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Antimicrobial Effectiveness of $[Cu(pzdc)(phen)_2]\cdot 5.5H_2O$ Complex on Some Microorganisms

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This study was conducted to find new chemical substances that are highly effective on selected microorganisms from among eight Cu(II) pyrazine-2,3-dicarboxylate complexes, which have been previously synthesized and characterized. The MIC value of the $[Cu(pzdc)(phen)_2]\cdot 5.5H_2O$ complex (8) was determined to be in the range of 15-40 μg/ml for all bacteria (except the clinical isolate *Pseudomonas aeruginosa*). However, the minimum microbiocidal concentrations (MMC) (30-200 μg/ml) were determined to be much higher than the MIC values. In time–kill assays, all the bacteria were fully finished after 120 minutes at 18 μg/ml and after 240 minutes at 4.5 μg/ml from 5.54 log. the initial number of bacteria at 37 ^{0}C in the lake water. However, all the bacteria were not fully inhibited even at 18 μg/ml at 10 ^{0}C. The same effect was not seen on *Candida albicans* in spite of the low MIC value (25 μg/ml). $[Cu(pzdc)(phen)_2]\cdot 5.5H_2O$ complex has broad-spectrum biostatic activity against microorganisms at the MIC values. The mechanism of action is not yet clear.

Key words: Antimicrobial activity, Disinfectant, Pyrazine-2,3-dicarboxylate complex.

A significant percentage of clinical isolates, including species of methicillin-resistant *Staphylococcus aureus* (MRSA), *Pseudomonas aeruginosa*, *Streptococcus pneumoniae*, *Enterococcus faecalis*, *Acinetobacter baumannii* and *Morganella morganii* are resistant to commonly used antibiotics, such as new-lactams, glycopeptides, fluoroquinolones, linezolids and macrolides[1-6]. At the same time, these bacteria generates serious public health problems which are called nosocomial infections because of the infection and superinfection of hospitalized patients. Especially, the situation is critical in the case of treatment of hospital infections caused by *S. aureus* and *P. aeruginosa*. Every year, two million hospital infections occur in the United States, and 90.000 people are reported dead [7]. The annual prevalence of hospital infections in a report including the nine countries has been reported to be 4-10%. In Turkey, this ratio varies between 1% and 8.6%. The frequency of hospital acquired infection in intensive care units is reported to be 50%, with the death ratio being 16% [7]. The emergence of antibiotic resistance among pathogenic bacteria has led to major research efforts to find alternative antimicrobial therapeutics. The spread of resistant bacteria causes problems of infection control in society. Clearly, new antimicrobial agents that can be effective at low doses, with broad-spectrum or species-specific activity, are required to combat

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an ever-increasing resistance problem among microorganisms. New antimicrobial agents are required for not only human diseases but also plant and animals diseases, for disinfection or sterilization of environment, water and wastewater.

In recent studies on the production of antimicrobial agents, metal ions such as Cd(II), Pd(II), Ag(I) and Au(I) and biocations such as Co (II), Ni(II), Cu(II), Fe(III), Cr(III), Mn(III) and Zn(II) were shown possess high activity to inhibit the development of bacterial resistance[8,9]. Positively charged metal centers are preferred in the production of antimicrobial drugs because they facilitate interaction with negatively charged biomolecules such as nucleic acids, ATP and protein components. Metal ions exert their effect by exchange of their metals or by disturbing to the internal and external coordination of active region to the structural integrity of enzymes. Metal ions allows longer time interaction through strong covalent or ionic bonds with the target molecules, compared to organic antimicrobial agents[10].

Although antimicrobial agents containing metals have high activity, some of them can not be used as antimicrobials, disinfectants, antiseptics due to their toxicity, drug resistance, and cellular metabolism of the complexes. Among antimicrobial agents, only silver complexes can be used to prevent bacterial infections in the treatment of burns, and to prevent the development of ophthalmia neonatorum[11,12]. Silver sulfadiazine (AgSD), a sulfa drug, is used to prevent and treat infections of second- and third-degree burns. Silver is an effective antimicrobial agent with low toxicity and hence is important especially in the treatment of burn wounds[11,13].

