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Inhibition behavior on alkaline phosphatase activity, antibacterial and antioxidant activities of ternary methimazole–phenanthroline–copper(II) complex

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ABSTRACT

Methimazole (MeimzH), a sulfur containing compound, is an antithyroid drug commonly used to treat Graves' disease acting as an inhibitor of the enzyme thyroid peroxidase. A new ternary $[Cu(MeimzH)_2(phen)(H_2O)_2]Cl_2$ (Cu-Met-phen) complex of this drug containing also phenanthroline as a second ligand (phen) was synthesized and characterized by elemental analysis, dissolution behavior, thermogravimetric analysis, UV–Vis, diffuse reflectance, FTIR and EPR spectroscopies. As it is previously reported, the binary $[Cu(MeimzH)_2(H_2O)_2](NO_3)_2{\cdot}H_2O$ (Cu-Met) complex can act as an inhibitor of alkaline phosphatase (ALP). In this work, the inhibitory effect of methimazole, phenanthroline, $[Cu(phen)_2Cl]Cl$ (Cu-phen) and the new ternary complex has also been investigated and compared with Cu-Met together with *in vitro* tests of susceptibility against *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Staphylococcus epidermidis* and *Enterococcus faecalis* bacteria. To our knowledge, the antibacterial activity of methimazole is determined for the first time. For the ternary complex, the results show that the coordination of the drug increased its antibacterial effect especially against *E. faecalis* demonstrating a strong effect against *S. aureus* and *S. epidermidis*. The assays also indicate that in same strains the new complex show better activity than their ligands. The ability for the inhibition of alkaline phosphatase (ALP) was lower than that of the binary complex (Cu-Met) probably due to the absence of labile ligands around the metal center. Moreover, the antioxidant activity of the complexes decreases as follows: Cu-Met > Cu-Phen > Cu-Met-Phen.

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1. Introduction

Numerous metal ions are recognized to play specific and important roles in biological processes in the human body [1]. In particular copper(II) ions, the third most abundant transition metals in humans, is essential for many organisms. This metal is required for aerobic metabolism, and it can be found as active site or as structural component of a large number of enzymes [2–4]. Metal complexes focused on enzyme inhibition have been extensively studied in light of their promissory biological applications in medicine. In this context, sometimes copper(II) and most of the time copper(II) complexes are involved in inhibiting enzymes (i) ribonucleotide reductase, protein kinase C, topoisomerase III, in cancer applications; (ii) γ-aminolevulinate and synthase nitric oxide synthase (NOS), in heme related applications; (iii) carbonic anhydrase and angiotensin converting enzyme, in hypertension applications; (iv) monoamine oxidase, in neurological applications; (v) elastase, collagenase, lipoxygenase, cathepsin, and cyclooxygenase, in arthritis applications; (vi) leukemia virus reverse transcriptase, protease and integrase in viral applications; (vii) hexokinase and glycerol kinase in toxicity applications and (viii) dihydrofolate reductase in non viral applications [5]. In recent times, Li et al. [6] showed that copper complexes with multi-benzimidazole derivatives act as protein tyrosine phosphatases inhibitors (PTPs), and our group [7] demonstrated for the first time that methimazole and its copper(II) complexes behaved as alkaline phosphatase (ALP) inhibitors. In the recent years, the role of metal

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complexes has been increased in relation to antibacterial activity. Literature survey indicates that copper(II) and their metal complexes have been found to exhibit antimicrobial activities becoming the complexation an effective strategy for the improvement of the biological activities of the ligands [8–11]. Literature points out that various organic ligands can be converted into more bacteriostatic on complexation in comparison with the unchelated ones [12,13]. Another plan consists on an appropriate ligand selection based in their chemical and biochemical characteristics. In this sense, 1,10-phenanthroline (phen) is a strong field bidentate ligand that forms very stable chelates with many first row transition metals [14]. It has long been known that 1,10-phenanthroline has antibacterial, antifungal and antiviral properties [15]. It has also been reported that copper(II) ion is essential for bacteria, but it becomes toxic at higher concentration. For instance, cells exposed to copper surfaces accumulate large amounts of copper(II) ions suffering and losing its integrity [16,17]. Zoroddu et al. [15] demonstrated that for the simple copper(II)–phenanthroline complexes ($Cu(phen)^{+2}$, $Cu(phen)_2^{+2}$) there is an increase in the biocidal activity in terms of their minimum inhibitory concentration (MIC) values and several phenanthroline derived complexes have been demonstrated to have antimicrobial activity [18–20]. Related to the ability of this ligand as inhibitor of the alkaline phosphatase (ALP) activity, it is well documented that this chelating agent inhibits *Escherichia coli* alkaline phosphatase by removal of its zinc ion on complexation [21].

On the other hand, sulfur containing ligands are established to be an important class of donor ligands for the most part of transition metal ions. At present, one of the best known types is the "sulfonamide" group which began to be recognized when sulfonylamide showed for the first time antibacterial activity [22]. Other ligands that constitute an important class of sulfur donor functional group are the thiosemicarbazones because of their highly demonstrated medicinal properties [23,24]. In this work we have selected the sulfur containing ligand methimazole (MeimzH), which behaves as an antithyroid drug commonly used to treat Graves' disease that moderate thyroid activity acting as an inhibitor of the enzyme thyroid peroxidase.

This work is an extension of previously reported studies [7] on the aspects related to unknown potential activities of methimazole and its copper complexes, their ability as ALP inhibitors including the measurements of their bactericidal capacities. The main purpose was to ascertain whether the differences in the chemical arrangement and in the coordination environment of copper(II) can affect its biological activities. For this reason, in the present investigation, the activity of the ligands and their respective copper(II) complexes was compared. Another driving force of this investigation consists in the development of new antibacterial drugs in order to avoid emerging bacterial resistance to the currently available antibiotics.

To the best of our knowledge, this is the first manuscript which includes the synthesis, characterization, antibacterial and SOD like activities and enzymatic inhibition behavior on ALP of the ternary $[Cu(MeimzH)_2(phen)(H_2O)_2]Cl_2$ (Cu-Met-phen) complex and measurements of the antimicrobial activity of methimazole which has not yet been reported in the literature.

