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Antibacterial effect and evaluation of the inhibitory effect against efflux pump in *Staphylococcus aureus* by abietic acid: In vitro and in silico assays

Maria Gabriely de Lima Silva [a], Lucas Yure Santos da Silva [a], Thiago Sampaio de Freitas [b], Janaina Esmeraldo Rocha [b], Raimundo Luiz Silva Pereira [b], Saulo Relison Tintino [b], Maria Rayane Correia de Oliveira [a,c], Anita Oliveira Brito Pereira Bezerra Martins [a], Maria Carolina Pacheco Lima [d], Gabriel Costa Alverni da Hora [e], Cicero L.G. Ramalho [f], Henrique Douglas Melo Coutinho [b,*], Irwin Rose Alencar de Menezes [a,*]

[a] Laboratory of Pharmacology and Molecular Chemistry (LFQM), Department of Biological Chemistry, Regional University of Cariri-URCA, Crato, Ceará, Brazil
[b] Laboratory of Microbiology and Molecular Biology, Department of Biological Chemistry, Regional University of Cariri-URCA, Crato, Ceará, Brazil
[c] Graduate Program in Biotechnology-Northeast Biotechnology Network (RENORBIO); State University of Ceará (UECE), Fortaleza, Ceará, Brazil
[d] Department of Medicinal Chemistry, University of Utah, Salt Lake City 84112, United States
[e] Department of Chemistry, University of Utah, Salt Lake City 84112, United States
[f] CECAPE - College of Dentistry. Juazeiro do Norte - CE, 63024-015, Brazil

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ABSTRACT

Abietic acid is a diterpene found in resins mainly from diverse species of genus Pinus. The present study evaluated the antibacterial and inhibitory effect against the NorA and MepA efflux pump of Staphylococcus aureus by abietic acid using in vitro and in silico assays. The microdilution bacterial assay was used to evaluate antibacterial activity in standard bacteria (SA 25923 and EC 25922) and clinical isolates bacteria multiresistant (SA-10 and EC-06). Their association with antibiotics ampicillin, gentamicin, and ciprofloxacin was also estimated. Staphylococcus aureus (SA-1199B and SA-K2068) was used with a NorA and MepA pump machine, respectively, to verify the inhibitory effect using MIC methodology proposed by CLSI and Ethidium Bromide, an indicator of an efflux pump. Molecular dynamics and molecular docking calculations were used to evaluate and validate the interaction of abietic acid with NorA and MepA efflux pumps. The results demonstrated a significate reduction of MIC values to EC 25922 and SA 10 and showed a synergistic effect when combined with increased gentamicin susceptibility against multiresistant strains. The abietic acid showed direct activity against Staphylococcus aureus overexpressing gene of efflux pump, demonstrating the possibility of interference in the efflux pump NorA (SA 1199B) and MepA (SA K2068) mediated by hydrogen bonds and hydrophobic interactions. Together, these findings are promisors validating the potential antimicrobial activity and the possibility of using abietic acid as antibiotic adjuvant resistance breakers (ARBs) to treat infections caused by multiresistant bacteria. However, other studies are necessary to confirm this potential using the in vivo model.

1. Importance

The growing increase in bacterial infectious diseases is related to infections acquired in hospitals and communities and the indiscriminate use of antibiotics. This favors the development of bacteria with resistance against drugs used as antibiotics. This study sought natural products extracted from plants of the Pinus genus, a new compound with antibacterial effects capable of fighting these infections. Therefore, these findings are promisors for validating the potential antimicrobial activity and the possibility of using abietic acid as antibiotic adjuvant resistance breakers (ARBs) combined with therapeutic antibiotics to treat infections caused by multiresistant bacteria. However, other studies are necessary to confirm this potential using the in vivo model.

2. Introduction

The increase of bacterial infectious diseases may be related to infections acquired in the hospital and community environments, reduced

* Corresponding authors.
E-mail addresses: hdmcoutinho@gmail.com (H.D.M. Coutinho), irwin.alencar@urca.br (I.R.A. de Menezes).

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defensive capacity of the organism, or progressively developed different resistance mechanisms. Antimicrobial resistance to antibiotics is understood as the adaptive capacity that microorganisms develop to grow in the presence of an antibiotic capable of killing them [1]. This natural selection process is often a consequence of the misuse of antimicrobials and represents a public health problem worldwide for both humans and animals [2]. Among the adaptation and resistance mechanisms, the most consistent is the expression of efflux pumps through plasmid transfer or quorum sensing by pathogenic bacteria with a resistance profile [3]. Gram-positive and Gram-negative bacteria, such as *Staphylococcus aureus* and *Escherichia coli*, are among the bacteria with the most significant capacity to induce the expression of the efflux pump [4] or specific adaptive processes such as the formation of biofilms that increase virulence that facilitates growth in the presence of antibiotics [5].

Escherichia coli is classified as Gram-negative bacteria that inhabit the commensal microbiota of the human and animal intestine. However, its virulent forms can cause enterohemorrhagic infections in the digestive tract, kidney damage resulting from hemolytic-uremic syndrome (HUS), sepsis in the bloodstream, prostate infections, and colonize other organs, where they are capable of causing a variety of illnesses [6]. *Staphylococcus aureus* is a Gram-positive bacteria belonging to the Staphylococcaceae family, known to cause infections in a wide variety of local and systemic lesions in humans and animals. Infections caused by this bacterium, usually acquired in hospital environments, can be fatal [7], principally when NorA or MepA efflux pumps are characterized in this organism. This pump is responsible for the moderate resistance of *S. aureus* to fluoroquinolone antibiotics [8].

