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The metabolic effects of native bioactive compounds with antitumor activity

315.01. Medical biochemistry

Summary of doctoral thesis in medical sciences

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SUMMARY

THE RESEARCH CONCEPTUAL FRAMEWORK

Relevance and importance of the investigated issue. Currently, oncological diseases represent an ongoing challenge in modern medicine. The research of new drugs with anti-neoplastic activity is a major task of modern medicinal chemistry [1, 2]. One of the most significant issues in treating neoplastic diseases is the high toxicity of chemotherapeutic agents. To date, there are known several hundred efficient antitumor agents used to treat various malignant pathologies, and nearly all of these compounds used in clinical practice are non-selective and toxic [2, 3, 4].

In recent years, special attention has been given to Schiff bases - important chemical compounds that have found wide applications in various fields such as inorganic, analytical, and medical chemistry due to their ability to form numerous stable complexes when coordinated with transition metal ions [9].

Schiff bases refer to imine linkages formed through nucleophilic substitution reactions that occur between an amine and a carbonyl compound, which are aldehydes and ketones, wherein the carbonyl group is replaced by an imine or azomethine group. Their compound group contains electrons, making them ideal candidates for the development of new antitumor drugs in therapeutic chemistry [2, 9, 10]. Among the organic substances used as ligands, a significant role is attributed to thiosemicarbazones (TSC), compounds with valuable pharmacological and therapeutic properties, including antimicrobial, antifungal, antitumor, detoxifying, antioxidant, and antidiabetic activities [2, 11-13]. It should be noted that the biological activity of free thiosemicarbazones is less pronounced than that of coordination compounds with ligands and metals [2, 17]. These compounds are typically obtained through the condensation reaction between aldehydes or ketones and thiosemicarbazones.

Synthetic macrocyclic metal chelates play a significant role in supramolecular coordination chemistry. Complexes of metal ions with thiosemicarbazones have proven to be more potent antitumor agents than cisplatin. They inhibit the proliferation of tumor cells by arresting the cell cycle [2, 17]. Coordination compounds containing copper and iron have shown to be more active antitumor therapeutic agents than free semicarbazone and thiosemicarbazone, capable of disrupting cellular metabolism and signaling pathways. The cytotoxic activity has been observed to depend not only on the metal ion but also on the substituent group's position on the aromatic ring [2, 18-23].

Particularly valuable are the scientific research efforts concerning the directed synthesis of 3d metal coordination compounds, conducted at the Department of Inorganic Chemistry and Physics at the State University of Moldova. Over the past years, under the guidance of Professor Aurelian Gulea, a series of novel 3d metal compounds with chelating and macrocyclic ligands have been obtained through the condensation of thiosemicarbazone with aldehydes and ketones. These compounds exhibit significantly superior antitumor properties compared to doxorubicin, a widely used oncology drug. However, to date, there is a lack of in-depth and detailed research on the influence of these compounds on cellular viability, potential cytotoxic and antiproliferative effects in multifactorial cancer, as well as their effects on normal cells. This research could serve as a foundation for developing new drugs with potent anticancer effects and minimal or even devoid of adverse effects.

The aim of the research was to study the influence of native copper coordination compounds with thiosemicarbazones on cellular viability and to assess their biochemical mechanisms of action in order to select compounds with minimal toxic effects.

Research objectives:

1. Investigating the effects of copper coordination compounds with thiosemicarbazones on the *in vitro* viability and proliferation of C6 rat cancer cells.
2. Evaluating the action of copper coordination compounds with thiosemicarbazones on *in vitro* viability and proliferation of normal rat hepatocytes.
3. Studying the influence of copper coordination compounds with thiosemicarbazones on hematological indices *in vivo* on healthy laboratory animals.
4. Estimating the action of native copper coordination compounds with thiosemicarbazones on oxidative stress indices and the antioxidant system *in vivo* on healthy laboratory animals.

Scientific research methodology.

An experimental preclinical study was conducted *in vitro* on specific cell systems (C6 rat glioma cells, normal rat hepatocytes) and *in vivo* on laboratory white rats (Rattus *norvegicus* var. Albicans), adhering to all scientific rigor and ethical research principles. Cell viability, proliferation capacity, and blood and metabolic changes upon administration of native copper coordination compounds (CCT) were evaluated. To achieve the purpose and objectives of the thesis, a series of micro-research methods were developed based on existing methodologies.

The obtained data were statistically processed using functions and modules of the Statistical Package for the Social Sciences (SPSS Inc. Chicago, IL, USA, version 23). To test significant differences between the studied indices of compared groups, the post hoc test for multiple comparisons Games-Howell following One-Way ANOVA and the nonparametric Kruskal-Wallis test were applied. The nonparametric correlation coefficient *ρ Spearman* was used to establish relationships.

The research was conducted at the Biochemistry Laboratory of the "Nicolae Testemițanu" State University of Medicine and Pharmacy within the framework of the following projects: 1) a doctoral project with the theme "Metabolic Effects of Native Bioactive Compounds with Antitumor Action", funded by the Ministry of Education and Research of the Republic of Moldova (Government Decision no. 1071 of 22.09.2016 and Ministry of Education and Research Order no. 842 of 26.09.2016); 2) "Identification of Cellular and Molecular Biochemical Mechanisms of Action of Novel Native Bioactive Compounds and Substantiation of Their Use in Chemoprevention and Treatment of Certain Tumoral Processes" (code 15.817.04.05F 2015-2019); 3) "New Innovative Products with Outstanding Performance in Medicine (Bio-Pharmaceutics). Elucidation of Molecular and Cellular Mechanisms of Action of These New Products and Substantiation of Their Use to Enhance the Treatment of Pathologies" (code 20.80009.5007.10, 2020-2023). The study was positively reviewed by the Research Ethics Committee of the "Nicolae Testemițanu" State University of Medicine and Pharmacy (minutes no. 73, issue no. 65, dated 26.04.2017).

The novelty and scientific originality of the obtained results. The individual effects of various types of copper coordination compounds (CCT) on the *in vitro* viability and proliferation capacity of rat C6 glioma cancer cells were investigated. This cell culture closely resembles human glioma, allowing for relative result extrapolation to humans. The *in vitro* cytotoxic potential of CCT

on normal rat hepatocytes was also studied. Hepatocytes are involved in the metabolism of different drugs and consequently serve as the target of pharmaceutical substances' toxic action. In both cases, the action's dependence on the type of CCT – benzothiazolic, phenyl, and allyl – and the administered dose was identified.

In healthy laboratory animals, the novel native CCT's effects on blood cells (number and properties) were elucidated *in vivo*. Blood plays a role in transporting CCT and their metabolites, reflecting metabolic changes induced by CCT in organs and tissues. The changes in the hemoleucogram of healthy rats were selective, dependent on the CCT type and the laboratory animal's sex.

To establish some mechanisms of CCT action, markers of oxidative stress and the antioxidant system in the blood of healthy rats were evaluated *in vivo*. A consistent modulatory action of CCT was observed, which could constitute possible mechanisms of the tested compounds' antitumor action.

Optimized procedures for determining the action of native bioactive compounds were developed.

Approval of scientific results

The research results have been presented, discussed, and approved at various national and international scientific forums: a) *University Days and Annual Scientific Conferences on Fundamental Problems of Medicine*: Normal and Pathological Physiology, Biochemistry, Pharmacology, and Clinical Pharmacology. "Nicolae Testemițanu" State University of Medicine and Pharmacy, Chisinau, Moldova, October 18-20, 2017; October 15-19, 2018; October 15-18, 2019. b) *Congress commemorating the 75th anniversary of the founding of "Nicolae Testemițanu" State University of Medicine and Pharmacy*. Fundamental Problems of Medicine: Physiology, Pathophysiology, Biochemistry, Pharmacology, and Laboratory Medicine. Chișinău, Moldova, October 21-23, 2020. c) *Annual Scientific Conferences "Research in Biomedicine and Health: Quality. Excellence and Performance"*. Fundamental Problems of Medicine: Physiology, Pathophysiology, Biochemistry, Pharmacology, and Laboratory Medicine. "Nicolae Testemițanu" State University of Medicine and Pharmacy, Chisinau, Moldova, October 20-22, 2021; October 19-21, 2022. d) *International Chemical Engineering and Materials Symposium* (SICHEM 2020), Polytechnic University of Bucharest, Romania, September 17-18, 2020. e) *8th International Medical Congress for Students and Young Doctors (MedEspera)*, "Nicolae Testemițanu" State University of Medicine and Pharmacy, Chisinau, Moldova, September 24-26, 2020. f) *5th International Scientific and Practical Conference "Medical Drugs for Humans: Modern Issues of Pharmacotherapy and Prescription of Medicine"*, National University of Pharmacy, Kharkiv, Ukraine, March 11-12, 2021. g) *National Scientific Symposium with International Participation: Modern Biotechnologies - Solutions for the Challenges of the Contemporary World*, Chisinau, Moldova, May 20-21, 2021. h) *International Scientific Conference on "Applications of Chemistry in Nanosciences and Biomaterials Engineering"*, Academy of Romanian Scientists, Polytechnic University of Bucharest, Romania, June 25-26, 2021. i) *4th International European Conference on Interdisciplinary Scientific Researches*, Institute of Economic Development and Social Researches, Warsaw, Poland, August 8-9, 2021. j) *International Scientific Conference on "Applications of Chemistry in Nanosciences and Biomaterials Engineering - NanoBioMat"*, Academy of Romanian Scientists, Polytechnic University of Bucharest, June 22-24, 2022. k) *5th edition of the International Scientific Conference on Microbial Biotechnology*, Chisinau, Moldova, October 12-13, 2022. l) *International Scientific Conference on "Applications of Chemistry in Nanosciences and Biomaterials Engineering - NanoBioMat"*, Academy of Romanian Scientists, Polytechnic University of Bucharest, November

24-26, 2022. m) *The 47th FEBS Congress "Together in Bioscience for a Better Future"*, Tours, France, July 8-12, 2023.

