UNIVERSITY OF BUCHAREST
FACULTY OF CHEMISTRY
DOCTORAL SCHOOL OF CHEMISTRY

DOCTORAL THESIS SUMMARY

SYNTHESIS AND CHARACTERIZATION OF NEW COMPLEXES WITH SOME BIOCATION AND PENTAAZAMACROCYCLIC LIGANDS

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INTRODUCTION

Having in view consideration their interesting chemical properties as well as the biological (antimicrobial, cytotoxic and antiviral) activity, the design and synthesis of the new types of polyaza macropolycyclic compounds have received much attention in the last years.

Bisazamacrocyclic ligands have the ability to generate stable complexes with different metal ions having thus a considerable potential for the design of therapeutic agents and diagnostics; they have rigid and stereospecifically oriented functional groups for optimal interaction to give rise the biological activity.

Since antiviral, antitumor and antimicrobial activities were demonstrated for complexes with bismacrocyclic ligand we have the purpose to synthetise and characterise of new species of Ni(II), Cu(II) and Zn(II) complexes with bismacrocyclic ligands.

OBJECTIVES

- The synthesis of new dinuclear complexes of Ni(II), Cu(II) and Zn(II) with bismacrocyclic ligands having an aromatic linker between bispentaazamacrocyclic moieties and the corresponding ligands;
- Characterization of dinuclear complexes and ligand by: microanalytical, ESI-MS, IR, UV-Vis, ^{1}H NMR, ^{13}C NMR and EPR spectra, magnetic data at room temperature and molar conductivities, the thermal behaviour and cyclic voltammetry;
- The antimicrobial activity screening against some Gram-negative, Gram-positive the well as fungal pathogenic strains (the inhibition of both planktonic and adherent on inert susbtratum microbial strains);
- The cytotoxicity evaluation (determination of apoptosis/necrosis by double staining with annexin V/propidium iodide and the determination of cell cycle phases by flow cytometry)

THESIS CONTENT

The doctoral thesis is structured in three parts.

The first chapter systematize the literature data concerning template condensation in the presence of formaldehyde /1/ that leads to a series of complexes with saturated mono- /2/ and bismacrocyclic /3/ ligands. The use of some of these complexes in different fields based on biological activity (antiviral /4/, antitumor /5/, antimicrobial /6/), catalytic effects /7/ or other properties (highly selective CO_2 capture /8/, the production of radiopharmaceuticals /9/ and obtaining porous material /10/) are presented as well.

The second chapter contains original contributions on the synthesis and characterization of 27 new complexes of ale Ni(II), Cu(II) și Zn(II). In addition, the three new ligands 1,2-, 1,3- and 1,4-bis(N,N-1,3,6,9,12-pentaazacyclotridecane)-benzene were obtained by one complex treatment with an excess of sodium sulfide nonahydrate.

The complexes features have been assigned from microanalytical, electrospray ionization tandem mass spectrometry, IR, UV–vis, ^{1}H NMR, ^{13}C NMR and EPR spectra as well as magnetic data at the room temperature, the thermal behaviour and the cyclic voltammetry.

The interest in these compounds with bismacrocyclic ligands appears from the fact that may show biological activity comparable or better than free ligand. As a result, in this paper are presented the results of antimicrobial activity assay concerning some microorganisms and their adherence at the inert substratum as well as cytotoxicity evaluation. The biological activity was correlated with log P_{ow} values.

The third chapter comprise analytical methods, techniques work and elemental chemical analysis data.

ORIGINAL CONTRIBUTIONS

Synthesis of complexes and ligands

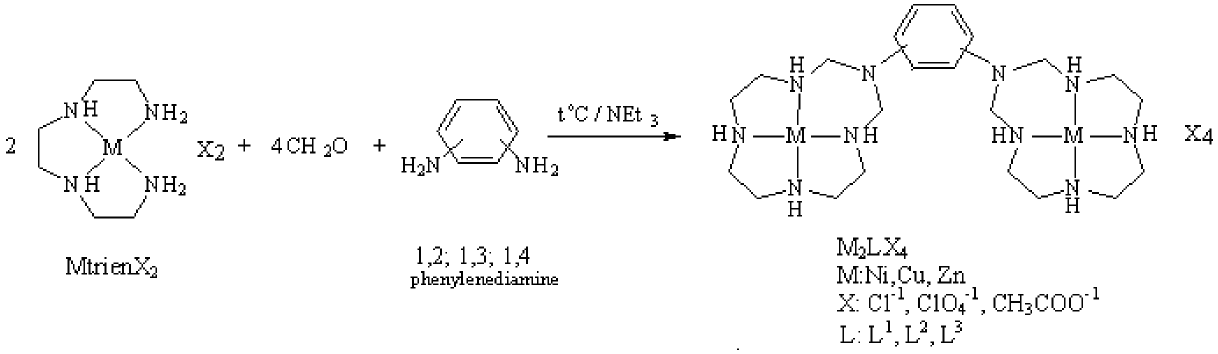
The one-pot reactions in excess of formaldehyde between nickel(II) copper(II) zinc(II) chloride perchlorate acetate 3,6-diazaoctane-1,8-diamine and 1,2-, 1,3- or 1,4-phenylenediamine in alkaline medium produced the species with bispentaazamacrocyclic saturated ligands (Scheme 1).

The aromatic amines and triethylenetetramine selection for the template condensation has been taken into account the presence of amine groups which may be involved in the condensation process with formaldehyde, and on the other hand the possibility to obtain complexes with an azamacrocyclic ligand which may have a high thermodynamic and kinetic stability. It started from 1,2-, 1,3- and 1,4-phenylenediamine to study how the mutual position of amino groups affect the species properties.

The preference for tetragonal stereochemistries such square planar and octahedral ones with strong field ligands recommend Ni(II) and Cu(II) as template in this type of reactions. In addition, the stereochemistries adopted by these ions are easily identified by using electronic and RPE spectroscopy,as well as magnetic measurements.

Formaldehyde have been utilized for the preparation of complexes because of hers high reactivity, the reactions with this compound taking place under mild conditions and does not require the use of anhydrous solvents; also is useful to insert methylene for cyclic compounds formation.

Complexes of nickel(II), copper(II), or zinc(II) with 1,2-, 1,3- and 1,4-bis(N,N-1,3,6,9,12-pentaazacyclotridecane)-benzene ligands were synthesized according to Scheme 1.



Scheme 1 Synthetic route to prepare complexes

The free ligand was synthesized by one of complexes treatment with sodium sulfide, followed by thin liquid chromatography purification and DMSO recrystallization.

Complexes of Ni(II), Cu(II) and Zn(II) with ligand (1,2-bis(N,N-1,3,6,9,12-pentaazacyclotridecane) -benzene)

The new series of species was prepared by the one pot condensation starting from aromatic linker 1,2-phenylenediamine. The chemical analyses are in accord with following formulas proposed for complexes:

$M_2L^1X_4{\cdot}nH_2O$ M = Ni(II), X = Cl, n = 5 (**1**)
M = Cu(II), X = Cl, n = 0 (**2**)
M = Zn(II), X = Cl, n = 5 (**3**)
M = Ni(II), X = ClO_4, n = 2 (**4**)
M = Cu(II), X = ClO_4, n = 4 (**5**)
M = Zn(II), X = ClO_4, n = 0 (**6**)
M = Ni(II), X = CH_3COO, n = 4 (**7**)
M = Cu(II), X = CH_3COO, n = 2 (**8**)
M = Zn(II), X = CH_3COO, n = 0 (**9**)

Mass spectra recorded by electrospray ionization tandem MS (ESI–MS) technique operating in the positive confirm complexes and ligand formation by identify the peak corresponding to the molecular ion and resulting from these species fragmentation in the presence of accelerated electrons. The molecular formula was confirmed by comparison the molecular weight of the compound with the values of the m / z values identified by mass spectrum. The peaks were assigned to species resulted from fragmentation and/or the addition of groups resulted from the cleavage of solvent, by removing of a different number of chloride, perchlorate, acetate anions or metal ions as well by bonds cleavage of the amine, methylene or ethylene groups. The complexes formation by condensation reaction is clearly evidenced by mass spectrometry study for the compound (8) (Fig. 1) the spectrum, indicates the presence of the molecular ion $[Cu_2C_{30}H_{56}N_{10}O_8+8H]^+$ with all four acetate groups, and bispentaazamacrocyclic as indicates structure formation the peak at m/z 454,36 (100 %).

