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In vitro and *in silico* evidences about the inhibition of MepA efflux pump by coumarin derivatives

Ana Luíza A.R. Martin [a,d], Raimundo Luiz Silva Pereira [b], Janaína Esmeraldo Rocha [b], Pablo A.M. Farias [d], Thiago S. Freitas [b], Francisco Rodrigo de Lemos Caldas [f], Fernando G. Figueredo [b,d], Nadghia Figueiredo Leite Sampaio [b,d], Jaime Ribeiro-Filho [e], Irwin Rose de Alencar Menezes [b], Guilherme Andrade Brancaglion [c], Daniela Carvalho de Paulo [c], Diogo T. Carvalho [c], Micheline Azevedo Lima [g], Henrique D.M. Coutinho [b,*], Marta M.F. Fonteles [a]

[a] *Federal University of Ceará - UFC, Brazil*
[b] *Regional University of Cariri - URCA, Brazil*
[c] *Federal University of Alfenas - UNIFAL, Brazil*
[d] *Faculty of Medicine Estácio Juazeiro do Norte – Estácio Juazeiro do Norte, Brazil*
[e] *Osvaldo Cruz Foundation – FIOCRUZ, Brazil*
[f] *Instituto de Educação de Ciências e Tecnologia do Ceará – IFCE, Brazil*
[g] *Federal University of Paraíba - UFPB, Brazil*

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ABSTRACT

The discovery of antibiotics has significantly transformed the outcomes of bacterial infections in the last decades. However, the development of antibiotic resistance mechanisms has allowed an increasing number of bacterial strains to overcome the action of antibiotics, decreasing their effectiveness against infections they were developed to treat. This study aimed to evaluate the antibacterial activity of synthetic coumarins *Staphylococcus aureus* in vitro and analyze their interaction with the MepA efflux pump in silico. The Minimum Inhibitory Concentration (MIC) determination showed that none of the test compounds have antibacterial activity. However, all coumarin derivatives decreased the MIC of the standard efflux inhibitor ethidium bromide, indicating antibacterial synergism. On the other hand, the C14 derivative potentiated the antibacterial activity of ciprofloxacin against the resistant strain. In silico analysis showed that C9, C11, and C13 coumarins showed the most favorable interaction with the MepA efflux pump. Nevertheless, due to the present in silico and in vitro investigation limitations, further experimental research is required to confirm the therapeutic potential of these compounds in vivo.

1. Introduction

The discovery of substances with antimicrobial activity has undoubtedly transformed the adverse outcomes of uncomplicated bacterial infections and contributed, to this day, to the control of morbidity and mortality in humans. However, the speed at which bacteria develop resistance mechanisms to existing antibacterials is greater than the speed at which science can develop drugs to combat this resistance [1].

Genetic alterations such as mutation and uptake of genetic material by horizontal transfer from other bacterial strains are the most common way of acquiring resistance [2]. The main mechanisms by which bacteria resist the effect of antibacterial drugs are the expression of efflux pumps that are responsible for removing the drug that has penetrated the bacterial cell; the production of inactivating enzymes that promote the degradation of the pharmacophoric group of the antibacterial, preventing its pharmacological action; reduction of penetration of the antibacterial into the bacteria or alteration of the target site of the antibacterial [3,4]. Some bacteria develop several of these mechanisms simultaneously, becoming resistant to multiple drugs, thus reducing the therapeutic possibilities for treatment. Among the various means of

* Corresponding author.
E-mail address: hdmcoutinho@gmail.com (H.D.M. Coutinho).

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bacterial resistance, transmembrane efflux pumps have been commonly associated with resistance to various antibiotics and other chemotherapeutic agents [5–8].

The efflux mechanisms play a significant negative role in the treatment of *S. aureus* infections as they act on various drugs, reducing the concentration of the antibacterial agent. These proteins are physiological in bacterial cells and have the capacity to promote both the influx and efflux of components [9], including heavy metals and antibiotics. These pumps function dependent on two transport systems: the co-transport system and the ATPase system [10]. Several efflux pumps have been identified in *S. aureus* strains [11,12]: the Major Facilitator Superfamily (MFS) pumps - NorA, NorB, NorC, and MdeA; the ATP-Binding Cassette (ABC) pumps - AbcA; the Small Multidrug Resistance (SMR) family - SepA; and the Multidrug and Toxic Compound Extrusion (MATE) Superfamily - MepA, among others.