2. Materials and methods

2.1. Reagents and instrumentation

All chemicals were of analytical grade. Copper(II) chloride dihydrate was obtained from Merck, Methimazole, 1,10-phenanthroline monohydrate, para-nitrophenyl phosphate (p-NPP), alkaline bovine intestinal ALP, anhydrous lactose and all the other analytical grade chemicals used were purchased from Sigma.

FTIR spectra of powdered samples were measured with a Bruker IFS 66 FTIR-spectrophotometer from 4000 to 400 cm^{-1} in the form of pressed KBr pellets. Electronic absorption spectra were recorded on a Hewlett–Packard 8453 diode-array spectrophotometer, using 1 cm quartz cells. Diffuse reflectance spectra were registered with a Shimadzu UV-300 instrument, using MgO as an internal standard. Elemental analyses for carbon, hydrogen and sulfur were performed using a Carlo Erba EA 1108 analyzer. For the dissolution capacity test a Hanson Research SR6 (Apparatus 2-Paddle Apparatus) equipment and a Spectrophotometer Metrolab 1700 were used. To record the spectra of the compounds at different temperatures, a Bruker ESP300 spectrometer operating at X and Q-bands and equipped with standard Oxford low temperature devices was used. The magnetic field was measured with a Bruker BNM 200 gaussmeter, and the frequency inside the cavity was determined by using a Hewlett–Packard 5352B microwave frequency counter. A computer simulation of the EPR spectra was performed using the programs SimFonia (WINEPR SimFonia 1996).

2.2. Preparative

$[Cu(MeimzH)_2(phen)(H_2O)_2]Cl_2$ (Cu-Met-phen) a solution was prepared by mixing 1 mmol (0.1142 g) of methimazole dissolved in 5 mL of water with 1 mmol (0.1802 g) of o-phenanthroline dissolved in 5 mL of ethanol. To this solution, 1 mmol (0.1705 g) of solid copper(II) chloride dihydrate was slowly added under continuous stirring. The resulting mixture was stirred at room temperature for 2 h. After that, a light green precipitate was formed which was separated, filtered and washed several times with ethanol. After this procedure it was dried in an oven at 60 °C. *Anal.* Calc. for $C_{20}H_{24}N_6O_2S_2Cl_2Cu$: C, 41.48; H, 4.15; N, 14.52; S, 11.06. Found: C, 41.15; H, 4.27; N, 14.78; S, 11.38%. Yield: 75%.

2.3. Dissolution profiles of the complexes

The dissolution profiles were performed according to the United States Pharmacopeia (USP 30). Capsules No. 3 were prepared with homogenous mixture (20 mg of the complex and 130 mg of anhydrous lactose) according to methimazole formulation [25]. Dissolution testing of capsules was performed in distilled water (pH 5–6), HCl 0.1 M, sodium lauryl sulfate 0.01 M and buffer KH_2PO_4 (pH 6.8, 0.2 M) and simulated gastric medium NaCl (2 g/L) and concentrated HCl (6.0 mL/L), final pH value of 1.2. The dissolution medium was 500 mL at 37 °C, stirred at 100 rpm. For dissolution profiles, 10.0 mL aliquots were withdrawn at 5, 10, 20, 30, 40, 50 and 60 min. The solutions were immediately filtered off through 0.45 μm nylon filter. One milliliter of the filtered aliquots was pipetted into 25 mL volumetric flask for each compound with fresh dissolution fluids in each case. Drug release (DR%) was assayed by electronic spectroscopy, UV absorbance was measured at 252 nm. Cumulative percentages of the dissolved drug from the tablets were calculated and plotted versus time.

2.4. Stability studies

In order to determine the stability of the compounds during the preparation of the solutions for the *in vitro* measurements, the variation of the UV–Vis spectra with time was performed. The dissolution has been carried out in water and with DMSO (dimethylsulfoxide). In both cases the copper(II) d–d electronic absorption bands in the visible spectral range were monitored.

2.5. SOD assays

A non-enzymatic assay was used to determine SOD mimetic activity. In this method, the system (PMS)/(NADH) produces the superoxide anion radical. The system contains 0.5 mL of sample, 0.5 mL of 1.40 mM NADH, 0.5 mL of 300 μM NBT, in 0.05 M phosphate buffer (pH 7.5). After incubation at 25 °C for 15 min, the reaction starts by the addition of 0.5 mL of 120 μM PMS [26]. The solutions of Cu-Met-phen and Cu-phen complexes were prepared in hot dimethylsulfoxide (DMSO) before adding the phosphate buffer to obtain the desired final concentrations. The final DMSO:-buffer concentration ratio never exceeded 5:100. Then, the reaction mixture was incubated for 5 min at 25 °C. The results were determined by reading the absorbance at 560 nm. The amount of complex that produced a 50% inhibition of NBT reduction (IC_{50}) was obtained from a plot of percentage of inhibition versus complex concentration. Kinetic constant value (k), which is independent of both detector concentration and nature, was calculated according to: *k*McCF = *k*detector x [detector]/IC_{50}(compound), where *k*NBT (pH 7.8) = 5.94 x 10^4 mol^{-1} L s^{-1} and [detector] = detector concentration [27].

2.6. Alkaline phosphatase specific activity

The effect of copper(II) cation and the other copper(II) complexes on ALP activity was determined by UV–Vis spectroscopy. The reaction was started by the addition of the substrate para-nitrophenyl phosphate (p-NPP) and the product p-nitrophenol was monitored by the absorbance changes at 405 nm. Briefly, the experimental conditions for ALP specific activity measurement were as follows: 1 μg/mL of bovine intestinal ALP and 5 mM of p-NPP were dissolved in the incubation buffer (55 mM glycine + 0.55 mM $MgCl_2$, pH 10.4) and held for 10 min. The effects of the compounds were determined by addition of different concentrations (1–1000 μM) of each one to the pre-incubated mixture. The solutions of the complexes were prepared in DMSO before adding the buffer to obtain the desired final concentrations. The effect of each concentration was tested at least in triplicate in three different experiments.

