Synthesis, Characterization and Biological Activity of the Manganese Complexes with α-ketoglutaric Acid and 1-(*o*-tolyl) Biguanide

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This paper presents the synthesis and characterization of three complexes of Mn (II,III), with the alfa-ketoglutaric acid (H_2A) and 1- (o-tolyl) biguanide (TB) as ligands. The complexes corresponding formulas are: $[Mn(TB)_2(H_2A)]Cl_2 \cdot 0.5C_2H_5OH$ (C1), $[Mn_2(TB)(A)(H_2A)_2(H_2O)_2(NO_3)_2](NO_3)_2 \cdot C_2H_5OH$ (C2) and $[Mn_2(TB)(A)(H_2A)_2(H_2O)_2(CH_3COO)_2](CH_3COO)_2 \cdot 3H_2O$ (C3), where A is H_2A without 2 protons. C1-C3 compounds were characterized by standard physico-chemical methods: chemical elemental analysis, IR spectroscopy, UV-Vis-NIR spectroscopy, molar conductivity and thermal analysis. For the obtained C1-C3 complexes, but also for the ligands (H_2A and TB) was tested the antibacterial activity against Staphylococcus aureus ATCC 25923 and Pseudomonas aeruginosa ATCC 27853 and antitumor activity on HeLa tumour cells. It has been observed a moderate cytotoxic effect of the complexes C1-C3 on growth and metabolic activity of HeLa cells, while the ligands show a very weak effect on them. The obtained complexes inhibits the adhesion on the substrate of bacterial strains Staphylococcus aureus and Pseudomona aeruginosa, which recommends them for possible therapeutic applications.

Keywords:αλφα-ketoglutaric acid, 1-(o-tolyl) biguanide, manganese complexes, biological activity

Manganese, at proper levels, it is vital to the human body, being used for its many functions, including the metabolism of carbohydrates, cholesterol, amino acids (glutamine, the most abundant amino acid in the body) and certain vitamins (E and B1).

Moreover, manganese helps in the proper functioning of the liver and thyroid enzymes, for absorbing nutrients (helps body to use the key nutrients such as biotin, thiamine, ascorbic acid and choline), wound healing and bone growth [1].

Biguanides are important compounds because of their biological properties, of which the most important are antibacterial, antifungal, hypoglycemic, antimalaria and anti-tumor activities [2-9].

Many coordination complex where the ligand is biguanide have biological properties, which explains the great interest in the study of these compounds.

Thus, are known complexes of Cu(II) having chlorhexidine as ligand [10,11], complexes of Fe(III), Ni(II) and Cu(II) with N, N-dimethyl biguanide and its derivatives as ligands [12-14].

There were synthesized and characterized complexes of manganese(IV) having as ligand biguanide or dibiguanide, as follows: $[\Delta\text{-}Mn(bigH)_3](ClO_4)_4 \cdot H_2O$, [15], $[Mn(bigH)_3]_2SO_4(NO_3)_6 \cdot 3H_2O$, [16], $[Mn(bigH)_3](NO_3)_6$, [17], where bigH: biguanide, $C_2H_7N_5$, $[Mn(C_{10}H_{24}N_{10})_2(OH)_2](OH)_2 \cdot 2H_2O$, [18] and $C_{10}H_{24}N_{10}$ is hexametylene dibiguanide.

α -ketoglutaric acid is a very important agent in the synthesis and degradation of amino acids and protein, in lipid and carbohydrate metabolism, with an essential biological role in the Krebs cycle [19]. Lanthanide complexes with α-ketoglutaric acid as ligand, have been synthesized [20].

The next complexes $[M_2^{III}M^{II}L_6(NO_3)_6(OH_2)_6](NO_3)$, where M^{III}= Ce and M^{II}= Cu, Co, Ni (L is α-ketoglutaric acid) have antibacterial and antioxidant activity [21].

Considering the biological properties of α-ketoglutaric acid and 1-(*o*-tolyl) biguanide and at the same time the biochemical role of manganese, the goal was to obtain and characterize new complexes of Mn (II and III) with the mentioned ligands.

