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ANTIMICROBIAL, ANTIBIOFILM, SYNERGISTIC ACTIVITIES, TOXICITY *in vitro* AND *in vivo* OF COPPER(II) COMPLEXES WITH THIOSEMICARBAZONA AGAINST ORAL PATHOGENS

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Trabalho de Conclusão de Curso
apresentado ao Instituto de Ciências
Biomédicas da Universidade Federal de
Uberlândia como requisito parcial para
obtenção do título de bacharel em
Biomedicina

Orientador: Prof. Dr. Carlos Henrique
Gomes Martins

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Santiago

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Dedico este trabalho a todas as pessoas que
contribuíram de maneira significativa para a
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Antimicrobial, antibiofilm, synergistic activities, toxicity *in vitro* and *in vivo* of copper(II) complexes with thiosemicarbazone against oral pathogens

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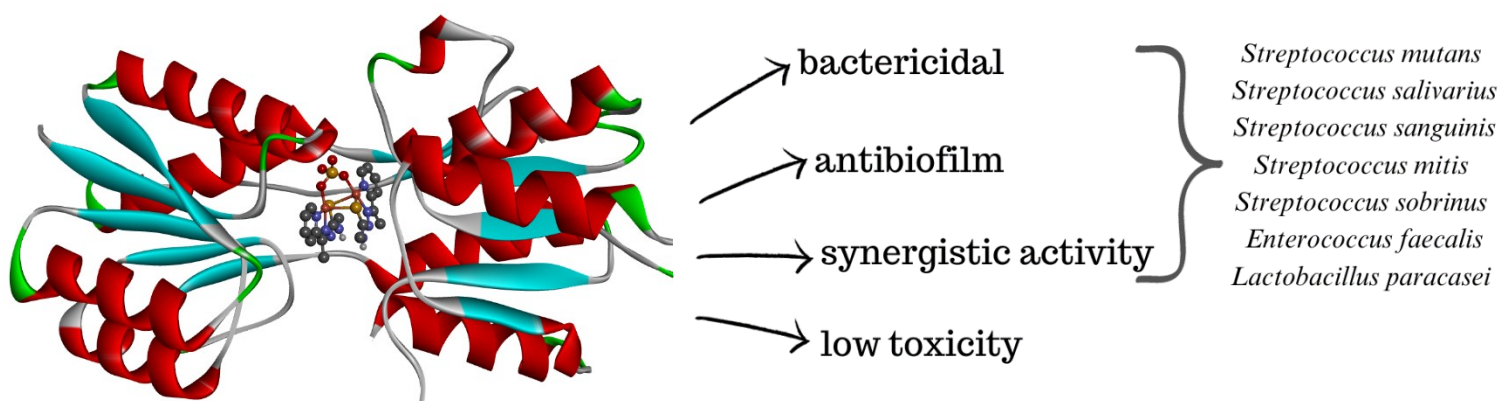
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Abstract

Caries represents a prevalent public health concern worldwide. This oral condition involves intricate interactions such as biofilm formation and contributes to the development of additional pathological conditions. In the pursuit of antimicrobial agents, metallic compounds have garnered significant attention because of their large biological activity, including antibacterial features. In this scenario, Copper(II) with thiosemicarbazone Schiff base encoded $[\text{CuCl}(\text{atc-Me})]$ (**1**) and $[\{\text{Cu}(\mu\text{-atc-Me})\}_2\mu\text{-SO}_4]$ (**2**) have shown promising antimicrobial activity as showed by previously result. To evaluate their anticariogenic potential, assays such as Minimum Inhibitory Concentration (MIC), Minimal Bactericidal Concentration (MBC), Minimum Inhibitory Concentration of Biofilm 50% (MICB₅₀) and synergistic activity with chlorhexidine and for the first time the toxicity of thiosemicarbazone was evaluate in *Caenorhabditis elegans*. The lowest value of MIC was 0,78 $\mu\text{g/mL}$ for *Streptococcus mutans*, *S. mitis*, *S. sanguinis* and *S. sobrinus* and MBC presented values of bactericidal activity. The synergic activity with chlorhexidine demonstrated a synergic activity for *S. mutans* and *Enterococcus faecalis*. Cytotoxic evaluation in human fibroblast cultures revealed that complexes **1** and **2** showed IC₅₀ equivalent to 2.9 $\mu\text{g/mL}$ and 4.9 $\mu\text{g/mL}$ respectively, being higher than the most promising antibacterial concentrations. The toxicity in *C. elegans* reveals a lethal concentration that kills 50% of larvae 3x higher than MIC for complexes **1** and **2**. The binding energy analyzed by molecular docking for compounds **1** and **2** revels values in a range of -4.78 to -7.79 kcal/mol, respectively. This work points towards an alternative for the cariogenic treatment, especially because of the great activity against biofilm and toxicity above what is necessary.

Keywords: copper complexes, thiosemicarbazone, oral pathogens, antibiofilm activity, cytotoxicity, *C. elegans*, molecular docking

Graphical abstract



Introduction

Dental caries is the most common oral disease, harming about 8% of the world's population in the primary dentition and 35% in the permanent dentition (1). According to Listl et al. (2) the direct and indirect costs for dental diseases on a global scale were around \$442 billions in 2010. Pathogenesis involves interactions between tooth structure, bacterial biofilm formation, diet, host and duration of bacteria and host contact (3). The microorganisms that represent caries are diversity, including *Prevotella* sp., *Aggregatibacter actinomycetemcomitans*, *Enterococcus faecalis*, *Candida* spp., *Streptococcus mitis*, *S. sanguinis*, *S. mutans*, *S. salivarius*, *S. sobrinus*, *Lactobacillus paracasei*, among others (4-6).

The gold standard treatment for caries is the extraction of biofilm, there are three main options for this removal: selective removal of carious tissue for soft and firm dentin and gradual and non-selective removal, both for hard dentin (7). However, it is a fact that prevention is the best way, so the focus is on inhibiting biofilm formation and for this reason antimicrobial compounds are used in toothpastes and mouthwashes (8). Chlorhexidine has been used for years to treat oral infections, however the adverse effects of this antimicrobial are described by Silvestri and McEnery-Stonelake (9) as tooth stains, loss of taste, hives and even some cases of anaphylaxis.

Considering that even with the introduction of new antibacterial drugs, bacteria will continue to develop resistance mechanisms to avoid antibiotic activity. Therefore, the best way to overcome this problem is to discover compounds that can achieve multiple targets, since the bacteria would have to undergo many mutations to acquire resistance (10). A plenty of studies reported in literature has shown that metallic compounds, such as silver, palladium, zinc and copper could be an alternative as antibacterial agents, since those compounds can reach several targets at the same time (11-15). On other hand, synthetic organic compounds as thiosemicarbazones are well-known for their antitumor, antibacterial, antifungal and antiviral activity (16).

According to Nakahata et al. (17) the combination of an organic compound with a metal ion can be a possibility of new antimicrobial drugs (18-20). Especially when a new trend of research is the modification of classical antibiotics by metal replace. The concept of focusing research towards improving antibiotic effectiveness through their integration with metals is based on the understanding that specific antibiotics necessitate a form of "partnership" with metals in order to produce the desired outcome, these are called as metalloantibiotics (18,19).

The choice of copper (II) as the metal center is due to the fact that it is an essential metal, which can provide less toxic components than other metals and is already know to have antimicrobial activity (17). Furthermore, copper(II) combined with thiosemicarbazone could possible afford interesting biological properties results and bring advantages like an alternative mechanism of action, multiple targets and prevention of resistance, benefits that are hard to achieve through purely organic arrangements (21-23).

To evaluate the anti-cariogenic potential of copper (II) complexes with thiosemicarbazone (**Figure 1**) this study performed antibacterial, antibiofilm and synergic activity with chlorhexidine in representative caries bacteria. This is the first study to perform *in vivo* toxicity of this molecule. Furthermore, docking

simulations were carried out to obtain more information about the binding mode of the compounds to cariogenic bacteria and the differences between the compounds in terms of molecular interaction.