2.7. Antibacterial assays

Antimicrobial activity was evaluated by the minimum inhibitory concentration (MIC) on five strains of bacteria derived from the American Type Culture Collections (ATCC), namely *E. coli* (ATCC 35218), *Pseudomonas aeruginosa* (ATCC 27853), *Staphylococcus aureus* (ATCC 25923), *Staphylococcus epidermidis* (ATCC 1263) and *Enterococcus faecalis* (ATCC 29212). The MICs were determined using the agar-dilution method. The cultivation/assay medium for all strains was Mueller Hinton Broth or Agar (MHB, MHA) [28]. The inoculum of bacterial strains was prepared from 18 h-old broth cultures. A McFarland 0.5 suspension was prepared for each microorganism and a 1:10 dilution was made prior to inoculation (~10^7 colony forming units (CFU) per milliliter) [29,30]. For the agar-dilution method methimazole and the complexes were dissolved in 50% aqueous dimethylsulfoxide (DMSO). Aqueous solutions of the metal salt and the ligands were prepared. All these solutions were sterilized by filtration. Serial of twofold dilutions were prepared from the stock solution in molten MHA medium and cooled down to 45 °C to obtain the desired final concentrations. Dosage of each chemical started from 2.93 μg mL^{-1} (micrograms per milliliter) and continued until 1500 μg mL^{-1} (stopping criterion). Then, the inoculum of 2 μL of the germ suspensions was streaked onto the plates and incubated aerobically at 37 °C for 24 h [1,2,31]. Inhibition of bacterial growth in the plates (45 × 15 mm) containing tested solutions was judged by comparison with growth in blank control plates. The MIC was defined as the lowest dilution of the complex that inhibited the visible growth of the test organism. A single colony or a faint haze caused by the inoculum was considered to be no growth. Each analysis was carried out three times [1,2,32].

3. Results and discussion

3.1. Syntheses

The mixed-ligand compound of general formula $[Cu(MeimzH)_2(phen)(H_2O)_2]Cl_2$ was synthesized by a straight forward synthesis in which the simultaneous reaction of methimazole with 1,10-phenanthroline monohydrate and copper(II) chloride dehydrate in a 1:1:1 molar ratio, yielded a green microcrystalline powder. All the attempts to grow single crystals of diffractometric quality for this complex failed. The new chelate coordination complex is soluble in water as well as in dimethylsulfoxide (DMSO) and dimethylformamide (DMF) but insoluble in methanol and ethanol. The analytical data C, H, N and TGA measurements confirm the composition of the complex and the stoichiometry proposed. Evidence of the mode of bonding of the ligands was gathered from the FTIR spectra, while their geometry was assumed from the diffuse reflectance spectra and EPR spectroscopies.

The complex $[Cu(phen)_2Cl]Cl$ named as Cu-phen and structurally characterized by X-ray measurements was obtained as a by-product of the main reaction and it was used for comparative purposes.

3.2. FTIR spectroscopy

Methimazole is a heterocyclic thione, which presents thione–thiol tautomerism in the molecule being the thione tautomer the dominant species in the solid state. The predominance of the thione form in the spectrum of the free ligand is established by the nonappearance of the ν(SH) band at ca. 2500 cm^{-1} and the existence of the ν(NH) band at 3108 cm^{-1} (Table 1) [33,34]. The spectra of the copper complex also confirm that methimazole is coordinated *via* the thione sulfur atom giving rise to four characteristic bands in the regions 1570–1395, 1420–1260, 1140–940 and 800–700 cm^{-1} which correspond to the called "thioamide I, II, III, IV bands", respectively [35]. It is well established that bands I and II have a little C=S contribution and they are originated mainly in C=N and NH deformation modes but bands III and IV have a significant C=S content. These bands are used to suggest the heterocyclic thione coordination mode in the complex. The insignificant changes in the position of the ν(NH) band on complexation

Table 1
Assignments of some characteristic FTIR bands (cm^{-1}) of methimazole (Met) and Cu-Met-phen complex.

MeimzH	Phen	Cu-Met-phen	Assignments	
3108 s,br		3115 s	ν(NH)	
1462 s		1470 s	I	Thioamide bands
1271 s		1283 s	II	
1084 m		1108 m	III	
766 m		748 m	IV	
		722 m		
	1642 m	1627 m		Phen
	1616 m	1607 m	ν(arC–C),	
	1505 m	1516 m	ν(C=N)	
	879 sh	871 vs	δ_H (out of plane)	
	852 s	855 vs		
	738 s	736 m		

vs = very strong, s = strong, m = medium, w = weak, sh = shoulder.

(Table 1) is an indication that no interaction occurs *via* the nitrogen atom of the ligand. All of the "thioamide bands" are moved to some degree being the most important changes the ones observed in the thioamide IV band. This band has the largest proportion of ν(CS) activity and its splitting and downfield shift are consistent with ligand thione-S donation in the complex [36,37].

Bands corresponding to the vibrational modes of phenanthroline were also detected. The spectra of the complex displayed new bands between 1630 and 1575 cm^{-1} which can be assigned to the vibrations ν(arC–C) and ν(C=N) of the aromatic rings of the o-phenanthroline ligand. Strong bands in the 900–700 cm^{-1} region in spectra of aromatic hydrocarbons have been identified with motions of the ring hydrogen atoms. In o-phenanthroline, and also in its complex, two strong bands appeared approximately at 850 and 725 cm^{-1} (Table 1) [38,39].

The observed FTIR changes suggest the presence of sulfur and nitrogen donor atoms from both ligands in the equatorial environment around the metal center.

3.3. Diffuse reflectance and stability studies by UV–Vis spectroscopy

The diffuse reflectance spectrum of Cu-Met-phen showed a broad band with a maximum at 750 nm (Fig. 1(A), solid line) which is assigned to the d–d transitions of the copper(II) ion (450–830 nm region, rather asymmetric due to d^9 configuration Jahn–Teller effect) [40]. The prominent band located at 370 nm could be tentatively assigned to charge transfer S → Cu(II) transitions characteristic to equatorial S-bonding from methimazole ligand [41,42]. For comparative purposes, Fig. 1(A), dash line showed diffuse reflectance spectrum of Cu-phen complex.

