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*Original scientific paper*

# Synthesis and efficacy of copper(II) complexes bearing *N*(4)-substituted thiosemicarbazide and diimine co-ligands on plasmid DNA and HeLa cell lines

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*Abstract*: This present work deals with the synthesizes of nine novel thiosemicarbazone copper(II) complexes {[Cu(L)$_2$]Cl **C3**, [Cu(L)(bpy)]Cl **C4**–**C6**, [Cu(L) (phen)]Cl **C7**–**C9** (where, L = H(L1)–H(L3), H(L1) = (E)-*N*-methyl-2--(1-phenyl-2-((5-(pyridin-3-yl)-4*H*-1,2,4-triazol-3-yl)thio)ethylidene)hydrazinecarbothioamide, H(L2) = (E)-*N*-ethyl-2-(1-phenyl-2-((5-(pyridin-3-yl)-4*H*--1,2,4-triazol-3-yl)thio)ethylidene) hydrazinecarbothioamide, H(L3) = (E)-*N*--phenyl-2-(1-phenyl-2-((5-(pyridin-3-yl)-4*H*-1,2,4-triazol-3-yl)thio)ethylidene) hydrazinecarbothioamide, bpy = 2,2′-bipyridyl and phen = 1,10-phenanthroline) with improved pharmacological results. The synthesized complexes were characterized by various spectral-analytical techniques. The structure of the copper(II) complexes **C1**–**C9** was proposed by EPR spectroscopy. It confirmed the square planar coordination around Cu(II) complexes. The antibacterial screening of the complexes revealed that complexes **C7** and **C8** demonstrated significant activity against Gram-positive (*B. thuringiensis*) and Gram-negative (*E. coli*) bacteria. The concentration-dependent DNA cleavage activity of supercoiled (SC) pUC18 DNA exhibited complete DNA degradation effect on complex **C6** at a minimum concentration of 40 μM. *In vitro* cytotoxic results showed that the mixed ligand copper(II) complexes **C4**, **C5** and **C7** exhibited higher effects on human cervical cancer cell lines, HeLa, when compared to cisplatin. Hence, the results obtained from each biological screening indicated the superior biological efficacy of the mixed ligand copper(II) complexes bearing diimine moieties. It could be considered as a promising alternative to an existing anticancer drug.

*Keywords*: thiosemicarbazone; heterocyclic compounds; copper(II) complexes; diimine; cytotoxicity.

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## INTRODUCTION

Over a few decades, platinum-based metal compounds have had a tremendous starting point in the development of anticancer drugs. However, the clinical success of cisplatin, oxaliplatin and carboplatin exhibited limited action against various cancers due to severe side effects, immune efficiency, less pharmacological stability and toxicity.[1,2] As a result, several efforts were made to prevail with the development of thiosemicarbazone copper(II) complexes to reduce the toxicity and increase specificity. Thiosemicarbazones metal complexes are an important group of compounds in the field of inorganic and bioinorganic chemistry. Moreover, these compounds were synthesized by simple and cost-effective procedures with small changes in their structures. Such changes in the thioamide nitrogen and its metal complexes provided interesting results due to their π-delocalization over the ring system.[3,4]Among them, some developed biologically active thiosemicarbazone compounds, such as triapine (3--aminopyridine-2-carboxaldehyde thiosemicarbazone), di-2-pyridylketone thiosemicarbazone, 2-benzoylpyridine thiosemicarbazone, di(2-pyridyl)ketone 4,4--dimethyl-3-thiosemicarbazone, di(2-pyridyl)ketone 4-cyclohexyl-4-methyl-3--thiosemicarbazone, 2-formyl thiosemicarbazone, 2-formyl-4-(*m*-amino)phenylpyridine thiosemicarbazone and 2-formyl(*m*-amino)phenylpyridine thiosemicarbazones, are currently under various stages of preclinical trials and have emerged for the development a new selective anticancer drug.[5–7]

Recently, Haribabu and co-workers synthesized *in vitro* anticancer compounds, *i.e*., water-soluble ruthenium metal complexes with substituted thiosemicarbazone derivatives. These mono and binuclear complexes showed significant toxicity against human lung carcinoma (A549) and human liver carcinoma cell lines (HepG-2).[8] More recently, Kallus (2019) *et al* demonstrated that iron(III) and copper(II) complexes of biotin-conjugated thiosemicarbazone were quite interesting owing to their improved *in vivo* anticancer activity against CT-26 colon cancer-bearing mice.[9] Presently, DNA interaction and anticancer activities of copper(II) complexes were extensively studied in association with both metabolism and oxidative DNA damage. Based on the assumption, investigations of copper-thiosemicarbazone-based complexes have been widely studied. In addition to this, metal complexes with diimine moieties have demonstrated potential *in vitro* anticancer activity with strong interaction and also capable to be exploited as DNA-targeted anticancer drug.[10,11] Based on these observations, the present study was undertaken to synthesize and characterize new thiosemicarbazone-based copper(II) complexes derived from (*E*)-*N*-methyl-2-(1-phenyl-2--((5-(pyridin-3-yl)-4*H*-1,2,4-triazol-3-yl)thio)ethylidene) hydrazine carbothioamide H(L1), (E)-*N*-ethyl-2-(1-phenyl-2-((5-(pyridin-3-yl)-4*H*-1,2,4-triazol-3--yl)thio)ethylidene)hydrazine carbothioamide H(L2) and (E)-*N*-phenyl-2-(1--phenyl-2-((5-(pyridin-3-yl)-4*H*-1,2,4-triazol-3-yl)thio)ethylidene)hydrazinecarbo-

