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ANTIMICROBIAL ACTIVITY OF COPPER(II) COMPLEXES

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ABSTRACT

Two novel copper(II) 5-chlorosalicylate complexes with either 1,10-phenantroline or its methyl derivative 2,9-dimethyl-1,10-phenanthroline (neocuproine) have been prepared and studied. A potential antimicrobial or antifungal activity of both complexes has been tested on prokaryotic *Escherichia coli* and eukaryotic *Saccharomyces cerevisiae* model organisms. Crystal structure of $[Cu(phen)(5\text{-}Clsal)(5\text{-}ClsalH_2)]_2$ a dimeric structure, whereas the second complex of formula $[Cu(H_2O)(5\text{-}Clsal)(Neo)]$ has been shown to be monomeric. Our results confirmed the toxic effect of prepared copper complexes as well as bioactive ligands on the yeast and bacteria growth. The effect of copper complexes was stronger compared to the solutions of free ligands. Our preliminary results showed that the complex $[Cu(H_2O)(5\text{-}Clsal)(Neo)]$ exhibited higher antimicrobial activity compared to the complex $[Cu(phen)(5\text{-}Clsal)(5\text{-}ClsalH_2)]_2$.

Keywords: 5-chlorosalicylic acid, antimicrobial activity, copper complex, 1,10-phenanthroline, neocuproine

INTRODUCTION

A new generation of antimicrobial drugs is urgently needed because the effectiveness of antibiotics is increasingly compromised by the rise of drug resistance and multiple drug resistant bacteria, which become new clinically common and represent a serious threat to public health **(Neville *et al.*, 2013)**. Metal-based drugs represent a novel group of antimicrobial agents with potential applications for the control of various infectious diseases. For scientific researchers working in the field of bioinorganic chemistry, the copper represents one of the most interesting biometals for the preparation of new metal-based drugs with strong potential for therapeutic applications **(Duncan, White, 2012)**.
A considerable attention has been given to the copper-based complexes with different types of organic bioactive ligands such as nitrogen-donor heterocyclic ligand, 1,10-phenanthroline or its derivates. Combination of copper(II) molecules with the cleavage reagent such as 1,10-phenanthroline constitutes another direction in the development of reactive molecules for design specificities which are part of their ability finely tune the metal toxicity **(Gallagher *et al.*, 1996; Efthimiadou *et al.*, 2008)**. Copper complexes with phenanthrolines show cytotoxicity which is attributed to their ability to cleavage of DNA. The mechanism of the toxicity is probably based on cycling reactions between Cu(II) and Cu(I) oxidation states resulting in the formation of reactive radical species. Under aerobic conditions, this redox cycling leads to the generation of oxidative damages by the production of highly reactive free hydroxyl radicals. Hydroxyl radical is a very reactive particle which easily reacts with its immediate environment within the cell including membrane lipids, proteins and nucleic acids thus generates various dangerous species and products. It is generated through the specific Fenton reaction, which in turn is responsible for the DNA damage. Based on these arguments, it has been argued that 1,10–phenanthrolines and its derivates, a class of chelators, have antibacterial, antifungal, antiviral properties, and have been applied as topical antimicrobials **(Zoroddu *et al.*, 1996; Jomová, Valko, 2011; Čongrádyová *et al.*, 2011; 2012; 2013)**.
In our work we report synthesis and structural characterization of two novel copper complexes containing 5-chlorosalicylate anions and bioactive ligands, 1,10-phenanthroline and neocuproine, as well as a study of possible antimicrobial activity of the prepared complexes using eukaryotic and prokaryotic model organisms such as *Saccharomyces cerevisiae* and *Escherichia coli*. A difference between the effect of Cu(II)-complex compound containing 1,10-phenanthroline or neocuproine and unbound ligands on the growth of model organisms was evaluated as well.

MATERIAL AND METHODS

Synthesis of copper(II) complexes

Complexes under study were prepared similarly to previously described method **(Ranford *et al.*, 1993)**. 5-chlorosalicylic acid (1.0 mmol) was slowly added to an ethanolic solution of copper(II) acetate (1.0 mmol). After a minute 1,10-phenantroline (1.0 mmol) was added to the reaction mixture under stirring. The total volume of reaction mixture reached 50 mL and the resulting solution turned to green. The complex containing phenanthroline derivate neocuproine was prepared in the same molar proportions. The resulting solution turned to yellow. The reaction mixtures were stirred at ambient temperature. The precipitates (products) were filtered off under vacuum and dried at room temperature. The mother liquids were left to crystallize at ambient temperature and the crystals suitable for X-ray structure determination were separated and dried. The X-ray and EPR characterization of the complex was already briefly presented **(Jomová *et al.*, 2011)**.

