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Synthesis and characterization of new copper(II) complex compounds with chlorhexidine. Part I

Research Article

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Abstract: Three new copper(II) complex compounds with chlorhexidine diacetate as a ligand have been prepared and characterized by elemental and thermogravimetrical analyses, molar conductances, magnetic susceptibility measurements, infrared, electronic and EPR spectra. The complexes correspond to the formulas: $[Cu_2(CHX)Cl_4]\cdot 2C_2H_5OH$, $[Cu_2(CHX)Br_4]\cdot 2C_2H_5OH$ and $[Cu_2(CHX)(CH_3COO)_2](CH_3COO)_2\cdot 2C_2H_5OH$, where CHX = chlorhexidine, their composition and stereochemistry depending on the reaction conditions and the metal salt used. Chlorhexidine acts as neutral tetradentate NNNN donor, coordinating through the four imine nitrogen atoms. Investigations on antimicrobial activity *in vitro* show that all the complexes are active against the tested microorganisms, the complex with chloride being more active against Gram negative bacteria than chlorhexidine diacetate..

Keywords: *Biguanides • Chlorhexidine • Copper(II) complexes*

1. Introduction

The biological activity of biguanides and their metal complexes is well known and intensely studied, some of them being used as antihyperglycemic, antimalarial, antibacterial and antifungal agents [1-4].

The most used antiseptic of the biguanide class is chlorhexidine, 1,1'-hexamethylene-bis-[5-(*p*-chlorophenyl)-biguanide] (Fig. 1), its mechanism of antimicrobial action being probably the disruption of the microbial membrane [5-7]. At relatively low concentrations, the action of chlorhexidine is bacteriostatic, whilst at higher concentrations its action is bactericidal.

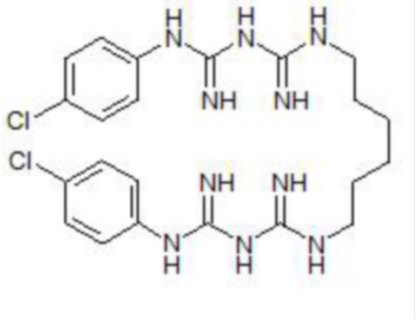

Figure 1. Chlorhexidine (CHX)

The activity spectrum of chlorhexidine and its salts (diacetate, digluconate and dichloride) is very broad, but they are more effective against Gram positive bacteria, such as *Mutans streptococcus* and for this reason they are the most widely used agents against dental plaque [6,7].

Chlorhexidine is also used in non-dental applications: for general skin cleaning, as a surgical scrub and for pre-operative skin preparation [5].

On the other hand, several metal ions, especially Cu^{2+}, Zn^{2+}, Sn^{2+} or Ag^{+} have shown their effectiveness against many harmful microorganisms [8-10]. Moreover, many studies have found a potentiation of antimicrobial activity when antiseptics were combined with some metal ions [11,12].

Although chlorhexidine is widely used as an antiseptic for the skin and oral cavity, a series of side effects such as discoloration of teeth, tongue, restorations and dentures, soreness of the oral mucosa and temporary taste disturbances have been observed.

For limiting the side effects of chlorhexidine, without reducing the bacteriostatic action, we have proposed the synthesis, characterization and determination of the antimicrobial activity of some complexes obtained from chlorhexidine diacetate and different copper(II) salts.

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The present paper reports the preparation, analytical and spectral characterization and *in vitro* investigations of antimicrobial activity of three complex compounds of chlorhexidine diacetate with copper(II) chloride, bromide and acetate, respectively.

2. Experimental Procedure

2.1. Preparation of the complexes

$[Cu_2(CHX)Cl_4]\bullet 2C_2H_5OH$ (**1**): Chlorhexidine diacetate monohydrate (0,6435 g, 1 mmol) were dissolved in 30 mL ethanol; with stirring. Subsequently, $CuCl_2\bullet 2H_2O$ (0.3408 g, 2 mmol) was added into the solution and was stirred at 35-40°C for 3 h. The blue precipitate was filtered off, washed with ethanol and dried in air.

$[Cu_2(CHX)Br_4]\bullet 2C_2H_5OH$ (**2**): $CuBr_2$ (0.4480 g, 2 mmol) were dissolved in a minimum amount of ethanol and was added dropwise to a solution of chlorhexidine diacetate (0.6435 g, 1 mmol); while stirring. The resulting mixture was stirred at room temperature for 3 h. The green precipitate was filtered off, washed with ethanol and dried in air.

