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Antimicrobial Activity of Copper (II) Complex with 1,2-bis [(1,3-diphenylpyrazol-4-yl)methyl] Diaminoethane

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ABSTRACT

***Background*:** Multi-resistant strains multiply daily, populate farms, hospitals and other ecological niches around the world, and cause serious infections in animals and humans, often leading to a fatal outcome. Researchers of all profiles are investigating intensively to find new substances with antimicrobial activity. In the period between 1981 and 2002, 163 new chemical compounds were approved for use as drugs. Synthesized compounds have become much more interesting than the natural ones in the production of new antimicrobial agents. Some of these synthesized compounds are Copper (II) complex. The antimicrobial properties of copper were known in ancient Egypt (2000 BC), where it was used to sterilize water and wounds. Copper is still interesting for today's research.

***Materials, Methods & Results*:** Antimicrobial activity was tested using a microdilution method according to Clinical and Laboratory Standards Institute. The percentage of surviving bacteria was calculated in comparison to the number of bacteria placed in each well. Based on these results, using the Excel software package from Microsoft Office 2007, graphs were generated that showed the percentage of surviving bacteria depending on the corresponding effective concentrations of the tested substance. The function, which was used to approximate the experimental results, was determined using the Power Trendline supplement from the Microsoft Excel program. Cytotoxicity (growth inhibition) was evaluated by tetrazolium colorimetric MTT assay, after exposure of cells to the tested compound for 48 h. Inhibition of growth was expressed as a percentage of cytotoxicity and calculated according to the following equation: (1-A test/A control) x 100. $MBC_{99.9}$ and MIC_{99} of the test substance were lowest for *Arcanobacterium haemolyticum* being 0.2 mg/L and 0.0054 mg/L, respectively. The highest values were obtained for *Arcanobacterium pyogenes* and methicillin-resistant *Staphylococcus aureus* (MRSA) 488.002 mg/L and 20.2 mg/L. MIC_{80} for all four strains ranged from 0.00002 to 0.0023 mg/L. Measured values for MIC_{99} are 0.00545 mg/L for *A. haemolyticum*, 0.0443199 mg/L for *R. equi*, 0.0520712 mg/L for *S. aureus* and 2.36378 mg/L for *A. pyogenes*. Values for $MIC_{99.9}$ ranged from 0.236134 to 488,002 mg/L. Most of the MIC values obtained in this study are significantly lower than those reported by other researchers. The values we obtained were lower as compared to MIC values for standard antibiotics, which were considered acceptable by the relevant institutions. This speaks in favor of a stronger antibacterial effect of our tested substances. In regards to cytotoxicity, the obtained MIC_{80} doses were lower than toxic, whereas MIC_{90} could be classified as low-toxic (less than 0.0625 µM), except of *Arcanobacterium pyogenes* only. According to the IC50 values, the compound Cu (L) Br2·MeOH was 6.4-fold and 4.8-fold more potent against HCT116 cells compared to normal lung fibroblasts and SW620 cells, respectively.

***Discussion*:** Copper (II) complex with an arylpyrazole ligand exhibits strong antibacterial properties, and it shows bacteriostatic effect at concentrations where there is no cytotoxic effect in normal human cells. The emergence of multi-resistant strains of pathogenic bacteria is a growing problem worldwide. Therefore, each new compound with potential antimicrobial activity, especially if it is not cytotoxic in effective dosage, deserves the attention of the scientific community. In this paper, we presented a newly synthesized substance with such properties.

Keywords: Cu (L)Br_2·MeOH, minimal bactericidal concentration, minimal inhibitory concentration, gram positive bacteria.

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INTRODUCTION

Extensive production and consumption of antibiotics has led to the worsening of the problem of resistance of bacteria and the emergence of strains of pathogenic bacteria that are resistant to almost all antibiotics [20]. Despite the great progress in science and medical technology, infective diseases remain the leading cause of morbidity and mortality worldwide [17]. The development of microbial resistance to antibiotics reduces the therapeutic effect of the available antimicrobial agents, which leads to increased rates of treatment [17]. Synthesized compounds have become more interesting than the natural ones in the production of new antimicrobial agents. By improving technology and enhancing knowledge, synthesized compounds will likely lead to better results in future [19,28].

Antimicrobial activity of a large number of synthesized copper compounds is now the topic of abundant research around the world. [3,5,9,22,24]. Mechanisms of action of these compounds on microorganisms are still unclear.