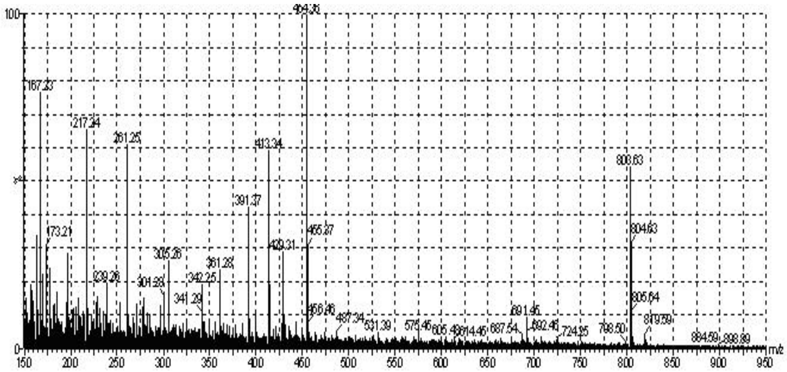


Fig. 1. Mass spectrum of complex $[Cu_2L^1](CH_3COO)_4{\cdot}2H_2O$ (**8**)

The thermal behaviour of chloride /11/ and acetate /12/ derivatives indicates the crystallization nature of water molecules /13/, elimination of these up to 175 ºC for complex $[Zn_2L^1Cl_4]\cdot 5H_2O$ (**3**), the chloride anions elimination as hydrochloric acid up to 330 ºC for complex $[Cu_2L^1Cl_4]$ (**2**), the paracyanide species as intermediate for compound $[Ni_2L^1]Cl_4\cdot 5H_2O$ (**1**)) /14/ and acetate into carbonate transformation for acetate derivatives, respectively. The TG and DTA curves corresponding to all complexes indicate that oxidative degradation of bisazamacrocyclic ligand consists in at least two processes. The final product of decomposition was in all cases the metal(II) oxide as powder X-ray diffraction indicated.

The IR spectra of the complexes were compared with those of the ligand, 1,2-phenylenediamine and the complexes with triethylenetetramine. In the spectra of the complexes and the ligand has noticed the absence of the two bands characteristic of the primary amine group /15/, being an indication of the fact that they were involved in the condensation process. The IR spectra showed also a single broad absorption around 3250 cm^{-1} which arise from secondary amine stretching vibration /16/. This band is shifted to lower wavenumbers in comparison with free ligand indicating that the secondary amine nitrogen atoms are involved in coordination /17/. It was identified also the characteristic bands of acetate and perchlorate groups and on the basis of these values it was established their behaviour as ligands or as free ion /18,19/.

^{1}H NMR spectra provided useful information about the Ni (II) complexes with square-planar stereochemistry and Zn (II) complexes which are diamagnetic. In the complexes spectra were identified signals corresponding to different types of protons, depending on their neighboring. The amine group coordination was further supported by ^{1}H NMR spectra of these complexes /20/. Thus, the resonance that arises from the NH group is upfield-shifted relative to the signal of the free ligand /21/.

Electronic spectra correlated with ***magnetic data at the room temperature*** provided useful information concerning the oxidation state of the metallic ion and stereochemistry and the ligand field nature for the Ni (II) and Cu(II) complexes. The electronic spectra of Ni(II) complexes displays a broad band with maximum in 430-490 nm range (Fig.2) /22/, characteristic pattern for the square-planar stereochemistry. As expected, the square-planar Ni(II) complexes are diamagnetic.

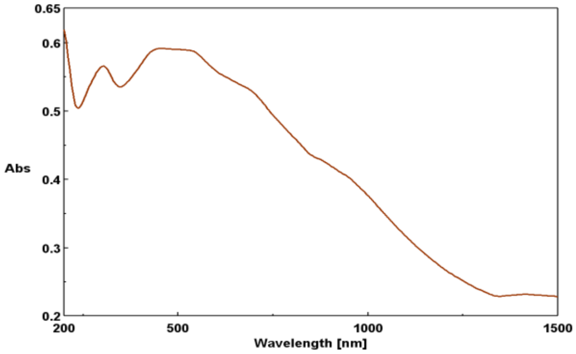


Fig.2. The electronic spectra of the complex $[Ni_2L^1]Cl_4{\cdot}5H_2O$ (**1**)

The electronic spectrum of $[Ni_2L^1(OH_2)_2](ClO_4)_4$ (**4**) complex exhibits bands in visible and near infrared regions with a characteristic pattern for a square pyramidal stereochemistry of Ni(II) with the high spin configuration. The slightly higher experimental $(\chi T)_{HT}$ value in comparison with calculated one for dinuclear species of 2.00 cm^3 K mol^{-1} furthermore sustains the proposed stereochemistry.

In the case of Cu (II) complexes for which the ground therm is orbitaly non-degenerate as a result Jahn-Teller effect the $(\chi T)_{HT}$ lies in the range 0.75 - 0.77 cm^3 mol^{-1} K and does not provide information concerning the stereochemistry.

EPR spectra confirmed the octahedral or square plan stereochemistry proposed for the of Cu (II) complexes and provide information concerning the presence of a rhombic or axial distortion, an interaction between the paramagnetic centres for square planar species and a large degree of covalent σ bonds between the Cu (II) and the nitrogen atoms of the bisazamacrocyclic ligand /23/. For example, in the case of complex $[Cu_2L^1](CH_3COO)_4{\cdot}2H_2O$ (**8**) (fig. 3) g and A values have indicated the presence of uncoupled electron in $d_{x^2-y^2}$ orbital, a significant degree of covalence, a low interaction between the paramagnetic ions and square planar stereochemistry.

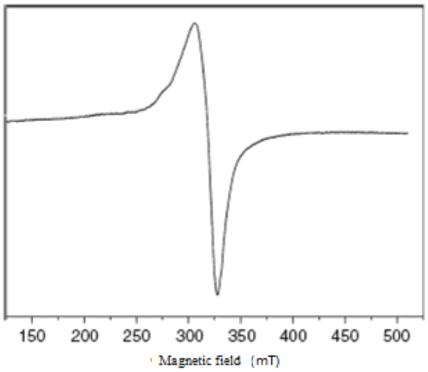


Fig.3. X-band powder EPR spectrum of complex $[Cu_2L^1](CH_3COO)_4{\cdot}2H_2O$ (**8**)

Electrochemical behaviour has been studied in order to obtain information related to the modification of the interaction with the electrons carriers or oxido-reduction enzymes from the composition of pathogenic microorganisms or tumor cells that can change the oxidation state of the metallic ions. The cyclic voltammograms have indicated an anodic wave that can be assigned to Ni(II)/Ni(III) couple for nickel compounds (Fig. 4), cathodic waves that can be assigned to Cu(II)/Cu(0) couple and one reduction waves in the cathodic range for Zn(II)/Zn(0) couple and the different behavior in comparison with complexes with triethylenetetramine as ligand.

