Research Article

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Antimicrobial and antifungal activities of bifunctional copper(II) complexes with non-steroidal anti-inflammatory drugs, flufenamic, mefenamic and tolfenamic acids and 1,10-phenanthroline

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Abstract: Copper(II) complexes represent a promising group of compounds with antimicrobial and antifungal properties. In the present work, a series of Cu(II) complexes containing the non-steroidal anti-inflammatory drugs, tolfenamic acid, mefenamic acid and flufenamic acid as their redox-cycling functionalities, and 1,10-phenanthroline as an intercalating component, has been studied. The antibacterial activities of all three complexes, [Cu(tolf-O,O′)$_2$(phen)] (**1**), [Cu(mef-O,O′)$_2$(phen)] (**2**) and [Cu(fluf-O,O′)$_2$(phen)] (**3**), were tested against the prokaryotic model organisms *Escherichia coli* (*E. coli*) and *Staphylococcus aureus* (*S. aureus*) and their antifungal activities were evaluated towards the yeast, *Saccharomyces cerevisiae* (*S. cerevisiae*). The antibacterial activity of both strains has been compared with the antibiotic Neomycin. The calculated IC_{50} values revealed slight differences in the antibacterial activities of the complexes in the order **1** ~ **3** > **2**. The most profound growth inhibition of *E. coli* was observed, at its highest concentration, for the complex **1**, which contains chlorine atoms in the ligand environment. The trend obtained from IC_{50} values is generally in agreement with the determined MIC values. Similarly, the complex 1 showed the greatest growth inhibition of the yeast *S. cerevisiae* and the overall antifungal activities of the Cu(II) complexes were found to follow the order **1** > **3** ≫ **2**. However, for complex **2**, even at the highest concentration tested (150 μM), a 50% decrease in yeast growth was not achieved. It appears that the most potent antimicrobial and antifungal Cu(II) complexes are those containing halogenated NSAIDs. The mechanisms by which Cu(II) complexes cause antibacterial and antifungal activities can be understood on the basis of redox-cycling reactions between cupric and cuprous species which lead to the formation of free radicals. The higher efficacy of the Cu(II) complexes against bacterial cells may be due to an absence of membrane-protected nuclear DNA, meaning that on entering a cell, they can interact directly with its DNA. Contrastingly, for the complexes to interact with the DNA in yeast cells, they must first penetrate through the nuclear membrane.

Keywords: Cu(II) complexes, antimicrobial activity, antifungal activity, NSAID, *Escherichia coli*, *Saccharomyces cerevisiae*

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1 Introduction

It has been reported that in the USA alone, more than 50,000 patients die annually from multiresistant bacterial infections [1]. Since the antibiotic resistance of various bacteria is becoming a serious medical threat globally, there is an urgent requirement to discover new classes of antibiotics, of which redox metal-based complexes with biologically active ligands are promising examples [2–7]. Copper is one of the most abundant

elements in the human body and is also a redox active metal, whose positive effects on human health were first noted as early as the late 19th century, when it was reported by Doctor Luton that a mixture of copper chloride and sodium salicylate was effective in the treatment of rheumatoid arthritis, rheumatic fever and other disorders [8]. This observation was later augmented during the first half of the 20th century in Finland, where miners who worked in copper mines were found to suffer a considerably lower incidence of arthritis than the majority of population.

Copper occurs most commonly in biological systems in either +2 or +1 oxidation states (referred to as cupric or cuprous species, respectively) [9]. To date, there is no direct proof that Cu(III) species exist in biological systems, which would require the presence of a redox active cofactor. Most common are those complexes containing copper in the +2 oxidation state and these show inhibitory effects toward the growth of a variety of bacteria, fungi and viruses [10–13]. Copper(II) complexes containing coordinated, structurally flat ligands, such as 1,10-phenanthroline, exhibit cytotoxic effects which have been attributed predominantly to their ability to cleave DNA, as a result of either complete or partial intercalation of the phenanthroline moiety in between the DNA base pairs [14–16]. In addition to this intercalation mechanism, a process of redox cycling, with the formation of DNA damaging ROS, may occur.