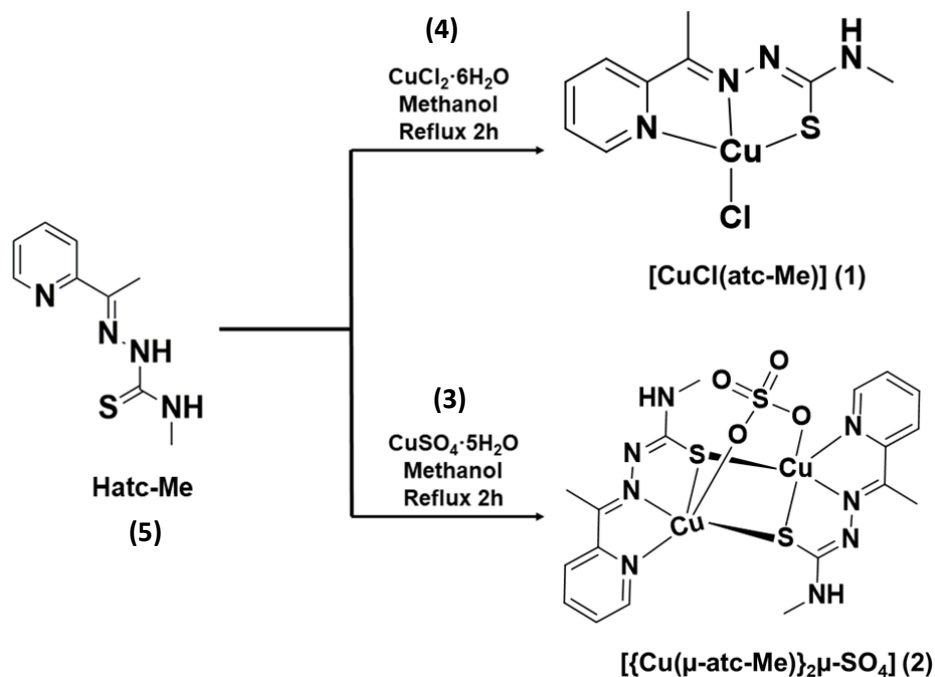
Material and methods

Synthesis of Copper (II) Complexes

The compounds studied here were previously synthesized and characterized as described by Souza et al. (13). The complex [CuCl(atc-Me)] (**1**) was prepared by adding a solution of CuCl₂·5H₂O (**4**) (56.13 mg, 0.25 mmol) to a solution of Hatc-Me (**5**) (52.07 mg, 0.25 mmol) dissolved in 15 mL of MeOH. The solutions were stirred under reflux at 60°C for 2 hours, which resulted in the precipitation of dark green solids which were filtered, washed with n-hexane and dried. For the synthesis of the complex [$\{\text{Cu}(\mu\text{-atc-Me})\}_2\mu\text{-SO}_4$] (**2**) the metallic precursor CuSO₄·5H₂O (**3**) (62.42 mg, 0.25 mmol) was added to a solution of Hatc-Me (**5**) (52.07 mg, 0.25 mmol) dissolved in 10 mL of methanol. The solution was maintained under constant stirring and reflux at 60°C for 2 hours. The green solids obtained from the reaction were filtered and washed with n-hexane. Both complexes were produced in good yields.

[CuCl(atc-Me)] (**1**). Yield: 61% (78.2 mg). Anal. Calcd for C₉H₁₁ClCuN₄S (306.27 g/mol) C, 35.29; H, 3.62; N, 18.29 %. Found: C, 35.18; H, 3.72; N, 17.81. IR ($\nu_{\text{max}}/\text{cm}$): 3307 $\nu(\text{N-H})$, 1618, 1600 $\nu(\text{C=N})$ + $\nu(\text{C=C})$, 1058 $\nu(\text{N-N})$, 771 $\nu(\text{C-S})$. UV-Vis, DMSO solution concentration: 1.56×10^{-5} M [λ_{max} (ϵ , L/mol.cm⁻¹): 405 nm (17564); 300 nm (26730); 617 nm (300). Molar conductivity (1×10^{-3} M in DMSO): 0.10 $\mu\text{S/cm}$.

[$\{\text{Cu}(\mu\text{-atc-Me})\}_2\mu\text{-SO}_4$] (**2**). Yield: 57% (92 mg). Anal. Calcd for C₁₈H₂₂Cu₂N₈O₄S₃ (637.70 g/mol) C, 33.90; H, 3.48; N, 17.57 %. Found: C, 34.12; H, 3.61; N, 17.85. IR ($\nu_{\text{max}}/\text{cm}$): 3203 $\nu(\text{N-H})$, 1598, 1564 $\nu(\text{C=N})$ + $\nu(\text{C=C})$, 1082 $\nu(\text{N-N})$, 771 $\nu(\text{C-S})$. UV-Vis, DMSO solution concentration: 3.06×10^{-5} M [λ_{max} (ϵ , L/mol.cm⁻¹): 410 nm (12908); 305 nm (17091); 621 nm (645). Molar conductivity (1×10^{-3} M in DMSO): 0.56 $\mu\text{S/cm}$.



Scheme 1. Synthetic routes for preparation of the copper(II) complexes.

Bacterial strains

A panel of representative caries associated bacteria was assessed from the ‘American Type Culture Collection’ (ATCC): *Streptococcus mutans* (ATCC 25175), *S. salivarius* (ATCC 25975), *S. sanguinis* (ATCC 10556), *S. mitis* (ATCC 49456), *S. sobrinus* (ATCC 33478), *Enterococcus faecalis* (ATCC 4082) and *Lactobacillus paracasei* (ATCC 11578). These microorganisms are part of the culture collection of Laboratory of Antimicrobial Testing (LEA) – University of Uberlândia in cryopreservation at -20°C.

Minimal Inhibitory Concentration (MIC) and Minimal Bactericidal Concentration (MBC)

The MIC was defined as the lowest concentration of the antimicrobial agent capable of inhibiting bacterial growth. The method used was microdilution in a microplate according to the methodology recommended by the ‘Clinical and Laboratory Standards Institute’ (24), with adaptation, using resazurin as an indicator of bacterial growth. The MIC determination for each microorganism was performed in triplicate.

Complexes **1** and **2** and the compounds **3**, **4** and **5** were dissolved in dimethylsulfoxide- DMSO (Sigma Aldrich - St. Louis, MO, USA) and Brain Heart Infusion - BHI (Difco – Sparks, MD, USA) was added. The complex concentration was evaluated from 0.195 to 400 µg/mL. The cultures of *E. faecalis*, *L. paracasei*, *S. mutans*, *S. salivarius*, *S. mitis*, *S. sobrinus* and *S. sanguinis* cultivated in BHI agar added with defibrillated sheep blood (5%) were transferred to the BHI broth until obtaining a turbidity equal to the 0.5 of the McFarland scale, checked in densitometer (Densimat®, Biomérieux). The concentration of inoculum in each well was 5.10⁵ CFU/mL.

Culture control was performed, controls for the sterility of the broth, complex, and the solvent used for complex solubilization was carried out. Chlorhexidine served as the positive control from 0.115

to 59 µg/mL. The microplates were incubated in a microaerophilia incubator for 24 hs at 37 °C with 10% CO₂, except for *E. faecalis* and *S. salivarius*, which were incubated aerobically.

After the incubation period 30 µL of resazurin (Sigma Aldrich - St. Louis, MO, USA) was added, at the standardized concentration of 0.02%, prepared in an aqueous solution, was added to each well, and the microplates will be incubated again for 30 minutes. For the reading, the color change was observed through a redox reaction, with a shift from blue (no bacterial growth) to pink (bacterial growth) (25).