There were obtained: $[Mn(TB)_2(H_2A)]Cl_2 \cdot 0{,}5C_2H_5OH$ **(C1)** – light pink, $[Mn_2(TB)(A^{2-})(H_2A)_2(H_2O)_2(NO_3)_2](NO_3)_2 \cdot C_2H_5OH$ **(C2)** - beige and $[Mn_2(TB)(A^{2-})(H_2A)_2(H_2O)_2(CH_3COO)_2](CH_3COO^-)_2 \cdot 3H_2O$ **(C3)** - brown**.**

Experimental part

Materials

In the synthesis of the three complexes were used: α-ketoglutaric acid - $C_5H_6O_5$ (Alfa Aesar), 1-(*o*-tolyl)biguanide - $C_9H_{13}N_5$ (Sigma Aldrich), manganese chloride (II) - $MnCl_2 \cdot 4H_2O$, manganese nitrate (II) - $Mn(NO_3)_2 \cdot 9H_2O$, manganese acetate (II) - $Mn(CH_3COO)_2$, (manganese salts are from Sigma Aldrich) and ethanol - C_2H_5OH (Chemical Company).

Complex compounds synthesis

Complex compounds have been synthesized by dissolving the substances (the two ligands and the metallic salt) in ethanol, followed by the reaction and then the obtained complexes were washed with ethanol and ethyl ether. The molar ratio for metal salt: α-ketoglutaric acid: 1-(*o*-tolyl) biguanide was 1:1:1, using 1 mmol of each reagent. The synthesis of coordination compounds had good yields and led to pure compounds, which necessitated no further purification.

Chemical and spectral analysis

The content of carbon, nitrogen and hydrogen of the synthesized complexes was determined by micro-combustion with an Elemental Analyzer Flash 2000. The presence of chlorine in the C1 complex was evidenced with $AgNO_3$ and the percentage of manganese was

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determined with a Perkin Elmer atomic absorption spectrophotometer, Analyst 400.

Electronic spectra were recorded at room temperature by the diffuse reflectance method, using MgO as standard in the 200-1500 nm domain with a Jasco V670 spectrophotometer.

FTIR spectra were recorded in the 4000-200 cm^{-1} domain with a Nicolet FT-IR IS 50 spectrophotometer. Thermal analyzes were carried out with a STA 449 F1 Jupiter in a dynamic air atmosphere, 20 mL/min and at a heating rate of 10°C/min, in the range of 25-900°C. Electrical molar conductivity was determined using a 10^{-3}M solution of DMF at 25°C with a PCD 6500 CybernScan conductometer.

Biological activity

The biological activity was tested on HeLa tumour cells for both the ligands and synthesized complexes, at a concentration of 500 μg/mL for 24h incubation at 37°C in an atmosphere of 5% CO_2 - MTT assay.

Method principle: in certain circumstances, cellular oxidoreductases NAD(P)H-dependent (NAD(P)H = nicotinamide-adenine-dinucleotide phosphate), may reflect the number of viable cells from a culture. These enzymes can reduce the tetrazolium MTT reagent (3-(4,5-dimethylthiazol-2-yl) -2,5-diphenyltetrazolium bromide) to insoluble formazan, which has a purple colour (fig. 1). Formazan colour intensity was assessed by measuring optical density at 570 nm with a Filter Max F5 Multi-Mode Microplate Reader spectrophotometer.

Tetrazolium dye reduction method is commonly used for measuring the cytotoxicity of the compounds, as well as cell proliferation. MTT method is used in dark condition, because MTT is sensitive to light.

The antimicrobial activity of the ligands and synthesized complexes was determined *in vitro* against *Staphylococcus aureus* and *Pseudomona aeruginosa* strains. To determine the MIC, the microdilution technique was used, in a binary liquid medium (broth) by successive dilution of the complex combination solution having a concentration of 10 mg/mL, using a 96-well plate. Also, the influence of DMSO solvent was quantified, as shown in the dilution scheme.

To determine the influence of the tested compounds on the adhesion of microbial biofilm at the inert substrate, the cells were cultured in 96-well plates with nutrient broth, in the presence of compounds of interest; they were incubated at 37°C for 24 h. The plates were emptied, washed two times with sterile physiological water and after, the fixing of cells for 5 min with 0.1 mL methanol 80% was made. The methanol was removed by inverting. Adhered cells were stained with crystal violet alkaline solution 1% (0.1 mL/well) for 15 min. Staining solution was removed and the plates were washed under running water. Microbial Biofilms formed on the plastic plates were resuspended in 33% acetic acid (by bubbling) and the color intensity was assessed by measuring the absorbance at 492 nm. Absorbance measurement was made with a Filter Max F5 Multi-Mode Microplate Reader spectrophotometer.