In this context, some *Staphylococcus aureus* stands out among the bacteria that can acquire this resistance mechanism. This gram-positive bacterium has been the target of infections over the years, responsible for skin conditions, respiratory tract, endocarditis, osteomyelitis, and septicemia [13]. The development of multidrug resistance developed by this microorganism, including the expression of the MepA efflux pump [14–16], has generated concern in the scientific community and increased the search for the discovery and synthesis of compounds that can reverse this resistance and restore the effectiveness of these antibacterial drugs.

Efflux pumps constitute an important mechanism of bacterial resistance in *S. aureus* strains, reducing the effectiveness of ciprofloxacin, for example. Ciprofloxacin is reported as a substrate, primarily for the efflux pumps of the MFS (Major Facilitator Superfamily) and MATE (Multidrug and Toxic Compound Extrusion) families [17]. A study evaluating bacterial resistance to ciprofloxacin demonstrated that 70% of tested MRSA isolates are phenotypically resistant to ciprofloxacin, and the presence of efflux pumps, including MepA and NorA, can promote resistance to ciprofloxacin [18]. Tolerance facilitates the development of resistance against ciprofloxacin in *S. aureus*, mainly due to the increased expression of pumps in these strains [19].

The presence of efflux pumps in *S. aureus* strains severely compromises infection treatment, necessitating a continuous search for and discovery of potential pump inhibitors, substances with antibiotic properties and/or the ability to modify the action of antibiotics, thereby reversing bacterial resistance.

The search for natural or synthetic products with an antibacterial activity that inactivates bacterial resistance mechanisms, such as efflux pumps, has recently increased to minimize antibiotic resistance. In this context, coumarins, secondary metabolites of several plant species, and coumarin-derived compounds have demonstrated significant antibacterial activity against *Staphylococcus aureus* [20–22]. However, few studies have evaluated the ability of these compounds to potentiate the antibacterial activity of conventional antibiotics against bacterial strains with a known resistance mechanism.

Molecular docking is a tool used in several areas of pharmacological and toxicological research [23–25] and shows promise in predicting the interaction between molecules and their target proteins. In this context, in silico research is an important resource in the study of interactions of different derivatives with antibiotic resistance targets, including efflux pumps.

Therefore, this study aimed to evaluate the antibacterial activity of synthetic coumarins *Staphylococcus aureus* in vitro and analyze their interaction with the MepA efflux pump in silico.

2. Results

2.1. Antibacterial activity of coumarin derivatives

The coumarin-derived compounds showed clinically ineffective antibacterial activity against *S. aureus* K2068 since they all presented minimum inhibitory concentrations above 1024 μg/mL. In contrast, the ciprofloxacin antibacterial control, with an MIC of 57 μg/mL, confirmed its antibiotic efficacy (Table 1). However, the tested compounds significantly reduced ($p < 0.0001$) the MIC of ethidium bromide, indicating that they act as efflux modulators in this strain. Of note, the compounds C10, C11, C13, and C14 showed even more significant efflux-inhibiting activity than the standard compound chlorpromazine (Fig. 1a). On the other hand, when associated with ciprofloxacin, only the C14 caused a significant decrease in the antibiotic MIC ($p < 0.0001$), indicating that this compound could be modulating the MepA-mediated antibiotic resistance in the tested strain. Curiously, coumarin derivatives C9, C10, C11, and C13, showed antagonistic effects when associated with the same antibiotic (Fig. 1b).

2.2. In silico inhibition of the MepA efflux pump

This study assessed the coumarin derivatives for their potential as MepA inhibitors. The natural substrate ethidium bromide and the classical inhibitor Chlorpromazine are used in the docking procedure to compare the binding affinity. The docking results showed that all tested compounds present favorable binding energy with interactions ranging between −6.6 kcal/mol and −8.3 kcal/mol calculated by Autodock Vina and Docking Score ranging between −326.3 and −238.5 kcal/mol by Molegro (Table 2) corroborating with results presented in Fig. 1a where all compounds promoted the reduction of MIC, indicating the interference in the efflux machine. The compounds C11, C9, and C13 showed significant efflux inhibitory capacity and predicted binding potency similar to that of the natural substrate ethidium bromide. Thus, they present a lead chemical structure in the search for new antibacterial drugs (see Table 3).

The Person's correlations between the data from the docking analysis and the MIC values obtained in the in vitro bioassay MIC are shown in Fig. 2. This analysis shows the highest correlation coefficient between the MIC and interactions ($r = -0.64$); docking score ($r = -0.61$), affinity ($r = -0.75$) and positive correlation with Ki ($r = 0.84$). These data present plausive results in the possible docking conformations of ligand into the binding site of the MepA efflux pump.

