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Chemical, spectroscopic characterization, molecular modeling and antibacterial activity assays of a silver (I) complex with succinic acid

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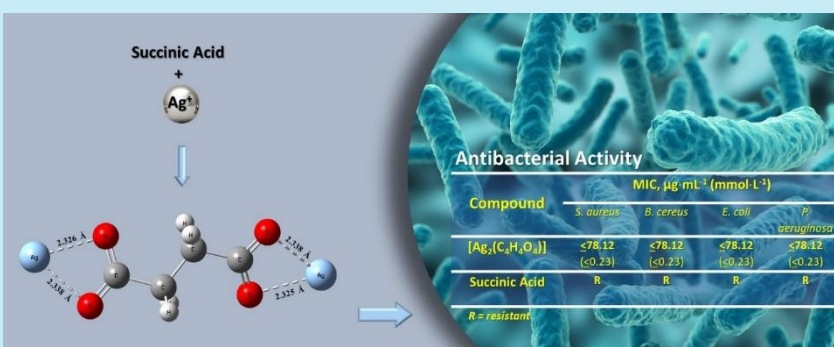
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ABSTRACT: A silver(I) complex with succinic acid in the form of succinate is presented. Chemical characterization confirms the molecular composition $\text{Ag}_2\text{C}_4\text{H}_4\text{O}_4$ for the complex. Infrared spectra suggest a bidentate coordination of both carboxylate groups of succinates to the two Ag(I) ions. Density functional theory (DFT) studies were used in the structures of succinic acid and Ag(I) succinate complex with coordination formula $[\text{Ag}_2(\text{C}_4\text{H}_4\text{O}_4)]$ in order to optimize them to their minimum energy. The studies confirmed that each carboxylate group of the succinate anion is coordinated to one silver atom by the two oxygen in a bidentate mode and the bond lengths $\text{O}\cdots\text{Ag}$ theoretically determined range from 2.325 to 2.338 Å. The complex $[\text{Ag}_2(\text{C}_4\text{H}_4\text{O}_4)]$ showed *in vitro* antibacterial activity against the bacterial strains of *Staphylococcus aureus*, *Bacillus cereus*, *Escherichia coli* and *Pseudomonas aeruginosa* complex. Anti-*Mycobacterium tuberculosis* analyses were also performed and the $[\text{Ag}_2(\text{C}_4\text{H}_4\text{O}_4)]$ complex was shown to be active over *M. tuberculosis* H37Rv strain with MIC_{90} of $23.94 \mu\text{g mL}^{-1}$ while succinic acid itself showed a value higher than $25.00 \mu\text{g mL}^{-1}$.



1. Introduction

According to recent reviews^{1,2}, the bacterial antibiotic resistance is increasing at an alarming rate. The indiscriminate use of antibiotics is largely responsible for the occurrence of resistant bacteria.

One of the most common bacterial strains is *Escherichia coli*, which is usually found in intestines of warm-blooded organisms. Most strains of *E. coli* are harmless, but some can cause severe food poisoning. It is transmitted to humans mostly by the ingestion of contaminated foods, such as raw meat, milk and vegetables. *Escherichia coli* can cause neurological complications (seizures, stroke and coma) in about 25% of patients with hemolytic uremic syndrome and chronic kidney sequelae in about 50% of survivors. The *E. coli* resistance to the standard antibiotic treatment with fluoroquinolones is now widespread. In some countries, this treatment has shown to be ineffective in more than 50% of patients³.

There has been a great increase in resistance to first-line drugs to treat infections caused by *Staphylococcus aureus*. People infected with *S. aureus* resistant to methicillin are estimated to be 64% more likely to die than people infected with a nonresistant strain of the same bacteria³.

Tuberculosis (TB) is a disease responsible for more than 1.6 million deaths annually and remains a remarkable public health case of concern worldwide. The number of infections is growing up in developed countries, especially for immunosuppressed patients (such as people with diabetes and HIV/AIDS), individuals receiving antitumor therapy and diabetic individuals⁴. Despite the improvement of TB treatment, it is greatly affected by growth of resistant strains of *Mycobacterium tuberculosis*⁵. In 2017, a range of 483,000–639,000 people worldwide developed TB resistant to rifampicin, the most effective first-line drug, according to the World Health Organization Tuberculosis Report⁴. More alarming is that, within this group, 82% developed multidrug-resistant TB⁶. Therefore, the challenge is the search for new substances with antimicrobial activities with a remarkable effectiveness when tested against bacteria (mainly the resistant strains), compared to those drugs used nowadays.