In this context, considering the importance of the NorA or MepA efflux pump as a mediator of antibiotic resistance and the ineffectiveness of drugs used in treating infections caused by efflux pump-inducing bacteria, the search for new medicines with antibacterial activity are of great importance [9]. Natural products extracted from plants can act as structural models for synthesizing new substances or active prototypes to develop novel chemical entities with antibacterial effects that can originate new possibilities of mechanisms of action against the bacterial target [10]. One promissory alternative for a break of bacterial resistance is a combination of isolate natural compounds with therapeutic antibiotics that are now being exploited in different studies [11-18]. The literature demonstrates that derivates isoprenoids represent a vast number of molecules with functional chemical groups, such as hydroxyl and carbonyl groups, responsible for plants' physiological processes ranging from pigments to fragrances and precursors of sex hormones [19]. However, the most commercially valuable isoprenoids are flavorings as monoterpenes class that present a range of biological properties especially, involved in antimicrobials actions as observed to limonene [15], estragole [20], carvacrol and thymol [16], and α-pinene [18,21]. Other studies showed that terpenes of higher molecular weight as diterpenes and sesquiterpenes classes also illustrate potential agents against antimicrobial resistance [22,23].

Among these natural products, abietic acid (AA) is a diterpene compound found as a major component in resins from the Pinus genus and many other species of conifers [24]. Abietic acid has been reported to show various activities: as antiviral [25], antibacterial [24], antimycotic [26], antiparasitic [27], antioxidant [28], anti-inflammatory [29], wound healing [30], anti-allergic [31], anticonvulsant activities [32] and inhibitory effects in the cancer cell [33].

So, given the increased bacterial resistance to therapeutics drugs, the research of new antibiotic adjuvant is necessary. The diversity of activity present in literature data to abietic acid and your antibacterial activity can be represented a pharmacological potential significant against bacterial resistance. The paper aims to verify the antibacterial effect and the inhibitory effect of abietic acid against efflux pump in *Staphylococcus aureus* by in vitro and in silico assays.

3. Materials and methods

3.1. Bacterial cultures

In this study, we have used strains standard ATCC of *Staphylococcus aureus* (SA 25923) and *Escherichia coli* (EC25922), and your respective multiresistant strains SA10 and EC06 were obtained from clinical isolates and present resistance profiles described in Table 1. Bacterial strains were stocked at 4 °C on Brain Infusion Heart agar (BHIA, Himedia, India). Before the assay, the cells were activated and grown at 37 °C in Brain Heart Infusion (BHI, Himedia, India) for 24 h. The strains were suspended at turbidity equivalent to 0.5 on the McFarland scale, corresponding to 10^5 CFU. The *Staphylococcus aureus* strains carrying genes the NorA (SA 1199B) and MepA (SA-K2068), kindly provided by Prof. S. Gibbons (University of London), were used in bacterial-resistance efflux pump inhibition assays. These bacteria were maintained in Heart Infusion Agar (HIA, Difco) medium at 4 °C and, posteriorly, samples of strains were transferred to test tubes containing sterile saline, and turbidity was assessed using a value of 0.5 on the McFarland scale, corresponding to 10^5 CFU.

3.2. Drugs

The abietic acid, the antibiotics – Norfloxacin (Nor), Ampicillin (Amp), Subactan (Sub), Gentamicin (Gen) and ciprofloxacin (Cip) –, the Ethidium Bromide (EtBr) and the efflux pump inhibitors – carbonyl cyanide 3-chlorophenylhydrazone (CCCP) and Chlorpromazine (CPZ) – were obtained from Sigma Chemical Corp., St. Louis.

3.3. Antimicrobial susceptibility testing (MIC)

The Minimal Inhibitory Concentrations (MICs) of abietic acid and antibiotics against bacterial isolates were evaluated by the agar dilution technique, according to the established guidelines of the Clinical and Laboratory Standards Institute (CLSI). The stock solutions of compounds were previously prepared in DMSO or saline followed by dilution in sterile water to a final concentration of 1024 μg/mL. For the MIC determinations, 100 μL of bacterial inoculum were suspended in saline solution, followed by the addition of 900 μL brain heart infusion (BHI) in Eppendorf tubes and transferred to 96-well microdilution plates. Then, the bacterial suspensions (10^5 CFU/mL) were exposed to abietic acid that was 2-fold serial diluted in concentrations ranging from 0.5 to 1024 μg/mL. The plates were incubated at 37 ºC for 24 h. The bacterial growth was evaluated by adding 20 μL of resazurin (0.01 % w/v in sterile distilled water) to each well. The growth is observed by a color change from blue to pink. The MICs values were defined as the lowest

Table 1
Minimal inhibitory concentration (MIC, μg/mL) of abietic acid against strains standard ATCC and multiresistant of *Staphylococcus aureus* and *Escherichia coli*.

	Strains standard ATCC		Strains multi-resistant of clinical isolated			
	MIC SA 25923	MIC EC 25922	MIC SA-10	Resistance profile	MIC EC-06	Resistance profile
Abietic Acid (AB)	1024 μg/mL	64 μg/mL	102 μg/mL	Ca, Cef, Cf, Cro	1024 μg/mL	Amc, Amox, Amp, Asb, Azi, Ca, Cef, Cf, Cip, Cla, Clin, Eri, Lev, Mox, Oxa, Pen

Amc - Amoxicillin + Ac. clavulanic; Amox - Amoxicillin; Amp - Ampicillin; Asb - Ampicillin + Sulbactam; Azi - Azithromycin; Ca - Cefadroxil; Cef - Cephalexin; Cf - Cephalotin; Cip - Ciprofloxacin; Cla - Clarithromycin; Clin - Clindamycin; Cro - Ceftriaxone; Eri – Eritromicin; Lev - levofloxacin; Mox - Moxifloxacin; Oxa - oxacillin; Pen - Penicillin.

concentration at which no bacterial growth. The antibacterial assays were performed in triplicates, and the results were expressed as the geometric mean of MIC value.

3.4. Modulatory activity of antibiotic resistance assay by MIC reduction

This technique consists of the same procedure as the previous assay with some modifications. The bacterial inoculum of multiresistant strains (SA10 and EC06) were transferred to 96-well microdilution plates and incubated with Ciprofloxacin, gentamicin, or ampicillin, diluted with ranging from 512 μg/mL to 0.5 μg/mL, and supplemented a subinhibitory concentration (MIC/8) of abietic acid. The control plates were prepared with only the 10 % BHI medium and bacterial inoculum of multiresistant strains (SA10 and EC06). The synergic effect was observed by MIC reduction of antibiotic and evaluated through the use of Resazurin. The antibacterial assays were performed in triplicates, and the results were expressed as the geometric mean of MIC value.