Yeast strain and growth conditions

Yeast strain of *Saccharomyces cerevisiae* BY 4741 comes from EUROSCARF collection. Cells were grown in YPD medium containing 1 g of yeast extract (HiMedia, India), 2 g of peptone (Serva, Germany) and 2 g of glucose (Lachema, Czech Republic) per 100 mL of distilled water. For a solid medium, 2 g of agar (Serva, Germany) were added. The media were sterilized by autoclaving at 0.1 MPa and 120 °C for 30 min. Single colony of yeast cells was inoculated in 10 mL of YPD medium and incubated at 30 ºC for 16 h under continual shaking at 150 rpm. The cells from overnight pre-culture were transferred to 50 mL of fresh YPD media, so that the culture reached the optical density value OD_{600} corresponding to circa 2.10^8 cells/mL. The culture served as inoculum for all tests done.

Bacterial strain and growth conditions

Bacterial strain of *Escherichia coli* was obtained from the Institute of Plant Genetics and Biotechnology, Slovak Academy of Sciences in Nitra. The bacterial cells were grown overnight in Luria-Bertani medium containing 1 g of tryptone (Biolife, Italia), 0,5 g of yeast extract (HiMedia, India), 1 g of NaCl (CentralChem, Slovakia) per 100 mL of distilled water; and the mixture was autoclaved at 0.1 MPa for 30 min at 120°C. For solid medium, 2 g of agar

(Biolife, Italia) were added. An antibiotic selection was needed for prepared media therefore they were supplemented with 50 µg/mL ampicilin (Serva, Germany). Overnight liquid culture was composed of single colony of bacterial strain inoculated in 10 mL LB media with antibiotic in 100 mL Erlenmeyer flasks, which was incubated at 37ºC for 16 h under continual shaking at 150 rpm. The cells from overnight pre-culture were transferred to 50 mL of fresh LB media, so that the culture reached the optical density value OD_{600} corresponding to a cell density of approximately 1 x 10^9 cells/mL. The culture served as inoculum for all tests done.

Determination of antimicrobial activity

The antimicrobial activity of the prepared copper(II)-complex with 1,10-phenanthroline and copper(II)-complex with neocuproine was tested on the yeast cells inoculated in liquid YPD medium and bacterial cells inoculated in liquid LB medium with antibiotic. The complex $[Cu(phen)(5\text{-}Clsal)(5\text{-}ClsalH_2)]_2$ was dissolved in dimethyl sulfoxide (DMSO) and added to the prepared inoculated liquid media to reach concentrations 0.01, 0.02, 0.03, 0.05, 0.1, 0.2 and 0.5 mM. The complex $[Cu(H_2O)(5\text{-}Clsal)(Neo)]$ also dissolved in DMSO was added to the prepared inoculated liquid media to reach concentrations 0.01, 0.02, 0.03, 0.04 mM. The bacterial cells were incubated with shaking at 37ºC and yeast cells were incubated with shaking at 30ºC. The cell growth was assessed by measuring of optical density of the culture at 600 nm at regular time intervals within 8 hours of cultivation. Each measurement was done in triplicate and data represent mean values.

RESULTS AND DISCUSSION

Current interest in copper complexes comes from their potential use as antimicrobial, antiviral, anti-inflammatory or even antitumour agents as well as from their intercalating properties **(Sousa *et al.*, 2012)**. It has been shown, that the most of the antitumour drugs used nowadays were firstly considered as antimicrobial agents. However, after the recognition of their antitumour value, their characterization substantially increased over the following years **(Almeida *et al.*, 2008)**. The most extensively studied N-heterocyclic chelating agents are 1,10- phenanthroline with its derivates like neocuproine. The biological activity of phenanthroline and metal – phenanthroline complexes represents novel highly active antifungal and antibacterial agents **(Coyle *et al.*, 2004; Kumar *et al.*, 2008; Liu *et al.*, 2013)**. Based on these arguments, different copper compounds containing phenanthroline and neocuproine ligands have been prepared and studied as potential antimicrobial agents able to counteract antibiotic resistant microorganisms or future antitumour drugs.

