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Ferulic acid derivatives inhibiting *Staphylococcus aureus* tetK and MsrA efflux pumps

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ABSTRACT

Background: Bacterial resistance to multiple drugs has recently emerged as a serious health problem. Concomitantly, the characterization of new substances with potential antimicrobial activity has been less frequent in the drug development industry. The overexpression of genes encoding efflux pumps that expel antimicrobial drugs from the intracellular environment, lowering these to subinhibitory concentrations, are among the resistance mechanisms predisposing microorganisms to high drug resistance. *Staphylococcus aureus* is a bacterium found in the normal microbiota of the skin and mucous membranes, and is an opportunistic microorganism capable of causing infections with high rates of morbidity and mortality. TetK is an efflux pump characterized by its ability to provide bacterial resistance to antibiotics from the tetracycline class. This study aimed to evaluate the inhibitory effect of ferulic acid and four of its esterified derivatives against resistant *Staphylococcus aureus* strains.
Method: Ferulic acid derivatives were obtained by esterification and then characterized by hydrogen and carbon-13 nuclear magnetic resonance analysis. The minimum inhibitory concentrations (MIC) of ferulic acid and its esterified derivatives, ethidium bromide, and antibiotics were obtained using the microdilution test, while the efflux pump inhibition test was conducted by examining reduction in the MICs.
Results: Propylferulate was seen to reduce the minimum inhibitory concentration (MIC) of both the control substance ethidium bromide and the tested antibiotic, indicating that this compound is promising for the use of efflux pump inhibition of IS-58 strains.
Conclusions: This study provides strong evidence that the molecular basis for this activity is potentially due to the MsrA and TetK efflux pumps. However, further investigations are necessary to prove this hypothesis and elucidate the potentiating mechanism of the modulatory effect.

1. Introduction

Staphylococcus aureus is appointed as a causative agent of infections, especially those associated with catheter and valve implant sites, since, for being associated with the skin, can migrate through the catheter until it reaches the blood circulation, becoming one of the main causes of hospital infections [1]. Bacterial strains resistant to methicillin, termed MRSA, are described as one of the pathogens that present the greatest

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difficulty in treatment in the hospital environment [2].

Studies have demonstrated the development of bacterial resistance to be associated with the indiscriminate use of antibiotics [3, 4]. A widely studied mechanism of bacterial resistance is the extrusion of a compound mediated by an efflux system [5, 6]. In these systems, the bacteria expel drugs against their concentration gradient by using energy, where this extrusion system is mediated by hyphrophobic proteins. The energy needed to expel these drugs can be obtained from the conversion of ATP or ionic gradients [7, 8].

The TetK, NorA [9] and MsrA proteins [10] have been described as proteins from the *S. aureus* bacterium responsible for the efflux of antibiotics. Therefore, *S. aureus* became resistant to tetracycline and erythomycin through an extrusion mechanism, this being the overexpression of efflux proteins located in its plasma membrane [11, 12].

Tetracycline is capable of inhibiting bacterial protein synthesis by forming a link with the 30S ribosome subunit, which blocks the binding of aminoacyl-tRNA, which normally results in the appropriate addition of new amino acids in the protein chain, thus blocking protein formation [13, 14, 15]. Erythromycin, an antibiotic belonging to the macrolide class, acts in the translation and amino acid addition process during bacterial protein synthesis, where the overexpression of the MsrA efflux pump is indicated in bacterial resistance development to this antibiotic [16, 17].

Ferulic acid (AF), 3-(4-hydroxy-3-methoxyphenyl)-2-propenoic acid, a secondary metabolite, belongs to the phenolic compound class, and is considered one of the most common phenolic acids found in natural species, found mainly in some fruits, such as oranges and tomatoes, and also present in cereals, such as rice and corn [18, 19, 20, 21, 22, 23]..

The ferulic acid molecule presents cis-trans isomerism, with the most abundant form in nature being the trans isomer. Both isomers have proven results in the treatment of several pathologies such as cancer, diabetes, neurodegenerative and cardiac diseases, in addition to having antimicrobial, anti-inflammatory and, especially, antioxidant activities, responsible for its main benefits and applications [20, 21]. Due to its ability to interrupt radical chain reactions by resonance, followed by polymerization, ferulic acid offers protection against UV radiation [24, 25, 26]. The investigation of ferulic acid's antimicrobial potential was proposed after indirect studies showed that ferulic acid had a high antibacterial activity against *L. monocytogenes* [27].

Given the above, this study had as its objective to evaluate the antibacterial activity of ferulic acid and its four derivatives, esterified with the methanol (methyl ferulate), ethanol (Ethyl ferulate), propanol (Propyl ferulate) and butanol (Butyl ferulate) alcohols, in tetK and MrsA efflux pump expressing *Staphylococcus aureus* strains, with the aim of establishing relationships between the structure of these compounds and their activities through their action on efflux pump mechanisms.

2. Materials and Methods

2.1. Chemical products

Ferulic acid, 4-hydroxy-3-methoxycinnamic acid, trans-4-hydroxy-3-methoxycinnamic acid, ferulic acid Sigma Aldrich absolute methyl alcohol 200, 99.8% Sigma Aldrich absolute ethyl alcohol 200, 99.5% Sigma Aldrich absolute propyl alcohol 200, 99.7% Sigma Aldrich n-butanol alcohol, absolute butyl alcohol 200, 99.8% Sigma Aldrich dicyclohexylcarbodiimide nickel (ii) carbonate hydroxide tetrahydrate Sigma Aldrich Carbonyl Cyanide m-ChloroPhenyl-hydrazone Sigma Aldrich.

2.2. General procedure for the synthesis of esterified derivatives

The procedure used followed the methodology from Narender [28], for the Fischer esterification of ferulic acid with methyl, ethyl, propyl and butyl alcohols. Ferulic acid (60 mg, 0.3092 mmol) was mixed with the respective alcohols (10 mL: MeOH, EtOH, PropOH and ButOH) and dicyclohexylcarbodiimide (DCC, 54 mg, 0.2621 mmol) in catalytic quantities of 4– N, N–dimethylaminopyridine (DMAP). The reaction mixture was maintained under reflux and magnetic stirring with heating at 50°C for 4 h. Following this period, the N, N-dicyclohexylurea formed was filtered off, the solvent was removed on a rotary evaporator at room temperature, and the crude residue was purified with a chromatographic column (silica gel, hexane/EtOAc: 70/30).

