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Research Article

Antimicrobial and Antibiofilm Activities of 4,5-Dihydro-1H-pyrazole-1-carboximidamide Hydrochloride against *Salmonella* spp.

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In the present study, the antimicrobial and antibiofilm activities of two 4,5-dihydro-1H-pyrazole-1-carboximidamide hydrochloride, (trifluoromethyl) phenyl-substituted (compound **1**) and bromophenyl-substituted (compound **2**), were evaluated against four *Salmonella* spp. serotypes through broth microdilution and biofilm-forming activity. Further, the cytotoxicity of the compounds was evaluated by cell viability assays using cultures of HeLa and Vero cell lines, and the mutagenic potential was assessed by the Ames test. In the broth microdilution test, compound **1** inhibited 90% of the strains tested at the minimum inhibitory concentration of 62.5 $\mu g\,mL^{-1}$. Furthermore, both compounds prevented biofilm formation, with a reduction of up to 5.2 $\log_{10}$. HeLa and Vero cells exhibited 100% viability in the presence of compound **1**. In contrast, low cell viability was observed in the presence of 15 $\mu g\,mL^{-1}$ of compound **2**. Furthermore, no mutagenic potential was detected at any of the tested concentrations of compound **1**.

1. Introduction

Salmonellosis is an enteric infection caused by bacteria of the genus *Salmonella* and is among the primary causes of food-borne illness and is a major public health problem worldwide [1, 2]. According to the Ministério da Saúde [3], salmonellosis symptoms include fever, abdominal pain, diarrhea, nausea, and vomiting while immunocompromised individuals present a rapid progression to septicemia that can lead to death [4]. Data from the Centers for Disease Control and Prevention (CDC) estimate that approximately 1.35 million illnesses and 420 deaths annually occur due to non-typhoidal *Salmonella* annually in the United States [5]. The costs of treating salmonellosis have a great economic impact on the medical, food, and agricultural industries. Millions of dollars are spent each year on food-borne infections, justifying research for control and prevention [6, 7].

Studies have shown that *Salmonella* infection occurs following ingestion of contaminated protein-rich food, such as beef, pork, and poultry [4, 8]. Another important factor in *Salmonella* transmission and infection is the ability of this microorganism to adhere and form biofilms on different

surfaces [9]. These biofilms become a source of *Salmonella* dissemination during food processing in industrial and domestic environments [10, 11]. The presence of extracellular matrix confers biofilms with a high resistance to antimicrobial and sanitizing agents, hindering their removal and elimination [10].

In medicinal chemistry, compounds containing pyrazoline substructures and their derivatives have attracted interest due to their promising biological activities, such as antimicrobial, anti-inflammatory, antidepressant, and antitumor activities [12–15]. Different substituents of these moieties have been assayed in the search for more active and selective molecules. Halogenated substituents have been widely used to improve the antimicrobial activity of pyrazole compounds and have been studied by several researchers worldwide [14, 16, 17]. In this context, Sid et al. [18] reported that there is an improvement in lipophilicity with the insertion of bromo-substituted compounds and fluorine atoms and that the latter also provides greater permeability for cellular membranes, facilitating transport in biological systems.

As part of our research interest in synthesis and biological evaluation of heterocyclic compounds [19–28], herein we report the antimicrobial and antibiofilm activities, of two 4,5-dihydro-1H-pyrazole-1-carboximidamide hydrochlorides ((trifluoromethyl) phenyl-substituted and bromophenyl-substituted), against four pathogenic *Salmonella* spp. serotypes.

2. Materials and Methods

2.1. Synthesis of Pyrazoline Hydrochlorides. All the pyrazoline hydrochlorides were synthesized according to the procedure reported by us recently [29] with some modifications (Scheme 1). To a 50 mL vial, a mixture of 3-(hetero) aryl-1-(2-hydroxyphenyl)enones (1.0 mmol), aminoguanidine hydrochloride (0.22 g, 2.0 mmol), and potassium hydroxide (0.11 g, 2.0 mmol) in ethanol (15 mL) were added. The US probe was placed in the reaction vial, which was sonicated for 30 min. After the completion of the reaction, the excess of aminoguanidine hydrochloride was filtered off and in the filtrate 10% hydrogen chloride (15 mL) was added. The mixture was subsequently extracted with chloroform (3×30 mL) and organic phase was dried over $MgSO_4$ and filtered and the solvent was evaporated under reduced pressure. The residue was purified by recrystallization from ethyl acetate-methanol (10 : 1), resulting in the pyrazoline hydrochlorides as crystalline solids.