The purpose of this study is to find new chemicals that can be used in the war againts microorganisms. For this purpose, complexes that were synthesized and characterized by Yesilel *et al*.[14, 15, 16, 17] were selected, and the antimicrobial effects of these complexes were investigated.

MATERIALS AND METHODS

Microorganisms and metal complexes used in this study

The bacteria (seven wild type and six clinical isolates) and yeast used in this study are listed in Table 1. The clinical isolates were collected from the Faculty of Medicine, University of Ondokuz Mayis. All the clinical isolates were isolated from patients in the hospital. The eight pyrazine-2,3-dicarboxylate complexes, the pyrazine-2,3-dicarboxylic acid (H_2pzdc) and phenanathroline (phen) ligands used in this study are shown in Table 2. The preparation of the metal complexes, and their characterization steps have been previously presented in articles published by Yesilel *et al*[14, 15, 16, 17]. The Cu(pzdc)(phen)$_2$]·5.5H_2O complex is shown at Fig. 1[15].

Minimum Inhibitory Concentration (MIC) tests of complexes

The stock solutions of metal complexes were prepared with distilled water (30 mg/ml). The in-vitro susceptibility tests (MIC tests) were determined by broth microdilution carried out in accordance with the guidelines of the National Committee for Clinical Laboratory Standards[18]. Bacteria were incubated in nutrient agar (NA-Merck) at 37 ^{0}C for 24 hours, and yeast was incubated for 48 hours. A few colonies were emulsified in 5 ml Nutrient Broth (NB-Merck), and adjusted spectrophotometrically to 0.1 absorbance at OD_{600}. The final inoculum was adjusted to 10^5 CFU/mL with 60 ml NB. From the resulting suspension, a 1.8 ml aliquot was transferred into first tube, added 1 ml into another tubes, then 200 µL of metal complexes was transferred into the first tube. The samples were serially diluted for 1/2 (from 3000 to 5.9 µg/ml). The samples were then incubated at 37 ^{0}C for 24 hours. The same study was repeated after narrowing the gap. The MIC was defined as the lowest concentration that completely prevented visible growth of the bacteria after overnight incubation at 37°C. The study was carried out in three replicates. Minimum Microbiocidal concentration (MMC) was determined only in the Cu(pzdc)(phen)$_2$]·5.5H_2O complex. To determine minimum microbiocidal concentration (MMC) of Cu(pzdc)(phen)$_2$]·5.5H_2O complex, 100 µl from each tube which did not show growth in the MIC tests, and were incubated 37 ^{0}C for 24 h in nutrient agar medium. After incubation, the MBC value was determined as the lowest concentration that did not show growth in petri dishes.

Effect of [Cu(pzdc)(phen)$_2$]·5.5H_2O complex on growth of bacteria

Each strain was grown in 5 ml NB, for 18

hours at 37 ^{0}C, in a shaking incubator at 175 rpm. After incubation, the cultures were adjusted spectrophotometrically to 0.1 absorbance at OD_{600} and were used as starter cultures. The diluted overnight cultures (1:250 in NB) were incubated again (175 rpm, 37°C) until they reached an OD_{600} 0.1, and defined concentrations of complexes (9 and 18 µg/ml) were added, incubated for 4 hours with the complex, and the turbidity of cultures growing in NB was determined at 600 nm at several time points by measuring the absorbance using an UV/Vis spectrophotometer (Thermo-Germany). The procedure was repeated three times. The average values were obtained.