thioamide H(L3) with a varying suitable substitutions at the terminal nitrogen. Thus, the present study was aimed at achieving the biological effect of these compounds for clinical application using the disc diffusion method, agarose gel electrophoresis and MTT assay.

## EXPERIMENTAL

### *Materials and methods*

All the reagents and solvents used for the synthesis of ligands and copper(II) complexes were of analytical or spectroscopic grade. The commercially available chemicals were purchased from Sigma–Aldrich, such as 4-methyl-3-thiosemicarbazide, 4-ethyl-3-thiosemicarbazide, 4-phenyl-3-thiosemicarbazide, phenacyl bromide, 5-(3-pyridyl)-4*H*-1,2,4-triazole-3--thiol, 2,2′-bipyridyl, 1,10-phenanthroline and copper(II) chloride. The chemicals Tris–HCl, boric acid, tris-base, ethidium bromide, EDTA (ethylenediaminetetraacetic acid) disodium salt used for the DNA cleavage studies were procured from Merck. The chemicals used for biological studies, such as penicillin, streptomycin, chloramphenicol, 10 % fetal bovine serum (FBS) and MTT (3-(4,5-dimathylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) were acquired from Himedia, India. The pUC18 DNA was obtained from GeNei, Bangalore, India, and stored at –20 °C. The cervical cancer cell lines (HeLa) were purchased from the National Centre for Cell Science (NCCS), Pune, India.

The molar conductance of the ligands as well as the complexes was analyzed using Elico CM 183 EC-TDS analyzer in $1\times10^{-3}$ M solution in DMF at room temperature. The elemental analyses (C, H, N and S) were obtained from Vario EL-III elemental analyzer. The $^{1}$H- and $^{13}$C-NMR spectra of free thiosemicarbazone ligands were recorded on a 300 MHz spectrophotometer using $CDCl_3$ or DMSO-$d_6$ as solvent and TMS as an internal standard. The electronic spectra of the synthesized ligands and copper(II) complexes were taken using a JASCO V-630 UV–Vis spectrophotometer within the range 200–1000 nm using $1\times10^{-3}$ M solution in DMF. The FT-IR spectra were recorded on a Shimadzu IR Tracer 100 spectrophotometer as KBr discs in the range 4000–400 $cm^{-1}$. The electrochemical performance of the copper(II) complexes was realized using an electrochemical analyzer on 600 C version 5.01 in DMF using platinum and Ag–AgCl as a working and reference electrode, respectively. Tetrabutylammonium perchlorate (TBAP, 0.1 M) was used as supporting electrolyte. The ESI-MS spectrum of the copper(II) complex was identified using Bruker Daltonics. The X-band EPR spectra of the copper(II) complexes were taken at room temperature as well as frozen solutions in DMF by Varian ESR-112 Sspectrometer and ESR JEOL JES-FA200, respectively.

Analytical and spectral data of the complexes are given in Supplementary material to this paper.

### *Evaluation of the biological activity*

*Minimum inhibitory concentration (*MIC*)*. The antibacterial activity of the synthesized thiosemicarbazone ligands H(L1)–H(L3) and copper(II) complexes **C1**–**C9** were tested using the method described earlier.[12] The minimum inhibitory concentration of the synthesized compounds (as 5 and 50 μg $ml^{-1}$) against *B. thuringiensis* (Gram-positive) and *E. coli* (Gram-negative) was evaluated in mm using the disc diffusion method in nutrient agar medium. Chloramphenicol and DMF were used as the positive and negative control, respectively. Filter paper of about 6 mm diameter was dipped into the compounds and placed in the nutrient agar plate inoculated with microorganisms under sterile conditions. Then the plates were incubated at 37 °C for 24 h to observe the zone of inhibition.

*DNA Cleavage activity*. The DNA cleavage efficiency of the thiosemicarbazone ligands and their copper(II) complexes were assessed by agarose gel electrophoresis.[13] The experiments were performed at 40 and 60 μM concentration of the complexes treated with SC pUC18 DNA under the experimental conditions in 5 mM Tris–HCl/50 mM NaCl buffer at pH 7.2 and incubated for 1 h at 37 °C in the presence of an activating agent (ascorbic acid). Later, the sample was mixed with the gel loading buffer (25 % bromophenol blue, 0.25 % xylene cyanol, 30 % glycerol) and loaded on to 1.2 % agarose gel containing 0.5 mg/mL ethidium bromide for 1 h. After electrophoresis, the extent of DNA cleavage of the copper(II) complexes was visualized and photographed by Spectroline ultraviolet transilluminator (Uvitec GeNeiTM fire reader, India) gel documentation system. A complete conversion of SC DNA (Form I) to NC DNA (Form II) and linear (Form III) was monitored.