A well-known strategy to obtain active compounds against bacteria includes preparation and uses of silver(I) complexes and Ag nanoparticles. Silver(I) is known for its antimicrobial activities for a long time in medicine and materials sciences^{7–12}. The Ag(I) sulfadiazine complex, for example, has been clinically used as antibacterial and antifungal drug for more than 50 years. This complex is an insoluble compound which slowly liberates Ag(I) ions when used as a cream to treat bacterial infections in severe burns⁸.

Silver(I) ions interact with DNA¹³ or with S-donor ligands in vital enzymes and inactivate them^{14,15}. Consequently, there is an increase of pyrimidine dimerization by a photodynamic process and interruption of DNA replication. So, Ag(I) complexes with N- and O-donor ligands have increased ability to replace such molecules by the S-donor ligands of target bacterial proteins^{10,16,17}.

Succinic acid (Fig. 1) is a nonhygroscopic acidulant of relatively low acid strength largely used in food and beverage industries. It is also a precursor to produce some polyesters and a component of alkyd resins heavily used in automotive and electronics industries¹⁸. The ligand is very well studied and has no toxicity or mutagenicity. So, if the complex is to be applied *in vivo* in the future, safety on the ligand is ensured. Furthermore, succinic acid has a chemical structure with two carboxylate groups, which are particularly good coordination points to prepare metal complexes. Succinic acid is an intermediate metabolic of the Krebs cycle. So, it could be expected that this characteristic promotes a higher intracellular absorption of the complex when compared to AgNO₃ and Ag(I) complexed to other ligands reported in the literature.

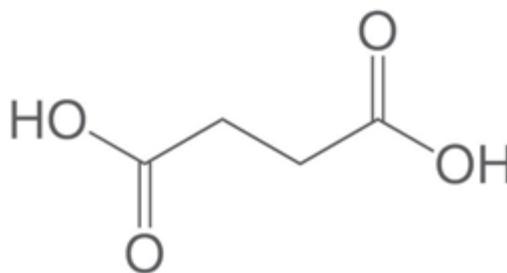


Figure 1. Chemical structure of succinic acid.

Succinic acid complexes have already been described in the literature, such as the interaction of neodymium(III) and iron(III) with it and some of its derivatives¹⁹. Sladkov *et al.*²⁰ studied complexation of uranyl (UO₂) and plutonyl (PuO₂) with succinic acid in aqueous acid solutions.

In the present work, synthesis and characterization combining experimental and density functional theory (DFT) studies of an Ag(I) complex with succinic acid in the form of succinate are described. Antibacterial assays of the complex against the Gram-positive bacteria *Staphylococcus aureus* ATCC 25923 and *Bacillus cereus* ATCC 14579 and the Gram-negative *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853 were also performed and are described in this manuscript.

2. Experimental

2.1 Materials

Succinic acid and silver nitrate were analytical grade chemical products from Sigma/Aldrich laboratories. All other chemicals were purchased from different sources. The reagents were used as received.

2.2 Synthesis

An aqueous solution (20 mL) with 3.0 mmol of succinic acid was adjusted to pH 7.0 with KOH; 20 mL of another aqueous solution containing 6.0 mmol of Ag(I) nitrate was added under stirring to the succinic solution in a dark room. A white precipitate was immediately formed. The solid was collected by filtration and left to dry in a desiccator with P₄O₁₀ protected from light. *Anal. Calc. for [Ag₂(C₄H₄O₄)] (%) C 14.5, H 1.2. Found (%) C 14.6, H 1.0. Yield: 75%.*

2.3 Instrumental methods

Carbon, hydrogen and nitrogen (CHN) elemental analyses were performed using a CHNS-O 2400 series II (Perkin Elmer) analyzer. Infrared (IR) spectra were obtained on a FTIR Cary 630 Agilent

spectrophotometer, equipped with attenuated total reflectance (ATR) sampling apparatus. The resolution was set at 4 cm⁻¹. Thermal analyses were performed on a thermoanalyzer TG/DTA simultaneous SDT Q-600 (TA Instruments) under the following conditions: aluminum crucible, synthetic air (100 mL min⁻¹), heating rate of 10 °C per min and temperature range from 30 to 1000 °C.