$[Cu_2(CHX)(CH_3COO)_2](CH_3COO)_2\bullet 2C_2H_5OH$ (**3**) was prepared in the same way as the complex (**2**), using chlorhexidine diacetate monohydrate (0.6435 g, 1 mmol) and $Cu(CH_3COO)_2\bullet H_2O$ (0.4000 g, 2 mmol). The violet precipitate was filtered off, washed with ethanol and dried in air.

All the complex compounds are soluble in DMF and DMSO and partly soluble in acetone.

2.2. Physico-chemical analyses

The purity of the hydrazones and their complexes was confirmed by C, H and N analyses, using a Carlo Erba 1180 analyzer. The copper content was determined by standard procedure [13]. Thermogravimetric analysis was carried out in static air atmosphere, at a heating rate of 10°C/min, using a LABSYS 1200 SETARAM derivatograph. Molar conductance measurements were made on a Consort C-533 conductometer. Infrared spectra (in KBr pellets) were recorded on a BIORAD FTIR 135 spectrophotometer, in the range 4000-400 cm^{-1}. UV-VIS diffuse reflectance spectra were measured on a UV-VIS Jasco 650 spectrophotometer, in the range 200-900 nm. The EPR spectra were recorded on a MiniScope MS 200 spectrophotometer, on powdered samples, in X band. Magnetic susceptibility measurements were performed by the Faraday method, on a Gouy balance at room temperature, using $Hg[Co(SCN)_4]$ as calibrant.

Elemental analysis, molar conductance and yield (η) :

$[Cu_2(CHX)Cl_4]\bullet 2C_2H_5OH$ (**1**), blue ; exp.%: C: 35.72; H: 4.65; N: 16.08; Cu: 14.34; calc.%: C: 35.96; H: 4.84; N: 16.14; Cu: 14.75. λ_M (in DMF) = 43 $\Omega^{-1}cm^2mol^{-1}$; η = 70%.

$[Cu_2(CHX)Br_4]\bullet 2C_2H_5OH$ (**2**), green; exp.%: C: 29.52; H: 4.15; N: 13.08; Cu: 11.95; calc.%: C: 29.84; H: 4.01; N: 13.39; Cu: 12.24. λ_M (in DMF) = 45 $\Omega^{-1}cm^2mol^{-1}$; η = 92%.

$[Cu_2(CHX)(CH_3COO)_2](CH_3COO)_2\bullet 2C_2H_5OH$ (**3**), violet; C: 42.22; H: 5.65; N: 14.28; Cu: 13.20; calc.%: C: 42.43; H: 5.61; N: 14.56; Cu: 13.31. λ_M (in DMF) = 95 $\Omega^{-1}cm^2mol^{-1}$; η = 63%.

3. Results and Discussion

3.1. Infrared spectra

The assignments of some important infrared bands of the chlorhexidine diacetate monohidrate and its copper(II) complexes are listed in Table 1.

The IR spectrum of the solid chlorhexidine diacetate monohydrate shows, in the range of 2900-3400 cm^{-1}, a medium absorption band, at 3326 cm^{-1}, due to the stretching vibrations N-H of the groups Alkyl-NH-Aryl and $(Alkyl)_2NH$ and another band, at 3180 cm^{-1}, due to the stretching vibration of the group =NH [1,14,15]. A ν(OH) absorption band, appearing in the same region, overlaps with the N-H vibration, leading to the enlargement of the band at 3326 cm^{-1}.

The symmetric and asymmetric N-H stretching modes of NH_2^+ group give a medium absorption band at 3140 cm^{-1}, while the deformation vibration band of the same bonding appears at 1613 cm^{-1} [14,16]. The presence of these two bands indicates the protonation of the chlorhexidine in its diacetate salt, in solid state.

The strong band, with a maximum at 1644 cm^{-1} may be assigned to the stretching vibration of the imine function, ν(C=N) [14,17]. Its position and intensity are of great importance in justifying the coordination of chlorhexidine to the copper ion.

Other characteristic bands, at 1574 and 1337 cm^{-1}, due to δ(NH) + ν(C-N) and at 1249 cm^{-1}, assigned to the stretching vibration C_{aliph}-N [1,15] are also affected by coordination.

The IR spectrum of the chlorhexidine diacetate ligand also shows bands due to the stretching vibrations of the acetate group: 1536 cm^{-1} - ν_{as}(COO) and 1417 cm^{-1} - ν_{sym}(COO) [18].