Effect of $[Cu(pzdc)(phen)_2]\cdot 5.5H_2O$ on survival of microorganisms in lake water

The effect of $[Cu(pzdc)(phen)_2]\cdot 5.5H_2O$ complex on survival of bacteria and yeast in lake water was tested at different temperatures (37 °C and 10 °C) and at different concentrations (18, 9, 4.5 µg/ml). For this purpose, *E. coli, S. aureus, P. aeruginosa* and *C. albicans* were used. Bacteria were incubated in NB (Merck) at 37 °C for 24 hours, and yeast was incubated for 48 hours. The bacteria and yeast were added into 100 ml of filtered-autoclaved lake water (final concentration approximately 5×10^5 CFU/ml for bacteria and 3×10^4 CFU/ml for yeast), the complex was transferred into the lake water (final concentrations of 18, 9 and 4.5 µg/ml), and incubated at different temperatures (37 and 10 °C) for six hours. The samples were serially diluted in Ringer's solution at several time points of incubation, including 240 minutes and plated onto NA. The colonies were counted after incubation at 37 °C for 24 h.

Data analyses

The JMP software was used for drawing graphics. Tests were carried out in independently three replicates.

RESULTS AND DISCUSSION

Resistant bacteria such as *S. aureus* and *P aeruginosa* that are known to cause hospital infections have developed resistance against antibiotics[19]. New active substances are needed not only for human pathogens, but also for microorganisms that cause disease in plants and animals, and for disinfection of both water and environment. Therefore, the development of new antibiotics and new subtances with antimicrobial activity is most urgent.

The antimicrobial activities of eight pyrazine-2,3-dicarboxylate complexes, which were previously synthesized and characterized[14,15, 16, 17], were investigated in this study, and the MICs of the complexes are presented in Table 3. $[Cu(pzdc)(phen)_2]\cdot 5.5H_2O$ clearly inhibited all test organisms at concentrations of 40 µg/ml, but the MIC for the clinical isolate *P. aeruginosa* was 100 µg/ml. The other seven complexes had values in the range of 400 - 3000 µg/ml against all the strains.

Table 1. Microorganisms used in this study

		Microorganisms	
Wild type(Prokaryote)	Gram(+)	*S. aureus*	ATCC6538
		S. epidermidis	ATCC12228
		B. cereus	ATCC7064
		E. faecalis	ATCC29212
	Gram(-)	*P. aeruginosa*	ATCC27853
		E. coli	ATCC25922
		S. typhimurium	LT2
Clinical isolates(Prokaryote)	Gram(+)	*MRSA-Methicillin resistant S. aureus*	
		MRCNS- Methicillin resistant coagulase negative *Staphylococcus*	
	Gram(-)	*P. aeruginosa*	
		E. coli	
		M. morganii	
		A. baumannii	
Eukaryote		*C. albicans* ATCC 10231	

$[Cu(pzdc)(phen)_2]\cdot 5.5H_2O$ complex is active against both wild-type and clinically isolated bacteria. The effectiveness of this substance did not change importantly according to the cell wall structure of bacteria. Gram (-) bacteria, due to the presence of the outer membrane, are known to be more resistant compared to the Gram (+) ones[20]. Some substances are not effective because they can not pass through the outer membrane of Gram (-) bacteria. However, they can be said to be more effective on the Gram-positive bacteria based on the MIC values of *S. aureus* (both wild-type and the clinical isolates, 15 µg/ml) and *B. cereus* (15 µg/ml). Furthermore, there are not any differences between prokaryotic and eukaryotic microorganisms in terms of the effectiviness of the $[Cu(pzdc)(phen)_2]\cdot 5.5H_2O$ complex.