The synthesized products and ferulic acid were characterized by ^{1}H and ^{13}C NMR, including the DEPT 135° technique. The analyzes were performed on a Bruker Avance DPX 300 spectrometer, operating at the frequency of 300 MHz for ^{1}H and 75 MHz for ^{13}C. The spectra were obtained in a CD_3OD solvent and the chemical shifts (δ) were expressed in ppm, with tetramethylsilane (TMS) being used as an internal standard. The signal multiplicities in ^{1}H NMR were indicated following the convention: s (singlet), sl (broad singlet), d (doublet), dd (double doublet), t (triplet), q (quartet) and m (multiplet).

2.3. Substance preparation

The substances were initially solubilized in dimethyl sulfoxide (DMSO), then diluted in water for the microbiological tests. The DMSO concentration contained in the samples is not considered toxic to cells, this being below 10%. The efflux pump inhibitor Carbonyl Cyanide m-ChloroPhenyl-hydrazone (CCCP) was used, after its dissolution in 50% Methane/Water and concentration adjustment to 1024 μg/mL.

The antibiotic norfloxacin, specific for the NorA pump, was used. This was initially dissolved in DMSO, adjusting the concentration to 10 mg/mL, being subsequently diluted in water, decreasing the concentration to 1024 μg/mL. Ethidium bromide was diluted in water to a concentration of 1024 μg/mL.

2.4. Natural material origin and preparation

First, the substances were solubilized in a small amount of dimethyl sulfoxide (DMSO), only to favor the dissolution mechanism. Thereafter the substances, were diluted in distilled water. Below 10% v/v, DMSO concentrations are not considered toxic to cells [29].

2.5. Culture media

Heart Infusion Agar (HIA, Difco laboratorises Ltda.) and Brain Heart Infusion (BHI, difco Laboratories Ltda.) culture media, prepared according to the procedures described by the manufacturers, were used at a concentration of 10% v/v.

2.6. Microorganisms

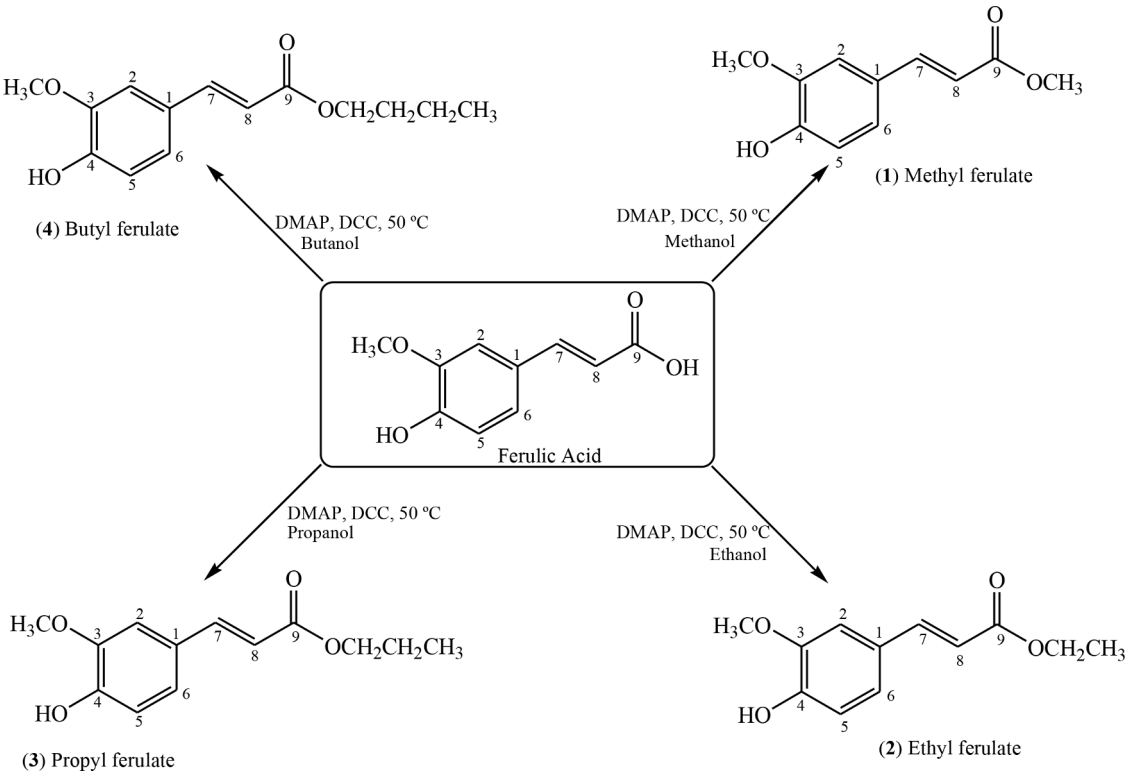
The RN4220 and IS-58 S. aureus strains, resistant to tetracycline and azithromycin by the tetK and Mrs(A) efflux proteins, respectively, were used. All strains were initially kept on agar, then transferred to a stock and kept in Heart Infusion Agar slants (HIA, Difco) at 4 °C.

2.7. Origin and preparation of the antibiotics and ethidium bromide

The antibiotics tetracycline, specific for the tetK pump, and azithromycin, specific for the Mrs(A) efflux pump, were used. The antibiotics were initially dissolved in DMSO to a concentration of 10 mg/mL and, subsequently, diluted in water, decreasing the concentration to 1024 μg/mL. Ethidium bromide was diluted directly in water, until the 1024 μg/mL concentration was reached. Both the antibiotics as well as ethidium bromide were obtained from Sigma-Aldrich.

2.8. Inoculum preparation and standardization

Test tubes containing sterile saline solution were used to bring a small amount of the inocula to a concentration corresponding to 1.5 ×



Scheme 1. Synthesis of esterified ferulic acid derivatives.