In order to evaluate the stability of the complex in the same experimental conditions than those of the *in vitro* studies, it was dissolved in DMSO and in water. Fig. 1(B) shows the time-dependent changes in the visible range of the spectra. Under these conditions, the chemical species formed on dissolution of the light green complex was stable in time. The electronic spectra in DMSO showed a prominent broad band in the range 500–810 nm with a maximum at 740 nm (ε = 97.54 $M^{-1}cm^{-1}$) (Fig. 1(B)). These results demonstrated that during the manipulation time of the samples on the bioactivity assays there is a great possibility that the cationic complex displays the same coordination mode than in the solid compound. In aqueous solution the UV–Vis spectrum of the complex also displayed a broad band with maximum at 658 nm (ε = 143.5 $M^{-1}cm^{-1}$) (Fig. 1(C)). The observed hypsochromic shift of the band is probably related to significant solvent effects when it changes from a polar aprotic solvent (DMSO) to a polar protic solvent as water. No strong absorptions attributable to S → Cu(II) LMTC transitions are observed probably due to the presence of solvent bands.

3.4. Electron spin resonance spectra

The X and Q-band EPR powder spectra of the complex were measured at room temperature (Fig. 2). The polycrystalline sample of Cu-Met-phen showed rhombic symmetry [43]. The spectral simulation predicted the spin Hamiltonian parameters and the hyperfine coupling constants of respectively g_1 = 2.048, g_2 = 2.141 and g_3 = 2.204 (g_0 = 2.131); $A_3 = 170 \times 10^{-4}$ cm^{-1}, $A_1 = A_2 = 65 \times 10^{-4}$ cm^{-1} ($A_0 = 100 \times 10^{-4}$ cm^{-1}). The calculated parameters are in concordance with a S_2N_2 donor environment in the equatorial plane of the distorted octahedral coordination [44–46]. In correspondence with the proposed equatorial coordination mode the value of the g_3 parameter lower than 2.3 suggests a considerable covalent bonding [47,48]. In order to distinguish the geometry, the ***R*** parameter, which is a measure of the exchange interaction between copper centers in the polycrystalline compound, was

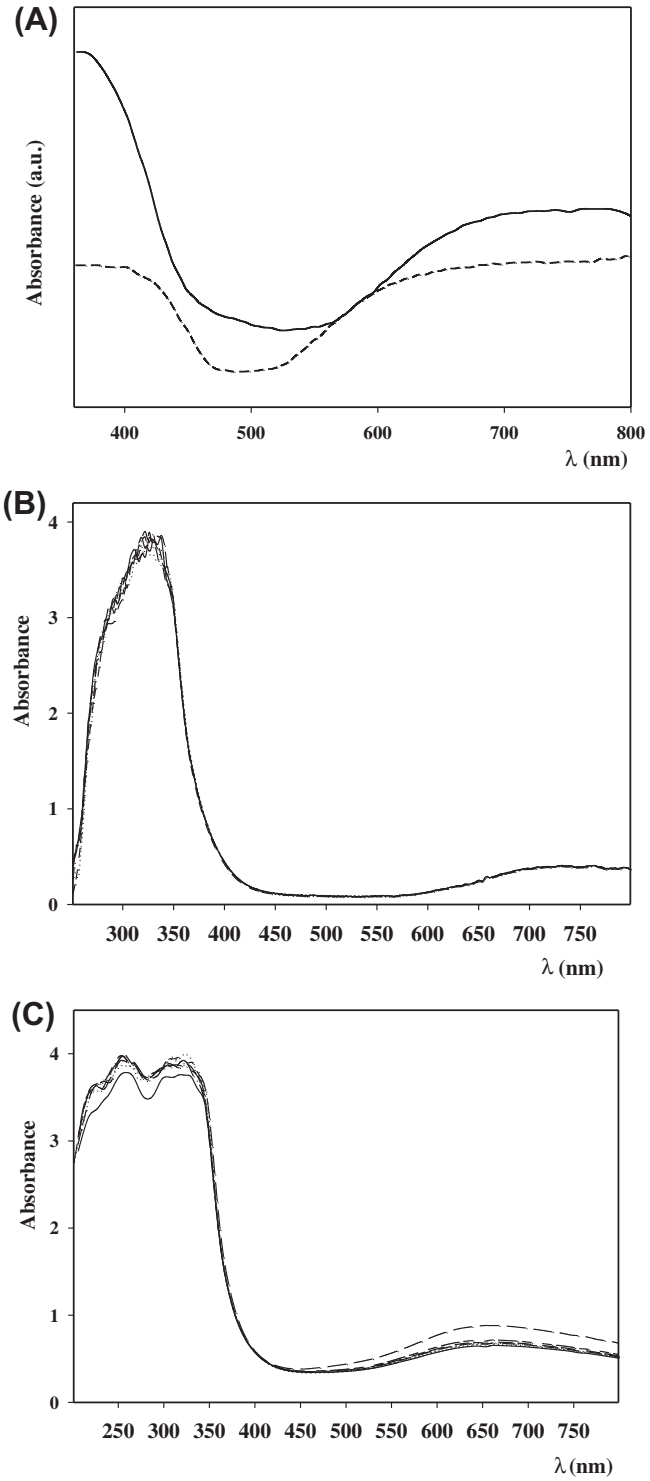

Fig. 1. (A) Solid line: reflectance spectrum of the Cu-Met-phen; short dash line: Cu-phen. (B) UV–Vis absorbance spectrum in time (10 min) of the Cu-Met-phen in DMSO solvent (3.98×10^{-3} mM, ε = 97.54 $M^{-1}cm^{-1}$). (C) UV–Vis absorbance spectrum in time (10 min) of the Cu-Met-phen in H_2O solvent (5.06×10^{-3} mM, ε = 143.5 $M^{-1}cm^{-1}$).

calculated using the equation $\boldsymbol{R} = (g_2 - g_1)/(g_3 - g_2)$ and taking into account that $g_3 > g_2 > g_1$. A value of 0.58 was obtained for ***R*** indicating that the unpaired electron is located mostly on $dx^2 - y^2$ ground state of the copper(II) atom being the elongated rhombic-octahedral geometry the most probable one [16].

