5. *González-Fernández C., Mercedes B.* Linking microalgae and cyanobacteria culture

SYNTHESIS AND BIOLOGICAL ACTIVITY OF COPPER(II), NICKEL(II) AND COBALT(III) COORDINATION COMPOUNDS WITH 2-BENZOYLPYRIDINE N(4)-ALLYL-S-METHYLISOTHIOSEMICARBAZONE

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Rezumat

Lucrarea conţine descrierea sintezei N(4)-alil-S-metilizotiosemicarbazonei 2-benzoilpiridinei (HL) şi a cinci compuşi coordinativi ai cuprului(II), nichelului(II) şi cobaltului(III) cu acest ligand. Compuşii noi obţinuţi au fost studiaţi cu ajutorul spectroscopiei IR şi RMN (^{1}H şi ^{13}C), analizei elementale, conductivităţii molare şi susceptibilităţii magnetice. Pentru compuşii sintetizaţi a fost determinată *in vitro*

activitatea antibacteriană şi antifungică faţă de spectrul larg de tulpini standard de *Staphylococcus aureus* (ATCC 25923), *Escherihia coli* (ATCC 25922), *Klebsiella pneumonae* şi *Candida albicans*. Activitatea antiproliferativă *in vitro* a ligandului şi complecşilor a fost testată pe celule leucemiei mieloide umane HL-60 şi HeLa.

Cuvinte-cheie: compuşi coordinativi, 2-benzoilpiridina, izotiosemicarbazona, activitatea biologică.

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Introduction

Thiosemicarbazones, isothiosemicarbazones and their coordination compounds have been attracting attention of the researchers for a number of years, because of their interesting physico-chemical and biological properties.

The data about S-alkylisothiosemicarbazones are rather scarce, but a recent study showed that they possess biological activity. In scientific reviews have been mentioned a wide range of biological properties of isothiosemicarbazones and their metal complexes, such as antiviral, antimicrobial, antibacterial, cytotoxic, antituberculous, antioxidant and antidiabetic activities [1-7]. Therefore, the aim of this work was the synthesis and study of biological properties of 2-benzoylpyridine N(4)-allyl-S-methylisothiosemicarbazone and it copper(II), nickel(II) and cobalt(III) coordination compounds.

Materials and methods

General

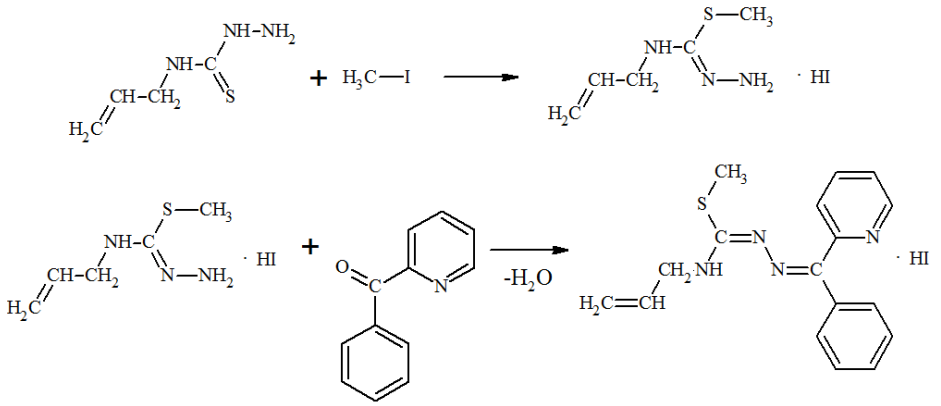
All commercially available reagents and chemicals were of analytical- or reagent-grade purity and used as received. The ^{1}H and ^{13}C NMR spectra were recorded on a Bruker DRX-400 NMR spectrometer, using $CDCl_3$ as a solvent. The chemical shifts (δ) in ppm were measured relative to tetramethylsilane (TMS). Infrared spectra of the compounds were recorded on a Bruker ALPHA FTIR spectrophotometer at room temperature in the range of 4000-400 cm^{-1}. Magnetochemical research was made at room temperature using Gouy method [8]. The determination of metal content in the synthesized coordination compounds was performed using titration methods according to the literature procedures [9-12]. Melting point of the isothiosemicarbazone was measured using capillary method [13]. Molar conductivity values were determined in 10^{-3} mol/L methanol solutions using slidewire bridge R-38.