Publications on the thesis topic. A total of 28 scientific papers have been published on the subject of the thesis, including 6 articles, out of which 2 are in international databases (SCOPUS, Biomedicines 2023: Citescore 3.0; IF - 4.757), 1 article in an internationally reviewed journal, 2 articles in journals from the National Registry of specialized journals, category B, including 1 article in the Moldovan Journal of Health Sciences, and 14 abstracts in national and international scientific conferences and congresses. Additionally, 3 invention patents and 5 innovation certificates have been obtained.

Keywords: local copper coordination compounds with thiosemicarbazones, viability, proliferation, cytotoxicity, oxidative stress, antioxidant system.

1. MECHANISMS OF ACTION OF LOCAL COPPER COORDINATION COMPOUNDS WITH THIOSEMICARBAZONES

Chapter I provides a literature review with the aim of highlighting the aspects that reflect the relevance of the conducted research. *Subchapter I* describes the impact of using copper coordination compounds with thiosemicarbazones on oxidative stress and the antioxidant system. Increasing oxidative stress can serve as a potential pathway for cancer cell destruction, thus the development of new molecules with antioxidant properties capable of inhibiting cancer cell proliferation remains a highly pertinent issue. The therapeutic potential of the diverse range of molecules (both ligands and metal complexes) can be fully utilized through the design of new and effective anticancer agents. *Subchapter 2* emphasizes the multifunctional applications based on copper-induced cell death.

2. MATERIALS AND RESEARCH METHODS FOR BIOCHEMICAL MARKERS IN THE EXPERIMENTAL PRECLINICAL STUDY OF LOCALLY SYNTHESIZED COPPER COORDINATION COMPOUNDS WITH THIOSEMICARBAZONES

During the period 2018-2022, an experimental preclinical research was conducted within the Biochemistry Laboratory and the Department of Biochemistry and Clinical Biochemistry at the "Nicolae Testemițanu" State University of Medicine and Pharmacy. The research involved both *in vivo* studies on laboratory animals and *in vitro* studies on cell cultures, conducted in accordance with contemporary principles in the biological standardization of experiments.

Compounds under investigation. The study focused on newly synthesized indigenous copper coordination compounds (table 1). These biologically active compounds, Schiff bases with chelating and macrocyclic ligands, in combination with *3d* metals, were synthesized at the Department of Inorganic Chemistry and Physics of the State University of Moldova under the guidance of academician Aurelian Gulea [24]. High-purity reagents from "Sigma-Aldrich" were used for the synthesis of the tested chemical compounds.

Table 1. **Newly Synthesized Indigenous Copper Coordination Compounds with Thiosemicarbazones Included in the Study**

Copper Coordination Compounds with Benzothiazolic Thiosemicarbazones	
CMA-18	Chloro-{1-(1,2-benzothiazol-3-yl)-2-[1-(pyridin-2-yl) ethylidene] diazanido} copper
CMD-8	Chloro-{4-ethyl-2-[phenyl (pyridin-2-yl)methylidene] hydrazine-1- carbothioamido} copper
MG-22	Chloro-{*N*'-(4-methoxyphenyl)-N,N-dimethylcarbmimido thioato} copper
Copper Coordination Compounds with Phenyl Thiosemicarbazones	
CMC-34	Chloro-{*N*'-[phenyl(pyridin-2-yl) methylidene]-N-pyridin-2-ylcarbamohydrazonothioato} copper
CMJ-33	Chloro-{4-(3-methoxyphenyl)-2-[1-(pyridin-2-yl) ethylidene] hydrazine-1-carbothioamido} copper
CMT-67	Nitrato-{*N*-phenyl-*N*'-(pyridin-2-ylmethylidene) carbamohydrazonothioato} copper
Copper Coordination Compounds with Allyl Thiosemicarbazones	
CMG-41	Nitrato-{*N*'-[phenyl(pyridin-2-yl) methylidene]-*N*-prop-2-en-1- ylcarbamohydrazonothioato} copper
TIA-123	Chloro-{*N*'-[phenyl(pyridin-2-yl) methylidene]-*N*- prop-2-en- 1-ylcarbamohydrazonothioato} copper
TIA-160	Acetato-{2-({[(methylsulfanyl)(prop-2-en-1-ylamino) methylidene] hydrazinylidene} methyl) phenolato}copper

General Research Design

The summary of the study design is depicted in Figure 1. The investigation of the biological effects of Copper Coordination Compounds (CCT) was carried out using two experimental models: a) *in vitro* on hepatocyte cultures obtained from healthy rat livers and standardized C6 rat glioma cell cultures (ref-CCL-107); b) *in vivo* on laboratory animals (Ratta albicans).

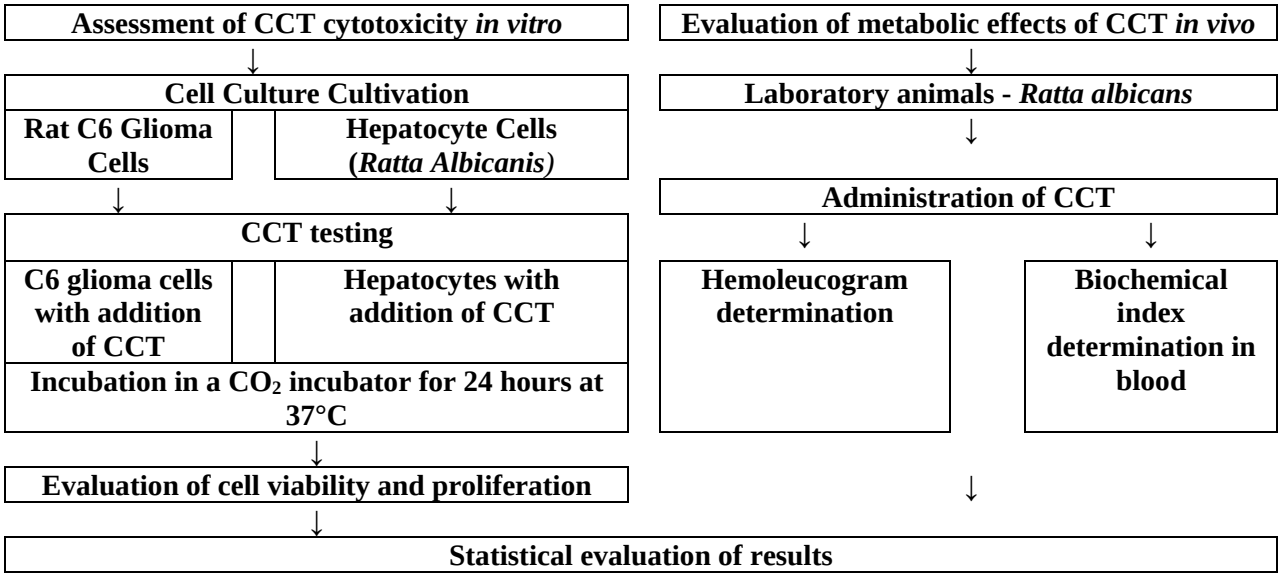


Figure 1**. Study Design**

Evaluation of cell viability and proliferation in hepatocyte and C6 glioma cell cultures was performed using the MTT assay and resazurin assay.

Methods for investigating biochemical indices. All enzyme activity and substance content determination procedures were carried out using adapted techniques for the Synergy H1 microplate reader (BioTek Instruments, USA) and the Power Wave HT spectrophotometer (BioTek Instruments, USA). The following indices were evaluated:

a) ***Oxidative stress indices:*** reactive oxygen metabolites, pro-oxidant-antioxidant balance, advanced protein oxidation products, malondialdehyde, nitric oxide metabolites.
b) ***Antioxidant system indices:*** total antioxidant activity, total antioxidant capacity, total antioxidant substances mass, average antioxidant activity, superoxide dismutase, and catalase activity.
c) ***Glutathione and thiol-disulfide metabolism indices:*** glutathione peroxidase, glutathione-S-transferase, glutathione reductase, total glutathione, thiol-disulfide homeostasis (total thiol groups and free thiol groups, as well as thiol groups of proteins).

