $C_\beta$ with values of $\delta_C$ 144.4 and 142.9 ppm for APCHAL and ACLOPHENYL, respectively, characteristic of enones. In addition, the $^1H$ signals in C-sp2 were observed, forming a doublet with coupling values of 12.12 - 15.96 Hz for the Hα and $H_\beta$ characteristics of *trans* hydrogens. This information justifies the prediction for the synthesis of chalcones in the generation of the double bond conjugated with C═O (Bandeira et al., 2019). The molecular structures of the chalcones APCHAL and ACLOPHENYL are shown in Fig. 1, and the $^1H$ and $^{13}C$ NMR spectra are given in the supplemental material.

### 3.2. Antibiotic activity study

By one hand, the MICs obtained for APCHAL with standard and resistant bacterial strains of *Staphylococcus aureus* (SA) and *E. coli* (EC) were: 1024 μg/mL with SA ATCC and 1024 μg/mL with SA 10; 1024 μg/mL with EC ATCC and 1024 μg/mL with EC 06. By the other hand, the MICs obtained for ACLOPHENYL with standard and resistant bacterial strains were: 512 μg/mL with SA ATCC and 1024 μg/mL with SA10; 1024 μg/mL with EC ATCC and 1024 μg/mL with EC 06. The subinhibitory concentration for APCHAL chalcone was 128 μg/mL for all tested strains, corresponding to 188 μL of volume of the compound in the Eppendorf microtube. The ACLOPHENYL had a subinhibitory concentration of 64 μg/mL for SA ATCC, which represents 94 μL of the volume of this compound in the Eppendorf microtube. For the other strains, the

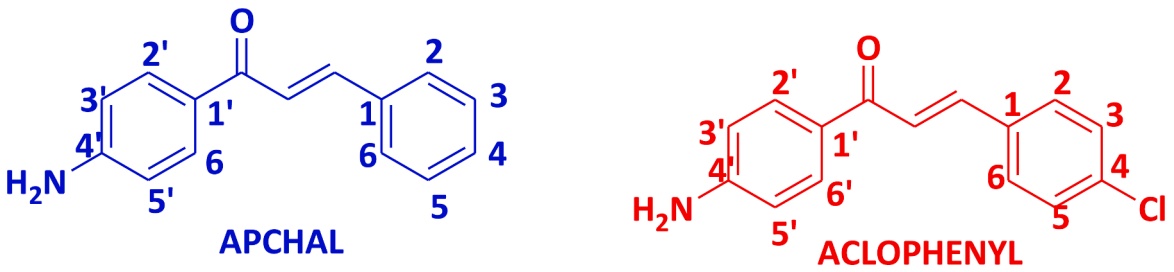



**Fig. 1.** Molecular structures of the APCHAL and ACLOPHENYL synthetic chalcones.

ACLOPHENYL follows the same pattern of the APCHAL.

Figs. 2 and 3 show, respectively, the MIC of the antibiotics gentamicin and penicillin in the presence and absence of the APCHAL and ACLOPHENYL chalcones.

Two different antibiotic classes were used to test the modulation effect: gentamicin, an aminoglycoside, and penicillin, a beta-lactam. The effect was tested in two models using Gram-positive and Gram-negative bacteria. Regarding the *Staphylococcus aureus* Gram-positive model, the APCHAL chalcone presented synergy with the gentamicin and antagonism with penicillin. APCHAL reduced the MIC of gentamicin by almost 70%. Gentamicin is an antibiotic that acts by inhibiting intracellular protein production (Cushnie et al., 2016). Penicillin, as a β-lactam, has a different mechanism of action. It acts outside the cell, preventing the formation of cross-bridge in the cell wall (Kapoor et al., 2017). This fact may explain the difference in the synergistic pattern found. Differently, on the *E. coli* Gram-negative model, APCHAL exhibits an antagonism to aminoglycosides and indifference to β-lactams. The loss of effectiveness in this bacterial model may be explained both by structural differences between Gram-positive and Gram-negative bacteria, which alter permeability and by drug interaction with their targets (Zengin and Baysal, 2014).

When comparing the effects of antibiotic-modifying activity between the two substances tested, ACLOPHENYL and APCHAL, a loss of synergistic effect is noted with gentamicin. This is due to the addition of a chlorine to the structure of the substance (APCHAL to ACLOPHENYL) in the Gram-positive model, while in penicillin the results were indifferent or antagonistic. For *E. coli*, there was a total lack of effect, synergistic or antagonistic, between ACLOPHENYL and antibiotics. The structure of ACLOPHENYL, when compared to APCHAL, differs only by a chlorine ligand in position 4 of the chalcones. Nevertheless, this single molecular change was able to reverse the synergistic effect that is observed with APCHAL when associated with gentamicin. In addition, compounds that exhibit structural similarity do not necessarily bind to the target receptor in the same way (Silverman, 2004).