Results and discussions

Elemental analysis

To establish the formulas for the synthesized complexes, an elemental analysis was conducted. It has been found a good correlation between the percentages of carbon, nitrogen, hydrogen and manganese, experimentally obtained and calculated (table 1).

Thermal analysis

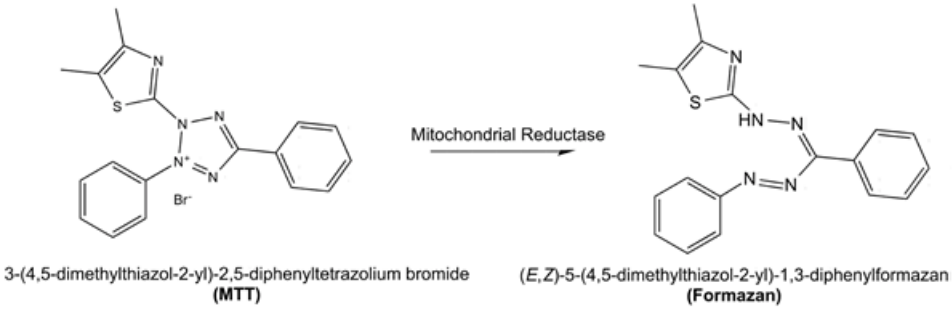
The thermogravimetric analysis has provided information that confirmed the proposed formulations for complex combinations, the presence of water and alcohol molecules and thermal effects that come togheter with the mass loss processes.

For C1 complex, in a first stage (<180°C) 0.5 C_2H_5OH crystallization molecules are lost (experimental loss 3.62%, calculated 3.40%). Chlorine anions are lost in an endothermic process, in the range of 180-220°C (experimental loss 10.79%, calculated 10.48%). Ligand decomposition is done by many oxidative processes between 220-760°C.

In case of C2 complex, up to 160°C, are removed the coordinating water and crystallization alcohol (7.95% exp., 7.68% calc.). Then, a series of processes up to 600°C follows, where the nitrate ions are removed as nitrogen oxides and the ligand decomposition takes place.

The crystallization and coordination water of the C3 complex is eliminated up to 120°C (8.67% exp., 8.46% calc.). Above this temperature, the ionic acetate is oxidized to CO_2 and H_2O and then ligand oxidation takes place up to 600°C.

For all three compounds, the mass loss from the last step, accompanied by a strong exothermic peak is due to the oxidation of carbonaceous mass resulted by decomposition of the ligands. The residue is Mn_2O_3 for all complexes, allowing the determination of the manganese percentage; experimental 8.04%, 8.12%, calculated for C1,



3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide **(MTT)**

(*E,Z*)-5-(4,5-dimethylthiazol-2-yl)-1,3-diphenylformazan **(Formazan)**

Fig. 1. Schematic representations of reducing MTT reagent and obtaining formazan

	Nitrogen %		Carbon %		Hydrogen %		Manganese %	
	exp.	calc.	exp.	calc.	exp.	calc.	exp.	calc.
C1	20.87	20.68	42.36	42.55	5.17	5.21	8.25	8.11
C2	11.64	11.81	29.70	29.25	3.72	3.68	10.14	10.29
C3	6.72	6.58	36.48	36.13	4.83	4.83	10.09	10.33

Table 1
THE CONTENT OF C, H, N AND Mn IN C1, C2, C3 COMPLEXES

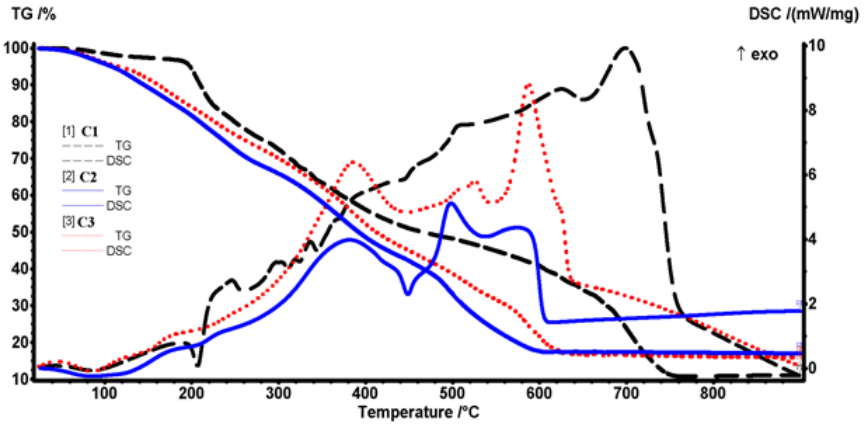


Fig. 2. Complex compounds thermal analysis C1(black), C2(red), C3(blue)

10.37% experimental, 10.3% calculated for C2, experimental 10.61%, 10.34%, calculated for C3.