*2.1.1. 3-(2-Hydroxyphenyl)-5-(4-(trifluoromethyl)phenyl)-4,5-dihydro-1H-pyrazole-1-carboximidamide Hydrochloride (**1**).* Whitish solid; 63% yield; m.p. 294–296°C; ^{1}H NMR (300 MHz, DMSO-d_6): δ (ppm) 9.93 (bs, 1H), 8.04 (bs, 4H), 7.79–7.77 (m, 3H), 7.45–7.43 (m, 2H), 7.38–7.32 (m, 1H), 7.03–7.01 (m, 1H), 6.93–6.88 (m, 1H), 5.98 (dd, 1H, $J = 11.4$, 3.0 Hz, H_X), 4.18 (dd, 1H, $J = 18.5$, 11.4 Hz, H_M), 3.49–3.40 (*m*, 1H, H_A); ^{13}C NMR (75 MHz, DMSO-d_6): δ (ppm) 157.9, 156.9, 153.0, 144.3, 132.7, 129.7, 128.7 (*q*, $J = 31.7$ Hz), 126.3, 126.0 (q, $J = 4.0$ Hz), 124.1 (*q*, $J = 272.4$ Hz), 119.4, 117.1, 115.7, 59.1, 45.9; HRMS (ESI-TOF MS) calculated for $[C_{17}H_{16}F_3N_4O]^+$: 349.1271; found: 349.1252 (Figures S1 and S2 for compound **1** in supplementary materials, pg S2).

*2.1.2. 5-(4-Bromophenyl)-3-(2-hydroxyphenyl)-4,5-dihydro-1H-pyrazole-1-carboximidamide Hydrochloride (**2**).* Yellowish solid; 68% yield; m.p. 277–279°C; ^{1}H NMR (300 MHz, DMSO-d_6): δ (ppm) 9.99 (*s*, 1H), 8.01 (bs, 4H), 7.78–7.75 (m, 1H), 7.61–7.58 (*m*, 2H), 7.37–7.32 (*m*, 1H), 7.19–7.15 (*m*, 2H), 7.03–7.01 (*m*, 1H), 6.93–6.88 (*m*, 1H), 5.85 (dd, 1H, $J = 11.2$, 3.0 Hz, H_X), 4.13 (dd, 1H, $J = 18.4$, 11.3 Hz, H_M), 3.46–3.38 (*m*, 1H, H_A); ^{13}C NMR (75 MHz, DMSO-d_6): δ (ppm) 158.0, 156.9, 152.9, 139.2, 132.7, 132.0, 129.7, 121.3, 119.5, 117.1, 115.8, 58.9, 45.8; HRMS (ESI-TOF MS) calculated for $[C_{16}H_{16}BrN_4O]^+$: 359.0502; found: 359.0492 (Figures S3 and S4 for compound **2** in supplementary materials, pg S3).

2.2. Salmonella Serotypes and Isolates. Fifty *Salmonella* isolates of the Enteritidis, Infantis, Braenderup, and Worthington serotypes isolated from chicken carcasses and belonging to the bacteriology collection of the Laboratory of Applied Microbiology (LMA) of UFGD were tested. Two reference strains, *Salmonella enterica* serotype Enteritidis (ATCC 13076) and *Salmonella enterica* serotype Typhimurium (ATCC 14028), from the American Type Culture Collection were included in all tests.

2.3. Minimum Inhibitory Concentration. The antimicrobial activity of compounds **1** and **2** was determined by the broth microdilution test, following document M07-A9 of the Clinical and Laboratory Standards Institute with some modifications [30].

After cultivation in Trypticase Soy Agar (TSA) at 37°C for 24 h, the bacteria were standardized to 1.5×10^8 colony-forming units (CFU).mL^{-1}. A serial dilution (1 : 2) was performed from the concentration of 1.000 μg mL^{-1} to the concentration of 1.9 μg mL^{-1}. Chloramphenicol was used as reference, considering sensitive ≤8, intermediate 16, and resistant ≥32. A 0.1% triphenyl tetrazolium chloride (TTC) solution was used to interpret the results. The minimum inhibitory concentration (MIC) was defined as the lowest concentration of the compound capable of inhibiting bacterial growth. The MIC50 and MIC90 values were calculated defined as the concentration at which the compounds were able to inhibit 50% and 90% of the total isolates tested. The tests were performed in triplicate at two different time points.