In the IR spectra of the three complexes, the band assigned to ν(C=N) shows a positive shift, indicating coordination of the imine nitrogen atoms to the metal ion [1,14,16]. This supposition is also supported by the upward shift of the band due to δ(NH) + ν(C-N) and the downward shift of the band assigned to C_{aliph}-N. Although the posibility of coordination through amino nitrogens also exists, many studies on complex compounds with biguanides have shown

Table 1. Characteristic bands in the IR spectra of the ligand and its copper(II) complexes (ν_{max}, cm^{-1})

Assignments	Ligand	Complex (1)	Complex (2)	Complex (3)
ν(OH) H_2O/C_2H_5OH	3338 m	3457 s	3448 s	3270 m
ν(NH) Alkyl-NH-Aryl $(Alkyl)_2NH$	3326 m	3336 s	3332 m	3243 m
ν(=NH)	3180 m	3284 s	3282 s	~3200 s
ν_{as} (NH_2^+) ν_{sym} (NH_2^+)	3140 m	-	-	-
ν(C=N)	1644 s	1671 vs	1667 vs	1651 s
$\delta(NH_2^+)$	1613 m	-	-	-
δ(NH) + ν(C-N)	1574 s 1337 m	1596 m 1341 m	1593 m 1340 m	1586 m 1335 m
$\nu(C=C)_{arom.}$	1550 s 1487 vs	1555 s 1493 m	1551 vs 1491 s	1556 s 1489 s
ν_{as}(COO)	1536 m	-	-	1560 s 1530 s
ν_{sym}(COO)	1417 s	-	-	1403 vs
$\nu(C_{aliph}$-N)	1249 m	1231 m	1232 m	1238 m
ν(Cu-N)	-	629 w	613 m	610 w
ν(Cu-O)	-	-	-	665 w

that the metal-N (imino) formation takes priority, probably due to the increase of stability by π-conjugation [19,20].

The changes occurring in the IR spectra of the complexes in the region 3000-3350 cm^{-1}, the most important being the disappearance of the band due to $\delta(NH_2^+)$ are in accordance with the deprotonation of the chlorhexidine and involvment in complexation as neutral ligand [14].

Supplementary bands appearing in the IR spectra of the complexes at low wavenumbers may be assigned to the copper-nitrogen: ν(M-N) (610-629 cm^{-1}) and copper-oxygen vibrations (from acetate, only for the complex **3**): ν(M-O) (665 cm^{-1}) [15].

The strong absorption bands due to the vibrations of the acetate group are present only in the spectrum of the complex (**3**): two maxima for ν_{as}(COO) at 1560 and 1530 cm^{-1} and only a maximum, at 1403 cm^{-1} for ν_{sym}(COO). The values of $\Delta = \nu_{as}(COO) - \nu_{sym}(COO)$, 157 cm^{-1} and 127 cm^{-1} are in the range of ionic and bidentate acetate ligand, respectively [18].

The free ν(OH) band is observed in the IR spectra of the three complexes between 3200-3457 cm^{-1} and it is due to the presence of one or two molecules of ethanol, as we can establish from the thermal analysis [1,14,15].

3.2. Thermogravimetric analysis

The observation of the TG curves of the three complexes indicates that their decomposition started at 50°C and was completed at 800°C. The first weight loss between 50 and 85°C corresponds to the elimination of the ethanolic alcohol, which is in agreement with the results obtained from the IR spectra. Futher weight loss continued with elimination of chloro benzene, resulting from the decomposition of the chlorhexidine (85-170°C), elimination of the coordinated chloride (complex 1), coordinated bromide (complex 2) and ionic and coordinated acetate (complex 3) at 170-400°C and finally removal of the remainder of chlorhexidine between 400 and 800°C [21,22].

Based on the analytical, IR and thermogravimetrical analyses we can conclude on the composition of the complex compounds and on the behaviour of the chlorhexidine ligand.

Thus, the elemental analysis shows that copper(II) complexes with chlorhexidine are in 2:1 metal:ligand ratio. Chlorhexidine acts as neutral tetradentate NNNN donor ligand in all the three complexes. The complex **3** contains ionic and bidentate acetate. Molar conductances determined in DMF solutions 10^{-4} M indicate a non-electrolytic nature for the complexes (**1**) and (**2**) and 1:2 electrolyte for the complex (**3**), in accordance with the proposed formulas.

3.3. Magnetic and electronic spectral data

The magnetic moment has lower values for all the complexes: 1.43 MB for the complex (**1**), 1.50 MB for the

complex (**2**) and 1.45 MB for the complex (**3**), indicating metal-metal interactions and supporting their dinuclear dimeric structure [23].

