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IN VITRO STUDY OF COPPER COORDINATION COMPOUNDS WITH THIOSEMICARBAZONE ACTION ON ANTIOXIDANT ENZYMES

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ABSTRACT

Introduction Thiosemicarbazones represent a class of organic compounds with significant pharmacological potential, known for their antitumor, antimicrobial, and antiviral activities. Recently, researchers have increasingly focused on how these compounds influence cellular redox balance, particularly through modulation of antioxidant system activity. Thus, the study aimed to evaluate the antioxidant properties of selected thiosemicarbazones via *in vitro* experiments.

Material and methods The research was performed on peripheral blood samples collected from 10 clinically healthy individuals. The compounds were tested at two concentrations (10.0 µmol/L and 1.0 µmol/L) to assess their impact on the antioxidant enzymes superoxide dismutase (SOD) and catalase (CAT).

Results The results revealed that certain thiosemicarbazones can impact the activity of SOD, and CAT in particular manner, thereby affecting cellular capacity to neutralize reactive oxygen species.

Conclusions Due to their ability to stimulate antioxidant responses, thiosemicarbazones emerge as promising platforms for the development of targeted therapeutic agents, particularly in cancer and degenerative disease treatment. In the current context of pursuing low-side-effect therapies that maintain cellular homeostasis, investigating the influence of thiosemicarbazones on the antioxidant system is a highly innovative research direction.

Keywords Copper coordination compounds with thiosemicarbazones, the supernatant, antioxidant enzymes.

EVALUAREA *IN VITRO* A ACȚIUNII COMPUȘILOR DE COORDONARE AI CUPRULUI CU TIOSEMICARBAZONE ASUPRA ENZIMELOR ANTIOXIDANTE

Introducere Tiosemicarbazonele reprezintă o clasă de compuși organici cu potențial farmacologic semnificativ, recunoscuți pentru activitățile lor antitumorale, antimicrobiene și antivirale. În ultimul timp, cercetătorii au acordat o atenție sporită modului în care acești compuși influențează echilibrul redox celular, în special prin modularea activității sistemului antioxidant. Astfel, studiul și-a propus evaluarea proprietăților antioxidante ale unor tiosemicarbazone selectate prin experimente *in vitro*.

Material și metode Cercetarea s-a desfășurat pe probe de sânge periferic, recoltate de la 10 indivizi clinic sănătoși. Compușii au fost testați la două concentrații (10,0 µmol/L și 1,0 µmol/L), în vederea evaluării impactului acestora asupra enzimelor antioxidante superoxid dismutaza (SOD) și catalaza (CAT).

Rezultate Rezultatele au evidențiat că anumite tiosemicarbazone pot influența în mod specific activitatea SOD și a CAT, afectând astfel capacitatea celulară de neutralizare a speciilor reactive de oxigen.

Concluzii Prin capacitatea lor de a stimula răspunsuri antioxidante, tiosemicarbazonele se impun ca platforme promițătoare pentru dezvoltarea de agenți terapeutici – țintă, în special în tratamentul cancerului și al bolilor degenerative. În contextul actual, al terapiei cu efecte adverse reduse, ce mențin homeostazia celulară, investigarea influenței tiosemicarbazonelor asupra sistemului antioxidant reprezintă o direcție de cercetare inovatoare.

Cuvinte-cheie Compuși de coordonare ai cuprului cu tiosemicarbazone, supernatant, enzime antioxidante.

INTRODUCTION

Reactive oxygen species (ROS) are essential regulators of normal cellular functions. However, their dysregulation is associated with the onset of various disorders, including multifactorial diseases. Compared to healthy cells, malignant cells exhibit higher levels of ROS due to an intensified metabolism. While elevated ROS levels can promote tumor development, they also represent a vulnerability in cancer cells. Exposure to additional oxidative stress (OS) makes these cells more susceptible to cell death, thereby providing an opportunity for selective therapeutic strategies (1).