2.4. Bacterial Cell Viability. Based on the results of the antibacterial activity, a representative of each serotype (Braenderup, Enteritidis, Worthington, and Infantis), in addition to *S.* Enteritidis (ATCC 13076) and *S.* Typhimurium (ATCC 14028), were selected to evaluate the impact of compounds **1** and **2** on bacterial cell viability. The compounds were tested at concentrations of 250, 125, and

62.5 μg mL^{-1}. After the 24 h of the broth microdilution test described above, serial dilutions of the compounds were prepared in saline solution (0.85%), and plating on Müeller Hinton agar using the drop plate technique [31]. The plates were incubated for 24 h at 37°C, the number of CFUs was counted, and the results were converted to $\log_{10}$ CFU [32]. The experiment was carried out in triplicate.

2.5. Effect of Compounds 1 and 2 on Biofilm Formation. Considering the antibacterial activity of the compounds **1** and **2**, their effects were evaluated on biofilm formation according to the methodology of Costa et al. [33] with modifications. *Salmonella* strains were cultured in Tryptone Soy Broth (TSB) for 18 h at 37°C under 80 rpm. Subsequently, the cells were centrifuged at 3000 g for 10 min, washed three times with phosphate-buffered saline (PBS), and resuspended in TSB to a concentration of 1×10^8 CFU/mL.

In order to evaluate the effect of compounds on *Salmonella* strains biofilm formation, the compounds were added at the same time of *Salmonella* inoculum. For that, 100 μL of inoculum and 100 μL of compounds 1 and 2 at concentrations 250, 125, and 62.5 μg mL^{-1} were added to the wells of 96-well microplates. The microplates were incubated with orbital shaking at 80 rpm for 48 h. The wells were washed three times with PBS to remove weakly adhered cells and then were scraped using a micropipette tip to remove the biofilms.

The resulting cell suspensions were homogenized by vortexing for 5 min to disaggregate the cells and serial dilutions were prepared, which were plated on Müller Hinton agar using the drop plate technique [31]. Plates were incubated at 37°C for 24 h. After incubation, the CFUs were counted, and results were converted into $\log_{10}$ CFU. The experiment was conducted in triplicate at two different time points.

2.6. Cytotoxicity Assay. The cytotoxicity of compounds **1** and **2** was evaluated following the methodology proposed by Capoci et al. [34] using HeLa and Vero cells. HeLa and Vero cells were grown in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS) and in RPMI 1640 medium, respectively. Cell concentrations were adjusted to 2×10^5 cells.mL^{-1}, added to 96-well microplates, and incubated under 5% CO_2 at 37°C for 24 h. After the incubation period, adherent cells were washed and treated with the compounds at concentrations of 500, 150, and 15 μg mL^{-1} and then incubated under the same conditions. The culture media and the cell suspensions were used as the growth controls (blanks).

Cytotoxicity was evaluated by measuring the reduction of MTS (3-[4, 5-dimethylthiazol-2-yl]-5-[3-carboxymethoxyphenyl]-2-[4-sulfophenyl]-2H-tetrazolium) in DMEM without phenol red. After a 3 h incubation at 37°C, the formazan absorbance was measured at 490 nm using a microplate reader. The percentage of cell viability (CV%) was calculated using the following formula:

$$\text{CV\%} = \left(\frac{\text{At}}{\text{Ac}}\right) \times 100, \quad (1)$$

where At and Ac correspond to the absorbance of the tested substance and the control (untreated cells), respectively [35]. The cytotoxicity of the compounds 1 and 2 was presented as the average of three independent experiments with three replicates.

2.7. Mutagenicity Assay. Mutagenicity was assessed by the Ames test, a microsuspension method described by Kado et al. [36]. The *Salmonella* Typhimurium TA98 and TA100 strains were standardized to a concentration of 1×10^8 CFU.mL^{-1}. The assays were performed in the presence and absence of the S9 exogenous microsomal fraction.