The aim of this study was to investigate the antimicrobial activity of the Cu (II) complex with an arylpyrazole ligand on some gram-positive bacteria that are major pathogens in humans and animals, and its cytotoxicity in normal and malignant cells.

MATERIALS AND METHODS

Investigated compound

The investigated compound was copper (II) complex of the formula Cu (L)Br_2·MeOH, where L stands for bidentate NN 2-bis[(1,3-diphenylpyrazol-4-yl)methyl]diaminoethane (Figure 1). The complex was prepared by mixing the warm methanolic solutions of L and $CuBr_2$ [16].

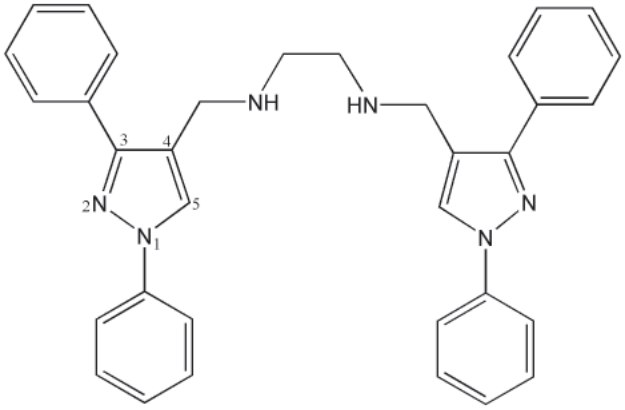

Figure 1. Chemical structure of bidentate NN 2-bis [(1,3-diphenylpyrazol-4-yl)methyl] diaminoethane.

Solvent

The substance examined in a previous study showed solubility only in dimethyl sulfoxide (DMSO)[1] and dimethylformamide (DMF)[1]. After preliminary examination, DMF solvent was chosen because it showed less toxicity compared to DMSO for the tested bacteria.

Microorganisms

The study included four strains of bacteria originating from animals: Methicillin-resistant *Staphylococcus aureus* (MRSA); A*rcanobacterium pyogenes*; *Arcanobacterium haemolyticum* and *Rhodococcus equi*.

All tested bacterial strains are autochthonous isolates originating from calves. At the same time, with the exception of *Arcanobacterium haemolyticum*, they are a leading cause of infectious animal diseases. All tested bacterial strains are important human pathogens, except of *Rhodococcus equi*, which is rarely identified as a causative agent in humans.

Antimicrobial testing

In a preliminary examination of the modified agar well diffusion method [2,29] we examined the effect of the complex on the autochthonous isolates of *S. aureus*, *A. pyogenes*, *A. haemolyticum* and *R.equi*.

Under the same experimental conditions, we examined the effects of conventional antibiotics streptomycin and vancomycin at the following concentrations: 64, 32, 16, 8, 4, 2, 1, 0.125 and 0.015 mg/L. Parallel testing has favored the complex effects over both examined antibiotics on all strains tested, but at doses lower or equal to 1 mg/L. Analog testing with vancomycin showed that it mostly has better effects than the complex at concentrations higher than 1 mg/L.

MIC corresponded to the lowest concentration of the tested substance that produced a measurable zone of inhibition [2,30]. The limit for the visible growth (MIC) was determined to be below 1 mg/L for all isolates tested. Accordingly, for the microdilution broth method we have chosen the test substance concentrations less than 1 mg/L.

Microdilution broth method for susceptibility testing of bacteria

Antibacterial effects of the Cu (L) Br_2·MeOH were tested by the microdilution broth method pursuant to the 2009 CLSI Guidelines [12]. Modifications to the standard implicated higher concentration of the

used DMF (25%) and quantification of the results was performed by determining the number of surviving bacteria from each experimental microwell and by comparing it with the number of bacteria that were introduced into the experimental well.

Preparation of the target dilutions

DMF was used as a solvent and as a diluent so its concentration could be maintained constant in testing wells [14]. In this way, the only variable component in the system was the concentration of the tested substance. The solvent was used at concentration of 25% due to good solubility of the substance in these experimental conditions. The tested substance was dissolved in DMF to form a series of concentrations from 2500 mg/L (stock solution) to 0.00015 mg/L. Every second dilution was chosen for the testing, starting from 2440 mg/L (working concentration). This concentration is reduced four times by the methodology [12] of the CLSI standard (effective concentration). Consequently, the initial concentration in our experiment was 0.610 mg/L.