In the present work, we report the antibacterial and antifungal activities of Cu(II) complexes bearing 1,10-phenanthroline as an intercalating ligand and the non-steroidal anti-inflammatory drugs: tolfenamic acid, mefenamic acid and flufenamic acid, to provide redox-cycling functionalities (Figure 1). The antibacterial activities of these Cu(II) complexes were gauged against the frequently studied prokaryotic model organisms, *E. coli* and *S. aureus*, and their antifungal activities were determined against *S. cerevisiae*, a yeast that is widely employed in the baking and brewing industries.

2 Experimental

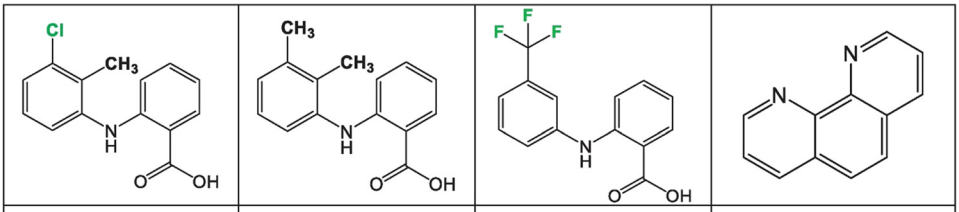
2.1 Materials

Copper acetate, methanol, 1,10-phenanthroline, flufenamic acid, tolfenamic acid and mefenamic acid were all obtained from Merck (Germany). To evaluate antimicrobial activity of Cu(II) complexes, *E. coli* DH5α and *S. aureus* ATCC25923 were used, as obtained from the Institute of Plant Genetics of the Slovak Academy of Sciences in Nitra. *S. cerevisiae* CCY27-22-6 was used in order to determine the antifungal activities of the Cu(II) complexes. The particular strain was obtained from the Institute of Chemistry of the Slovak Academy of Sciences, Bratislava.

2.2 Preparation of Cu(II) complexes with phenanthroline and non-steroidal anti-inflammatory drugs

[Cu(tolf-O,O′)$_2$(phen)] (**1**). To 50 mL of Cu(II) acetate (0.200 g) methanol solution (0.02 M) 0.198 g of phenanthroline monohydrate was added. The solution was stirred for 10 min and then 0.523 g of solid tolfenamic acid was added to achieve the 1:2 ratio of phenanthroline: tolfenamic acid. The resulting olive-coloured mixture was stirred for a further 24 h until a precipitate was formed, which was filtered off and air-dried for one month. Suitable monocrystals for X-ray analysis were obtained by slow evaporation of the solvent.

[Cu(mef-O,O′)$_2$(phen)] (**2**). To 50 mL of Cu(II) acetate (0.200 g) dissolved in methanol (0.02 M) 0.198 g of phenanthroline monohydrate was added. The solution was stirred for 10 min and then 0.483 g of solid mefenamic acid was added to achieve the 1:2 ratio of phenanthroline: mefenamic acid. The resulting mixture was stirred for



Tolfenamic acid	Mefenamic acid	Flufenamic acid	1,10-phenathroline

Figure 1: The structures of ligands used for preparation of studied Cu(II) complexes.

24 h until a precipitate was formed, which was filtered off and air-dried for one month. Suitable monocrystals for X-ray analysis were obtained by slow evaporation of the solvent.

[Cu(fluf-O,O′)$_2$(phen)] (**3**). The Cu(II) complex with flufenamic acid was prepared analogously to above described complexes. To 50 mL of Cu(II) acetate (0.200 g) dissolved in methanol (0.02 M) 0.198 g of phenanthroline monohydrate was added. The solution containing copper acetate and phenanthroline in methanol was stirred for 10 min. Into this solution, 0.483 g of solid flufenamic acid was added to achieve the 1:2 ratio of phenanthroline: flufenamic acid. The reaction mixture was stirred for 24 h until a precipitate was formed, which was filtered off and air-dried for one month. Suitable monocrystals for X-ray analysis were obtained by slow evaporation of the solvent.