The determination of the minimum bactericidal concentration (MBC) was carried out before adding resazurin, 10 µL was withdrawn from each well was inoculated onto a new blood agar. The medium was incubated at 37°C in an aerobic or microaerophilic incubator for 24 hours. The growth of microorganisms at concentrations greater than MIC will indicate that the metabolites in the MBC are bacteriostatic, while the absence of growth at higher concentrations of MIC will indicate that the samples are bactericidal. The MBC determination for each microorganism was performed in triplicate

Minimum Inhibitory Concentration of Biofilm 50% (MICB₅₀)

Selection of the best inoculum concentration and incubation time for the antibiofilm activity assay was accomplished by standardizing biofilm formation (data not shown). Only one microorganism representative of each MIC value was chosen (*L. paracasei*, *E. faecalis*, *S. salivarius* and *S. mutans*). The complexes [CuCl(atc-Me)] (**1**) and [{Cu(µ-atc-Me)}₂µ-SO₄] (**2**) were elected because of their better MIC results.

The antibiofilm activity was evaluated by determination of the Minimum Inhibitory Concentration of Biofilm - MICB₅₀ (26). The method used was microplate dilution recommended by the Clinical and Laboratory Standards Institute (24), with modifications. The experiments were performed in triplicates and the results demonstrated graphically.

The method used to inhibit the formation of biofilm was similar to the MIC assay conducted for planktonic cells. Two plates for each complex (one for optical density reading and other for counting of CFU/mL) were prepared with serial dilutions of the complexes **1** and **2**, the final concentration ranging from 0.195 to 400 µg/mL. Chlorhexidine at concentrations between 0.115 to 59 µg/mL was assessed as positive control. Each strain was added at concentration of 10⁶ CFU/mL. Bacterial strains with BHI broth was positive control, controls for the sterility of the broth, sample, and the solvent DMSO (Sigma-Aldrich; 1%), was carried out. The microplates were incubated at 37°C for 24 hours with appropriate atmospheres (aerobiosis/microaerophily).

Each well was washed three times with sterile Milli-Q water and fixed with methanol. Antibiofilm activity was measured by MICB₅₀ determined by optical density (OD) and by counting the colony forming unit (CFU/mL). Based on the procedures described by (27) OD was quantified in the biofilm by adding 200 µL of crystal violet (0.2%) to the microplate wells. After 15 min at room temperature, excess dye was removed with tap water and dried in air at room temperature. Next, acetic acid at 33% was added to each well to re-solubilize the dye bound to the cells. After 30 min were measured the OD of the microplates at 595nm using a microtiter plate reader (GloMax[®], Promega). The percentage of inhibition was calculated by

using the equation: $1 - (A_{595nm}/A_{595nm}) \times 100$, where A_{595nm} and A_{595nm} are the absorbance values of the wells treated with the samples and the control, respectively (26).

For counting the CFU, after the incubation period, the entire volume was carefully aspirated from the wells of the microplate and washed with water to completely remove the non-adherent cells. Then, BHI broth was added to each well and the microplate was submitted to sonication for 15 min. Dilutions of 10^0 to 10^{-7} were performed for all wells and 50 μ L of each dilution was plated in BHI agar added with defibrinated sheep blood (5%). Which were incubated in a microaerophilia incubator for 24 hs at 37 °C with 10% CO₂, except for *E. faecalis* and *S. salivarius*, which were incubated aerobically. After incubation, the colonies were counted and the results were expressed in Log¹⁰ by Colony Forming Units per milliliter (CFU/mL) and shown graphically.

Fractional Inhibitory Concentration Index (FICI)

To assess the *in vitro* antibacterial effectiveness of the combination of chlorhexidine with complexes **1** and **2** the same bacteria of the MICB₅₀ was chosen, *S. mutans*, *S. salivarius*, *E. faecalis* and *L. paracasei*. The experimental approach followed the methodology outlined by Chaturvedi et al. (28) with specific adaptations pertaining to the culture medium and incubation conditions. Synergy tests were performed in triplicate, and the concentrations of the tested samples and chlorhexidine were standard with 4xMIC against 1×10^6 CFU/ml inoculum.

The FIC index was determined algebraically, with FIC_A denoting the MIC of drug A in the combination divided by the MIC of drug A alone, and FIC_B representing the MIC of drug B in the combination divided by the MIC of drug B alone. The cumulative FIC value (Σ FIC) was calculated as Σ FIC index = FIC_A + FIC_B (28). Interpretation of fractional inhibitory concentration index values revealed the following classifications: FICI index values ≤ 0.5 indicated synergism, values > 0.5 and < 1.0 signified an additive effect, values ≥ 1.0 and < 4.0 suggested indifference, and values ≥ 4 were indicative of antagonism (29)

Cytotoxicity assessment

The GM07492A cell line (non-tumor human lung fibroblasts) was utilized in the present study. Cells were cultured in Ham F-10 + Dulbecco's Modified Eagle's medium (1:1; Sigma-Aldrich) supplemented with 10% fetal bovine serum (Nutricell), antibiotics (0.01-mg/ml streptomycin and 0.005-mg/ml penicillin; Sigma-Aldrich), and 2.38-mg/mL HEPES (Sigma-Aldrich), at 37°C with 5% CO₂.

The evaluation of cytotoxicity was carried out through the colorimetric assay of toxicology *in vitro* - Kit XTT® (Roche Diagnostics) according to the manufacturer's guidelines. For carrying out the experiments, 1×10^4 cells were seeded in microplates containing 96 wells. The concentrations tested were determined according to the solubility limit. After solubilization in dimethyl sulfoxide (DMSO; Sigma-Aldrich; 1%), complexes **1**, **2** and compound **5** were tested at concentrations ranging from 3.9 to 500 μ g/mL, and compounds **3**, **4** and chlorhexidine in concentrations ranging from 7.8 to 1000 μ g/mL. Negative (no treatment), solvent (1% DMSO) and positive (25% DMSO) controls were included. The treatment and analysis procedures were conducted as described by Soares et al. (30). The experiments were performed in triplicate, and the results are expressed as IC₅₀ (half maximal inhibitory)

Toxicity assessment in *Caenorhabditis elegans*

The strain used of *Caenorhabditis elegans* AU37, a mutant strain that is sterile at 25°C, according to Singulani et al. (31). The complexes **1** and **2** were evaluated at concentrations of 11.71 to 375 µg/mL and the compounds **3**, **4** and **5** at 46.87 to 1500 µg/mL. Dimethyl sulfoxide (DMSO) was used as solvent (final concentration ≤1%).

C. elegans AU37 was grown on Nematode Growth Medium (NGM) plates seeded with *Escherichia coli* OP50 and incubated at 16° C for three days. After incubation, the supernatant was washed with the bleaching solution (sodium hypochlorite + NaOH) to synchronize the larvae at the L4 stage. The plates containing larvae synchronized in the L4 phase were washed with M9 and the supernatant was placed in 15 mL conical tubes. Subsequently, 20 µL of the larval suspension was added to each well of a 96-well flat-bottom microplate, along with 80 µL of BHI medium + antibiotics (Streptomycin, ampicillin and Kanamycin) and 100 µL of each sample to be tested, and incubated for 24h at 25° C. Counting live and dead larvae was performed on an Evos microscope and the percentage of mortality was calculated.

Molecular Docking

Molecular docking studies were performed using autodock4.2 (32) with [CuCl(atc-Me)] (CCDC 2063732) and [$\{Cu(\mu\text{-atc-Me})\}_2\mu\text{-SO}_4$] (CCDC 2083951). CHD (CCDC 988972) was obtained from The Cambridge Crystallographic Data Centre, Hatc-Me ligand was drawn in GABEDIT (33) and optimized with semi-empirical method PM7 using MOPAC2016 program (34). The crystal structures of *S. mutans* (PDB 5C2O), *S. mitis* (PDB 3LE0), *S. sanguinis* (PDB 4N82), *L. paracasei* (PDB 6CHK) and *E. faecalis* (PDB 4LRL) bacteria were obtained from Protein Data Bank (PDB). During molecular docking simulation, the re-dock procedure was made with protein and itraconazole. The procedure of molecular docking was performed using the AutoDockTools 1.5.6 interface along with autogrid4 and autodock4.2 (32), which uses the Lamarckian generic algorithm (LGA) to recognize the putative binding site and the orientation of the ligand. As per the pre-requisites of LGA, the water molecules were removed from the protein, polar hydrogens were added, followed by the merging of non-polar hydrogen and the addition of Gasteiger charges (35). The ligands were drawn in GABEDIT (32) and optimized with semi-empirical method, PM7, using MOPAC2016 program (34). The LGA in Autodock was used with 20 runs for docking simulation, while an initial population (150 individuals), maximum energy evaluations (25,000,000), maximum generations (27,000), one individual surviving into next generation, genetic algorithm docking runs (150) and a ranked cluster analysis was performed using an RMS tolerance of 2.0 Å on each docking calculation. Ligand interactions were generated using the Discovery Studio Visualizer program (36).