$[Cu(pzdc)(phen)_2]\cdot 5.5H_2O$ was synthesized from pyrazine-2,3-dicarboxylic acid, 1,10-phenanthroline, and $Cu(CH_3COO)_2\cdot H_2O$. If we look at the results of Table 3 individually, the MIC values of pyrazine-2,3-dicarboxylic acid for six microorganisms were 312.5 µg/ml and for eight microorganisms, the value was 625 µg/ml. In addition, the MIC values of 1,10-phenanthroline monohydrate were in the range 15-312.5 µg/ml and $CuCl_2 2H_2O$ were in the range 312,5-625 µg/ml for all tested bacteria and yeast. The complex is also highly active compared to the parent compounds (from 9 and 10) shown by the 8- to 64-fold lower MIC value (Table 3). $[Cu(pzdc)(phen)_2]\cdot 5.5H_2O$ was more potent than pyrazine-2,3-dicarboxylic acid[10] in all cases and nearly equipotent with the 9 against *E. coli, S. typhimurium, B. cereus* and some clinical isolates (*E. coli, A. baumannii, M. morganii,* and *C. albicans*). The synthesized complex was found to have lower MIC value according to the ligands and the main matter. Our results clearly showed that $[Cu(pzdc)(phen)_2]\cdot 5.5H_2O$ possesses broad antibacterial and anticandidal effects against the microorganisms used in the study. After this study, the MIC values of $[Cu(pzdc)(phen)_2]\cdot 5.5H_2O$ complex were determined that it did not kill microorganisms both using plate count and microscope. Moreover, the minimum microbiocidal concentration (MMC) of $[Cu(pzdc)(phen)_2]\cdot 5.5H_2O$ was investigated, MMC values are compared in Table 3. There is a big difference between the MIC (15-40 µg/ml) and MMC (30-200 µg/ml) values. Therefore, the mechanism of action of this complex is thought to be associated with both cell wall integrity and replication mechanisms.

The bacteriocidal potential of $[Cu(pzdc)(phen)_2]\cdot 5.5H_2O$ was assessed in time-kill assays with one Gram-positive organisms (*S. aureus*) and two Gram-negative bacteria (*E. coli* and *P. aeruginosa*). According to Pankey and Sabath[21], a compound is defined as bacteriocidal if it effects a 3-log or greater decrease in bacterial cell density after 24 hours of incubation. According to the results of the MIC, the effect of the $[Cu(pzdc)(phen)_2]\cdot 5.5H_2O$ complex, which was determined to be highly effective in NB, 1ts effect on survival of microorganisms in lake water was examined. The effect of temperature on the activity of the complex, the effect of different concentrations of the complex on its antimicrobial activity, and its effectiveness against the microorganisms in lake water as a natural environment were investigated. The data obtained from these experiments are presented in the Figures 2-6. The lake-water experiments showed that temperature is very important for the effectiveness of complex. While *E. coli, S. aureus* and *P. aeruginosa* were completely inhibited at a concentration of 4.5 µg/ml at 37 °C in a period of 240 minutes, these bacteria were not inhibited even at a concentration of 18 µg/ml at 10 °C in the same time interval (Fig. 3-5). Another interesting observation encountered in this study was the effect of the complex on the survival of *C. albicans*

Table 2. Metal complexes used in this study

	Complexes and Ligant	Ref.
1	$[Cu_2(\mu\text{-pzdca})_2(pen)_2]\cdot 2H_2O$	16
2	$\{[Cu(\mu\text{-pzdca})(bipy)]\cdot H_2O\}n$	16
3	$[Cu(pzdca)(H_2O)(dmpen)_2]$	15
4	$\{[Cu(\mu\text{-pzdca})(tmen)]\cdot H_2O\}n$	16
5	$[Cu(pzdca)(H_2O)(en)2]\cdot H_2O$	16
6	[Cu(µ3-pzdca)(mea)]n	17
7	$[Cu_2(pzdca)_2(H_2O)_2(dmen)_2]\cdot 6H_2O$	14
8	$[Cu(pzdca)(phen)_2]\cdot 5.5H_2O$	14
9	1,10 phenanthroline monohydrate	14
10	Pyrazine-2,3-dicarboxylic acid (ligand)	14

[Abbreviations: pzdca=Pyrazine-2,3-dicarboxylic acid, pen=1,3-propanediamine, bipy=2,2'-bipyridine, dmpen =2, 2-dimethylpropane-1,3-diamine, tmen=N,N,N',N'-tetramethylethylenediamine, en=ethylenediamine, dmen =N,N'-dimethylethylenediamine, phen=1,10-phenanthroline, mea= monoethanolamine]

Table 3. Minimal inhibition concentrations (MIC) and Minimum microbiocidal concentration values (MMC of 8) of complexes and ligand for wild type and clinical isolate (µg/ml)