The EPR spectra of the solid complex after dissolution in DMSO (g_1 = 2.068, g_2 = 2.070 and g_3 = 2.288 (g_0 = 2.1420);

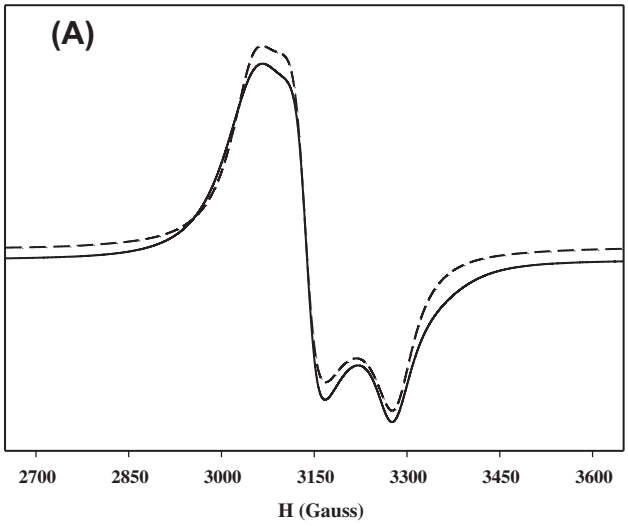

Fig. 2. EPR powder spectra at 290 K (X band) of the complex Cu-Met-phen (solid line) and simulated spectrum (WINEPR SimFonia (dash line)).

$A_3 = 165 \times 10^{-4}\ cm^{-1}$, $A_1 = A_2 = 2 \times 10^{-4}\ cm^{-1}$ ($A_0 = 56.33 \times 10^{-4}\ cm^{-1}$) and water ($g_1 = 2.071$, $g_2 = 2.071$ and $g_3 = 2.320$ ($g_0 = 2.154$); $A_3 = 164 \times 10^{-4}\ cm^{-1}$, $A_1 = A_2 = 5 \times 10^{-4}\ cm^{-1}$ ($A_0 = 58 \times 10^{-4}\ cm^{-1}$) show spectra of approximately axial symmetry in agreement with the data obtained from the UV–Vis spectra.

Taking into account all the spectroscopic results obtained for the complex, it is possible to assume that the copper(II) ion exhibits a octahedral geometry, where two N atoms (phen), two S atoms (two methimazole molecules) in the equatorial plane and two oxygen atoms arising from two water molecules in the axial position, are coordinated to the metal center.

3.5. Dissolution assay

The purpose of the present study was to characterize the dissolution performance of the danantizol (methimazole) and the Cu-Met-phen complex in different dissolution media. Fig. 3 shows the dissolution profile for methimazole (danantizol) (Fig. 3(A)) and Cu-Met-phen complex (Fig. 3(B)) using 100 rpm.

The percentage of the dissolution of methimazol increases with the time in all cases until a constant value gets an asymptote where the value is maintained constant for several minutes reaching to thermodynamic equilibrium. The greatest percentage of the dissolution of commercial drug (danantizol) was achieved using HCl as solvent, resulting in thermodynamic equilibrium between 20 and 60 min. When others solvents were used this percentage decreases slightly for H_2O and gastric medium while the lower solubility was achieved for sodium lauryl sulfate (LSNa) and the buffer solution and in both cases it was quite similar.

For the complex (Fig. 3(B)), the dissolution rate increased with time arriving at the thermodynamic balance in all cases. The highest percentage of dissolution was achieved using HCl as a solvent, producing the thermodynamic equilibrium between 40 and 60 min. It was further noted that the simulated gastric medium acted as a good solvent reaching thermodynamic equilibrium faster than that in HCl. For the other solvents, the solubility of the ternary complex studied was substantially similar when using sodium lauryl sulfate (LSNa) and buffer solution, and the smallest percentage of dissolution was produced in an aqueous medium.

3.6. Antioxidant activity

The beneficial role of Cu in several diseases has been attributed to its redox activity/in particular, as catalytic site in enzymes as

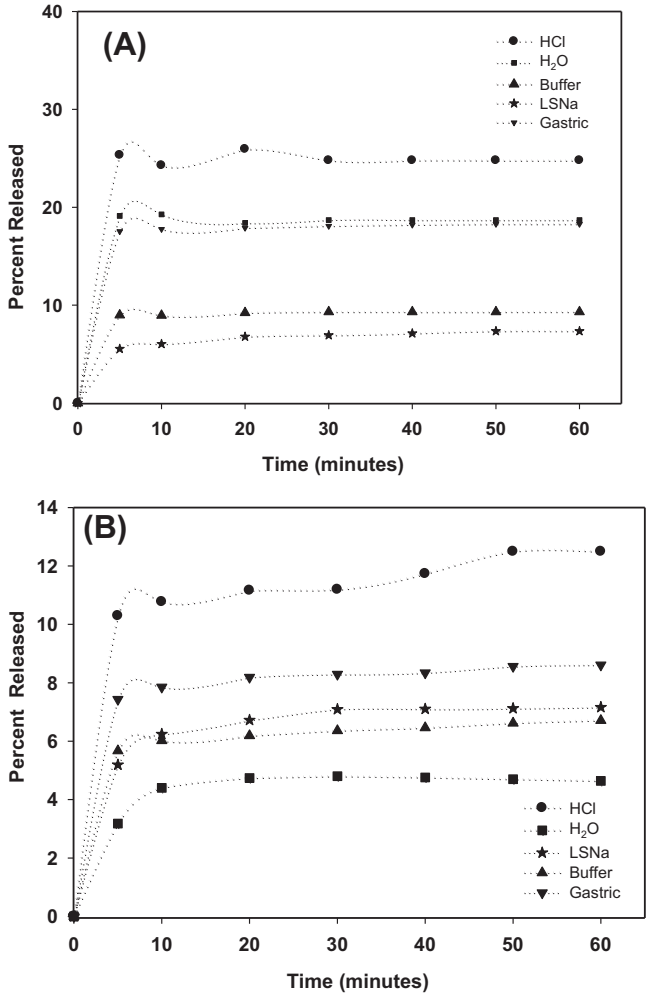

Fig. 3. Dissolution profile for methimazole (danantizol) (A) and Cu-Met-phen complex (B) using 100 rpm.