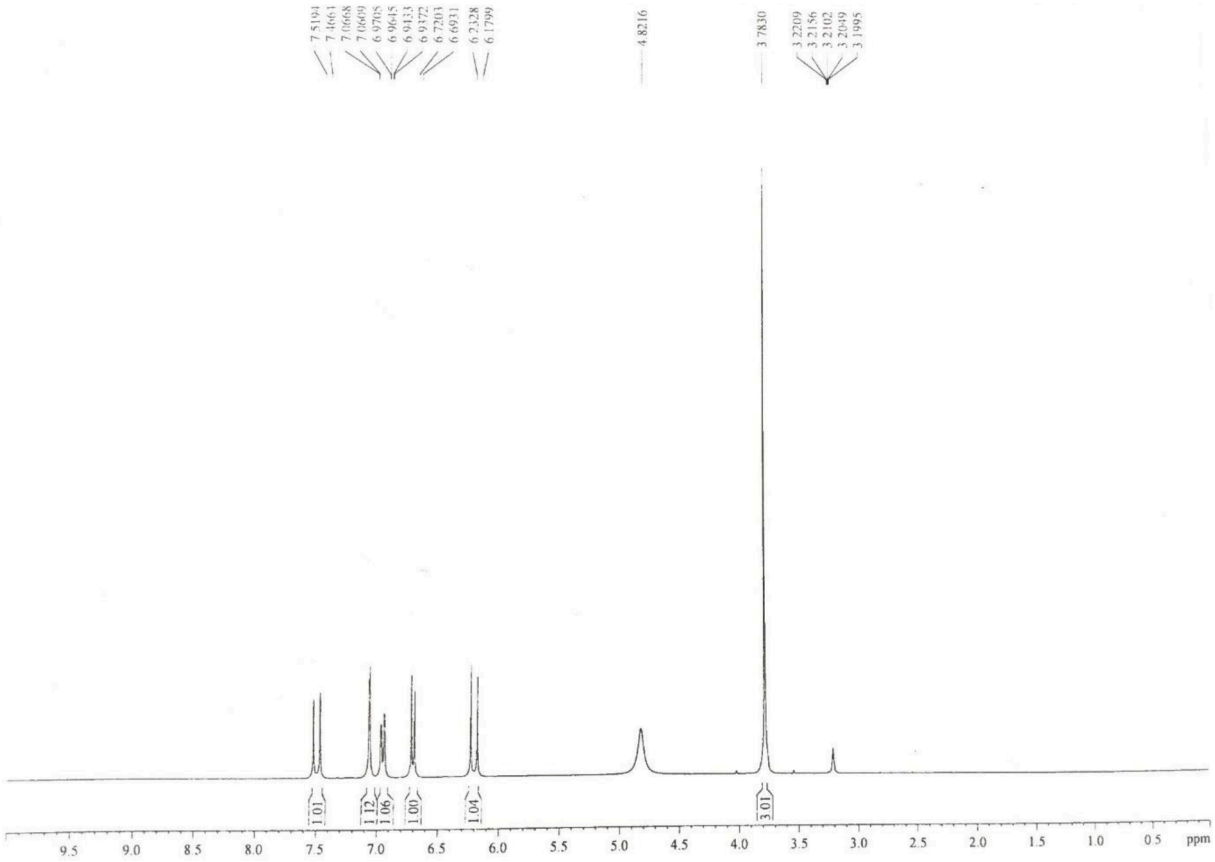


Fig. 1. ^{1}H NMR spectral (300 MHz, CD_3OD) of the ferulic acid

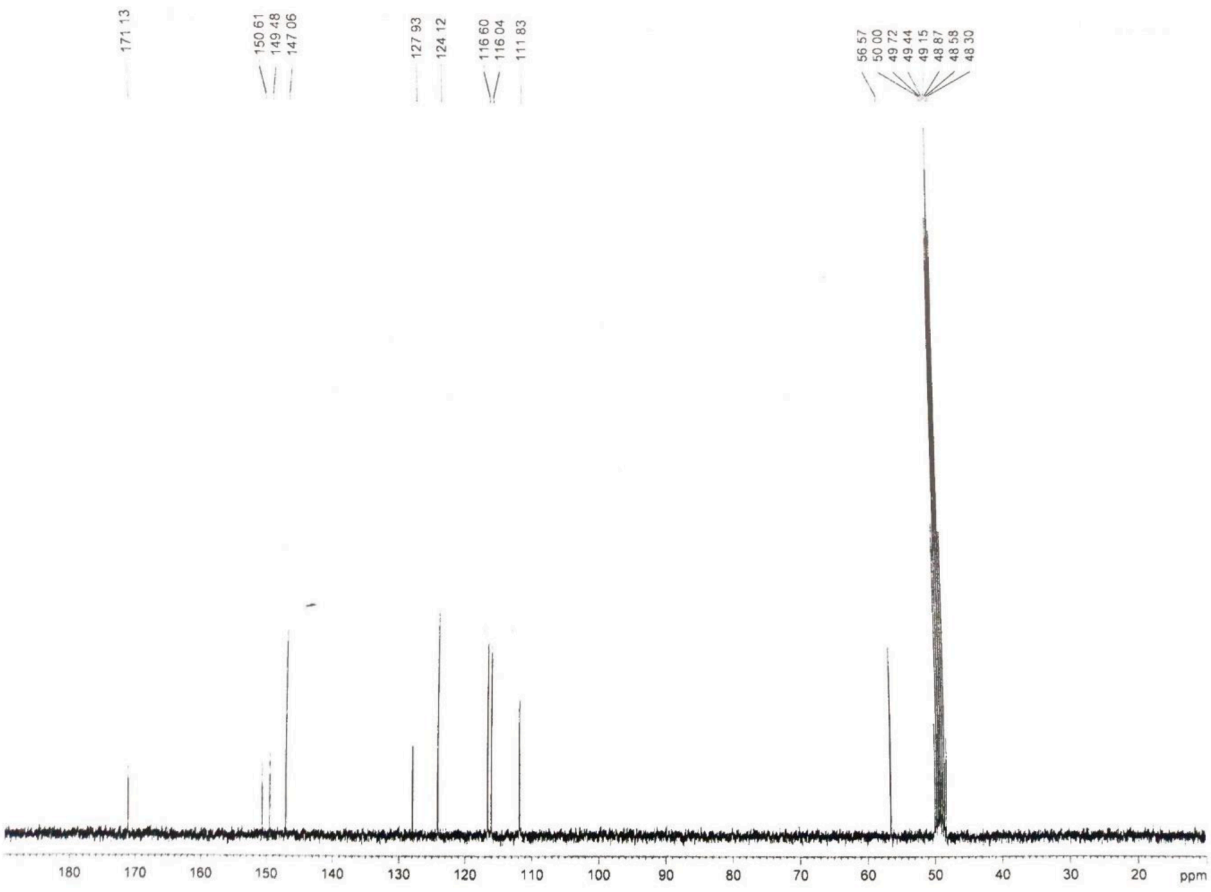


Fig. 2. ^{13}C NMR spectral (75 MHz, CD_3OD) of the ferulic acid.

10^8 CFU (Colony Forming Units), which were used both in the MIC test of the substances and the efflux pump inhibition assays.

2.9. Minimum Inhibitory Concentration Assays

The minimum inhibitory concentration of ferulic acid and its esterified compounds were tested to confirm the level of bacterial resistance reversal, with the aim of analyzing whether this mechanism was due to the presence of a pump. Inocula in saline solution with a bacterial concentration corresponding to 1.5×10^8 CFU (Colony Forming Unit) were distributed in eppendorfs, using 100 μL of the inoculum and 900 μL of 10% v/v BHI liquid culture medium. The eppendorf content was then horizontally distributed into a standard 96-well microdilution plate, with 100 μL in each well, for a total of 10 wells. Subsequently, the substances were microdiluted (1:1) up until the penultimate cavity. Nothing was added to the last cavity, with this well being defined as the growth control. The concentrations range from 1024 μg/mL to 0.5 μg/mL [30].

After 24h, the plates were read by visualizing the medium color change using a 20 μL aliquot of resazurin (7-hydroxy-3H-phenoxazine-3-one 10-oxide). Resazurin has the characteristic of changing the medium color from blue to red in the presence of bacterial growth and remaining in blue in the absence of bacterial growth. The tests were performed in triplicates.

2.10. Efflux pump inhibition assays by the MIC reduction of ethidium bromide and antibiotics