	Microorganisms		1	2	3	4	5	6	7		8	9	10	11
			(MIC)	(MIC)	(MIC)	(MIC)	(MIC)	(MIC)	(MIC)	(MIC)	(MMC)	(MIC)	(MIC)	(MIC)
Wildtype	Gram(-)	*E. coli*	800	600	1400	1500	2500	1400	<3000	40	<100	30	625	625
		P. aeruginosa	1500	1250	1400	1500	2750	<3000	<3000	20	100	312.5	625	625
		S. typhimurium	900	900	2250	1750	<3000	2500	<3000	30	<100	30	625	625
	Gram(+)	*E. faecalis*	1500	750	1500	1000	<3000	<3000	<3000	20	60	312.5	312.5	312.5
		B. cereus	1000	750	2500	1500	2750	2500	<3000	15	30	20	312.5	312.5
		S. epidermidis	1000	750	2500	1500	2000	2750	<3000	30	40	156	625	312.5
		S. aureus	800	750	1000	1500	1250	1700	<3000	15	30	250	625	312.5
Clinicalisolates	Gram(+)	*MRSA*	1250	750	1500	1750	<3000	1700	<3000	15	40	75	312.5	625
		MRCNS	600	500	750	1000	1000	2500	<3000	20	60	40	312.5	625
	Gram(-)	*E. coli*	800	1500	1750	1750	<3000	1400	<3000	35	70	30	625	625
		P. aeruginosa	1500	1500	<3000	<3000	<3000	<3000	<3000	100	<200	312.5	312.5	625
		A. baumannii	700	1250	2750	3000	1500	2500	<3000	35	60	40	625	625
		M. morganii	1500	400	1750	<3000	2750	<3000	<3000	30	<100	40	312.5	625
Eucaryote		*C. albicans*	<3000	<3000	<3000	<3000	<3000	<3000	<3000	25	60	30	625	625

1-$[Cu_2(<\text{-pzdca})_2(pen)_2]\cdot 2H_2O$,
2-$\{[Cu(<\text{-pzdca})(bipy)]\cdot H_2O\}n$,
3-$[Cu(pzdca)(H_2O)(dmpen)2]$,
4-$\{[Cu(<\text{-pzdca})(tmen)]\cdot H_2O\}n$,
5- $[Cu(pzdca)(H_2O)(en)2]\cdot H_2O$,
6-[Cu(<-pzdca)(mea)]n,
7-$[Cu_2(pzdca)_2(H_2O)_2(dmen)_2]\cdot 6H_2O$,
8-$[Cu(pzdca)(phen)_2]\cdot 5.5H_2O$,
9-1,10-phenanthroline monohydrate (phen),
10- Pyrazine-2,3- dicarboxylic acid (ligand).
11- $CuCl_2.2H_2O$

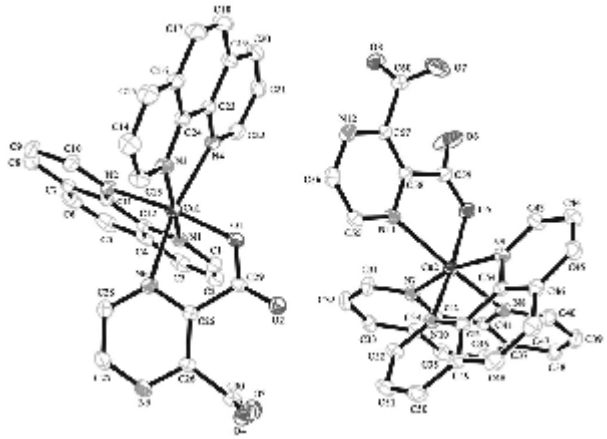

Fig. 1. Structure of the [Cu(pzdca)(phen)$_2$]·5.5H$_2$O complex[27]

