Biomedicine & Pharmacotherapy 179 (2024) 117291

Contents lists available at ScienceDirect

Biomedicine & Pharmacotherapy

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

Thiazine-derived compounds in inhibiting efflux pump in *Staphylococcus aureus* K2068, *mepA* gene expression, and membrane permeability alteration

Priscilla R. Freitas [a], Ana C.J. de Araújo [a], Isaac M. Araújo [a], Ray S. de Almeida [a], João A.O. Borges [a], Cícera L.R. Paulo [a], Cícera D.M. Oliveira-Tintino [a], Gustavo M. Miranda [b], José B. Araújo-Neto [c], Igor J.S. Nascimento [d], João X. Araújo-Júnior [d], Julia M.A. Silva [e], Tereza C.L. Balbino [e], Edeildo F. Silva-Júnior [f], Thiago M. Aquino [f], Francisco J.B. Mendonca-Junior [g], Emmanuel S. Marinho [h], Hélcio S. Santos [h], Clara Mariana Gonçalves Lima [i], Ahmad J. Obaidullah [j], Talha Bin Emran [k,l,m], Francisco A.B. Cunha [a], Irwin R.A. Menezes [a], Saulo R. Tintino [a], Henrique D.M. Coutinho [a,*]

[a] *Department of Biological Chemistry, Universidade Regional do Cariri, Crato-CE, Brazil*
[b] *Instituto Gonçalo Moniz (IGM) - Fiocruz Bahia, Brazil*
[c] *Postgraduate Program in Biological Sciences, Biosciences Center, Federal University of Pernambuco, Recife, PE 50740-570, Brazil*
[d] *Laboratory of Medicinal Chemistry, Institute of Pharmaceutical Sciences, Federal University of Alagoas, Maceió, AL 57072-900, Brazil*
[e] *Department of Microbiology, Aggeu Magalhães Institute, Oswaldo Cruz Foundation (Fiocruz), Recife, Brazil*
[f] *Biological and Molecular Chemistry Research Group, Institute of Chemistry and Biotechnology, Federal University of Alagoas, Maceió, AL 57072-900, Brazil*
[g] *Laboratory of Synthesis and Drug Delivery-LSVM, Paraiba State University, João Pessoa, PB 58071-160, Brazil*
[h] *Laboratory of Chemistry of Natural and Synthetic Product, State University of Ceará, UECE, Fortaleza, CE 60741-835, Brazil*
[i] *Department of Food Science, Federal University of Lavras, Lavras, Minas Gerais CEP 37200-900, Brazil*
[j] *Department of Pharmaceutical Chemistry, College of Pharmacy, King Saud University, P.O. Box 2457, Riyadh 11451, Saudi Arabia*
[k] *Department of Pathology and Laboratory Medicine, Warren Alpert Medical School, Brown University, Providence, RI 02912, USA*
[l] *Legorreta Cancer Center, Brown University, Providence, RI 02912, USA*
[m] *Department of Pharmacy, Faculty of Allied Health Sciences, Daffodil International University, Dhaka 1207, Bangladesh*

ARTICLE INFO

Keywords:
Bacterial resistance
Mechanism of action
MepA efflux pump

ABSTRACT

Staphylococcus aureus is a bacterium responsible for resistance to multiple drugs and the efflux system is widely studied among the resistance mechanisms developed by this species. The present study evaluates the inhibition of the MepA efflux pump by thiadiazine-derived compounds. For this purpose, thiadiazine-derived compounds (IJ-14 to IJ-20) were tested against *S. aureus* K2068 strains. Microdilution tests were initially conducted to assess the Minimum Inhibitory Concentration (MIC) of the compounds and their efflux pump inhibition activity. In addition, fluorimetry tests were performed using BrEt emission and tests were conducted to inhibit the expression of the mepA gene. This involved comparing the bacterial gene expression with the antibiotic alone to the gene expression after combining compounds (IJ-17 and IJ-20) with the antibiotic. Furthermore, membrane permeability assessment tests and *in silico* molecular docking tests were performed. It was observed that the IJ17 and IJ20 compounds exhibited direct activity against the tested strain. The IJ17 compound produced significant results in the gene inhibition tests, which was also evidenced through the membrane permeability alteration test. These findings suggest that thiadiazine-derived compounds have promising effects against one of the main resistance mechanisms, with the IJ17 compound presenting observable mechanisms of action.

* Corresponding author.
E-mail addresses: aobaidullah@ksu.edu.sa (A.J. Obaidullah), talha_bin_emran@brown.edu (T. Bin Emran), hdmcoutinho@gmail.com (H.D.M. Coutinho).

https://doi.org/10.1016/j.biopha.2024.117291

Available online 14 August 2024

1. Introduction

Infections caused by antibiotic-resistant bacteria have become a problem today due to the high levels of pathologies associated with these microorganisms [1]. In this scenario, the large number of infections is associated with the development of multi-resistant bacteria, among which it is possible to highlight the *Staphylococcus aureus* species (*S. aureus*) [2].

There are different types of bacterial resistance mechanisms, such as inactivation through enzymes, alteration of membrane permeability, modification of the drug binding target and the efflux mechanism [3]. The efflux system is specifically an important factor which results in drug ineffectiveness, and through this an extrusion of antibiotics from the bacterial cell occurs. This mechanism can be specific to a given antibiotic or even cause the withdrawal of different types of antibacterials, which can be called the multi-drug resistant (MDR) efflux system [4,5].

The efflux system occurs through the activity of proteins located in the membrane of bacterial cells which actively transport antibiotics through ATP hydrolysis to the extracellular environment [6]. In this context, there are different types of efflux pumps, including the MepA efflux pump expressed through the *mepA* gene and responsible for the extrusion of fluoroquinolas and quaternary ammonium compounds [7]. Given its clinical importance, the search for new agents which act to inhibit the efflux pump of *S. aureus* strains has significantly increased [8].

Furthermore, heterocyclic compounds, such as thiadiazines, are an important class of compounds used for developing therapeutic agents due to their biological activity [9]. According to the literature, thiadiazines and their analogues demonstrate antifungal, antiparasitic and antibacterial activities [10–13]. On the other hand, there are still no reports in the literature demonstrating the activity of these compounds inhibiting the MepA efflux pump and their activity against the *S. aureus* K2068 bacterial strain.

With the aim of finding new products for bacterial resistance cases related to the efflux pump mechanism, the present study aims to evaluate the inhibition of the MepA efflux pump, the inhibition of the *mepA* gene, and the assessment of membrane permeability alteration by thiadiazine-derived compounds against the *S. aureus* K2068 bacterial strain.

2. Results

2.1. Antibacterial activity

Only the IJ17 and IJ20 compounds showed antibacterial action against the S. Aureus K2068 strain in performing the MIC of the synthesized compounds, with an MIC of 256 μg/mL and 768 μg/mL, respectively. The IJ14, IJ15, IJ16, IJ18 and IJ19 compounds presented an MIC ≥1024 μg/mL, showing that they did not present direct antibacterial activity against the tested strain.