To each tube, 50 μL of phosphate buffer or the S9 fraction, 5 μL of compound **1** at different concentrations (5000, 1500, 500, 150, and 50 μg.plate^{-1}), and 50 μL of bacterial suspension were added. After the tubes were preincubated for 90 minutes at 37°C, 2 mL of top agar was immediately added, and the mixture was poured onto plates with minimal agar. The plates were incubated at 37°C for 48–66 hours, and the revertant colonies (His+) were counted. DMSO was used as a negative control, while 4-nitrophenylenediamine (NPD) (10 μg.plate^{-1}) was used as positive control for the TA98 strain and sodium azide (2.5 μg.plate^{-1}) in the absence of S9 and 2-aminoanthracene in the presence of S9 were used as positive controls for the TA100 strain. The mutagenicity index (MI) was calculated according to the following formula:

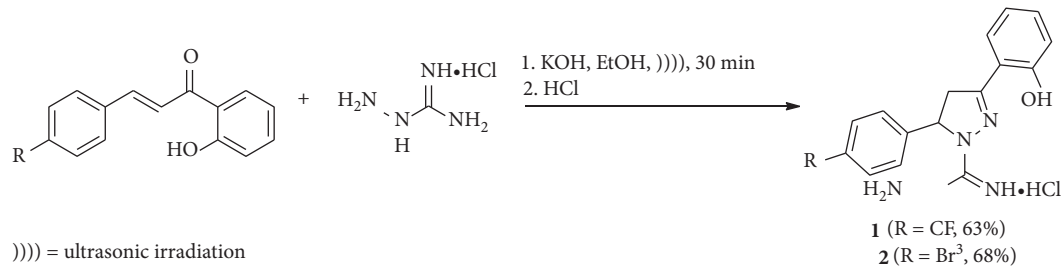
$$\text{MI} = \left(\frac{\text{no.of induced revertants}}{\text{no.of pontaneous revertants}}\right). \quad (2)$$

The compound was considered to have mutagenic potential when the MI was equal to or greater than 2.0 for at least one of the concentrations tested and when there was a dose-response relationship between the concentrations and the number of induced revertants. Concentrations with an MI below 0.7 were considered cytotoxic [37].

2.8. Statistical Analysis. The results of the biofilm and bacterial cell viability tests were analyzed by GraphPad Prism® 5.01, using analysis of variance (ANOVA), and the means were compared by Tukey's test, with a p value < 0.05 considered significant. The Ames test data were analyzed by ANOVA using the statistics program Salanal® 1.0, with a p value < 0.05 considered significant.

3. Results and Discussion

A series of amidino pyrazolines were synthesized and were subjected to a screening of biological activities. Two of these compounds showed promising results and were selected to continue further biological tests. The selected compounds were named as follows: compound **1**: 3-(2-hydroxyphenyl)-5-(4-(trifluoromethyl)phenyl)-4,5-dihydro-1H-pyrazole-1-carboximidamide hydrochloride and compound **2**: 5-(4-bromophenyl)-3-(2-hydroxyphenyl)-4,5-dihydro-1H-



Scheme 1: Ultrasound-promoted synthesis of pyrazoline hydrochlorides.

pyrazole-1-carboximidamide hydrochloride (Scheme 1). The synthesis was accomplished by cyclocondensation reactions between facile prepared chalcones (3-(hetero) aryl-1-(2-hydroxyphenyl)enones) and commercially available aminoguanidine hydrochloride, using ethanol as solvent and potassium hydroxide as base, promoted by ultrasonic irradiation. Compounds **1** and **2** were isolated in 63 and 68% of yields, respectively, after liquid-liquid extraction and purification by recrystallization. The success of the synthesis is evidenced by the results from nuclear magnetic resonance (NMR) and high-resolution mass spectrometry (EMAR) analyses. For compound **1**, the ^{1}H NMR spectrum shows a broad singlet at 9.93 ppm attributed to the OH hydrogen and another broad singlet at 8.04 ppm due to the four equivalent hydrogens attached to the nitrogens. The eight arylic hydrogens generate five multiplets at 7.79–7.77 (3H), 7.45–7.43 (2H), 7.38–7.32 (1H), 7.03–7.01 (1H), and 6.93–6.88 (1H). The hydrogen attached to the C5 of the pyrazoline ring (H_X) gives a doublet of doublets at 5.98 ppm due to the spin coupling with the diastereotopic hydrogens H_A and H_M ($J = 11.4$ and 3.0 Hz). H_M and H_A, attached to the C4 of the pyrazoline ring, appear as a doublet of doublets at 4.18 ($J = 18.5$ and 11.4 Hz) and multiplet at 3.49–3.40 ppm (partially covered by the peak from residual H_2O from the DMSO-d_6), respectively. The identity of compound 1 is also confirmed by the ^{13}C NMR spectrum that shows 16 signals at the expected chemical shifts for the 16 nonequivalent carbons present in the structure. The ^{13}C NMR spectrum also shows three quadruplets due to the C-F couplings. Unequivocally, the HRMS analyses confirm the identity of the proposed elemental composition for compound **2** because the comparison between the theoretical exact m/z for $[C_{17}H_{16}F_3N_4O]^+$ (m/z 349.1271) and the experimental one (m/z 349.1252) gives only 5.30 ppm of discrepancy. For compound **2**, the ^{1}H NMR spectrum shows the OH hydrogen and the four NH hydrogens as broad singlets at 9.99 and 8.01 ppm, respectively. The spectrum shows six multiplets at 7.78–7.75 (1H), 7.61–7.58 (2H), 7.37–7.32 (1H), 7.19–7.15 (2H), 7.03–7.01 (1H), and 6.93–6.88 (1H) due to the eight arylic hydrogens. H_X gives a doublet of doublets at 5.85 ppm due to the spin coupling with the diastereotopic hydrogens H_A and H_M ($J = 11.2$ and 3.0 Hz). H_M and H_A appear as doublet of doublets at 4.13 ($J = 18.4$ and 11.3 Hz) and multiplet at 3.46–3.38 ppm (partially covered by the peak from residual H_2O from the DMSO-d_6), respectively. The ^{13}C NMR spectrum also shows 13 peaks at the expected chemical shifts for the nonequivalent carbons of the proposed structure. Also, the HRMS analyses confirm the identity of the proposed elemental composition for compound **2** because the comparison between the theoretical exact m/z for $[C_{16}H_{16}BrN_4O]^+$ (m/z 359.0502) and the experimental one (m/z 359.0492) gives only 2.60 ppm of discrepancy. The purity of compounds **1** and **2** is evidenced by the narrow range of their melting points and by the cleanness of their ^{1}H NMR spectra.