Mediums

Thioglycollate broth and plate count agar (Torlak)[2] were prepared according to the manufacturer's instructions. Thioglycollate medium has proven to be better than cationic regulated nutrient broth because it allowed 25% DMF without deposits.

Preparation of the bacterial suspensions (McFarland 0.5)

Bacterial suspension equivalent to 0.5 McFarland was prepared in saline, compared to the standard (Biomerieux)[3] ad oculi. The density was checked spectrophotometrically at a wavelength of 625 nm and adjusted if necessary to the desired absorbance from 0.08 to 0.1 (Agilent 8453 UV-Visible Spectrophotometer)[4]. The resultant suspension was diluted 1:100 and inoculated in microtiter plates. In this way, the inoculum density ranging from 1 to $5x10^5$ organisms/mL of the suspension was provided [11]. Evaluation of the number of formed colonies (colony forming units, CFU) from such a suspension was done by counting colonies on agar surface at dilutions 10^{-4} and 10^{-5} [11].

The procedure of inoculation of the experimental wells:

Microtiter plates with "U" bottom (Spektar)[5] were used in the experiment and inoculated with 20 µL DMF with desired concentration of the test substance, 20 µL of the Thioglycollate broth and 40 µL of the standardized bacterial suspension. Thus, it was provided that every experimental well contained 25% DMF with the test substance, 25% of the thioglycollate and 50% of bacterial suspension of 0.5 McFarland. The microtiter plates inoculated in this way were termostated for 24 h at 36°C.

The modification to the standard that we performed was determined by the nature of the substance. Any attempt to reduce the concentration of DMF led to the sedimentation of substances.

Control tests

- Sterility control test: 20 µL of the test substance solution, 20 µL of the Thioglycollate broth and 40 µL of normal saline (without bacteria).
- Growth inhibition control test: 20 µL of the DMF, 20 µL of the Thioglycollate broth and 40 µL of the bacterial suspension that was used to inoculate the wells (without substance).

As the DMF has antimicrobial properties, the continulus control of inhibition growth was performed to establish whether the inoculum can survive the solvent activity.

After the overnight incubation, 40 µL of each microtiter plate well content was diluted 1:10 and 1:100, and than inoculated onto two 90-mm Petri dishes with bacteria count agar in order to check the number of surviving bacteria [28]. The percentage of surviving bacteria was calculated in terms of the number of bacteria added into the wells. Inhibition control gave an insight into the effect of the DMF solvent on bacterial inoculum.

Cytotoxicity testing

- Cell lines

SW620 (colon, adenocarcinoma, human), HCT116 (colon carcinoma, human) and MRC-5 (human fibroblast) cells were grown in RPMI 1640 medium, supplemented with 10% of fetal calf serum[6] and antibiotics: 100 IU/mL of penicillin and 100 µg/mg of streptomycin[7]. Cell lines were cultured in 25 cm^2 flasks[8], at 37°C in the atmosphere of 100% humidity and 5% of CO_2 (Heraeus)[9]. Exponentially growing viable cells were used throughout the assays.

- MTT assay

Growth inhibition was evaluated by tetrazolium colorimetric MTT assay (Sigma)[1]. The assay is

based on the cleavage of the tetrazolium salt MTT, (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide), to formazan by mitochondrial dehydrogenases in viable cells. Exponentially growing cells were harvested, counted by trypan blue and plated into 96-well microtiter plates (Costar)[8] at optimal seeding density of $5x10^3$ cells per well to assure logarithmic growth rate throughout the assay period [18]. Viable cells were plated in a volume of 90 μL per well, and preincubated in complete medium at 37°C for 24 h to allow cell stabilization prior to the addition of substances. Tested substances, at tenfold of the required final concentration, were added (10 μL/well) to all wells except to the control ones, and microplates were incubated for 48 h. The wells containing cells without tested substances were used as control. Three hours before the end of the incubation period, 10 μL of MTT solution was added to all wells. MTT was dissolved in medium at 5 mg/mL and filtered to sterilize and remove a small amount of insoluble residue present in some batches of MTT. Acid-isopropanol (100 μL of 0.04 N HCl in isopropanol) was added to all wells and mixed thoroughly to dissolve the dark blue crystals. After a few minutes at room temperature, to ensure that all crystals were dissolved, the plates were read on a spectrophotometer plate reader (Multiskan MCC340, Labsystems)[10] at 540/690 nm. The wells without cells containing complete medium and MTT only acted as blank [18].