2.2. Analysis of antibacterial activity through efflux pump inhibition

Efflux pump inhibition tests were carried out from the data obtained from the MIC using the microdilution method, as described in the methodology, where the value of "n" used corresponds to 3. EtBr was used to evaluate the efflux pump expression, and the antibiotic Ciprofloxacin in association with each compound, in addition to CCCP being used as a pump inhibitor.

The associations made between the compounds and ethidium bromide demonstrate that all products had significant activity, acting to reduce the MIC of ethidium bromide. The IJ20 compound deserves to be highlighted, as it reduced the MIC from 50.8 μg/mL (SD = 1,49), indicating that the standard deviation is 1,49, to 0.57 μg/Ml (SD = 1), achieving a lower MIC than that of the CCCP inhibitor, which was 8 μg/mL (SD = 1). In addition, the IJ14, IJ16 and IJ17 compounds presented an MIC of 3.17 μg/mL, and the IJ15 compound presented an MIC of 5.03 μg/Ml (SD = 1,49), thereby showing a greater reduction in the MIC of ethidium bromide than that shown by associating ethidium bromide and the inhibitor used, as seen in Fig. 1.

The IJ18 and IJ19 compounds associated with ethidium bromide also showed significant activity, in which a reduction in the minimum inhibitory concentration of ethidium bromide was observed to 25.39 μg/mL and 32 μg/mL for each of the associations, respectively (Fig. 1).

The analysis performed associating Ciprofloxacin and compounds derived from thiadiazine also demonstrated a reduction in the MIC of the antibiotic, as shown in Fig. 2. This was evidenced through the antibiotic's MIC of 16 (SD = 1) being reduced when associated with the IJ14, IJ15, IJ17 and IJ20 compounds with an MIC of 3.17, 6.24, 3.17 and 4 μg/mL, respectively.

2.3. Assessment of inhibition of MepA efflux pumps by fluorescence emission

In evaluating the fluorescence emission in the S. aureus K2068 strain, it was observed that all compounds were able to increase the fluorescence emission of EtBr compared to the negative control, as shown in Fig. 3.

2.4. mepA quantitative real-time PCR

From the implementation of the described methodology and the calculation of ΔΔCT, it was observed that the IJ20 compound did not inhibit the expression of the mepA gene. However, the IJ17 compound showed a gene expression inhibition of approximately 3702 times when compared to the relative value of the antibiotic control, as shown in Fig. 4. The control indicated by preparation 2, containing only culture medium and bacterial inoculum, did not show expression of the mepA gene used.

2.5. Assessment of membrane permeability alteration

In terms of evaluating membrane permeability alteration, it was observed that the IJ17 compound showed significant results across all tested concentrations, allowing the entry of the Sytox Green dye used for fluorescence assessment. On the other hand, the IJ20 compound did not exhibit significant results in all three condition variations compared to the bacteria and Sytox Green control, as shown in Fig. 5.

2.6. Molecular docking

The IJ17 compound showed the best binding energy (-7.68 Kcal/mol) and inhibition constant (2.36 μM) in docking with mepA, followed by IJ20, which also showed significant results (-7.46 Kcal/mol and 3.42 μM, respectively). The affinity of the other ligands with the analyzed efflux pump followed the order: IJ14 > IJ16 > IJ15 > IJ18 > IJ19 > CCCP. CCCP was tested as a reference inhibitor, and showed the best evaluation only in the ligand efficiency parameter (Table 1).

The interactions of IJ17 with *mepA* are divided into conventional hydrogen bonding (Gln380 – 2.10 Å), carbon-hydrogen bonding (Phe373 – 3.77 Å), π-sulfur interaction (Cys294 – 4.60 Å), π-π stacked interaction (Phe373 – 4.37 Å), π-alkyl interaction (Ala247 – 4.47 Å), and van der Waals interactions (≅ 5.00 Å) (Fig. 6).

The residues with which IJ20 interacted indicate that its best position and that of IJ17 occur in a similar region of the MepA protein. Conventional hydrogen bonds were identified (Gln380 – 2.09 Å, Gly67 – 1.85 Å, Met66 – 3.09 Å), carbon-hydrogen bond (Phe373 – 3.33 Å), attractive charge (Glu295 – 5.14 Å), π-π stacked interaction (Phe373 – 4.43 Å), π-alkyl interaction (Ala247 – 4.64 Å, Cys294 – 4.65 Å), and van der Waals interactions (Fig. 7).

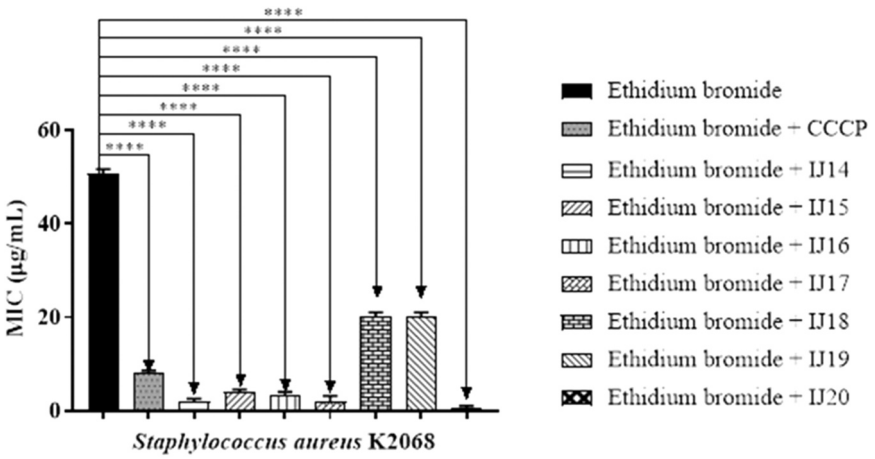

Fig. 1. Activity of thiadiazine-derived compounds associated with EtBr against *Staphylococcus aureus* K2068 strains. (n=3).“****” $p < 0.0001$ indicates significant differences between groups. Statistical significance was determined by one-way ANOVA and Bonferroni posthoc test.

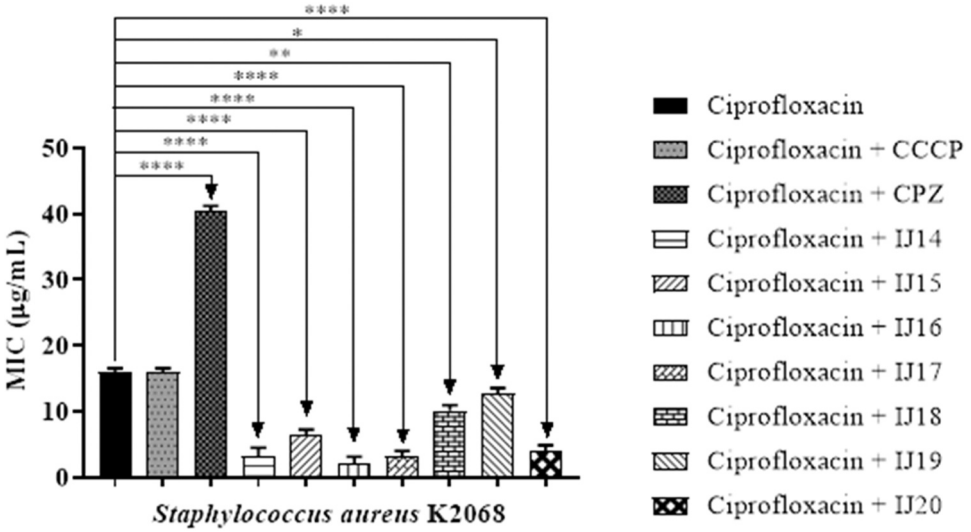

Fig. 2. Activity of thiadiazine-derived compounds associated with Ciprofloxacin against Staphylococcus aureus K2068 strains. “**” p 0,0034 indicates significant differences between groups. Statistical significance was determined by one-way ANOVA and Bonferroni posthoc test. (n=3).“*” p 0,0481 indicates significant differences between groups. Statistical significance was determined by one-way ANOVA and Bonferroni posthoc test.