We have prepared two types of the Cu(II) complex compounds containing 5-chlorosalicylic acid in combination with either 1,10-phenanthroline or its methylated derivate, 2,9-dimethyl-1,10-phenanthroline (neocuproine). The prepared green crystal of copper complex with phenanthroline was studied by means of the X-ray structural analysis which revealed a dimeric structure of the formula $[Cu(phen)(5\text{-}Clsal)(5\text{-}\ ClsalH_2)]_2$ **(Jomová *et al.*, 2011)**. The X-ray structural analysis of the yellow crystal of $[Cu(H_2O)(5\text{-}Clsal)(Neo)]$ revealed a monomeric structure (Kucková et al., 2013, in preparation).

Both organic ligands, 1,10-phenanthroline and neocuproine, have a planar structure which enables them to intercalate into DNA and disrupt the DNA structure. As already mentioned the organic ligands may finely tune the metal toxicity **(Gallagher *et al.*, 1996; Efthimiadou *et al.*, 2008)** mediated through the Cu(II)/Cu(I) redox cycling and the production of reactive radicals. The radicals are responsible for damage to biomolecules and subsequent cell death. In the recent years, many scientific researches focus on exploration and development of copper(II) complexes with biologically active ligands which exhibit interesting properties. Several studies show that 1,10-phenanthroline, derivates of phenanthroline and metal–phenanthroline complexes display antitumour, antifungal and antibacterial activities and have the potential to induce apoptosis in fungal, bacterial and mammalian cells **(Neville *et al.*, 2013; Liu *et al.*, 2013; Pillai, Sreekanth, 2013)**.

In the presented biochemical tests various concentrations of the prepared copper complexes with the model organisms have been used for each complex. The complexes differing in both the structure and the coordination environment have significantly different solubility in the DMSO solvent which did not allow us to test the same concentration range. Since a solubility of the dimeric phenanthroline complex $[Cu(phen)(5\text{-}Clsal)(5\text{-}ClsalH_2)]_2$ was higher compared to the monomeric neocuproine complex the tested concentrations of the phenanthroline complex ranged from 0.01 mM to 0.5 mM. Conversely, poor solubility of neocuproine complex $[Cu(H_2O)(5\text{-}Clsal)(Neo)]$ allowed us to test the maximum concentration of the complex up to 0.04 mM.

Our results showed that the cell growth was inhibited in a dose-dependent manner (figures 1 and 2). Interestingly, the yeast cells exhibited a high sensitivity to the low concentrations of neocuproine complex $[Cu(H_2O)(5\text{-}Clsal)(Neo)]$. While the 0.04 mM concentration of this complex inhibited the growth of yeast cells completely, such an effect was not achieved even at almost ten times higher concentration of the phenanthroline complex $[Cu(phen)(5\text{-}Clsal)(5\text{-}ClsalH_2)]_2$ (figure 1). Demonstrable inhibitory effect on the microbial growth showed two highest concentrations of phenanthroline complex (0.2 mM and 0.5 mM) (figures 1 and 2). The low concentrations of phenanthroline complex (from 0.01 to 0.05 mM) exhibited comparable effect being ineffective to suppress cell growth.

To find out the effect of free nitrogen ligand on the growth of microbial cells and to compare it to the effect of the complex compound we studied the highest 0.5 mM and 0.04 mM concentrations for phenanthroline and neocuproine complex/free ligand, respectively (figures 3 – 6). We have observed that both the free phenanthroline and Copper(II) phenanthroline complex $[Cu(phen)(5\text{-}Clsal)(5\text{-}ClsalH_2)]_2$ reduced the microbial growth for the given concentration (figures 3 – 4). The figure 4 shows a slightly higher sensitivity of the bacterial cell culture against free phenanthroline ligand compared to its bound form however, the difference is not significant. A comparable effect of both free and bound form of phenanthroline on the growth of microbial cells indicates that an intercalation of phenanthroline ligand can be a key mechanism of action for the phenanthroline complexes.