We generated interaction maps to understand better the relationship between the stability of the ligand-receptor interaction, the inhibition potential, and in vitro antibacterial activity. The procedure was validated by re-docking into its active site with acceptance of the calculated value of root mean square deviation (RMSD) less than 2.0 Å. The ligand's most relevant interaction with the enzyme's binding site involves van der Waals and hydrogen bond interactions.

The coumarin ring of the C11 compound has an affinity for isoleucine, a nonpolar amino acid. In contrast, the carbonyl group has an affinity for the polar amino acid threonine through dipole-dipole interactions and hydrogen bonding. On the other hand, hydrogen bonds mediate the interaction of the glycosidic group with asparagine, a polar amino acid (Fig. 3d).

We found an accumulation of energy involving hydrogen bonds (ASH 183, ALA197; ASN205; SER175; THR201) and van der Waals

Table 1
Antibacterial and modulating activity of coumarins against SA-K2068.

Substance	*S. aureus* K2068			
	MIC (μg/mL) Alone	MIC (μg/mL) + EtBr	MIC (μg/mL) Alone	MIC (μg/mL) + CIPRO
C9	>1024	128	>1024	71,8
C10	>1024	90,5	>1024	80,6
C11	>1024	114	>1024	90,5
C13	>1024	71,8	>1024	71,8
C14	>1024	114	>1024	50,8
CPZ	128	–	45	–
CIPRO	–	–	57	–
EtBr	181	–	–	–