2.4 Computational simulations

All the methods described in this section were based in previous work²¹. The chemical structures of succinic acid and the Ag(I) succinate complex [Ag₂(C₄H₄O₄)] were optimized to the minimum of energy by the application of DFT with B3LYP^{22–24}. The basis sets 6-31+G(d,p)^{25–27} were applied for carbon (C), hydrogen (H) and oxygen (O) atoms. The LANL2DZ²⁸ effective core potential basis set was used for Ag atoms. Frequency calculations were employed to confirm that the optimized structures were at their minimum energy and no imaginary frequencies were found. Gaussian program 09 was used to perform all the calculations²⁹. Gauss View 5.09 program was used to generate some structures³⁰.

2.5 Determination of the minimal inhibitory concentration (MIC)

Determination of the minimum inhibitory concentration (MIC) of the free ligand and [Ag₂(C₄H₄O₄)] was performed using different reference bacterial strains, *S. aureus* ATCC 25923, *B. cereus* ATCC 14579, *E. coli* ATCC 25922 and *P. aeruginosa* ATCC 27853, as described in CLSI 2016^{31,32}. The bacterial strains were inoculated in tubes containing 10.0 mL of brain heart infusion (BHI KASVI) and incubated for 18 h at 35–37 °C. Sufficient inoculums of each bacterial suspension were added in new tubes of sterile BHI medium until reaching 1.0 turbidity of the McFarland nephelometric scale (~ 3.0 × 10⁸ CFU mL⁻¹). A volume of 50 µL of the stock solution (20 mg mL⁻¹) of the free ligand and [Ag₂(C₄H₄O₄)] suspended in 20% dimethyl sulfoxide (DMSO) aqueous solution were added from the second well (B) of the 96-well microplate plus 50 µL of sterile

BHI medium, followed by serial dilutions (5.0 to 0.078 mg mL^{-1}) and $100 \text{ }\mu\text{L}$ of the microorganism suspensions, on the McFarland 1.0 scale, were added to each well, reaching turbidity 0.5 McFarland ($\sim 1.5 \times 10^8 \text{ CFU mL}^{-1}$) in a final volume of $200 \text{ }\mu\text{L well}^{-1}$. In the first well of the microplate, used as a growth control, $50 \text{ }\mu\text{L}$ of sterile BHI medium, $50 \text{ }\mu\text{L}$ of 20% aqueous DMSO solution and $100 \text{ }\mu\text{L}$ of bacterial suspensions were added, on scale 1.0 McFarland. The microplates were incubated for 18 h at $35\text{--}37 \text{ }^\circ\text{C}$ in a humid chamber under agitation at 150 rpm. After the incubation period, $15 \text{ }\mu\text{L}$ of 0.02% resazurin in sterile aqueous solution was added to each hole in the plates. After 3 h of reincubation, the reading was performed. When active (or able to replicate), the bacterial cells convert the “blue” resazurin to “pink” resorufin. The lower concentration that resulted in the inhibition of bacterial growth (or blocked the conversion of resazurin to resorufin) was considered the MIC value. The tests were performed in triplicate.

2.6 Anti-*Mycobacterium tuberculosis* analyses

The MIC_{90} of compounds against the standard *M. tuberculosis* H₃₇Rv strain was determined with resazurin microtiter assay (REMA). Briefly, the compounds were dissolved in Middlebrook 7H9 broth, oleic albumin dextrose catalase (OADC) and glycerol 0.5%. The solutions of the compounds, in a range concentration of 0.09 to 25 mg mL^{-1} , were placed in a microplate (96-well) containing the bacterial inoculum, adjusted to 10^5 CFU mL^{-1} . The plates were incubated for seven days at $37 \text{ }^\circ\text{C}$, 5.0% CO_2 atmosphere. An aqueous solution of resazurin (0.01%) was added and the fluorescence was read at 530/590 nm after incubation of 24 h. The MIC_{90} is the lowest concentration of the compound, which inhibits 90% of bacterial growth³³. Three replicates were performed.