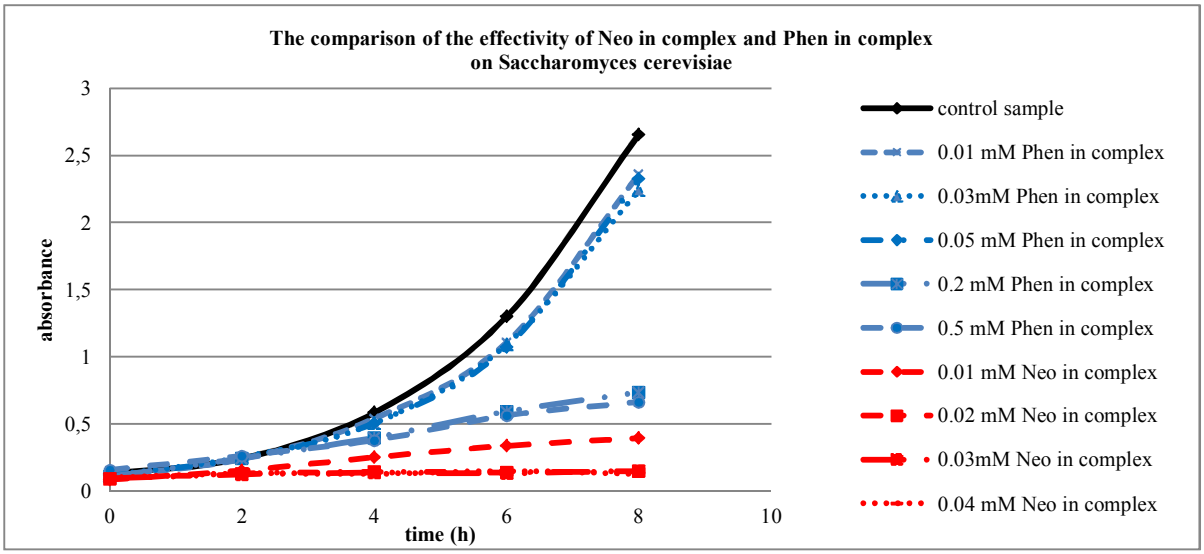

Figure 1 The growth of *S. cerevisiae* yeast cell culture within 8 hours cultivation in the presence of various concentrations of $[Cu(H_2O)(5\text{-}Clsal)(Neo)]$ and $[Cu(phen)(5\text{-}Clsal)(5\text{-}ClsalH_2)]_2$ complexes

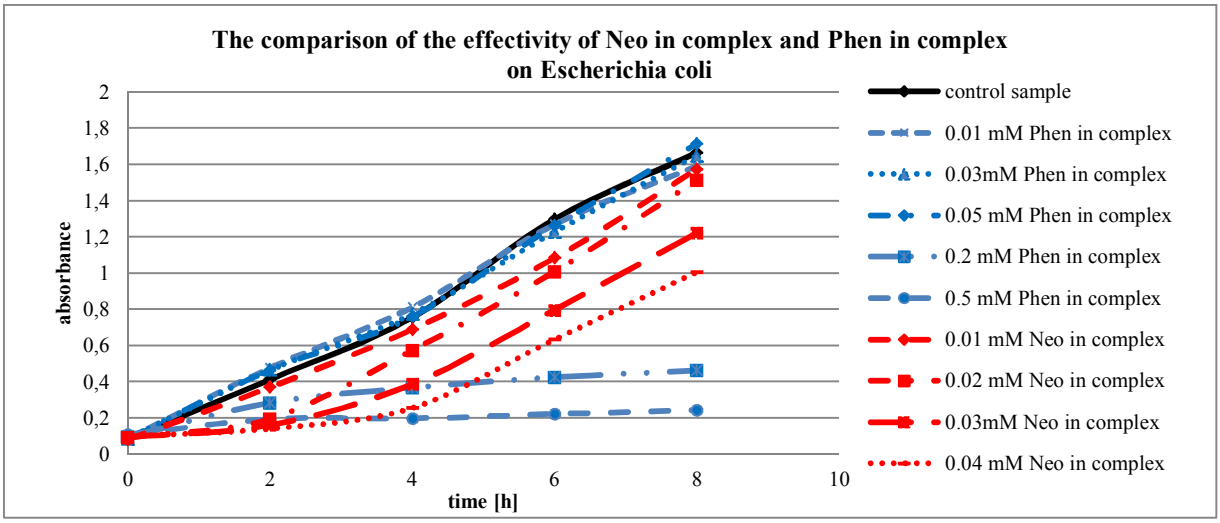

Figure 2 The growth of *E. coli* bacterial cell culture within 8 hours cultivation in the presence of various concentrations of [Cu(H_2O)(5-Clsal)(Neo)] and [Cu(phen)(5-Clsal)(5-ClsalH$_2$)]$_2$ complexes