3.5. Analysis of efflux pump inhibition by MIC reduction

To investigate the activity of the abietic acid as efflux pump inhibitors is previously determined the MIC of norfloxacin and ethidium bromide against the *S. aureus* strains that express the NorA and MepA machines pump. For this assay, suspensions of strains SA-1199B and SA-K2068 corresponding to 0.5 of the McFarland scale were vortexed with 1350 μL of brain heart infusion (BHI). Then, 150 μL of this bacterial inoculum was transferred to 96-well plates and vortexed with a solution of EtBr or norfloxacin at concentrations ranging from 1024 μg/mL to 0.5 μg/mL in the presence of chlorpromazine (101 μg/mL), carbonyl cyanide m-chlorophenylhydrazone (CCCP) (2 μg/mL) or abietic acid in subinhibitory concentrations (1/8 MIC). The positive control was prepared with a bacterial suspension, and EtBr (8 μg mL^{-1}) or norfloxacin. The blank controls were prepared with abietic acid in saline solution. The saline inoculum in brain heart infusion (BHI) was used as growth control. These tests were transferred to 96-well microdilution plates, and serial dilutions of 100 μL were performed. The microtitre plates were incubated at 37 °C for 24 h, and bacterial growth was revealed through the use of Resazurin. All tests were executed in triplicates, and the final results were described as a geometric mean of the replicates [32].

3.6. Docking and molecular dynamics studies

The docking procedure and three-dimensional (3D) structure efflux pump MepA and NorA were performed conform established by Morais Oliveira-Tintino at al. [34] and Santos et al. [35,36]. All structures were carried out using the virtual screening workflow available at the Autodock Tools (http://autodock.scripps.edu). Molecular docking was carried out using the Autodock Vina algorithm. The rigid docking procedure was carried out using Autodock Vina with grid box defined as a 20Åx20Åx20Å box around the geometrical center of the model structure. The default settings of Autodock Vina with number of docking runs and number of solutions obtained was set at 50 runs and conformations, repectivly, the number of output conformations was set to one. For comparison, docking of the ligands was also performed Molegro Virtual Docker [37]. with grid coordinates of 5 Å the of geometrical center of best predocked 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, maximum iteration of 2000 with scaling factor of 0.50 and crossover rate of 0.90. The most favorable binding free energy poses were analyzed using the Discovery Studio visualizer program version 3.1 [38].

Molecular Dynamics (MD) simulations were used to evaluate the interaction of the abietic acid with the protein structures of MepA and NorA. The abietic molecules were positioned at the active sites of each protein. The simulations were carried out using the program AMBER 20 [39]. The Antechamber [40] was used to generate the parameters for the abietic acids using the General AMBER Force Field 2 (GAFF2) [41]. At the same time, the all-atom ff19SB force field [42]was employed to describe the protein molecules. OPC water molecules [43] and Cl- counterions (2 for MepA, 13 for NorA) were added to solvate and neutralize the charge of the octahedron simulation boxes. The distance between the solute and the edges of the boxes was set to 10 Å. Periodic boundary conditions were applied in each direction. The systems were minimized using the steepest descent method, with restraints on heavy atoms for the first 1000 steps. Then, the restraints were steadily removed through an eight-step equilibration process of 50 fs each. The final step was an equilibration of 100 fs without any restraints. Then, the simulations were produced using a 2 fs time-step, using Langevin dynamics [44] to keep the temperature at 300 K with a time constant of 1 ps, and Berendsen barostat [45] to hold the pressure at 1 bar. Finally, an 8 Å cutoff was used to calculate the long-range electrostatics using the Particle-mesh Ewald method [46]. Both simulations were performed during 4 μs under NPT conditions. The CPPTRAJ package [47] was used for the analysis, and the MM-PBSA [48] method to estimate ligand-binding affinities.

3.7. Statistical analysis

The results were expressed as geometric means and statistical analyses by one-way ANOVA and subsequent post hoc test with Holm Sidak's correction or a two-way ANOVA followed by Bonferroni's post hoc test using GraphPad (GraphPad Software, San Diego, CA, USA). The obtained p-Values below 0.05 were considered significant.

4. Results

4.1. Intrinsic antibacterial activity effect and modulatory activity against clinical ciprofloxacin-resistant strains

The antibacterial activity of abietic acid was obtained by microdilution method against strains standard ATCC of *Staphylococcus aureus* and *Escherichia coli* (SA 25923 and EC25922). The respective multiresistant strains (SA10 and EC06) are presented in Table 1. The MIC results showed a clinically relevant inhibitory effect to SA-10 and EC-25922.

To verify the modulatory effect of the abietic acid against Gram-positive and Gram-negative bacteria was used three different antibiotic classes gentamicin (aminoglycoside), ampicillin (beta-lactam), and ciprofloxacin (fluoroquinolone). So, our findings firmly indicate a significant modulating activity for *Escherichia coli* (EC-06) for both antibiotics: gentamicin, with MIC reduction of 102–64 μg/mL; and ciprofloxacin with a decrease of MIC of 50.8–20.2 μg/mL; thus causing an increase the activity of these antibiotics (Fig. 1A). However, for the Gram-positive strain *Staphylococcus aureus* (SA-10), the results presented modulating activity for all antibiotics tested: a MIC reduction of 20.2–12.7 μg/mL to ampicillin; 16–0.5 μg/mL gentamicin; and 40–16 μg/mL to ciprofloxacin (Fig. 1B). In this essay, we use Ampicilin with subactan and chlorpromazine to evidence possibles resistance mechanisms by efflux pump and the presence of β-lactamase. So, the results of the abietic acid did not demonstrate a significant effect against the β-lactamase activity. However, they show relevant effects similar to chlorpromazine, a pump efflux inhibitor.