A preparation similar to that described for the MIC assay was followed, however, 150 μL of the inocula were added to eppendorfs, plus the investigated substance with a volume corresponding to its sub-inhibitory concentration (MIC/8), and the final volume was completed to 1.5 mL. For the control standard, 150 μL of the inocula were added to an eppendorf and its volume was made up to 1.5 mL with 10% BHI solution. Then, eppendorf solutions were vertically distributed in 96-well microdilution plates, with 100 μL of the eppendorf content being transferred to each well. Thereafter, the microdilution (1:1) of ethidium bromide or the antibiotic was performed with 100 μL of the compound, distributed up to the penultimate cavity. No solution was added to the last well, as this was the growth control. Well concentrations ranged from 1024 μg/mL to 0.5 μg/mL. After 24h, the plates were read by visualizing the medium color change, characterized by the addition of 20 μL resazurin (7-hydroxy-3H-phenoxazine-3-one 10-oxide). Experiments were performed in triplicates.

The MIC decrease of ethidium bromide or the specific antibiotic is suggested to be indicative of efflux pump mediated bacterial resistance inhibition, this being a selective test for strains carrying an efflux pump [36].

2.11. Statistical analysis

Each experiment was performed in triplicates, and the results were normalized by calculating their geometric means. Error deviation and standard deviation of geometric means were revealed. Statistical analyses were performed using the GraphPad Prism 5.02 software (GraphPad Software, Inc., La Jolla, CA, USA). Differences between treatments with antibiotics in the absence or presence of derived compounds were examined using a One-way analysis of variance (ANOVA). Significant differences were analysed by Tukey's post hoc test and were considered statistically significant when $p < 0.0001$.

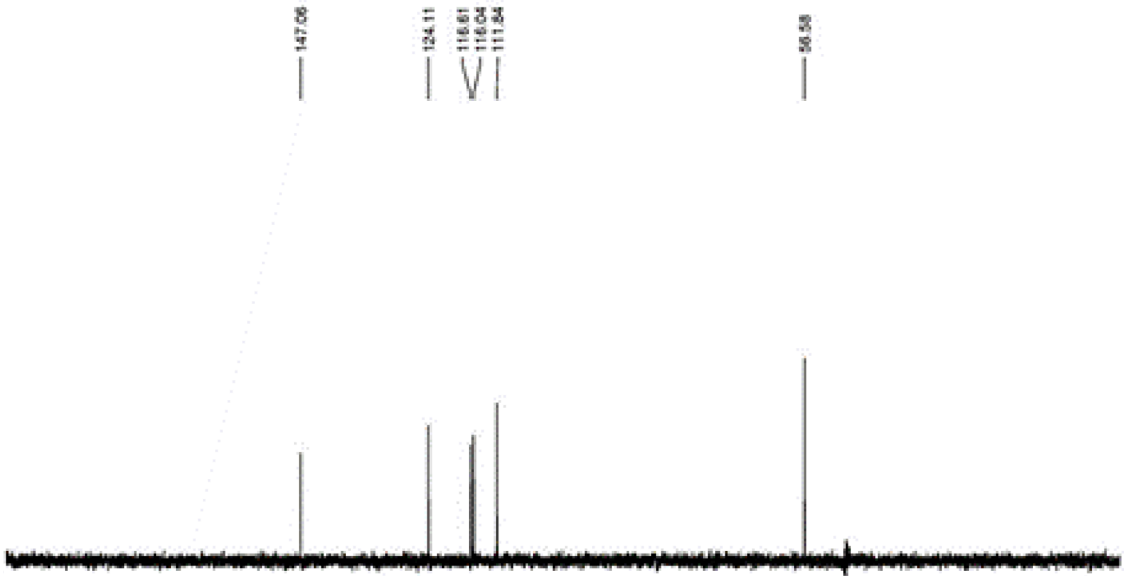


Fig. 3. ^{13}C NMR – DEPT 135 spectral (75 MHz, CD_3OD) of the ferulic acid.