Inhibition of growth was expressed as a percentage of cytotoxicity and calculated according to the formula: $(1\text{-}A_{test}/A_{control}) \times 100$.

The substance potency was expressed as the IC_{50} (50% inhibitory concentration) [18].

Statistical analysis

The experimental results obtained are presented in Table 1. Based on these results, using the Excel software package (Microsoft Office 2007)[11], graphs were generated that showed the percentage of surviving bacteria depending on the corresponding effective concentrations of the tested substance. The function, which was used to approximate the experimental results, was determined using the Power Trendline supplement from the Microsoft Excel program.

The equation determined using the Power Trendline was:

$$y = c*x\text{^}b \quad (1)$$

where

$$c = EXP (INDEX (LINEST (LN (y), LN (x) ,,) ,1,2)) \quad (2)$$

and

$$b = INDEX (LINEST (LN (y),LN (x),,),1). \quad (3)$$

R2 values were also determined from

$$RSQ = INDEX (LINEST (LN (y), LN (x), TRUE,TRUE), 3,1), \quad (4)$$

and its values indicate that the approximations are accurate.

Approximations were calculated for an increased range of concentrations of the test substance, which enabled determination of the concentration values that were not encompassed in the experiment.

The calculated approximation values for MIC_{80}, MIC_{90}, MIC_{99} and $MBC_{99.9}$ of the tested substance are shown in Table 2. MIC_{80} values implied that 80% of added bacteria were killed (pharmacological MIC, not epidemiological MIC). According to the standard, MIC_{80} and $MBC_{99.9}$ values are considered prominent values of the inhibition (Table 3).

RESULTS

Experimentally obtained results

The percentage of surviving bacteria at selected effective concentrations of test substance ranged from 20% for *Arcanobacterium pyogenes* at concentration of 0.00238281 mg/L to 0.06% for *Arcanobacterium haemolyticum* at the concentration of 0.610 mg/L (Table 1).

The results of the mathematical approximation

$MBC_{99.9}$ and MIC_{99} of the tested substance were lowest for the *Arcanobacterium haemolyticum* being 0.2 mg/L and 0.0054 mg/L, while the highest values were determined for the *Arcanobacterium pyogenes* and *Staphylococcus aureus*, being 488.002 mg/L and 20.2 mg/L, respectively. (Table 2)

Prominent values for MIC and MBC

$MBC_{99.9}$, MIC_{99}, MIC_{90} and MIC_{80} values were determined by mathematical approximation (Table 3 and Figure 2).

Table 1. Percentage of surviving bacteria treated with Cu (L) Br_2·MeOH (experimental data).

Effective concentration of the tested substance [mg / L]	The percentage of surviving bacteria			
	Staphylococcus aureus	*Arcanobacterium pyogenes*	*Arcanobacterium haemolyticum*	*Rhodococcus equi*
0.610	0.434783	2.00000	0.069204	0.277778
0.1525	0.564972	2.85714	0.10000	0.307692
0.038125	1.13636	5.71429	0.288184	1.07527
0.00953125	1.88679	17.6991	0.769231	2.77778
0.00238281	3.44828	20.0000	1.72414	6.15385

Table 2. Percentage of surviving bacteria treated with Cu (L) Br_2·MeOH (mathematical interpolation and extrapolation).

Effective concentration of the tested substance [mg / L]	The percentage of surviving bacteria*			
	Staphylococcus aureus	*Arcanobacterium pyogenes*	*Arcanobacterium haemolyticum*	*Rhodococcus equi*
488.002		0.1 ($MBC_{99.9}$)		
20.2895	0.1 ($MBC_{99.9}$)			
2.36378		1 (MIC_{99})		
1.98045				0.1 ($MBC_{99.9}$)
0.236134			0.1 ($MBC_{99.9}$)	
0.0520712	1 (MIC_{99})			
0.0443199				1 (MIC_{99})
0.0114496		10 (MIC_{90})		
0.00545128			1 (MIC_{99})	
0.00230125		20 (MIC_{80})		
0.000991822				10 (MIC_{90})
0,000315998				20 (MIC_{80})
0.000133636	10 (MIC_{90})			
0.000125845			10 (MIC_{90})	
0,0000404718			20 (MIC_{80})	
0,0000221847	20 (MIC_{80})			
approximation	$y = 0.3196 \cdot x^{-0.386}$	$y = 1.4501 \cdot x^{-0.432}$	$y = 0.0414 \cdot x^{-0.611}$	$y = 0.1513 \cdot x^{-0.606}$
R^2	0.9859	0.9905	0.9826	0.9605

*The percentage of the surviving bacteria at appropriate effective concentrantions of the Cu (L) Br_2 MeOH based on the analytical data obtained; approximated functions and the accuracy of the aproximations are provided in the last two rows of the table.