Statistical analysis of the results was performed using the SPSS (Statistical Package for the Social Sciences) software, version 23.0. *In vitro* experimental data were presented as **arithmetic mean ± standard deviation**; *in vivo* data were presented as **median and interquartile range (IQR)**. Levene's test and Kruskal-Wallis test with a significance threshold of $p < 0.05$ were applied. *Post-hoc testing for multiple comparisons* was conducted using Games-Howell after One-Way Anova. The degree of correlation between two variables was estimated using the Spearman rank correlation coefficient (ρ).

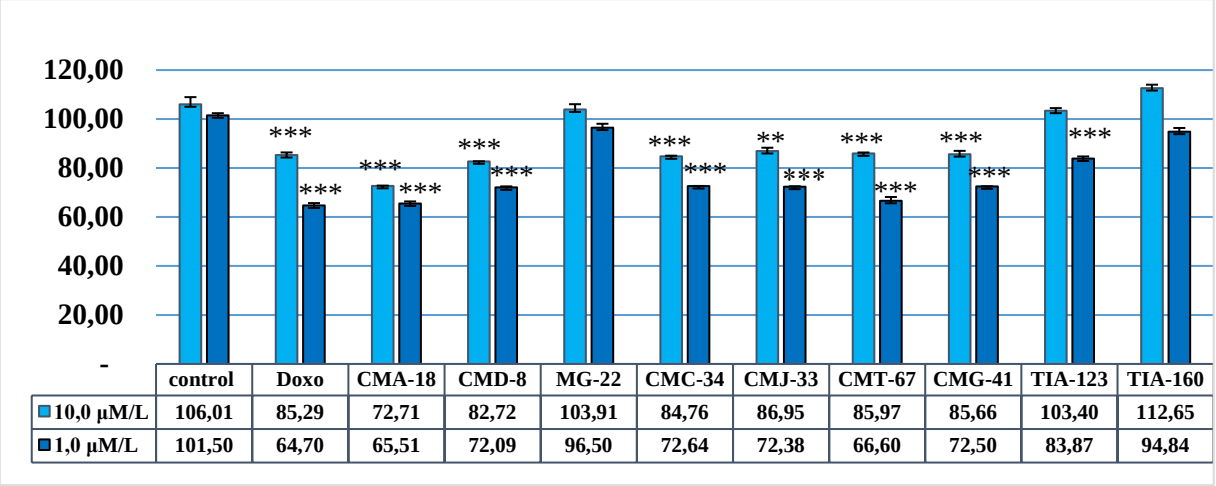
3. THE EVALUATION OF THE ACTION OF COPPER COORDINATIVE COMPOUNDS WITH THIOSEMICARBAZONES *IN VITRO*

3.1. Evaluation of the cytotoxic potential of copper coordinative compounds with thiosemicarbazones *in vitro* in rat c6 glioma cell culture

The MTT and resazurin tests are commonly employed to investigate the biological effects of potential drug compounds by assessing their capacity to impact cell viability (MTT) and proliferation (resazurin) *in vitro*.

The analysis of the MTT test results revealed a statistically significant decrease ($p< 0.001$) in cell viability upon administration of the reference compound, DOXO, at both 10.0 μM/L (-14%) and 1.0 μM/L (-25%) doses compared to the control.

When administered at a dose of 10.0 μM/L, several compounds exhibited similar effects to DOXO: CMD-8 (-17%, $p< 0.001$), CMC-34 (-15%, $p< 0.001$), CMJ-33 (-13%, $p< 0.001$), CMT-67, and CMG-41 (-14%, $p< 0.001$). The impact of CMA-18 was more pronounced (-27%, $p< 0.001$), whereas MG-22, TIA-123, and TIA-160 showed no effect (figure 2). Reducing the administered dose by 10 times potentiated the effects of CCT on the viability of C6 glioma cells. Thus, CMA-18 and CMT-67 reduced C6 cell viability by approximately 35% ($p< 0.001$ in both cases), CMD-8, CMC-34, CMJ-33, and CMG-41 by about 27% ($p< 0.001$ in all cases), and TIA-123 only by 14% ($p< 0.001$). MG-22 and TIA-160 did not influence the viability of C6 glioma cells even at this dose.

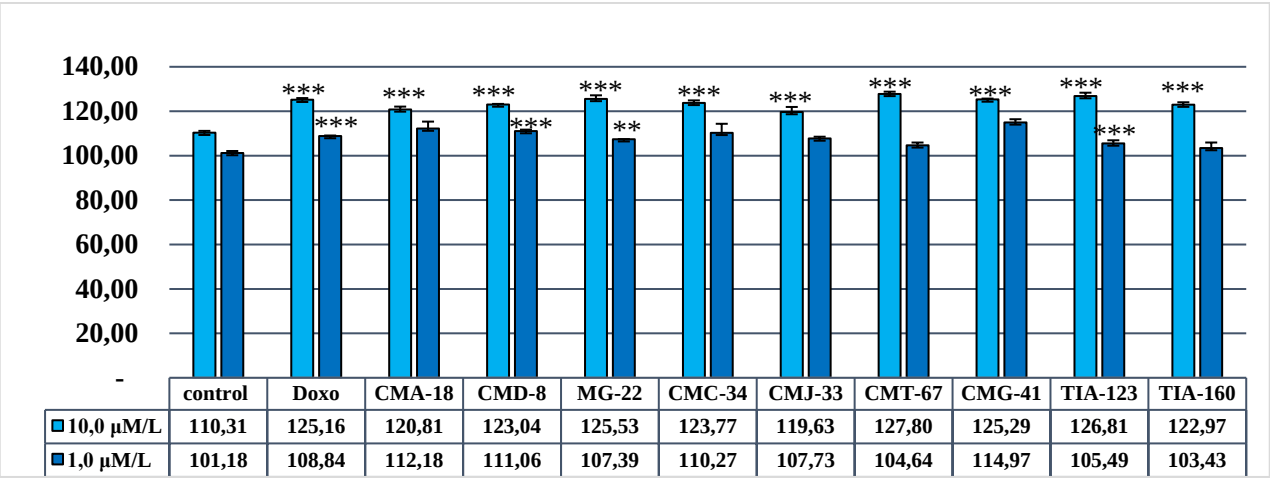


	control	Doxo	CMA-18	CMD-8	MG-22	CMC-34	CMJ-33	CMT-67	CMG-41	TIA-123	TIA-160
10,0 µM/L	106,01	85,29	72,71	82,72	103,91	84,76	86,95	85,97	85,66	103,40	112,65
1,0 µM/L	101,50	64,70	65,51	72,09	96,50	72,64	72,38	66,60	72,50	83,87	94,84

Figure 2. **Influence of CCT at doses of 10.0 µM/L and 1.0 µM/L on the viability of rat C6 glioma cells tested *in vitro* using the MTT assay**

Note: Statistical significance compared to the control-group: ** − p< 0.01; *** − p< 0.001.

The results of the resazurin assay revealed that the proliferation during the incubation period of the control culture of rat C6 glioma cells was 10.31%. Significant statistical changes in the proliferation of glioma cells were observed when cells were treated with both doses of the compounds. CMA-18 with (+9.5% - 10.9%, p< 0.001), CMD-8 (+9.8% -11.5%, p< 0.001) and TIA-123 (+4.3% - 15%, p< 0,001). Additionally, administration of MG-22 at a dose of 10.0 µM/L resulted in a proliferation increase of 13.8%, p< 0.001, while at a dose of 1.0 µM/L the increase was 6.1%, p< 0.01. Compounds CMC-34, CMT-67, CMG-41, and CMT-67 at a dose of 10.0 µM/L significantly enhanced proliferation by 11.5% - 15.9%, (p< 0.001), while at a dose of 1.0 µM/L the effect was non-significant, ranging from 2.2% to 13.6%, (p> 0.05). Similarly, the coordinative compound CMJ-33 induced a non-statistically significant proliferation at a dose of 10.0 µM/L by 8.4% (p> 0.05), and at a dose of 1.0 µM/L by 6.5% (p< 0.001) (figure 3).



	control	Doxo	CMA-18	CMD-8	MG-22	CMC-34	CMJ-33	CMT-67	CMG-41	TIA-123	TIA-160
10,0 µM/L	110,31	125,16	120,81	123,04	125,53	123,77	119,63	127,80	125,29	126,81	122,97
1,0 µM/L	101,18	108,84	112,18	111,06	107,39	110,27	107,73	104,64	114,97	105,49	103,43

Figure 3. **Proliferation of rat C6 glioma cells (CCL-107) under the influence of CCT at doses of 10.0 µM/L and 1.0 µM/L evaluated using the resazurin assay**

Note: Statistical significance compared to the control-group: ** − p< 0.01; *** − p< 0.001.