Synthesis of the 2-benzoylpyridine N(4)-allyl-S-methylisothiosemicarbazone (HL)

The synthesis of 2-benzoylpyridine N(4)-allyl-S-methylisothiosemicarbazone was done starting form N(4)-allyl-3-thiosemicarbazide that was obtained by the reaction between allyl isothiocyanate and hydrazine hydrate as described in [14].

2-Benzoylpyridine N(4)-allyl-S-methylisothiosemicarbazone (HL) **(Scheme 1)** was prepared according to a modification of the procedure described in the literature [14].

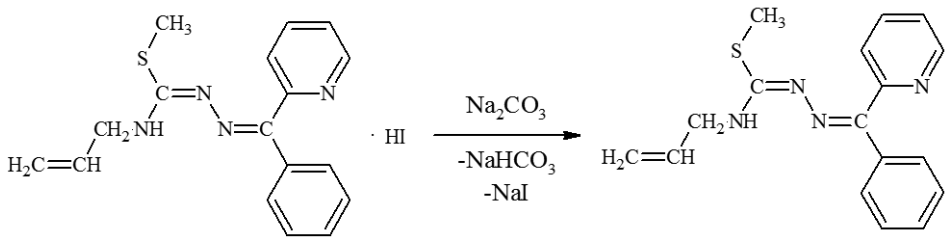
N(4)-Allyl-3-thiosemicarbazide (1.31 g, 10 mmol) was dissolved in 20 mL of ethanol with constant stirring. After that iodomethane (1.56 g, 11 mmol) was added. The mixture was stirred at room temperature for 2 hours and 2-benzoylpyridine (1.83 g, 10 mmol) was added. The solution was stirred at 70 ^{0}C for 30 min. After the reaction mixture was cooled to room temperature, the yellow solid was isolated by filtration, washed with ethanol and dried *in vacuo*.



Scheme 1. Synthesis of 2-benzoylpyridine N(4)-allyl-S-methylisothiosemicarbazone hydroiodide.

Sodium carbonate (1.06 g, 10 mmol) was added to the solution of 2-benzoylpyridine N(4)-allyl-S-methylisothiosemicarbazone hydroiodide (4.38 g, 10 mmol).

After the reaction mixture was cooled to room temperature, the 2-benzoylpyridine N(4)-allyl-S-methylisothiosemicarbazone was extracted with chloroform from the reaction mixture. After evaporation yellow solid was obtained (**Scheme 2**).



Scheme 2. Neutralization of 2-benzoylpyridine N(4)-allyl-S-methyl-isothiosemicarbazone hydroiodide.

Yellow solid. Yield: 76%; m.p.: 80-82 ^{0}C; FW: 310.42 g/mol;

Main IR peaks (cm^{-1}): ν(N^{4}H) 3002, ν(C=C allyl) 1644, ν(C=N^1) 1613, ν(C-N$_{pyr}$) 1365, ν(CH$_3$–S) 1082, ν(C–S) 639.

1st tautomeric form (**HL**(A) on **Scheme 3**): ^{1}H NMR ($CDCl_3$; δ, ppm): 8.80 (d, 1H, CH aromatic pyridinic); 7.34 (d, 1H, CH aromatic pyridinic); 7.99 (t, 1H, CH aromatic pyridinic); 7.64 (t, 1H, CH aromatic pyridinic); 7.50 (m, 7H, CH aromatic); 5.92 (m, 1H, CH from allyl moiety); 8.98 (br, 1H, NH); 5.21 (m, 2H, CH_2=C); 4.50 (t, 2H,

CH_2-N); 2.96 (s, 3H, CH_3). ^{13}C NMR ($CDCl_3$; δ, ppm): 151.22 (C-S); 146.05, 139.01, 128.82, 126.25, 123.96 (C aromatic-pyridinic); 142.61 (C=N - azomethinic); 130.51, 130.42, 129.55, 128.48 (C aromatic-phenilic); 134.00 (CH from allyl moiety); 117.01 (CH_2=); 45.44 (CH_2-N); 13.26 (CH_3).