Results

The antibacterial activity of all samples was assessed by MIC and MBC. The complex **1**, demonstrated MIC results of 6.25 µg/mL for *E. faecalis* and *S. salivarius*, 12.5 µg/mL for *L. paracasei*, and 1.56 µg/mL for *S. sobrinus*, *S. mitis*, *S. sanguinis*, and *S. mutans*. Meanwhile complex **2**, exhibited MIC values of 12.5 µg/mL for *E. faecalis*, 6.25 µg/mL for *S. salivarius* and *L. paracasei* and 0.78 µg/mL for *S. sobrinus*, *S. mitis*, *S. sanguinis*, and *S. mutans*. Notably, both complexes demonstrated bactericidal effects at the MBC against all tested bacterial species. In contrast, compounds [CuSO₄·5H₂O] (**3**), [CuCl₂·2H₂O] (**4**) and Hatc-

Me (**5**) displayed MIC results between 400 µg/mL and 100 µg/mL for all tested species. These results are shown in **Table 1**.

The antibiofilm activity was calculated as MIC_{B50}, with a range value from 0.78 to 12.5 µg/mL (**Figure 2**). Complex [CuCl(atc-Me)] (**1**) displayed MIC_{B50} values of 0.78 µg/mL for *L. paracasei*, 3.12 µg/mL for *S. mutans* and *S. salivarius*, and 6.25 µg/mL for *E. faecalis*. Meanwhile, complex [{Cu(µ-atc-Me)}₂µSO₄](**2**) presented results of 1.56 µg/mL for *S. mutans* and *S. salivarius*, 6.25 µg/mL for *L. paracasei*, and 12.5 µg/mL for *E. faecalis*. Chlorhexidine was used as positive control and the data are in **Figure 3**.

The Fractional Inhibitory Concentration Index (FICI) for the complex [CuCl(atc-Me)] (**1**) was 0.74, representing an active interaction for *E. faecalis* and 0.24 for *S. mutans* demonstrating a synergic interaction. The complex [{Cu(µ-atc-Me)}₂µSO₄] (**2**) had an ICIF of 0.44 for *E. faecalis* and 0.45 for *S. mutans* indicating a synergic interaction for both, demonstrated in **Table 2**.

The results obtained from the cytotoxic evaluation of the samples in non-tumor human cells are presented in **Table 3**. After 24 hours of treatment, complexes **1** and **2** showed IC₅₀ equivalent to 2.9 µg/mL and 4.9 µg/mL, respectively. The toxicity with *C. elegans* (**Figure 4**) exhibits values of larvae mortality (%) during three days of counting and the complexes **1** and **2** had an 50% Lethal Concentration (LC₅₀) at 46,875 µg/mL on the second day. The difference between dead and alive larvae are in **Figure 5**.

The molecular docking study was carried out to investigate and compare the biological potential of complexes **1** and **2** and ligand **5** with to the positive control chlorhexidine (CHD), in order to identify interactions with the active site of the structures of the *S. mutans*, *S. mitis*, *S. sanguinis*, *L. paracasei* and *E. faecalis* bacteria. Re-docking was performed using the AutoDock4 program to validate structure-activity relationships. The results, expressed in interaction energy values (ΔG), obtained for the **1**, **2**, **5** and CHD with the studied bacteria are shown in **Table 4**. The analysis of the most favorable energy conformation of complexes **1** and **2** with *S. mutans*, *S. mitis*, *S. sanguinis*, *L. paracasei* and *E. faecalis* bacteria are shown in **Figures 6** and **7** respectively.

Discussion

Dental caries can be related to severe pain and their presence can be incapacitating. In many cases, without an early and suitable treatment can leave to tooth loss (3). In the present study, MIC results reveals that the complexed (**1** and **2**) have better grown inhibition activity, to 0.78 µg/mL for 12.5 µg/mL comparing to the compounds **3**, **4** and **5**, ranging values to 200 for 400 µg/mL (**Table 1**). These results can be supported with Khan et al. (37), their study tested a structure with copper (II) and thiosemicarbazone in Gram-positive and Gram-negative bacteria. MIC values of the complexed samples with *Staphylococcus aureus* and *S. pyogenes* was 32 µg/mL and 64 µg/mL, respectively and 32 µg/mL for *Salmonella typhimurium* and *Escherichia coli*. For thiosemicarbazone alone, the MIC was 512 µg/mL for Gram-positives and 128 µg/mL for Gram-negatives. Similar results was observed in Azam et al. (38), the ligand used was azo-azomethine with Cu(II) against *S. aureus* and *E. coli*. MIC values for the complex was 128 µg/mL (*S. aureus*) and 64 µg/mL (*E. coli*), for the ligand alone was 512 µg/mL and 256 µg/mL, respectively. As observed in the

present study, the antibacterial activity of complexed samples was in concentrations much lower than others, demonstrating a big anticariogenic potential with this chemical combination.

For the present study, MBC values shows that the complexes **1** and **2** are bactericidal for all microorganisms in low concentrations and the compounds **3**, **4** and **5** were bactericidal or bacteriostatic at 200 µg/mL and 400 µg/mL. In the study of Azam et al. (38) the MBC of the ligand alone was bactericidal at 512 µg/mL and for the complex with copper (II) was bactericidal at 128 µg/mL for *S. aureus* and bacteriostatic for *E. coli*. In this analysis, it is obvious the great potential of copper (II) complexes with thiosemicarbazone with bactericidal action at very low concentrations.

Concerning biofilm, the complexes **1** and **2** were able to significant inhibit both biomass and cell viability. The bests results, showed as MICB₅₀, were for complex **1** for *L. paracasei* at 0.78 µg/mL and for complex **2** for *S. mutans* and *S. salivarius* at 1.56 µg/mL. The results of MICB₅₀ are resembling to MIC, demonstrating that is not necessary to increase the concentration too much to have a combined action of killing planktonic cells and biofilm reduction. In a study with thiosemicarbazone complex of Li et al. (39), a similar result was against *Candida glabrata* at 2 µg/mL with MIC of 0.25 µg/mL. The ability to inhibit biofilm in lowest concentrations is important because a greater quantity may be related to a greater possibility of toxicity to humans. Most of well-known antibiotics and treatments can't inhibit biofilms because of their levels of toxicity. (40)

The Fractional Inhibitory Concentration Index of complexes **1** and **2** with chlorhexidine for *S. mutans* had a synergistic activity; against *E. faecalis* complex **1** had an active action while complex **2** had synergistic activity. This is the first study to use copper (II) complex with thiosemicarbazone and chlorhexidine to evaluate the synergism of them, Namiecińska (18) defends that research into new molecules for treatment needs to look for synergistic activity with classic pharmacological agents. Due to the lack of articles in the literature about synergism effect of thiosemicarbazones, this trial can not be discussed in depth, the only study that evaluate thiosemicarbazone synergism was Moreira (41). They evaluated thiosemicarbazone complexes with meropenem against *Enterobacter cloacae* (CCBH10982), the ICIF values were varied but some concentrations had a synergistic effect. Based on that, more experiments are necessary to understand how thiosemicarbazones react with antimicrobials.

The cytotoxicity assessment reveals an IC₅₀ of 2.9 µg/mL for complex **1** and 4.9 µg/mL for complex **2**. The compounds **3**, **4** and **5** demonstrate lower toxicity, similar results were in the study of Kate et al. (42) the cytotoxicity assessment was in HeLa cells with thiosemicarbazone and copper (II), the toxicity for the ligand was lower than the copper(II) complex. These results are similar with Neville study (43) where they get greater toxicity of copper(II) complexes against HK-2 renal cells and in ovarian cancer cell line A2780 . It is essential to comprehend the real level of toxicity making more assays, such as *in vivo* because they represent greater similarity with reality.