3. Results and discussion

3.1 IR spectra

Some bands of the IR spectrum of the complex change significantly when compared to the succinic acid and its potassium salt indicating coordination of

succinate to the metal by the carboxylate group. Figure 2 shows the IR spectra of succinic acid, K^+ -succinate (the anionic form of succinic acid) and the $[\text{Ag}_2(\text{C}_4\text{H}_4\text{O}_4)]$ complex. The spectrum of succinic acid shows a very large stretching O–H band in the range $3300\text{--}2500 \text{ cm}^{-1}$. It also shows a combined absorption pattern in the range $3300\text{--}2500 \text{ cm}^{-1}$, with a broad O–H band superimposed on the sharp stretching bands of C–H. The reason for a so broad O–H stretching band of succinic acid is certainly because carboxylic acids usually exist as hydrogen-bonded dimers. This band disappears in the spectra of both potassium salt and complex. The second change is in the stretching and bending vibrations C=O, C–O and O–H in the region $1300\text{--}1700 \text{ cm}^{-1}$. The C=O stretching mode of succinic acid occurs at 1675 cm^{-1} , the O–H bend at 1408 and 890 cm^{-1} and the C–O stretch at 1304 cm^{-1} . For K^+ -succinate, the COO^- ions generate a strong asymmetric stretching vibration ($\nu\text{COO}^-_{\text{as}}$) at 1559 cm^{-1} and a weak symmetric stretching vibration ($\nu\text{COO}^-_{\text{sym}}$) at 1392 cm^{-1} . These bands are located at 1507 cm^{-1} ($\nu\text{COO}^-_{\text{as}}$) and at 1385 cm^{-1} ($\nu\text{COO}^-_{\text{sym}}$) in the silver-succinate complex. The difference between $\nu\text{COO}^-_{\text{as}}$ and $\nu\text{COO}^-_{\text{sym}}$ ($\Delta\nu$) depends on the type of coordination between the metal and the carboxylate. The carboxylate group keeps the C_2 symmetry when coordinating as a bridging or a bidentate group. The metal atom is equally associated with the two oxygen atoms in the succinate salt. The difference $\Delta\nu$ between the COO^- asymmetric and symmetric stretching frequencies in the carboxylate salt and in the complex is 167 and 122 cm^{-1} , respectively, suggesting a bidentate coordination of each carboxylate group to the two Ag(I) ions^{34,35}.

The structure of succinate molecule is highly symmetric. So, the IR bands of the two carboxylate groups would have the same frequencies and such bands are superimposed. Consequently, $\Delta\nu$ should be the same for both carboxylate groups. In summary, each carboxylate group is coordinated to one Ag(I) ion in a bidentate mode.

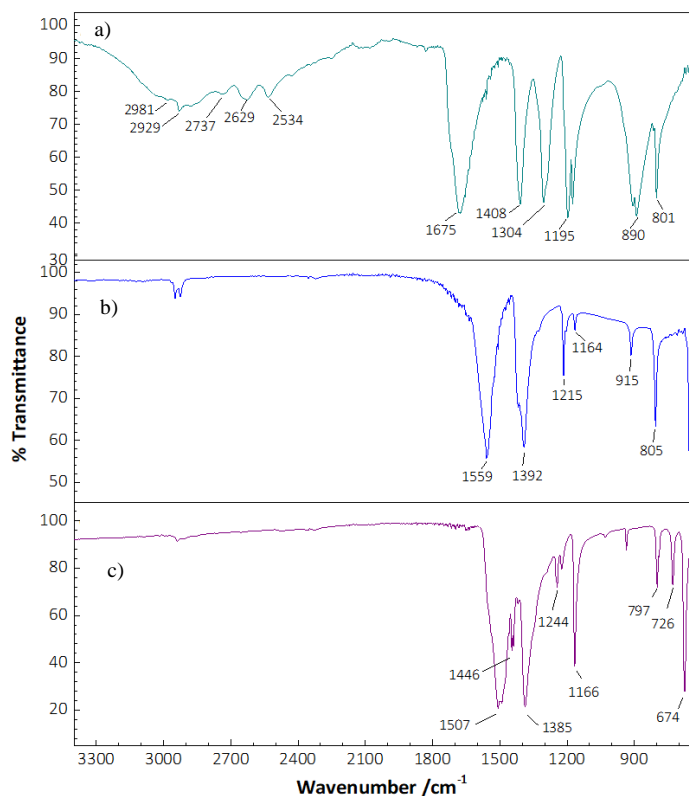


Figure 2. Infrared spectra of succinic acid (a), potassium succinate (b) and [Ag₂(C₄H₄O₄)] (c).