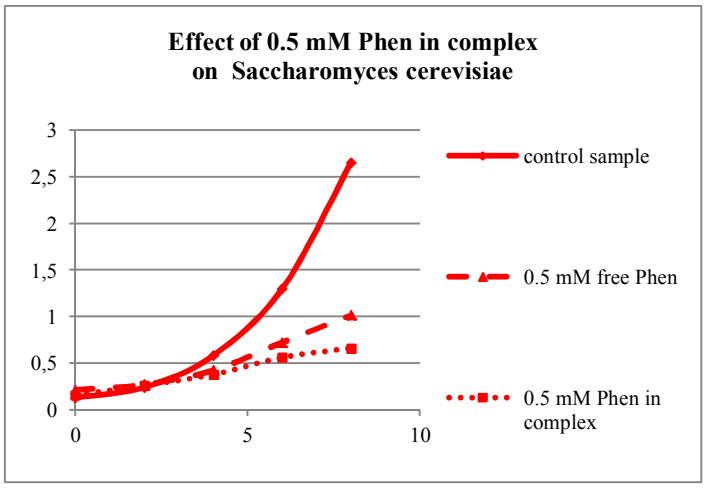

Figure 3 The growth of yeasts culture within 8 hours cultivation in the presence of the highest 0.5 mM concentration of the complex [Cu(phen)(5-Clsal)(5-ClsalH$_2$)]$_2$ and 0.5 mM concentration of unbound 1,10-phenanthroline as a positive control

For the given 0.04 mM concentration, the unbound neocuproine exhibited weaker antimicrobial response compared to its complex form [Cu(H_2O)(5-Clsal)(Neo)] (figures 5 – 6). Striking difference we observed in case of yeast cell culture (figure 5) indicating strong antifungal effect of very low concentration of the neocuproine Cu(II) complex. Generally stronger effect of neocuproine Copper(II) complex on the microbial growth compared to the effect of free neocuproine indicates that different mechanism than intercalation should participate in the toxic action of the molecule. It is known that a hindrance of methyl groups in neocuproine molecule does not allow effective intercalation.

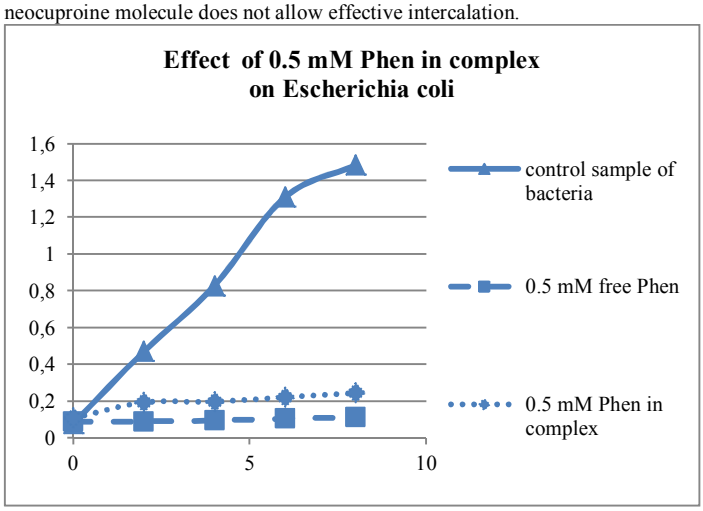

Figure 4 The growth of bacteria culture within 8 hours cultivation in the presence of the highest 0.5 mM concentration of the complex [Cu(phen)(5-Clsal)(5-ClsalH$_2$)]$_2$ and 0.5 mM concentration of unbound 1,10-phenanthroline as a positive control