SOD to eradicate the highly reactive pro-inflammatory superoxide radical anion $O_2^{-\cdot}$ [49]. The Cu(II)–Cu(I) redox cycle involves the dismutation of $O_2^{-\cdot}$ by Cu as follows:

$$Cu(II) + O_2^{-\cdot} \rightleftharpoons Cu(I) + O_2$$

$$Cu(I) + O_2^{-\cdot} + 2H^+ \rightleftharpoons Cu(II) + H_2O_2$$

In which the net reaction is : $2O_2^{-\cdot} + 2H^+ \rightleftharpoons H_2O_2 + O_2$

It is well known that the superoxide generated in biological systems is able to damage tissues because of its ability to act as a weak base ($pK_a = 4.8$) and for this reason SOD enzyme acts at first avoiding degenerative processes producing oxygen and H_2O_2. It is also well established that the injurious effects of H_2O_2 in cells are diminished by the action of certain enzymes like catalase and glutathione-peroxidase [49].

In a previous work, we manifested the relevance to measure the superoxide scavenger power of methimazole and its Cu-Met complex [50] due to free radical-mediated oxidative stress that has been implicated in numerous autoimmune disorders as Graves' disease in which hyperthyroidism results in a marked increase of antioxidant enzymes including superoxide dismutase. We also demonstrated that methimazole did not show SOD activity while Cu-Met exhibited a strong superoxide radical scavenging capacity. In order to elucidate structure–activity relationships between Cu-Met and the new ternary complex the SOD mimic effect was determined (Fig. 4). In addition, with comparative purpose, we were

looking for information about SOD activity for the well known Cu-phen complex. To our knowledge, only a catalytic activity as an intermediate of the phenanthroline–copper(I) complex [51] was found and for this reason we included their SOD mimic activity in the measurements. A representative plot of inhibition with increasing concentration of the complexes is given in Fig. 4. The SOD activity data for the complexes have been compiled in Table 2 along with the SOD activity values of other ternary copper(II) complexes. It should be noted that the reported IC_{50} values are dependent both on the detector used (usually cytochrome c Fe(III) or nitroblue tetrazolium (NBT)) and on its concentration. So, the IC_{50} values are not appropriate for comparisons with the literature. For that reason in Table 2 the kinetic constant value (k_{McCF}), which is independent of both detector concentration and nature, was calculated for all the complexes [26].

The tested complexes exhibited catalytic activity towards the dismutation of superoxide anions. As can be seen, Cu-phen ($k_{McCF} = 1.06 \times 10^6$ mol^{-1} L s^{-1}) and Cu-Met-phen ($k_{McCF} = 4.71 - \times 10^5$ mol^{-1} L s^{-1}) exhibited lower superoxide radical scavenging activity than Cu-Met ($k_{McCF} = 5.94 \times 10^6$ mol^{-1} L s^{-1}). The superoxide dismutase activity has been related to some structural characteristics of the copper(II) complexes. It has been suggested that a better interaction between superoxide ion and Cu(II) in mixed ligand complexes are induced due to stronger axial bonds which leads to an increase of the catalytic activity [52]. For this reason a strong equatorial field opposes the interaction of the complex with the superoxide radical, not favoring the reduction process from copper(II) to copper(I) and in consequence the formation of the copper-intermediate superoxide adduct [53]. The observed order of SOD activities can be rationalized in terms of the coordination geometry of the complexes. Considering that the three complexes retain their coordination sphere after dissolution, the following points can be addressed: (i) a feasible explanation for the less SOD activity of the phenanthroline derived complexes, as compared to Cu-Met could be related to the less flexibility and steric effect of the phenanthroline ligands that produces a greater ligand field crowding over the central metal ion than the methimazole [53]; on the other hand, (ii) SOD activity depends strongly on the geometry around the copper ion being better in structurally flexible geometry. Based in this concept it can be proposed that Cu-Met have a more flexible structure with labile equatorial ligand in the metal-coordination sphere. The ternary complex has low SOD activity because the axial ligands produced medium to weak field interactions.

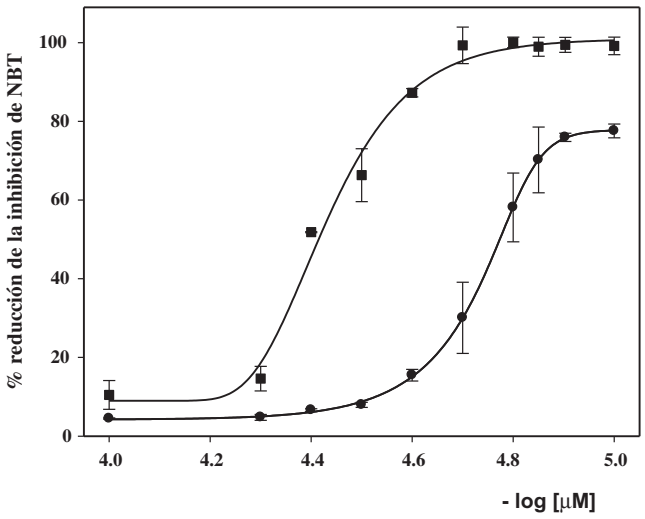

Fig. 4. Percentage of inhibition of the NBT reduction as a function of the negative logarithm of Cu(II) concentration in the nonenzymatic assay at pH 7.5 for species obtained after dissolution in hot DMSO (5 min) of the Cu-phen (●) and Cu-Met-phen (■).

3.7. Alkaline phosphatase assays

The alkaline phosphatases (ALPs) are a superfamily of homodimeric metalloenzymes (E.C.3.1.3.1) that exist widely in the animal and microorganism kingdoms which biological function consists in the hydrolysis of orthophosphoric monoesters linked to nucleotides, proteins and several other substrates (pH 8–11). It is a dimer containing three non-equivalent metal-binding sites in each subunit. Two of them are occupied by zinc ions, one with a catalytic role and the other one with a structural function. The third metal is a Mg(II) cation, also playing a structural role. Their functional groups included an imidazole group of histidine and amino group of lysine in the active site and it has also been suggested that a tyrosine residue is relevant for the enzyme's function [54]. In particular, ALP is a ubiquitous enzyme relevant in physiological functions and in medical diagnosis [55,56].