Table 3. Prominent values for MIC and MBC of Cu (L) Br_2·MeOH in mg/L and mmol /L for treated bacteria (mathematical interpolation and extrapolation).

Bacteria species	$MBC_{99.9}$		MIC_{99}		MIC_{90}		MIC_{80}	
	[mg / L]	[mmol / L]	[mg / L]	[mmol / L]	[mg / L]	[mmol / L]	[mg / L]	[mmol / L]
Staphylococcus Aureus	20.2895	0.0259899	0.0520712	0.000066700655	0.000133636	0.0000001711811	0.0000221847	0.00000002841
Arcanobacterium Pyogenes	488.002	0.6251066	2.36378	0.00302788	0.0114496	0.000146664	0.00230125	0.00000294778
Arcanobacterium Haemolyticum	0.236134	0.0003024761	0.00545128	0.0000069828224	0.000125845	0,000000161201	0.0000404718	0.00000005184
Rhodococcus Equi	1.98045	0.00253686	0.0443199	0.0005677162	0.000991822	0.00000127048	0.000315998	0.000000404777

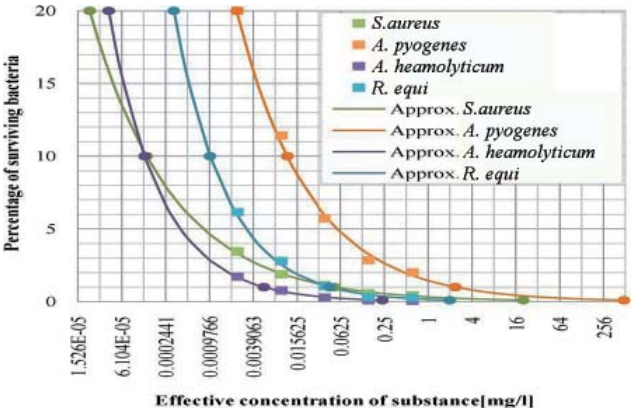

Figure 2. Comparative review of the experimental and approximative effect values of the Cu (L) Br2·MeOH on four bacterial isolates.

The results of cytotoxicity

After the completion of synthesis, compound was also evaluated for its *in vitro* cytotoxic activity against two human tumor cell lines, and against normal lung fibroblasts (MRC-5). Cytotoxic activity was evaluated using the standard MTT assay, after exposure of cells to the tested compound for 48 h Cytotoxicity (%) of compound Cu (L) Br_2·MeOH against human colon carcinoma cell lines (HCT116 and SW620) and normal human lung fibroblasts (MRC5). Cells were treated with Cu (L) Br2·MeOH in a concentration range from 10^{-8} M to 10^{-4} M presented in the lower part of the graph (Figure 3). Cytotoxicity was evaluated after 48 h by MTT assay. Each point represents a mean value of quadruplicate from two independent experiments.

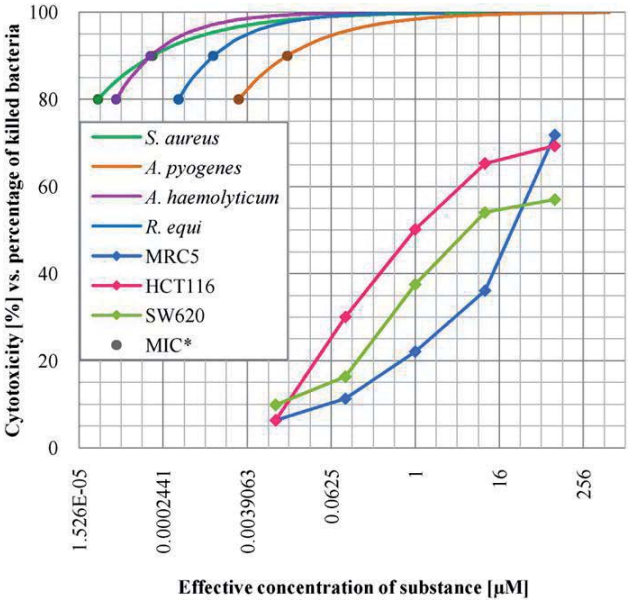

Figure 3. Comparative review of bacteriostatic and cytotoxic effect of the Cu (L) Br_2·MeOH.