3. Discussion

From the obtained results, it is possible to highlight that this is the first study on compounds derived from thiadiazine in which the inhibition of the *mepA* efflux pump is evaluated, as well as its direct action on the *S. aureus* K2068 bacterial strain.

In relation to thiadiazine analogues, a study by Araújo et al. [13] presents synthesized analogues and demonstrates their direct antibacterial activity by performing MIC against multi-resistant strains of *S. aureus* and *Pseudomonas aeruginosa*.

In evaluating the inhibition of the efflux system activity, it was possible to observe that all compounds have inhibition activity, which was observed in the microdilution tests and consolidated through fluorimetry. Considering that EtBr is an intercalating nucleic acid agent, greater fluorescence occurs when it is bound to DNA. The intracellular concentration of EtBr increases through the inhibition of efflux pumps, and consequently increases its binding to DNA and its fluorescence intensity. Therefore, the increase in fluorescence in the groups treated with IJ14–20 thiadiazine derivatives indicates that the *mepA* efflux pump was possibly inhibited [14–16].

Such action is also observed through the use of other synthetic products, such as those observed through the activity of limonene against another *S. aureus* bacterial strain (RN4220) which has another *mepA* efflux pump, in which the compound also showed the ability to inhibit the efflux system observed through increased fluorescence emission [17].

The activity of the compounds against the efflux mechanism can occur through gene expression inhibition, direct blocking of the efflux pump, or using compounds that competitively bind to the protein to prevent drug action. Additionally, alteration in structural integrity and membrane permeability may be indirectly related to the action of efflux pump inhibitors [18–20].

In addition to analyzing the inhibition of the *mepA* efflux pump, potential mechanisms of action of the IJ17 and IJ20 compounds were

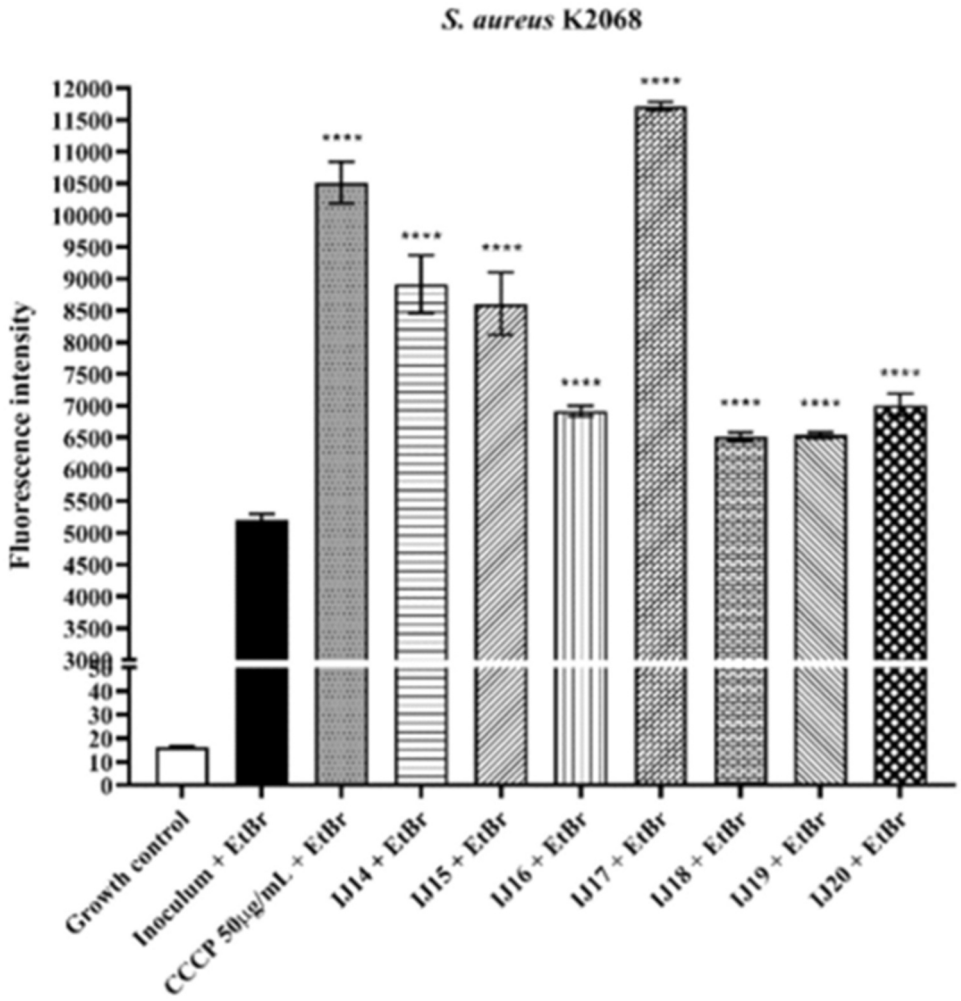

Fig. 3. Evaluation of MepA efflux pump inhibition by IJ14, IJ15, IJ16, IJ17, IJ18, IJ19, and IJ20 derivatives (50 µg/mL). Assessed by fluorescence emission. *S. aureus* K2068 strain carrying MepA efflux pump. (n=3) "****" = $p < 0.0001$ vs inoculum + EtBr.

evaluated. Since there are no reports in the literature regarding the action of thiadiazines or their derivatives in gene inhibition, it was observed that IJ17 directly inhibited gene expression among the tested compounds. Then in the assays, it was observed that the control containing only culture medium and bacterial inoculum did not express the studied gene, *mepA*. This can be explained considering that *mepA* efflux proteins are not constitutively expressed; thus, their expression occurs in response to the presence of antibiotics [21].

Furthermore, the analysis of the assessment of membrane permeability alteration by these compounds revealed that another mechanism of action is involved with the IJ17 compound: alteration of membrane permeability, demonstrated by Sytox Green emission and fluorescence uptake. This can be explained because Sytox Green does not have the ability to cross the cell membrane when it is intact; however, when alterations occur, this compound is able to penetrate the cell and bind to genetic material, allowing its uptake and subsequent fluorescence emission [22,23].