There is a good concordance between the metal content spectrophotometrically determined and the metal found in the residue from the thermal analysis, for all analyzed complexes. The thermograms of the three complexes are shown in figure 2.

UV-Vis-NIR spectra

The stereochemistry of the C1, C2 and C3 complexes was assessed by using UV-Vis-NIR spectra compared to those of the ligands (α-ketoglutaric acid and 1-(*o*-tolyl) biguanide). Complexes electronic spectra are shown in figure 3.

All complexes have absorption bands in the 231-346 nm domain, assigned to H_2A and TB organic ligands, which are due to π-π* and n-π* transitions. These bands are slightly shifted due to the coordination of the ligand to the metallic ions.

In C1 complex, manganese is divalent, while in C2 and C3 is trivalent.

For the C1 complex, spectral band at 685 nm (14600 cm^{-1}) was assigned to the transition $^6A_{1g} \rightarrow {}^4T_{1g}$ and the one at 420 nm (23810 cm^{-1}) to the transition $^6A_{1g}, \rightarrow A_{1g}$, ^4E_g (G), in accordance with the octahedral symmetry (O_h). Both bands are spin forbidden.

The C2 complex combination spectrum band at 415 nm (24100 cm^{-1}) is assigned to the load transfer, the one from 520 nm (19230 cm^{-1}) to $^5B_{1g} \rightarrow {}^5E_g$ transition and the one at 680 nm (14710 cm^{-1}) to $^5B_{1g} \rightarrow {}^5A_{1g}$ transition.

The assignments spectral bands for C3 are: the band at the 420 nm (23810 cm^{-1}) is assigned to the load transfer, while the bands at 510 nm (19610 cm^{-1}) and 685 nm (14600 cm^{-1}) to $^5B_{1g} \rightarrow {}^5E_g$ and $^5B_{1g} \rightarrow {}^5A_{1g}$ transition.

For C2 and C3 complexes the proposed symmetry is tetragonally distorted octahedral [22].

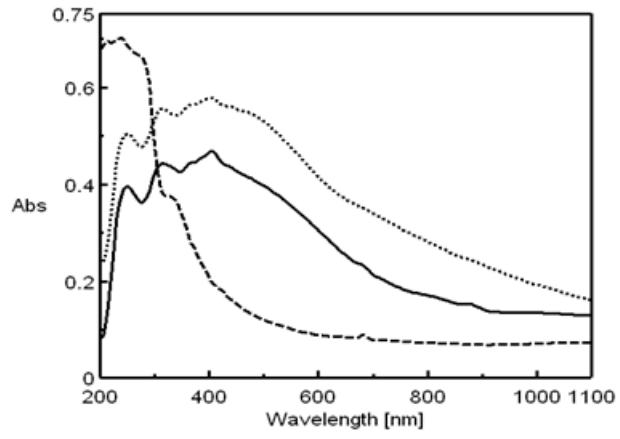


Fig. 3. UV-Vis spectra for C1 (dashed line), C2 (continuous line), C3 (dotted line)

FTIR spectra

FTIR spectra of C1-C3 complexes and of the ligands were analyzed in order to establish how the ligands are coordinating with the metalic ions [23]. The spectral characteristic bands and their assignments are presented in table 2. To determine the coordinating way of the α-ketoglutaric acid the IR spectrum of sodium α-ketoglutarate was analyzed, the difference between the positions of bands n(COO^-)asim and n(COO^-)sim being 181 cm^{-1}.

In the complex combination spectra a band shifting is observed due to the valence vibration of the imines group, ν(C=N), at 1610 cm^{-1}, namely: for C1 at 1634 cm^{-1}, for C2 at 1570 cm^{-1} and for C3 at 1580 cm^{-1}. This is in agreement with the TB ligand coordination to metallic ions by the unshared electron pair of the iminic nitrogen [24].