Compound **1** and **2** were tested against *Salmonella* spp., and both exhibited antimicrobial activity. For compound **1**, MIC50 and MIC90 against all *Salmonella* species were 62.5 μg mL^{-1}. For compound **2**, MIC50 and MIC90 against all *Salmonella* species tested were 125 μg mL^{-1}, with the exception of *S.* Infantis, which was more sensitive to the compound, with MIC50 of 62.5 μg mL^{-1}. With regard to internal control, all *Salmonella* spp. tested resulted in being sensitive to chloramphenicol, with MIC of 4 μg mL^{-1}.

The greater antimicrobial activity of compound **1** was attributed to the presence of fluorine atoms in the molecule that promote increased biological activity due to the intrinsic properties of fluorine, small atomic radius, high electronegativity, and stability [12, 38–41]. The high electronegativity of fluorine may enhance the electrophilicity of neighboring groups in the molecule and the substitution of a methyl group by a trifluoromethyl group, resulting in increased lipophilicity. This lipophilicity provides greater permeability in cell membranes and facilitates the absorption and transport of these molecules within biological systems, improving its properties [42]. Furthermore, literature data describe that the antimicrobial effect of fluorine is associated with the inhibition of intracellular acid, inhibition of the transformation and use of some nutrients, affecting the synthesis and storage of polysaccharides, and inhibition of enzymes related to bacterial pathogenicity, such as catalase, phosphatase, and others [43].

In addition, the effect of the compounds on the number of viable bacterial cells was evaluated and the results showed a significant reduction against all the evaluated serotypes. At a concentration of 62.5 μg.mL-1, compound 1 showed reduction ranging from 3, 3–6, 6 $\log_{10}$ CFU, while at a concentration of 250 μg.mL-1 reduction ranging from 7, 7–11, 39 $\log_{10}$ CFU. Regarding compound 2, the reduction ranged from 0, 5–3 $\log_{10}$ CFU at a concentration of 62.5 μg.mL-1, and from 5, 3–8 $\log_{10}$ CFU at a concentration of 250 μg.mL-1, which corroborate the MIC50 and MIC90 values (Figures 1(a) and 1(b)).