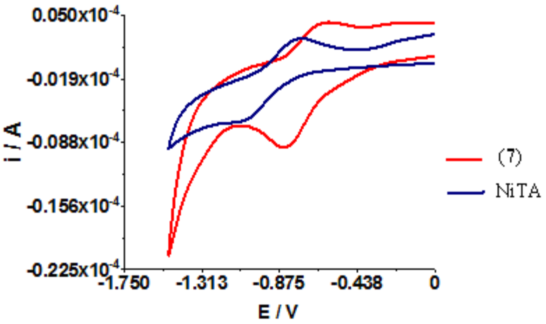


Fig.4. Cyclic voltammograms for complexes $[Ni_2L^1](CH_3COO)_4{\cdot}4H_2O$ (**7**) and Nitrien$(CH_3COO)_2$ recorded in DMSO at Pt electrode (concentration 10^{-3} M, supporting electrolyte 0.1 M $[Bu_4N]ClO_4$, reference electrode Ag/AgCl/KCl sat., scan rate 0.1 V s^{-1})

Molar conductivities were shown in the case of Zn (II) complexes the non-electrolyte nature for $[Zn_2L^1Cl_4]{\cdot}5H_2O$ (**3**) and $[Zn_2L^1(CH_3COO)_4]$ (**9**) and 1:4 electrolyte behaviour for $[Zn_2L^1](ClO_4)_4$ (**6**), thus confirming the presence of perchlorate in the ionization sphere.

The antimicrobial activity of the new complexes, ligand and trien complexes was assayed against Gram-positive, Gram-negative pathogenic bacteria as well as against *C. krusei* fungal stain which consisted in the inhibition of some microorganisms (MIC) and the adherent cells grown in biofilms developed in plastic wells. Concerning the antimicrobial activity, our results demonstrated that the newly synthesized complexes exhibited an improved antimicrobial activity in comparasion with the ligand and trien complexes.

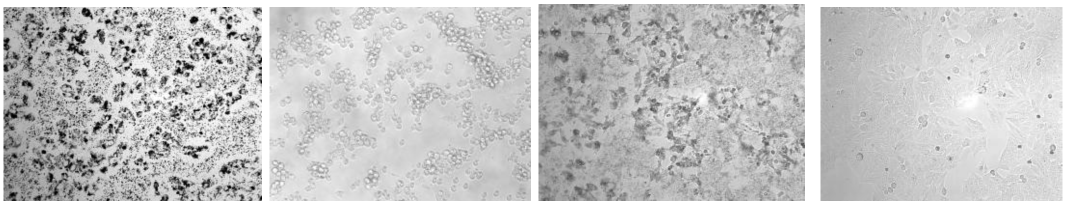
The tests revealed a good antibacterial activity only in the case of complex $[Zn_2L^1(CH_3COO)_4]$ (**9**) with a MIC value 7.81 şi 31.25 μg mL^{-1} in the case of Gram-negative strains (*E. coli* şi *K. pneumoniae*). The assays revealed a very good antifungal activity for the complexes $[Ni_2L^1]Cl_4·5H_2O$ (**1**) and $[Cu_2L^1](CH_3COO)_4·2H_2O$ (**8**) with a MIC value of 15.62 μg mL^{-1} in the case of *C. krusei* strain.

Taking in account the differences in the physiology and susceptibility to antibiotics of biofilm-embedded microorganisms, the complexes were investigated concerning their ability to inhibit adherent cells grown in biofilms developed in plastic wells. From this point of view, it was observed that the complexes interact differently with the biofilm formed by the microbial strains, the effect being either inhibitory or stimulatory. The complex $[Zn_2L^1Cl_4]·5H_2O$ (**3**) exhibited the most evident inhibitory effect upon adherence ability of *E. coli* on the entire concentrations range (1,000–1.95 μg mL^{-1}), whilst in the case of *B. subtilis, K. pneumoniae* and *E. cloacae* the anti-biofilm activity was expressed only at higher concentrations of 31.25 μg mL^{-1}. This aspect is important considering that genetic resistance of different microbial strains to antimicrobials is amplified when pathogenic microorganism is developing in biofilm. The increased activity of Zn(II) complex $[Zn_2L^1Cl_4]·5H_2O$ (**3**) could be explained considering its non-electrolyte nature resulted from both bismacrocycle and acetate coordination. This increases the compound lipophilicity, allowing an easier across of the lipoid membrane of the pathogenic microorganism.

Antimicrobial activity was correlated with ***log Pow values.*** As result the compounds lipophilicity were determined in order to obtain information concerning the compounds ability to penetrate the microorganisms lipid layers. The log P values obtained for complexes $[Ni_2L^1(OH_2)_2](ClO_4)_4$ (**4**) and $[Cu_2L^1](ClO_4)_4·4H_2O$ (**5**) indicate their hydrophilic character and suggesting thus their inability to penetrate the microbial lipid layers through passive diffusion.

The ***cytotoxicity evaluation*** of the ligand and complexes with both trien and bisazamacrocyclic ligand was performed using the human tumor cell line HCT 8 (human ileocecal adenocarcinoma). It is to be pointed that complexes with trien did not exhibit any cytotoxicity.

The preliminary step was consistent with the view of cells in the presence and absence of these compounds with optical microscopy in order to determine the morphology of the cells changes. Most of the compounds did not induce toxic effects on the HCT 8 cells with the exception of compounds $[Ni_2L^1(OH_2)_2](ClO_4)_4$ (**4**), $[Cu_2L^1](ClO_4)_4{\cdot}4H_2O$ (**5**) and $[Ni_2L^1](CH_3COO)_4{\cdot}4H_2O$ (**7**) that showed both cytotoxicity observed by the changing of the cells morphology in culture and the viability decrease evidenced with the trypan blue test (Fig. 5).



(a) (b) (c) (d)

Fig. 5. . Inverted microscopy images of HCT 8 grown in the presence of the newly synthetized compounds. The cells were exposed to 100µg/ml compounds and then observed for the morphological changes of the cells and their nuclei $[Ni_2L^1(OH_2)_2](ClO_4)_4$ (**4**) (a), $[Cu_2L^1](ClO_4)_4{\cdot}4H_2O$ (**5**) (b), $[Ni_2L^1](CH_3COO)_4{\cdot}4H_2O$ (**7**) (c), control (d)

Flow cytometry technique was used to determine the cell viability, in order to identify the percentage of viable cells by means of morphological, physico-chemical or biological changes of population under different phases of the cell cycle. The same compounds (**4**), (**5**) and (**7**) exhibit the highest percentage of cells blocked in mitosis phase and the appearance of apoptotic cells.

Complexes of Ni(II), Cu(II) and Zn(II) with ligand (1,3-bis(N,N-1,3,6,9,12-pentaazacyclotridecane) -benzene)

The second series of complexes was synthesized by the same method, except that 1,3-phenylenediamine was used instead of 1,2-phenylenediamine. The chemical analyses toghether with data provided by physico-chemical methods provided for the formulas proposed for the new complexes.

$M_2L^2X_4{\cdot}nH_2O$ M = Ni(II), X = Cl, n = 4 (**10**)
M = Cu(II), X = Cl, n = 2.5 (**11**)
M = Zn(II), X = Cl, n = 1.5 (**12**)

M = Ni(II), X = ClO_4, n = 2 (**13**)
M = Cu(II), X = ClO_4, n = 6 (**14**)
M = Zn(II), X = ClO_4, n = 2 (**15**)
M = Ni(II), X = CH_3COO, n = 2.5 (**16**)
M = Cu(II), X = CH_3COO, n = 1 (**17**)
M = Zn(II), X = CH_3COO, n = 10 (**18**)

Mass spectrometry technique was confirmed bispentaazamacrocyclic the structure by identification for most species through the ion $[C_{22}H_{44}N_{10}+6H]^+$ with m/z 454. The enhanced peaks intensity in comparation with those observed for the first series indicates that these species are more stable /24/, aspect that can be explained by the spatial arrangement of the macrocyclic units in the 1,3 positions of the benzene ring.