2.3 Determination of the antibacterial activity for Cu(II) complexes

To investigate the antibacterial activity of the complexes, the prokaryotic model organism *E. coli* DH5α and *S. aureus* were used, as obtained from the Institute of Plant Genetics of the Slovak Academy of Sciences in Nitra. DH5-strain alpha was used because it appears to be suitable for high efficiency transformation in a wide variety of routine applications such as plasmid isolation and other procedures.

Bacterial cells were incubated in a liquid LB (Lysogen Broth, Luria-Bertani) medium containing 0.5% yeast extract, 1% tryptone and 1% NaCl. For solid LB media, the composition was enriched with 2% agar. The media were sterilized at 125°C for 30 min. Prior to inoculation of the bacterial cells, an ampicillin selection agent (50 mg/mL) was added to the medium.

The bacterial culture was seeded under sterile conditions into 20 mL of prepared LB medium, and the cultivation was performed in a 250 mL Erlenmeyer flask at 37°C over 16 h, with continuous stirring at 150 rpm (Heidolf Unimax 1010, Germany). Once grown, the culture was diluted with pure LB medium to achieve an optical density (OD_{600}) of 0.1, which represents a concentration of 1–2 × 10^8 cells/mL. The optical density was determined spectrophotometrically (Varioskan Flash, Thermo Scientific). Thus prepared, the inoculum was pipetted into 25 mL Erlenmeyer flasks.

To each flask was added different concentrations of the substance to be tested, but this additional volume was maintained to equal exactly 1% of the total volume of the test culture. The cultures were incubated at 37°C, with continuous stirring at 150 rpm.

The effect of the studied substance on the bacterial cell growth was monitored from an absorbance measurement made every two hour, over a period of 24 h. The growth profiles of bacterial cultures, determined as a function of multiple concentrations of each test substance, were compared with those for a control culture where no test substances were present. Data were obtained in triplicate and used for the calculation of the IC_{50} values (i.e. the concentration of the test substance that causes 50% inhibition of bacterial growth).

Since its fraction in the medium did not exceed 1%, the effect of the DMSO solvent on the growth curve could be excluded in the consideration of the antibacterial activities of the Cu(II) complexes **1–3** [17].

In order to determine minimum inhibitory concentration (MIC) values of the Cu(II) complexes against *E. coli* and *S. aureus*, the rapid *p*-iodonitrotetrazolium chloride (INT) colorimetric assay was used [18]. Briefly, both *E. coli* and *S. aureus* were diluted with sterile broth (Müller and Hinton) to reach final inocula of cca. 10^6 CFU mL^{-1}. Stock solutions of copper(II) complexes were dissolved in DMSO to reach the concentration of 25 mg mL^{-1}. 100 µL solution of each Cu(II) complex was diluted with redistilled and deionized water (Merck Millipore) in a 96 well microtiter microplates (Thermo Fischer scientific) to reach final concentrations ranging from 3 to 300 µL mL^{-1}. As a positive control against bacterial strains, 1 mg mL^{-1} solution of the antibiotic Neomycin (Sigma-Aldrich, FRG) was applied with final concentrations in the range 2.5–270 µL mL^{-1}. Individual bacteria (100 µL) were added to wells and incubated at 36.8°C for 24 h. *p*-Iodonitrotetrazolium chloride (45 µL of 0.2 mg mL^{-1} solution) from Sigma Aldrich (FRG) was used to indicate the bacterial growth at 36.8°C for 24 h. *p*-Iodonitrotetrazolium undergoes colour change from colourless to red upon its reduction by active organisms. The values of MIC were obtained visually as the lowest concentrations resulting in growth inhibition.