Radchenko et al. [24] describe how specific substrate-binding residues in the DinF-BH and NorM-NG transporters, members of the multidrug and toxic compound extrusion (MATE) superfamily, and similar to *mepA*, can be important for efflux inhibition. In line with the docking results, this analysis demonstrates the predominant role of hydrophobic residues (e.g., alanine, phenylalanine, and methionine), followed by uncharged polar residues (i.e. glutamine).

We additionally observed interactions of IJ17 and IJ20 with Glu295, an acidic (or negatively charged) residue. MATE efflux pumps have conserved acidic residues, which indicates their participation in the transport of Na+ or H+ ions, providing the necessary energy for the transporters function [25]. These preliminary data show that the inhibition of *mepA* by thiadiazine-derived compounds can occur through both competition with substrates and by energetic disruption.

These differences in the mechanism of action between the two evaluated compounds may arise due to their structural differences. The IJ17 compound, N-benzyl-5-(3-chlorophenyl)-6 H-1,3,4-thiadiazine-2-amine, contains the 3-Cl radical, while the IJ20 compound, N-benzyl-5-(4-hydroxyphenyl)-6 H-1,3,4-thiadiazine-2-amine, has the 4-OH radical added to its composition. Thus, even though the compounds have similar structures and derive from the same compound, it does not indicate that they will bind to the same target to carry out their action. Furthermore, this factor can be explained by the added radical for the synthesis of the IJ17 compound, where chlorine is capable of promoting alteration in membrane permeability [26–28].

4. Materials and methods

4.1. Obtainment of materials

The chemical structures were synthesized through an initial mixture of thiosemicarbazone (1 mmol) and 2-bromoacetophenone (1 equiv) in methanol, where the compounds (IJ14, IJ15, IJ16, IJ17, IJ18, IJ19, and IJ20) were obtained by adding radicals to each compound, as described by Freitas et al. [26].

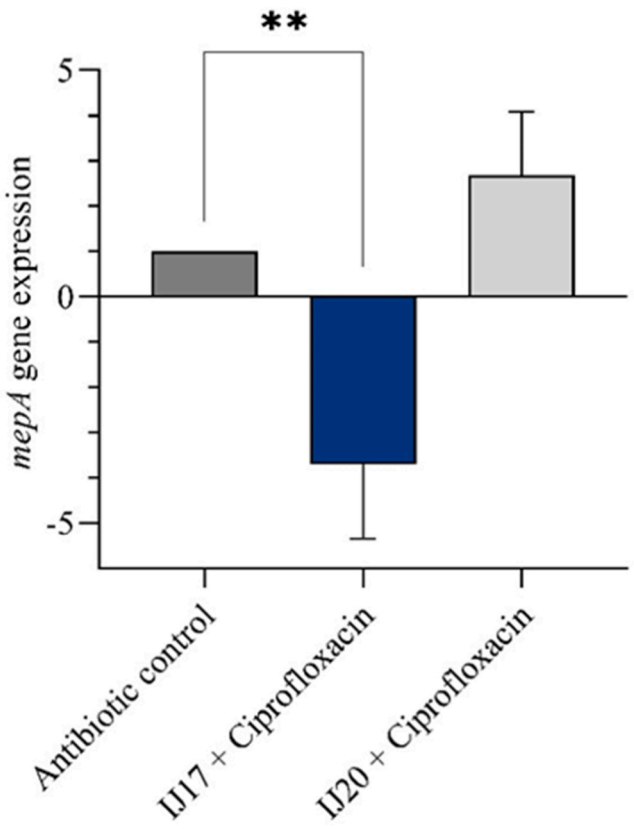

Fig. 4. Evaluation of the expression of the mepA gene in the presence of thiadiazine-derived compounds (IJ17 and IJ20) in combination with the antibiotic compared to the growth control. (n=3) "**" p 0,0317 indicates significant differences between groups. Statistical significance was determined by one-way ANOVA.

4.2. Microorganisms and inoculum preparation for microdilution tests

The *S. aureus* (SA) K2068 bacterial strain was used to carry out the tests, provided by Prof. S. Gibbons of the University of London. The SA K2068 strain expresses the *mepA* efflux pump, responsible for the extrusion of multiple drugs, among which hydrophilic fluoroquinoles can be highlighted.

The bacterial strains which were initially kept under refrigeration were cultured in Petri dishes containing Mueller Hinton Agar culture medium and incubated at 37ºC for a period of 24 hours. After breeding, a portion of the bacteria was used to prepare the bacterial inoculums for the microdilution tests. These were done in triplicate in test tubes containing sterile saline solution until reaching 0.5 on the McFarland scale, which corresponds to 1.5 $\times 10^8$ Colony-Forming Units/mL. Then, an inoculum was prepared in Phosphate buffered saline (PBS) until reaching 0.5 on the McFarland scale to test the inhibition of efflux pumps by EtBr fluorescence emission after spiking.

4.3. Preparation of the substances

Ethidium Bromide (EtBr), Carbonyl Cyanide m-Chlorophenylhydrazone (CCCP) and the antibiotic Ciprofloxacin were used for the tests, with all of these products being purchased from Sigma Aldrich, Louis, USA. In addition to the products used, the IJ14–20 substances were also diluted to carry out the tests.

The substances used were prepared to obtain an initial concentration of 1024 μg/mL. Therefore, 10 mg of each substance was initially weighed to dilute the products. For ethidium bromide, the heavy material was diluted in sterile distilled water, the CCCP was diluted in a solution prepared with 50 % methanol and 50 % sterile distilled water and the antibiotic, and finally 500 μL of dimethyl sulfoxide (DMSO) and

Table 1
Values recorded in the molecular docking with the MepA efflux pump.

Ligands	Binding Energy[1]	Inhibition Constant[2]	Ligand Efficiency[3]
IJ14	−7.30	4.48	−0.37
IJ15	−7.25	4.83	−0.35
IJ16	−7.27	4.67	−0.33
IJ17	−7.68	2.36	−0.37
IJ18	−6.95	8.00	−0.33
IJ19	−6.75	11.18	−0.31
IJ20	−7.46	3.42	−0.36
CCCP	−6.42	19.82	−0.46

[1] Expressed in Kcal/mol. [2] Expressed in μM. [3] Binding energy ÷ heavy atoms.