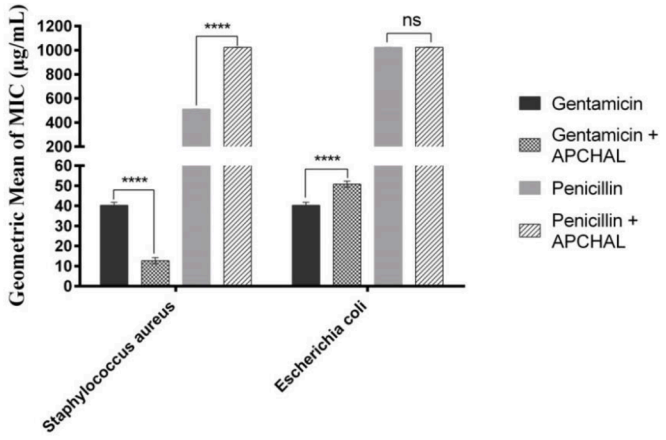



**Fig. 2.** Minimum inhibitory concentration (MIC) of the antibiotics gentamicin and penicillin in the presence and absence of the chalcone APCHAL towards *S. aureus* 10 and *E. coli 06*.

A possible explanation for the loss of synergism is that the chlorine addition in ACLOPHENYL reduces its membrane permeability. Studies have shown that there is a good correlation between physicochemical properties, such as octanol/water partitioning coefficients and membrane permeability (Lipinski et al., 1997). Drugs with an octanol/water partitioning coefficient (logP) close to 2 are generally readily adsorbed while compounds with logP $>$ 4 have lower permeability (Artursson et al., 2001). The logP for APCHAL and ACLOPHENYL are, respectively, 2.89 and 3.56. In other words, the chlorine addition slightly reduces the membrane permeability and may explain the difference in the synergistic effect.

The evaluation of inhibition of the efflux pump for both compounds was performed against two strains of *S. aureus*: SA-1199B, which overexpresses the NorA gene encoding the NorA efflux protein (Fig. 4) and the multi-drug resistant (MDR) mutant strain SA-K2068, which presents the MepA efflux pump (Fig. 5).

Ethidium bromide (EB) is a dye that presents antibiotic activity due to its characteristic of DNA intercalating, promoting damage to the DNA structure, causing cell death (Shabestari et al., 2017). The only mechanism used by bacteria to resist the action of the ethidium bromide is the efflux pump (Pal et al., 2020). Its use associated with standard efflux pump inhibitors is a technique used in several studies to investigate the presence or absence of efflux pumps (Limaverde et al., 2017; Tintino et al., 2018, 2016).

In our tests, we utilized two standard efflux pump inhibitors, the CCCP and the chlorpromazine, each with its own inhibition mechanism. The CCCP acts by causing a disturbance in the electrochemical potential due to a decrease in the production of adenosine triphosphate (ATP) leading to efflux pump inhibition. Whereas the chlorpromazine acts via competitive inhibition on antibiotics, interacting directly with the efflux pumps and inhibiting them [25,42]. Thus, the presence of the efflux pump will be visualized when the minimum inhibitory concentration of ethidium bromide associated with a standard inhibitor is lower than the MIC of the bromide control, indicating the inability of the bacterial cell to use the efflux mechanism.

We can evidence the presence of efflux pumps in strains 1199B and K2068. In strain 1199B, both chlorpromazine and CCCP associated with bromide showed MICs that were lower than the control, showing the presence of efflux pumps with distinct mechanisms of the expulsion of intracellular bromide. In strain K2068, we have efflux pumps sensitive only to CCCP in the presence of ethidium bromide, showing that a likely competitive inhibition mechanism is not effective for this bacterium in relation to ethidium bromide. This fact leads us to infer that the most effective mode of action by which the two chalcones inhibit the NorA and MepA efflux pumps is related to the inhibition of the generator mechanisms of the motive force that acts as a propellant of the respective pumps. These results were corroborated by the study conducted by Gupta et al. [42] who showed that the IMRG4 synthetic chalcone has antibacterial activity against several multi-resistant strains of *Staphylococcus aureus*. Gupta et al. [42] also showed that IMRG4 inhibits the NorA efflux pump by interference in the proton motive force, preventing the generation of energy necessary for the pump operation (Gupta et al.,

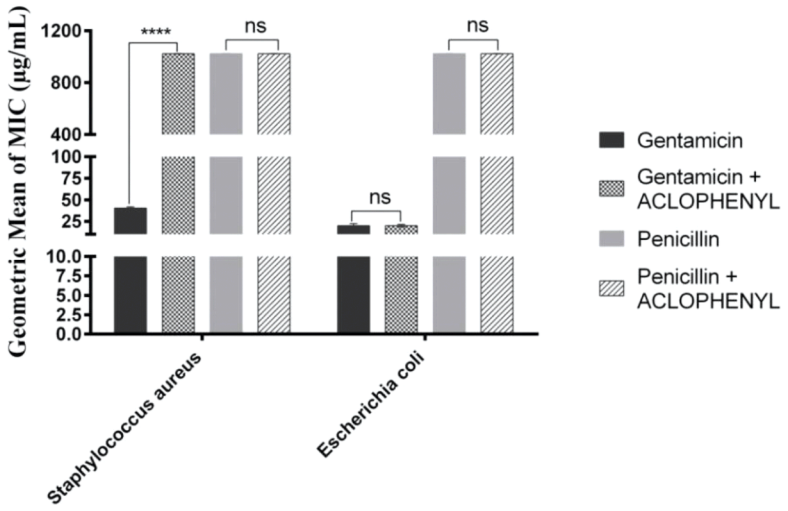



**Fig. 3.** Minimum inhibitory concentration (MIC) of the antibiotics gentamicin and penicillin in the presence and absence of the chalcone ACLOPHENYL towards *S. aureus* 10 and *E. coli 06*.