2.4 Determination of antifungal activity of Cu(II) complexes

A model organism *S. cerevisiae* CCY 27-22-6 was used in order to determine the antifungal activities of the Cu(II)

complexes. The particular strain was obtained from the Institute of Chemistry of the Slovak Academy of Sciences, Bratislava. Yeast cells were cultured in a liquid YPD (Yeast Extract–Peptone–Dextrose) medium containing: 2% peptone, 2% glucose and 1% yeast extract. In the case of solid YPD media, the composition was enriched by adding 2% agar. The media were sterilized for 30 min at 125°C and the yeast culture was inoculated under sterile conditions into 20 mL of the prepared liquid YPD medium. The cultivation was made over 16 h in a 250 mL sterile Erlenmeyer flask maintained at 30°C, with continuous stirring at 150 rpm (Heidolf Unimax 1010, Germany). Once grown, the culture was diluted with pure YPD medium, so that the optical density OD_{600} reached value of 0.1, which corresponded to 1–2 × 10^8 cells/mL. The optical density was determined spectrophotometrically (Varioskan Flash, Thermo Scientific). Thus prepared, the yeast culture was used as a vegetative inoculum for all yeast cell assays, inoculums, and was pipetted into 25 mL Erlenmeyer flasks. To each Erlenmeyer flask was then added the complex to be studied at different concentrations so that the volume of the solution occupied 1% of the total volume. The cultures were incubated at 30°C and stirred continuously at 150 rpm. The change in yeast culture growth caused by the tested complex was monitored from spectrophotometric measurements, made every two hour, over a 24 h period. The growth of bacterial cultures in the presence of the tested complexes was compared with a control culture in which these complexes were absent. Each measurement was performed in triplicate and the data obtained were used to calculate IC_{50} values (i.e. the concentration of the test substance required to cause a 50% inhibition in the growth of the yeast culture).

3 Results and discussion

The aim of the present work was to evaluate both antibacterial and antifungal properties of selected bifunctional Cu(II) complexes, bearing NSAID ligands to provide redox-cycling functionalities and with 1,10-phenanthroline as an intercalating agent. The antimicrobial activities of the Cu(II) complexes were determined using the prokaryotic organisms, *E. coli* and *S. aureus*, and the eukaryotic organism, *S. cerevisiae*, was used for the determination of antifungal activity.

3.1 Structure of Cu(II) complexes with NSAIDs and 1,10-phenanthroline

The molecular structures of the copper(II) complexes [Cu(tolf-O,O′)$_2$(phen)] (**1**), [Cu(mef-O,O′)$_2$(phen)] (**2**) and [Cu(fluf-O,O′)$_2$(phen)] (**3**) are shown in Figure 2. All three complexes are monomeric and crystallize in the monoclinic space group, and in each case, the Cu(II) ion is chelated in the equatorial plane by the two 1,10-phenanthroline nitrogen atoms and a total of four carboxylate oxygen atoms from two molecules of the particular NSAID [14]. The equatorial plane of all three complexes (2N2O) has a regular planar geometry. Conversely, the axial positions of the Cu(II) complexes are distorted, due to the limited bonding capacity of the bidentate, tolfenamte, mefanamate and flufenamate anions. In the solid state, the molecular structures of the Cu(II) complexes are stabilized by intermolecular hydrogen bonds, which are, however, disrupted upon their introduction into the fluid environment.