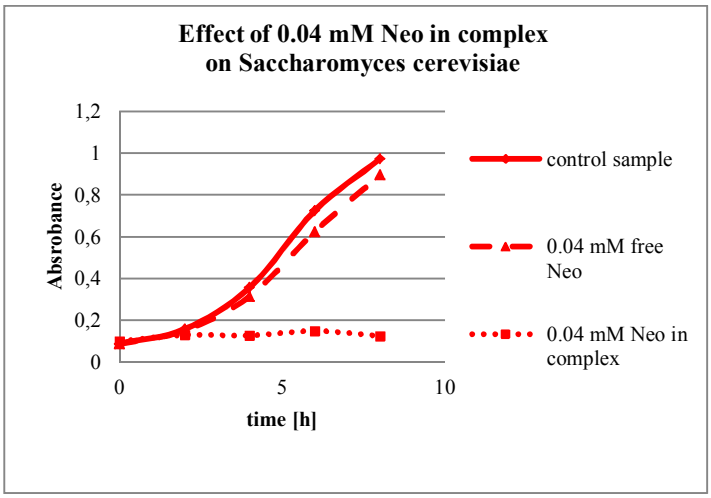

Figure 5 The growth of the bacteria culture within 8 hours cultivation in the presence of the highest 0.04 mM concentration of complex [Cu(H_2O)(5-Clsal)(Neo)] and 0.04 mM concentration of unbound neocuproine as a positive control

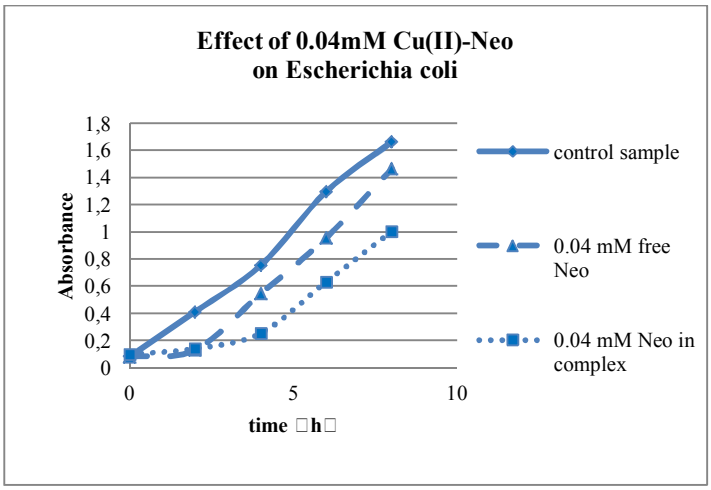

Figure 6 The growth of the yeasts culture within 8 hours cultivation in the presence of the highest 0.04 mM concentration of complex [Cu(H_2O)(5-Clsal)(Neo)] and 0.04 mM concentration of unbound neocuproine as a positive control

Our results are consistent with the results of other scientists who have established that copper complexes with neocuproine are highly toxic towards various types of microorganisms including bacteria and yeasts, and that the chelation with copper is essential for the toxic effect **(Zoroddu *et al.*, 1996)**. It has been reported that the higher activity of the copper(II) 2,9-dimethyl derivative

complexes is attributed to the key position of the methyl groups proximate to the copper coordination site, which increases redox potential by creating steric hindrance on coordination. The high redox potential in the planar cupric bidentate chelate facilitates the reduction by biological reductants of the less stable copper(II)-complexes to more stable tetrahedral copper(I)-complexes. This fact shows that very subtle differences in the chemical arrangement can result in appreciable differences in the antimicrobial activity **(Zhu, Chevion, 2000; Neville *et al*., 2013)**.

CONCLUSION

Our preliminary results with newly synthetized copper phenanthroline-salicylate complex and copper(II)-neocuproine-salicylate complex showed antibacterial and antifungal activities. In both cases it was shown that the effect of complexes $[Cu(phen)(5\text{-}Clsal)(5\text{-}ClsalH_2)]_2$ and $[Cu(H_2O)(5\text{-}Clsal)(Neo)]$ on the growth of eukaryotic and prokaryotic organisms was slightly stronger as the activity of free ligands. However, free 1,10– phenanthroline has shown stronger antibacterial activity compared to its complex $[Cu(phen)(5\text{-}Clsal)(5\text{-}ClsalH_2)]_2$. A comparison of the effectiveness of the two prepared complexes indicates that the $[Cu(H_2O)(5\text{-}Clsal)(Neo)]$ complex showed greater antimicrobial activity than the $[Cu(phen)(5\text{-}Clsal)(5\text{-}ClsalH_2)]_2$ complex.

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