In a previous work we determine for the first time the inhibition of the alkaline phosphatase activity by copper(II)–methimazole complexes. While this type of determination was very common for vanadium coordination complexes due their relevance for insulin mimetic effects, there were not many reports for copper (II) complexes. In these measurements we found that copper(II) cation did not display significant inhibitory effect up to 100 μM concentration while Cu-Met complex behaved as a stronger inhibitory agent ($IC_{50} = 42$ μM) being a better enzymatic inhibitor than several oxovanadium complexes. It is also been reported for *E. coli* phosphatase alkaline, that when a 2×10^{-7} M enzyme was incubated with 2×10^{-3} M phenanthroline, the activity was completely abolished after 40 min of incubation. This behavior was attributed to the removal of the zinc(II) cation rather than through the formation of a mixed complex. Since our ternary complex presented in its coordination sphere two ligands, it seemed interesting to determine the enzymatic inhibitory ability of this complex and verified whether copper complexation enhances or not the inhibitory effect demonstrated by them. In Fig. 5 it can be observed the effects of copper(II) cation, phenanthroline, Cu-phen (determined for the first time) and Cu-Met-phen on ALP activity from bovine intestinal mucose. As it can be seen, only phenanthroline inhibited the 50% of the enzymatic activity at a concentration of 700.2 μM while Cu-phen and Cu-Met-phen complexes presented small inhibitory and similar effects. There are different mechanisms for enzymatic inhibitions. Metal complexes can inhibit enzymes through ligand-substitution mechanism with biomolecular targets. Metal ions can bind residues in proteins (nitrogen, sulfur or selenium atoms of the histidine, cysteine, or selenocysteine) [57]. Other possibilities occur *via* coulombic or electrostatic interaction between the inhibitors and the active site or inducing protein aggregation, etc. Analyzing our results, we can propose that bovine intestinal ALP inhibition occurs through a similar mechanism to that reported for the *E. coli* ALP when phenanthroline is used as inhibitor. In our study, phenanthroline behave more effectively on bovine intestinal ALP than on *E. coli* ALP. If we evaluate the relationship between activity and the copper(II) complexes structures, the lower activity of the complexes containing phenanthroline could probably be related to the additional stability given by the "chelate effect" blocking the phenanthroline. The ability of complexing cinc(II) cation from the active site of the enzyme is apparently canceled by complex formation with copper(II). The maximum inhibition achieved at the higher concentrations (Fig. 5) would be related to electrostatic interaction because both complexes presented similar effects. In contraposition, the strong effect of the Cu-Met complex could be attributed to the presence of labile water molecules ligands that possibly exchange rapidly

Table 2
IC_{50} values and kinetic catalytic constants: comparison with the literature.

Complex	IC_{50}	k_{McCF} [mol^{-1} L s^{-1}][a]	Ref.
Native enzyme (SOD)	0.21×10^{-6} M	8.5×10^{8}	[70]
Cu-Met	3.0×10^{-6} M	5.94×10^{6}	[50]
Cu-phen	16.8×10^{-6} M	1.06×10^{6}	this work
Cu-Met-phen	37.8×10^{-6} M	4.71×10^{5}	this work
$[Cu(ada)(phen)(H_2O)]\cdot 2H_2O$[b]	24×10^{-6} M	1.39×10^{5}	[71]
$[(Phen)_2Cu–Im–Zn(Phen)_2](BF_4)_3$	15×10^{-6} M	2.22×10^{5}	[72]
[Cu(SAA)(phen)][c]	50×10^{-6} M	6.60×10^{5}	[54]
$[Cu(IDA)(phen)(H_2O)]\cdot 2H_2O$[d]	62×10^{-6} M	5.37×10^{4}	[73]

[a] k_{McCF} for complexes were calculated taking into account the following constant values: k_{NBT} (pH 7.8) = 5.94×10^{4} mol^{-1} L s^{-1}, $k_{Cyt\ c}$ (pH 7.8; 21 °C) = 2.6×10^{5} mol^{-1} L s^{-1}.
[b] H_2ada = adipic acid.
[c] SAA = salicylidene anthranilic acid.
[d] H_2IDA = iminodiacetic acid.

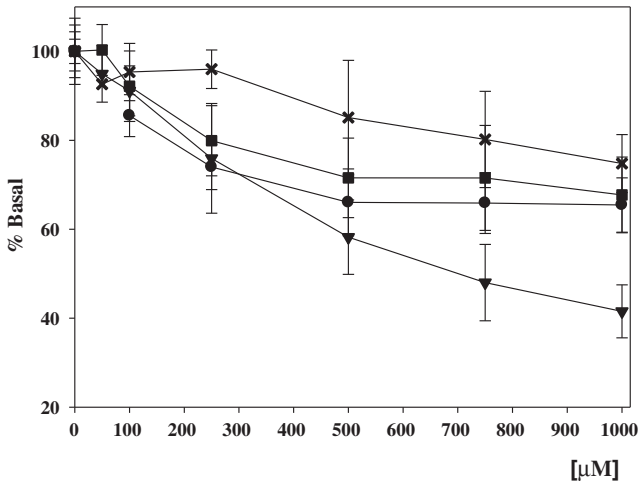

Fig. 5. Effect of copper(II) ion (**x**), phenanthroline (Phen) (▼), Cu-phen (●) and Cu-Met-phen (■) on ALP activity from bovine intestinal mucose. Initial rate was determined by incubation of the enzyme at 37 °C for 10 min in the absence or presence of variable concentrations of the inhibitors. Basal activity was 5.2 ± 0.2 nmol pNP min^{-1} μg^{-1} protein.

favoring the adoption of a more appropriate symmetry to interact with the enzyme [7].

3.8. Microbiological assays

The results of the antibacterial activity assays are given in Table 3. It is established that the antimicrobial activity was considered to be significant when the MIC values are near 100 μg/mL or less. When the MIC values are in the 100–500 μg/mL range, the antibacterial activity was considered moderate when these values are found between 500 and 1000 μg/mL, the antibacterial activity was considered weak. In addition, values of MIC over 1000 μg/mL indicate no antibacterial activities [58]. Methimazole (determined for the first time) shows no activity at the tested concentrations. Besides, phenanthroline shows the lowest MIC values against *E. coli*, *S. aureus* and *S. epidermidis*. The susceptibility of microorganisms decrease in the order *E. coli* > *S. aureus* > *E. faecalis* > *P. aeruginosa* and these results are in agreement with previous reports of phenanthroline antimicrobial activity [59,60].