4.2. Assessment of efflux pump inhibition of NorA or MepA by MIC reduction test

The evaluation of the inhibitory capacity of the efflux pump was performed against two strains of *S. aureus* SA-1199B (overexpresses the NorA gene encoding) and multi-drug resistant (MDR) mutant strain SA-

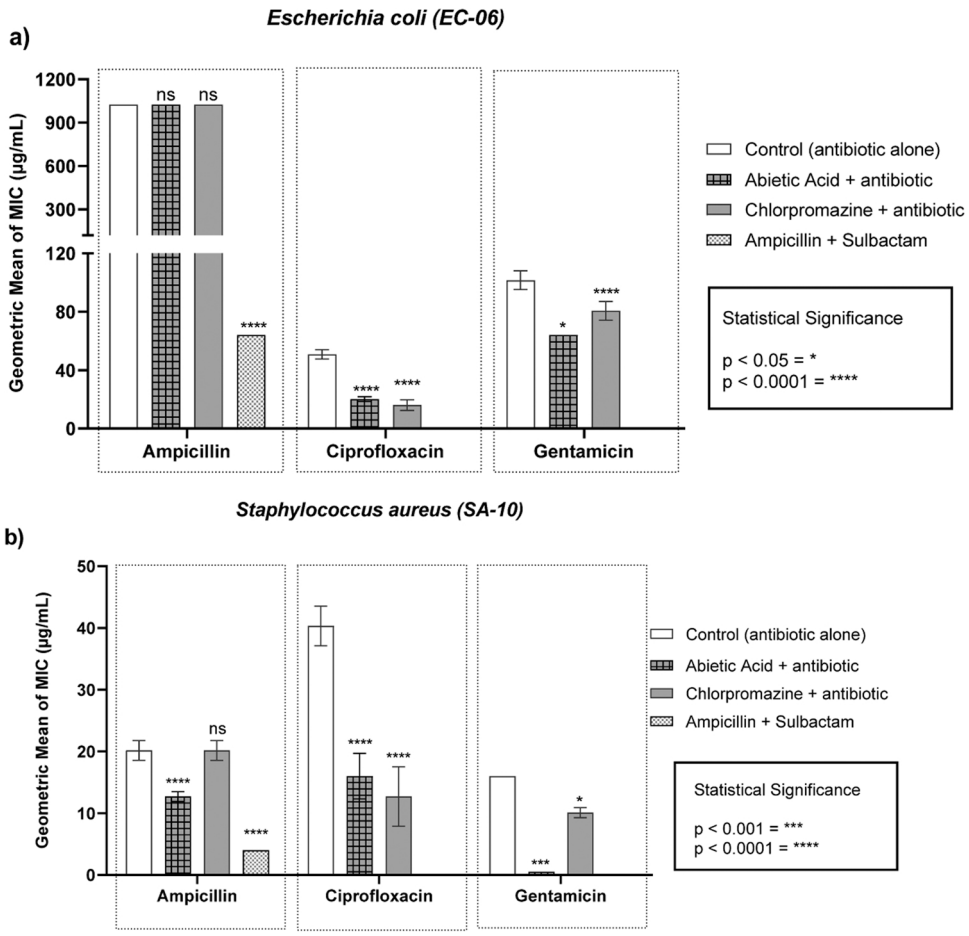

Fig. 1. Minimum inhibitory concentration (MIC) of the antibiotics gentamicin, ciprofloxacin, and ampicillin in the presence and absence of the abietic acid. A) *Escherichia coli* (EC-06) e B) *Staphylococcus aureus* (SA-10). The value was expressed as the geometric mean of three simultaneous experiments. (***) Statistically significant values in ANOVA analysis; ns: no significance.

K2068 (overexpresses the MepA gene encoding). Initially, we performed a MIC against SA-1199B, and K2068 was determined for all the molecules (Table 2). Interestingly, except for chlorpromazine, all other compounds demonstrated a slight activity (MIC $< 512\ \mu g\ mL^{-1}$) (Table 2).

Evaluation of the modulatory effect of fluoroquinolone resistance was procedure using the SA-1199B strain. The observed results show that abietic acid increases the activity of norfloxacin, reducing the antibiotic MIC from 362.04 to 161.27 µg/mL (Fig. 2A). Effects similar were observed to chlorpromazine (362.04–203.09 µg/mL) and CCCP (80–64 µg/mL) that are known to your inhibitory capacity in pump efflux as NorA and MepA. Abietic acid can also modulate resistance to EtBr by reducing the MIC from 80 to 32 µg/mL (Fig. 2B), which confirms the potential for inhibition of the efflux pump since EtBr is a known substrate that interacts exclusively with efflux pumps like NorA or MepA.

The modulatory activity of abietic acid on resistance to ciprofloxacin or EtBr was also evaluated using the SA-K2068 strain that overexpressing efflux pump MepA. The results showed a significant change in MICs when abietic acid was tested in combination with antibiotics ciprofloxacin 101.6–64 µg/mL (Fig. 3A) or EtBr with MIC reduction 256–64 µg/mL (Fig. 3b). Similar results in the MIC are observed when

Table 2
Minimal inhibitory concentration (MIC, µg/mL) of abietic acid, ethidium bromide, and two pump inhibitors against two *S. aureus* overexpress the efflux pump gene encoding.