Table 1
Spectral data for ferulic acid obtained under the conditions: ^{1}H NMR (300 MHz, CD_3OD) and ^{13}C NMR (75 MHz, CD_3OD).

Data Carbon	Multiplicity	δ_C (ppm) Analysis	Yamadio et al	δ_H (ppm) Analysis	Yamadio et al
1	C	127.9	127.6	-	-
2	CH	111.8	111.6	7.1 (d, J = 1.7 Hz	7.5 (d, J = 1.7 Hz)
3	C	149.5	150.1	-	-
4	C	150.6	149.6	-	-
5	CH	116.0	116.4	6.7 (d, J = 8.0 Hz)	7.6 (d, J = 8.0 Hz)
6	CH	124.1	123.8	6.9 (dd, J = 8.0 e J = 1.7 Hz)	7.1 (dd, J = 8.0 e J = 1.7 Hz)
7	CH	147.0	149.1	7.5 (d, J = 16.0 Hz)	6.3 (d, J = 16.0 Hz)
8	CH	116.6	115.6	6.2 (d, J=16.0 Hz)	6.3 (d, J=16.0 Hz)
9	C	171.0	171.1	-	-
OCH_3-3	CH_3	56.6	56.3	3.8 (s)	3.9 (s)

3. Results

3.1. Ferulic acid esters

The synthetic reaction method caused structural changes in the carboxylic group from ferulic acid (Scheme 1) as expected, with ^{1}H NMR (Fig. 1), ^{13}C NMR (Fig. 2) and ^{13}C NMR - DEPT 135° (Fig. 3) analyzes for ferulic acid being in accordance with literature data [28], as seen in Table 1, in which the displacements (δ in ppm) and coupling constants (*J*) confirm the substrate identification. The good quality of the spectra indicate the considerable purity degree of the derivatives, which reflect the efficiency of the synthesis of the derivatives. The hydrogenation pattern of the carbons was determined by the ^{13}C NMR - DEPT 135° technique, in combination with the variation of the signal multiplicities in the ^{1}H NMR spectrum (Fig. 4). The alkyl groups (methyl, ethyl, propyl and n-butyl) incremented in the structure of ferulic acid, resulting from the esterification reactions, were confirmed by the appearance of characteristic signals in the ^{13}C NMR - DEPT 135° spectra (Figure 4) of the derivatives: methyl ferulate (1), ethyl ferulate (2), propyl ferulate (3) and n-butyl ferulate (4), since only carbonyl carbon was been altered.

3.2. Microbiological assays: minimum inhibitory concentration of the compounds

Data analysis revealed that all compounds obtained MIC values greater than 1024 μg/mL, indicating these do not present clinically relevant activity against the tested strains [31]. Demonstrated that thymol, a compound similar to ferulic acid, was able to affect the integrity of the bacterial membrane leading to cell death [30].

3.3. Effects on S. aureus efflux mechanism

The butyl ferulate compound showed a non-significant MIC reduction from 16 μg/mL to 12 μg/mL with the RN-4220 strain. When associated with CCCP, an ethidium bromide MIC reduction from 16 μg/mL to 8 μg/mL was observed. As for the IS-58 strain, only the propyl ferulate compound, at subinhibitory concentrations in association with ethidium bromide, presented a MIC reduction from 16 μg/mL to 12.7 μg/mL; the remaining compounds obtained antagonistic effects when associated with ethidium bromide (Figures 5 and 6).

3.4. Antibiotic potentiating effects over tetK and MrsA pumps

The ethyl ferulate and propyl ferulate compounds showed, at subinhibitory concentrations in association with erythromycin, MIC reductions from 161 μg/mL to 128 μg/mL against the RN-4220 strain. However, ferulic acid presented greater synergism than the esterified compound series, reducing the MIC from 161 μg/mL to 64 μg/mL. In terms of the IS-58 strain, a MIC reduction from 101.6 μg/mL to 50.8 μg/mL for propyl ferulate, and a reduction from 101.6 μg/mL to 80.6 μg/mL for buthyl ferulate was observed. In both tests, a significant antibiotic MIC reduction occurred when these were in association with CCCP (Figures 7 and 8).

Antibiotic MIC reduction is also a method used to assess pump inhibition, however, it is not as conclusive as using ethidium bromide, since other resistance mechanisms exist when only the antibiotic MIC inhibition is investigated [32].