In vitro *cytotoxicity analysis*

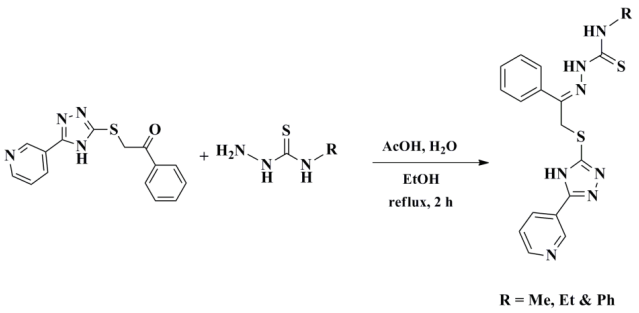
*Maintenance of the cell lines*. The human cervical carcinoma cell line (HeLa) was routinely maintained in Eagle's minimal essential medium (MEM) containing penicillin (100 U $mL^{-1}$) and streptomycin (100 μg $ml^{-1}$) and supplemented with 10 % fetal bovine serum (FBS, Himedia, India) in a humidified atmosphere of 5 % $CO_2$ incubator at 37 °C. Cisplatin (Platinex) was used as the positive control and the cells without treatment as the negative control.

*MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay*. The complex stimulated cell toxicity study was realized using human cervical cancer cell line (HeLa). It is mainly based on the metabolic reduction of soluble MTT to insoluble formazan crystal by mitochondrial enzyme activity of viable cells.[14] The HeLa cell line was seeded onto 96 well plates with a seeding density of $2\times10^5$ cells/well and kept in a $CO_2$ incubator at 37 °C for attachment of the cells. After 16 h, the test sample at 5 μM concentration was added and incubated for 24 h. To the cells were added 20 μL of MTT (5 mg $mL^{-1}$ in PBS buffer) and kept for 4 h. Then the supernatant was completely removed and 50 μL of DMSO was added into each well to dissolve the precipitants. The absorbance was measured at 570 nm using an Elisa plate reader (Read well Touch, Robonik, India) with a reference wavelength at 690 nm. The assay was performed in triplicate and the values were compared with that of the positive control (cisplatin).

*Synthesis of thiosemicarbazone ligands*

*Preparation of 1-phenyl-2-((5-(pyridin-3-yl)-4*H*-1,2,4-triazol-3-yl)thio)ethanone (HL)*. The starting material HL was synthesized by stirring (2 h) in a 1: 1 molar ratio 5-(3-pyridyl)-4*H*-1,2,4-triazole-3-thiol (0.18 g, 1 mmol) and phenacyl bromide (0.20 g, 1 mmol) in dimethylformamide (2 mL) and ethanol (10 mL). Then, the obtained colorless solid was set aside under cold conditions for a day. It was then filtered and washed with cold ethanol and dried *in vacuo* over anhydrous $CaCl_2$.

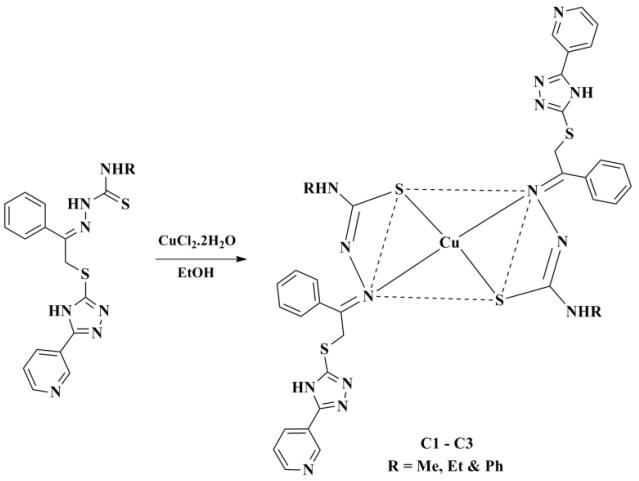
*Preparation of H(L1)–H(L3)*. The compound H(L1) was synthesized using the following procedure: 1-phenyl-2-((5-(pyridin-3-yl)-4*H*-1,2,4-triazol-3-yl)thio)ethanone (0.30 g, 1 mmol) in ethanol was mixed with 4-methyl-3-thiosemicarbazide (0.12 g, 1 mmol) in water (20 mL). To this, a few drops of glacial acetic acid were added and the mixture was refluxed for 2 h at 90 °C in a water bath. The resulting clear yellow solution was set aside under cold condition for 24 h in a refrigerator. Thereafter the obtained yellow solid was filtered, dried and recrystallized from chloroform. The similar method was applied for the synthesis of H(L2) and H(L3), but using 4-ethyl-3-thiosemicarbazide or 4-phenyl-3-thiosemicarbazide, respectively, instead of 4-methyl-3-thiosemicarbazide (Scheme 1).