3.2 Thermogravimetric measurements

Figure 3 shows the thermogravimetric curve for the [Ag₂(C₄H₄O₄)] complex, with only one well-defined mass loss. The loss of about 34% occurred in the range 260–310 °C and corresponds to the net mass loss. The final residue percentage is in accordance with the formation of metallic silver. Differential scanning calorimetry (DSC) analysis of [Ag₂(C₄H₄O₄)] (Fig. 4) shows the occurrence of only one exothermic event at 304 °C, which corresponds to oxidation of the ligand leading to the formation of the residue of Ag⁰.

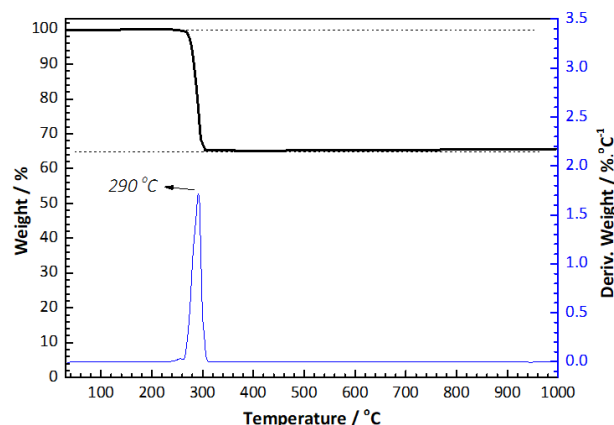


Figure 3. Thermogravimetric curves (TG and DTG) for [Ag₂(C₄H₄O₄)].

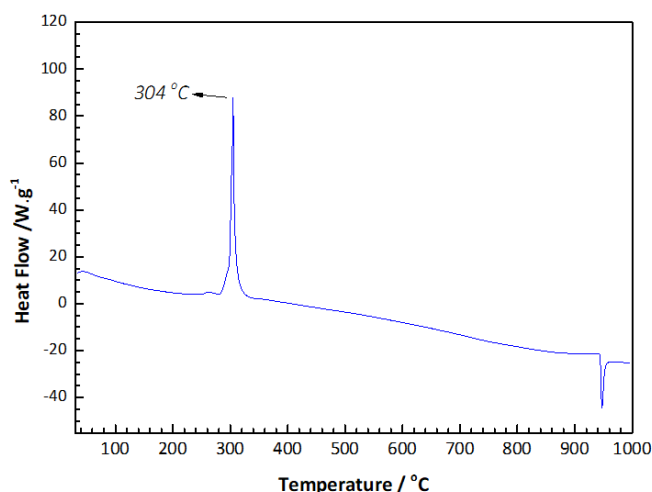


Figure 4. Differential scanning calorimetry (DSC) for [Ag₂(C₄H₄O₄)].

3.3 Electronic and structural properties by density functional theory (DFT)

Succinic acid and [Ag₂(C₄H₄O₄)] were theoretically studied and their structural parameters after complexation and their frontier molecular orbitals (FMO) were determined. The structural parameters calculated were the bond lengths for the metal and oxygen (O···Ag) (Fig. 5).

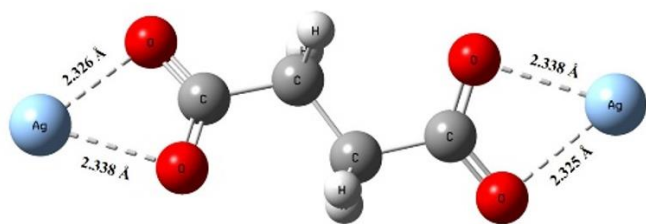


Figure 5. Structural formula for $[\text{Ag}_2(\text{C}_4\text{H}_4\text{O}_4)]$ and the bond length values for the $\text{O}\cdots\text{Ag}$ bonds.