Comparative review of bacteriostatic and cytotoxic effect of the tested substances

Concentrations that cause the MIC_{80} were compared to the ones that cause cytotoxicity in the same graph (Figure 3). In the upper part of the graph, the values of MIC_{80} and MIC_{90} are highlighted for all four species tested. At doses below 1 μM, e.g. 0.001 mmol / L, the test substances express moderate or low cytotoxic effect on normal human cells. Bacteriostatic concentrations (MIC80) are not in domain of cytotoxicity for normal human cells. Obviously, the MIC_{80} belongs to the doses lower than toxic and MIC_{90}, for *Arcanobacterium pyogenes* only, belongs to the low dose of toxicity (less than 0.0625 μM).

The IC50 values the compound Cu (L) Br2·MeOH

IC_{50} is the concentration (μM) of compound required to inhibit the cell growth by 50% compared to an untreated control. Values are means of two independent experiments. Coefficients of variation were less than 10%.

According to the IC_{50} values, the compound Cu (L) Br_2·MeOH was 6.4-fold and 4.8-fold more potent against HCT116 cells compared to normal lung fibroblasts and SW620 cells, respectively (Figure 4).

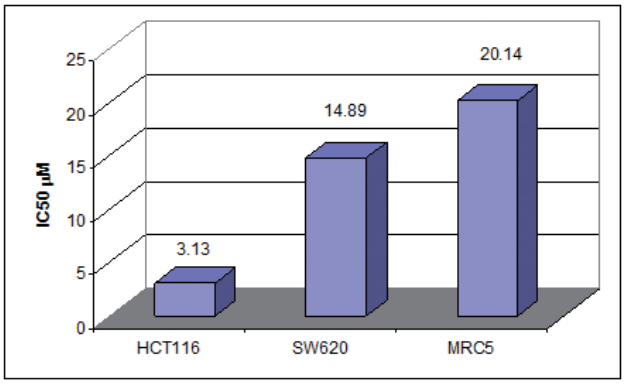

Figure 4. Cu (L) Br_2·MeOH IC50 value.

DISCUSSION

MIC_{80} and MIC_{90} values obtained in our research were in nanograms/L and micrograms/L. MIC_{99} values were expressed in micrograms, except for the *A. pyogenes*, which had a value of 2.36378 mg/L. $MBC_{99.9}$ values ranged from 0.236134 mg/L to 488.002 mg/L (Tables 2 and 3). Even the most resistant species had MIC_{99} of 2.36 mg/L, and MIC_{80} of 0.00230 mg/L. The relations between MIC_{80} and $MBC_{99.9}$ values are much more than four-fold (even more than 100 times!). Such an extremely large range between MIC_{80} and $MBC_{99.9}$ indicates a bacteriostatic effect of the metal complex that we examined (Table 2, Figure 2).

The authors apply various methods for reading MIC values of synthesized compounds. Because of different interpretation of the term MIC (microbiological, epidemiological, and pharmacological) in the literature, the researchers should precisely define what MIC is and how it is determined (*ad oculi*, spectrophotometrically, ELISA reader in dilution method or lack of visible growth) to ensure the comparability of the results. Even if CFU counting is not compulsory according to relevant standards, in case of working with an unknown substance, MIC should be quantified by counting CFU because of better accuracy. Qualifications like "blurring" and "visible growth" relativize the results. In addition, growth quantification permits the application of mathematical models that provide valuable information about the exact values that are important and were not obtained by experiment. The values of MIC_{80}, MIC_{90}, MIC_{99} and $MBC_{99.9}$ that we obtained, are the result of a precise method of counting the surviving bacteria on the plate and mathematical approximation (Table 2). In determining the absorbance, the dead bacteria also contribute to the result. Contrary to that, by counting CFU, the dead bacteria do not influence the result because only living bacteria can form a visible colony [10, 28].