3.5. Statistical Analysis of Microbiological Results

The results were expressed as the geometric mean ± standard

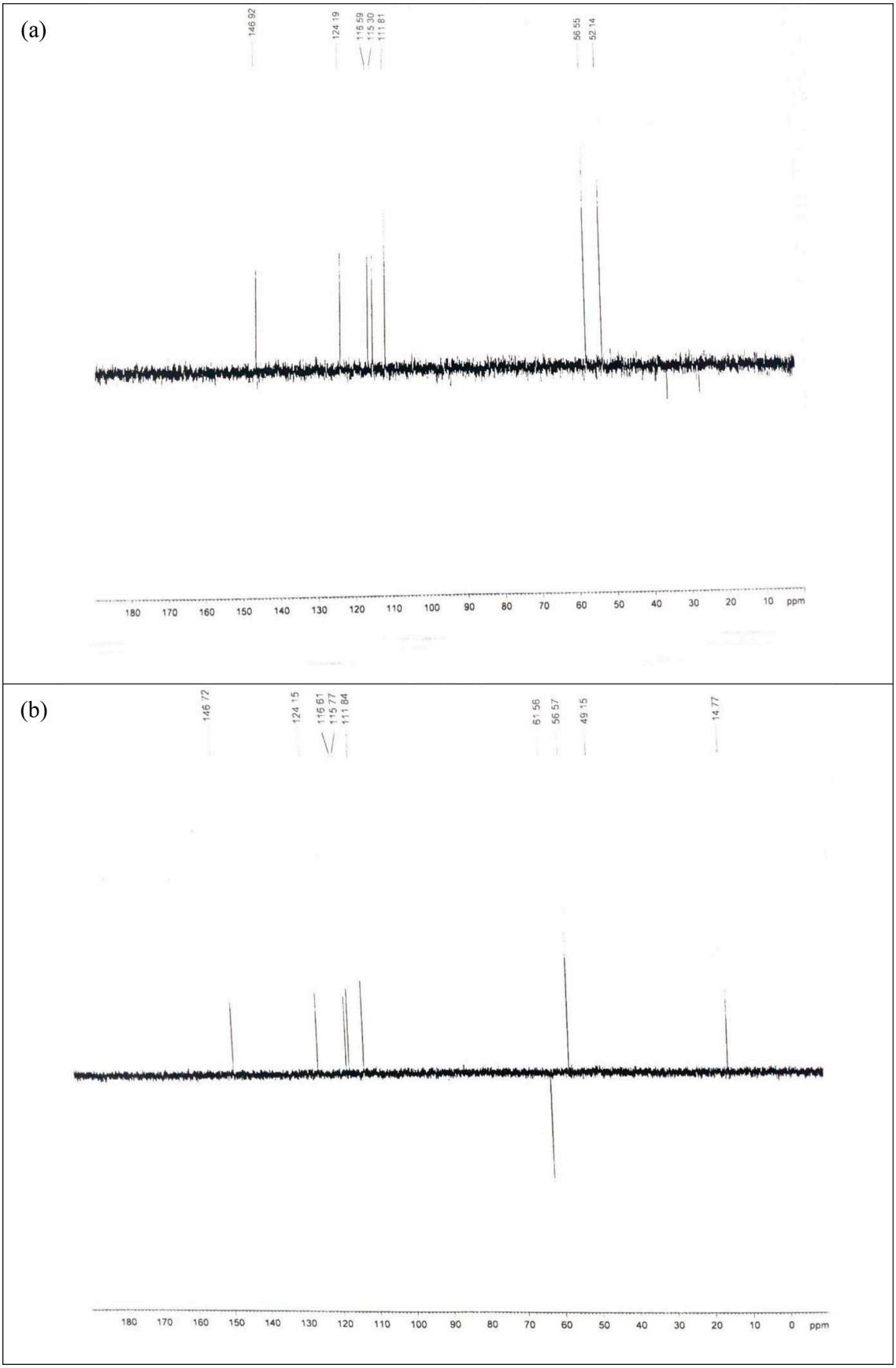


Fig. 4. ^{13}C NMR – DEPT 135 (75 MHz, CD_3OD). (a) Methyl ferulate. (b) Ethyl ferulate. (c) Propyl ferulate. (d) n-butyl ferulate.

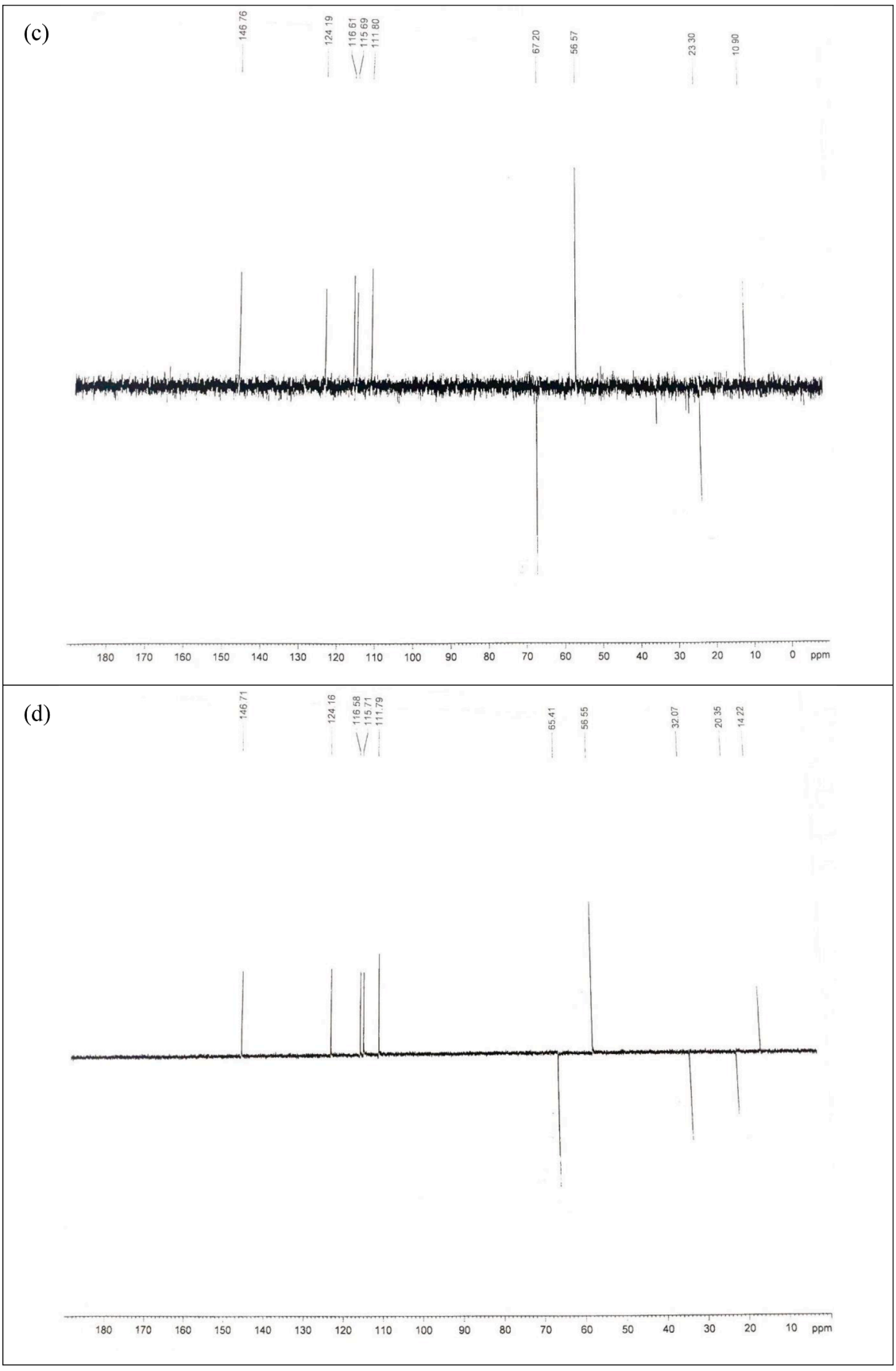


Fig. 4. (*continued*).