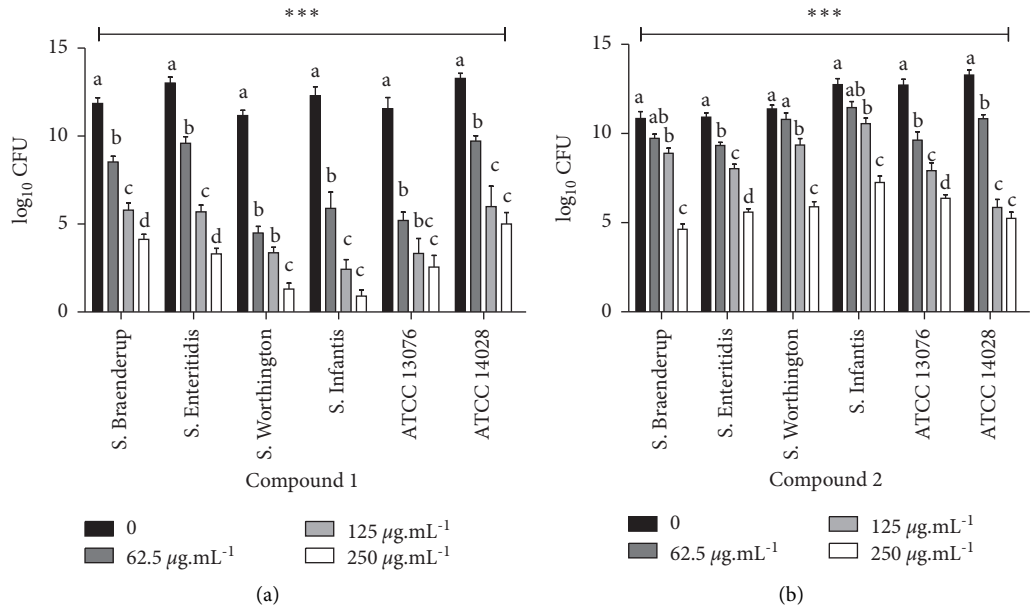


FIGURE 1: Quantification of viable cells in $\log_{10}$ CFU of *Salmonella* spp. treated with different concentrations of 4,5-dihydro-1H-pyrazole-1-carboximidamide hydrochlorides (trifluoromethyl)phenyl-substituted, compound **1** and bromophenyl-substituted, compound **2**, using ANOVA and Tukey's test. $^{***}(p < 0.05)$ indicates a significant difference. Different letters for each concentration represent a significant difference between them by the means test ($p < 0.05$).

As biofilms are more resistant than bacteria in planktonic form, the effect of the compounds on biofilm formation was also evaluated, and compounds 1 and 2 reduced the number of cells present in the biofilm of *Salmonella* spp. in all tested concentrations. At the highest concentration (250 μg.mL-1), compound 1 was able to elicit decrease 2.1–4.9 log10 in the formation of biofilms, while compound 2 at the same concentration decreased 3.8–5.2 log10 (Figures 2(a) and 2(b)).

The antibiofilm activity of the compounds can be attributed to halogenated substituents, which generate changes in relative binding affinities to the bacterial surface. Halogenated substitutes reduce affinity to components of the bacterial structure, such as lipopolysaccharide, and this may facilitate the compounds' ability to cross the outer membrane, enhancing their antimicrobial activity [44, 45]. Additionally, they are known to control biofouling and to be effective in penetration and disinfection of biofilms [46]. Compound **2** showed more highly significant effect in reducing biofilm formation by *Salmonella*, which can be related to the effect of the bromine atom, which possibly promoted the breakdown of the extracellular matrix secreted during the biofilm formation process to allow increased permeability of this compound to these strongly adherent cells. The presence of bromine as an important factor for antibiofilm activity was also reported by Galarraga-Vinueza et al. and Campana et al. [47, 48]. In our study, we observed that fluoro- and bromo-substituted pyrazoline compounds were effective against *Salmonella* spp. in different *in vitro* analyses. The microdilution test enabled the determination of the minimum concentration that inhibited the multiplication of the bacterial cells, while the bacterial cell viability test showed the decrease in CFUs. To the best of our knowledge, there are not results describing regarding the action of such compounds against biofilm formation in the literature, advocating an innovative application of pyrazoline compounds for this purpose.

Due to the antibacterial and antibiofilm activity of our compounds, their cytotoxic potential was tested to examine the potential toxic effects. Compound **1** showed 100% viability for HeLa and Vero cells after 24 h of treatment in all concentrations, indicating that it does not present cytotoxicity *in vitro*. Compound **2**, however, at the lowest concentration reduced viability to 23% and 19% for HeLa and Vero cells, respectively (Figure 3).

In a study by Altintop et al. [14] p-bromophenyl compounds derived from thiazolyl-pyrazolines showed high cytotoxicity, whereas the fluoro-substituted compounds had low cytotoxicity. These data indicate that cytotoxicity is not related to the pyrazoline moiety.