Keto groups from H_2A is involved in the coordination in all analyzed complexes, as confirmed by the shifting of the corresponding band ν(C=O), below 1720 cm^{-1}: 1690 cm^{-1} for C1, 1670 cm^{-1} and 1680 cm^{-1} for C2 and C3.

For C1 complex, α-ketoglutaric acid coordinate to the metal ion in form as H_2A, while in C2 and C3 complexes, one molecule is in bridging coordinated, as A^{2-}, and the other two are coordinated as H_2A .

The presence of both ionic and monodentate nitrate in C2 is demonstrated by its characteristic bands at: 1405, 1315, 1042, 878 and 711 cm^{-1}, while in C3, ionic and monodentate acetate is evidenced by bands at 1565, 1405, 1605, and 1312 cm^{-1}.

The bands between 247-272 cm^{-1}, respectively 410-448 cm^{-1} have been attributed to the formation of the Mn-O and Mn-N links.

The presence of a sharp band around 3350 cm^{-1} for C1 and C2 complexes was attributed to ethanol as crystallization molecule. Coordination water in C2 complex and the one for coordinating and crystallization from C3 is confirmed by the presence of the 3 characteristic bands at approx. 3380 cm^{-1}, 600 cm^{-1}, 700 cm^{-1}.

The *molar conductance* was determined in a solution of N, N-dimethylformamide having a concentration of 10^{-3}M at 25°C. For C1 complex was obtained a value of 134.5 $S \cdot cm^2 mol^{-1}$, for C2 161 $S \cdot cm^2 mol^{-1}$, while C3 has a molar conductivity of 142.5 $S \cdot cm^2 mol^{-1}$. These values indicates an electrolyte type 1:2 for all analyzed complexes [25].

Biological activity

The results of MTT assay showed that the HeLa cells metabolism varies, depending on the type of the material.

So, H_2A and TB ligands shows a very weak effect on HeLa tumour cells, under the tested conditions, while C1, C2, C3 complexes showed a mild cytotoxic effect on them. Figure 4 shows the absorbance values at 570 nm for the analyzed samples (at a 500μg/mL conc. for 24h incubation

Assignments	1-(*o*-tolyl) biguanide	α-ketoglutaric acid	C1	C2	C3
$\nu(C=O)_{keto}$		1720vs	1690s	1670s	1680s
$\nu(COOH)as$		1692vs	1650s	1610s	1635s
$\nu(C=N)$	1610vs		1634vs	1570vs	1580vs
$\nu(COO^-)asim$				1537vs	1538vs
$\delta(NH_2)$ $\delta(NH)+\nu(C-N)$	1577s 1270m		1531s 1252m	1515s 1257s	1520s 1255s
$\delta(CH)+\nu(C=C)$	1481vs		1487vs	1470m	1490m
$\nu(COOH)sim$		1406s	1434s	1360vs	1345vs
$\nu(COO^-)sim$				1389vs	1399vs
$\Delta=\nu_{(COO^-)as}-\nu_{(COO^-sim)}$				148	139
$\nu_3(NO_3)$				1405vs 1315vs	
$\nu_1(NO_3)$				1042m	
$\nu_2(NO_3)$				878m	
$\nu_4(NO_3)$				711m	
$\nu(C=O)_{acetate}$					1565s 1605fi
$\nu(C=O)_{acetate}$					1405vs 1312s
$\nu(Mn-N)$			448w	410w	420ws
$\nu(Mn-O)$			247m	272m	265m
$\nu(OH)_{alcohol}$			3350m	3326m	
$\nu(OH)_{water}$				3380m	3340m
ρr coord. water				745m	717m
ρw coord. water				607s	616s

Table 2
IR BANDS AND THEIR ASIGNMENTS FOR THE SYNTHESIZED COMPLEXES AND LIGANDS

at 37°C) and for the untreated control, indicating the metabolic activity of the HeLa cells cultures by the MTT method.

Evaluation of the antimicrobial activity of the new

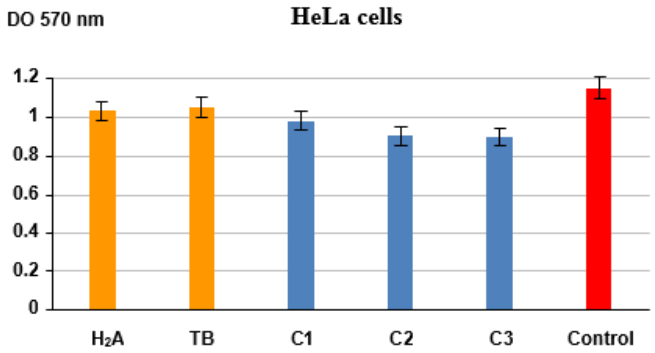


Fig. 4. Absorbance values at 570 nm for HA, TB, C1, C2, C3 for HeLa culture cells

complexes and the ligands was made on *Pseudomona aeruginosa* and *Staphylococcus aureus* species. It has been noticed that the solvent used for dilution (DMSO) does not influence the antimicrobial activity of the tested compounds at the concentrations of work.