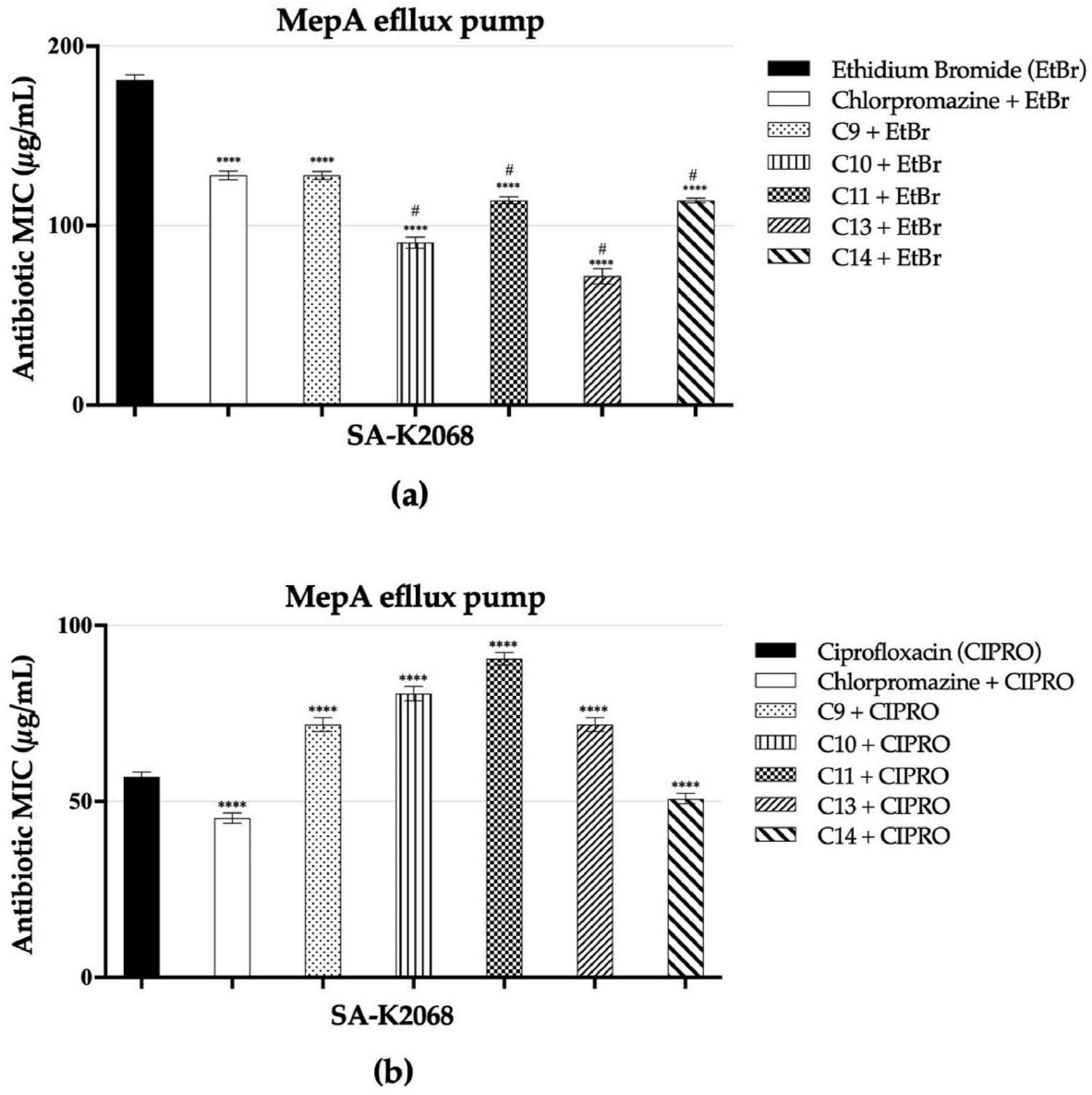


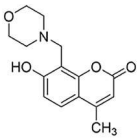
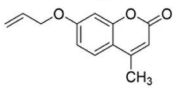
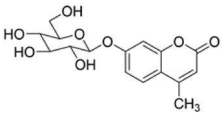
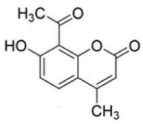
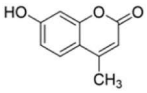
Fig. 1. Minimum inhibitory concentrations of compounds C9, C10, C11, C13, and C14 in association with ethidium bromide (a) and ciprofloxacin (B), against the multidrug-resistant strain SA-K2068, which expresses the MepA efflux pump. These values represent the geometric mean ± SEM (standard error of the mean). Two-way ANOVA, followed by the Bonferroni test. ****p < 0.0001 vs control; #p < 0.0001 vs chlorpromazine; CIPRO = ciprofloxacin; C9 = 7-hydroxy-4-methyl-8-(morpholin-4-ylmethyl)-2H-chromen-2-one; C10 = 7-(Allyloxy)-4-methyl-2H-chromen-2-one; C11 = 7-(alpha-D-Galactopyranosyloxy)-4-methyl-2H-1-benzopyran-2-one; C13 = 8-Acetyl-7-hydroxy-4-methyl coumarin; C14 = 7-Hydroxy-4-methyl-2H-chromen-2-one; EtBr = ethidium bromide.

Table 2
The energies resulting from the interaction with the MepA efflux pump.

Ligand	MIC + EtBr	Molegro			Autodock Vina		
		Interaction (Kcal/mol)	HBond (Kcal/mol)	Docking Score	Affinity (Kcal/mol)	Ki (μM)	LE
Ethidium Bromide (EtBr)	143.66	−116.9	−4.4	−323.6	−8.3	0.82	−035
Chlorpromazine	90.51	−112.1	0	−332.7	−6.8	10.37	−0.32
C11	90.51	−124.6	−2.6	−367.4	−8.3	0.82	−0.35
C09	101.55	−113.9	−3.3	−289.3	−7.6	2.69	−0.38
C13	50.80	−96.7	−5.0	−241.1	−7.0	7.40	−0.44
C10	45.26	−104.7	−2.5	−264.9	−6.6	14.53	−0.41
C14	71.84	−92.3	−7.5	−238.5	−6.6	15.50	−0.51

Ki = inhibition constant obtained from the binding energy (ΔG-Affinity) using the formula: Ki = exp(ΔG/RT), where R is the universal gas constant (1.985 × 10−3 kcal mol−1 K−1) and T is the temperature (298.15 K); Hbond = hydrogen bond energy of interaction; LE = Ligand efficacy.

Table 3
Chemical structure of evaluated coumarins.

Compounds	Chemistry Structure	Name IUPAC
C9		7-hydroxy-4-methyl-8-(morpholin-4-ylmethyl)-2H-chromen-2-one
C10		7-(Allyloxy)-4-methyl-2H-chromen-2-one
C11		7-(alpha-D-Galactopyranosyloxy)-4-methyl-2H-1-benzopyran-2-one
C13		8-Acetyl-7-hydroxy-4-methylcoumarin
C14		7-Hydroxy-4-methyl-2H-chromen-2-one

interaction, which represents the need for a balance in hydrophilicity/hydrophobicity and contributes to the stabilization of the MepA/ligand complex.

3. Discussion

Previous research has demonstrated that coumarin derivatives present significant antibacterial activity against gram-positive and gram-negative bacterial strains with no known resistance mechanism [26–32] However, the coumarin derivatives investigated by this study showed no significant antibacterial activity against the SA-K2068 strain of *Staphylococcus aureus*, whose antibiotic resistance was associated with the expression of the MepA efflux pump.

The MepA protein belongs to the MATE family of efflux pumps that mediate the extrusion of multiple drugs and toxins. Its energy source for substrate transport is the proton gradient through the sodium antiport [33–35]. As common substrates of this efflux pump, antibiotics are expelled from the bacterial cell, compromising their effectiveness. Therefore, compounds capable of inhibiting the activity or expression of this protein can potentially increase the effectiveness of antibiotics and, as such, be helpful in the fight against bacterial resistance [36–38].