EC-06											
Antibiotic (ANT)			Chlorpromazine+ANT			Abietic acid+ANT			Ampicilin+sulbactan		
	Mean of MIC	SD	Mean of MIC	SD	MF (%)	Mean of MIC	SD	MF (%)	Mean of MIC	SD	MF (%)
Ampicillin	1024	*0*	1024	0	***0***	1024	0	***0***	64	0	***93.75***
Ciprofloxacin	50.8	*3.21*	16	3.68	***68.50***	20.16	1.61	***60.31***	0	0	***100***
Gentamicin	101.59	*6.42*	80.63	6.42	***20.63***	64	0	***37.00***	0	0	***100***
SA-10											
Ampicillin	20.16	1.61	20.16	1.61	***0.00***	12.7	0.8	***37.00***	4	0	***80.16***
Ciprofloxacin	40.32	3.21	12.7	4.82	***68.50***	16	3.68	***60.32***	0	0	***100***
Gentamicin	16	0	10.08	0.8	***37.00***	0.5	0	***96.88***	0	0	***100***

Mean of MIC = geometric mean of minimum inhibitory concentrations (µg/mL), SD = standart erro of triplicate; MF(%) = percentage of modulation factors in comparison with compound alone.

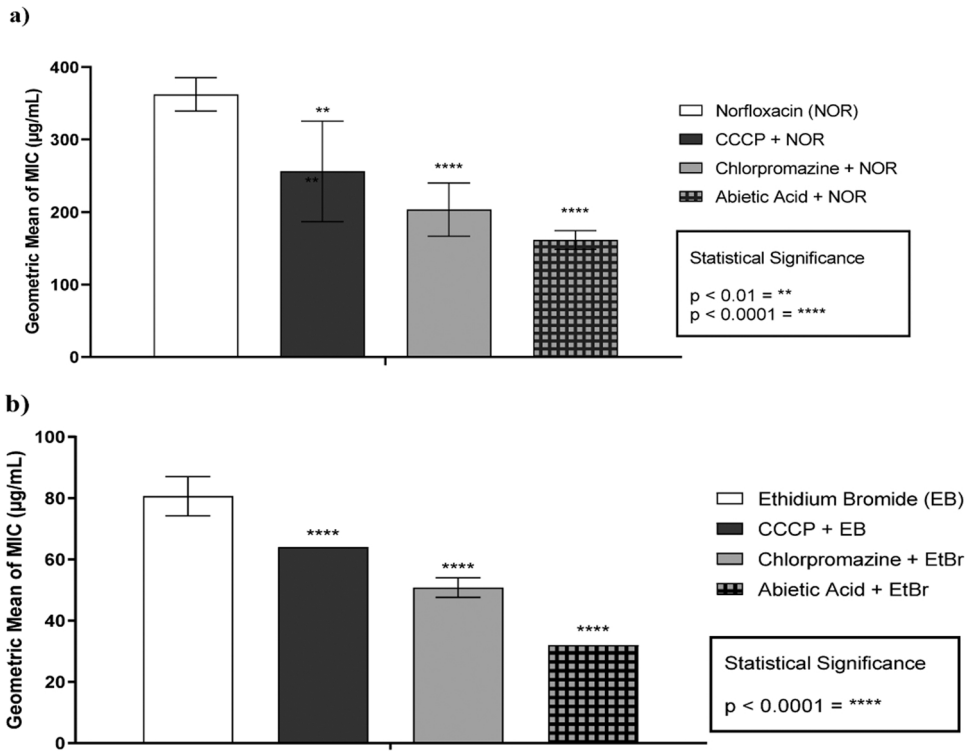

Fig. 2. MIC of ciprofloxacin (a) and ethidium bromide (b) alone and in association with the NorA efflux pump inhibitors or abietic acid against *S. aureus* SA-1199B. The value was expressed as the geometric mean of three simultaneous experiments. (***) Statistically significant values in ANOVA analysis; ns: no significance.

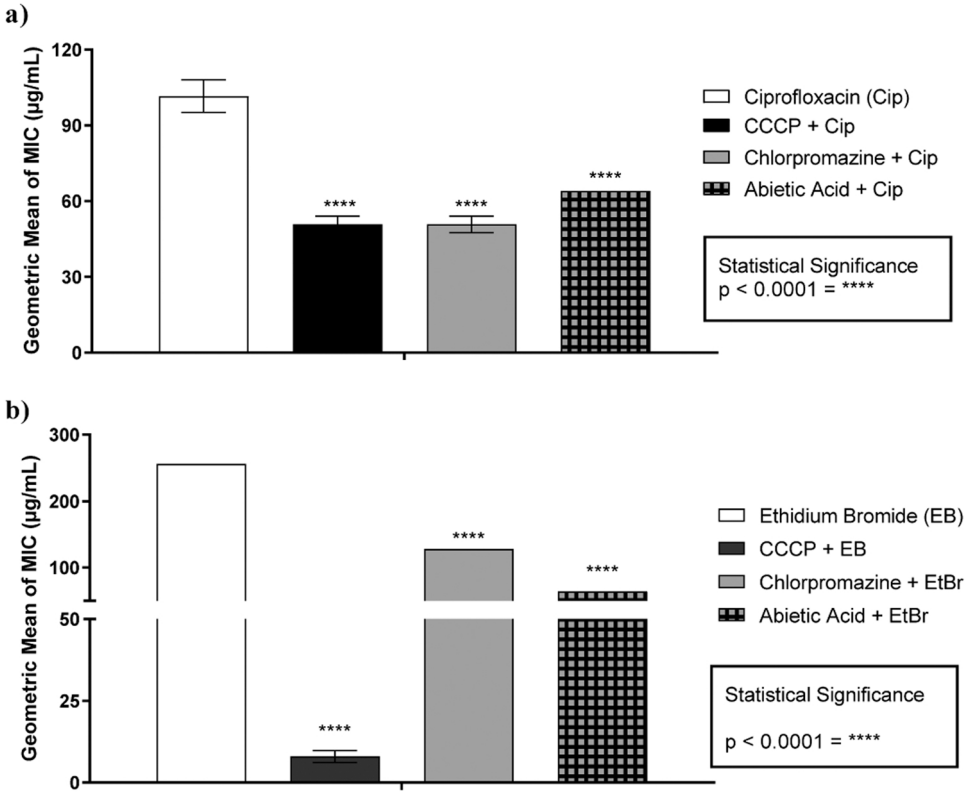

Fig. 3. MIC of ciprofloxacin (a) and ethidium bromide (b) alone and in association with the MepA efflux pump inhibitors or abietic acid against *S. aureus* SA-K2068. The value was expressed as the geometric mean of three simultaneous experiments. (***) Statistically significant values in ANOVA analysis; ns: no significance.

chlorpromazine (256–128 μg/mL) or CCCP (256–8 μg/mL) were added to the bacterial broth with EtBr. All these results indicate the modulation of resistance to these antibacterial agents by the interference of pump machine function.