Scheme 1. Schematic representation of synthesis of thiosemicarbazone ligands.

*Synthesis of copper(II)* bis *complexes (**C1**–**C3**)*

To a 2 mmol ethanolic solution of ligand H(L1), a solution of copper(II) chloride dihydrate (1 mmol in ethanol) was added over 1 h under constant stirring to obtain a clear green solution. The resulting solution was set aside overnight at room temperature. Subsequently, the obtained green precipitate was washed several times with cold ethanol and dried to afford complex CI (Scheme 2). A similar method was applied for the synthesis of complexes **C2** and **C3** using H(L2) and H(L3), respectively, instead of H(L1).

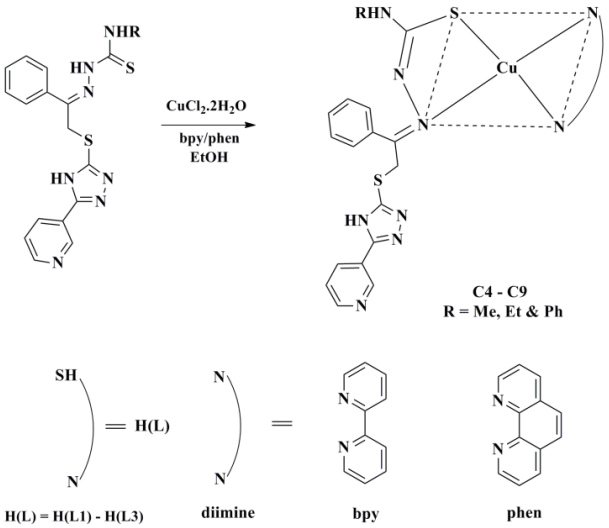



Scheme 2. Schematic representation of the preparation of copper(II) bis complexes **C1**–**C3**.

*Synthesis of mixed ligand copper(II) complexes (**C4**–**C9**)*

To a 2 mmol ethanolic solution of ligand H(L1), a solution of 2,2′-bipyridyl (1 mmol in ethanol) was added over 1 h under constant stirring. To this solution, copper(II) chloride dihydrate was added dropwise and again stirred for 30 min. Subsequently, the obtained green precipitate was washed several times with cold ethanol and dried. A similar method was applied for the synthesis of complexes **C5** and **C6** using H(L2) and H(L3), respectively, instead of H(L1) (Scheme 3).

Complexes **C7**–**C9** were prepared in a similar manner to **C4**–**C7**, respectively, using 1,10-phenanthroline instead of 2,2′-bipyridyl (Scheme 3).




Scheme 3. Schematic representation of the preparation of the mixed ligand copper(II) complexes **C4**–**C9**.

## RESULTS AND DISCUSSION

### *$^{1}$H- and $^{13}$C-NMR spectra of the thiosemicarbazone ligands H(L1) – H(L3)*

The structures of the free thiosemicarbazone ligands were verified by their $^{1}$H- and $^{13}$C-NMR spectra in DMSO-$d_6$. The $^{1}$H-NMR spectra of thiosemicarbazone ligand H(L1) is given in the Supplementary material, Figs. S-1 and S-2. All the synthesized thiosemicarbazone ligands H(L1)–H(L3) showed a singlet of the hydrazinic proton at $\delta$ 10.88 and 11.08 ppm for the methyl and ethyl substituted thiosemicarbazone ligands H(L1) and H(L2) except for H(L3) that appeared in $\delta$ 11.60 ppm as a result of the inductive effect of the electron withdrawing phenyl group.[15,16] A sharp singlet of azomethine proton was observed at around $\delta$ 8.55 ppm. The signals at around $\delta$ 7.50–6.90 ppm were attributed to the aromatic protons of the ligands. The chemical shift of $CH_2$ of the free ligand was found at about $\delta$ 4.50 ppm. The signals found near $\delta$ 6 ppm were assigned to methyl and ethyl protons. Similarly, in the $^{13}$C-NMR spectrum, signals of C=N and C=S appeared within the region of $\delta$ 145 and 175 ppm, respectively. The signals due to aromatic carbons were observed in the expected region ($\delta$ 140–112 ppm). The signals corresponding to the $CH_2$ carbon showed a signal near to 38.5 ppm.[17] The rest of the methyl and ethyl carbon appeared in the downfield region. Hence, it was clear that the data obtained from the $^{1}$H-NMR and $^{13}$C-NMR data were in good agreement with each other and confirmed the structures of the thiosemicarbazone ligands. The data of the other thiosemicarbazone derivatives, H(L2) and H(L3), are given in the Supplementary material.