With the aim to evidence the modifications that appear at heating, the thermodynamic effects that accompany them, and the nature of evolved gases, the thermal behavior of these derivatives was investigated by simultaneous ***thermogravimetric/dynamic scanning calorimetry*** (TG/ DSC) coupled with mass spectrometry (MS). Processes as water elimination, fragmentation and oxidative degradation of the organic ligand as well as chloride elimination (Fig.6) /25/ were observed during the thermal decomposition.

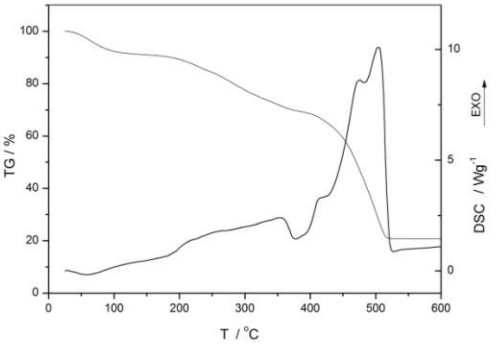


Fig.6. TG and DSC curves of $[Ni_2L^2]Cl_4\cdot 4H_2O$ (**10**)

These decomposition processes gives rise to fragments as H_2, C, C_6H_8, CN_2H_3, and HCO_2. Moreover, the oxidation of some fragments generates products as HO, H_2O, and CO_2 in the evolved gaseous products. The organic ligand fragmentation followed by the aromatic ring reduction by H_2 leads to C_6H_8 fragment. Besides, the aromatic ring reduction catalyzed by complexes is well known /26/.

The final product of decomposition was metal(II) oxide except for compound $[Cu_2L^2Cl_4]\cdot 2.5H_2O$ (**11**). According with powder X-ray diffraction profile (Fig. 7), the final product formed is a mixture of CuO and CuCl. The copper(I) chloride was identified by additional peak that appears at 2θ of 28.5°.

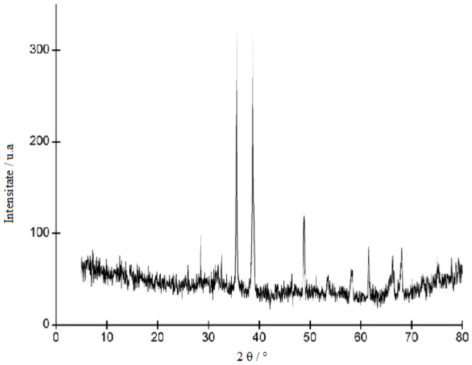


Fig. 7. Powder X-ray diffractogram for complex $[Cu_2L^2Cl_4]\cdot 2.5H_2O$ (**11**) residue

The difference observed between the experimental and calculated of residue can be explained by the fact that $CuCl_2$ formed as intermediate decompose with CuCl generation and a large amount of this compound volatilizes /27/.

The ***IR spectra*** of complexes exhibit bands that can be assigned to some moieties provided both by 3,6-diazaoctane-1,8-diamine and 1,3-phenylenediamine, respectively. The IR spectra showed also a single broad absorption which arises from the secondary amine stretching vibration. This band is shifted to lower wavenumbers in comparison with the metal-free ligand indicating the ligand coordination through secondary amine nitrogen. For carboxyl group two bands can be noticed for all complexes and the difference between these bands for complexes $[Cu_2L^2(CH_3COO)_4]\cdot H_2O$ (**17**) and $[Zn_2L^2(CH_3COO)_4]\cdot 10H_2O$ (**18**) is in agreement with unidentate nature of acetate; in $[Ni_2L^2](CH_3COO)_4\cdot 2.5H_2O$ (**16**) the acetateis in the ionization spfere /18/; for perchlorate compounds the characteristic bands indicate the presence of perchlorate also in the ionization spfere /19/.

The ***^{1}H NMR spectra*** of compounds consist of two singlets corresponding to NH and -NCH$_2$N- /27/ protons, and one triplet arising from -NCH$_2$CH$_2$N- /28/ fragment provided by 3,6-diazaoctane-1,8-diamine for bismacrocycle. The aromatic protons are responsible for the doublet

/29/. The amino group coordination is further supported by ^1H NMR spectrum of complexes where the resonance assigned to NH group is upfield-shifted relative to the signal of the free ligand.

The ***^{13}C NMR spectra*** of $[Zn_2L^2(CH_3COO)_4]\cdot10H_2O$ (**18**) showed characteristic peaks of all carbon atoms of the bispentaazabiscrocyclic and acetate ligands respectively.

Based on data obtained from the ***electronic spectra*** following stereochemistry we have been proposed: square-planar ($[Ni_2L^2]Cl_4\cdot4H_2O$ (**10**), $[Cu_2L^2](ClO_4)_4\cdot6H_2O$ (**14**), $[Ni_2L^2](CH_3COO)_4\cdot2.5H_2O$ (**16**)), distorted octahedral ($[Cu_2L^2Cl_4]\cdot2.5H_2O$ (**11**) (Fig.8), $[Cu_2L^2(CH_3COO)_4]\cdot H_2O$ (**17**)) and square pyramidal with a high spin configuration ($[Ni_2L^2(OH_2)_2](ClO_4)_4$ (**13**)).

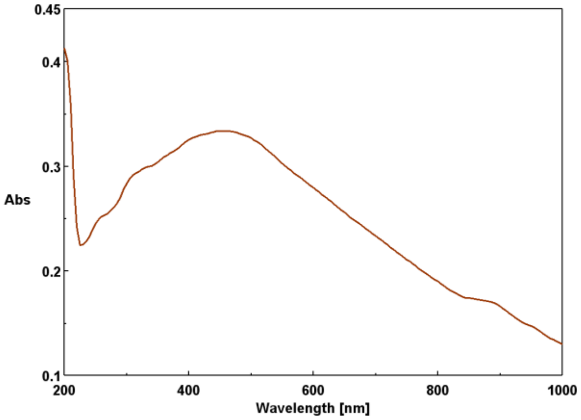


Fig.8. The electronic spectrum of the complex $[Cu_2L^2Cl_4]\cdot2.5H_2O$ (**11**)

The experimental $(\chi T)_{HT}$ value of complex $[Ni_2L^2(OH_2)_2](ClO_4)_4$ (**13**) is close to that calculated for a dinuclear species of this metallic ion /30/.

EPR spectroscopy confirmed the octahedral or square planar stereochemistry proposed for Cu (II) complexes combinations (Fig. 9) and indicating also the presence of an axial distortion, interaction between paramagnetic centers for square planar species, a high degree of σ covalency in the plan between the Cu (II) and the nitrogen atoms of the ligand azamacrocycle and the appearance of hyperfine coupling for some combination respectively.

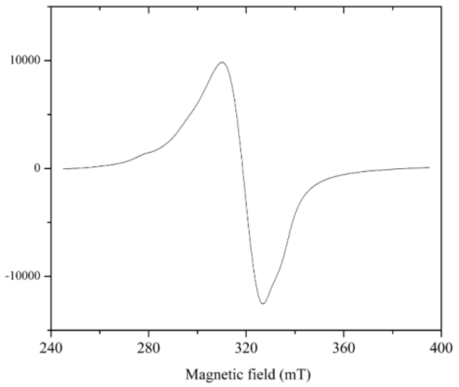


Fig.9. Powder EPR spectrum of complex $[Cu_2L^2](ClO_4)_4\cdot 6H_2O$ (**14**)

The molar conductivity indicated the nature non-electrolyte for $[Zn_2L^2Cl_4]\cdot 1.5H_2O$ (**12**) and $[Zn_2L^2(CH_3COO)_4]\cdot 10H_2O$ (**18**) while for the compound $[Zn_2L^2(OH_2)_4](ClO_4)_4$ (**15**) this value indicates a 1:4 electrolyte type behavior.