4.3. Docking and Molecular Dynamics of abietic acid on the active sites of NorA and MepA

For the molecular docking study, two different docking algorithms were proposed to be validated by self-docking. As a result, the comparison of docked positions has exhibited root mean square deviation (RMSD) values of 0.85 Å for the position with the best interaction energy of each algorithm. This procedure was adopted by the absence of X-ray crystal structures of these efflux pumps. Table 3 shows the best interaction energy of the docked compounds from the molecular docking procedure.

As mentioned before, EtBr is a known substrate of efflux pumps as present in the S. aureus 1199B and K2068 and, carbonyl cyanide 3-chlorophenylhydrazone (CCCP) and chlorpromazine are knowledge in literature as an inhibitor of these pumps conform effect observed in the reduction of the MIC of EtBr (Figs. 2 and 3). A similar inhibitor effect is observed when the abietic acid is used in an association, causing a reduction of the function of the pump machine for both targets. Table 2 demonstrated the binding energy of interactions against MepA and NorA targets compared to abietic acid (AB), carbonyl cyanide 3-chlorophenylhydrazone (CCCP), and chlorpromazine displayed favorable binding energy value by ~3 kcal/mol of difference. The observed trend in the binding free energy of abietic acid was found to be more consistent with the NorA efflux pump corroborating with the antimicrobial assay, which can be explained by hydrogen bond interactions and aromatic–aromatic interactions involving the stabilizations of NorA-AB complex. Similar results were also observed with MepA, where the binding free energy of abietic acid was more consistent than chlorpromazine. However, as observed in the antimicrobial assay, the CCCP presents a more significant inhibitor powder that can be explained by better hydrogen bond interactions that contribute to stabilizing the complex with the MepA efflux pump.

Furthermore, A complete description of all interactions of the abietic acid with MepA and NorA active site is shown as a 2D protein-ligand interaction diagram (Fig. 4). We found pi–Alkyl, Alkyl, van der Waals and hydrogen bonds interactions involved in stabilizing the complexes for both targets. As it happens to other efflux pumps, such as NorA, there is a hydrophobic patch in the distal site, consisting of several amino acids, such as PHE153, VAL149, ALA146, etc. binding site, displaying a hydrogen bond interaction of 2.25 Å with ASN205. van der Waals interactions with residues THR201, SER175, VAL176, ASN179, SER32, and many others are also present. A complete description of all interactions of the chalcone is shown as a 2D protein-ligand interaction diagram in Fig. 4.

In order to analyze these interactions and calculate their free energy contributions, Molecular Dynamics simulations were performed with the abietic acid in the active site of each protein. Each receptor's active site was defined as the amino acids within a radius of 5 $\AA$ from the ligand (Fig. 5).

Table 3
The binding affinities of the best poses for all compounds of both efflux pumps.

Compounds	Strains multiresistant	
	MIC 1199B	MIC K2068
Ethiduim bromide (EtBr)	80.63 μg/mL	256 μg/mL
Carbonyl cyanide 3-chlorophenylhydrazone (CCCP)	8 μg/mL	8 μg/mL
Chlorpromazine (CPMZ)	≥ 1024 μg/mL	≥ 1024 μg/mL
Abietic Acid (AB)	322.54 μg/mL	406.37 μg/mL

Using the MM-PBSA method, we were able to calculate the total binding free energy for each protein: − 4.13 kcal/mol for the MepA and − 8.35 kcal/mol for the NorA. The standard error of the mean was 0.75 and 0.87 kcal/mol, respectively. Furthermore, we also calculated the contribution of each residue and ligand in the complexes' interaction and its binding energy, as shown in Fig. 6 and Fig. 7.

The residues of MepA that contribute to the binding energies with the abietic acid are MET249, ILE289, MET290, LEU292, PHE372, THR375, GLN379. The MET290 stands out as the amino acid that most promotes the binding energy with the abietic acid. On the other hand, the GLU286 creates some challenges for binding the ligand, probably due to its strong positive charge. For the NorA, most of the residues interactions favor the binding with the ligand: ILE219, SER223, GLY226, PHE227, LEU230, TYR243, VAL247, LEU286, ILE302, and especially the PHE244. Interestingly, for both efflux pumps, a neutral and nonpolar residue is the one that most contributes to the binding energy with the abietic acid.

5. Discussion

The results clearly demonstrated that abietic acid presents two significant developments: synergic effect when combined with ampicillin, gentamicin, and ciprofloxacin against multiresistant strains and also presented promissory results against strain which overexpresses the efflux pump NorA (SA-1199B) and MepA (SA-K2068), resulting in a decrease of bacterial resistance. The antimicrobial activity of abietic acid was described previously against *Staphylococcus aureus*, *Escherichia coli* [49], *Staphylococcus pseudintermedius* [24], and *Streptococcus mutans* [48]. The proposed mechanism of antibacterial activity can be explained by the hydrophobicity of the rigid hydrophenanthrene skeleton and carboxylic functionality that interacts with the lipid component of the bacterial cell membrane altering the membrane functions [49] and lysis of cell membranes. Others studies were showed low concentrations of abietic acid produce bacteriostatic effects.