*Electronic spectra*

The electronic spectra of thiosemicarbazone ligands H(L1)–H(L3) and their respective copper(II) complexes **C1**–**C9** provided good evidence for the geometry of the complexes. The electronic spectra and the absorption spectral data of ligand H(L1) and complex **C5** are given in the Supplementary material, Figs. S-3 and S-4, respectively. The electronic spectra of thiosemicarbazone ligands in DMF displayed two intense absorption bands near 240 and 350 nm that are assigned to $\pi\rightarrow\pi^*$ and $n\rightarrow\pi^*$ transitions, respectively.[18] The band around 240 nm corresponding to the azomethine chromophore experienced a bathochromic shift as a result of the donation of the lone pair of electrons to the central metal ion. The absorption band of the thioamide chromophore near 350 nm experienced blue shift owing to thioenolization.[11,18] In the electronic spectra of copper(II) complexes displayed three to four bands. A significant low intensity d→d band was observed near 600–750 nm for all the copper(II) complexes. This might be due to the structural differences of the complexes through the extended conjugation of the present aromatic ring. However, complex **C9** exhibited a moderate intense band in the region 735 nm due to $^2B_{1g}\rightarrow{}^2A_{1g}$ transitions of square planar copper(II) complexes.[19] Noticeably, all the thiosemicarbazone copper(II) complexes in DMF were in concurrence with the $d^9$ electronic configuration of the Cu(II) ion.

*FT-IR spectroscopy*

The coordinating sites of the copper and free thiosemicarbazone ligands were attained from FT-IR spectroscopy. The characteristics FT-IR spectral data afforded suitable evidence for the coordination of ligand to the central metal ion through deprotonated sulfur, the azomethine nitrogen and *N*,*N*′-donor of heterocyclic bases. The free thiosemicarbazone ligands displayed ν(N(4)–H) band around 3055–3350 $cm^{-1}$.[19] In general, thiosemicarbazone can exist in thione––thiol tautomerism. A strong band around 800–856 $cm^{-1}$ in the ligand was attributed to the presence of –NH–C(=S)–N group that might be converted to C–S in the spectra of complexes as a result of enothiolization by loss of the N(2)H hydrogen and the formation of a new –C=N– group, found at around 1530–1560 $cm^{-1}$.[15] In addition, a characteristic high intensity C=N band of a ligand in the region of 1520–1540 $cm^{-1}$ could be shifted to higher frequencies (1550–1580 $cm^{-1}$) in the spectra of complexes, suggesting the coordination of azomethine to copper. The ν(N–N) band of thiosemicarbazone was found near to 970–990 $cm^{-1}$ and this might be increased by 20–25 $cm^{-1}$ in the complexes upon chelation that increases in the double bond character as well as a loss of electron density.[20] The infrared spectra of complexes showed medium bands around 420 and 550 $cm^{-1}$, which is consistent with ν(Cu–N) and ν(Cu–S), respectively.[17]

*EPR spectral study*

The EPR spectra of the thiosemicarbazone copper(II) complexes offer valuable information regarding the extent of delocalization of the unpaired electron in copper(II) complexes, the geometry and metal ligand bonding. The EPR spectra of the complexes were recorded at room temperature and at liquid nitrogen temperature (77 K) to facilitate the determination of EPR parameters, such as $\boldsymbol{g}_{\|}$, $\boldsymbol{g}_{\perp}$, $\boldsymbol{A}_{\|}$ and $\boldsymbol{G}$. The X-band EPR spectra of mixed ligand thiosemicarbazone copper(II) complex **C7** in DMF are presented in the Supplementary material, Fig. S-5). The X-band EPR spectra of the copper(II) complexes displayed three well-resolved peaks at $g_{\|}$ region, corresponding to nuclear spin $I = 3/2$. The thiosemicarbazone copper(II) complexes were found to be $\boldsymbol{g}_{\|}$ (2.252–2.268) > $g_{\perp}$ (2.056––2.074) > 2.0023, which revealed that the unpaired electron of the copper(II) complexes had a $d_{x^2-y^2}$ ground state that is characteristic of square planar geometry.[21] The exchange interaction parameter of the copper(II) complexes could be calculated using the following expression: $G = ((g_{\|} - 2)/(g_{\perp} - 2))$ and values within 3.55–4.54 were found, indicating negligible interaction in the copper(II) complexes according to Hathaway.[22] Meanwhile, $\boldsymbol{g}_{\|}$ values less than 2.3 were found for these complexes, suggesting the covalent nature of the copper(II) complexes.