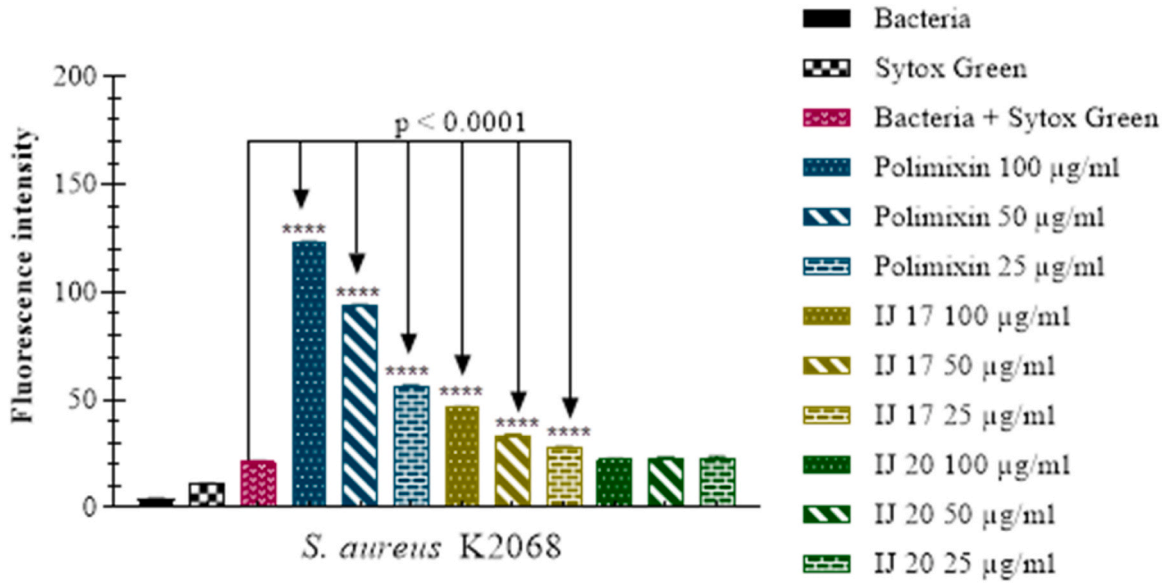

Fig. 5. Membrane permeability assessment using Sytox Green in combination with polymyxin antibiotic (positive control), IJ17, and IJ20 (test substances) against S. aureus K2068 strains. (n=3) $p < 0.0001$ indicates significant difference between groups. Statistical significance was determined by one-way ANOVA and Bonferroni post-hoc.

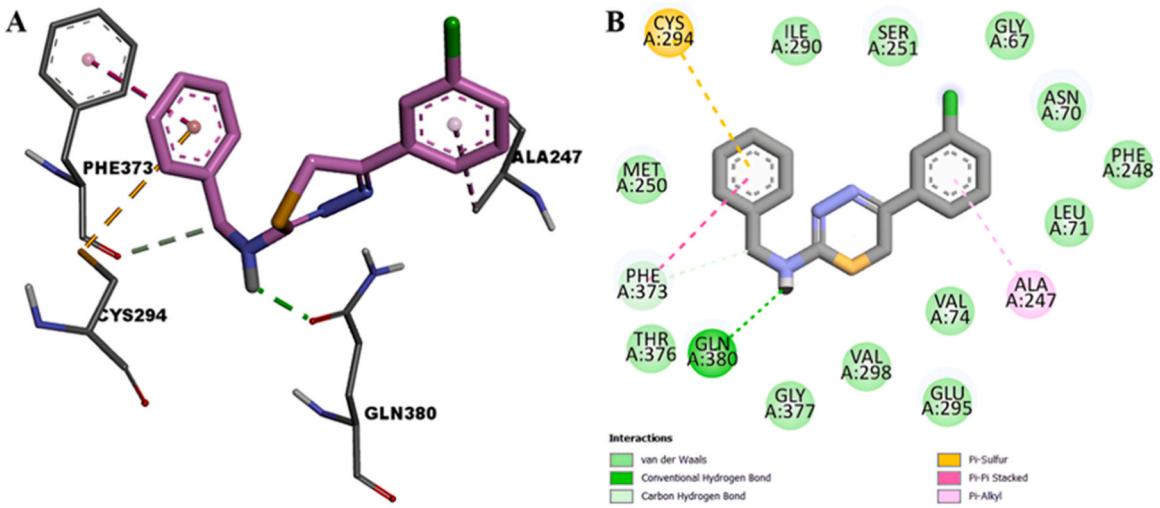

Fig. 6. Best pose of IJ17 (A) and interactions with MepA efflux pump (B).

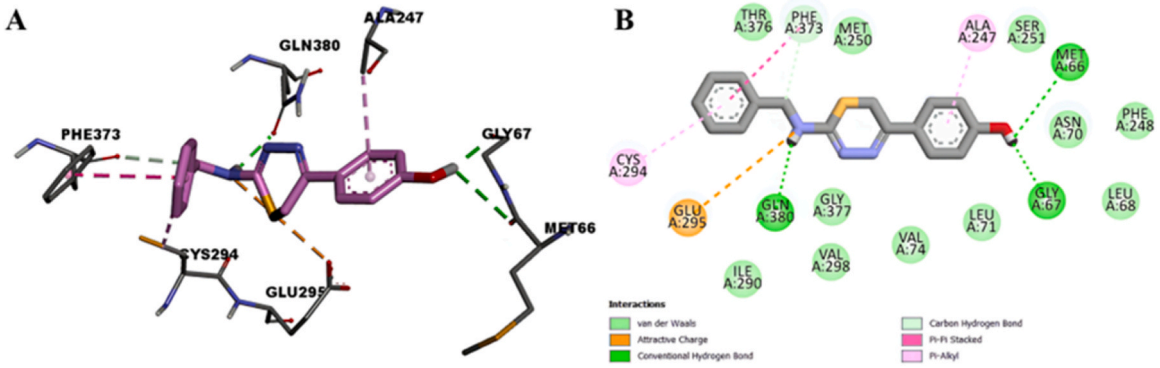

Fig. 7. Best pose of IJ20 (A) and interactions with MepA efflux pump (B).

9265 μL of sterile distilled water were added to each substance (IJ14–20) used in order to obtain the initial concentration.

4.4. Determination of the Minimum Inhibitory Concentration (MIC)

The Minimum Inhibitory Concentration test was conducted from the initial preparation of solutions in sterile microtubes with 900 μL of BHI culture medium prepared at a concentration of 10 %, and 100 μL of bacterial inoculum, a volume which corresponds to 10 % of the volume total of 1000 μL. After preparing the microtubes, 100 μL was transferred to sterile 96-well microdilution plates, and then 100 μL of each tested product was microdiluted until the penultimate well, resulting in a variation of 512 μg/mL to 8 μg/mL, considering the last well as a positive control for the test. In addition to this control, a negative control containing only 10 % BHI culture medium was also prepared. After filling the plates, they were incubated in an oven at 37°C for 24 hours. Next, 20 μL of sodium resazurin was added after this period and kept for a period of 1 hour at room temperature to observe bacterial growth. The entire test was performed in triplicate [29].

4.5. Assessment of antibacterial activity through efflux pump inhibition

The antibacterial activity through inhibition of the *mepA* efflux pump was evaluated by obtaining the MIC of the compounds [30,31]. A total volume of 1500 μL containing 10 % bacterial inoculum (the volume corresponding to the sub-inhibitory concentration - MIC/8), and the remaining volume of 10 % BHI was used to prepare the microtubes. Next, 100 μL of the solution was transferred to each well of the 96-well microdilution plates in numerical direction, and microdiluted with 100 μL of the antibiotic until the penultimate well, ranging from 512 μg/mL to 0.5 μg/mL. Microtubes were also prepared with the same conditions for microdilution with EtBr. Positive controls were used for the tests, and the last well of each column negative with 10 % BHI, and modulation controls for EtBr and the antibiotic containing only culture medium and inoculum in the prepared microtubes. The prepared plates were incubated at 37°C for 24 hours, and after this period a reading was carried out with sodium resazurin. The test was performed in triplicate.