Therapy based on the synergic association is an important strategy in modern medicine [50]. It permits old antimicrobial drugs with a history of bacterial resistance to restore their efficacy[17]. The interesting alternative therapeutics for this problem is the use of phytomedicines in combination with antibiotics. The terpenes are usually found in essential oil or resin and present bacteriostatic and bactericidal activities [51], with promising effects in reducing bacterial resistance [11,12,16,17]. The synergism between terpenes and antibiotics and their interference in the bacterial resistance is present in the literature for different associations: e.g., carvacrol or thymol [52,53] or eugenol [54] or estragole [55] with norfloxacin, thymol with tetracycline [56], 1,8 cineole with mupirocin [57], α-bisabolol [58] or D-limonene [59] or β-citronellol [60] in combination with gentamicin. The results in this work confirm synergic effects observed in the literature and corroborating with studies of Buommino et al. [61] and Helfenstein et al. [59] that reported the antimicrobial activity of abietic acid against differents strains with antibiotic resistance. Literatura data demonstrate a similar synergic effect after the combination of ciprofloxacin antibiotic with a metallic nanocomposite of CuFe2O4 @Ag [60], Fe3O4 @Ag [62], and ZnO@-Glu–TSC [63,64]. This association shows significant synergistic effects increasing the antibiotic efficacy with a reduction of MIC value and a decrease in expression of norA gene.

On the other hand, for the first time, the data presented show that the association of abietic acid with ethidium bromide promotes the reduction of MIC statistically significant, indicating that the efflux pump mechanism was inhibited, attributing this effect to a previously observed synergism. Other terpenes as estragole, eugenol, Carvacrol and Thymol, α-Pinene, and Limonene also demonstrate similar capacity of efflux pump inhibition [11,12,14,16-18,54,58]. These results confirm that both physical-chemical characteristics, lipophilicity, and hydrophobicity are essential to explain the possibility of interactions with these pumps. As observed in abietic acid, the carbon skeleton and

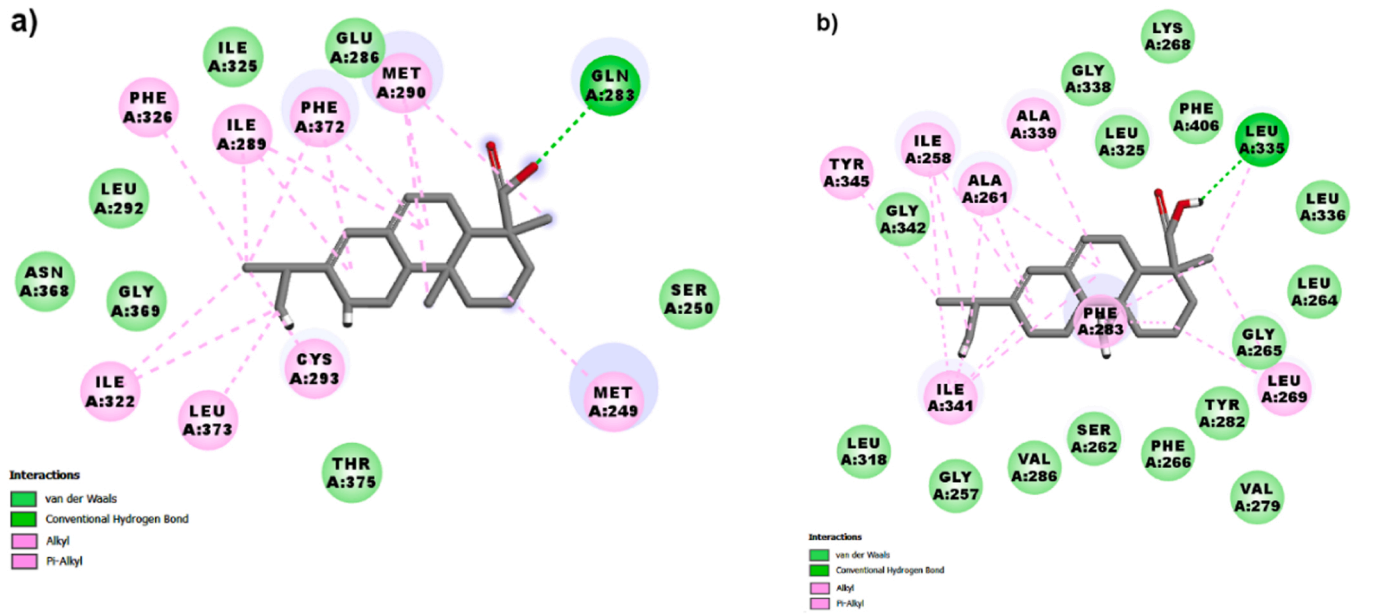

Fig. 4. Interaction maps showing the binding of Abietic acid with residues of binding sites in the MepA (a) and NorA (b).

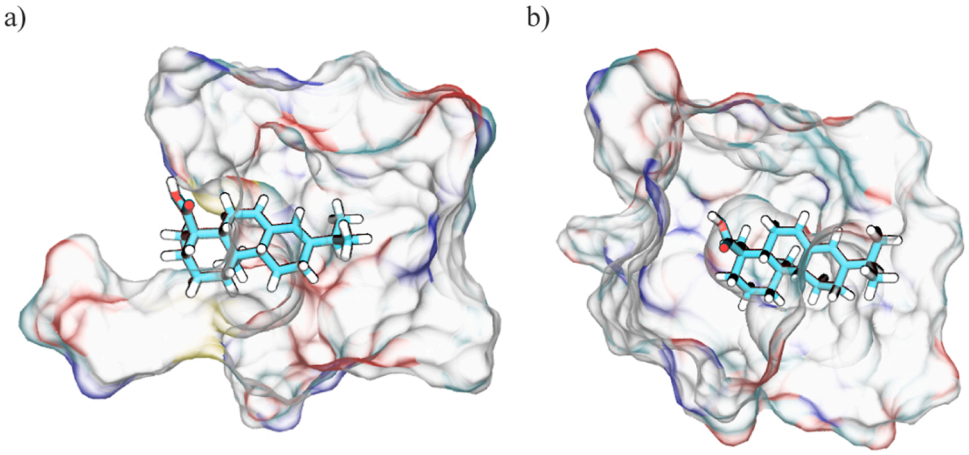

Fig. 5. Region of the binding of Abietic acid in the active sites in the MepA (a) and NorA (b). Residues within 5 Å of the ligand are represented as surfaces. Colors scheme according to the atom types (red = oxygen, blue = nitrogen, carbon = green, sulfur = yellow, white = hydrogen). Water molecules were omitted for better visualization.

hydroxyl groups are responsible for the binding with the efflux pump and essential to the cell membrane interactions. The docking and molecular dynamics results suggest the, possibly, the abietic acid can interact with NorA and MepA efflux pump by competition mechanism decreasing the effect of pump binding with antibiotic. The outcomes of the docking analysis reflect and agree with the MD and PBSA calculations. These findings suggest a possible use as antibiotic resistance breakers (ARBs), confirming the hypothesis proposed by (Laws et al., 2019). Therefore, the co-administration of ARBs as adjuvants to antibiotics associated with conventional antibiotics can bring health benefits, impacting the reduction of high levels of bacterial resistance.