2nd tautomeric form (**HL**(B) on **Scheme 3**): ^1H NMR ($CDCl_3$; δ, ppm): 9.23 (d, 1H, CH aromatic); 7.47 (d, 1H, CH aromatic); 8.32 (t, 1H, CH aromatic pyridinic); 7.81 (t, 1H, CH aromatic pyridinic); 7.50 (m, 7H, CH); 6.02 (m, 1H, CH from allyl moiety); 5.40 (m, 2H, CH_2=C); 9.57 (br, 1H, NH); 4.11 (t, 2H, CH_2-N); 2.16 (s, 3H, CH_3). ^{13}C NMR ($CDCl_3$; δ, ppm): 148.55 (C-S); 147.83, 135.31, 129.34, 128.17, 127.82 (C aromatic-pyridinic); 145.05 (C=N - azomethinic); 130.94, 130.54, 129.29, 125.46 (C aromatic-phenilic); 131.60 (CH from allyl moiety); 119.02 (CH_2=); 46.85 (CH_2-N); 16.36 (CH_3).

Synthesis of coordination compounds

The complexes (**I-III**) were obtained by stirring a hot solution of **HL** in ethanol with the corresponding copper salts in 1:1 molar ratio: $CuCl_2 \cdot 2H_2O$ **(I)**, $CuBr_2$ **(II)**, $Cu(NO_3)_2 \cdot 3H_2O$ **(III)**. Nickel coordination compound **(IV)** was synthesized similarly, but in 1:2 molar ratio, using $Ni(NO_3)_2 \cdot 6H_2O$ **(IV)**. The complex (**V**) was obtained by stirring a hot solution of **HL·HI** in ethanol with the cobalt salt $Co(CH_3COO)_2 \cdot 4H_2O$ in 1:2 molar ratio.

After cooling green (in case of complexes **I- III**) or brown (in case of complexes **IV-V**) precipitates of corresponding coordination compounds were filtered, washed with small amounts of cold ethanol and dried.

Antimicrobial and antifungal activities. Antimicrobial and antifungal activities were tested on a series of standard strains of gram-positive, gram-negative microorganisms and fungi. It was studied in vitro using the method of two-fold serial dilutions in liquid nutrient medium (meat infusion broth, pH = 7.0) [16]. The substances were dissolved in DMSO in such amounts that the solutions of concentration 10 mg/mL were obtained. The next dilutions were made using meat infusion broth.

***In vitro* antiproliferative activity.** Antiproliferative activity was tested on a series of tumor cells: human leukemia HL-60 cells, cervical cancer HeLa-cells.

Cell Cultures

Human cervical epithelial cells of line HeLa, human promyelocytic leukemia cells of line HL-60 were used in this study. HL-60 line was cultured as monolayer in Roswell Park Memorial Institute (RPMI) 1640 medium containing L-glutamine (2 nM), antibiotics penicillin-streptomycin (final concentration 100 IU/ml penicillin and 100 µg streptomycin /ml) and supplemented with fetal bovine serum (FBS) (10% v/v). Cell line HeLa was cultured in the Dulbecco's Modified Essential Medium (DMEM) with L-glutamine (4 mM), glucose (4.5 g/L), bovine albumin fraction (0,2% v/v), HEPES buffer (N-2 hydroxyethylpiperazine-N'-2-ethane sulfonic acid) (20 mM), antibiotics penicillin-streptomycin (final concentration 100 U/mL penicillin and 100 µg streptomycin /ml) and supplemented with FBS (10% v/v). Cells were maintained at 37°C in a 2-5% humidified CO_2 atmosphere in the incubator in 75-cm^2 culture dishes, and used for experiments between passage 5 and 16. The compounds were dissolved at the time of the experiments.

Cell proliferation Resazurin assay

Cells of line HeLa was trypsinized Trypsin-ethylenediaminetetraacetic acid (trypsin-EDTA) 0.05% (Invitrogen) and counted under an inverted microscope (OLYMPUS). The cell proliferation assay was performed using resazurin (7-hydroxy-3H-phenoxazin-3-one-10-oxide sodium salt) (SIGMA), which allowed us to measure the number of viable cells.