4.6. Inhibitory action of mepA efflux pumps assessed by increased EtBr fluorescence emission

The IJ14–20 derivatives were all tested at a concentration of 50 μg/mL. A solution containing bacterial inoculum and thiadiazine derivatives was initially prepared. A positive control also containing CCCP at a concentration of 50 μg/mL, and a negative control with only PBS with inoculum were used. The solutions were subsequently incubated for 1h30min in an oven at 37°C. Then, EtBr was added at a concentration of 100 μg/mL to all solutions.

No EtBr was added in the growth control solution. Soon after, the solutions were then incubated for another 1 hour. After incubation, they were subjected to centrifugation at 10,000 rpm for 2 minutes and washed with PBS to eliminate excess EtBr. The resulting pellet was resuspended and distributed into black 96-well plates. Plate reading was performed using a Cytation 1 fluorescence microplate reader from

BioTek® (Winooski, VT, USA) and using the Gen5™ 3.11 software program. An excitation at 530 nm was applied and the emission wavelength of 590 nm was measured. The following groups were read: growth control; negative control containing the inoculum and EtBr; positive control, composed of the inoculum, EtBr and CCCP; and the test that contained the inoculum, EtBr and thiadiazine-derived products (IJ14–20) [32].

4.7. MepA quantitative real-time PCR

Three conditions were prepared in triplicates in conical tubes to evaluate the inhibition of *mepA* gene expression after breeding the bacteria, namely: 1 – control containing the volume corresponding to the MIC/8 of the antibiotic, a portion of bacteria and the remaining volume for 5 mL of solution BHI prepared according to the manufacturer; 2 – control containing the volume for 5 mL of solution BHI prepared according to the manufacturer and a portion of bacteria; 3-volume corresponding to the MIC/4 of the antibiotic, MIC/8 of the thiadiazine-derived compounds (IJ-17 and IJ-20), a portion of bacteria and the remaining volume for 5 mL of BHI solution prepared according to the manufacturer. The tubes were incubated during the period at 37ºC for 24 hours. After the incubation period, extraction was performed using Trizol (Thermo Fisher Scientific) and quantitative analysis was performed using Gen5TM. Complementary DNA was obtained from SuperScript™ VILO™ Master Mix (Thermo Fisher Scientific) according to the manufacturer's instructions. Gene expression was evaluated using the SYBR™ Green PCR Master Mix (Thermo Fisher Scientific) using the 23 s gene as endogenous and the qTowerG3 device from Analytik Jena and its qPCRsoft software program. Both primers had an annealing temperature of 61°C. The primers used are described in Table 2. The relative mepA gene expression was determined using the ΔΔCT method [33].

4.8. Assessment of membrane permeability

The Sytox Green addition methodology for fluorescence measurement was employed to assess membrane permeability, adapted from the procedure described by Mello et al. [36]. From this methodology, an inoculum of 1.0 ×10ˆ8 CFU were prepared and distributed in 96-well microdilution plates with a black coloration. The plates were then treated with polymyxin, used as the positive control, and also treated with the IJ17 and IJ20 compounds, all at concentrations of 100 μg/mL, 50 μg/mL and 25 μg/mL. After the transfer, the plates were incubated for a period of 1 hour, followed by the addition of 100 μL of 1 μM Sytox Green. The plates were then further incubated for an additional 1-hour period. Readings were subsequently taken using a BioTek® Cytation 1 microplate reader (Agilent, CA, USA) with a fluorescent filter set at an excitation wavelength of 485 nm and an emission wavelength of 528 nm. The entire assay was performed in triplicate.

4.9. Molecular docking

We used the SWISS-MODEL server (protein model construction) and the MarvinSketch version 23.14 (ligand construction), Open Babel 2.4.1 (energy minimization), AutoDock Tools version 1.5.7 (receptor and ligand preparation), and AutoDock version 4.2.6 (molecular docking) [37–39] software programs to perform the *in silico* analyses. The *mepA* model was built using the Q2G140 sequence (UniProt database) and the 3W4T template (Protein Data Bank). The selection of the best template for building the protein model (carried out by the SWISS-MODEL server) was validated by checking the parameters related to Ramachandran Plots, MolProbity Results (MolProbity Score, Clash Score, Ramachandran Favored, Ramachandran Outliers, Rotamer Outliers, C-Beta Deviations, Bad Bonds, Bad Angles, and Twisted Prolines/Non-Prolines), level of confidence of sequence alignment (protein model and template) and Quality Estimation (QMEAN-DisCo method). The MMFF94 force field was used in the energy minimization of the ligand molecules. Hydrogens (merging non-polar) and Gasteiger charges were added to the macromolecule and ligands, and the ligand bonds and residues 48, 81, 156, 161, 183, 291, 302, and 423 of the transporter were kept flexible [40].

A grid box (50 Å × 50 Å × 50 Å) was positioned at the center of the protein cavity (x: −28.441, y: 0.029, z: 11.606). The Genetic Algorithm (Lamarckian) was used in the docking process with the following parameters: 100 runs, 2500,000 evaluations, 27,000 generations, population size of 150, and RMSD (Root Mean Square Deviation) limit of 2.0 Å. The conformation with the lowest binding energy was selected for each ligand.

4.10. Statistical analysis

Data were expressed as geometric means, as mean ± standard deviation and evaluated with ANOVA analysis of variance, followed by Bonferroni's post-hoc test, with significant differences being considered at $p<0.05$. The tests regarding the expression assays for efflux pump genes were carried out in triplicate and relative gene expression was performed using the ΔΔC method. All analyzes were performed using the GraphPad prism 9.2.0 program.

5. Conclusions

The inhibition of the MepA efflux pump by thiadiazine-derived compounds was analyzed in detail in this study. These compounds yielded significant results, demonstrating the inhibition of these proteins when all tested compounds were evaluated through microdilution with ethidium bromide and confirmed by ethidium bromide fluorescence emission. Furthermore, it was possible to elucidate the mechanisms of action of the IJ17 compound through gene expression inhibition and alteration of membrane permeability. Such results have a significant contribution, considering this is the first study to describe the action of thiadiazine-derived compounds against strains of *Staphylococcus aureus* K2068, making them potential candidates for cases of bacterial resistance.

Funding

The authors extend their appreciation to the Researchers Supporting Project number (RSPD2024R620), King Saud University, Riyadh, Saudi Arabia.

CRediT authorship contribution statement

Gustavo Marinho: Visualization, Investigation. **Clara Lima:** Visualization, Resources. **Helcio Santos:** Writing – original draft, Software, Methodology. **igor Nascimento:** Writing – original draft, Investigation. **Talha Bin Emran:** Resources, Project administration. **josé Araújo neto:**

Table 2
Primers for the expression of the *mepA* and 23 s genes.