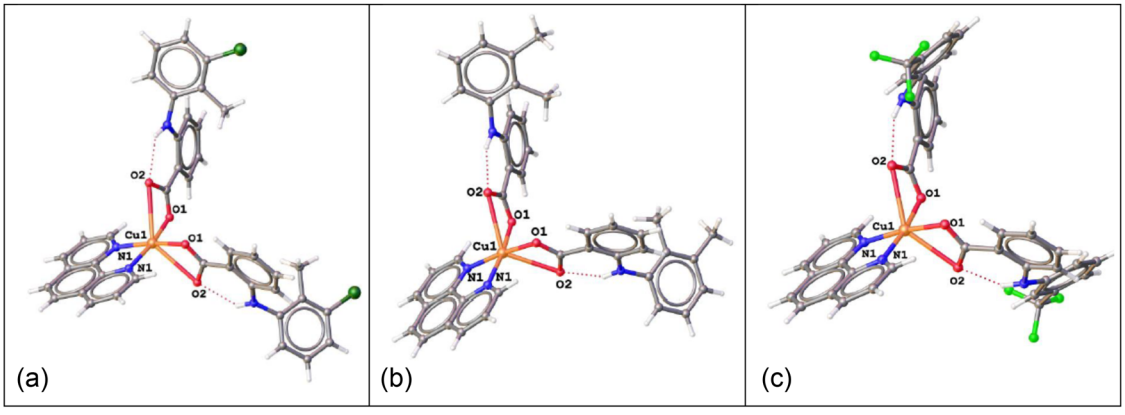


Figure 2: Molecular structures of the Cu(II) complexes. (a) Molecular structure of complex [Cu(tolf-O,O′)$_2$(phen)] (**1**). (b) Molecular structure of complex [Cu(mef-O,O′)$_2$(phen)] (**2**). (c) Molecular structure of complex [Cu(fluf-O,O′)$_2$(phen)] (**3**).

3.2 Antibacterial activity

Antibacterial activity of all three Cu(II) complexes was studied against *E. coli* and *S. aureus*. The effect of the Cu(II) complexes on the growth of prokaryotic *E. coli* was investigated over a 24 h period. During the initial 12 h, data were recorded at two hourly intervals. The concentration range employed for all three Cu(II) complexes was 10–150 µmol. At the lowest concentration tested (10 µmol), a slight decrease in absorbance for complexes **1** and **3** was observed, but practically no effect for the complex **2**, as compared with the control (Figures 3–5). A gradual decrease in the absorbances, with increasing concentrations of the Cu(II) complexes, indicates a sensitivity of the bacterial culture to their presence, which is manifested by an inhibition of cell culture growth.

From the measured absorbance values determined following 24 h of cell culture growth, the IC_{50} values were calculated for each complex (Table 1). The IC_{50} value corresponds to the concentration of the complex that causes 50% inhibition of bacterial growth. Although, at first sight, the graphs (Figures 3–5) indicate the effects of the Cu(II) complexes to be similar, the calculated IC_{50} values reflect slight activity differences in the order of **1** ~ **3** > **2**. The most profound growth inhibition was observed at the highest concentration of the complex **1**, which contains coordinated NSAIDs, each bearing a chlorine atom. A slightly lower inhibitory activity was observed for complex **3**, which contains NSAIDs, each with three fluorine atoms. The lowest antibacterial activity was observed for the complex **2**, with its methyl-substituted NSAID ligands. From the growth curves of all three Cu(II) complexes, and at all concentrations tested, the stationary phase of bacterial culture growth of *E. coli* is apparent after 12 h. For the complex **3**, at concentrations of 80 µmol and higher, decreases in the absorbance are measured after 12 h (Figure 5), which indicates bacterial cell death.

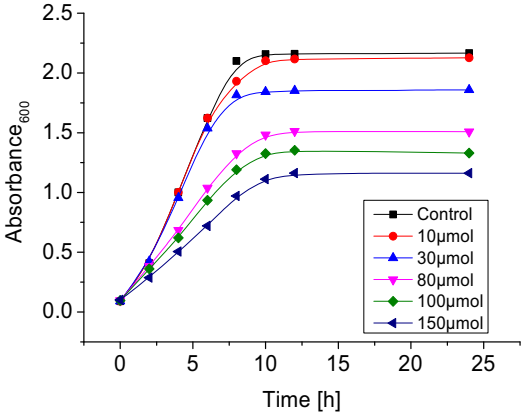


Figure 4: Growth curves of *E. coli* during the course of a 24 h cultivation in the presence of 10; 30; 80; 100; and 150 µmol of the complex **2**.