The ***antimicrobial activity*** assayed against the same bacterial and fungal microorganisms, both reference and clinical isolates as in the case described above as well as against two new strains highly resistant to antibiotics, *S. aureus MRSA* that produces hard to treat due to the fact that develops resistance to methicillin, and *E. coli ESBL* which produces beta-lactamase that confer resistance to most antibiotics (penicillins, cephalosporins, cephamycins, and carbapenems). The antibacterial activity of Ni (II) complexes was better than that shown by the compounds Cu(II) and Zn (II) and also against a large number of microbial strains at a lower MIC value. Compound $[Zn_2L^2(CH_3COO)_4]\cdot 10H_2O$ (**18**) was the only one who showed both antibacterial and antifungal activity. The assays revealed a good antimicrobial activity for the complexes $[Ni_2L^2]Cl_4\cdot 4H_2O$ (**10**) and $[Cu_2L^2Cl_4]\cdot 2.5H_2O$ (**11**) with a MIC value of 62.5 and 31.25 μg mL^{-1} in the case of *E. coli* ESBL tested strain. The complexes $[Cu_2L^2Cl_4]\cdot 2.5H_2O$ (**11**) and $[Zn_2L^2Cl_4]\cdot 1.5H_2O$ (**12**) exhibited the most evident inhibitory effect upon the adherence ability of the *E. coli* ESBL strain, up to the concentration of 7.81 şi 62.5 μg mL^{-1}. The good activity of complexes $[Cu_2L^2Cl_4]\cdot 2.5H_2O$ (**11**) could be explained considering its non-electrolyte nature resulted from both bismacrocycle and chloride coordination that increases the ***lipophilicity.*** On the other hand, the lipophilic character resulted from the positive charge of the metallic ion shielding through the ligands, probably allows an easier passage through the lipid membrane of the pathogenic microorganisms. The antimicrobial activity of some complexes correlate well with their ***hydrophilic*** character. Moreover, the antimicrobial activity may be related to other factors besides the diffusion ability through the cell membranes such as solubility, stereochemistry, thermodynamic and kinetic stability, as well as the combined effect of the ligand and the metal ion to inactivate a specific part of the pathogenic microorganisms.

The cytotoxic activity determinated by assessing of the HCT 8 cells viability after treatment with the complexes for 24 hours indicates that these compounds induced no toxic effect with the exception of $[Cu_2L^2](ClO_4)_4 \cdot 6H_2O$ (**14**) and $[Zn_2L^2(OH_2)_4](ClO_4)_4$ (**15**) that increased the percent of cells found in G2/M phase, an important aspect that can block the mitosis by DNA inhibition (Fig.10).

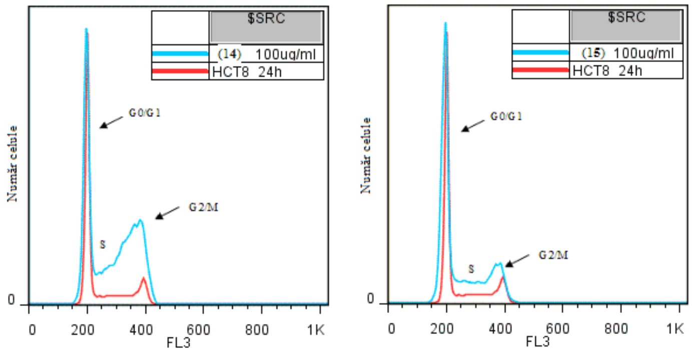


***Fig.* 10.** Cell cycle analysis of cells treated with 100µg mL^{-1} $[Cu_2L^2](ClO_4)_4 \cdot 6H_2O$ (**14**), $[Zn_2L^2(OH_2)_4](ClO_4)_4$ (**15**). The cells were harvested, stained with propidium iodide and subjected to flow cytometric analysis for cell distributions at each phase of cell cycle.

Complexes of Ni(II), Cu(II) and Zn(II) with ligand (1,4-bis(N,N-1,3,6,9,12-pentaazacyclotridecane) -benzene)

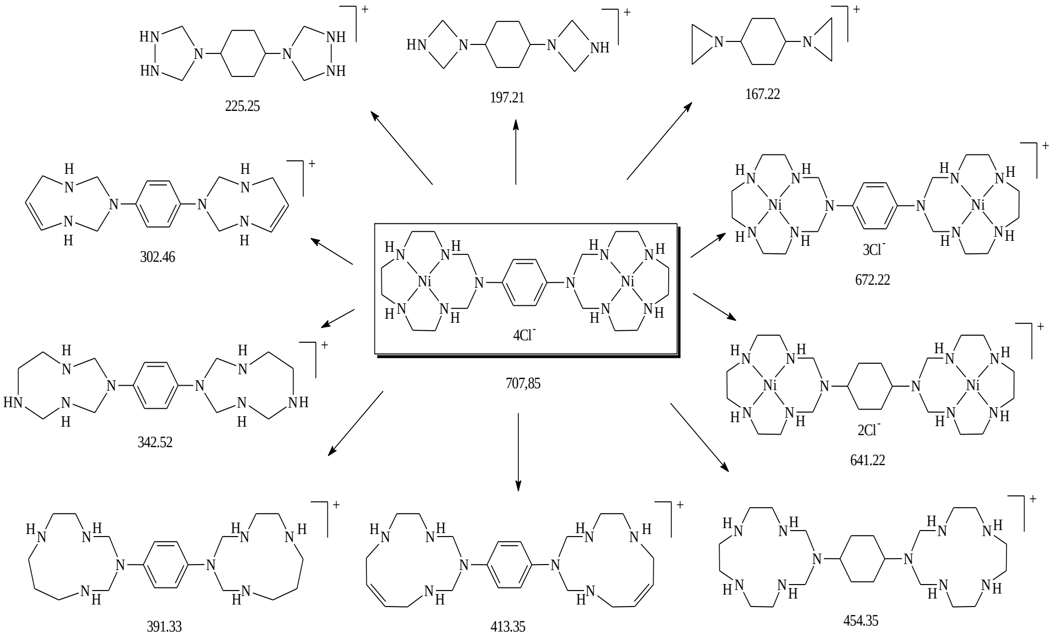
Another series of complexes was obtained by the same "one pot" condensation reaction by using the aromatic amine 1,4-phenylenediamine. By correlating the results of the analytical, thermal and spectral data the following formulas have been proposed:

$M_2L^3X_4 \cdot nH_2O$ M = Ni(II), X = Cl, n = 0 (**19**)
M = Cu(II), X = Cl, n = 3 (**20**)
M = Zn(II), X = Cl, n = 0 (**21**)
M = Ni(II), X = ClO_4, n = 6 (**22**)
M = Cu(II), X = ClO_4, n = 0 (**23**)
M = Zn(II), X = ClO_4, n = 0 (**24**)

M = Ni(II) X = CH_3COO, n = 3.5 (**25**)
M = Zn(II), X = CH_3COO, n = 2 (**26**)
M = Zn(II), X = CH_3COO, n = 1.5 (**27**)

Mass spectra having a high intensity of the peaks indicated a higher stability of the species produced by the impact with the accelerated electrons /31/, as in the case of ligand where the presence of the molecular ion m/z 454 has at 100 % intensity.

On the basis of ESI-MS spectrum for the compound $[Ni_2L^3Cl_2]Cl_2$ (**19**) it was proposed a fragmentation scheme starting from the former bisazamacrocyclic structure by removing a number of different chloride anions, metal ions bonds cleavage between the amine, methylene or ethylene groups with smaller macrocyclic formation (Scheme 2).