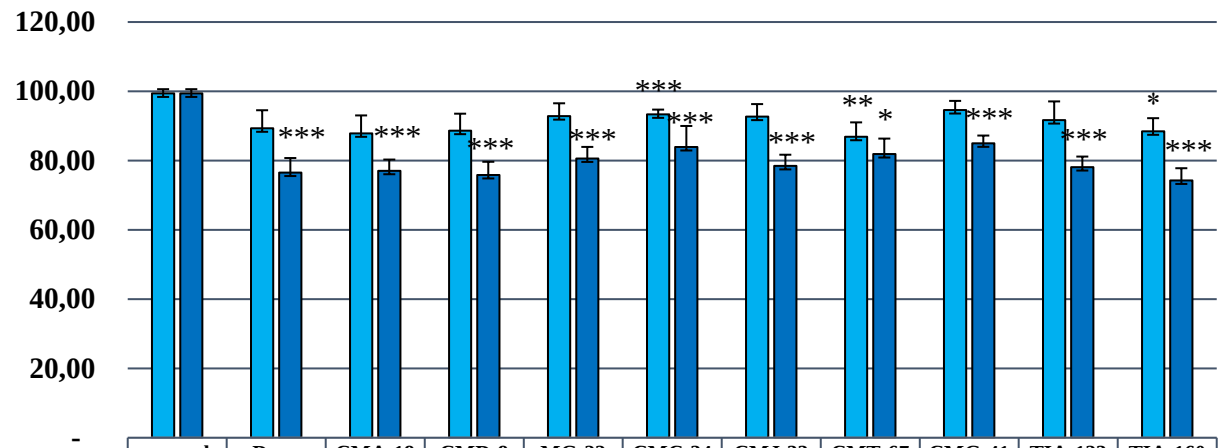
3.2. Evaluation of the cytotoxic potential of CCT *in vitro* in hepatocyte culture

Taking into consideration the liver's role in detoxifying endo- and exogenous compounds, including drugs, the organ is a primary target of the toxic effects of medicinal substances, especially those used in chemotherapy. Based on these premises, we conducted a study on the effects of CCT on the viability and proliferation of normal hepatic cells obtained from healthy rats (*ratta albicans*).

The results of the MTT test indicated that hepatocytes treated with CCT exhibited relatively high viability, suggesting low cytotoxicity of the tested compounds (figure 4).

Supplementation of doxorubicin at a concentration of 10.0 μM/L in the hepatocyte culture induced a non-significant decrease in hepatocyte viability (10%, $p > 0.05$). Analysis of the MTT test results indicated that the viability of cells treated with CCT at a concentration of 10.0 μM/L decreased significantly only under the influence of CMC-34 (5.8%, $p < 0.001$), CMT-67 (12.5%, $p < 0.01$), and TIA-160 (11.0%, $p < 0.01$). Meanwhile, other CCT compounds insignificantly reduced hepatocyte viability by 5% - 11% ($p > 0.05$). Based on the research findings, it can be concluded that CCT at a concentration of 10.0 μM/L did not exhibit a toxic effect on hepatocytes, which largely maintained their viability, possibly due to preserved metabolic functions.

Upon administering CCT at a dose of 1.0 μM/L, it was observed that all compounds significantly reduced viability: CMG-41 (14.5%, $p < 0.001$), CMC-34 (17.4%, $p < 0.001$), CMT-67 (17.6%, $p < 0.05$), MG-22 (18.8%, $p < 0.001$), CMJ-33 (21.06%, $p < 0.001$), TIA-123 (21.4%, $p < 0.001$). Compounds CMA-18, DOXO, CMD-8, and TIA-160 exhibited a statistically significant decrease of 14% - 22%, $p < 0.001$, demonstrating the most favorable effect on hepatocytes, which maintained their functions and viability to a greater extent.



	control	Doxo	CMA-18	CMD-8	MG-22	CMC-34	CMJ-33	CMT-67	CMG-41	TIA-123	TIA-160
10,0 μM/L	99,38	89,32	87,87	88,63	92,83	93,34	92,69	86,88	94,59	91,69	88,44
1,0 μM/L	99,38	76,54	77,06	75,85	80,62	83,92	78,46	81,89	84,97	78,13	74,26

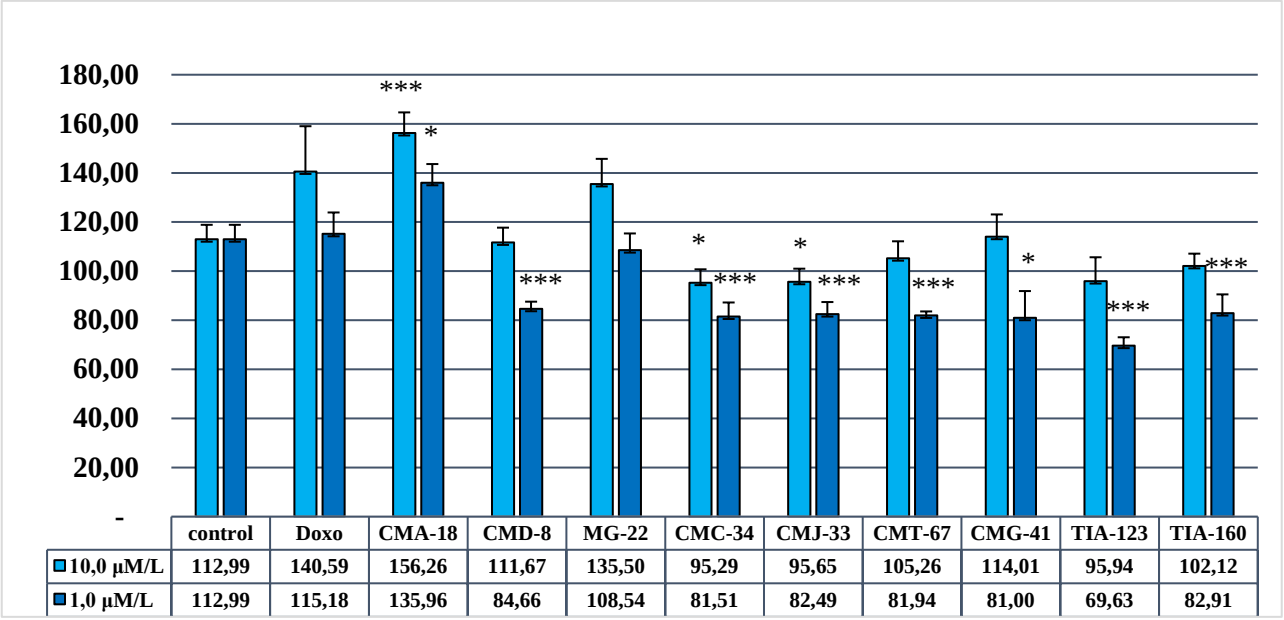
Figure 4. **The results of the *in vitro* viability and cytotoxicity assessment under the action of CCT (10.0 μM/L and 1.0 μM/L) on rat hepatocytes are as follows:**

Note: Statistical significance compared to the control-group: * − $p < 0.05$; ** − $p < 0.01$; *** − $p < 0.001$.

The results of the resazurin test revealed that the proliferation during the incubation period of the control hepatocyte culture was 12.99%. The effect of CCT 10.0 μM/L on the proliferation of the hepatocyte culture, evaluated using the resazurin test, established statistically significant changes with treatment of CMA-18 (+38%, p< 0.001), CMC-34 (-16%, p< 0.05), and CMJ-33 (-15%, p< 0.05). A statistically insignificant increase was observed with CMG-41 (+1%, p>0.05), MG-22 (+20%, p> 0.05), and DOXO (+24%, p> 0.05), while a statistically insignificant decrease was recorded with CMD-8 (1.17%, p> 0.05), CMT-67 (7%, p> 0.05), TIA-160 (9.62%, p> 0.05), and TIA-123 (15.09%, p> 0.05) (figure 5).

The resazurin test of the influence of CCT at a dose of 1.0 μM/L on the proliferation of the hepatocyte culture indicated a statistically insignificant increase induced by DOXO (+2%, p> 0.05), and a statistically insignificant decrease induced by MG-22 (4%, p> 0.05). A statistically significant increase in the proliferation of the hepatocyte culture was observed when hepatocytes were treated with CMA-18 (+20%, p< 0.05), while a statistically significant decrease was recorded with CMD-8 (25%, p< 0.001), CMJ-33 (27%, p< 0.001), TIA-160 (26.62%, p< 0.001), CMT-67 (27.48%, p< 0.001), CMC-34 (27.87%, p< 0.001), CMG-41 (28.31%, p< 0.05), and TIA-123 (38.37%, p< 0.001).

The research results allow us to conclude that the tested compounds at a dose of 1.0 μM/L exert unfavorable effects on cell viability and proliferation. Increasing the concentration leads to the annihilation of these effects. Thus, at a dose of 10.0 μM/L, the compounds CMD-8, MG-22, CMG-41, and TIA-123 do not affect the viability and proliferation of normal hepatocytes, while CMA-18 stimulates their proliferation without influencing viability.



	control	Doxo	CMA-18	CMD-8	MG-22	CMC-34	CMJ-33	CMT-67	CMG-41	TIA-123	TIA-160
10,0 μM/L	112,99	140,59	156,26	111,67	135,50	95,29	95,65	105,26	114,01	95,94	102,12
1,0 μM/L	112,99	115,18	135,96	84,66	108,54	81,51	82,49	81,94	81,00	69,63	82,91

Figure 5. **Proliferation of hepatocytes under the influence of CCT at doses of 10.0 μM/L and 1.0 μM/L evaluated through the resazurin test**

Note: Statistical significance compared to the control-group: * − p< 0.05; *** − p< 0.001.