Gene	Primer F	Primer R	Reference
mepA	GGCAAATAAAGGCCGTATGA	CAGTCGCTTGAAGCATACCA	[34]
23 s	ACGGAGTTACAAAGGACGAC	AGCTCAGCCTTAACGAGTAC	[35]

F: Forward; R: Reverse

Software, Methodology. **Ahmad Obaidullah:** Writing – original draft, Visualization. **Priscilla Freitas:** Conceptualization. **JULIA Silva:** Resources, Methodology. **Irwin R. Alencar Menezes:** Software, Investigation. **João araujo junior:** Resources, Methodology. **francisco Cunha:** Writing – original draft, Supervision. **Isaac Araújo:** Investigation, Data curation. **edeildo silva junior:** Resources, Methodology. **Henrique Douglas Coutinho:** Writing – original draft, Supervision, Project administration. **Ana Carolina Araújo:** Conceptualization. **Teresa Balbino:** Resources, Methodology. **Saulo Tintino:** Writing – original draft, Supervision, Project administration. **joão BORGES:** Resources, Investigation. **Francisco J.B. Mendonca-Junior:** Resources, Methodology. **Ray almeida:** Investigation, Formal analysis. **Thiago aquino:** Resources, Methodology. **Cicera Oliveira-Tintino:** Validation, Investigation. **Cicera Paulo:** Software, Investigation. **emmanuel Marinho:** Writing – original draft, Software, Methodology.

Declaration of Competing Interest

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

Data Availability

Data will be made available on request.

Acknowledgements

The authors extend their appreciation to the Researchers Supporting Project number (RSPD2024R620), King Saud University, Riyadh, Saudi Arabia.

Informed Consent Statement

Not applicable.

Disclaimer/Publisher's Note

The statements, opinions and data contained in all publications are solely those of the individual author(s) and contributor(s) and not of MDPI and/or the editor(s). MDPI and/or the editor(s) disclaim responsibility for any injury to people or property resulting from any ideas, methods, instructions or products referred to in the content.

Supplementary Materials

No applicable.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.biopha.2024.117291.

References

[1] S.R. Tintino, V.C. d Souza, J.M. d Silva, C.D. d M. Oliveira-Tintino, P.S. Pereira, T. C. Leal-Balbino, A. Pereira-Neves, J.P. Siqueira-Junior, J.G. Costa, F.F. da; Rodrigues, Effect of Vitamin K3 inhibiting the function of NorA efflux pump and its gene expression on *Staphylococcus aureus*, Membranes *10* (2020) 130.

[2] S. Jang, Multidrug efflux pumps in *Staphylococcus aureus* and their clinical implications, J. Microbiol *54* (2016) 1–8.

[3] L. Fernández, R.E. Hancock, Adaptive and mutational resistance: role of porins and efflux pumps in drug resistance, Clin. Microbiol Rev. *25* (2012) 661–681.

[4] S.S. Costa, M. Viveiros, L. Amaral, I. Couto, Multidrug efflux pumps in *Staphylococcus aureus*: an update, Open Microbiol. J. *7* (2013) 59.

[5] S. Hassanzadeh, M.R. Pourmand, R. Mashhadi, K. Ghazvini, Epidemiology of efflux pumps genes mediating resistance among *Staphylococcus aureus*; a systematic review, Micro Pathog. *139* (2020) 103850.

[6] T.S. Freitas, J.C. Xavier, R.L. Pereira, J.E. Rocha, F.F. Campina, J.B. de Araújo Neto, M.M. Silva, C.R. Barbosa, E.S. Marinho, C.E. Nogueira, In vitro and in silico studies of chalcones derived from natural acetophenone inhibitors of nora and mepa multidrug efflux pumps in *Staphylococcus aureus*, Micro Pathog. *161* (2021) 105286.

[7] N.J.S. Araújo, A.R.P. da Silva, M.S. Costa, C.A.P. Silva, T.S. de Freitas, E.O. de Sousa, J.M. Barbosa Filho, Y.M.L.S. de Matos, H.D.M. Coutinho, J.C. Andrade-Pinheiro, Evaluation of the antibacterial activity of hecogenin acetate and its inhibitory potential of NorA and MepA efflux pumps from *Staphylococcus aureus*, Micro Pathog. *174* (2023) 105925.

[8] C.R. d S. Barbosa, N.S. Macêdo, Z.S. Silveira, J.E. Rocha, T.S. Freitas, D.F. Muniz, I. M. Araújo, C.D. de Morais Oliveira-Tintino, E.S. Marinho, M.N. da Rocha, Evaluation of the Antibacterial and Inhibitory Activity of the MepA Efflux Pump of *Staphylococcus aureus* by Riparins I, II, III, and IV, Arch. Biochem. Biophys. (2023) 109782.

[9] R. Nechak, S.A. Bouzroura, Y. Benmalek, N. Boufroua, B.N. Kolli, S.P. Martini, E. Duñach, Synthesis, identification and antimicrobial activity of substituted thiazolines and 1, 3, 4-thiadiazines from dehydroacetic acid, Synth. Commun. *49* (2019) 1895–1905.

[10] M. Čačić, V. Pavić, M. Molnar, B. Šarkanj, E. Has-Schon, Design and synthesis of some new 1, 3, 4-thiadiazines with coumarin moieties and their antioxidative and antifungal activity, Molecules *19* (2014) 1163–1177.

[11] L. Monzote Fidalgo, A.M. Montalvo Álvarez, L.F. Geigel, R.P. Pineiro, M. S. Navarro, H.R. Cabrera, Effect of thiadiazine derivatives on intracellular amastigotes of *Leishmania amazonensis*, MIOC *99* (2004) 329–330.

[12] S.A. El Bialy, A.M. Abdelal, A.N. El-Shorbagi, S.M. Kheira, 2, 3-Bis (5-Alkyl-2-Thiono-1, 3, 5-Thiadiazin-3-Yl) propionic acid: one-pot domino synthesis and antimicrobial activity, Arch. der Pharm.: Int J. Pharm. Chem. *338* (2005) 38–43.

[13] A.C.J. de Araújo, P.R. Freitas, I.M. Araújo, G.M. Siqueira, J.A. de Oliveira Borges, D.S. Alves, G.M. Miranda, I.J. dos Santos Nascimento, J.X. de Araújo-Júnior, E. F. da Silva-Júnior, Potentiating-antibiotic activity and absorption, distribution, metabolism, excretion and toxicity properties (admet) analysis of synthetic thiadiazines against multi-drug resistant (Mdr) strains, Fund. Clin. Pharm. (2023).

[14] J.M. Blair, M.A. Webber, A.J. Baylay, D.O. Ogbolu, L.J. Piddock, Molecular mechanisms of antibiotic resistance, Nat. Rev. Microbiol *13* (2015) 42–51.

[15] S. Gibbons, M. Oluwatuyi, G.W. Kaatz, A Novel inhibitor of multidrug efflux pumps in *Staphylococcus aureus*, J. Antimicrob. Chemother. *51* (2003) 13–17.

[16] P.N. Markham, E. Westhaus, K. Klyachko, M.E. Johnson, A.A. Neyfakh, Multiple novel inhibitors of the NorA multidrug transporter of *Staphylococcus aureus*, Antimicrob. Agents Chemother. *43* (1999) 2404–2408.