Scheme 2 The fragmentation proposed for$[Ni_2L^3Cl_2]Cl_2$ (**19**) based on the values m/z identifiedin the ESI-MS spectrum

The ***thermal behaviour*** of chloride and acetate complexes was investigated by simultaneous TG-DTA analysis and the final residues were examined by powder X-ray diffraction. The species isolated after the water elimination were also isolated and characterized.

IR spectra reveals the secondary amine group coordination and provided information about ligands (bisazamacrociclic, acetate and perchlorate).

^{1} H NMR spectra of all Zn(II) compounds exhibitthe signal proton of secondary amines group shifted to up field.

The acetate group coordination in complex $[Zn_2L^3(CH_3COO)_4]\cdot1.5H_2O$ (**27**) was evidenced by ***^{13} C NMR spectrum*** where the peaks assigned to carboxylate group appear up field shifted compared to $[Ni_2L^3](CH_3COO)_4\cdot3,5H_2O$ (**25**).

A square pyramidal with the high spin configuration ($[Ni_2L^3Cl_2]Cl_2\cdot4H_2O$ (**19**)), a square-planar ($[Ni_2L^3](ClO_4)_4\cdot6H_2O$ (**22**), $[Ni_2L^3](CH_3COO)_4\cdot3.5H_2O$ (**25**)) and distorted octahedral ($[Cu_2L^3Cl_4]\cdot3H_2O$ (**20**), $[Cu_2L^3(OH_2)_4](ClO_4)_4$ (**23**), $[Cu_2L^3(CH_3COO)_4]\cdot2H_2O$ (**26**)) stereochemistry was proposed based on ***electronic spectra*** and ***magnetic data*** at room temperature.

EPR spectroscopy confirmed the proposed stereochemistry and indicated axial distortion, a high degree of σ covalency plane between the Cu(II) and the nitrogen atoms of the ligand and the appearance of hyperfine coupling. EPR spectra of complex $[Cu_2L^3Cl_4]\cdot3H_2O$ (**20**), recorded at room temperature in X- and Q-band, are shown in Figure 11. X-band spectrum is characteristic for a Cu(II) complex with isolated centres and an axial symmetry with hyperfine coupling. Q-band spectrum reveals the distribution of these parameters, so-called "*g*-*A*-strain effect", due to small variation in copper local site geometry, and hyperfine coupling. Both spectra show also an additional signal, well separated in Q-band, due to the presence of a structural paramagnetic defect.

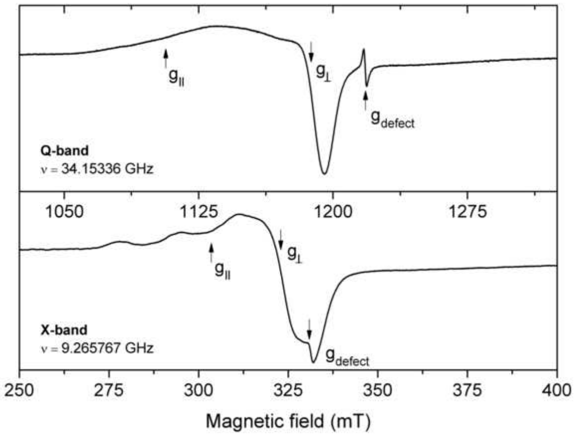


Fig. 11. Q and X-band powder EPR spectra of complex $[Cu_2L^3Cl_4]\cdot3H_2O$ (**20**)

The ***cyclic voltammograms*** of complexes $[Ni_2L^3Cl_2]Cl_2\cdot4H_2O$ (**19**) and $[Cu_2L^3Cl_4]\cdot3H_2O$ (**20**) show no anodic wave, behaviour that indicate that the macrocyclic ligand stabilizes the metallic ion as M(II). The complex $[Zn_2L^1Cl_4]\cdot 5H_2O$ (**21**) was investigated only in the cathodic potential range. The cyclic voltammograms displays a quasireversible reductions corresponding to a two-electron reduction process $Zn^{2+} + 2e^- \rightarrow Zn^0$, that in the reverse scan exhibits the typical anodic stripping of the electrodeposed metallic zinc.

The molar conductivity indicated non- electrolyte behavior for complexes (**20**), (**27**) and electrolyte type for (**19**), (**21**) and (**24**) respectively.

The activity against antibiotic-resistant strains highly increased compared to prior series. It is to be pointed out the very good activity of compound $[Ni_2L^3Cl_2]Cl_2\cdot 4H_2O$ (**19**) against *S. aureus* MRSA 1648 and of compound $[Zn_2L^3]Cl_4$ (**21**) on *E. coli* ESBL 1576, strains with acquired resistance of epidemiological importance to a beta-lactam antibiotic.

The most susceptible strains to the tested compounds were *E. coli* followed by *S. aureus*, while less susceptible were *B. subtilis*, *P. aeruginosa* and the fungal strains. Concerning the antibiofilm activity, the most efficient compound against the adherent microbial cells was the complex $[Cu_2L^3Cl_4]\cdot 3H_2O$ (**20**) (fig. 12) which inhibited the biofilm formed by Gram negative, Gram-positive bacterial and fungal strains, followed by $[Zn_2L^3]Cl_4$ (**21**) and thereafter complex $[Cu_2L^3Cl_4]\cdot 3H_2O$ (**20**).

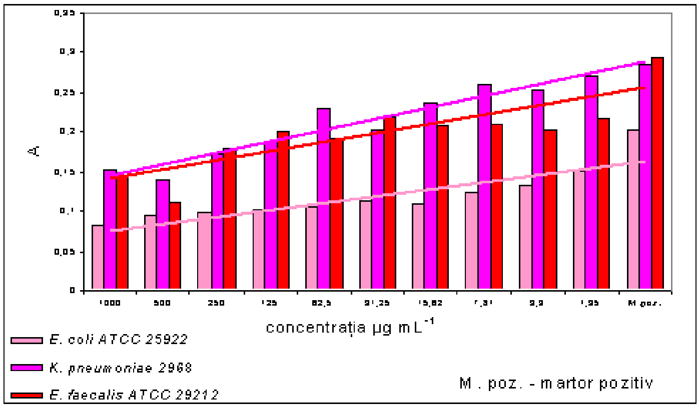


Fig.12. The influence of the complex $[Cu_2L^3Cl_4]\cdot 3H_2O$ (**20**) on the adherence to substrates of the bacterial strains *E coli* ATCC 25922, *K. pneumoniae* 2968 şi *E. faecalis* ATCC 29212

All Cu(II) complexes and $[Zn_2L^3(CH_3COO)_4]\cdot 1.5H_2O$ (**27**) inhibited biofilm adhesion to the substratum fungal strain *C. krusei* wide on the entire range of concentrations (1000 -1.95 mg mL^{-1}) (Fig. 13).