Ethidium bromide is an efflux pump substrate widely used in experimental research targeting the discovery of new efflux pump inhibitors. In this context, evidence indicates that compounds capable of reducing the minimum inhibitory concentration of this substrate against resistant bacteria potentially act as efflux pump inhibitors [6,39].

This study demonstrated that the coumarin derivatives significantly reduced the MIC of this substrate against the MepA-expressing strain SA-K2068 of *S. aureus*, indicating that these compounds may be acting by inhibiting the MepA-mediated EtBr efflux.

Among the test compounds, C13 showed the most potent inhibitory activity. This compound has phenolic hydroxyl and carbonyl groups that can favor intermolecular dipole-dipole interactions and hydrogen bonding with the polar residues of threonine and asparagine in the MepA efflux pump. The in silico study by Tiwari (2020) showed that the carbonyl oxygen present in the coumarin ring forms a hydrogen bond with the hydroxyl group, which could justify the activity observed in vitro in this study [40].

We used chlorpromazine as a standard efflux inhibitor since it was found to potentiate the effectiveness of several antibiotics against *S. aureus* [41–43]. It has been hypothesized that this compound act by impairing

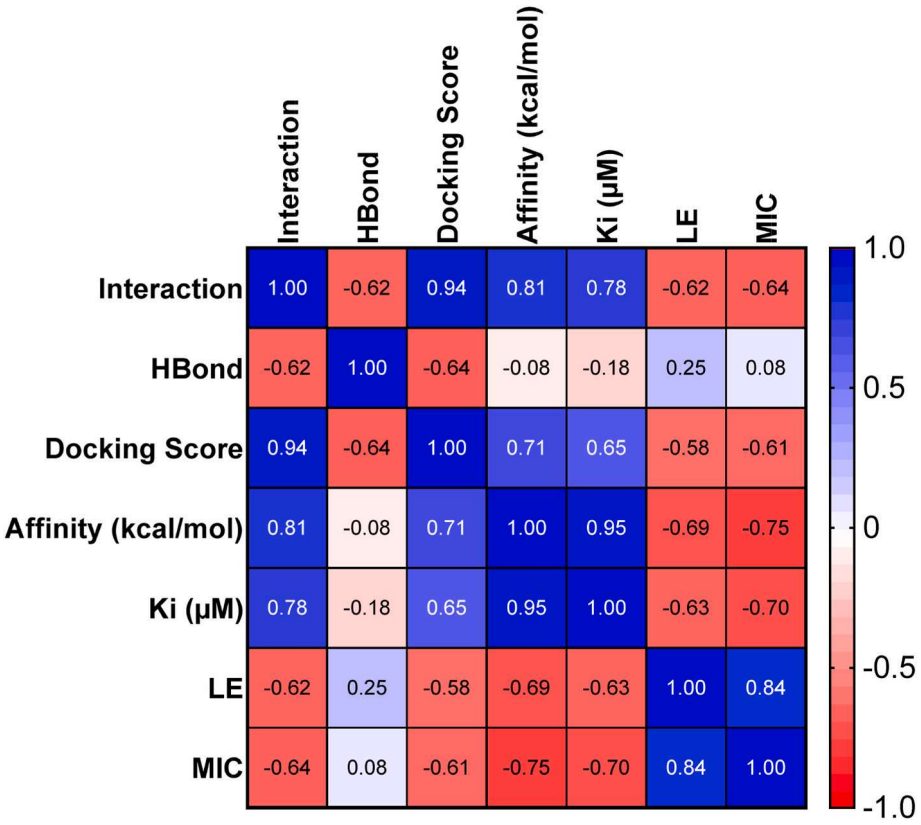


Fig. 2. Pearson's correlation between the MIC (in vitro assay) and docking parameters.