The symmetry of coordination polyhedron was established by means of the electronic and EPR spectral studies. For all the compounds, the electronic spectra were recorded in solid state.

The chlorhexidine diacetate exhibits absorption bands in the UV region, at 208 nm (48100 cm^{-1}), 255 nm (39215 cm^{-1}), 298 nm (33500 cm^{-1}) and 344 nm (29000 cm^{-1}), which can be assigned to n - σ^*, π - π^* and n - π^* transitions, respectively [24]. The electronic spectra of the copper(II) complexes show, in the UV region, the same characteristic bands of the ligand. There is a slight shift of these bands during the formation of the complexes.

In the visible region, the complexes exhibit a broad, structureless band, with or without shoulders, due to the *d-d* transitions of Cu^{2+} ion.

The bands observed in the ultraviolet-visible spectrum of the complex (**1**) are presented in Fig. 2.

The strong bands with maxima at 213, 259, 289 and 330 nm are due to n - σ^*, π - π^* and n - π^* transitions from chlorhexidine ligand, while the maximum and the shoulder in the visible region may be assigned to the following *d-d* transitions, assuming a D_{4h} symmetry [25]:

663 nm (15080 cm^{-1}) ($^2B_{1g} \rightarrow {}^2E_g$) and 850 nm (11760 cm^{-1}) ($^2B_{1g} \rightarrow {}^2B_{2g}$).

The EPR spectrum of this complex, recorded on a powdered sample, in X band, shows axial symmetry, with $g_{||} > g_{\perp} : g_{//} = 2.202$ and $g_{\perp} = 2.058$ (Fig. 3). This trend in the values of the experimental EPR parameters is in accordance with the planar square geometry proposed for the complex [26].

The values of the bonding parameters, $K_{||} = 0.37$ and $K_{\perp} = 0.52$ indicate an important covalent character for the metal-

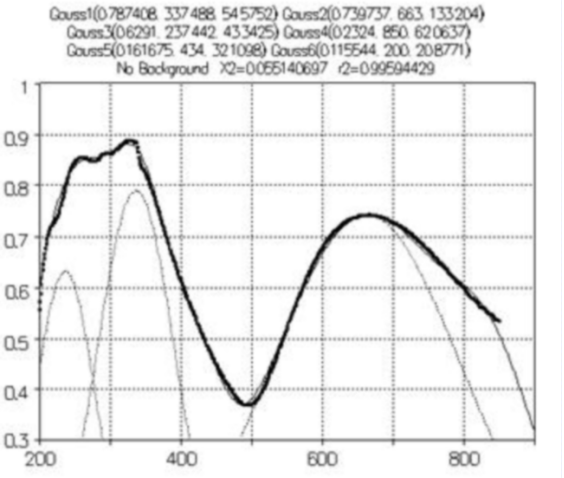


Figure 2. UV-VIS diffuse reflectance spectrum of the complex $[Cu_2(CHX)Cl_4]\cdot 2C_2H_5OH$ (1)

observed in the visible region (Fig. 4) may be assigned to the transitions: 2B2R2B1 (15210 cm-

For the complex (**2**), the EPR spectrum shows an isotropic signal, with $g_{iso} = 2.088$. In such conditions, the maxima observed in the visible region (Fig. 4) may be assigned to the transitions: 2B2→2B1 (15210 cm^{-1}/657 nm) and 2B2→2E (11760 cm-1/850 nm) in a distorted tetrahedral D2d symmetry [25].

Another strong band, at 418 nm (23920 cm^{-1}) is a ligand ; metal charge transfer band (LMCT) [25]. The maxima observed in the ultraviolet region (Fig. 5) are those characteristic of the chlorhexidine ligand.