In the case of Gram-positive species *Staphylococcus aureus*, complex compounds had a similar activity as the ligands (C2 and C3 complexes) and better than them for C1, activity wich was measured by MIC of 0.75 mg/mL. Complexes activity against Gram-negative bacteria *Pseudomona aeruginosa* is better (MIC 2.5 mg/mL) than for the H_2A ligand, but weaker than for TB ligand. Minimum inhibitory concentrations for the ligands and complexes, against the two bacterial strains are shown in figures 5 and 6.

As for the ability to inhibit microbial biofilm adhesion to

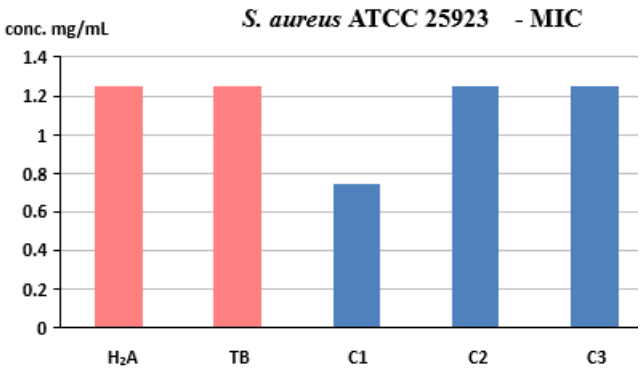


Fig. 5. Minimum inhibitory concentration for ligands, C1, C2, C3 against *S. aureus*

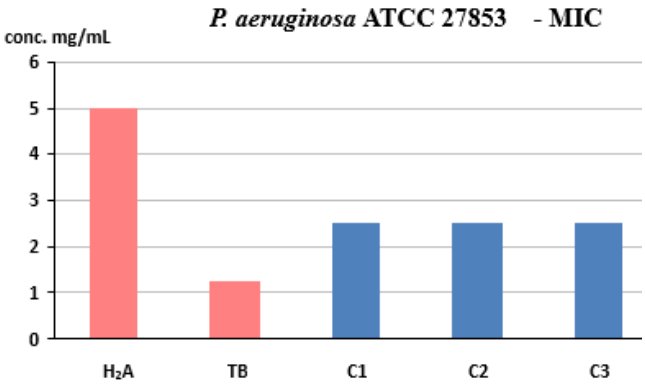


Fig. 6. Minimum inhibitory concentration for ligands, C1, C2, C3 against *P. aeruginosa*

the substrate, both ligands and complexes inhibits this process in a dose-dependent manner up to a minimum biofilm eradication concentration, (MBEC) of 0.04 mg/mL (H_2A, C2, C3), 0.09 mg/mL (TB) and 0.18 mg/mL (C1), in case of *Staphylococcus aureus*.

Also for *Pseudomona aeruginosa*, all compounds can

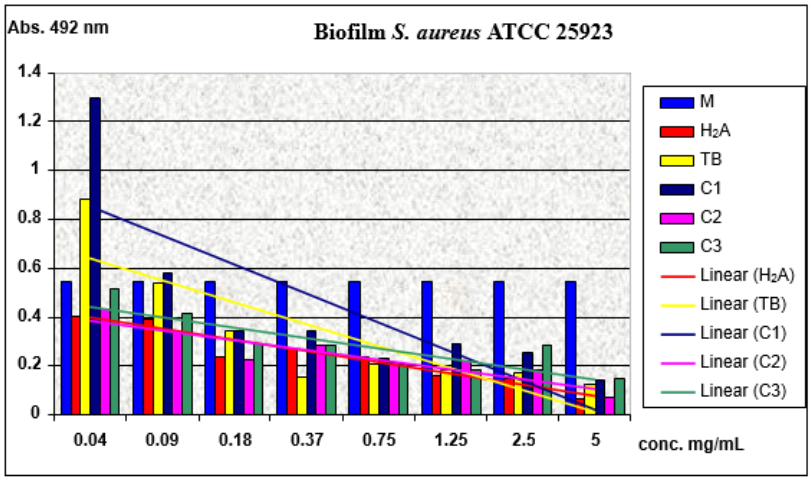


Fig. 7. The influence of H_2A, TB, C1, C2, C3 on the ability of adhesion to the inert substrate of *S. aureus*