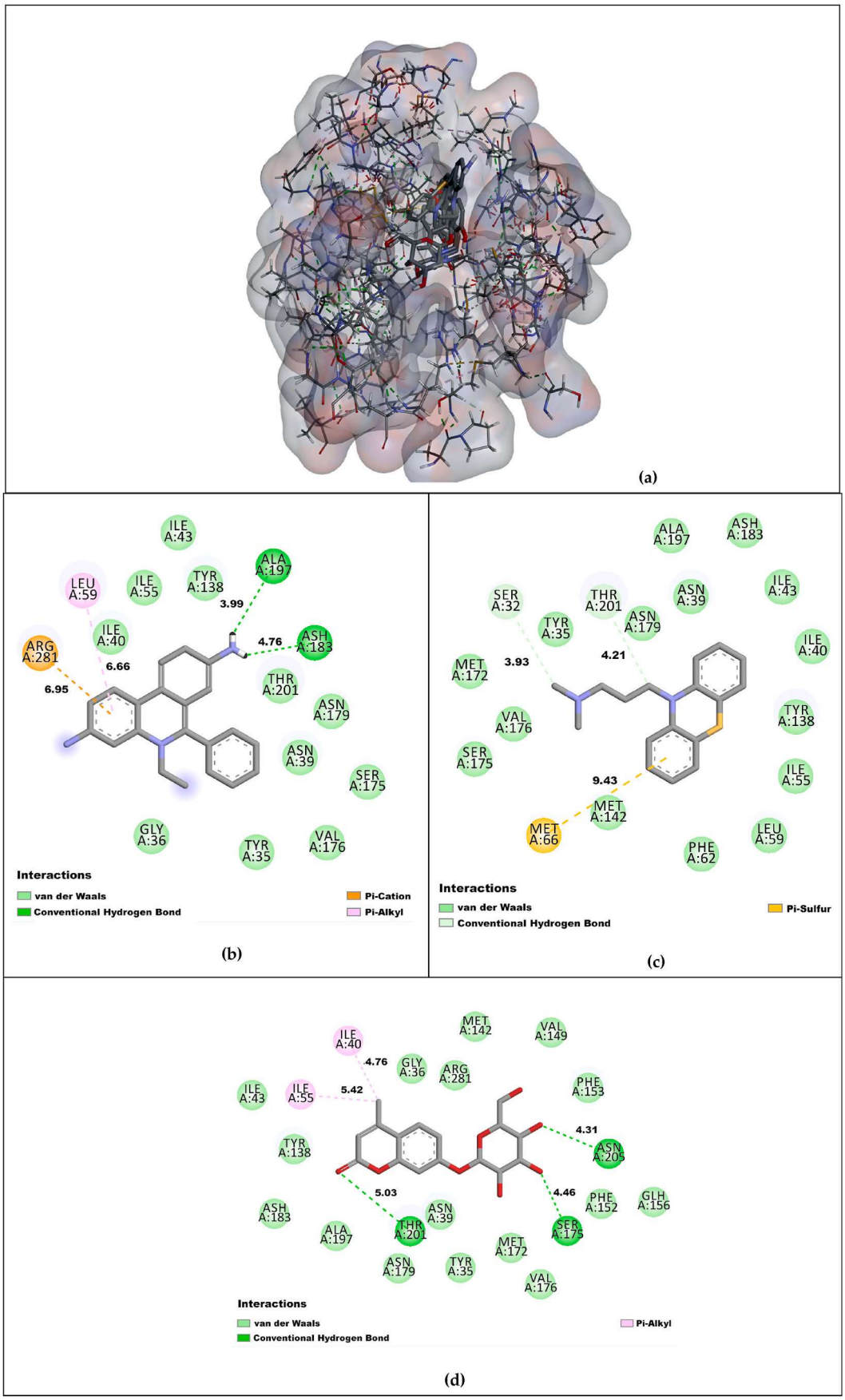


Fig. 3. Schematic diagram of ligand-receptor complexes (a). Ethidium Bromide (b) and Chlorpromazine (c) and Derived Coumarin C11(d) 2D docking interactions maps.

the K+ flux through the *S. aureus* membrane, inducing structural changes in the cell wall, or interfering with the H + -dependent energy supply to the bacterial efflux pump [41,44,45]. We observed that coumarins C10 (7-(Allyloxy)-4-methyl-2H-chromen-2-one), C11 (7-(alpha-D-Galactopyranosyloxy)-4-methyl-2H-1-benzopyran-2-one), C13 (8-Acetyl-7-hydroxy-4-methyl coumarin), and C14 (7-Hydroxy-4-methyl-2H-chromen-2-one) showed antibiotic-enhancing properties comparable and even superior to that observed for chlorpromazine in the ethidium bromide test.

The molecular docking analysis demonstrated that the C11 compound showed a promising MepA inhibitory capacity in silico. It was found that the hydrophobic interaction mediates the affinity of this coumarin to the pump between the coumarin ring and nonpolar isoleucine residues, which causes the stabilization of the coumarin ring by hydrophobic forces [40]. In addition, the interaction between the carbonyl oxygen of the coumarin ring forming a hydrogen bond with the hydroxyl group could also be mediated by a polar hydrogen bond between the threonine amino acid and the carbonyl group of coumarin, as well as by the polar interaction between the sugar moiety present in the compound and the amino acid asparagine, through hydrogen bonding.