Considering that, this is the first study to test the toxicity of thiosemicarbazone with copper (II) in *C. elegans*. It's extremely important to understand that mammal testing is very expensive and demand a long time to be done. In contrast to *in vitro* testing, toxicity assessments using *C. elegans* leads information from an entire organism possessing an intact and metabolically active digestive, endocrine, sensory,

reproductive and neuromuscular system (44). The Lethal Concentration that killed 50% (LC₅₀) *C. elegans* for complexes **1** and **2** was 46,875 µg/mL in the second day of counting. Despite the demonstration that the toxicity of complexed samples are higher than that of ligands and ions alone. The LC₅₀ was found at a concentration 3x higher than the highest MIC value. This means that the antibacterial activity is not because of the toxicity but because of the biological activity of the complex and based on this assay the concentrations of MIC, MICB₅₀ and synergism are safe to be used.

The molecular docking results were analyzed using the RMSD (Root Mean Square Deviation) value. RMSD values in the range of 0–2 Å are considered valid for molecular docking results (45). Furthermore, during the analysis, it was observed that the standard interactions of the ligand with the protein, extracted from the PDB itself, were maintained. This consistency indicates that the protocol used was able to preserve the known interactions between the ligand and the protein of interest. The results of the binding energy between the compounds and all bacteria tested from molecular docking calculations (Table 4) showed that complex **2** present more favorable binding energies (ΔG in the range of -5.01 to -7.79 kcal mol⁻¹) than complex **1** (ΔG in the range of -4.78 to -6.53 kcal mol⁻¹) and compound **5** (ΔG in the range of -5.07 to -6.85 kcal mol⁻¹). Consequently, compound **2** is able to interact with more efficiency the active site of bacteria. These results are very similar to those obtained for biological assays, where complex **2** also generally presented the best results in the *in vitro* antibacterial assay.

Analysis of the most favorable energetic conformation between complex **2** and *L. paracasei* shows the presence of hydrogen bonds with the amino acid residues Gln and Phe. Furthermore, π - π , π -S and π -alkyl interactions are also observed with the amino acids His and Arg. These interactions is justified by the high interaction energy between the compound and the protein structure, since the presence of stronger interactions stabilize the active site and increase the energy.

Figure 6. 2d predicted binding mode of minimum energy docked poses of **1** with the active site of *S. mutans*, *S. mitis*, *S. sanguinis*, *L. paracasei* and *E. faecalis* bacteria. For clarity, the non-polar hydrogen atoms of the metal complex are omitted. Dashed green lines indicate hydrogen bond, dashed purple and pink lines indicate hydrophobic and dashed orange lines indicate sulfur interactions. Unbound green amino acids indicate van der waals interactions.

Figure 7. 2d predicted binding mode of minimum energy docked poses of **2** with the active site of *S. mutans*, *S. mitis*, *S. sanguinis*, *L. paracasei* and *E. faecalis* bacteria. for clarity, the non-polar hydrogen atoms of the metal complex are omitted. Dashed green lines indicate hydrogen bond, dashed purple and pink lines indicate hydrophobic and dashed orange lines indicate sulfur interactions. Unbound green amino acids indicate van der waals interactions.

Conclusion

Based on the results obtained, the anticariogenic effect of copper (II) complex with thiosemicarbazone is very promising because of their low values of MIC/MBC and MICB₅₀. In addition, the complexes showed cytotoxicity on human cells at concentrations higher than those that revealed more promising antibacterial activity. The present work describes for the first time the synergistic activity of copper(II) complex with thiosemicarbazone and chlorhexidine against cariogenic bacteria with promising results and *in vivo* toxicity

assay with *C. elegans*. The molecular docking results are in accordance with the biological *in vitro* assays in which the compound **2** is the best candidate compared with Hatc-Me and complex **1**. In conclusion, this complex can be a future promise for anticariogenic products.

Author contributions **SLS**: investigation, methodology, formal analysis, data-curation, conceptualization writing-original draft. **MBS**: methodology, supervising. **NBSS**: methodology, formal analysis, writing-review, supervising. **ALOS**: methodology, formal analysis. **NOA**: methodology, writing-review. **DCT**: methodology, writing-review. **RACS**: methodology, writing-original draft. **CGO**: methodology, writing-original draft. **CHGM**: writing-original draft, supervision, validation, revision, funding acquisition, project administration,

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Declarations

Conflict of interest: The authors declare that they don't have competing interests to declare that are relevant to the content of this article.

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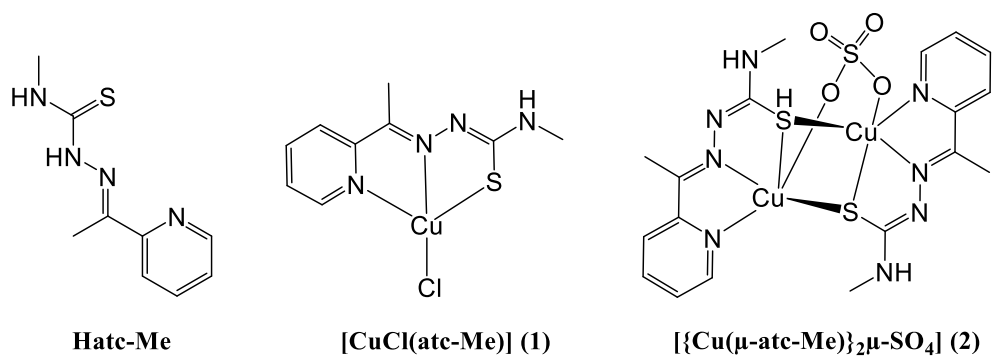


Fig 1. Chemical structure of Hatc-Me (5) and complexes **1** and **2**.

Table 1: Results of antibacterial activity Minimum Inhibitory Concentration (MIC)/ Bactericidal Minimum Inhibitory (MBC) of samples [CuCl(atc-Me)] (**1**), [$\text{Cu}(\mu\text{-atc-Me})_2 \mu\text{-SO}_4$] (**2**), $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ (**3**), $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$ (**4**), Hatc-Me (**5**) and Chlorhexidine against dental caries bacteria.

Samples	Cariogenic Bacteria $\mu\text{g/mL}$						
	<i>Enterococcus faecalis</i> ATCC 4082	<i>Streptococcus salivarius</i> ATCC 25975	<i>Streptococcus sobrinus</i> ATCC 33478	<i>Streptococcus mitis</i> ATCC 49456	<i>Streptococcus sanguinis</i> ATCC 10556	<i>Streptococcus mutans</i> ATCC 25175	<i>Lactobacillus paracasei</i> ATCC 11578
[CuCl (atc-Me)] 1	6.25 / 6.25	6.25 / 6.25	1.56 / 1.56	1.56 / 1.56	1.56 / 1.56	1.56 / 1.56	12.5 / 12.5
[$\text{Cu}(\mu\text{-atc-Me})_2 \mu\text{-SO}_4$] 2	12.5 / 12.5	6.25 / 6.25	0.78 / 0.78	0.78 / 0.78	0.78 / 0.78	0.78 / 0.78	6.25 / 6.25
$\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ 3	400 / >400	400 / 400	400 / 400	200 / 400	400 / 400	400 / 400	100 / 200
$\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$ 4	400 / >400	400 / >400	400 / 400	200 / 400	400 / 400	400 / >400	200 / 400
Hatc-Me 5	>400 / >400	400 / 400	200 / 400	200 / 200	200 / 200	400 / 400	200 / 400
Chlorhexidine	14.75 / 14.75	1.84 / 1.84	1.84 / 1.84	7.35 / 7.35	3.68 / 3.68	1.84 / 1.84	1.84 / 1.84