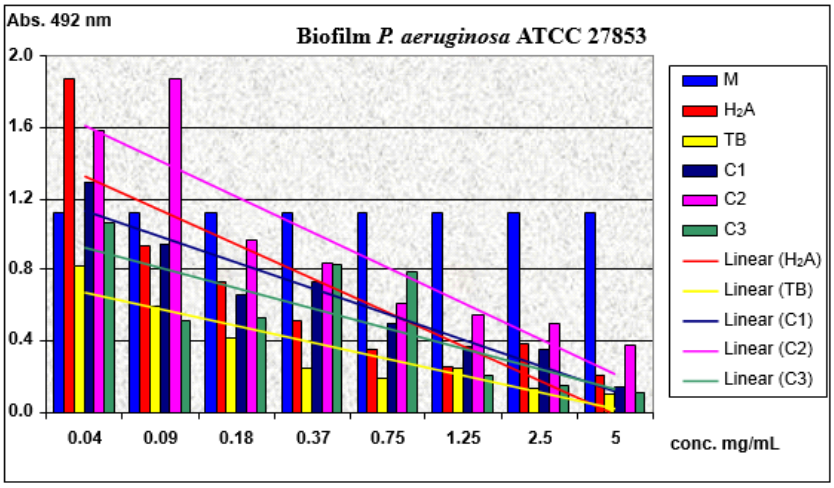


Fig. 8. The influence of H_2A, TB, C1, C2, C3 on the ability of adhesion to the inert substrate of *P. aeruginosa*

inhibit biofilm to a minimum concentration of biofilm eradication of 0.04mg/mL (TB, C3), 0.09mg/mL (H_2A, C1) and 0.18 mg/mL (C2).

The influence of ligands and complexes on the ability of adhesion to the inert substrate is shown in figures 7 and 8.

Conclusions

Three manganese complexes with α-ketoglutaric acid and 1- (*o*-tolyl) biguanide as ligands.

Based on the were synthesized and characterized elemental analysis, UV-Vis-NIR, IR spectra, thermal analysis and electric molar conductivity the formula for C1, C2, C3 complexes have been proposed.

Both α-ketoglutaric acid and 1-(*o*-tolyl) biguanide act as bidentate ligands in all three complexes. In all the synthesized complexes, TB ligand coordinates to the metallic ion through the iminic nitrogen atoms and H_2A ligand by oxygen atoms from the ketonic groups in alpha position and the carboxyl group (the structures of the two ligands are shown in fig. 9). C2 and C3 complexes are dinuclear and have a ligand in bridge, namely α-ketoglutaric acid deprotonated.

Biological activity was tested on HeLa tumour cells both

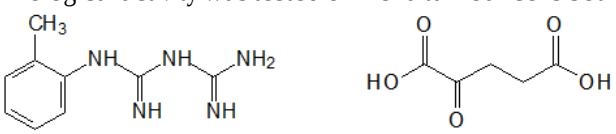


Fig. 9. Ligands TB and H_2A structures

for complexes and the ligands used in the synthesis. It has been found better cytotoxic effect of complexes C1, C2, C3 than the one of the ligands on HeLa cells.

The biological activity against bacteria *Staphylococcus aureus* and *Pseudomona aeruginosa* of the obtained complexes is comparable or better than the one of the ligands. As the complexes are electrolyte type, their activity can result from electrostatic interaction of the cationic complex of the species with negatively charged components of the membrane and their inactivation. On the other hand, antimicrobial activity may be associated with the stereochemistry, manganese valence, as well as the combined effect of the ligand and the metallic ion to inactivate a specific component involved in the pathogenesis of the microorganism.

The different activity of C2 and C3 complexes can be explained by the presence of various anions nitrate ion in C2 and acetate ion in C3.

Acknowledgement: This paper is supported by the UEFISCDI through PN-II-PT-PCCA-2013-4-0891 project: Innovative dental products with multiple applications no. 229/2014

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Manuscript received: 11.05.2017