It is renowned that a $CuN_4$ chromophore with square planar geometry is expected to display $\boldsymbol{g}_{\|}$ and $\boldsymbol{A}_{\|}$ values within the range of 2.220 and (180–200)×$10^{-4}$ $cm^{-1}$, respectively.[23,24] The replacement of any coordinated nitrogen by sulfur/ /nitrogen would increase the $\boldsymbol{g}_{\|}$ value and decrease the $\boldsymbol{A}_{\|}$ likewise. At this point, the observed values for copper(II) bis complexes ($CuN_2S_2$) **C1**–**C3** and mixed ligand copper(II) complexes ($CuN_3S$) **C4**–**C9** were found to be $\boldsymbol{g}_{\|}$ (2.252 to 2.268) and $A_{\|}$ (158 to 163×$10^{-4}$ $cm^{-1}$), revealing the presence of square planar geometry. Furthermore, the geometrical distortions of the copper(II) complexes were measured using the $\boldsymbol{g}_{\|}/\boldsymbol{A}_{\|}$ ratio. In general, the values for square planar complexes fall between 130–138×$10^{-4}$ cm. The values for the synthesized copper(II) complexes were in the range (138–142)×$10^{-4}$ cm, suggesting that the complexes reliably have perfect square planar geometry with the absence of any significant distortion from planarity.[25] This was further supported by molar conductivity measurement in DMF the obtained values of which fall in the neutral and 1:1 electrolyte range of copper(II) bis complexes and mixed ligand copper(II) complexes, respectively. [26]

*High resolution mass spectra*

The stoichiometric composition of the synthesized thiosemicarbazone copper(II) complexes was identified using high-resolution mass spectroscopy and their molecular ion peaks was used to authenticate the proposed formulae of the complexes. The high-resolution mass spectra of complex **C9** is given in the Supplementary material, Fig. S-6. It showed a molecular ion peak at *m*/*z* 765.16,

which corresponds to the existence of 99 % of the relative abundance of $[C_{34}H_{26}CuN_9S_2]^+Cl^-$ species with an ethanol adduct. Thus, the *m/z* of **C9** confirmed the stoichiometry of complex **C9** as a [Cu(L3)(phen)]Cl type.

*Electrochemistry*

The electrochemical actions of the thiosemicarbazone copper(II) complexes were studied using cyclic voltammetry and differential pulse voltammetry (Supplementary material, Fig. S-7 and Table S-I). The measurements were performed using a platinum working electrode and TBAP as the supporting electrolyte in DMF. The Cu(II)/Cu(I) redox potential (DPV) of the bis copper(II) complexes and mixed ligand copper(II) complexes of bpy/phen could be in the following order: 0.601 (**C1**) > 0.529 (**C2**) > 0.471 (**C3**); 0.637 (**C4**) > 0.598 (**C5**) > 0.446 (**C6**); 0.643 (**C7**) > 0.612 (**C8**) > 0.587 (**C9**). The Cu(II)/Cu(I) redox potential values of the mixed ligand copper(II) complexes **C4**, **C5**, **C8** and **C9** were comparatively more positive than the copper(II) bis complexes, which might be attributed to the existence of electron donating –NMe, –NEt groups and diimine co-ligands. It was shown that the incorporation of –NMe, –NEt and bpy/phen in the coordination would predominantly raise the Cu(II)/Cu(I) redox potential rather than –NPh electron withdrawing substituents. Thus, in the case of copper(II) bis complexes **C1** and **C2**, were slightly more positive upon $–NMe_2$ and $–NEt_2$ substitution. Similarly, **C3** with two electron withdrawing groups ($–NPh_2$) shows lower redox potentials than **C1** and **C2**. Therefore, the mixed ligand copper(II) complexes facilitating Cu(II)→Cu(I) reduction were more feasible than the copper(II) bis complexes.[24,27]

*Pharmacological results*

In vitro *antibacterial activity*. The comparative results of antibacterial activity of thiosemicarbazone ligands H(L1)–H(L3) and their copper(II) complexes **C1**–**C9** in association with a zone of inhibition with the standard positive control (chloramphenicol) are presented in the Supplementary material, Table S-II. The tabulated antibacterial results suggested the following: the thiosemicarbazone derivatives displayed minimal activity against *E. coli* at 50 μg $ml^{-1}$ concentration and showed no activity on the other tested Gram-positive bacteria, specifically *B. thuringiensis*. Only the copper(II) complexes **C7** and **C8** showed higher antibacterial activity and act as a potent bacteriostatic agent against both bacterial strains in comparison with chloramphenicol. This was due to the existence of *N*(4)-substituted thiosemicarbazones and diimine co-ligands.[12] On the other hand, all the other complexes were found to exhibit only moderate activity. This could be explained based on the chelation theory.[11] On chelation, the polarity of the copper(II) complexes would be reduced owing to the overlapping of the donor groups of a ligand with the orbital and partial positive charge of the copper. Moreover, it enhances the delocalization of π-electrons over the chelating ring system as well

as the lipophilicity of the complexes.[28] This might enhance the penetration of lipid layer and therefore affects the growth of the microorganisms.