Since it has no cytotoxic potential, compound **1** was subjected to the Ames test to evaluate its mutagenic potential. The *Salmonella* Typhimurium TA98 and TA100 strains were tested and the assays were performed in the presence and absence of the S9 exogenous microsomal fraction (Table 1). This experiment indicated that there was no significant ($p < 0.05$) increase in the number of revertant colonies of *S. typhimurium* TA98 and TA100 strains in the presence and absence of metabolic activation at the concentrations tested (50–5000 μg.plate^{-1}) when compared to the negative control. The concentrations tested also did not present MI ≥ 2, indicating that compound **1** has no potential to cause gene base-pair substitution and *frameshift* mutations. However, at 5000 μg.plate^{-1} cytotoxicity was observed for TA98 in an assay without metabolic activation and for TA100 in the presence and absence of metabolic activation.

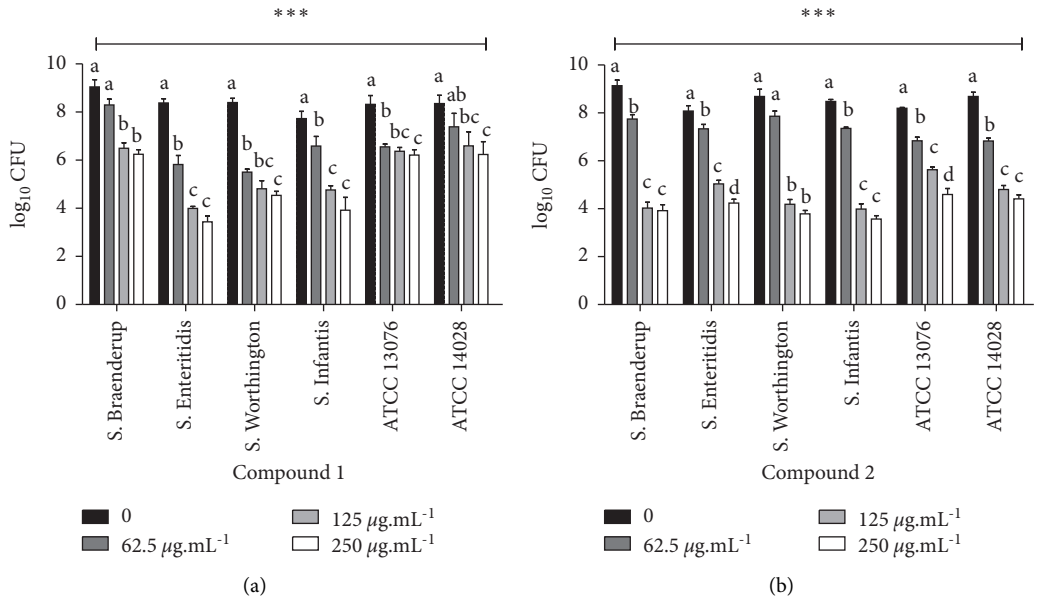


Figure 2: Antibiofilm activity of 4, 5-dihydro-1H-pyrazole-1-carboximidamide hydrochlorides (trifluoromethyl)phenyl-substituted, compound **1** and bromophenyl-substituted, compound **2**, using ANOVA and Tukey's test. ***($p < 0.05$) indicates a significant difference. Different letters for each concentration represent a significant difference between them by the means test ($p < 0.05$).