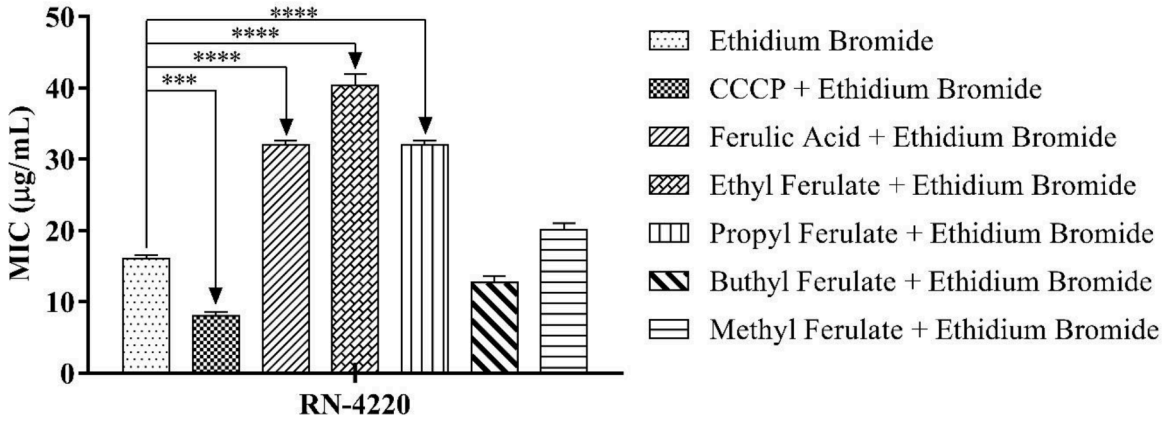


Fig. 5. Minimum Inhibitory Concentration (MIC) of ethidium bromide alone or in association with the compounds under analysis (ferulic acid and its esterified derivatives) against S. *aureus* RN-4220 strains. **** $p < 0.0001$ indicates significant differences between groups. Statistical significance was determined by a one-way ANOVA and Tukey's post-hoc test.

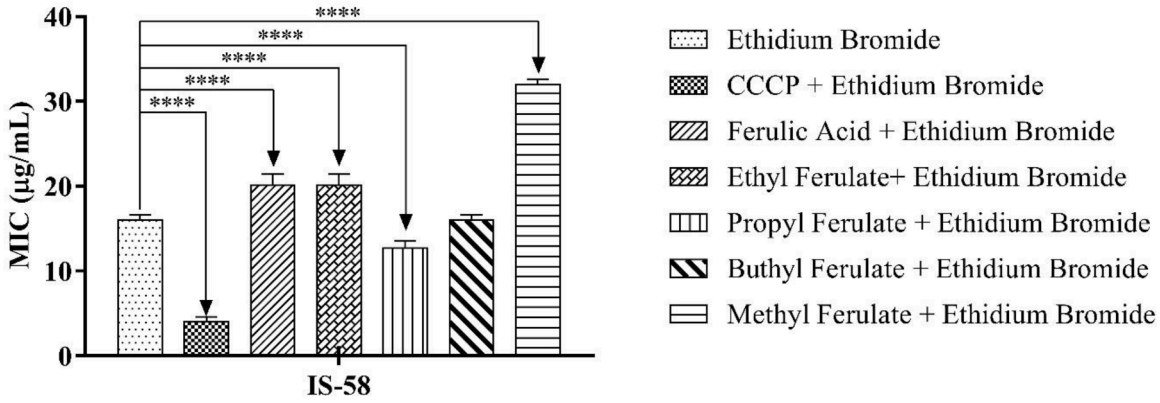


Fig. 6. Minimum Inhibitory Concentration (MIC) of ethidium bromide alone or in association with the compounds under analysis (ferulic acid and its esterified derivatives) against *S. aureus* IS-58 strains. **** $p < 0.0001$ indicates significant differences between groups. Statistical significance was determined by a one-way ANOVA and Tukey's post-hoc test.

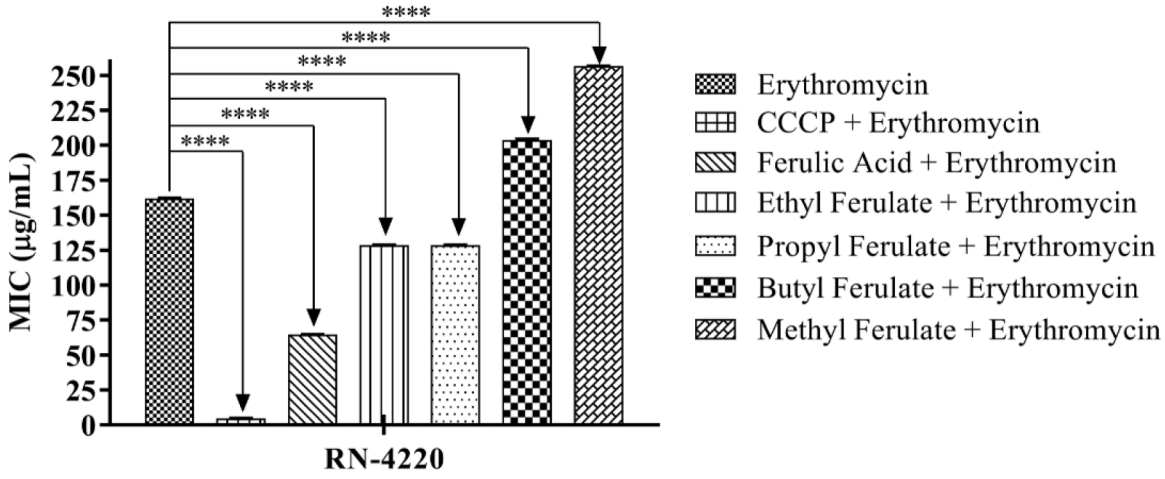


Fig. 7. Minimum Inhibitory Concentration (MIC) of erythromycin in association with the studied compounds (ferulic acid and its esterified derivatives) against the multiresistant RN-4220 *S. aureus* strain **** $p < 0.0001$ indicates significant differences between groups. Statistical significance was determined by a one-way ANOVA and Bonferroni's post hoc test.

deviation, statistically evaluated through an analysis of variance (ANOVA), followed by Tukey's post hoc test, using the GraphPad Prism 7.0 software. Differences were considered significant when $p < 0.0001$.