4. THE INFLUENCE OF COPPER COORDINATIVE COMPOUNDS WITH THIOSEMICARBAZONE ON HEMOLEUCOGRAM INDICES IN HEALTHY RATS *IN VIVO*

4.1. Evaluation of the impact of copper coordination compounds with thiosemicarbazone on hemoleucogram indices in rats *in vivo*

It was observed that **benzothiazolic CCT** administered to laboratory animals did not significantly alter the number of red blood cells, as well as the red cell-to-plasma ratio (HCT). However, a statistically significant change in the total hemoglobin content was observed. This change was significant when administering MG-22 to both females (+3%, $p< 0.01$) and males (-8%, $p< 0.05$), and when administering CMA-18 to males (-9%, $p< 0.01$). In other cases, only non-significant trends of varying magnitude were observed in both male and female animals, resulting in a decrease in total hemoglobin content by different extents (-11% to -5%). Similarly, the mean corpuscular hemoglobin concentration (MCH) was not altered, but an increase in hemoglobin content per unit of erythrocyte volume was observed when administering all CCT to females (CMA-18 - +7%, $p< 0.05$; CMD-8 - +9%, $p< 0.01$, and MG-22 - +8%, $p< 0.05$), and a decrease was noted when administering MG-22 to males (-4%, $p< 0.01$).

With the exception of CMD-8 in females (-7%, $p< 0.05$), the tested benzothiazolic CCT did not significantly influence the mean corpuscular volume (MCV), which varied within the range of (-1)-(-7%) for both females and males. The effects of benzothiazolic CCT on indicators reflecting variations in erythrocyte size, such as RDW and RDW-CV, were minor. In females, MG-22 significantly reduced RDW-CV (-17%, $p< 0.001$), while in males, RDW was increased by CMD-8 (+4%, $p< 0.05$) and decreased by MG-22 (-3%, $p< 0.05$).

Analysis of the impact of benzothiazolic CCT on leukocyte indices revealed that the total number of leukocytes (WBC) was not significantly altered by these substances, except for MG-22, which decreased WBC count by 22% in females and 16% in males ($p< 0.05$ in both cases).

Analysis of the results of the modifications in the red blood cell line revealed that phenyl CCT did not significantly alter the number of red blood cells and the plasma-to-formed elements ratio (HCT) in both females and males. However, phenyl derivatives of TSC had a statistically significant impact of small magnitude on other erythrocyte indices of the hemoleucogram, with more significant changes observed in females compared to males. Specifically, administration of all phenyl CCT in females led to a statistically significant decrease in the mean corpuscular volume of erythrocytes by 6% ($p< 0.01$) for CMC-34, 10% ($p< 0.05$) for CMJ-33, and 5% ($p< 0.05$) for CMT-67. The variations in erythrocyte dimensions resulted in changes in the amount of hemoglobin per unit of cell volume, which decreased due to administration of all phenyl CCT in females (CMC-34 - +10%, $p< 0.01$; CMJ-33 - +7%, and CMT-67 - +6%, $p< 0.05$ in both cases).

In males, conclusive changes were observed in three erythrocyte indicators – HGB, MCHC, and RDW. Only MCHC was modified by CMC-34 (-4%, $p< 0.05$), CMJ-33 (-2%, $p< 0.05$), and CMT-67 (-3%, $p< 0.01$). HGB was decreased by -14% for CMJ-33 and -5% for CMT-67 in males ($p< 0.05$ in both cases), MCHC was decreased by -4% for CMC-34, -2% for CMJ-34, and -3% for CMT-67 ($p< 0.05$ in all cases), and RDW was modulated by 1-3% ($p< 0.05$) for CMJ-33 and CMT-67.

Copper coordinative compounds with **allyl thiosemicarbazone** have shown significant impact on hemoleucogram indices in healthy rats of both sexes, affecting not only erythrocyte markers but also leukocyte and platelet markers.

In females, administration of CMG-41 resulted in a decrease in erythrocyte dimensions (MCV by -8%, $p< 0.05$ and RDW-CV by -1%, $p< 0.01$) associated with an increase in the average amount of hemoglobin per erythrocyte by 7% ($p< 0.01$). The total number of leukocytes was significantly altered, with various forms showing changes. Specifically, there was an enhancement in segmented neutrophils (+47%, $p< 0.01$), eosinophils (+68%, $p< 0.01$), and monocytes (+48%, $p< 0.05$), while lymphocyte count diminished (-11%, $p< 0.05$). Platelet count was significantly augmented (+43%, $p< 0.01$), but the mean volume of platelets decreased (-6%, $p< 0.05$).

Administration of TIA-123 to females led to practically all erythrocyte indices being altered. The number of erythrocytes (+6%, $p< 0.01$), hematocrit (+13%, $p< 0.01$), total hemoglobin amount (+3%, $p< 0.01$), and hemoglobin distribution per unit of cell volume (+7%, $p< 0.05$) all increased. Additionally, leukocyte indices were elevated. There was a statistically significant increase in the total number of leukocytes by 8% ($p< 0.05$) and eosinophils by 53% ($p< 0.01$). The impact on platelet indices was similar to that of CMG-41: the total platelet count increased by 14% ($p< 0.001$), while their mean volume diminished by 3% ($p< 0.05$).

TIA-160 exhibited a similar action to that of TIA-123 in females. The indicators of all blood cell lines – erythrocytic, leukocytic, and thrombocytic – were modified, but with varying amplitude and significance. The number of erythrocytes augmented significantly (+3%, $p< 0.01$), as did hematocrit (+16%, $p< 0.001$) and total hemoglobin content (+2%, $p< 0.001$), while RDW-CV lessened by 12% ($p< 0.001$). Similarly, the total number of leukocytes amplified (+2%, $p< 0.05$), including segmentates (+41%, $p< 0.05$), while eosinophils diminnished by 4% ($p< 0.01$). The effects on platelet indices were similar to those of other allyl thiosemicarbazone compounds: the total number of platelets increased by 21% ($p< 0.001$), and their mean volume decreased by 3% ($p< 0.01$).

The copper coordinative compounds with allyl thiosemicarbazone TIA-123 and TIA-160 did not significantly alter the platelet count, but induced trends of increase, except for TIA-160, which caused a 27% increase in platelets ($p< 0.05$). At the same time, the mean platelet volume was significantly decreased by TIA-123 by 4% ($p< 0.05$).

4.2. Comparative analysis of the influence of copper coordination compounds with thiosemicarbazones on the hemoleukogram indicators of rats *in vivo*

Comparative analysis of the identified changes in erythrocyte series markers in females and males allows us to conclude that healthy females are more sensitive to the action of tested CCT compared to males. Through the comparative analysis of the impact of CCT based on their chemical nature, we can deduce that aliltiosemicarbazones produce the most statistically significant modifications of erythrocyte markers, particularly TIA-123. Allyl thiosemicarbazones are followed by benzothiazolic thiosemicarbazones, with particularly numerous effects of MG-22. The least modifications were produced by phenylthiosemicarbazones, without distinctions between individual compounds.

Comparative analysis of the action of copper coordination compounds with phenylthiosemicarbazones on healthy females and intact males revealed a dependence on the sex of the animals, with numerous effects observed in females and a smaller number in males. Moreover,

significant differences in the number of effects produced by compounds of a certain type of CCT were not identified.

We can conclude that all investigated CCT exert a similar number of actions on platelet indices, without differences dictated by the type of CCT – whether benzothiazolic, phenyl, or allyl.

In general, the study highlighted a greater impact of CCT on hemoleucogram indicators in females, as well as a slightly lower number of effects of phenylthiosemicarbazonic compounds.

5. THE INFLUENCE OF COPPER COORDINATION COMPOUNDS WITH THIOSEMICARBAZONES ON THE INDICATORS OF OXIDATIVE STRESS AND THE ANTIOXIDANT SYSTEM IN THE SERUM OF HEALTHY RATS

5.1. Evaluation of the Activity of Copper Coordination Compounds with Thiosemicarbazones on the Markers of Oxidative Stress *in vivo*

The development of degenerative processes is correlated with the presence of harmful excess of free radicals, promoters of disastrous oxidative processes for the body. To evaluate the properties of the CCT included in the study and determine their nature – prooxidant, antioxidant, or modulator – their ability to induce oxidative stress (OS) and the accumulation of its products was assessed. The analysis of the obtained results was conducted considering the CCT group (benzothiazolic, phenyl, and allyl), as well as the sex of the laboratory animals.

The benzothiazolic derivatives of CCT did not exert prooxidant action, except for the induction of enhanced ROS production by CMA-18 (+83%, $p< 0.01$) and CMD-8 (+21%, $p< 0.01$) in females. Simultaneously, CMD-8 decreased AOP significantly in females by up to 23% ($p< 0.001$) from the specific values of the control animals. CMA-18 also induced an inconclusive trend of DAM (+30%, $p> 0.05$) and PPOA (+12%, $p> 0.05$) enhancement in females, and for CMD-8, this trend was 21% for both indicators ($p> 0.05$) in females (table 2).