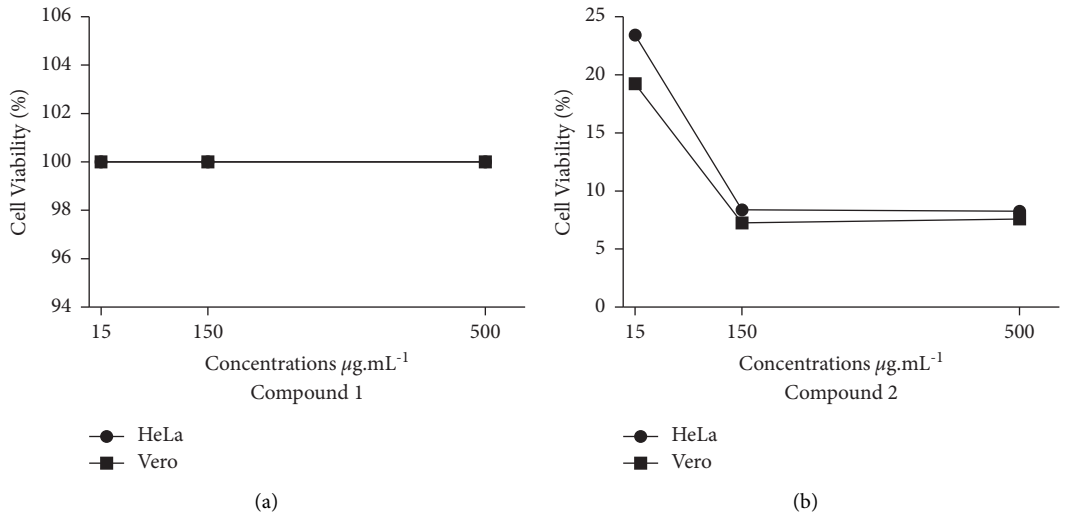


Figure 3: Percentage of cell viability of HeLa and Vero cell lines treated with different concentrations of 4, 5-dihydro-1H-pyrazole-1-carboximidamide hydrochlorides (trifluoromethyl)phenyl-substituted, compound **1**, and bromophenyl-substituted, compound **2**.

Altintop et al. [14] also investigated the mutagenicity of substituted thiazolyl-pyrazoline derivatives against *S.* Typhimurium TA 98 and TA100, with and without metabolic activation, and did not observe any mutagenic potential.

Furthermore, for both compounds, we performed an in silico prediction of the pharmacokinetics properties [49]. For this calculation, we used the open-source software Molinspiration [50] and OSIRIS Property Explorer [51].

Lipinski's rule of 5 [52] predicts that for a potentially drug candidate to present a good pharmacokinetic profile it has to meet some of the following requirements, such as MW (molecular weight) ≤ 500, ClogP ≤5.0 or MLogP ≤4.15, HBA (hydrogen bond acceptors) ≤ 10, and HBD (hydrogen bond donors) ≤ 5. Veber and coworkers [53] introduced other two important criteria for this type of prediction, that is, to have TPSA (topological polar surface area) ≤ 140 Å^2 and 10 or fewer rotatable bonds. Compound **1** and compound **2** satisfied Lipinski and Veber criteria (Table 2). The results from in silico prediction showed that both compounds may present good absorption and permeation after oral administration, turning them good drug candidate.

Table 1: Revertants per plate (mean and standard deviation) of *S.* typhimurium TA98 and TA100 strains after treatment with the (trifluoromethyl)phenyl-substituted compound in the presence and absence of metabolic activation.

Treatment (μg.plate-1)	TA98		TA100	
	−S9	+S9	−S9	+S9
0.0*	11 ± 1	13 ± 2	73 ± 9	80 ± 13
50	11 ± 3	15 ± 1	76 ± 7	91 ± 6
150	11 ± 1	10 ± 3	80 ± 8	89 ± 3
500	13 ± 2	12 ± 5	79 ± 10	103 ± 10
1500	8 ± 1	11 ± 5	68 ± 10	91 ± 7
5000	3 ± 1	7 ± 2	54 ± 4	9 ± 1
C+	295 ± 6[a]	306 ± 8[b]	912 ± 5[c]	987 ± 9[b]

*0.0: negative control: dimethyl sulfoxide (DMSO); positive controls: 4-nitrophenylenediamine (10 μg.plate^{-1})[a]; 2-AA aminoanthracene (0.625 μg.plate^{-1})[b]; sodium azide (2.5 μg.plate^{-1})[c].

Table 2: In silico pharmacokinetics properties predictions for compound.

Property	Value	
	Compound 1	Compound 2
*M*LogP	3.47	3.39
*C*LogP	2.71	2.59
MW	348.33	359.23
tPSA[a] (Å^2)	85.70	85.70
HBA[b]	5	5
HBD[c]	4	4
NRB[d]	4	3

[a]tPSA: topological polar surface area; [b]HBA: H-bond acceptors; [c]HBD: H-bond donors; [d]NRB: number of rotatable bonds.

4. Conclusions

The (trifluoromethyl)phenyl-substituted (**1**) and bromophenyl-substituted (**2**) 4,5-dihydro-1H-pyrazole-1-carboximidamide hydrochlorides assayed in this study exhibited promising antimicrobial activity. Both produced log reductions and were able to reduce biofilm formation. Besides, to these biological activities, compound **1** has displayed no cytotoxic or mutagenic potential. Taking into account challenges and difficulties in design novel and efficient drugs able to control the action of pathogenic microorganisms, these bioactive compounds could be used as alternatives for treatment, disinfection, and sanitizing agents in the food industry. In this sense, future in vivo studies will be necessary to confirm this potential and assess their risk to the human health.

Data Availability

The data used to support the findings of this study are included in the article. In addition, the Supplementary Materials include the spectral data of the compounds.

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this study.

Supplementary Materials

The supplementary information features H1-NMR, C13-NMR spectra of the synthesized compounds (). (*Supplementary Materials*)

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