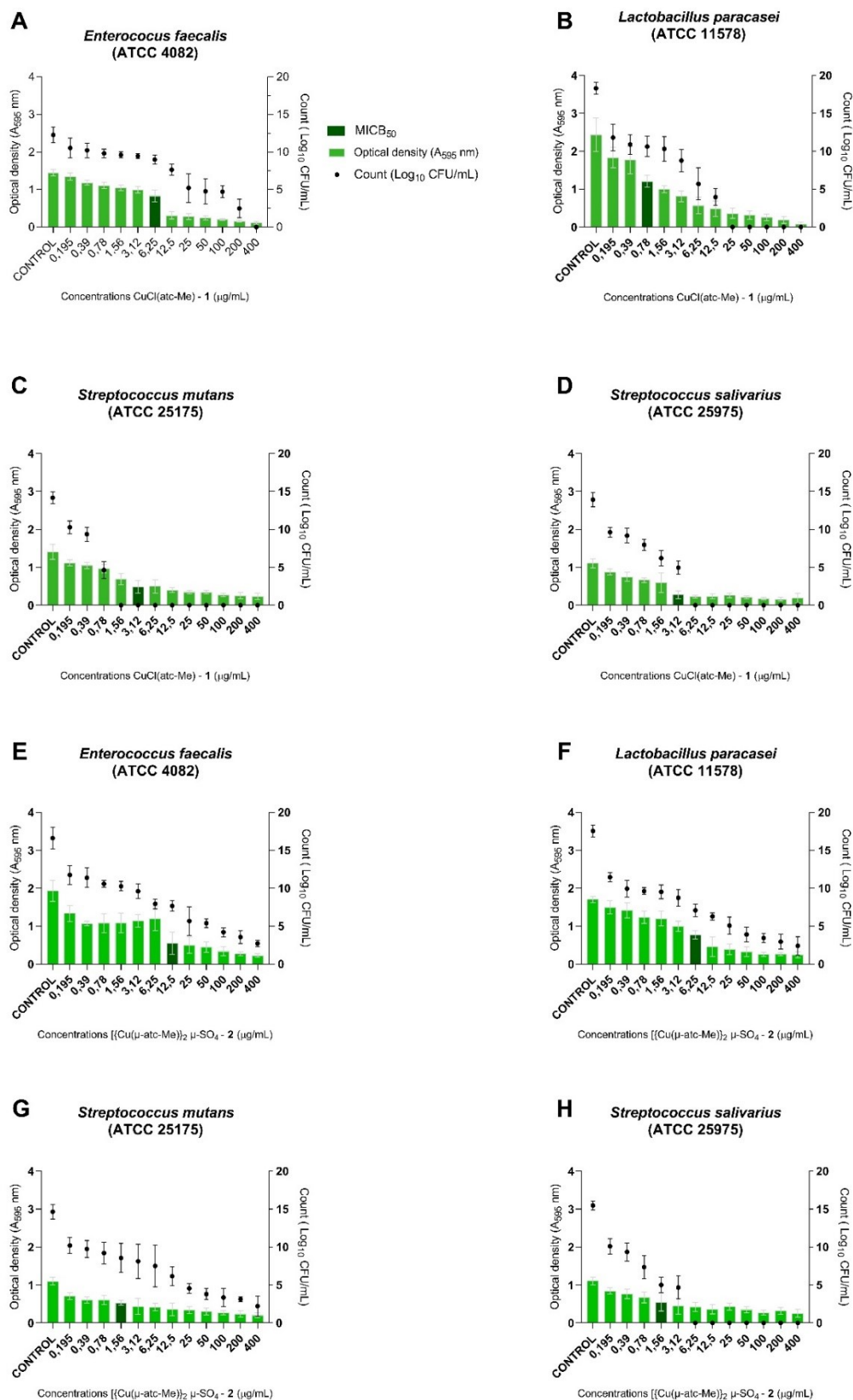


Figure 2: Antibiofilm activity of samples [CuCl (atc-Me)] (1) and [{Cu (μ-atc-Me)}₂ μ-SO₄] (2) against cariogenic bacteria. Optical density and counting of CFU/mL with Minimal Inhibitory Concentration Biofilm 50% (MICB₅₀). **A-D:** values from sample [CuCl (atc-Me)]. **E-H:** values from sample [{Cu (μ-atc-Me)}₂ μ-SO₄].

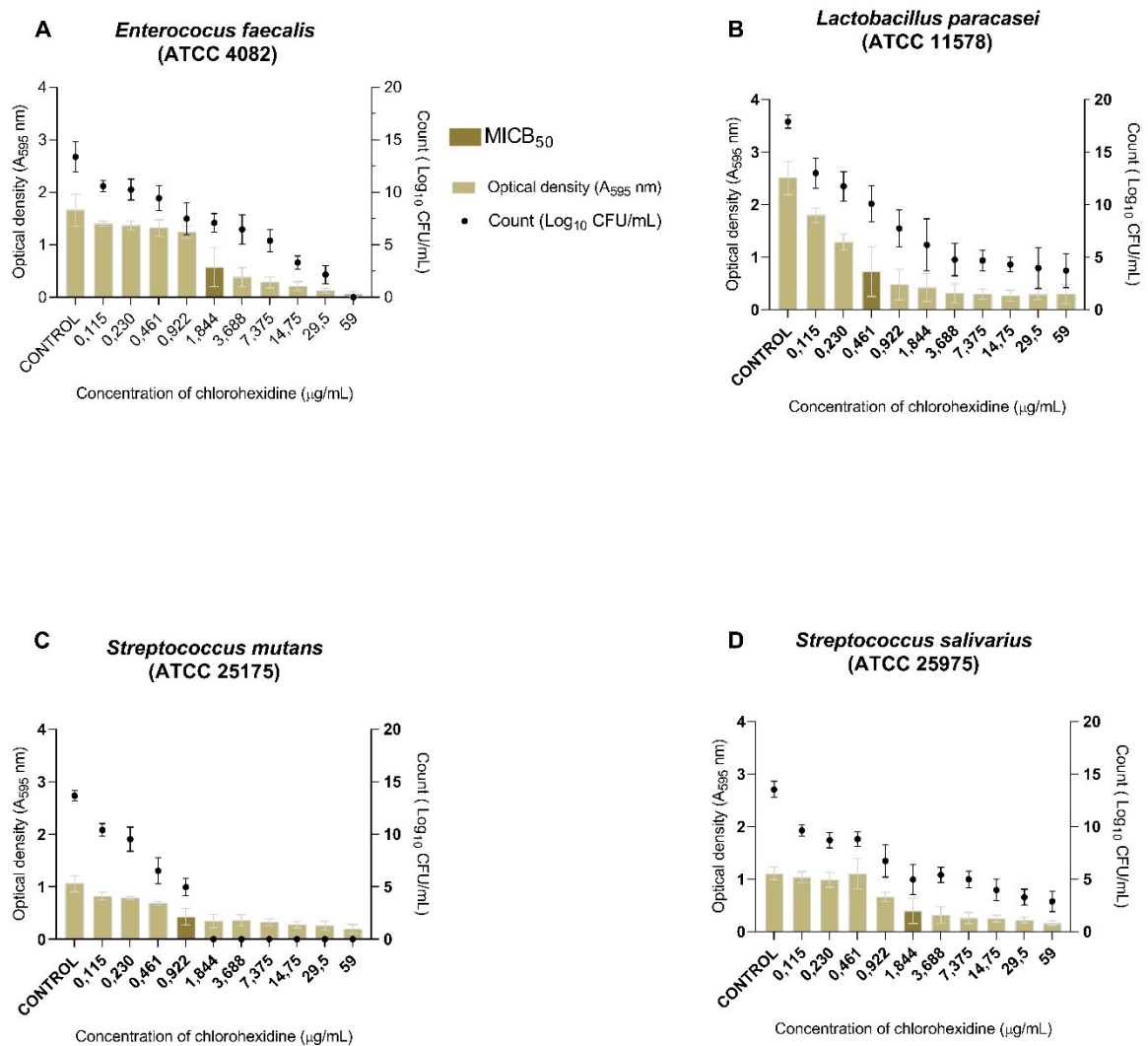


Figure 3: Antibiofilm activity of chlorhexidine (positive control) against cariogenic bacteria. Optical density and counting of CFU/mL with Minimal Inhibitory Concentration Biofilm 50% (MICB₅₀).