Minimum inhibitory concentrations for both Gram-negative *E. coli* and Gram-positive *S. aureus* bacteria were determined. The standard antibiotic Neomycin was used as a control for antibacterial activity of both strains. The results are summarized in Table 2. From the obtained results, we can see a relatively good correlation with above-presented IC_{50} values for *E. coli*. The best inhibitory activity against both *E. coli* as well as *S. aureus* has been obtained for complex **1**, followed by complex **3**. Complex **2** shows the least activity against both strains. From the obtained results, it can be concluded that all three Cu(II) complexes exhibit better inhibitory activity

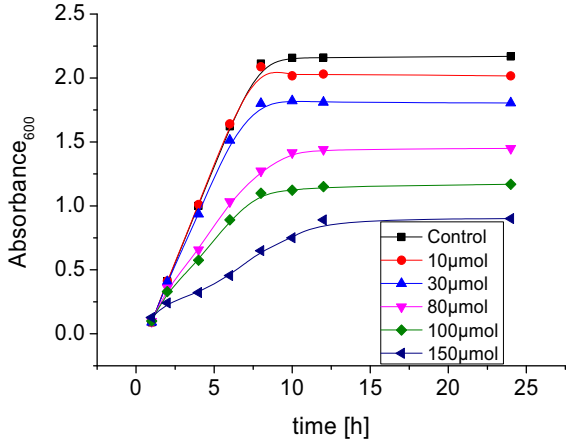


Figure 3: Growth curves of *E. coli* during the course of a 24 h cultivation in the presence of 10; 30; 80; 100; and 150 µmol of the complex **1**.

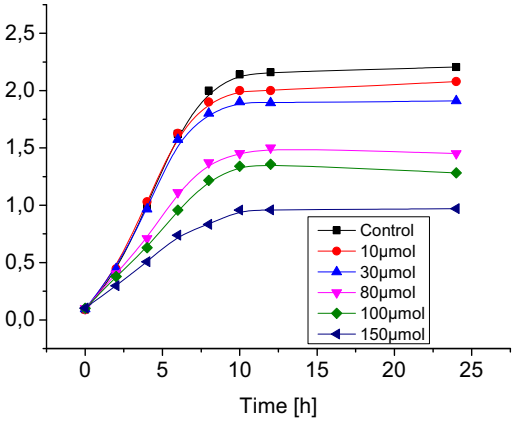


Figure 5: Growth curves of *E. coli* during the course of a 24 h cultivation in the presence of 10; 30; 80; 100; and 150 µmol of the complex **3**.

Table 1: Inhibitory concentrations of complexes **1–3** required to cause a 50% decrease in the growth of *E. coli* bacterial culture (IC_{50}) at 24 h

Complex	IC_{50} (%)
[Cu(tol-O,O′)$_2$(phen)] (**1**)	112.4
[Cu(mef-O,O′)$_2$(phen)] (**2**)	148.2
[Cu(flu-O,O′)$_2$(phen)] (**3**)	120.1

Table 2: Minimum inhibitory concentrations (μg mL^{-1}) of Cu(II) complexes against *S. aureus* and *E. coli*

Complex	Gram (+) *S. aureus*	Gram (−) *E. coli*
[Cu(tol-O,O′)$_2$(phen)] (**1**)	65.72	74.25
[Cu(mef-O,O′)$_2$ (phen)] (**2**)	95.62	105.56
[Cu(flu-O,O′)$_2$(phen)] (**3**)	77.29	80.66
Neomycin	9.35	3.25

against *S. aureus*. This can be due to the absence of outer membrane of Gram-positive bacteria *S. aureus*, which makes them more susceptible to certain antibiotics or molecules with antibiotic affects such as Cu(II) complexes. Further modifications in ligand sphere (both redox-cycling and intercalating functionalities) are necessary to achieve better antibacterial activity of Cu(II) complexes.

3.3 Antifungal activity

The effect of the Cu(II) complexes on the growth of the eukaryotic model microorganism *S. cerevisiae* was studied over the period of 20 h. During the initial 12 h, as with the *E. coli* measurements, we recorded data at two hourly intervals. The complexes **1–3** were all measured in the same concentration range, 10–150 μM (Figures 6–8).