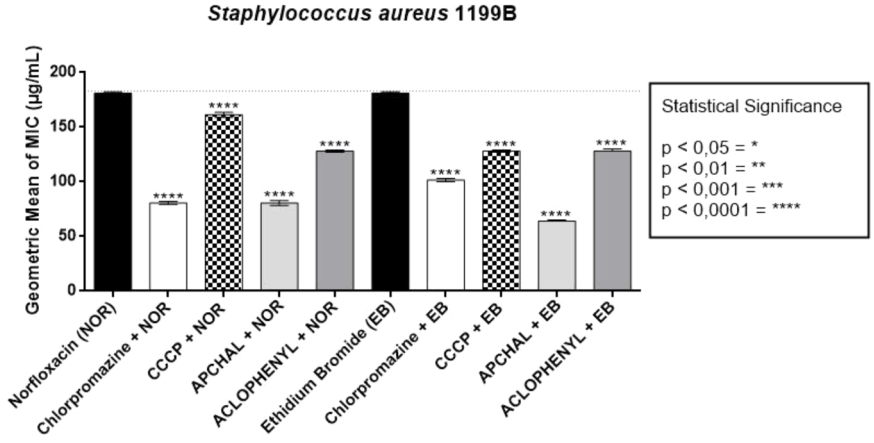



**Fig. 4.** MIC of norfloxacin and ethidium bromide alone and in association with the standard inhibitors and chalcones APCHAL and ACLOPHENY against the strain *S. aureus* 1199B

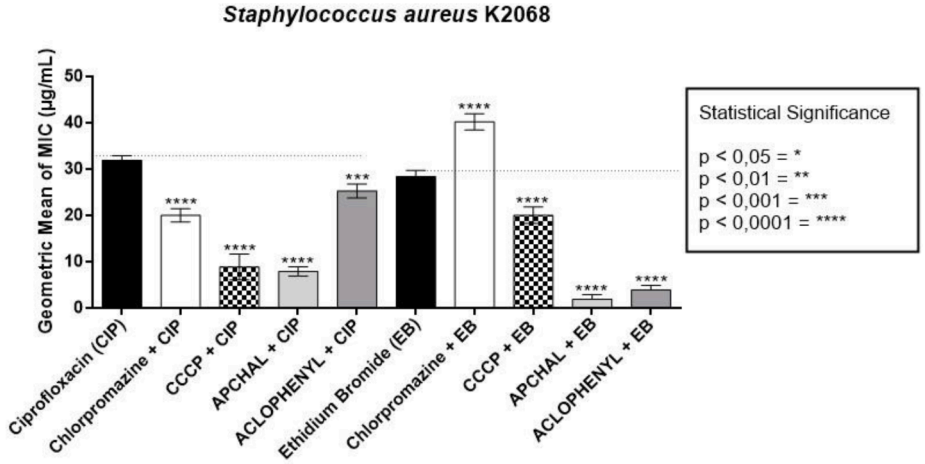



**Fig. 5.** MIC of ciprofloxacin and ethidium bromide alone and in association with the standard inhibitors and chalcones APCHAL and ACLOPHENY against the strain *S. aureus* K2068.

2019).

The standard inhibitors revealed the presence of efflux pumps sensitive to both inhibition mechanisms, both in strain 1199B with relation to norfloxacin and in strain K2068 in relation to ciprofloxacin. As can be seen from Figs. 4 and 5, both chalcones presented a synergistic effect with norfloxacin and ciprofloxacin against multiple drug-resistant *Staphylococcus aureus*, although the effect is much less pronounced with ACLOPHENYL. The synergistic effect of the APCHAL is particularly notable against the strain K2068. The less pronounced synergistic effect of ACLOPHENYL chalcone may be related to a lower efficacy in the main sensitivity mechanism presented by the efflux pumps, and this lower efficacy may be correlated to the structural difference of APCHAL and ACLOPHENYL chalcones.

Our docking studies show that both APCHAL and ACLOPHENYL bind to the binding site of the NorA model, although in different regions of the binding site. Fig. 6 shows the best poses of APCHAL (Pink) and ACLOPHENYL (Light Green), as well as the best pose of norfloxacin (Light Blue) on the binding site of the NorA model. 2D ligand-protein interaction diagrams along with contact distances for both molecules are available as Figures S5 and S6 of the Supplementary Material. Both APCHAL's phenyl, as well as ACLOPHENYL's chlorophenyl, are positioned, such as to facilitate π-π stacking interactions with the Phe139 residue. In addition, APCHAL makes close contacts with amino acid residues Ser214 and Ser218 in a similar fashion as norfloxacin. As can be seen from the figure, the best pose of APCHAL overlaps with that of norfloxacin, while ACLOPHENYL does not. This may explain the much greater synergistic effect of APCHAL compared to ACLOPHENYL. In other words, the bulky and electronegative chlorine changes the binding pose of ACLOPHENYL in a way that is less efficient in inhibiting the efflux pump when compared to APCHAL. According to Palazzotti et al. the description of the binding of ciprofloxacin to the orthosteric binding site of another NorA model is roughly equivalent to ours, although the preparation of the model is quite different (Palazzotti et al., 2019). One should note that their residue numbering is not equal to ours, with Phe139 appearing as Phe140 and Glu221 as Glu222 and so on. One should also note the difference in antibiotics (norfloxacin versus ciprofloxacin) when comparing these results.