In our research, the methicillin-resistant *Staphylococcus aureus* had the lowest MIC*80* value (0.000022 mg/L). The next one is *Arcanobacterium haemolyticum* (0.000041 mg/L), then *Rhodococcus equi* (0.000315 mg/L) and finally *Arcanobacterium pyogenes* (0.002301 mg/L) [Tables 2 and 3]. All other researchers reported significantly higher values for the MIC_{80} of the substances they examined.

Some authors tested enhydrin, polymatin B, allo-schkuhriolide from the leaves of *Smallanthus sonchifolius* against 2 referent strains of MRSA (ATCC 33591, ATCC 25923) and 15 autochthonous strains of clinical isolates MRSA using the microdilution broth method. We used this same method in our research. In their research, enhydrin appeared to be the only compound that showed antibacterial activity, with MICs ranging from 125 to 500 mg/L [4]. In our research, the MIC_{80} value was 0.000022 mg/L.

The group of authors, who investigated copper (II) and manganese (II) metal complexes activity

on the methicillin-resistant *Staphylococcus aureus* (MRSA), found a MIC_{80} of 12,1 μM [6], which is a much higher level than our results [Table 3]. The authors defined MIC_{80} as the lowest concentration of the investigated substances that inhibit the growth of 80% of the initial bacterial inoculum, as we did. The same group of authors [7] investigated the Ag (I) complex hydroxycoumarin-carboxylate and established a MIC_{80} value 0.63 mg/L for MRSA, which is also a much higher level than our result [Table 2].

The results reported by other authors investigating metal complexes of various organic compounds have been less attractive than the results that we obtained for the MIC_{80}.

Antimicrobial effects of the metal complexes (Cu, Ni, Co) investigated by the group of researchers [25] resulted in MIC_{80} values ranging from 1200 mg/L for *Staphylococcus aureus* to 2000 mg/L for the *Escherichia coli*. The authors defined the minimum inhibitory concentration as the lowest concentration of the ligand and its complexes that causes a reduction in absorbance by 80% (MIC_{80}) compared to control (without tested substance). The same group of scientists studied the effects of metal complexes (Cu, Ni, Co) of the bacteria using the same reading technique and obtained the lowest MIC_{80} value for *Pseudomonas aeruginosa* (90 mg/L) and highest value for *Escherichia coli* (400 mg/L) [26] which is much higher than our results.

MIC_{80} values for Cu(II) complex of pyrazolic ligand of all isolates that we tested are also much lower than the acceptable values for the MIC of standard antibiotics: for vancomycin from 2 to 16 mg/L, for penicillin from 0.12 to 0.25 mg/L and for oxacillin from 2 to 4 mg/L for reference strain *Staphylococcus aureus* ATCC 29213 [13]. In our study, we obtained a MIC_{80} value for the metal complex for *Staphylococcus aureus* 0.00002 mg/L (Table 2).

It should be pointed out that our MIC_{99} values obtained for *Arcanobacterium pyogenes* 2.364 mg/L, methicillin-resistant *Staphylococcus aureus* 0.0520712 mg/L, *Rhodococcus equi* 0.0443199 mg/L and *Arcanobacterium haemolyticum* 0.00545128 mg/L [Table 2] were more attractive as compared to the results reported by other researchers.

Some recent research [8] of antimicrobial activity of newly synthesized compounds without metal complexes, using broth microdilution tests, encompassed the following microorganisms: *E. coli*, *S. aureus*, *Pseudomonas aeruginosa* and *Streptococcus pyogenes*. The lowest concentration of a substance that led to at least 99% inhibition of growth of tested microorganisms was defined as MIC_{99}. Based on the results of the aforementioned paper, the most effective compound had a MIC_{99} 62.5 mg/L for *E. coli*, whilst the least effective compound had a MIC99 of 1000 mg/L for *S. aureus* and *S. pyogenes*. The MIC_{99} value for *S. aureus* obtained in our research was 0.0520712 mg/L.

The $MBC_{99.9}$ values we obtained for Cu(II) complex are as following: 0.236 mg/L for *Arcanobacterium haemolyticum*, 1.980 mg/L for *Rhodococcus equi*, 20.2895 mg/L for methicillin resistant *Staphylococcus aureus*, and 488.002 mg/L for *Arcanobacterium pyogenes* (Table 2).