The growth curves shown in Figures 6–8 exhibit a sigmoidal character. In contrast with the growth of the bacterial cells, in each experiment performed, the yeast cultures of *S. cerevisiae* all demonstrated exponential growth phases up to a period of 12 h. Based on these growth curves, the Cu(II) complexes under investigation can be ordered according to their antifungal activity as: **1** > **3** ≫ **2**. Similarly, due to its low antibacterial activity, the results showed that complex **2** exhibits minimal antifungal activity (Table 3). Hence, we may conclude that, overall, the order of antifungal activities for all three Cu(II)

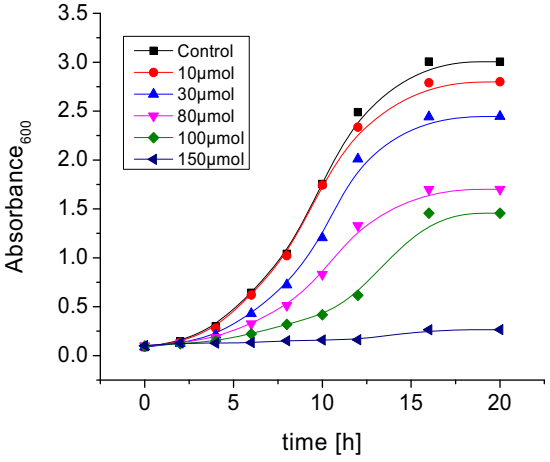


Figure 6: Growth curves of *S. cerevisiae* during a 24 h cultivation in the presence of 10; 30; 80; 100; and 150 μM of complex **1**.

complexes accords with their antibacterial activities against *E. coli*.

Based on our *in vitro* studies, which unequivocally confirmed that interaction of Cu(II) complexes with DNA takes place, we assumed that oxidative damage had occurred at the DNA level with subsequent inhibition of cell culture growth. The mechanism by which Cu(II) complexes cause toxicity can be understood on the basis of redox-cycling reactions between cupric and cuprous species which result in the formation of free radicals [14,19]. Under aerobic conditions, this redox cycling leads to the occurrence of oxidative damage by the production of superoxide radical anions and highly reactive hydroxyl radicals. The present results demonstrated that the antibacterial activities of the Cu(II) complexes were generally higher than their antifungal activities, other than the single exception of the rather high activity of complex **1**

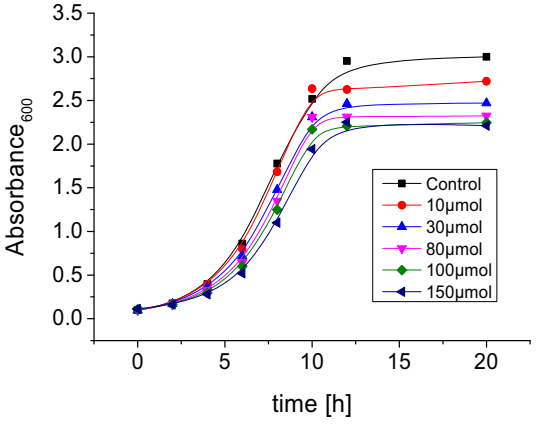


Figure 7: Growth curves of *S. cerevisiae* during a 24 h cultivation in the presence of 10; 30; 80; 100; and 150 μM of complex **2**.

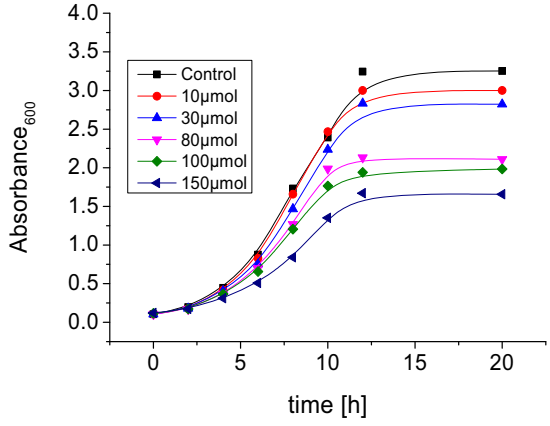


Figure 8: Growth curves of *S. cerevisiae* during a 24 h cultivation in the presence of 10; 30; 80; 100; and 150 μM of complex **3**.