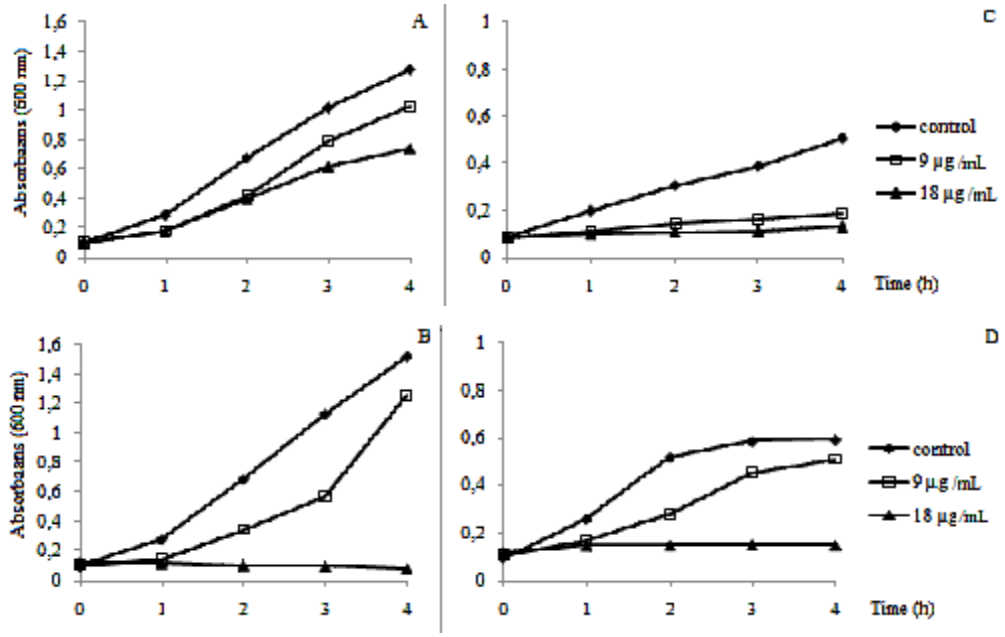

Fig. 2. Inhibition of the growth of microorganisms with the [Cu(pzdc)(phen)$_2$]·5.5H$_2$O complex. The complex was added to early logarithmic phase cultures at 0. hour (approximately 0.1 at 600 nm) (A- *E. coli*, B- *S. aureus*, C- *C. albicans*, D- *P. aeruginosa*)

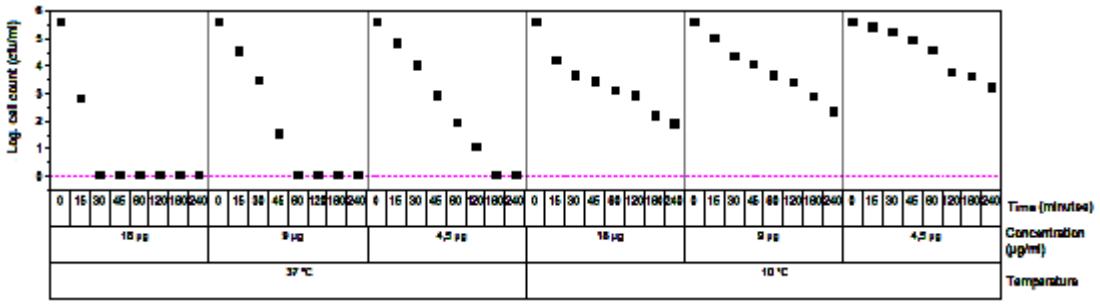

Fig. 3. Effect of the [Cu(pzdc)(phen)$_2$]·5.5H$_2$O complex on survival of *E. coli* in lake water

(Fig. 6). *C. albicans* was not inhibited even at 18 µg/ml, at 37 °C, in period of 240 minutes. None of the studied conditions showed any remarkable effect on *C. albicans* in lake water (Fig. 6). This results is quite interesting.