Additionally, APCHAL and ACLOPHENYL bind to the binding site of the MepA model in almost the same way, as can be seen from Fig. 7. Fig. 7 displays the best poses of APCHAL (pink) and ACLOPHENYL (light green), and the best pose of ciprofloxacin (light blue) on the binding site of the NorA model. 2D ligand-protein interaction diagrams along with contact distances for both molecules are available as Figures S7 and S8 of the Supplementary Material. The phenyl and chlorophenyl rings are positioned to facilitate π-π stacking interactions with the Phe61. It is observed that APCHAL and ACLOPHENYL molecules overlap almost completely with ciprofloxacin, with the three molecules making close contacts with the same residues, such as Met65, Met171, and Asn204. In other words, the MepA model cannot capture the effect of adding a chlorine atom. These results show that the chalcones can act as efflux pump inhibitors, as previously proposed for other chalcones (Garcia et al., 2020; Rezende-Júnior et al., 2020) through a combination of inhibition essays and docking studies.

## 4. Conclusions

The APCHAL chalcone presented a synergistic effect with gentamicin against a multi-drug resistant *Staphylococcus aureus* strain. However, when a chlorine atom was added to this structure (ACLOPHENYL), the synergistic effect was lost. The loss of a synergistic effect was associated with the presence of the chlorine atom in the molecular structure of the chalcone ACLOPHENYL. However, the presence of synergism or antagonism depends on factors such as the location of the halogen ligand, the types of associated antibiotic ligands to be combined, and the bacterial model involved. Docking studies have shown that the addition of the chlorine impairs the compound's ability to bind to the binding site of the efflux pump proteins, reducing the proposed ability to inhibit the pump. However, other mechanisms of action could not be ruled out.

## Credit authorship contribution statement

**M.M.R.S.:** Investigation, and writing-original draft. **H.S.S.:** Conceptualization, performed the NMR study, and writing-review and editing. **P.T.C.F.:** Supervision and writing-review and editing. **T.S.d.F.:** Investigation of the modulation of multidrug resistance efflux pump activity and revised the manuscript. **B.G.C.:** Investigation of the antimicrobial activity assays. **J.d.C.X.;** Statistical analysis. **P.N.B.:** Co-supervision and performed the synthesis of the chalcone. **M.M.C.d.S.:**

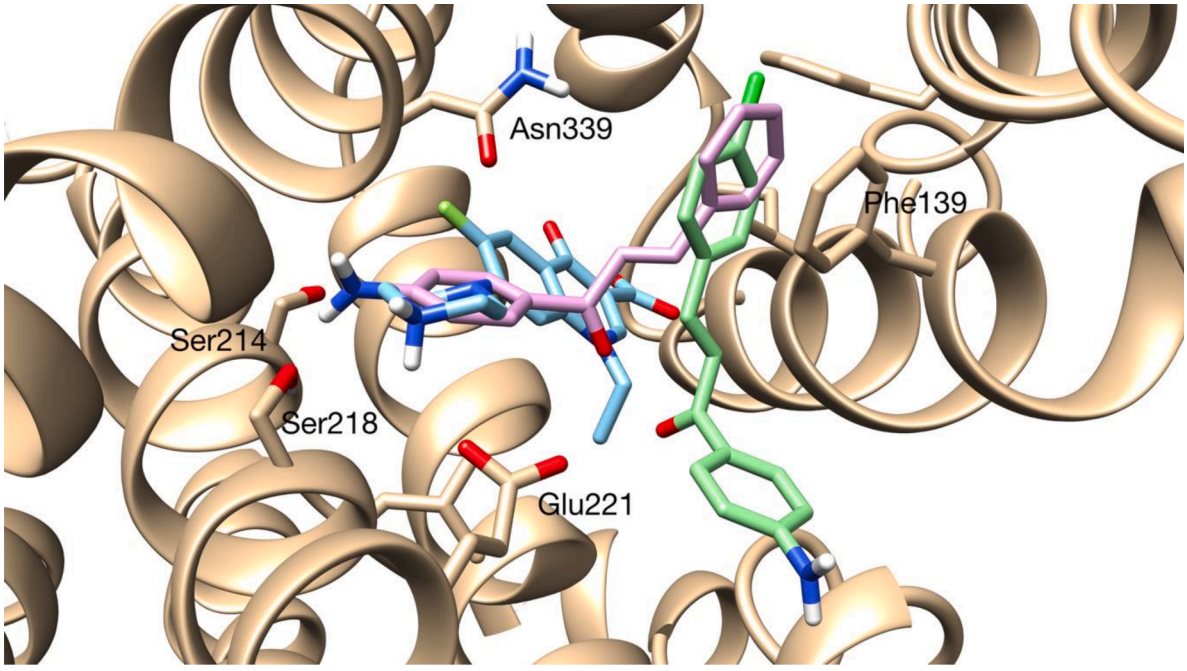



**Fig. 6.** The best poses of APCHAL (Pink) and ACLOPHENYL (Light Green), as well as the best pose of Norfloxacin (Light Blue) on the binding site of the NorA model.