In brief, plate out, in triplicate of $1 \cdot 10^4$ cells in a total of 100 µl medium in 96-well microtiter plates (Becton Dickinson and Company, Lincoln Park, NJ, USA) were incubated at 37 °C, 2% CO_2. Compounds were dissolved in dimethylsulfoxide to prepare the stock solution of 10mM. These compounds and doxorubicin was diluted at multiple concentrations with culture media, added to each well and incubated for 24 hours. Following each treatment, 20 µL resazurin indicator solution was added to each well and incubated for 4 hours. Subsequently, the absorbance was read with 570 nm and 600 nm filters. The measurement was made by imaging hybrid reader (Synergy H1, Biotek).

The percentage inhibition was calculated according to the formula:

$$100-((Abs570nm_{sample}-Abs600nm_{sample})/(Abs570nm_{control}-Abs\ 600nm_{control})\times 100)$$

The IC_{50} values were evaluated by statistical software.

Cell proliferation MTS assay

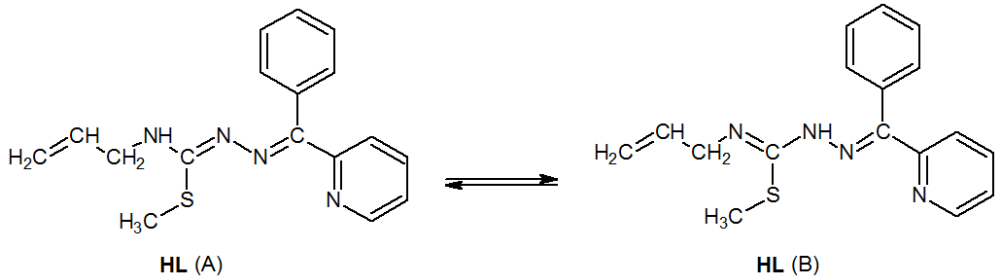
The cell proliferation assay was performed using 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) (Cell Titer 96 Aqueous, Promega, USA), which allowed us to measure the number of viable HL-60 cells. In brief, triplicate cultures of $1 \cdot 10^4$ cells in a total of 100 µl medium in 96-well microtiter plates (Becton Dickinson and Company, Lincoln Park, NJ, USA) were incubated at 37 °C, 5% CO_2. Compounds were dissolved in ethanol to prepare the stock solution of $1 \cdot 10^{-2}$ M. These compounds and doxorubicin (Novapharm, Toronto, Canada) was diluted at multiple concentrations with culture media, added to each well and incubated for 3 days. Following each treatment, 20 µL MTS was added to each well and incubated for 4 h. MTS is converted to water-soluble colored formazan by a dehydrogenase enzyme present in metabolically active cells. Subsequently, the plates were read at 490 nm using a microplate reader (Molecular Devices, Sunnyvale, CA).

Results and discussion

The pro-ligand **HL** and five new metal complexes, $Cu(HL)Cl_2$ (**I**), $Cu(HL)Br_2 \cdot H_2O$ (**II**), $Cu(HL)(NO_3)_2 \cdot 2H_2O$ (**III**), $Ni(HL)_2(NO_3)_2 \cdot H_2O$ (**IV**), $Co(L)_2I$ (**V**) were synthesized in ethanol.

The purity and structure of isothiosemicarbazone **HL** was determined using ^1H and ^{13}C NMR spectroscopy. The alkylation of sulfur atom is proved by the comparative analysis of 2-benzoylpyridine N(4)-allylthiosemicarbazone and N(4)-allyl-S-methylisothiosemicarbazone NMR spectra. A peak in the range of 177-179 ppm that is common for C=S of thiosemicarbazones disappears in the ^{13}C NMR spectrum of the **HL**. Also peaks of carbon atom in methyl group appear in the range 13-17 ppm that are not found in the spectra of corresponding thiosemicarbazone. Peaks of methyl protons appear in the ^1H NMR spectrum of **HL** in the range of 2.16-2.96 ppm [17]. All peaks in the spectra of isothiosemicarbazone

HL are double [18]. It indicates the presence of tautomeric forms of isothiosemicarbazone in solution.



Scheme 3. The tautomeric forms of the ligand HL.

The integral ratio between two tautomeric forms is 1:0.7 (**HL**(A):**HL**(B)).