It is well know that copper is an essential element but it also has been proved that at higher concentrations can became toxic to the microorganisms. Copper ion interferes with the biosynthesis of Fe–S clusters incrementing cluster scaffold and target proteins production. In connection with the toxic effect, many processes can occur: (i) cell damage due to copper ions accumulation, (ii) oxidation of sulfhydryl groups in proteins or in glutathione, (iii) inactivation of proteins by damaging Fe-S clusters in cytoplasmic hydratases, among others. It has also been suggested that *in vivo* copper ion toxicity in bacteria is related to oxidative DNA damage due to its ability to catalyze reactions (Fenton and Haber–Weiss reactions) that produces highly reactive oxygen intermediates [16,17]. In our determination, copper salt presented a moderate MIC value of 375 μg mL^{-1} (2200 μM) against all the studied strains being in the same order of magnitude than in previous reports [61].

As for the Cu-Met complex an increase of the antibacterial activity was observed in comparison to the free ligand upon complexation but their antibacterial activities are considered weak against all the tested strands. This complex showed the highest values of MICs. On the other hand, the Cu-phen complex showed a higher activity than the free ligand against *P. aeruginosa* and *E. faecalis*. Also, it behaved as a strong antibacterial agent against *E. coli*, *S. aureus*, *S. epidermidis*, *E. faecalis* but it behaved with moderate activity against *P. aeruginosa*. In the other tested complex, Cu-Met-phen, the coordination increased the antibacterial effect especially against *E. faecalis*, demonstrating a strong effect against *S. aureus* and *S. epidermidis* as well. For the others strains and taking into account the weak activities displayed by the Cu-Met complex, the inclusion of phenanthroline in the coordination sphere of Cu(II) improved their effect in 6–12% range against *S. epidermidis*, *S. epidermidis* and *E. faecalis*. Such an increased activity of the complexes can possibly be explained on the bases of Over-

Table 3
Minimum inhibitory concentrations (MICs) of the methimazole (Met) and copper(II) complexes Cu-Met-phen, Cu-phen, Cu-Met, $CuCl_2\cdot 2H_2O$ and phenanthroline (phen) for the reference strains, in μg mL^{-1} (μM).

	E. coli (ATCC 35218)	*P. aeruginosa* (ATCC 27853)	*S. aureus* (ATCC 25923)	*S. epidermidis* (ATCC 1263)	*E. faecalis* (ATCC 29212)
$CuCl_2\cdot 2H_2O$	375 (2.2×10^3)	375 (2.2×10^3)	375 (2.2×10^3)	375 (2.2×10^3)	375 (2.2×10^3)
Phen	12 (66.7)	375 (2.1×10^3)	24 (133.33)	12 (66.7)	94 (522.22)
Met	>1500 (13.16×10^3)	>1500 (13.16×10^3)	>1500 (13.16×10^3)	>1500 (13.16×10^3)	>1500 (13.16×10^3)
Cu-phen	47 (91.70)	187 (365)	24 (46.8)	24 (46.8)	12 (23.4)
Cu-Met	750 (1.6×10^3)	750 (1.6×10^3)	750 (1.6×10^3)	750 (1.6×10^3)	750 (1.6×10^3)
Cu-Met-phen	375 (648)	375 (648)	94 (162)	94 (162)	47 (81)

tone's concept and Tweedy's chelation theory [62–64]. According to Overtone's concept of cell permeability, the lipid membrane that surrounds the cell favors the passage of only lipid soluble materials so that liposolubility is an important factor which controls bactericidal activity. On the Tweedy's theory, chelation reduces the polarity of the central metal atom because of partial sharing of its positive charge with the ligand, which favors permeation of the complexes through the lipid layer of the membrane [65–67]. Together with this possibility, the presence of chloride ion in Cu-phen and Cu-Met-phen complexes may also contribute to their antimicrobial effect due to the hypochlorous acid formation. This acid decomposes forming hydrochloric acid and oxygen. Oxygen destroys microbes oxidizing cellular components. Chlorine compounds can also interact and combine with proteins and enzymes of membranes [68].

Although a direct comparison of the antimicrobial effect cannot be made due to some differences in the experimental methodologies, a comparison of the antimicrobial activity of $[Cu(MeimzH)_2(phen)(H_2O)_2]Cl_2$ (Cu-Met-phen) with copper(II) complexes of Schiff bases $(Cu(L)(OAc)_2H_2O$, 1,3-diphenyl-1H-pyrazole-4-carboxaldehyde and 4-amino-5-mercapto-3-methyl/H-1,2,4-triazole) having similar equatorial coordination sphere [69] led our complex to an improvement of their antibacterial activity mainly against *S. aureus*. As for copper(II) mixed complexes derived of *N*,*N*′-propanediyl-bis-benzenesulfonamide with phenanthroline ($[CuL_2(phen)_2]$ MICs values are in the same order of magnitude [22].

4. Conclusions

In the present work we have synthesized and characterized a ternary copper(II) complex with water, methimazole and phenanthroline as ligands. Considering all the spectroscopic results the structure can be assumed as an elongated rhombic-octahedral geometry around the copper(II) ion where two N atoms (phen), two S atoms (two methimazole molecules) are linked to the metal center in the equatorial plane and two oxygen atoms from two water molecules are located in the axial position. After dissolution, the bioactive species show axial EPR spectra indicating the presence of a less distorted structure retaining the coordination sphere of the solid phase. During our investigation, we have observed a relationship between the effectiveness of the biological activities of the phen containing complexes in comparison with the methimazole copper complex. It has been determined that Cu-phen acts as a better SOD mimic and ALP inhibitor than Cu-Met-phen and a plausible explanation of this behavior can be attributed to the more flexible structure. It has been demonstrated that methimazole has not antibacterial activity, the binary complex Cu-Met has weak activity against all the tested strands but the derived ternary complex, Cu-Met-phen, exhibits antimicrobial activity. The coordination increases the antibacterial effect especially against *E. faecalis*, exhibiting strong effect against *S. aureus* and *S. epidermidis* as well.

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