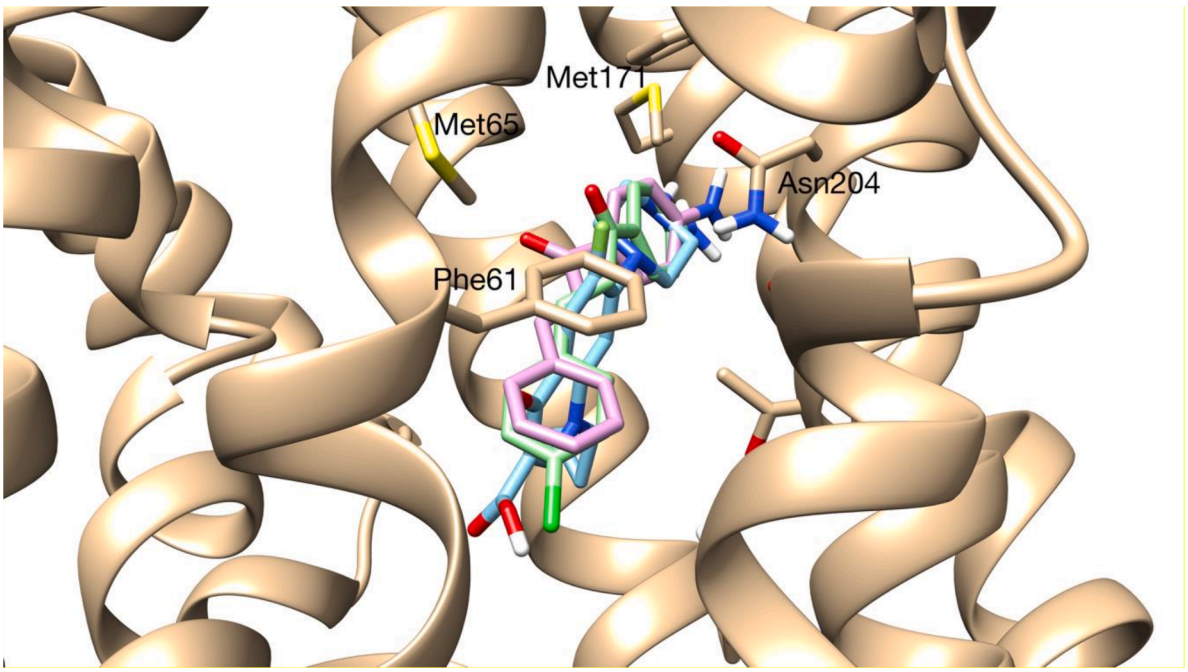
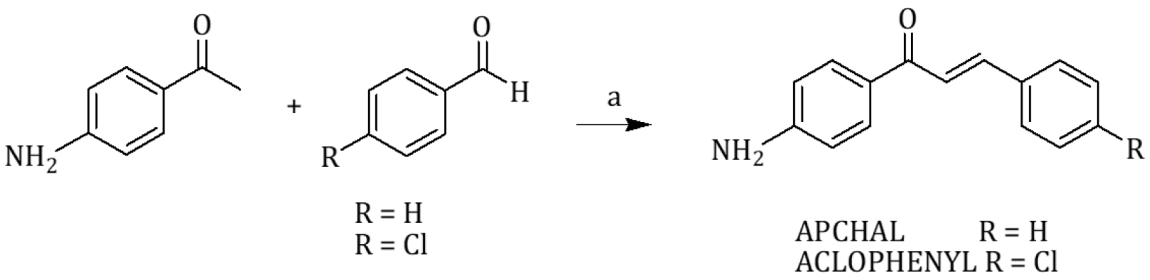



**Fig. 7.** The best poses of APCHAL (Pink) and ACLOPHENYL (Light Green), and the best pose of Ciprofloxacin (Light Blue) on the binding site of the MepA model.




**Scheme 1.** Preparation of chalcones a) NaOH 50% w /v, ethanol, r.t., 48 h.

Data curation. **R.L.S.P.:** Methodology. **A.M.R.T.:** Writing-review and editing. **C.R.d.S.B.:** Visualization. **C.E.S.N.:** Software, formal analysis, molecular docking, and writing-review and editing. **J.P.S.Jr.:** Project administration. **H.D.M.C.:** Methodology and project administration. **J. B.d.A.N.:** Resources and funding acquisition.

## Author contributions

Investigation, and writing-original draft, M.M.R.S.

Conceptualization, performed the NMR study, and writing—review and editing. H.S.S. supervision and revised the manuscript. P.T.C.F. performed the efflux pumps assays, and writing—review and editing, T. S.d.F. validation, M.M.R.S. carried out the antimicrobial activity assays, B.G.C.

Statistical analysis, J.d.C.X. performed the synthesis of the chalcone, P.N.B. data curation, M.M.C.d.S. methodology, R.L.S.P. funding acquisition, and writing—review and editing, A.M.R.T. visualization, C.R.d.S. B. software, formal analysis, molecular docking, and writing—review and editing, C.E.S.N. project administration, J.P.S.Jr. methodology and project administration H.D.M.C. resources and funding acquisition, J.B. d.A.N.

All authors have read and agreed to the published version of the manuscript."

## Funding

This work was supported by the Conselho Nacional de Desenvolvimento Científico e Tecnológico – CNPq [grant number 305719/2018-1].

## Declaration of Competing Interest

The authors declare no conflict of interest.

## Acknowledgments

We thank the Northeastern Center for the Use and Application of Nuclear Magnetic Resonance (CENAUREM) for the spectroscopy measurements.

## Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ejps.2020.105695.