By analyzing the results, it is possible to observe that each carboxylate group of the succinate coordinates to one Ag atom by the two O atoms in a bidentate mode and the theoretically determined bond lengths $\text{O}\cdots\text{Ag}$ were in the range from 2.325 to 2.338 Å. It is important to note that, for optimization, the Ag atom was placed close to the O atom and no imaginary frequencies were identified after optimization, showing that the structure determined in Fig. 6 was at minimum energy. The theoretically determined values for the $\text{O}\cdots\text{Ag}$ bond are similar to the crystal data found in the literature for a silver complex with mixed ligands 2-aminobenzonitrile and 4-methylbenzoic acid, where the carboxylate group is coordinated to silver in a bidentate chelate mode³⁶. The frontier molecular orbitals highest occupied molecular orbital (HOMO), lowest unoccupied molecular orbital (LUMO) and the energy gap for succinic acid and the $[\text{Ag}_2(\text{C}_4\text{H}_4\text{O}_4)]$ complex were evaluated and are also shown in Fig. 6.

The results indicate that the HOMO and LUMO orbitals of succinic acid, as well as the HOMO of $[\text{Ag}_2(\text{C}_4\text{H}_4\text{O}_4)]$, are located over all molecular structures. The exception was the LUMO of the complex which is most located over the Ag atoms. The results for the determined energy gap were

$\Delta E = 7.21$ eV for the ligand and $\Delta E = 3.32$ eV for the complex. The values of gap show that there is a significant decrease in the energy gap ($\cong 3.89$ eV) after complexation, which indicates that the complex is more reactive than the ligand.

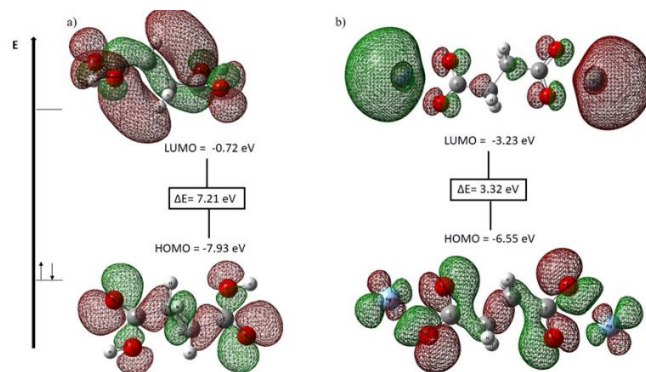


Figure 6. Frontier molecular orbitals and energy gap for: a) succinic acid and b) $[\text{Ag}_2(\text{C}_4\text{H}_4\text{O}_4)]$ complex.

3.4 Minimum inhibitory concentration (MIC)

The $[\text{Ag}_2(\text{succ})]$ shows its inhibitory activity against Gram-positive and Gram-negative bacteria used in the assay. The free ligand succ and DMSO aqueous solution did not show antibacterial activities. The results are summarized in Table 1.

The results suggest that antibacterial activity of $[\text{Ag}_2(\text{C}_4\text{H}_4\text{O}_4)]$ is due to the release of Ag^+ ions in the same way it was described for Ag-sulfadiazine and for other Ag complexes. Inhibition (in $\text{mmol}\cdot\text{L}^{-1}$) provided by $[\text{Ag}_2(\text{C}_4\text{H}_4\text{O}_4)]$ was higher than that observed by the starting salt AgNO_3 . It is already described in the literature that Ag^+ can link to the cell membrane and consequently inhibits cell divisions. Silver ions also binds to bacterial DNA and RNA and inhibits bacterial replication³⁷.

Table 1. Minimum inhibitory concentration (MIC) values of AgNO_3 , succinic acid and $[\text{Ag}_2(\text{succ})]$.

	<i>S. aureus</i> ATCC 25923		<i>B. cereus</i> ATCC 14579		<i>E. coli</i> ATCC 25922		<i>P. aeruginosa</i> ATCC 27853	
	MIC $\mu\text{g}\cdot\text{mL}^{-1}$	MIC $\text{mmol}\cdot\text{L}^{-1}$	MIC $\mu\text{g}\cdot\text{mL}^{-1}$	MIC $\text{mmol}\cdot\text{L}^{-1}$	MIC $\mu\text{g}\cdot\text{mL}^{-1}$	MIC $\text{mmol}\cdot\text{L}^{-1}$	MIC $\mu\text{g}\cdot\text{mL}^{-1}$	MIC $\text{mmol}\cdot\text{L}^{-1}$
$[\text{Ag}_2(\text{succ})]$	≤ 78.12	≤ 0.23	≤ 78.12	≤ 0.23	≤ 78.12	≤ 0.23	≤ 78.12	≤ 0.23
Succinic acid	R		R		R		R	
AgNO_3	≤ 78.12	≤ 0.45	≤ 78.12	≤ 0.45	≤ 78.12	≤ 0.45	≤ 78.12	≤ 0.45

R = Resistant. Student t-test was used to determine the statistical significance for *S. aureus*, *B. cereus*, *E. coli* and *P. aeruginosa* replicates. Results were expressed as the mean (***) $p < 0.005$.