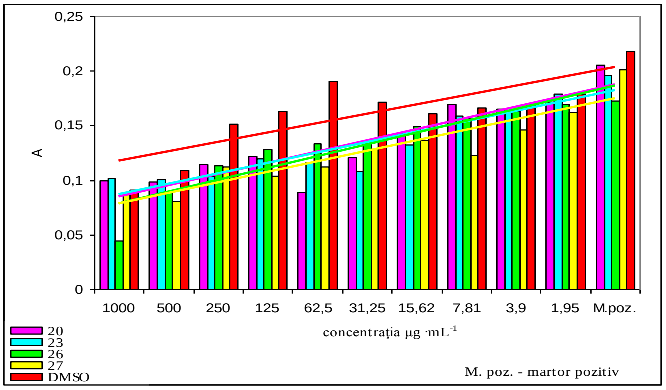


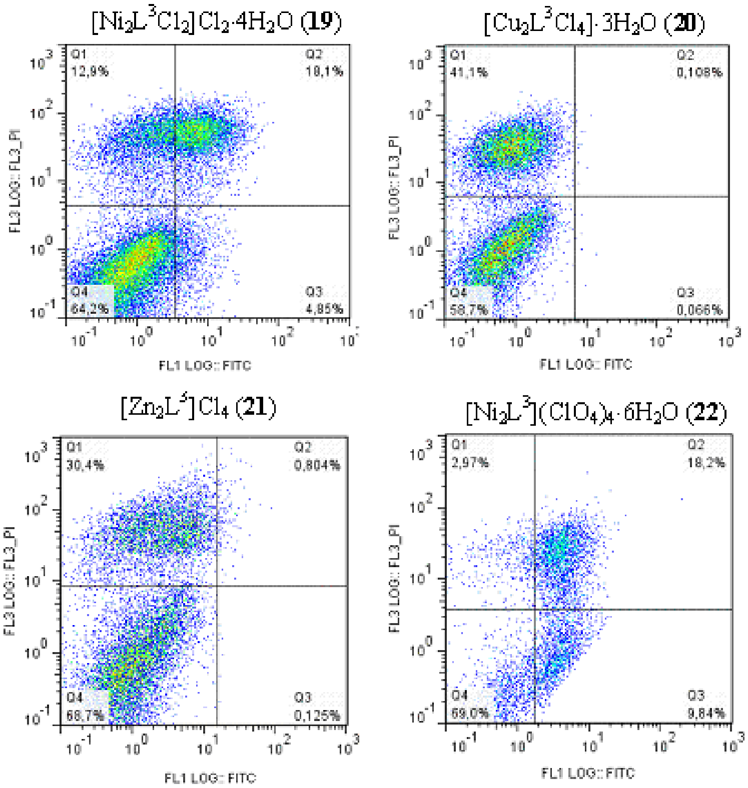
Fig.13. The influence of the complexes $[Cu_2L^3Cl_4]\cdot 3H_2O$ (**20**), $[Cu_2L^3(OH_2)_4](ClO_4)_4$ (**23**), $[Cu_2L^3(CH_3COO)_4]\cdot 2H_2O$ (**26**) and $[Zn_2L^3(CH_3COO)_4]\cdot 1.5H_2O$ (**27**) on the adherence to substrates of the bacterial strains *C.krusei* 963

As result, the compounds lipophilicity were determined in order to obtain information concerning their ability to penetrate the microorganisms' lipid layers. The log P_{wo} values obtained for complexes indicate their hydrophilic character suggesting their inability to penetrate the microbial lipid layers through passive diffusion.

Taking into account that the complex $[Cu_2L^3Cl_4]\cdot 3H_2O$ (**20**) was the most active from this series and that it exhibited the highest log P_{wo} value, it could hypothesize than: either the compound is internalized through porins and is reaching its intracellular target, or the complex interact with functional groups of the microbial wall, interfering thus with microbial cell viability and pathogenicity.

Tests for cytotoxicity of these compounds on cells HCT 8 by flow cytometry /33/ provided data relating to the detection of cells bloked in apoptosis/necrosis arrest. Only few cells were positive for annexin V-FITC after treatment with compounds $[Ni_2L^3Cl_2]Cl_2\cdot 4H_2O$ (**19**) and $[Ni_2L^3](ClO_4)_4\cdot 6H_2O$ (**22**) (4.85 and 9.84 % early apoptosis and 18.10 and 18.20 % late apoptosis) (Fig. 14). On the other hand, all compounds induced an intensive cell staining by propidium iodide, indicating that membrane integrity was severely damaged especially after the treatment with the compound $[Cu_2L^3Cl_4]\cdot 3H_2O$ (**20**) (41.10 %) and compound $[Zn_2L^3]Cl_4$ (**21**) (30.40 %).

Additionally, compound $[Zn_2L^3]Cl_4$ (**21**) increased the percent of cells arrested in G2/M phase, an important aspectthat can block the entry into mitosis by DNA is damaging (Fig. 15).



$[Ni_2L^3Cl_2]Cl_2\cdot 4H_2O$ (**19**)

$[Cu_2L^3Cl_4]\cdot 3H_2O$ (**20**)

$[Zn_2L^3]Cl_4$ (**21**)

$[Ni_2L^3](ClO_4)_4\cdot 6H_2O$ (**22**)

Fig.14. Dot plots quantification of HCT 8 cell viability in the presence of complexes (**19**)- (**22**), assayed by Annexin-FITC/PI Kit

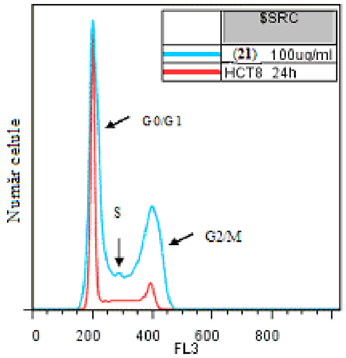


Fig. 15. Cell cycle analysis of cells treated with 100µg mL^{-1} $[Zn_2L^3]Cl_4$ (**21**). The cells were harvested, stained with propidium iodide and subjected to flow cytometric analysis for cell distributions at each phase of cell cycle.

These results could indicate the anti-tumor potential of this compound, further investigations being necesary in order to confirm this hypothesis.

Cytotoxic activity of this compound may be correlated with the cationic nature of this complex that favors its attraction with DNA strands. Following the closeness to DNA as with other cytostatics of inorganic nature, probably covalent bonds between Zn (II) and nucleobasis are formed, hydrogen bonds and / or π - π stacking interactions with nucleic acid components. All these interactions leads to DNA strands deformation and produce a long-range damage, lesions that are difficult to repair by natural systems that recognize and repair.

Conclusions

Finally, having in view the three derivatives of phenylenediamine which can be distinguished by the mutual position of the amino groups may conclude on the basis of all experimental data, that the derivative of the diamine ligand that involves the minimal steric hindrance and the longer distance between the two metal ions had the advantage of generating a higher capacity in terms of antimicrobial activity and cytotoxicity.

On the other hand, it is not surprising that the Zn(II) ion is present in the compounds with the higher performance, having in view the fact that the complex of paraxylylbiscyclam with this metallic ion represent the active species of Mozobil, an antiviral drug.

The good antimicrobial activity of some complexes exhibited by both planktonic and biofilm embedded cells are likely to recommend further research concerning the development of new materials or strategies in order to combat pathogenic biofilms frequently involved in the etiology of chronic infections and the treatment of infections caused by strains that have acquired resistance to antibiotics.

Bibliography

1. A. De Blas, G. De Santis, L. Fabbrizzi, M Licchelli, P. Pallavicini, *Pure Appl. Chem.*, **65** (1993) 455-459.

2. S.K. Jung, S.G. Kang, P. Suh, *Bull. Korean Chem. Soc.*, **10** (1989) 363-366.

3. H. Kim, S.G. Kang, S.K. Jeong, *Bull. Korean Chem. Soc.*, **4** (2012) 1329-32.

4. E. De Clercq , N. Yamamoto, R. Pauwels, J. Balzarini, M. Witvrouw, K. De Vreese, Z. Debyser, B. Rosenwirth, B. Rosenwirth, P. Peichl, R. Datema, D. Thornton, R. Skerlj, F. Gaul, S.Padmanabhan, G. Henson, G. Bridger, M. Abrams, *Antimicrob. Ag. Chemother.*, **38 (**1994) 668-674.

5. J.B. Rubin, J.B. Rubin, A.L. Kung, R.S. Klein, J.A. Chan, Y.P. Sun, K. Schmidt, M.W. Kieran, A.D. Luster, R.A. Segal, *PNAS,* **23** (2003) 13513-13518.

6. Z.A. Siddiqi , S. Kumar , M. Khalid , M. Shahid , *Spectrochim. Acta A Mol. Biomol. Spectrosc.*, **72** (2009) 970-974.