Coumarins appear to possess interesting potential in modulating the effects of antibiotics. A previous study with the same tested coumarin derivatives demonstrated that compound C13 was able to exhibit intrinsic antibacterial activity against multidrug-resistant strains of *S. aureus* 10 and *E. coli* 06. However, although the other tested coumarin compounds did not possess antibacterial activity on their own, they were able to enhance the effects of the antibacterials gentamicin and norfloxacin against both strains. The exception was the combination of compound C11 with norfloxacin against the *S. aureus* 10 strain, which showed indifference to the MIC value. Nonetheless, it significantly reduced the MIC of gentamicin for the same bacterial strain [46]. Studies evaluating the modulatory activity of coumarins against *S. aureus* (MRSA) demonstrated that these compounds were able to enhance the activity of levofloxacin and gentamicin [47–50].

Despite the shortage of studies reporting the role of coumarin derivatives as efflux pump inhibitors, Araújo et al. (2016) demonstrated that these compounds are effective in modulating the activity of antibiotics *S. aureus* strains expressing efflux pumps such as NorA, MsrA, and TetK), suggesting that coumarin-derived compounds can interact with efflux pumps, reducing bacterial resistance to several antibiotics [51].

In contrast with the ethidium bromide test, only the coumarin derivative C14 (7-Hydroxy-4-methyl-2H-chromen-2-one) reduced the MIC of ciprofloxacin and, as such, inhibited the MepA-mediated antibiotic resistance in the SA-K2068 strain of *S. aureus*. Curiously, the coumarins C9, C10, C11, and C13, which showed synergistic effects in association with ethidium bromide, presented antagonistic effects when combined with the fluoroquinolone antibiotic ciprofloxacin, showing no relevant impact on antibiotic resistance.

Several studies have demonstrated the antibiotic-modulating activity of natural products against MepA-expressing strains. Costa (2021) demonstrated that achardol synergistically interacted with ethidium bromide against *S. aureus* K2068 although it was not able to reduce the MIC of ciprofloxacin [38]. Similar findings were observed from the association of carvacrol with ethidium bromide and conventional antibiotics against the strain IS-58 of *S. aureus* [52]. On the other hand, limonene [53], chalcone (2E)-1-(4′-aminophenyl)-3-(phenyl)-prop-2-en-1-one [54,55], and 1,8-naphthyridines sulfonamides [56] showed synergistic effects in association with both ciprofloxacin and ethidium bromide against the same strain. Thus, it is possible to observe an absence of uniformity in the performance of new drug candidates as efflux pump inhibitors and bacterial resistance modulators.

Concerning antagonistic and synergistic interactions between efflux pump inhibitors and antibiotics, some mechanisms have been proposed, including change in membrane permeability to the antibiotic, chelation of efflux pump cofactors [57] or antibiotic functional groups, and inhibition of signaling pathways involved in efflux pump expression [58] among others. However, further studies are needed to elucidate the mechanisms underlying the effects of coumarin derivatives on antibiotic resistance.

4. Materials and methods

4.1. Drugs and strains

This study used the strain SA-K2068 of *Staphylococcus aureus*, which encodes the MepA efflux pump. This protein is primarily responsible for the multidrug resistance (MDR) phenotype in this mutant strain and the extrusion of a variety of substances, including hydrophilic fluoroquinolones and other drugs [14,16]. The strain was cultured overnight at 37 °C in Heart Infusion Agar slants (HIA, Disco) before use [59].

The standard antibiotic ciprofloxacin was initially dissolved in DMSO and later diluted in water 1024 μg/mL. Ethidium bromide was dissolved in water and prepared at a concentration of 1024 μg/mL. These substances were obtained from SIGMA Chemical Co. (St. Louis, USA).

4.2. Obtention of coumarins

The coumarins evaluated in this study were synthesized and provided by the Pharmaceutical Chemistry Research Laboratory (LQFar), Faculty of Pharmaceutical Sciences, Federal University of Alfenas (UNIFAL-MG).

These compounds (Table 2) were obtained following classical protocols of organic synthesis, as previously described by Martin [46].