The synthesis of the coordination compounds is reproducible with good yield. The obtained compounds are microcrystalline solids which are stable in air. The elemental analyses on copper, nickel and cobalt suggest the general formulae $Cu(HL)X_2 \cdot nH_2O$ ($X=Cl^-$, Br^-, NO_3^-; n=0-2), $Ni(HL)_2(NO_3)_2 \cdot H_2O$ and $Co(L)_2I$.

Table 1.Physical and analytical data of the metal complexes (I-V)

No.	Compound	Formula	η[a], %	Found / calculated, metal %	μ_{eff}[b], MB	λ[c]
I	$Cu(HL)Cl_2$ (**I**)	$C_{17}H_{18}Cl_2CuN_4S$	84	14.15/14.28	1.75	165
II	$Cu(HL)Br_2 \cdot H_2O$ (**II**)	$C_{17}H_{20}Br_2CuN_4OS$	82	11.41/11.52	1.80	179
III	$Cu(HL)(NO_3)_2 \cdot 2H_2O$ (**III**)	$C_{17}H_{22}CuN_6O_8S$	77	11.71/11.90	1.79	183
IV	$Ni(HL)_2(NO_3)_2 \cdot H_2O$ (**IV**)	$C_{34}H_{38}NiN_{10}O_7S_2$	83	7.29/7.14	2.94	172
V	$Co(L)_2I$ (**V**)	$C_{34}H_{34}CoIN_8S_2$	80	7.20/7.32	dia[d]	103

a – yield; b – effective magnetic moments at room temperature (293K); c – molar conductivity in methanol at room temperature, $\Omega^{-1} \cdot cm^2 \cdot mol^{-1}$; d – diamagnetic.

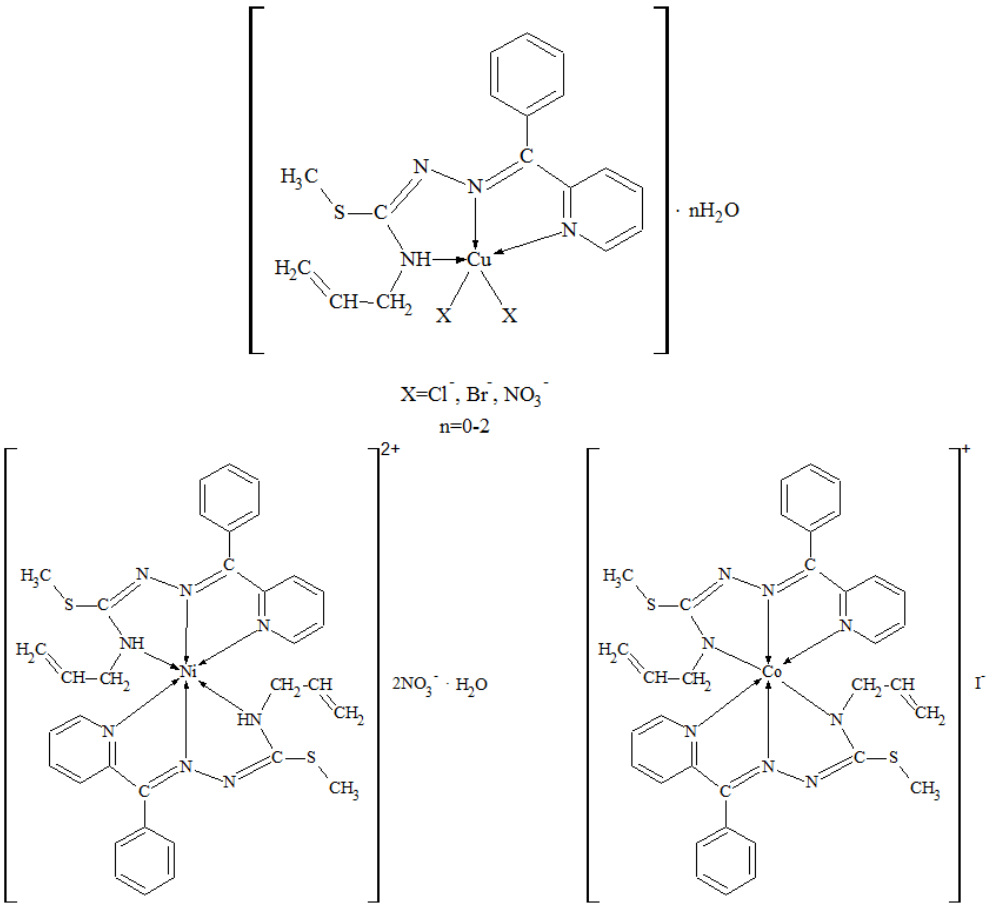
The way of the isothiosemicarbazone **HL** coordination to the central ions was elucidated from comparative analysis of IR spectra of complexes **I-V** and the pro-ligand **HL** [19-20].