7. K. Mochizuki, T. Sugita, F. Yamada, N. Mochizuki, K. Hayano, Y. Ohgami, *Inorg. Chim. Acta,* **362** (2009) 1204-1208.

8. H.S Choi, M.P. Suh, *Angew. Chem. Int. Ed.*, **48** (2009) 6865-6869.

9. C.A. Boswell, P. McQuade, G.R. Weisman, E.H. Wong, C.J. Anderson, *Nuclear. Med. Biol.*, **32** (2005) 29-38.

10. M.P. Suh, Y.E. Cheon, E.Y. Lee, *Coord. Chem. Rev.*, **252** (2008) 1007-1026.

11. R. Olar, M. Badea, D. Marinescu, L. Calu, C. Bucur, *J. Therm. Anal. Calorim.*, **105** (2011), 571-575.

12. C. Bucur, M. Badea, L. Calu, D. Marinescu, M.N. Grecu, N. Stănica, M.C. Chifiriuc, R.Olar, *J. Therm. Anal. Calorim.*, **110** (2012) 235-241.

13. D.A. Köse, G. Gökçe, S. Gökçeand, I. Uzun, *J. Therm. Anal. Calorim.*, **5** (2009) 247-251.

14. R. Olar, M. Badea, D. Marinescu, *J. Therm. Anal. Calorim.*, 99 (2010) 893-898.

15. S.G. Kang, K. Ryu, S.K. Jung, C.S. Kim, *Bull. Korean Chem. Soc.*, **17** (1996) 331-334.

16. A. Rastogi, R. Nayan, *J. Coord. Chem.*, **62** (2009) 3366-3376.

17. M. Salavati-Niasari, A. Amiri, *J. Mol. Cat. A Chem.*, **235** (2005) 114-121.

18. G.B. Deacon, J.R. Philips, *Coord. Chem. Rev.*, **33** (1980) 227–250.

19. B.J. Hathaway, Oxyanions din: G. Wilkinson, R.D. Gillard, J.A. McCleverty, Comprehensive Coordination Chemistry, Pergamon Press, Oxford, 1987.

20 R. Smith, D. Huskens, D. Daelemans, R.E. Mewis, C.D. Garcia, A.N. Cain, T. N.C. Freeman, C. Pannecouque, E. De Clercq, D. Schols, T. J. Hubin, S.J. Archibald, *Dalton Trans.*, **41** (2012) 11369-11377.

21 A.T. Balaban, M. Banciu şi I.I. Pogany, Aplicaţii ale metodelor fizice în chimia organică, Ed. Tehnica, Bucureşti, 1983.

22. A.B.P. Lever, Inorganic electronic spectroscopy, Elsevier, Amsterdam, London, New York, 1986.

23. B.J. Hathaway, D.E. Billing, *Coord. Chem. Rev.,* **5** (1970) 143-207.

24. W. Henderson, J.S. McIndoe, Mass spectrometry of inorganic, coordination and organometallic compounds, John Wiley & Sons, Chichester, 2005.

25. C. Bucur, R.C. Korošec, M. Badea, L. Calu, M.C. Chifiriuc, N. Grecu, N.Stănică, D. Marinescu, R. Olar, *J. Therm. Anal. Calorim.*, **113** (2013) 1287-1295.

26. S.A. Patil, U.V. Kamble, P.S. Badami, *Nucleos. Nucleot. Nucl.*, **29** (2010) 658-675.

27. G. De Micco, A.E. Bohé, D.M. Pasquevich, *J. Alloy. Compd.*, **437** (2007) 351-359.

28. R.A. Shiekh, S. Shreaz, L.A. Khan, A.A. Hashmi, *J. Chem. Pharm. Res.*, **2 (**2010) 172-185.

29. M. Salavati-Niasari, F. Davar, *Inorg. Chem. Comm.*, **9** (2006) 175-179.

30. J.R. Gispert, Coordination chemistry, Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim, 2008.

31. S. Chandra, L.K. Gupta, *Spectrochim. Acta Part A Mol. Biomol. Spectrosc.*, **60** (2004) 1751-1761.

32. C. Bucur, M. Badea, M.C. Chifiriuc, C. Bleotu, E.E. Iorgulescu, I.A. Badea, M.N. Grecu, V. Lazăr, O.I Patriciu, D. Marinescu, R. Olar, *J. Therm. Anal. Calorim.*, **115** (2014) 2179-2189.

33. D. Bratosin, Explorarea structurii şi funcțiilor celulare prin citometrie în flux, ed. Universitatii de Vest: Vasile Goldis, University Press, Arad, 2007.

Articles published

1. Investigation of thermal stability, spectral, magnetic and antimicrobial behavior for new complexes of Ni(II), Cu(II) and Zn(II) with a bismacrocyclic ligand, C. Bucur, R. Cerc Korošec, M. Badea, L. Calu, M.C. Chifiriuc, N. Grecu, N. Stanică, D. Marinescu, R. Olar, *J. Therm. Anal. Calorim.*, **113** (2013) 1287-1295 .

2. Studies on thermal, spectral, magnetic and biologic properties of new Ni(II), Cu(II) and Zn(II) complexes with a bismacrocyclic ligand bearing an aromatic linker,C. Bucur, M. Badea, M. C. Chifiriuc, C. Bleotu, E.E. Iorgulescu, I. A. Badea, M.N. Grecu, V. Lazăr, O.I. Patriciu, D. Marinescu, R. Olar, *J. Therm. Anal. Calorim.*, **115** (2014) 2179-2189.

3. Thermal behaviour of some new complexes with decaazabismacrocyclic ligand as potential antimicrobial species, C. Bucur, M. Badea, L. Calu, D. Marinescu, M.N. Grecu, N. Stănica, M.C. Chifiriuc, R.Olar, *J. Therm. Anal. Calorim.*, **110** (2012) 235-241.

4. Thermal behaviour of some new complexes with bismacrocyclic ligands as potential biological active species, R. Olar, M. Badea, D. Marinescu, L. Calu, C. Bucur, *J. Therm. Anal. Calorim.*, **105** (2011) 571-575.

Participation in scientific events

1. Synthesis, spectral, biological and thermal characterization of some new complexes with bismacrocyclic ligands, R. Olar, M. Badea, D. Marinescu, C. Cicioiu, ESTAC 2010, 22-27 august, Rotterdam, Olanda, Book of abstracts pg. 177, poster.

2. Thermal behavior of some new complexes of Ni(II), Cu(II) and Zn(II) with a bismacrocyclic ligand as biological active species, C. Bucur, L. Calu, R. Olar, M. Badea, D. Marinescu, CEEC-TAC1 2011, 7-10 septembrie, Craiova, Romania Book of abstracts pg. 404, poster.

3. Thermal behavior of some new complexes with decaaza bismacrocyclic ligand as potential antimicrobial species, C. Bucur, R. Olar, M. Badea, D. Marinescu, CEEC-TAC1 2011, 7-10 septembrie, Craiova, Romania Book of abstracts pg. 405, poster.

4. Thermal study on new Ni(II), Cu(II) and Zn(II) complexes with a decaazabismacrocyclic ligand as biological active species, C. Bucur, L. Calu, R. Olar, M. Badea, D. Marinescu, P. Rotaru, C. Chifiriuc, L. Marutescu, C. Bleotu, V.Lazar, ICTAC15 2012, 20-24 august, Osaka, Japan, poster.

5. New antimicrobials with a low citotoxicity based on complexes of Ni(II), Cu(II) and Zn(II) with a decaazabismacrocyclic ligand, C. Bucur, R. Olar, M. Badea, D. Marinescu, L. Marutescu, C. Balotescu, C. Bleotu, New Trends in Materials Science Workshop, 28-31 martie 2012, Bucuresti, poster.