So, the use of silver complex promotes a slower release of the silver ions when compared to silver nitrate. This characteristic increases the antibacterial effect of the compound. For example, the role of silver and sulfadiazine in the mechanism of action of silver sulfadiazine on burn wound infections was investigated³⁸. The efficacy of silver sulfadiazine is thought to result from its slow and steady reactions with serum and other sodium chloride-containing body fluids, which permits the slow and sustained delivery of silver ions into the wound environment. In this circumstance, a relatively minimum amount of sulfadiazine appears to be active.

Furthermore, the ligand succinic acid is an intermediate metabolic of the Krebs cycle. So, it is suggested that this characteristic promotes a higher intracellular absorption of the complex when compared to AgNO₃ and Ag(I) complexed to other ligands reported in the literature.

The results obtained in this work demonstrated that the [Ag₂(C₄H₄O₄)] complex showed significant growth inhibition activity of the tested bacterial species, when compared with other studies that report the inhibitory activity of bacterial growth by Ag(I) complexes with several ligands, as Ag(I) with furosemide (MIC = 0.39 mmol L⁻¹ for gram-positive bacteria strains)³², sulfathiazole (MIC 3.45 mmol L⁻¹ for gram-negative bacteria and 6.90 mmol L⁻¹ for Gram-positive bacteria strains) and sulfamethoxazole (MIC = 1.74 mmol L⁻¹ for gram-negative bacteria and 13.9 mmol L⁻¹ for gram-positive bacteria)³⁹. The results demonstrate the potential for using the [Ag₂(C₄H₄O₄)] complex as an antibacterial drug in the future.

3.5 Anti-*Mycobacterium tuberculosis* activity

The MIC₉₀ values for [Ag₂(C₄H₄O₄)], succinic acid and rifampicin were obtained through REMA. Rifampicin, the most effective first-line drug, was used as a control and showed a MIC₉₀ of 0.08 µg mL⁻¹. The silver complex with succinate was shown to be active with a MIC₉₀ of 23.94 µg mL⁻¹ while succinic acid itself presented a value higher than 25.00 µg mL⁻¹. In spite of being active, the [Ag₂(C₄H₄O₄)] complex has a MIC value lower than that of other antimycobacterial agents used in TB clinical treatments as ethambutol

(MIC₉₀ = 5.62 µg mL⁻¹) and *p*-aminosalicylic (MIC₉₀ = 1.25 µg mL⁻¹).

4. Conclusions

A new silver complex with succinic acid was obtained and characterized. On the basis of elemental and thermogravimetric analyses, the complex was formulated as [Ag₂(C₄H₄O₄)]. Infrared spectra showed that the value for Δν decreases for both carboxylate groups because of the high symmetry of succinate anion compared to the free succinic acid molecule. The reduction of the Δν value indicates coordination of each carboxylate group to each Ag(I) in a bidentate mode. The theoretical structures for succinic acid and the [Ag₂(C₄H₄O₄)] complex were optimized to the minimum of energy using DFT. The studies confirm that each carboxylate group of the succinate coordinates to one Ag atom by the two O atoms in a bidentate mode as suggested by the IR data and the bond lengths O...Ag theoretically range from 2.325 to 2.338 Å.

The complex shows inhibitory activity against the considered strains used in the assay with MIC values ≤ 0.23 mmol L⁻¹. Succinic acid, K⁺-succinate and DMSO aqueous solution were inactive. Anti-*Mycobacterium tuberculosis* analyses were also performed with [Ag₂(C₄H₄O₄)] and succinic acid. The [Ag₂(C₄H₄O₄)] complex was shown to be active presenting a MIC₉₀ of 23.94 µg mL⁻¹ while succinic acid itself presented a value higher than 25.00 µg mL⁻¹. The results obtained indicate the significant activity *in vitro* of the silver complex with succinic acid and warrants for additional studies in the search of safer silver-based antimicrobial drugs.

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