Table 2: Synergistic activity of complexes [CuCl (atc-Me)] (**1**) and [{Cu (μ-atc-Me)}₂ μ-SO₄] (**2**) with chlorhexidine against cariogenic bacteria. (A) Complex **1** or **2** (B) Chlorhexidine

Microorganisms	Complex	MIC alone (μg/mL)		MIC combination (μg/mL)		FIC		FICI	Outcome
		(A)	(B)	(A)	(B)	(A)	(B)		
<i>E. faecalis</i>	1	6.25	7.37	3.12	1.84	0.49	0.25	0.74	Aditve
	2	12.5	7.37	3.12	1.84	0.24	0.20	0.44	Sinergistic
<i>L. paracasei</i>	1	12.5	1.84	3.12	1.84	0.25	1	1.25	Indiferent
	2	6.25	1.84	6.25	3.68	1	2	3	Indiferent
<i>S. mutans</i>	1	1.56	1.84	0.19	0.23	0.12	0.12	0.24	Sinergistic
	2	0.78	1.84	0.19	0.46	0.20	0.25	0.45	Sinergistic
<i>S. salivarius</i>	1	6.25	1.84	6.25	3.68	0.5	1	1.5	Indiferent
	2	6.25	1.84	3.12	1.84				Indiferent

Table 3- IC₅₀ values obtained against GM07492A cell line after 24 hours of treatment with different concentrations of complexes **1**, **2**, **3**, **4** and **5**.

Complexes	IC ₅₀ (µg/mL)
1: [CuCl (atc-Me)]	2.9 ± 0.9
2: [{Cu(µ-atc-Me)} ₂ µ-SO ₄]	4.9 ± 0.2
3: CuSO ₄ ·5H ₂ O	207.3 ± 12.8
4: CuCl ₂ ·2H ₂ O	117.6 ± 25.5
5: Hatc-Me	> 500
Chlorhexidine	51.4 ± 11.7

IC₅₀ - complex concentration that inhibits 50% of cell viability; **GM07492A** - non-tumor human lung fibroblasts; Values are mean± standard deviation.

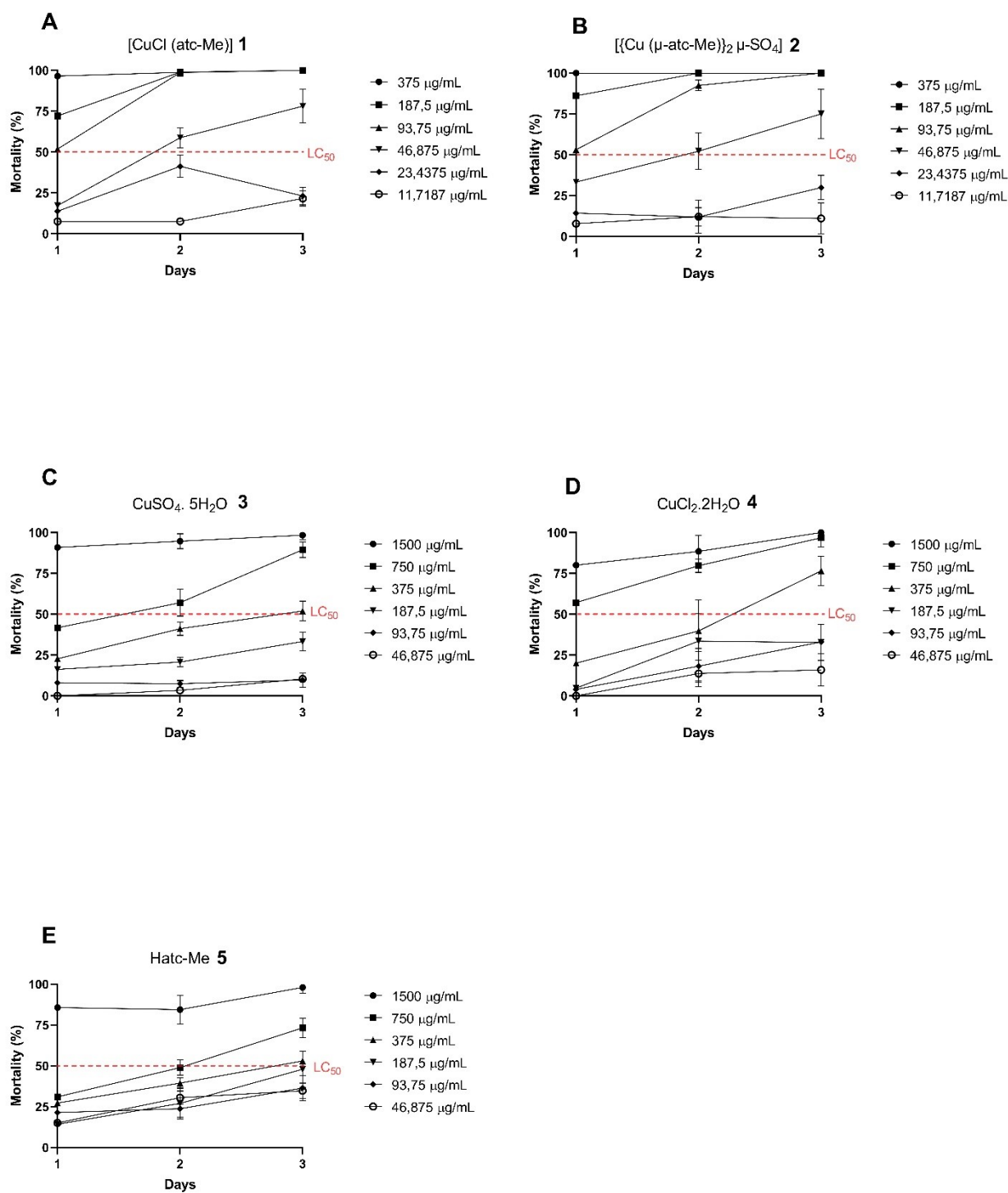
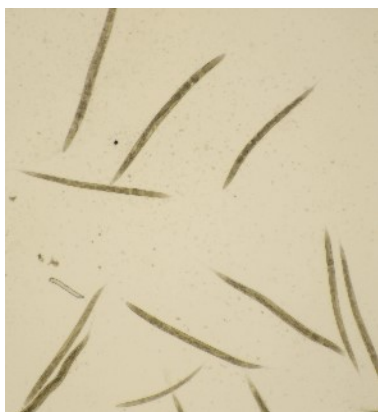


Figure 4: Values of mortality for *Caenorhabditis elegans* against complexes $[\text{CuCl}(\text{atc-Me})]$ (**1**), $[\{\text{Cu}(\mu\text{-atc-Me})\}_2 \mu\text{-SO}_4]$ (**2**), $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ (**3**), $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$ (**4**) and Hatc-Me (**5**) during 3 days of counting. LC_{50} is the lethal concentration that kills 50% of larvae.



A



B

Figure 5. Transmittance images obtained from the EVOS[®] inverted microscope. **A:** *C. elegans* larvae incubated with sample [CuCl(atc-Me)] (**1**) after 1 day at the concentration of 375 µg/mL. **B:** *C. elegans* larvae incubated after 1 day, positive control well. Needle-shaped larvae were considered dead, and those with a curvilinear shape were considered alive.

Table 4. Binding affinities (ΔG in kcal/mol) of free ligand, complexes and the standard drug.