It was determined that the isothiosemicarbazone **HL** behaves like a tridentate ligand with NNN-set of donor atoms. It coordinates to the central ions by pyridine, azomethine and deprotonated (in case of cobalt complex) or non-deprotonated (in case of copper and nickel complexes) thiocarbamide nitrogen atoms forming five-membered metallacycles. The proposed distribution of chemical bonds in the coordination compounds is shown in scheme 4.

The molar conductivity values of the coordination compounds (**I-IV**) are in the range 165 - 183 $\Omega^{-1} \cdot cm^2 \cdot mol^{-1}$ that indicates that complexes represent 1:2 electrolytes. The corresponding anion (Cl^-, Br^-, NO_3^-) can be either in the outer sphere or in the inner sphere as it can be easily substituted by the solvent molecule during dissolution process. The molar conductivity value of the cobalt coordination compounds (**V**) is 103 $\Omega^{-1} \cdot cm^2 \cdot mol^{-1}$ that indicates that this complex represents an 1:1 electrolyte.

The magnetochemical research showed that the synthesized copper coordination

compounds **(I-III)** have monomeric structure because the effective magnetic moments for the synthesized complexes (**I-III**) vary in the range of 1.75-1.80 μ_B which are close to the spin value for one unpaired electron. The nickel complex **(IV)** have octahedral structure, because its effective magnetic moment is 2.94 μ_B. The cobalt complex **(V)** are diamagnetic that indicates that cobalt (II) is oxidized by oxygen from air to cobalt (III) during the synthesis and this coordination compound have octahedral structure.



Scheme 4. Proposed distribution of chemical bonds in the metal complexes.

In order to find out the biological properties of these substances their antimicrobial, antifungal and antiproliferative activities were studied.

Table 2. The minimum inhibitory concentration (MIC) and minimum bactericide concentration (MBC) (µg/mL).

Compound	Escherichia coli		Klebsiella pneumoniae		Staphylococcus aureus		Candida albicans	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
HL	60	120	120	250	0.7	0.7	120	250
$Cu(HL)Br_2 \cdot H_2O$	30	30	120	250	7	7	250	250
$Cu(HL)(NO_3)_2 \cdot 2H_2O$	60	60	120	250	1.5	1.5	60	120
$Co(L)_2I$	120	120	120	250	15	30	60	60

The study of antimicrobial and antifungal activities (table 2) showed that **HL** and its coordination compounds possess bacteriostatic and bactericidal activities. The activity of **HL** towards gram-negative microorganisms and fungi is less pronounced than towards gram-positive microorganisms. The coordination of **HL** ligand to copper(II) ions results in reduction of minimum inhibitory concentrations and minimum bactericide concentrations towards *Escherichia coli*. The cobalt complex possesses better activity towards fungi *Candida albicans*.

The antitumor activity of synthesized compounds was studied on human leukemia HL-60 cells and cervical cancer HeLa cells. From the obtained data of HL-60 and HeLa cells inhibition it was calculated the IC_{50} value of tested substances.

Table 3. Antiproliferative activity of some synthesized compounds on human leukemia (HL-60) cells at three concentrations and their IC_{50} values.

Compound	Inhibition of cell proliferation (%)[a]			IC_{50}, μM
	10 μM	1 μM	0.1 μM	
HL	90	72	8	0.50
HL·HI	89	69	13	0.50
$Cu(HL)Cl_2$	98	95	30	0.28
$Co(L)_2I$	93	91	0	0.42
Doxorubicin	99	98	15	0.36

[a]SEM < ±4% of a single experiment in triplicate. The IC_{50} values were calculated using statistical software.