Table 3: Inhibitory concentrations of complexes **1–3** required to cause a 50% decrease in the growth of *S. cerevisiae* (IC_{50}) estimated after a 24 h period

Complex	IC_{50} (%)
[Cu(tol-O,O′)$_2$(phen)] (**1**)	95.5
[Cu(mef-O,O′)$_2$(phen)] (**2**)	—
[Cu(flu-O,O′)$_2$(phen)] (**3**)	147.2

against *S. cerevisiae*. Indeed, complex **1**, with its Cu(II)-coordinated chlorinated NSAID ligand, appears to be the most effective agent against both bacteria and fungi. The overall greater activity of the Cu(II) complexes against bacteria than fungi may be due to the fact that these agents can interact directly with the DNA upon entry into a bacterial cell, which, unlike a fungal cell, does not have membrane-protected nuclear DNA [20]. In contrast, for the complexes to interact with DNA in yeast cells, they must first penetrate through the nuclear membrane.

The results obtained demonstrate a correlation between the cytotoxic effects of the complexes toward the studied microorganisms and their observed SOD mimetic activities, as recently reported by our group (**1** ~ **3** > **2**) [14]. To recapitulate, briefly, the SOD mimetic activity of the studied complexes rests upon their ability to dismutate the superoxide radical anion and produce hydrogen peroxide, according to the reaction [9].

$$2O_2^{\bullet -} + 2H^+ \xrightarrow{SOD} H_2O_2 + O_2$$

Thus formed, hydrogen peroxide can give rise to formation of a highly reactive hydroxyl radical according to the Fenton reaction [14,21].

$$H_2O_2 \xrightarrow{Cu,Fe} {}^{\bullet}OH + OH^-$$

When generated in a living cell, hydroxyl radicals may disrupt the integrity of the bacterial membrane and cause damage to important cellular components such as bacterial DNA, RNA and proteins, resulting in a range of deleterious effects, and even cell death. Nonetheless, we note that the cytotoxic effects of the added complexes toward the studied microorganisms may not necessarily correspond with their SOD mimetic activities, because in actual living cells, hydroxyl radicals may also damage other structures and so the spectrum of possible action mechanisms is accordingly broadened [22,23].

4 Conclusions

In this paper, the antimicrobial and antifungal properties of copper(II) complexes containing biologically active non-steroidal anti-inflammatory drugs, tolfenamic acid, mefenamic acid and flufenamic acid, and 1,10-phenanthroline were studied. Based on the IC_{50} and MIC values, it can be concluded that Cu(II) complexes exhibit promising antibacterial activity against *E. coli* and *S. aureus*, of which the most powerful effect is observed for complex **1**, which contains a chlorine atom on each tolfenamic acid moiety. Interestingly, the most potent anticancer activities, against several cancer cell lines, have been observed for Cu(II) complexes containing halogenated NSAIDs. We suggest that, in addition to these complexes causing ROS-induced damage to bacteria, their NSAID halogen atoms are involved in hydrogen bond formation with particular hydrogen atoms at the DNA surface.

With respect to their antifungal activities, the results indicate that the Cu(II) complexes can be arranged in the order **1** > **3** ≫ **2**. The lower activity observed for Cu(II) complexes against *S. cerevisiae* can be accounted for by the presence of a nuclear membrane in yeast cells, which protects their DNA from attack by external agents such as Cu(II) complexes.

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Conflict of interest: The authors declare no conflicts of interest.

Ethical approval: The conducted research is not related to either human or animal use.

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