*Cleavage of pUC18DNA by the copper(II) complexes*. The effect of concentration-dependent DNA cleavage efficiency of the copper(II) complexes on pUC18 DNA in the presence of the activating agent was studied by agarose gel electrophoresis (Figs. 1 and 2). It is interesting to note that many approved anticancer agents can trigger cell death by damaging DNA. For this reason, the extent of DNA cleavage activity of thiosemicarbazone-based copper(II) complexes has been considered for the development of a new novel anticancer drug.[29] During electrophoresis, relatively fastest migration was observed in Form I. While, Form II occurs on the strand by the nick produced. If both strands were cleaved, linearized form (Form III) is generated that migrates in the midst of Forms I and II.

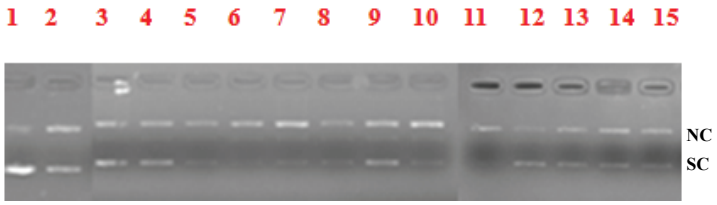



Fig. 1. a) Gel electrophoresis of copper(II) complexes (40 μM) in buffer containing 50 mM Tris/-–HCl/50 mM NaCl (pH 7.2) in the presence of ascorbic acid at 37 °C. Lane 1: DNA control; lane 2: DNA + ascorbic acid; lane 3: DNA + ligand HL+ ascorbic acid; lane 4: DNA + ligand H(L1)+ ascorbic acid; lane 5: DNA + $[Cu(L1)_2]$ + ascorbic acid; lane 6: DNA + [Cu(L1)(phen)]Cl + ascorbic acid; lane 7: DNA+[Cu(L1)(bpy)]Cl + ascorbic acid; lane 8: DNA + ligand H(L3) + ascorbic acid; lane 9: DNA + $[Cu(L3)_2]$Cl + ascorbic acid; lane 10: DNA + [Cu(L3)(phen)]Cl + ascorbic acid; lane 11: DNA + [Cu(L3)(bpy)]Cl + ascorbic acid; lane 12: DNA + ligand H(L2) + ascorbic acid; lane 13: DNA + $[Cu(L2)_2]$ + ascorbic acid; lane 14: DNA + [Cu(L2)(phen)]Cl + ascorbic acid; lane 15: DNA + [Cu(L3)(bpy)]Cl + ascorbic acid.

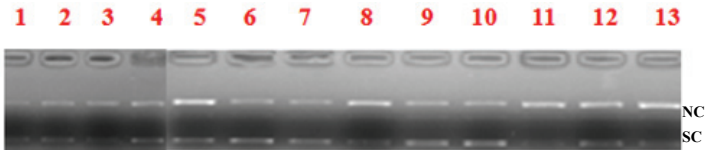



Fig. 2. b) Gel electrophoresis of copper(II) complexes (60 μM) in buffer containing 50 mM Tris–HCl/50 mM NaCl (pH 7.2) in the presence of ascorbic acid at 37 °C. Lane 1: DNA + ligand HL + ascorbic acid; lane 2: DNA + ligand H(L1) + ascorbic acid; lane 3: DNA + $[Cu(L1)_2]$ + ascorbic acid; lane 4: DNA + [Cu(L1)(phen)]Cl + ascorbic acid; lane 5: DNA + [Cu(L1)(bpy)]Cl + ascorbic acid; lane 6: DNA + ligand H(L3) + ascorbic acid; lane 7: DNA + $[Cu(L3)_2]$Cl + ascorbic acid; lane 8: DNA + [Cu(L3)(phen)]Cl + ascorbic acid; lane 9: DNA + [Cu(L3)(bpy)]Cl + ascorbic acid; lane 10: DNA + ligand H(L2) + ascorbic acid; lane 11: DNA + $[Cu(L2)_2]$ + ascorbic acid; lane 12: DNA + [Cu(L2)(phen)]Cl + ascorbic acid; lane 13: DNA + [Cu(L3)(bpy)]Cl + ascorbic acid.

As illustrated in Fig. S-7a, the cleavage activity of plasmid DNA at 40 μM concentration involved the effect of complete DNA degradation on **C6** into unnoticeable minor fragments in the presence of ascorbic acid. The predominant DNA cleavage efficiency of **C6** could be strong intercalation of the diimine moiety (bpy) and the generation of a large amount of reactive oxygen species by stabilizing Cu(I) species.[24] However, other complexes showed partial and moderate activity. In addition, under the same experimental conditions at 60 μM concentration resulting in the complete conversion of Form I to Form II by means of increasing concentration of copper(II) complexes. However, no cleavage activity was observed in the control DNA and DNA with ascorbic acid.