4.3. Molecular docking

The 3D structure and identification of potential efflux binding pockets of MepA were performed following the study by Ref. [56]. All chemical structure coordinates were generated using CORINA, and Gasteiger partial charges were determined. For the docking procedure, which was carried out using the Autodock Vina [60] and Molegro Virtual Docker (MVD) program [61]. The rigid docking procedure was carried out using Autodock Vina with a grid box defined as a 20Åx20Åx20 Å box around the geometrical center of the model structure. The default settings of Autodock Vina with the number of docking runs and the number of solutions obtained were set at 50 runs and conformations, respectively. The number of output conformations was assigned to one. The flexible docking runs were carried out using Molegro with grid coordinates of 5 Å the geometrical center of the best-predicted compound (coordinate x = −29.78, y = 49.65, z = 71.78, and box size with x = 46.00, y = 38.00 and z = 30.00). The protocols used in the docking procedure contemplate the MolDock optimizer as a search algorithm; runs were set to 10, the maximum population size of 50, the ultimate iteration of 2000 with a scaling factor of 0.50, and a crossover rate of 0.90. The docking results were viewed with the help of the UCSF Chimera visualization program and the Discovery studio (DS) was utilized for generation of a map of interaction detail.

4.4. Minimum inhibitory concentration (MIC) determination

The minimum inhibitory concentration (MIC) was determined to assess the antibacterial activity of the substances. For this purpose, 100 μL of bacterial inoculum, prepared in saline solution at a turbidity corresponding to 0.5 on the McFarland scale, was added to 900 μL of brain heart infusion (BHI) in microtubes. The solutions were then transferred to 96-well plates, followed by the addition of compounds at concentrations ranging from 0.5 to 512 μg/mL. The plates were incubated at 37 °C for 24 h, and bacterial growth was evaluated using Resazurin. The MIC was defined as the lowest concentration capable of inhibiting bacterial growth, according to the CLSI (2013) [62]. The antibiotic ciprofloxacin was used as positive control. Antibacterial assays were performed in triplicate, and results were expressed as the mean

geometric of replicates. Due to the technique of microdilution being based on a serial dilution by geometric progression (from 512 to 0.5 μg/mL), the MIC results were expressed as geometric means.

4.5. Efflux inhibition assay

The coumarin derivatives C9, C10, C11, C13, and C14, were combined with the efflux pump substrate ethidium (EtBr) and the antibiotic ciprofloxacin to evaluate their ability to modulate the MepA-mediated drug efflux in *S. aureus*. To this end, the compounds were added to the wells at the concentration of 128 μg/mL (1/8 MIC), and the bacterial suspension was prepared at the previously described conditions. Then, the wells were added with 100 μL of the efflux pump substrates at concentrations ranging from 0.5 to 512 μg/mL. The plates were incubated at 37 °C for 24 h, and the MICs of ciprofloxacin and ethidium bromide (EtBr) were assessed in a resazurin assay as previously described. Chlorpromazine was used as a standard efflux pump control. Wells were used as positive controls without adding the derivatives or chlorpromazine. Growth controls were obtained using wells containing the inoculum without drug treatment [63].

4.6. Statistical analysis

All results represented two independent experiments performed in triplicate and expressed as geometric means. A two-way ANOVA followed by Bonferroni's post hoc test was used for the statistical analysis using the GraphPad Prism 9.0 software.

5. Conclusions

In this study, an in silico molecular docking assay demonstrated that the hypothesis of possible interactions between the coumarins derivative and with MepA efflux pump was corroborated with a reduction of MIC observed for all compounds on the antibacterial assay indicating the interference in the efflux machine. The present model is reliable for the drug design and synthesis of novel candidates exploring the chemical space observed in compounds C9, C11, and C13 that showed the most favorable interaction with the MepA efflux pump. The in vitro study showed that all coumarin derivatives studied reduced the minimum inhibitory concentration of ethidium bromide. Against the SA-K2068 strain of *Staphylococcus aureus.* However, only the C14 coumarin produced synergic effects when associated with ciprofloxacin, and such has the potential to be targeted in antibiotic-resistance drug development.

Nevertheless, due to the present in silico and in vitro investigation limitations, further experimental research is required to confirm the therapeutic potential of this compound in vivo.

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CRediT authorship contribution statement

Ana Luíza A.R. Martin: Conceptualization. **Raimundo Luiz Silva Pereira:** Methodology. **Janaína Esmeraldo Rocha:** Methodology. **Pablo A.M. Farias:** Formal analysis. **Thiago S. Freitas:** Software. **Francisco Rodrigo de Lemos Caldas:** Investigation. **Fernando G. Figueredo:** Investigation. **Nadghia Figueiredo Leite Sampaio:** Methodology. **Jaime Ribeiro-Filho:** Writing – original draft. **Irwin Rose de Alencar Menezes:** Software. **Guilherme Andrade Brancaglion:** Methodology. **Daniela Carvalho de Paulo:** Formal analysis. **Diogo T. Carvalho:** Resources. **Micheline Azevedo Lima:** Project administration. **Henrique D.M. Coutinho:** Project administration. **Marta M.F. Fonteles:** Supervision.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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