[17] P.R. Freitas, A.C.J. de Araújo, C.R.S. Barbosa, D.F. Muniz, R.S. de Almeida, I.R. A. de Menezes, J.G.M. da Costa, F.F.G. Rodrigues, J.E. Rocha, F.N. Pereira-Junior, Inhibition of the MepA efflux pump by limonene demonstrated by in vitro and in silico methods, Folia Microbiol *67* (2022) 15–20.

[18] R. Ferrer-Espada, H. Shahrour, B. Pitts, P.S. Stewart, S. Sánchez-Gómez, G. Martínez-de-Tejada, A permeability-increasing drug synergizes with bacterial efflux pump inhibitors and restores susceptibility to antibiotics in multi-drug resistant *Pseudomonas aeruginosa* strains, Sci. Rep. *9* (2019) 3452.

[19] A. Sharma, V.K. Gupta, R. Pathania, Efflux pump inhibitors for bacterial pathogens: from bench to bedside, Indian J. Med Res *149* (2019) 129.

[20] A.T. Green, M. Moniruzzaman, C.J. Cooper, J.K. Walker, J.C. Smith, J.M. Parks, H. I. Zgurskaya, Discovery of multidrug efflux pump inhibitors with a novel chemical scaffold, Biochim. Biophys. Acta *1864* (2020) 129546.

[21] P. Blanco, S. Hernando-Amado, J.A. Reales-Calderon, F. Corona, F. Lira, M. Alcalde-Rico, A. Bernardini, M.B. Sanchez, J.L. Martinez, Bacterial multidrug efflux pumps: much more than antibiotic resistance determinants, Microorganisms *4* (2016) 14.

[22] B.L. Roth, M. Poot, S.T. Yue, P.J. Millard, Bacterial viability and antibiotic susceptibility testing with sytox green nucleic acid stain, Appl. Environ. Microbiol *63* (1997) 2421–2431.

[23] P. Lebaron, P. Catala, N. Parthuisot, Effectiveness of sytox green stain for bacterial viability assessment, Appl. Environ. Microbiol *64* (1998) 2697–2700.

[24] M. Radchenko, et al., Structural basis for the blockade of MATE multidrug efflux pumps, Nat. Commun. 6 (2015) 7995.

[25] T. Kusakizako, et al., Structural biology of the multidrug and toxic compound extrusion superfamily transporters, Biochim Biophys. Acta *1862* (2020) 183154.

[26] P.R. Freitas, A.C.J. d. Araújo, I.M. Araújo, G.M. Siqueira, D.S. Alves, J.A.O. Borges, G.M. Miranda, I.J.S. Nascimento, J.X. Araújo-Júnior, E.F. Silva-Júnior, T.M. de Aquino, F.J.B.M. Júnior, E.S. Marinho, H.S. d. Santos, S.R. Tintino, C.M.G. Lima, T. B. Emran, S.F. Ahmad, S.M. Attia, H.D.M. Coutinho, Admet analysis, antibacterial and modified antibiotic activities of thiadiazine derivatives, J. Biol. Regul. Homeost. Agents *38* (2024) 13.

[27] M.M.R. Siqueira, P. d T.C. Freire, B.G. Cruz, T.S. de Freitas, P.N. Bandeira, H.S. Dos Santos, C.E.S. Nogueira, A.M.R. Teixeira, R.L.S. Pereira, J.C. Xavier, Aminophenyl chalcones potentiating antibiotic activity and inhibiting bacterial efflux pump, Eur. J. Pharm. Sci. *158* (2021) 105695.

[28] Silverman, R.B.; Holladay, M.W. The Organic Chemistry of Drug Design and Drug Action: Academic press, 2014.

[29] M. Javadpour, M. Juban, W. Lo, S. Bishop, J. Alberty, C. Mann, J.L. Markhan, A new method for determining the minimum inhibitory concentration of essential oils, J. Appl. Microbiol *84* (1996) 538–544.

[30] H.D. Coutinho, J.G. Costa, E.O. Lima, V.S. Falcão-Silva, J.P. Siqueira-Júnior, Enhancement of the antibiotic activity against a multiresistant *Escherichia coli* by *Mentha arvensis* L. and chlorpromazine, Chemotherapy *54* (2008) 328–330.

[31] S.R. Tintino, C.D. Oliveira-Tintino, F.F. Campina, P.W. Limaverde, P.S. Pereira, J. P. Siqueira-Junior, H.D. Coutinho, L.J. Quintans-Júnior, T.G. da Silva, T.C. Leal-

Balbino, Vitamin K enhances the effect of antibiotics inhibiting the efflux pumps of *Staphylococcus aureus* strains, Med. Chem. Res. *27* (2018) 261–267.
[32] C.D. d M. Oliveira-Tintino, J.E.G. Santana, G.G. Alencar, G.M. Siqueira, S. A. Gonçalves, S.R. Tintino, d Menezes, I.R.A. Rodrigues, J.P.V. Gonçalves, V.B. P. Nicolete, R. Valencene, Nootkatone and their liposomal nanoformulations as potential inhibitors of NorA, Tet (K), MsrA, and MepA Efflux Pumps in *Staphylococcus aureus* Strains, Pharmaceutics *15* (2023) 2400.
[33] S.R. Tintino, P. Wilairatana, V.C.A. de Souza, J.M.A. da Silva, P.S. Pereira, C.D. M. Oliveira-Tintino, Y.M.L.S. de Matos, J.T.C. Júnior, V.Q. Balbino, J.P. Siqueira-Junior, Inhibition of the *norA* gene expression and the NorA efflux pump by the tannic acid, Sci. Rep. *13* (2023) 17394.
[34] S. Ashraf, D. Parrine, M. Bilal, U. Chaudhry, M. Lefsrud, X. Zhao, Proteomic comparison of ivermectin sensitive and resistant *Staphylococcus aureus* clinical isolates reveals key efflux pumps as possible resistance determinants, Antibiotics *11* (2022) 759.
[35] G.P. Machado, R.C. Silva, F.F. Guimarães, A. Salina, H. Langoni, Detection of *Staphylococcus aureus*, *Streptococcus agalactiae* and *Escherichia coli* in Brazilian mastitic milk goats by multiplex-PCR, Pesqui. Vet. Bras. *38* (2018) 1358–1364.
[36] E.O. Mello, S.F. Ribeiro, A.O. Carvalho, I.S. Santos, M. da Cunha, C. Santa-Catarina, V.M. Gomes, Antifungal activity of Pv D1 defensin involves plasma membrane permeabilization, inhibition of medium acidification, and induction of ros in fungi cells, Curr. Microbiol. *62* (2011) 1209–1217.
[37] A. Waterhouse, et al., SWISS-MODEL: homology modelling of protein structures and complexes, Nucleic Acids Res *46* (2018) W296–W303.
[38] N.M. O'Boyle, et al., Open Babel: An open chemical toolbox, J. Chemin. *3* (2011) 1–14.
[39] G.M. Morris, et al., AutoDock4 and AutoDockTools4: automated docking with selective receptor flexibility, J. Comput. Chem. *30* (2009) 2785–2791.
[40] B.D. Schindler, et al., Mutagenesis and modeling to predict structural and functional characteristics of the *Staphylococcus aureus* MepA multidrug efflux pump, J. Bacteriol. *195* (2013) 523–533.