Bacterial strains	[CuCl(atc-Me)] (1)	[{Cu(μ -atc-Me)} ₂ μ -SO ₄] (2)	Hatc-Me (5)	Chlorhexidine
<i>Streptococcus mutans</i>	−4.78	−6.71	−5.07	−7.92
<i>Streptococcus mitis</i>	−5.35	−5.01	−6.85	−3.03
<i>Streptococcus sanguinis</i>	−6.53	−7.58	−6.10	−9.49
<i>Lactobacillus paracasei</i>	−6.10	−7.79	−5.21	−8.46
<i>Enterococcus faecalis</i>	−5.02	−7.32	−5.78	−11.24

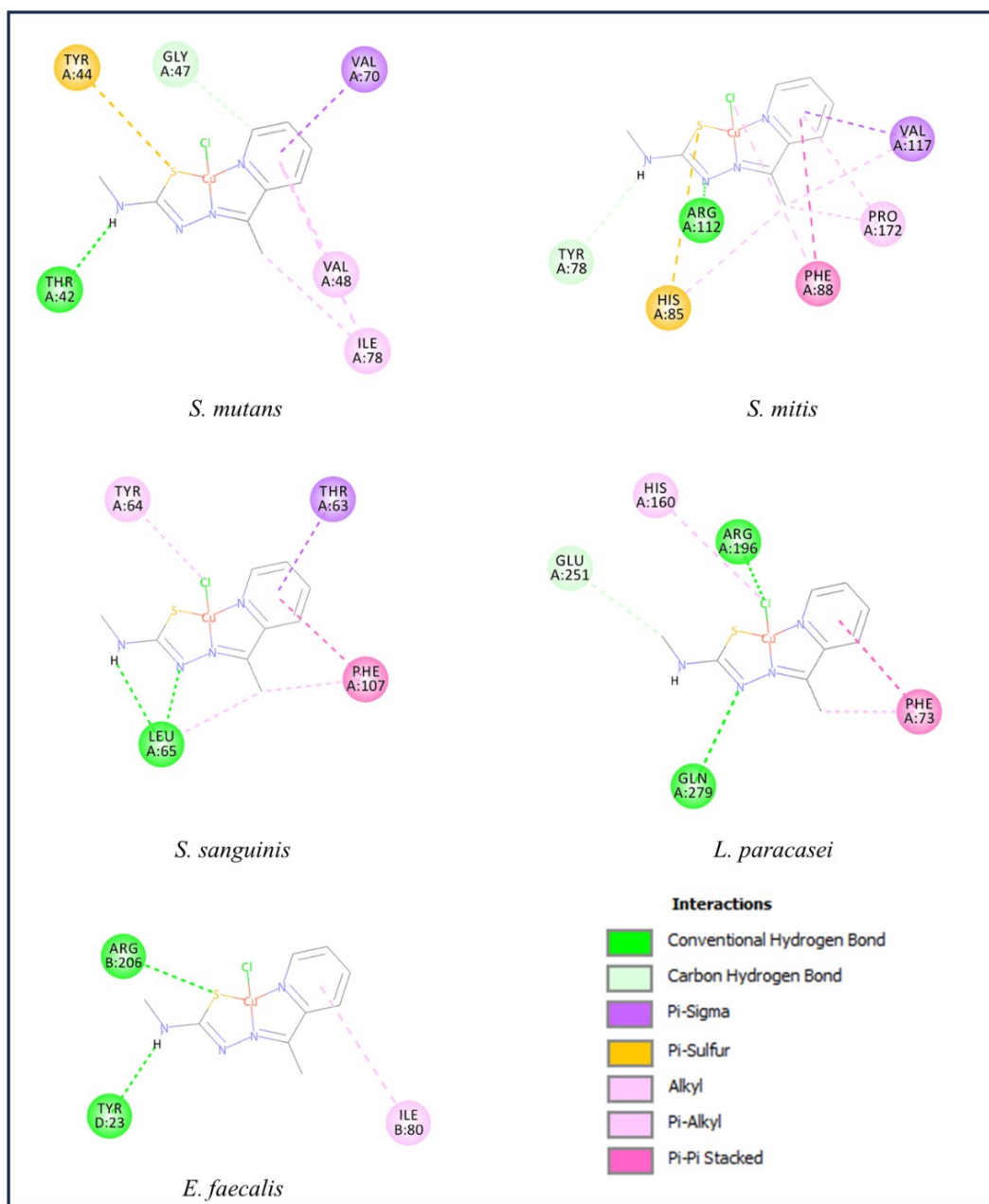


Figure 6. Interactions between complex $[\text{CuCl}(\text{atc-Me})]$ (1) and cariogenic bacteria.

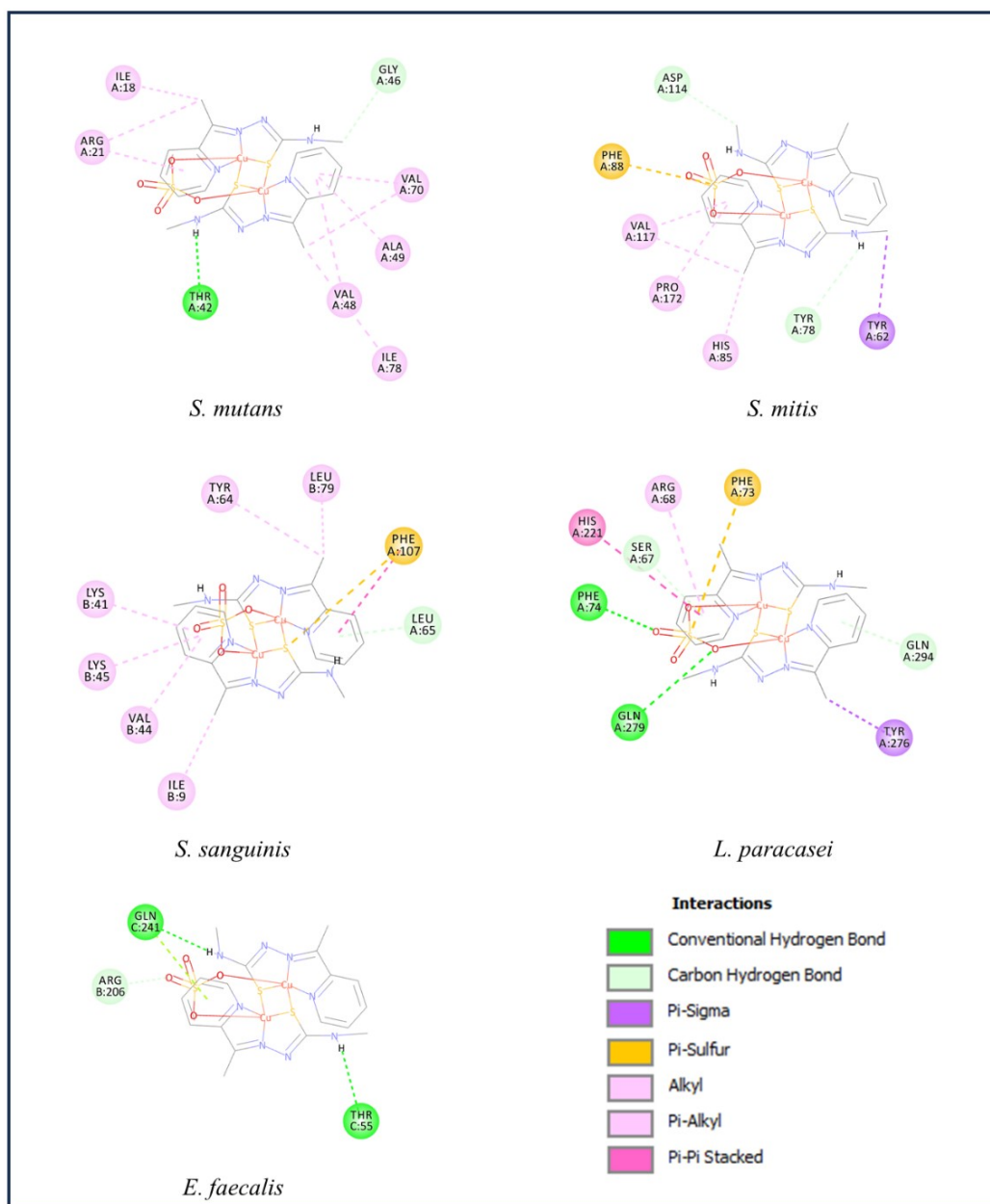


Figure 7. Interactions between complex $[\text{Cu}(\mu\text{-atc-Me})_2\mu\text{-SO}_4]$ (**2**) and cariogenic bacteria.