A concentration of 4.5 µg/ml of this complex in lake water showed a 3 log. decline within 45 minutes for *E. coli* and *S. aureus*, and in 180 minutes for *P. aeruginosa*. These results indicate that while 18 µg/ml of this complex had a bacteriostatic effect in NB, the same concentration had a bactericidal effect on microorganisms in lake water. When the activity of [Cu(pzdc)(phen)$_2$]·5.5H$_2$O complex was compared at two temperature, 37 and 10 °C, the complex was found to be very effective at 37 °C than at 10 °C. The reason for this effect is thought to be a the high levels of metabolic activity reached by microorganisms in their logarithmic growth phase, in addition to the temperature 37 °C being the optimum temperature required for the growth of microorganisms. The effectiveness of the complex is thought decreased at 10 °C due to the slower metabolism of the microorganisms.

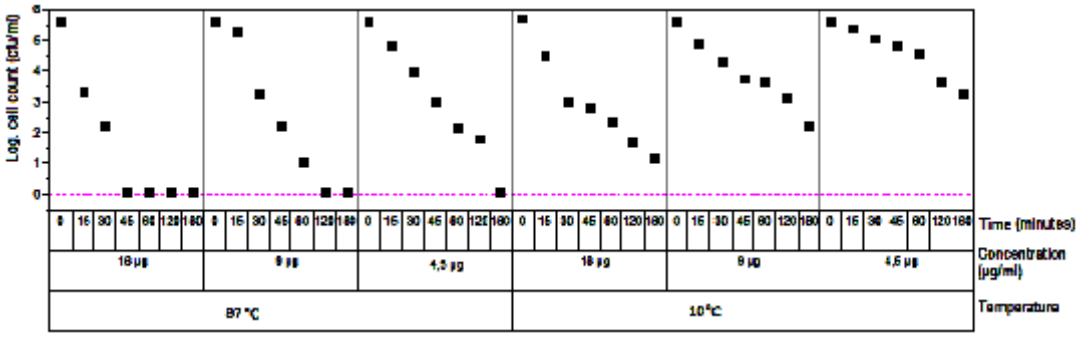

Fig. 4. Effect of the [Cu(pzdc)(phen)$_2$]·5.5H$_2$O complex on survival of *S. aureus* in lake water

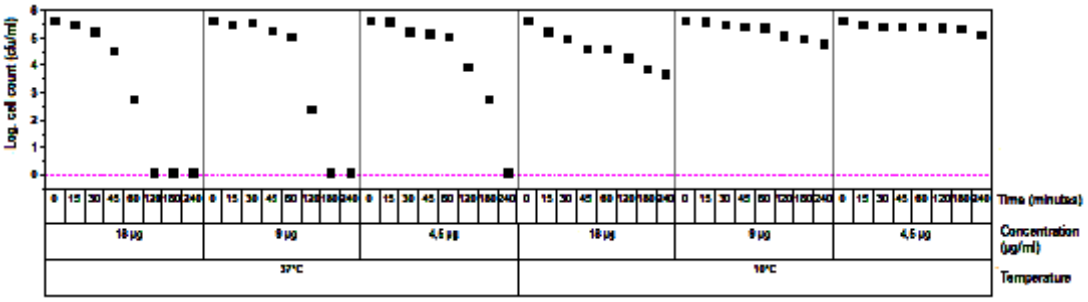

Fig. 5. Effect of the [Cu(pzdc)(phen)$_2$]·5.5H$_2$O complex on survival of *P. aeruginosa* in lake water