Similarly, the phenyl derivatives of CCT had an insignificant impact on oxidative stress. The identified changes, as in the case of benzothiazolic derivatives, were specific only to females. The compound CMJ-33 significantly amplified ROS (+24%, $p> 0.05$), concurrently inducing a significant trend of increased AOP (+99%, $p< 0.001$), DAM (+15%), and PPOA (+13%). CMT-67 showed trends of increase in ROS (+13%), AOP (+15%), and PPOA (+12%), conclusively affecting lipids, as evidenced by a true increase in DAM (+36%, $p< 0.01$). Imbalances in pro- and anti-oxidant processes induced CMC-34 in females, characterized by the trend of amplified ROS (+26%) and statistically insignificant diminish in AOP (-18%, $p> 0.05$) (table 2).

The allyl derivatives of CCT induced statistically significant changes only in AOP. A significant boost was observed in the indicator in females following the administration of CMG-41 (+19%, $p< 0.01$) and in males treated with TIA-123 (+31%, $p< 0.05$) and TIA-160 (+33%, $p< 0.05$) compared to control values. The increase observed in females was associated with trends of enhancement in ROS (+17%), DAM (+20%), and PPOA (+16%), while in males, the changes were not consistent. TIA-123 induced trends of decrease in all markers – ROS (-5%), DAM (-12%), and PPOA (-6%), while TIA-160 induced amplification – ROS (+5%), DAM and PPOA by 13%.

Table 2. **Levels of oxidative stress markers and prooxidant-antioxidant balance (PAB) in the serum of rats under the action of copper coordination compounds with thiosemicarbazone**

Study Groups - Males		MRO tert-butyl hydroperoxide, μM/L	BPA, arbitrary units	PPOA, μM/L	DAM, μM/L
Control Group	M	450.30 ; IQR 124.43 100%	103.69 ; IQR 13.10 100%	29.25; IQR 9.29 100%	6.96 ; IQR 1.89 100%
	F	276.50 ; IQR 29.63 100%	98.83 ; IQR 2.35 100%	33.67; IQR 8.67 100%	7.85 ; IQR 1.49 100%
Benzothiazolic CCTs					
CMA – 18	M	604.35 ; IQR 231.08 134%	105.39 ; IQR 28.94 102%	38.90 ; IQR 23.34 133%	7.71 ; IQR 3.08 111%
	F	505.60; IQR 150.1 * 183%	95.14 ; IQR 9.97 96%	37.59 ; IQR 3.98 112%	10.23 ; IQR 2.29 130%
CMD – 8	M	438.45 ; IQR 65.18 97%	102.08 ; IQR 29.39 98%	36.82 ; IQR 8,23 126%	8.48 ; IQR 3.42 122%
	F	335.75 ; IQR 51.35 * 121%	76.33 ; IQR 6.40*** 77%	40.60 ; IQR 6.15 121%	9.54 ; IQR 2.34 121%
MG – 22	M	438.45 ; IQR 88.88 79%	147.81 ; QR 33.89 142%	37.77 ; IQR 34.66 129%	6.36 ; IQR 1.89 91%
	F	363.40 ; IQR 100.73 131%	115.02 ; IQR 36.63 116%	3.54 ; IQR 4.07 117%	8.84 ; IQR 3.48 113%
CCT with phenyl					
CMC – 34	M	533.25 ; IQR 189.60 118%	98.43 ; IQR 12.95 95%	37.39 ; IQR 11.8 128%	7.79 ; IQR 2.49 112%
	F	347.60; IQR 37.5 ** 126%	81.57 ; IQR 27.16 82%	36.10 ; IQR 5.21 107%	9.24; IQR 1.99 118%
CMJ – 33	M	485.85 ; IQR 106.65 108%	120.63 ; IQR 86.01 116%	37.39 ; IQR 6.54 128%	7.10 ; IQR 3.43 102%
	F	343.65 ; IQR 33.58 124%	196.44;IQR 25.7*** 199%	38.03 ; IQR 2.81 113%	9.04 ; IQR 2.34 115%
CMT – 67	M	438.45 ; IQR 77.03 97%	111.25 ; IQR 16.98 107%	33.10 ; IQR 4.52 113%	9.68 ; IQR 3.93 139%
	F	316.00 ; IQR 73.08 113%	114.09 ; IQR 19.05 115%	37.70 ; IQR 5.2 112%	10.73 ; IQR 1.3** 136%
CCT with allyl					
CMG – 41	M	485.85 ; IQR 124.43 108%	132.52 ; IQR 53.10 128%	33.50 ; IQR 7.37 114%	6.92 ; IQR 1.79 99%
	F	323.90 ; IQR 37.53 117%	118.03; IQR 12.6 ** 119%	39.03 ; IQR 6.51 116%	9.44 ; IQR 1.25 120%
TIA – 123	M	426.60 ; IQR 100.73 95%	135.73 ; IQR 26.7 * 131%	27.40 ; IQR 3.90 94%	6.12 ; IQR 1.00 88%
	F	221.20 ; IQR 15.80 80%	111.68 ; IQR 19.88 113%	40.01 ; IQR 3.00 119%	8.05 ; IQR 1.64 102%
TIA – 160	M	474.00 ; IQR 82.95 105%	138.13 ; IQR 14.9 * 133%	32.98 ; IQR 10.8 113%	7.85 ; IQR 2.02 113%
	F	248.85 ; IQR 57.28 90%	130.22 ; IQR 41.15 132%	35.30 ; IQR 5.93 105%	9.54 ; IQR 1.94 121%

Note: Statistical significance compared to the control-group: * − $p< 0.05$; ** − $p< 0.01$; *** − $p< 0.001$.

5.2. Evaluation of the action of copper coordination compounds with thiosemicarbazones on the indicators of the antioxidant system in the blood serum of healthy rats

Table 3. **Influence of copper coordination compounds with thiosemicarbazones on the general indicators of the antioxidant system in the blood serum of healthy rats**

Study Groups		AAT with ABTS, μM/L	TAC, u/c	MA, u/c	AMA, u/c
Control groups	M	445.82 ; IQR 49.88 100%	9.18 ; IQR 0.96 100%	1.66 ; IQR 0.03 100%	5.54 ; IQR 0.47 100%
	F	483.72 ; IQR 57.43 100%	11.75 ; IQR 1.06 100%	1.94 ; IQR 0.01 100%	6.07 ; IQR 0.53 100%
Benzothiazolic CCTs					
CMA-18	M	311.49 ; IQR 41.84*** 70%	10.91 ; IQR 1.45 119%	1.73 ; IQR 0.09 104%	6.35 ; IQR 0.76 115%
	F	438.81 ; IQR 32.26 91%	13.70 ; IQR 1.82 117%	1.93 ; IQR 0.02 99,5%	7.09 ; IQR 0.93 117%
CMD-8	M	455.11 ; IQR 28.91 102%	9.55 ; IQR 1.13 104%	1.67 ; IQR 0.03 101%	5.77 ; IQR 0.59 104%
	F	430.70 ; IQR 7.92 89%	12.41 ; IQR 1.13 106%	1.94 ; IQR 0.02 100%	6.35 ; IQR 0.51 105%
MG-22	M	435.13; IQR 133.46 98%	9.47 ; IQR 1.62 103%	1.68 ; IQR 0.04 101%	5.72 ; IQR 0.82 103%
	F	437.70 ; IQR 23.78 90%	11.67 ; IQR 0.81 99%	1.95 ; IQR 0.01 100,5%	5.99 ; IQR 0.40 99%
CCT with phenyl					
CMC-34	M	348.84; IQR 80.10* 78%	10.80 ; IQR 2.07 118%	1.70 ; IQR 0.04 102%	6.39 ; IQR 1.00 115%
	F	426.66 ; IQR 25.56 88%	13.49 ; IQR 1.80 115%	1.94 ; IQR 0.01 100%	6.94 ; IQR 0.96 114%
CMJ-33	M	417.50 ; IQR 48.25 94%	9.76 ; IQR 0.88 106%	1.68 ; IQR 0.04 101%	5.83 ; IQR 0.51 105%
	F	436.22 ; IQR 45.73 90%	11.95 ; IQR 0.64 102%	1.94 ; IQR 0.01 100%	6.17 ; IQR 0.27 102%
CMT-67	M	429.26 ; IQR 27.62 96%	9.48 ; IQR 1.18 103%	1.67 ; IQR 0.05 101%	5.67 ; IQR 0.62 102%
	F	438.44 ; IQR 27.42 91%	12.44 ; IQR 1.93 106%	1.94 ; IQR 0.01 100%	6.43 ; IQR 0.96 106%
CCT with allyl					
CMG-41	M	434.38 ; IQR 6.41 97%	9.82 ; IQR 1.04 107%	1.68 ; IQR 0.02 101%	5.89 ; IQR 0.52 106%
	F	432.17 ; IQR 9.74 89%	12.14 ; IQR 0.82 103%	1.94 ; IQR 0.01 100%	6.26 ; IQR 0.41 103%
TIA-123	M	463.80 ; IQR 27.27 104%	8.99 ; IQR 0.32 98%	1.64 ; IQR 0.01 99%	5.47 ; IQR 0.21 99%
	F	433.28 ; IQR 16.58 90%	11.28 ; IQR 1.06 96%	1.94 ; IQR 0.01 100%	5.83 ; IQR 0.52 96%
TIA-160	M	451.08 ; IQR 17.77 101%	8.91 ; IQR 0.96 97%	1.65 ; IQR 0.03 99%	5.40 ; IQR 0.52 97%
	F	487.44 ; IQR 40.28 101%	11.66 ; IQR 0.57 99%	1.94 ; IQR 0.01 100%	6.04 ; IQR 0.29 99%

Note: Statistical significance compared to the control-group: * − p< 0.05; *** − p< 0.001.