In the case of the complex (**3**), because the analytical data indicate a tetracoordination around the copper ion and the EPR spectrum gives an isotropic signal with $g_{iso} = 2.086$ at room temperature and $g_{iso} = 2.087$ at 77 K, we can also interpret the electronic transitions in terms

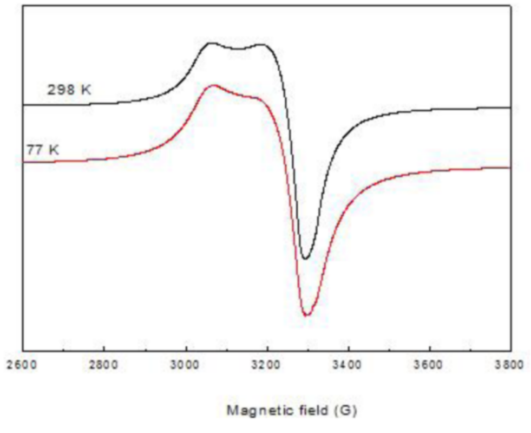


Figure 3. X-band EPR spectrum of the complex (1) at room temperature and at 77 K

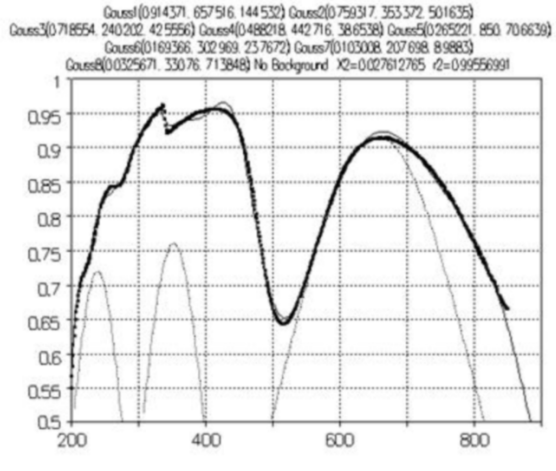


Figure 4. UV-VIS diffuse reflectance spectrum of the complex $[Cu_2(CHX)Br_4]\cdot 2C_2H_5OH$ (2)

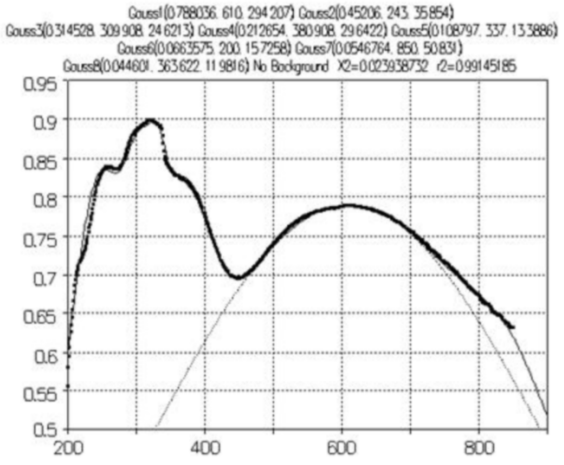


Figure 5. UV-VIS diffuse reflectance spectrum of the complex $[Cu_2(CHX)(CH_3COO)_2](CH_3COO)_2 \cdot 2C_2H_5OH$ (3)

of a distorted tetrahedral symmetry.

Thus, the absorption maximum at 610 nm (16380 cm^{-1}) and the shoulder at 845 nm (11800 cm^{-1}) in the visible region may be assigned to the transitions: $^2B_2 \rightarrow {}^2B_1$ and $^2B_2 \rightarrow {}^2E$, respectively [25].

The differences attributed for the symmetry of the complexes, despite the same tetracoordination of the copper(II) ion, may be explained by the size of the halide and acetate ligands; the square planar symmetry is achieved only in the complex with chloride, while the bulky bromide and acetate ligands determine the distortion from the planar symmetry and favor the tetrahedral symmetry.

According to the presented results, the following structures are proposed for the copper(II) complexes (Fig. 6).

3.4. Biological activity

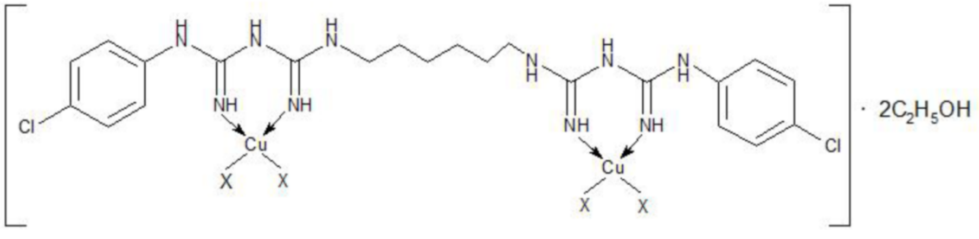
Agar disc diffusion method was adopted to evaluate the antibacterial and antifungal activity of the ligand and its copper(II) complexes against four bacterial and one fungal cultures [28-30].

3.4.1.Preparation of agar plates.