The proliferation of human leukemia HL-60 cells in presence of **HL** and synthesized coordination compounds is shown in scheme 5.

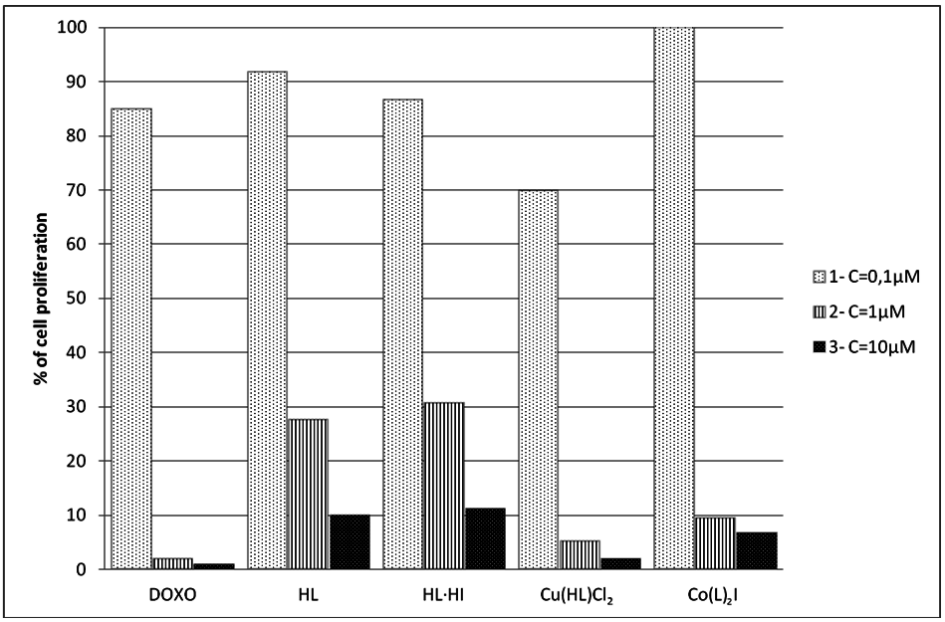
The isothiosemicarbazone **HL** and its coordination compounds manifest antitumor activity towards HL-60 cells at concentrations 10μM and 1μM. The activity at lower concentration (0.1 μM) decreases and practically disappears in case of cobalt complex. The activity of copper(II) coordination compound surpasses the activity of the pro-ligand **HL** at all studied concentrations and also surpasses the activity of doxorubicin, used in medical practice, at 0.1 μM concentration.

The study of antiproliferative activity towards cervical cancer HeLa cells showed that the synthesized coordination compounds possess higher activity towards these cancer cells than doxorubicin (Table 4). The activity of complexes at lower concentration (0.1 μM) practically disappears.

Table 4. Antiproliferative activity of some synthesized compounds on cervical cancer (HeLa) cells at three concentrations and their IC_{50} values.

Compound	Inhibition of cell proliferation (%)[a]			IC_{50}, μM
	10 μM	1 μM	0.1 μM	
$Cu(HL)Cl_2$	48.5	0	0	>10
$Cu(HL)Br_2·H_2O$	100	100	0	0.4
$Ni(HL)_2(NO_3)_2·H_2O$	85.5	17.8	0	5.2
$Co(L)_2I$	100	44.5	6.3	1.7
Doxorubicin	49.8	12.2	0	10.0

[a]SEM < ±4% of a single experiment in triplicate. The IC_{50} values were calculated using statistical software.



Scheme 5. Proliferation of HL-60 cells in presence of tested substances.

Conclusions

In this work 2-benzoylpyridine N(4)-allyl-S-methylisothiosemicarbazone was synthesized and studied using NMR spectroscopy. This ligand was used for synthesis of the copper(II), nickel(II) and cobalt(III) coordination compounds. It was determined that pro-ligand and its coordination compounds show antibacterial and antifungal activities in the range of concentration 0.7-250 μg/mL. The most vulnerable to synthesized substances is *Staphylococcus aureus*. The study of antitumor activity showed that synthesized substances inhibit proliferation of the human leukemia HL-60 and cervical cancer HeLa cells. The activity of tested substances towards HL-60 cells is less pronounced than towards HeLa cells.

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