4. Discussion

Ethidium bromide is a compound that is extruded from the bacterial cell through the efflux pump mechanism and is, thus, said to be an

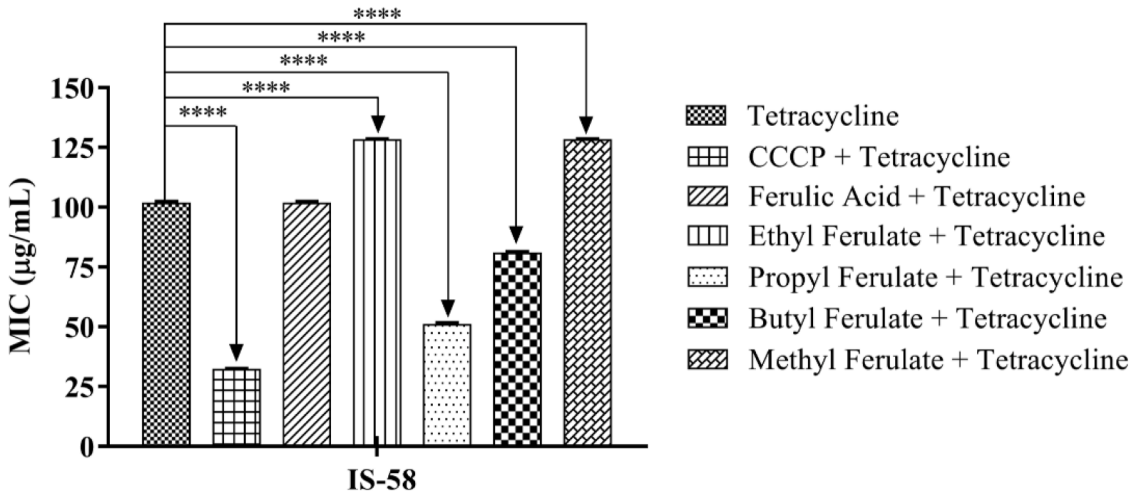


Fig. 8. Minimum Inhibitory Concentration (MIC) of tetracycline in association with the studied compounds (ferulic acid and its esterified derivatives) against the multiresistant IS-58 *S. aureus* strain **** $p < 0.0001$ indicates significant differences between groups. Statistical significance was determined by a one-way ANOVA and Bonferroni's post hoc test.

instrument used to evaluate the efficiency of these proteins [33, 34].

A correlation between the MIC reduction of ethidium bromide or antibiotics and the process of inhibition of bacterial resistance, mediated by efflux pump mechanisms, has been known to exist, with studies reporting that a MIC reduction ≥ 3 would be indicative of a resistance mechanism mediated by an efflux pump, when a potential inhibitor is associated with the pump's antibiotic substrate or ethidium bromide [35]. However, the results from this study did not show such an expressive MIC reduction, suggesting the compounds may only be capable of affecting the integrity of the plastic membrane and inhibit bacterial resistance by other mechanisms.

The MIC reduction of tetracycline or ethidium bromide, in the presence of the carbonyl cyanide m-chlorophenylhydrazone proton pump inhibitor (CCCP), is indicative of an efflux pump reversal [36]. The CCCP compound helps to elucidate the efflux system, since it acts as an energy-dependent decoupler preventing antibiotic efflux. Therefore, this is a positive control widely used to prove the existence of an efflux pump in a given strain, as was seen in the present study. However, given the results obtained herein, we believe ferulic acid and its derivatives act less so directly and more so indirectly on efflux proteins, that is, they may act on the bacterial plasma membrane or even secondarily to facilitate antibiotic entry.

Lipidsoluble substances present the possibility of altering membrane permeability, making these more susceptible to penetration by various substances [30]. Changes in membrane permeability may hinder or facilitate the entry of an antibiotic into the bacterial cell [37]. The compounds hypothesized as bacterial resistance inhibitors, ferulic acid and its derivatives, have a polar hydroxyl group, allowing for energy dissolution and favorable entropy, however, further studies are needed to prove this hypothesis, where these may associate with the bacterial plasma membrane, and may be able to enter the bacterial cell, a mechanism that can be further favored by the nonpolar structure of the benzene ring present in the molecule [38]. Signals membrane damage as a likely reason for pump inhibition, since efflux proteins are transmembrane proteins sensitive to changes in this segment [39].

It was observed in this study that despite similar chemical structures, the compounds had distinct biological activities. Synthesized compounds can improve the pharmacological properties and increase the therapeutic benefits of antimicrobial compounds capable of reversing bacterial resistance [40]. However, some compounds presented antagonistic properties to the bacterial resistance reversal process, acting by contributing to bacterial efflux. Moreover, the antagonism was seen to be more pronounced with ethidium bromide, raising the hypothesis that the esterified compounds may protect the bacteria from ethidium bromide toxicity.

5. Conclusion

Ferulic acid and some of its esterified derivatives presented a significant capacity for reducing the MIC of the antibiotic, however, the inhibition of an efflux pump mechanism could not be proven, thus the assumption of structural and/or functional damage to the cytoplasmic membrane was raised. Propyl ferulate showed a MIC reduction for both ethidium bromide and the antibiotic against the IS-58 strain, making propyl ferulate a promising compound to be used in efflux pump inhibition. However, further studies are necessary to prove this hypothesis and elucidate the mechanism which led to the restoration and enhancement of the reference antibiotic in this study.

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.

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