## References

Artursson, P., Palm, K., Luthman, K., 2001. Caco-2 monolayers in experimental and theoretical predictions of drug transport1PII of original article: S0169-409X(96) 00415-2. The article was originally published in Advanced Drug Delivery Reviews 22 (1996) 67–84.1. Adv. Drug Deliv. Rev. 46, 27–43. https://doi.org/10.1016/S0169-409X(00)00128-9.

Bandeira, P.N., Lemos, T.L.G., Santos, H.S., Carvalho, M.C.S.d., Pinheiro, D.P., Moraes Filho, M.O.d., Pessoa, C., Barros-Nepomuceno, F.W.A., Rodrigues, T.H.S., Ribeiro, P. R.V., Magalhães, H.S., Teixeira, A.M.R., 2019. Synthesis, structural characterization, and cytotoxic evaluation of chalcone derivatives. Med. Chem. Res. https://doi.org/10.1007/s00044-019-02434-1.

Bhat, B.A., Dhar, K.L., Puri, S.C., Saxena, A.K., Shanmugavel, M., Qazi, G.N., 2005. Synthesis and biological evaluation of chalcones and their derived pyrazoles as potential cytotoxic agents. Biorg. Med. Chem. Lett. 15, 3177–3180. https://doi.org/10.1016/j.bmcl.2005.03.121.

CLSI, 2008. CLSI - Clinical and Laboratory Standards Institute Reference Method for Broth Dilution Antifungal Susceptibility Testing of Yeasts, Approved Standard M27-A3., 3ª ed. Clinical and Laboratory Standards Institute, Wayne, PA, USA.

Coutinho, H.D.M., Costa, J.G.M., Lima, E.O., Falcão-Silva, V.S., Siqueira-Júnior, J.P., 2008. Enhancement of the Antibiotic Activity against a Multiresistant *Escherichia coli*by *Mentha arvensis*L. and Chlorpromazine. Chemotherapy 54, 328–330. https://doi.org/10.1159/000151267.

Cushnie, T.P.T., O'Driscoll, N.H., Lamb, A.J., 2016. Morphological and ultrastructural changes in bacterial cells as an indicator of antibacterial mechanism of action. Cell. Mol. Life Sci. 73, 4471–4492. https://doi.org/10.1007/s00018-016-2302-2.

Dan, W., Dai, J., 2020. Recent developments of chalcones as potential antibacterial agents in medicinal chemistry. Eur. J. Med. Chem. 187, 111980 https://doi.org/10.1016/j.ejmech.2019.111980.

Eichenberger, M., Lehka, B.J., Folly, C., Fischer, D., Martens, S., Simón, E., Naesby, M., 2017. Metabolic engineering of Saccharomyces cerevisiae for de novo production of dihydrochalcones with known antioxidant, antidiabetic, and sweet tasting properties. Metab. Eng. 39, 80–89. https://doi.org/10.1016/j.ymben.2016.10.019.

Felicetti, T., Cannalire, R., Pietrella, D., Latacz, G., Lubelska, A., Manfroni, G., Barreca, M.L., Massari, S., Tabarrini, O., Kieć-Kononowicz, K., Schindler, B.D., Kaatz, G.W., Cecchetti, V., Sabatini, S., 2018. 2-Phenylquinoline S. aureus NorA Efflux Pump Inhibitors: evaluation of the Importance of Methoxy Group Introduction. J. Med. Chem. 61, 7827–7848. https://doi.org/10.1021/acs.jmedchem.8b00791.

Felicetti, T., Mangiaterra, G., Cannalire, R., Cedraro, N., Pietrella, D., Astolfi, A., Massari, S., Tabarrini, O., Manfroni, G., Barreca, M.L., Cecchetti, V., Biavasco, F., Sabatini, S., 2020. C-2 phenyl replacements to obtain potent quinoline-based Staphylococcus aureus NorA inhibitors. J. Enzyme Inhib. Med. Chem. 35, 584–597. https://doi.org/10.1080/14756366.2020.1719083.

Fontaine, F., Hequet, A., Voisin-Chiret, A.-S., Bouillon, A., Lesnard, A., Cresteil, T., Jolivalt, C., Rault, S., 2014. First Identification of Boronic Species as Novel Potential Inhibitors of the Staphylococcus aureus NorA Efflux Pump. J. Med. Chem. 57, 2536–2548. https://doi.org/10.1021/jm401808n.

Garcia, T.R., de Freitas, T.S., dos Santos, H.S., Bandeira, P.N., Julião, M.S.S., Rocha, J.E., Nogueira, C.E.S., Pereira, R.L.S., Barreto, A.C.H., Freire, P.T.C., Coutinho, H.D.M., Teixeira, A.M.R., 2020. Structural, vibrational and electrochemical analysis and antibiotic activity study of chalcone (2E)-1-(3′,-methoxy-4′,-hydroxyphenyl)-3-(3-nitrophenyl)prop-2-en-1-one. J. Mol. Struct. 1216, 128358 https://doi.org/10.1016/j.molstruc.2020.128358.

Gupta, D., Jain, D., 2015. Chalcone derivatives as potential antifungal agents: synthesis, and antifungal activity. J. Adv. Pharm. Technol. Res. 6, 114–117. https://doi.org/10.4103/2231-4040.161507.

Gupta, V.K., Gaur, R., Sharma, A., Akther, J., Saini, M., Bhakuni, R.S., Pathania, R., 2019. A novel bi-functional chalcone inhibits multi-drug resistant Staphylococcus aureus and potentiates the activity of fluoroquinolones. Bioorg. Chem. 83, 214–225. https://doi.org/10.1016/j.bioorg.2018.10.024.