However, it is necessary to mention that all studied compounds induced tendencies of reduction in AAT evaluated with ABTS by 9-11% in females, except for TIA-160. Statistically non-significant

tendencies of increase in TAC were also observed in animals of both sexes upon administration of CMA-18 (17-19%) and CMC-34 (15-18%), as well as in AMA with 15-17% and 14-15%, respectively (table 3).

The analysis of the influence exerted by copper coordination compounds derived from thiosemicarbazone on the general indices of the antioxidant system in the serum of healthy rats revealed the absence of major effects (table 3). Only two statistically significant changes were identified in males – a decrease in total antioxidants evaluated by the ABTS method by 30% ($p< 0.001$) upon administration of CMA-18 and by 22% ($p< 0.05$) of CMC-34.

No statistically significant changes or noteworthy tendencies of changes were observed in the levels of antioxidant substances upon administration of all CCTs, in total antioxidant capacity, and in the average activity of antioxidants upon administration of CMD-8, MG-22, CMJ-33, CMT-67, CMG-41, TIA-123, and TIA-160. The latter compound did not affect the levels of total antioxidants evaluated by the ABTS method either.

The analysis of the results obtained for the evaluation of SOD and CAT activities revealed changes of varying amplitude and direction upon administration of CCTs, with only a limited number of them being statistically significant (table 4).

In males, the administration of CMD-8 resulted in a significant decrease in SOD activity (-36%, $p< 0.01$) and a simultaneous increase in CAT activity (+22%, $p< 0.05$), while the administration of CMC-34 led to a 48% enhancement in SOD activity ($p < 0.01$) and 38% in CAT activity ($p < 0.05$). On the other hand, CMA-18 and TIA-123 did not alter SOD activity but enhanced CAT activity in males by 18% ($p < 0.05$) and 34% ($p < 0.05$), respectively.

Similar trends, though not identical, were identified following the administration of other CCTs. For instance, CMJ-33 induced a tendency of decreased SOD activity by 16% ($p> 0.05$) in males and by 11% ($p > 0.05$) in females, while CMT-67 and CMG-41 resulted in a 15% diminish in SOD activity ($p> 0.05$) in males, but an insignificant amplification in CAT activity by 11% ($p> 0.05$) and 8% ($p> 0.05$) in females, respectively.

Tendencies of enhanced activity for both enzymes were induced by MG-22 in both males and females, as well as by TIA-160 in males. MG-22 led to a trend of increased SOD activity by 25% in males and 16% in females, along with a trend of increased CAT activity by 12% in males and 17% in females ($p> 0.05$, in all cases). TIA-160 amplified SOD activity by 14% ($p> 0.05$) and CAT activity by 29% ($p< 0.01$) in males (table 4).

We can conclude that CCTs induce a significant decrease or tendencies of decrease in SOD activity, with the exception of MG-22 and TIA-160 in males. CAT activity is increased to varying degrees by all studied CCTs compared to the level established in control animals. The observed changes may demonstrate a modulation of enzymatic activity adapted to the increased level of MRO (*described in subsection 5.1*) with the purpose of halting the initiation of oxidative stress at its early stages. Within the action of CCTs on SOD and CAT activities, no significant differences based on their chemical structure were noted, and only some individual effects influenced by the sex of the animals were observed.

Table 4. **The action of copper coordination compounds derived from thiosemicarbazones on markers reflecting the capacity to neutralize radicals in the blood serum of rats**

Study groups		SOD, u/c	Catalase, µM/L
Control groups	M	55.36 ; IQR 8.93; 100%	18.10 ; IQR 1.9; 100%
	F	67.28 ; IQR 9.37; 100%	17.50 ; IQR 3.41; 100%
Benzothiazolic CCTs			
CMA-18	M	49.82 ; IQR 9.22; 90%	21.40 ; IQR 1.65; 118% *
	F	64.16 ; IQR 8.48; 95%	19.60 ; IQR 5.25; 112%
CMD-8	M	35.68 ; IQR 9.15; 64% **	22.07 ; IQR 1.92; 122% *
	F	67.95 ; IQR 4.24; 101%	19.30 ; IQR 5.67; 110%
MG-22	M	69.15 ; IQR 25.77; 125%	20.27 ; IQR 2.33; 112%
	M	64.38 ; IQR 4.47; 116%	20.57 ; IQR 3.83; 117%
CCT with phenyl			
CMC-34	M	28.71 ; IQR 14.22; 52% **	25.00 ; IQR 3.46; 138% *
	F	65.05 ; IQR 3.35; 97%	19.00 ; IQR 2,36; 109%
CMJ-33	M	46.51 ; IQR 21.76; 84%	19.37 ; IQR 1.99; 107%
	F	67.51 ; IQR 8.26; 100,3%	19.52 ; IQR 4.13; 111%
CMT-67	M	46.86 ; IQR 11.40; 85%	19.15 ; IQR 1.05; 106%
	F	65.94 ; IQR 4.35; 98%	20.87 ; IQR 5.70; 119%
CCT with allyl			
CMG-41	M	38.79 ; IQR 12.85; 70%	22.07 ; IQR 3.90; 122%
	F	63.71 ; IQR 4.13; 95%	18.40 ; IQR 5.67; 105%
TIA-123	M	57.63 ; IQR 17.12; 104%	24.25 ; IQR 5.56; 134% *
	F	64.38 ; IQR 6.69; 96%	20.27 ; IQR 3.71; 116%
TIA-160	M	63.17 ; IQR 11.98; 114%	23.42 ; IQR 2.63; 129% **
	F	63.49 ; IQR 10.93; 94%	19.45 ; IQR 1.95; 111%

Note: Statistical significance compared to the control-group: * − $p< 0.05$; ** − $p< 0.01$

5.3. Synthesis of the impact of copper coordination compounds with thiosemicarbazones on the markers of oxidative stress and the antioxidant system in the blood serum of healthy rats.

The evaluation of individual markers of AOS allows us to identify the action of the studied compounds on different links of antioxidant protection.

Considerable trends of increased total glutathione content in females were observed upon administration of TIA-123 (+83%, $p< 0.001$), CMC-34 (+83%, $p< 0.05$), and CMT-67 (+87%), while a significant increase was observed with CMD-8 (+94%, $p< 0.01$), TIA-160 (+98%, $p< 0.001$), and moderately with CMA-18 and CMG-41 (both 52%, $p< 0.05$). In males, the trends of enhanced total glutathione content prevailed, recorded in the benzothiazolic group (96% - 140%, $p< 0.01$), while in the phenyl group, a significant increase was highlighted in the administration of CMC-34 and CMJ-33 (61% - 125%, $p< 0.05$). Upon administration of TIA-123 (+101%, $p< 0.001$), CMG-41 (+91%, $p< 0.01$), TIA-160 (+86%, $p< 0.001$), compared to the control group (table 5).

Table 5. **Changes in rat blood serum levels of total glutathione and thiol groups upon administration of copper coordination compounds with thiosemicarbazones**