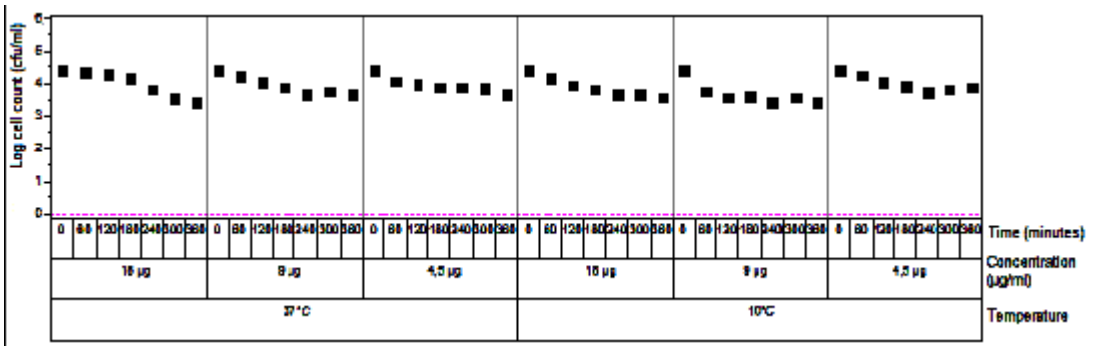

Fig. 6. Effect of the [Cu(pzdc)(phen)$_2$]·5.5H$_2$O complex on survival of *C. albicans* in lake water

When the $[Cu(pzdc)(phen)_2]\cdot 5.5H_2O$ complex is dissolved in water, it is composed of a mononuclear structure consisting of a Cu(II) ion, two 1,10-phenanthroline ligands and one pyrazine-2,3-dicarboxylate dianion. When the structure-activity relationship of the complex was examined in isolation, the MIC values of 1-10-phenanthroline is higher, in general, than metal complex. Yesilel *et al*.[22] reported that the MIC value of $[Co(pzdc)(phen)_2]_2\cdot 11H_2O$ was 2000-2250 µg/ml. $[Co(pzdc)(phen)_2]_2\cdot 11H_2O$ and $[Cu(pzdc)(phen)_2]\cdot 5.5H_2O$ have an identical molecular structure. Therefore, the difference between the two molecules is only that the current compound under study has Cu(II) instead of Co(II) as the central metal moiety. However, the effectiveness of the molecule cannot be said to be derived completely either due to Cu(II) or phenanthroline (Table 3). The structure of $[Cu(pzdc)(phen)_2]\cdot 5.5H_2O$ as a whole can be said to have a strong effect on microorganisms. In lake water studies, with an MIC value of 20 µg/ml against *C. albicans*, 1t was found to be virtually ineffective because *C. albicans* is in the logarithmic growth phase in NB and in the stationary phase in lake water.

2,3-diethyl pyrazine dicarboxylic acid which was produced from pyrazine dicarboxylic acid was found to prevent corrosion in steel[23]. The pyrazine ring is known to be a part of many polycyclic compounds of biological and industrial importance. In addition, pyrazine derivatives have been reported to possess antituberculotic, antifungal, and cytotoxic activities[24-26]. Especially, pyrazine-2,3-dicarboxylic acid derivatives were shown to be effective on *Mycobacterium tuberculosis* which causes the dreaded tuberculosis disease in the third-world countries and is beginning to be a problem again currently[27-29]. Pyrazine derivatives were seen as suitable candidates for the antimicrobial substances[30]. In addition, in some studies of the antimicrobial activity of various compounds, the hydrazinium salts of 2-pyrazinecarboxylate, pyrazine-2,3-dicarboxylate, pyrazine-3,5-dicarboxylate and imidazole-4,5-dicarboxylate have been found to be more effective than standard antibiotics against *E. coli, Salmonella typhi* and *Vibrio cholerae*[31,32]. In addition, pyrazine-2-carboxylic acid derivatives inhibited the growth of *Leuconostoc mesenteroides*[33].

According to the obtained data of the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cytotoxicity test, $[Cu(pzdc)(phen)_2]\cdot 5.5H_2O$ was found to have a IC_{50} value of 5 µg/ml against NS20Y neoroblastoma cells (detailed data did not given).

Thus, the $[Cu(pzdc)(phen)_2]\cdot 5.5H_2O$ complex is determined to be very effective even at low concentrations with 15-40 µg/ml being the MIC value against microorganisms. This complex can be applied as a disinfectant because of its very low MIC value, is very easily soluble in water, and is odorless and colorless at the effective concentration. This novel metal complex which is potent against antibiotic-resistant pathogens, should be further investigated. Combining the information obtained in this study with pharmacokinetic and pharmacodynamic data is necessary to provide an in vivo efficacy prediction that is more meaningful.

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