In vitro *cytotoxic evaluation by the MTT assay*

Encouraged by the positive results achieved from the antibacterial and oxidative DNA cleavage activities, the *in vitro* cytotoxicity of the thiosemicarbazone ligands and copper(II) complexes were tested against human cervical cancer cell line (HeLa). The percentage of cytotoxicity of each compound is displayed in Table S-III of the Supplementary material. The thiosemicarbazone ligands alone showed less cytotoxicity towards HeLa cell lines. Interestingly, complexation of thiosemicarbazone ligands with heterocyclic bases would predominantly enhance their cytotoxicity even at 5 μM concentration with 24 h exposure. In addition, it was clearly indicated that the cytotoxicity was time and dose-dependent. Thus, the activity of complexes **C4**, **C5** and **C7** were significantly superior when compared to cisplatin. It was also confirmed that the complexes were very specific to the cervical cancer cell line. Consequently, varying of heterocyclic bases with the existence of bulky *N*(4)-substituents at the thioamide nitrogen could improve their cell-killing activity through stronger and efficient oxidative cleavage.[12,13] Consequently the data obtained for each complex showed the quality of the results with minimum concentration (5 μM) and shorter incubation period (24 h) when compared to the results of similar observations.[12,31,32]

## CONCLUSIONS

In this study, the coordinating ability of the nine thiosemicarbazone-based copper(II) complexes **C1**–**C9** was derived from three sulfur-containing ligands H(L1)–H(L3) with copper and diimine co-ligands. It was concluded that the copper(II) bis complexes **C1**–**C3** coordinated to the central metal copper through two molecules, deprotonated thiolic sulfur and azomethine nitrogen. While the mixed ligand copper(II) complexes **C4**–**C9** coordinated through deprotonated thiolic sulfur and the *N*,*N*′-donor of the heterocyclic bases. Remarkably, SC pUC18 DNA efficiently cleaves the mixed ligand copper(II) complexes and completely degrades complex **C6** through intercalation of bpy/phen in the presence of an activating agent. The antibacterial studies of the compounds displayed superior activity of **C7** and **C8** at higher concentrations. However, the mixed ligand

copper(II) complexes **C4**, **C5** and **C7** exhibited an enhanced cytotoxic effect on cervical HeLa cell lines upon the existence of *N*(4)-substituted thiosemicarbazone ligands and diimine co-ligands. Hence, the remarkable biological activity of the mixed ligand copper(II) complexes **C7** should be further studied with different cancer cells to elucidate its mode of action.

## SUPPLEMENTARY MATERIAL

Additional data are available electronically from http://www.shd.org.rs/JSCS/, or from the corresponding author on request.

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## ИЗВОД

## СИНТЕЗА КОМПЛЕКСА БАКРА(II) СА *N*(4)-СУПСТИТУИСАНИМ ТИОСЕМИКАРБАЗИДОМ И ДИИМИНОМ КАО ЛИГАНДИМА И ИСПИТИВАЊЕ ЊИХОВЕ АКТИВНОСТИ ПРЕМА ПЛАЗМОИДНОЈ DNK И HeLa ЋЕЛИЈСКИМ ЛИНИЈАМА

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У овом раду описане су синтезе девет нових комплекса бакра(II) са тиосемикарбазонским типом лиганада, {[Cu(L)$_2$] **C1–C3**, [Cu(L)(bpy)]Cl **C4–C6**, [Cu(L)(phen)]Cl **C7–C9** (L = = H(L1)–H(L3), H(L1) = (*E*)-*N*-метил-2-(1-фенил-2-((5-(пиридин-3-ил)-4*H*-1,2,4-триазол-3--ил)тио)етилиден)хидразинкарботиоамид, H(L2) = (*E*)-*N*-етил-2-(1-фенил-2-((5-(пиридин-3--ил)-4*H*-1,2,4-триазол-3-ил)тио)етилиденхидра-зинкарботиоамид, H(L3) = (*E*)-*N*-фенил-2--(1-фенил-2-((5-(пирадин-3-ил)-4*H*-1,2,4-триазол-3-ил)тио)етилиден)хидразинкарботиоамид, bpy = 2,2′-бипиридил и phen =1,10-фенантролин). Синтетисани комплекси су окарактерисани применом различитих спектроскопских метода. Квадратно-планарна геометрија **C1–C9** комплекса је претпостављена на бази EPR спектроскопских мерења. На основу испитивања антибактеријске активности ових комплекса нађено је да **C7** и **C8** комплекси показују најбољу активност према Грам-позитивним (*B. thuringiensis*) и Грам-негативним (*E. coli*) бактеријама. Испитивање степена деградације суперколоидне (SC) pUC18 DNK у зависности од концентрације комплекса је показало да **C6** комплекс показује комплетну деградацију DNK при минималној концентрацији (40 μM). *In vitro* испитивања цитотоксичне активности синтетисаних комплекса су показала да, у поређењу са цисплатином, комплекси **C4, C5** и **C7** имају највећу активност према хуманој ћелијској линији канцера грлића материце (HeLa). На основу добијених резултата биолошких испитивања закључено је да најбољу активност показују комплекси бакра(II) који као лиганд имају дииминski остатак. Добијени резултати могу бити од значаја за синтезу нових алтернативних антиканцерних цитостатика у односу на оне који су до сада у медицинској примени.

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