Study groups		Total Glutathione, μM/L	Total thiol groups, μM/L	Free thiol groups, μM/L	Thiol groups of proteins, μM/g.prot
Martor	M	291.46 ; IQR 83.01 100%	77.38 ; IQR 6.21 100%	74.38 ; IQR 3.9 100%	4.47 ; IQR 1.48 100%
	F	366.71; IQR 104.07 100%	80.40 ; IQR 19.18 100%	59.7 ; IQR 14.9 100%	5.88 ; IQR 1.63 100%
Benzothiazolic CCTs					
CMA-18	M	614.51; IQR 78.31*** 211%	98.44 ; IQR 9.21*** 127%	70.24 ; IQR 8.5 94%	4.66 ; IQR 0.83 104%
	F	557.31 ; IQR 106.99* 152%	70.62 ; IQR 13.36 88%	64.60 ; IQR 5.1 108%	6.18 ; IQR 5.56 105%
CMD-8	M	699.03; IQR 161.68*** 240%	100.70;IQR 3.39*** 130%	74.00 ; IQR 9.4 99%	4.28 ; IQR 0.72 96%
	F	712.99 ; IQR 117.11 ** 194%	75.13 ; IQR 7.90 93%	64.22 ; IQR 3.4 107%	6.78 ; IQR 1.66 115%
MG-22	M	571.69; IQR 121.30*** 196%	102.20; IQR 7.71*** 132%	75.13 ; IQR 3.4 101%	4.14 ; IQR 2.21 93%
	F	614.51 ; IQR 98.91 *** 168%	77.01 ; IQR 6.39 96%	67.23 ; IQR 5.1 113%	6.61 ; IQR 0.78 112%
CCT with phenyl					
CMC-34	M	470.23 ; IQR 139.05 * 161%	97.31 ; IQR 6.77*** 126%	73.25 ; IQR 4.3 98%	4.10 ; IQR 1.05 92%
	F	670.86 ; IQR 187.80** 183%	75.51 ; IQR 11.84 94%	66.86 ; IQR 11.8 112%	7.51 ; IQR 1.34 70%
CMJ-33	M	655.62 ; IQR 275.78 * 225%	108,97;IQR 2,41*** 141%	77.39 ; IQR 11.5 104%	3.92 ; IQR 1.12 88%
	F	600.29; IQR 134.79 ** 164%	73.63 ; IQR 6.76 92%	63.47 ; IQR 4.5 106%	5.68 ; IQR 1.99 97%
CMT-67	M	542.84 ; IQR 265.06 186%	100.32;IQR 3.76*** 130%	72.50 ; IQR 22.0 97%	3.99 ; IQR 1.17 89%
	F	684.99 ; IQR 187.80** 187%	78.14 ; IQR 5.45 97%	67.98 ; IQR 2.1 114%	7.10 ; IQR 1.19 121%
CCT with allyl					
CMG-41	M	557.31 ; IQR 121.76** 191%	108.22 ; IQR 5.3*** 140%	68.74 ; IQR 3.0 86%	3.76 ; IQR 0.67 84%
	F	557.31 ; IQR 129.08 ** 152%	75.13 ; IQR 4.14 93%	65.36 ; IQR 4.5 109%	6.61 ; IQR 2.60 112%
TIA-123	M	585.82; IQR 135.81 *** 201%	102.20; IQR 8.65*** 132%	80.02 ; IQR 5.3 108%	3.71 ; IQR 0.63 83%
	F	670.86 ; IQR 119.21 *** 183%	78.14 ; IQR 16.55 97%	71.37 ; IQR 8.3 119%	7.75 ; IQR 0.79 132%
TIA-160	M	542.84; IQR 135.59 ** 186%	101.45;IQR10.15*** 131%	71.74 ; IQR 2.1 96%	4.27 ; IQR 0.64 95%
	F	726.86; IQR 171.71 *** 198%	102.20; IQR 8.65*** 132%	80.02 ; IQR 5.3 108%	3.71 ; IQR 0.63 63%

Note: Statistical significance compared to the control group: * − $p< 0.05$; ** − $p< 0.01$; *** − $p< 0.001$.

The administration of all CCTs led to a statistically significant increase in the total content of thiol groups in males (table 5). The increases were significant in magnitude, with benzothiazolic derivatives causing increases of 27-32% ($p< 0.001$), phenyl derivatives causing increases of 26-41% ($p< 0.001$), and allyl derivatives causing increases of 31-40% ($p< 0.001$). In females, all compounds

induced non-significant trends of decreased total thiol group levels (-8-12%, $p > 0.05$), except for TIA-160 which increased the total thiol group values by 32% ($p < 0.001$).

However, these increases were not driven by corresponding boost in total free thiol groups and/or protein thiol groups, as changes in these indices were statistically non-significant (Table 5). Analysis of the results for these respective markers, however, reveals some changes that should be considered informative. Total free thiol groups show a trend of enhancement upon administration of all benzothiazolic CCTs to females by approximately 8-19% ($p > 0.05$), concurrently increasing the content of free protein thiol groups by approximately 8-13% ($p > 0.05$). Phenyl derivatives of CCT induce a trend of decreased values of free protein thiol groups in both males and females (-8-30%, $p > 0.05$), except for CMT-67 which increased the content of these groups by 21% ($p > 0.05$), concurrently inducing a trend of increased content of free thiol groups by 14% ($p > 0.05$). Allyl derivatives of CCT exhibit a similar action to phenyl derivatives in males, inducing a trend of decreased free protein thiol groups (-5-17%, $p > 0.05$), while in females, an enhancement of approximately +12-32% was induced ($p > 0.05$), except for TIA-160 which caused a decrease of 12%.

In conclusion, the studied CCTs induce coordinated changes in individual markers of the antioxidant system, indicating an adaptive influence aimed at enhancing the activity of major antioxidant enzymes. This phenomenon is associated with the modulation of thiol quantities required for their optimal functioning and the maintenance of thiol-disulfide balance, associated with the functionality of other essential biomolecules.

Benzothiazolic derivatives of the studied CCTs had a less pronounced impact on oxidative/nitrosative stress and the antioxidant system compared to phenyl derivatives. A comprehensive analysis of our results suggests that the most numerous and pronounced effects on oxidative/nitrosative stress markers and the antioxidant system were exerted by allyl derivatives of CCTs.

In conclusion, the studied CCTs in this research exert moderate or minor effects on oxidative/nitrosative stress and the antioxidant system, preferably activating antioxidant enzymes (GPO and GR) and increasing the total content of thiol groups. This could be considered a favorable adaptive response of the organism to the action of CCTs.

GENERAL CONCLUSIONS

1. The majority of tested Copper Coordination Compounds (CCT) exhibit dose-dependent selective cytotoxicity based on the dose used (10.0 μM/L vs 1.0 μM/L) and cell type – C6 rat glioma cells vs normal rat hepatocytes.

a) All CCTs induced a decrease in viability of C6 glioblastoma cells at both concentrations of 10.0 μM/L (15-27%) and 1.0 μM/L (3-35%) after 24 hours of incubation. The proliferation of C6 glioblastoma cells was stimulated by CCTs similarly to DOXO at the 10 μM/L dose, while at the 1 μM/L dose, five of the tested substances (CMA-18, CMC-34, CMT-67, CMG-41, and TIA-160) did not induce cell proliferation in the glioma culture. Cumulatively, CMA-18, CMC-34, CMT-67, and CMG-41 significantly reduced viability without stimulating proliferation of C6 glioma cells.

b) At the 10.0 μM/L dose, CCTs did not have a significant effect on viability and proliferation of hepatic cells. However, at the lower concentration (1.0 μM/L), all CCTs significantly reduced hepatocyte viability to a level similar to that produced by DOXO and conclusively decreased their proliferation (25-38%), except for CMA-18, which induced the process.

2. Minor effects of copper coordination compounds with thiosemicarbazones on the hematological indices of healthy rats were observed, with the magnitude of effects largely dependent on the sex of the laboratory animals and to a lesser extent on the type of thiosemicarbazone compound – benzothiazolic, phenyl, or allyl. Healthy females were found to be more sensitive to the tested CCT action compared to males, showing significantly more pronounced alterations in hematological parameters. No significant differences were identified in the number of effects produced by compounds of a certain CCT type, except for the impact on erythrocyte markers, which were not significantly altered by phenylthiosemicarbazones.
3. The research results on the influence of copper coordination compounds with thiosemicarbazones on oxidative stress parameters and the antioxidant system evaluated *in vivo* on healthy laboratory animals generally revealed positive effects, often synergistic, providing the potential for modulating the organism's redox status. Investigated CCTs exerted moderate or minor effects on oxidative/nitrosative stress and the antioxidant system, primarily activating antioxidant enzymes (GPO and GR) and increasing the total content of thiol groups. This could be considered a favorable adaptive response of the organism to CCT action.
4. The study of the biochemical mechanisms of action of copper coordinating compounds with thiosemicarbazones for the selection of those with minimal toxic effects did not identify significant differences in their effects, all CCT exerting a small or moderate influence on the investigated markers.

PRACTICAL RECOMMENDATIONS

1. For the investigation of the effects of synthetic and natural chemicals as potential medicines, it is recommended to apply optimized methods, as the proposed modifications have allowed for the simplification of the analytical procedure, reduction of costs for expensive reagents, and the time required for analysis by simultaneous processing of a large number of biological samples, as well as increasing the reproducibility and precision of the micro-method of determination.
2. It is recommended to expand the research on the action of copper coordination compounds with benzothiazolic, phenyl, and allyl thiosemicarbazones for the evaluation of:

a) *in vitro* impact on oxidative stress and the antioxidant system in C6 glioma cell cultures and normal rat hepatocytes in order to establish the role of these processes in the viability and proliferation changes established in the current study.

b) *in vivo* action on oxidative stress and the antioxidant system in the brain and liver, as well as in other organs and tissues of healthy laboratory animals;

c) *in vivo* action on oxidative stress and the antioxidant system in induced gliomas in healthy laboratory animals.

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✓ **International**

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✓ **Participări cu postere la foruri ştiinţifice:**

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PANTEA VALERIANA

THE METABOLIC EFFECTS OF NATIVE BIOACTIVE COMPOUNDS WITH ANTITUMOR ACTIVITY

315.01. **MEDICAL BIOCHEMISTRY**

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