GraphPad Prism version 6.02 for Windows, GraphPad Software, La Jolla California USA, www.graphpad.com.

Holler, J.G., Slotved, H.-.C., Mølgaard, P., Olsen, C.E., Christensen, S.B., 2012. Chalcone inhibitors of the NorA efflux pump in Staphylococcus aureus whole cells and enriched everted membrane vesicles. Biorg. Med. Chem. 20, 4514–4521. https://doi.org/10.1016/j.bmc.2012.05.025.

Hospital, A., Andrio, P., Fenollosa, C., Cicin-Sain, D., Orozco, M., Gelpí, J.L., 2012. MDWeb and MDMoby: an integrated web-based platform for molecular dynamics simulations. Bioinformatics 28, 1278–1279. https://doi.org/10.1093/bioinformatics/bts139.

Kapoor, G., Saigal, S., Elongavan, A., 2017. Action and resistance mechanisms of antibiotics: a guide for clinicians. J. Anaesthesiol. Clin. Pharmacol. 33, 300–305. https://doi.org/10.4103/joacp.JOACP_349_15.

Katsori, A.M., Hadjipavlou-Litina, D., 2009. Chalcones in cancer: understanding their role in terms of QSAR. Curr. Med. Chem. 16, 1062–1081. https://doi.org/10.2174/092986709787581798.

Lepri, S., Buonerba, F., Goracci, L., Velilla, I., Ruzziconi, R., Schindler, B.D., Seo, S.M., Kaatz, G.W., Cruciani, G., 2016. Indole based weapons to fight antibiotic resistance: a structure–activity relationship study. J. Med. Chem. 59, 867–891. https://doi.org/10.1021/acs.jmedchem.5b01219.

Limaverde, P.W., Campina, F.F., da Cunha, F.A.B., Crispim, F.D., Figueredo, F.G., Lima, L.F., Datiane de, M., Oliveira-Tintino, C., de Matos, Y.M.L.S., Morais-Braga, M. F.B., Menezes, I.R.A., Balbino, V.Q., Coutinho, H.D.M., Siqueira-Júnior, J.P., Almeida, J.R.G.S., Tintino, S.R., 2017. Inhibition of the TetK efflux-pump by the essential oil of Chenopodium ambrosioides L. and α-terpinene against Staphylococcus aureus IS-58. Food Chem. Toxicol. 109, 957–961. https://doi.org/10.1016/j.fct.2017.02.031.

Lipinski, C.A., Lombardo, F., Dominy, B.W., Feeney, P.J., 1997. Experimental and computational approaches to estimate solubility and permeability in drug discovery and development settings. Adv. Drug Deliv. Rev. 23, 3–25. https://doi.org/10.1016/S0169-409X(96)00423-1.

Mariño, P.A., Pereira, D.B., Santi, G., de Souza, R.O., Faoro, D., de Oliveira, L.F.S., Machado, M.M., Paula, F.R., 2016. In vitro and in silico toxicity evaluation of bioactive 4′-aminochalcone derivatives. Drug Chem. Toxicol. 39, 147–152. https://doi.org/10.3109/01480545.2015.1056881.

Molinspiration, 2020. Molinspiration Cheminformatics Software (https://www.molinspiration.com/). (Accessed 18 September 2019).

Morris, G.M., Huey, R., Lindstrom, W., Sanner, M.F., Belew, R.K., Goodsell, D.S., Olson, A.J., 2009. AutoDock4 and AutoDockTools4: automated docking with selective receptor flexibility. J. Comput. Chem. 30, 2785–2791. https://doi.org/10.1002/jcc.21256.

Nandedkar, N., Deepali, J., Arote, Y., Patel, M., Kadam, V., 2013. Recent developments in synthesis and application of multifaceted chalcone compounds. Indo Am. J. Pharm. Res. 3, 7493–7505.

O'Neill, J., 2016. Tackling Drug-Resistant Infections Globally: final Report And Recommendations - The review on antimicrobial resistance.

Oliveira, A.C.d., Kovner, C.T., Silva, R.S.d., 2010. Nosocomial Infection in an Intensive Care Unit in a Brazilian University. Hospital. Rev. Latino-Am. Enfermagem 18, 233–239. https://doi.org/10.1590/S0104-11692010000200014.

Pal, S., Misra, A., Banerjee, S., Dam, B., 2020. Adaptation of ethidium bromide fluorescence assay to monitor activity of efflux pumps in bacterial pure cultures or mixed population from environmental samples. J. King Saud Univ. Sci. 32, 939–945. https://doi.org/10.1016/j.jksus.2019.06.002.

Palazzotti, D., Bissaro, M., Bolcato, G., Astolfi, A., Felicetti, T., Sabatini, S., Sturlese, M., Cecchetti, V., Barreca, M.L., Moro, S., 2019. Deciphering the Molecular Recognition Mechanism of Multidrug Resistance Staphylococcus aureus NorA Efflux Pump Using a Supervised Molecular Dynamics Approach. Int. J. Mol. Sci. 20, 16. https://doi.org/10.3390/ijms20164041.