6. Conclusions

The rise and alarming rate of antibiotic resistance require urgent global attention. At this point, several strategies have been developed to break bacterial resistance levels; thus, the search for new ARAs may represent a promising avenue. This study highlighted the observation that abietic acid inhibited the growth of standard gram-positive and gram-negative bacteria strains. Furthermore, when combined with the antibiotic, it demonstrated a significant synergistic effect against multidrug-resistant strains. Thus, these results allow us to infer two hypotheses: abietic acid may interfere with the function of the efflux pump mechanism, and the synergistic effect against multidrug-resistant bacteria may result from the interaction with the bacterial cell membrane, promoting permeability changes. However, further studies are needed to verify its effectiveness using in vivo models.

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CRediT authorship contribution statement

M.G.L.S., L.Y.S.S., T.S.F. and J.E.R. designed the study and performed the experiments. R.L.S.P. and S.R.T. isolated and identified the human strains. M.G.L.S., M.R.C.O. and A.O.B.P.B.M. drafted and wrote the manuscript. S.R.T., H.D.M.C. and I.R.A.M. edited and revised the

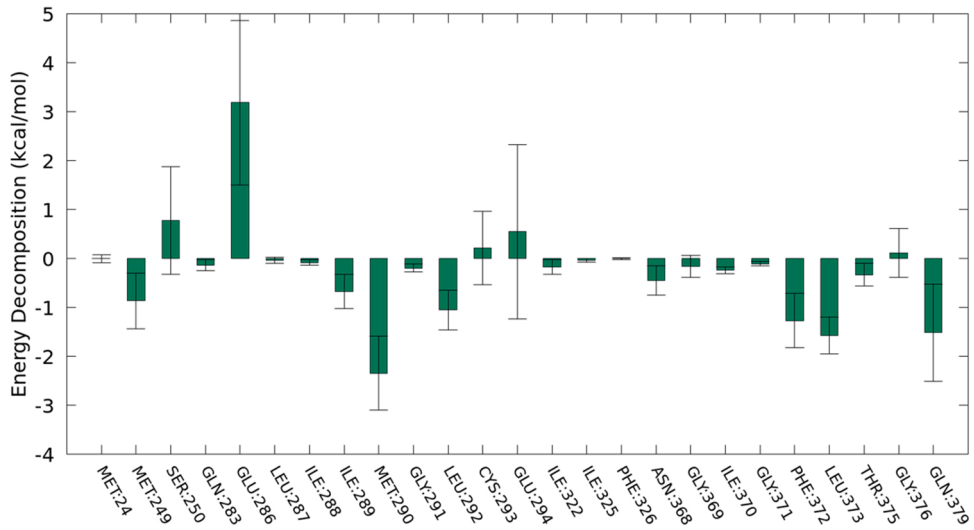

Fig. 6. Energy contribution from each residue in the ligand-protein complex with MepA efflux pump. The energy contribution of the adiabatic acid was − 17.859 (± 1.342) kcal/mol. Residues selected were within 5 Å of the ligand.

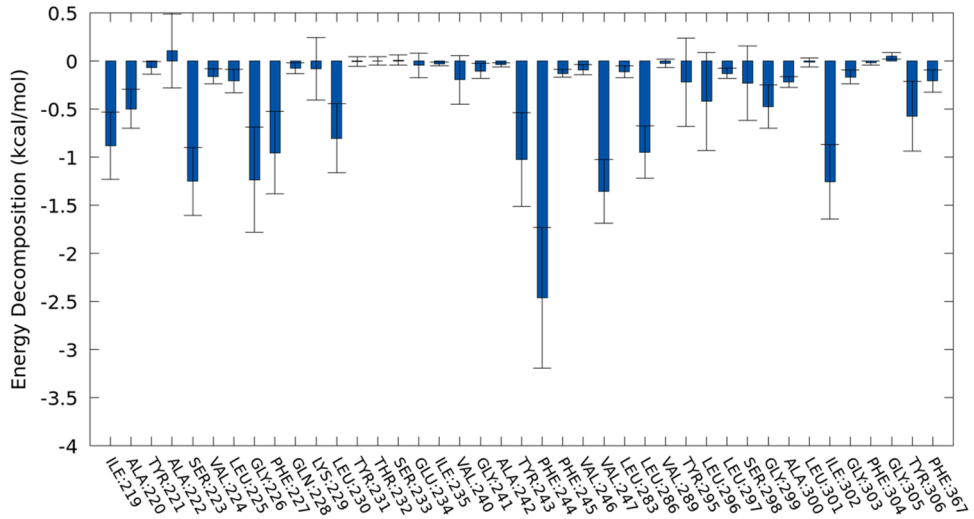

Fig. 7. Energy contribution from each residue and the abietic acid (LIG394) in the complex with NorA efflux pump. The energy contribution of the adiabatic acid was − 15.551 (± 1.356) kcal/mol. Residues selected were within 5 Å of the ligand.

manuscript. M.C.P.L and G.C.A.H. performed the computational simulations, data analysis, interpretation and writing. H.D.M.C. and I.R.A.M. collaborated intellectually with the conception, structuring, and orientation of experiments. All authors have read and agreed to the published version of the manuscript.

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.

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