Researchers, who applied a methodology most similar to that employed in our study [23], defined the minimum bactericidal concentration (MBC) as the concentration that led to a 99.9% reduction in CFU compared to the initial inoculum, which was determined by subculturing a part of the well-content onto the nutrient agar, as we have done. The authors obtained the $MBC_{99.9}$ value for all tested compounds (5-amino-8-hydroxy-1, 4-naphthoquinone, Naphthazarin, 5-acetamido-8-hydroxy-1, 4-naphthoquinone, and 2,3-diamino-1, 4-naphthoquinone) for all tested bacteria greater than 500 mg/L [23]. Similarly, they tested the antimicrobial activity of the newly synthesized compounds (lapachol and its derivatives) on the clinical isolates from patients blood with: methicillin resistant *Staphylococcus epidermidis* 228 and *Staphylococcus haemolyticus* 225 and on standard strains of *S. aureus* ATCC 29213 (methicillin-sensitive *Staphylococcus aureus*) and ATCC 33591 (methicillin-resistant *Staphylococcus aureus*), by the use of dilution broth method [21]. They obtained MBC99.9 values for all compounds tested on all bacteria with concentrations greater than 512 mg/L.

Better results were obtained by testing the antimicrobial activity of thiazole/benzothiazole sulfonamide in vitro on the panel of selected Gram positive and Gram-negative bacteria using the broth dilution method. In all cases, the values obtained for $MBC_{99.9}$ were equal to or greater than 100 mg/L [1].

Even better results were obtained for *in vitro* antimicrobial activity of the newly synthesized compounds dissolved in DMF at a concentration of 1 mg/L with the broth dilution method on reference strains of *Staphylo-*

coccus aureus (ATCC 6538), *Escherichia coli* (ATCC 8735), and *Pseudomonas aeruginosa* (ATCC 9027) [27]. $MBC_{99.9}$ values obtained for the tested compounds were in the range from 62.5 to 125 mg/L.

The best results were obtained on the azomethine derivatives. The $MBC_{99.9}$ values determined using broth dilution method were in the range from 6.25 to 100 mg/L [15].

In our experiment, the MBC99.9 for *Staphylococcus aureus* was 20.2895 mg/L, which is comparable with the listed results. For other bacteria we examined, we did not find a comparable report in the available literature. For *Arcanobacterium pyogenes*, $MBC_{99.9}$ was 488.002 mg/L, which is an acceptable result. For *Rhodococcus equi* MBC99.9 was 1.980 mg/L and for *Arcanobacterium haemolyticum*, $MBC_{99.9}$ was 0.236 mg/L, which are excellent results.

Cytotoxicity of compound Cu (L) Br_2·MeOH towards human tumor colon carcinoma, HCT116 and SW620, and towards normal lung fibroblasts (MRC5) was dose-dependent. The tested compound was the most active against HCT116 cells in the range of concentrations from 0.1 μM to 10 μM and 100 μM compared to MRC5 and SW620 cells, respectively (Figure 3).

Different sensitivity of cancer cell lines originating from the same tissue (human colon) reflects their inherent cell biological characteristics and metabolic activity. However, compound Cu(L)Br_2·MeOH induced moderate to high cytotoxicity towards normal human cells, MRC5, at concentrations above 1 μM, e.g. 0.001 mmol/L [18].

Cu(L)Br_2·MeOH was moderately to highly cytotoxic for normal human cells only above 1 μmol/L. The values obtained for MIC80 for all four tested bacteria, expressed in mmol/L, were lower than 1 μmol/L, moreover, were less than 0.0039 μmol/L (Figure 3). MIC_{99} for all tested bacteria, except for *Arcanobacterium pyogenes*, was lower than the concentrations of cytotoxic compounds, which provided the changes in normal human cells (Table 3). Unfortunately, the compound was cytotoxic for normal human cells at bactericidal concentrations. Only *Arcanobacterium haemolyticum* had $MBC_{99.9}$ value 0.0003024761 mmol/L that is out of cytotoxicity range.

Considering the obtained results, we believe that further investigation of pharmacological properties of Cu (II) complexes of pyrazolic ligand would be justified. Comparing the results we obtained examining the antibacterial and cytotoxic activity of copper complexes we can conclude that the investigated substance is not toxic for normal human cells at concentrations at which it is bacteriostatic.

CONCLUSIONS

In our results, MIC_{80} and MIC_{90} values were in nanogram/L and microgram/L, so we can conclude that copper (II) complex with an arylpyrazole ligand exhibits strong antibacterial properties, and it shows bacteriostatic effect at concentrations that do not produce any cytotoxic effect in normal human cells.

SOURCES AND MANUFACTURERS

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