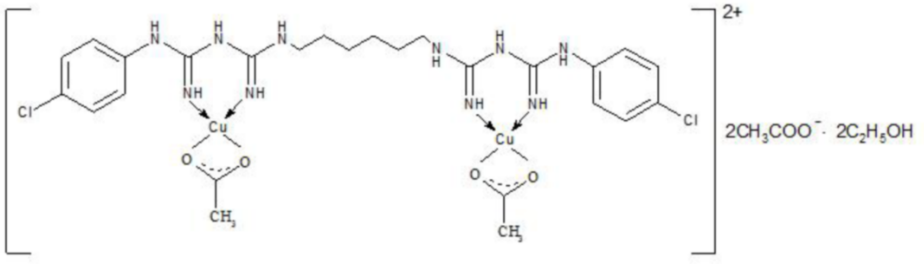
Agar plates for antibacterial activity were prepared by poured melted sterile Mueller Hinton agar medium into sterile petri plates (100 mm) and allowed to solidify. Agar plate for antifungal activity was prepared in the same way, using Sabouraud dextrose as Nutrient agar medium.

3.4.2. Organism cultures.

Antibacterial activity was tested against three Gram negative bacteria (*E. coli,* from a urine sample, *Pseudomonas aeruginosa serotype IV,* from sewage water samples, *Klebsiella pneumoniae,* from a saliva sample) and one Gram positive bacteria (*Staphylococcus*



(1) X = Cl; (2) X = Br



(3)

Figure 6. Proposed structures for the copper(II) complexes

Table 2. Biological activity for the chlorhexidine diacetate and Cu(II) complexes
A - ampicillin (10 μg/disc); C - ciprofloxacin (5 μg/disc); M - miconazol (30 μg/disc).

Microbial strain		Microbial culture inhibition diameter [mm]						
		A	C	M	Ligand	(1)	(2)	(3)
Gram negative bacteria	Escherichia coli	18	28	-	10	17	10	9
	Pseudomonas aeruginosa serotip VI	-	26	-	10	15	11	10
	Klebsiella pneumoniae	-	20	-	10	15	11	10
Gram positive bacteria	Staphyloccocus aureus	28	25	-	24	25	16	15
Fungi	Candida albicans	-	-	21	24	20	18	17

aureus, from a pharyngeal exudate). Antifungal activity was tested against *Candida albicans*, collected from a pharyngeal exudate. Each culture was streaked onto a non inhibitory agar medium (blood agar), to obtain isolated colonies. After incubation overnight 4-5 colonies were transferred with an inoculation loop to a tube of nonselective broth and vortexed. The turbidity of the bacterial suspensions was measured and adjusted to 0.5 McFarland units. The bacterial and fungal cultures were then uniformly streaked over the surface of the agar medium contained in Petri dishes.

3.4.3. Antibacterial screening.

A filter disc (Φ = 6 mm) was impregnated with 10 μL solution 10^{-3} M of the ligand or complex in acetone and than placed on an agar surface inoculated with bacterial and fungal cultures. At this concentration all the three complexes are soluble in acetone. Because the molecular weight of the complexes is between 867 and 1045, the amount of the compound/disc was 8.6-10.4 μg/disc. For the ligand, the amount was 6.4 μg/disc. Acetone was used to dissolve the compounds due to its fast evaporation, so that the effect on microbial culture is only due to the tested compound. The plates were incubated at 37°C, for 24 h for the bacteria and 72 h for the fungi.

The diameter of the zone of inhibition was then measured and compared with the standard antibiotics (ampicillin and ciprofloxacin for bacteria and miconazol for fungi).

The results are presented in Table 2.

Since chlorhexidine is a cationic agent, it is more active against Gram positive bacteria than Gram negative bacteria. Among the three complexes, complex (**1**) shows a significant increase of biological activity compared to chlorhexidine diacetate against Gram negative bacteria, but both chlorhexidine and this complex are less active against Gram negative bacteria than standard antibiotics. Against Gram positive bacteria and fungi, their activity is comparable with that of standard antibiotics and miconazol, respectively. The observed differences between the biological activity of the complexes will be explained after the testing of a larger number of Cu(II) complexes with chlorhexidine.

4. Conclusion

We have prepared three new copper(II) complex compounds with chlorhexidine diacetate, using copper(II) chloride, bromide and acetate, respectively. The screening data for the inhibition diameter of microbial culture show an increase of activity for the complex containing chloride against Gram negative bacteria comparatively with chlorhexidine diacetate.

We recommend using these copper complexes for the disinfection of metallic surfaces and medical instruments.

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