Rezende-Júnior, L.M., Andrade, L.M.S., Leal, A.L.A.B., Mesquita, A.B.S., Santos, A.L.P.A., Neto, J.S.L., Siqueira-Júnior, J.P., Nogueira, C.E.S., Kaatz, G.W., Coutinho, H.D.M., Martins, N., da Rocha, C.Q., Barreto, H.M., 2020. Chalcones Isolated from Arrabidaea brachypoda Flowers as Inhibitors of NorA and MepA Multidrug Efflux Pumps of Staphylococcus aureus. Antibiotics 9, 1–12. https://doi.org/10.3390/antibiotics9060351.

Rios, J.L., Recio, M.C., Villar, A., 1988. Screening methods for natural products with antimicrobial activity: a review of the literature. J. Ethnopharmacol. 23, 127–149. https://doi.org/10.1016/0378-8741(88)90001-3.

Santiago, R.N.S., Freire, P.T.C., Teixeira, A.M.R., Bandeira, P.N., Santos, H.S., Lemos, T. L.G., Ferraz, C.A.N., 2018. FT-Raman and FT-IR spectra and DFT calculations of chalcone (2E)-1-(4-aminophenyl)-3-phenyl-prop-2-en-1-one. Vib. Spectrosc. 97, 1–7. https://doi.org/10.1016/j.vibspec.2018.04.007.

Shabestari, M.H., Meijering, A.E.C., Roos, W.H., Wuite, G.J.L., Peterman, E.J.G., 2017. Chapter four - recent advances in biological single-molecule applications of optical tweezers and fluorescence microscopy. In: Spies, M., Chemla, Y.R. (Eds.), Methods Enzymol. Academic Press, pp. 85–119.

Sharma, A., Gupta, V.K., Pathania, R., 2019. Efflux pump inhibitors for bacterial pathogens: from bench to bedside. Indian J. Med. Res. 149, 129–145. https://doi.org/10.4103/ijmr.IJMR_2079_17.

Silverman, R., 2004. The Organic Chemistry of Drug Design and Drug Action Second ed. Elsevier Academic Press.

Sun, J., Deng, Z., Yan, A., 2014. Bacterial multidrug efflux pumps: mechanisms, physiology and pharmacological exploitations. Biochem. Biophys. Res. Commun. 453, 254–267. https://doi.org/10.1016/j.bbrc.2014.05.090.

Thai, K.M., Ngo, T.D., Phan, T.V., Tran, T.D., Nguyen, N.V., Nguyen, T.H., Le, M.T., 2015. Virtual Screening for Novel Staphylococcus Aureus NorA Efflux Pump Inhibitors From Natural Products. Med. Chem. 11, 135–155. https://doi.org/10.2174/1573406410666140902110903.

Tintino, C.D.d.M.O., Tintino, S.R., Limaverde, P.W., Figueredo, F.G., Campina, F.F., da Cunha, F.A.B., da Costa, R.H.S., Pereira, P.S., Lima, L.F., de Matos, Y.M.L.S., Coutinho, H.D.M., Siqueira-Júnior, J.P., Balbino, V.Q., da Silva, T.G., 2018. Inhibition of the essential oil from Chenopodium ambrosioides L. and α-terpinene on the NorA efflux-pump of Staphylococcus aureus. Food Chem 262, 72–77. https://doi.org/10.1016/j.foodchem.2018.04.040.

Tintino, S.R., Oliveira-Tintino, C.D.M., Campina, F.F., Silva, R.L.P., Costa, M.d.S., Menezes, I.R.A., Calixto-Júnior, J.T., Siqueira-Junior, J.P., Coutinho, H.D.M., Leal-Balbino, T.C., Balbino, V.Q., 2016. Evaluation of the tannic acid inhibitory effect against the NorA efflux pump of Staphylococcus aureus. Microb. Pathog. 97, 9–13. https://doi.org/10.1016/j.micpath.2016.04.003.

Tran, T.-.D., Do, T.-.H., Tran, N.-.C., Ngo, T.-.D., Huynh, T.-N.-P., Tran, C.-.D., Thai, K.-.M., 2012. Synthesis and anti Methicillin resistant Staphylococcus aureus activity of substituted chalcones alone and in combination with non-beta-lactam antibiotics. Biorg. Med. Chem. Lett. 22, 4555–4560. https://doi.org/10.1016/j.bmcl.2012.05.112.

Waterhouse, A., Bertoni, M., Bienert, S., Studer, G., Tauriello, G., Gumienny, R., Heer, F. T., de Beer, T.A P., Rempfer, C., Bordoli, L., Lepore, R., Schwede, T., 2018. SWISS-MODEL: homology modelling of protein structures and complexes. Nucleic Acids Res 46, W296–W303. https://doi.org/10.1093/nar/gky427.

Webber, M.A., Piddock, L.J.V., 2003. The importance of efflux pumps in bacterial antibiotic resistance. J. Antimicrob. Chemother. 51, 9–11. https://doi.org/10.1093/jac/dkg050.

WHO, 2009. WHO Guidelines on Hand Hygiene in Health Care: First Global Patient Safety Challenge Clean Care Is Safer Care. World Health Organization, Geneva, p. 270.

Zengin, H., Baysal, H.A., 2014. Antibacterial and Antioxidant Activity of Essential Oil Terpenes against Pathogenic and Spoilage-Forming Bacteria and Cell Structure-Activity Relationships Evaluated by SEM Microscopy. Molecules 19. https://doi.org/10.3390/molecules191117773.