OS results from an imbalance between ROS production and the antioxidant defense mechanisms that neutralize them. This disruption of redox homeostasis can profoundly damage vital cellular structures such as proteins, lipids, and genetic material, leading to systemic consequences and an increased risk of mutations. Such ROS-induced effects are thought to contribute to aging and may play a role in the initiation of cancer (2).

The accumulation of ROS, along with reactive nitrogen species (RNS) from both endogenous and exogenous sources, contributes to OS – a hallmark of many cancer cell types – characterized by redox imbalance and disrupted cellular signaling pathways. This redox imbalance is more pronounced in tumor cells than in normal cells and may contribute to oncogenic activation (3).

The superoxide anion, generated through metabolic processes or by the activation of oxygen under physical irradiation, is recognized as a primary type of ROS. This anion can interact with other molecules to generate secondary ROS, either directly or more commonly *via* enzyme- or metal-catalyzed mechanisms (4). Although the superoxide radical does not directly react with polypeptides, carbohydrates, or nucleic acids, and its role in lipid peroxidation remains unclear, it is primarily eliminated through dismutation. In this process, two superoxide molecules are converted into hydrogen peroxide (H_2O_2) and molecular oxygen (O_2), catalyzed by the enzyme superoxide dismutase (SOD). The resulting hydrogen peroxide is further broken down into water and oxygen by catalase (CAT) or peroxidase, thereby completing the detoxification of free radicals (5).

SOD is crucial in this elimination process, catalyzing the dismutation of superoxide radicals and working synergistically with CAT and peroxidase to maintain redox balance (6). These antioxidant enzymes are vital for cellular protection against ROS, including those oxidative processes implicated in chronic diseases such as cancer, cardiovascular, and neurodegenerative conditions. Recent studies emphasize the role of SOD in converting superoxide anion within various cellular compartments, a process vital for redox homeostasis and intracellular signaling. SOD also protects nitric oxide from oxidative inactivation, thereby preventing peroxynitrite formation and supporting endothelial and mitochondrial function (7).

Understanding the biological control mechanisms and metabolic processes across molecular, cellular, tissue, and systemic levels remains one of the major challenges in modern medicine, particularly in unraveling the pathogenesis of cancer. At the same time, there is growing interest in developing novel drugs and alternative in vitro methods for toxicological assessment that eliminate the use of animals, driven by increasing ethical considerations.

Copper(II) coordination compounds have attracted significant attention due to the redox properties and biological compatibility of copper ions, which give rise to a broad spectrum of biological activities. The pharmacological

efficacy of these metal-based compounds can be improved by modifying the ligand type and donor atoms. Copper(II) coordination compounds have demonstrated promising antitumor activity and significant therapeutic potential in the treatment of microbial infections, tuberculosis, malaria, fungal diseases, and inflammation (8).

The anticancer potential of the copper(II) coordination compounds is primarily attributed to their ability to induce intracellular ROS accumulation, which in turn activates cellular antioxidant defense mechanisms in response to OS. These findings support the exploration of ROS-inducing copper(II) coordination compounds as potential antiproliferative agents in cancer chemotherapy (10-13).

Control of OS is critical in both tumor development and response to anticancer treatments. Multiple carcinogenesis-related signaling pathways directly or indirectly influence ROS metabolism. The redox balance in cancer cells differs significantly from that seen in normal cells. Metabolic and signaling alterations result in enhanced ROS levels, often counterbalanced by an upregulated antioxidant system. This dual role of ROS, as both a barrier to and a driver of tumor progression, has important implications for therapeutic strategies targeting ROS modulation (1).

In this context, investigating the biochemical impact of such compounds proves highly relevant. Copper(II) coordination compounds represent a promising direction for the development of new effective treatments for multifactorial disorders, including tumors, chronic inflammatory diseases, autoimmune pathologies, especially through their modulation of antioxidant mechanisms.

The aim of this study was to assess the *in vitro* antioxidant properties of thiosemicarbazones with significant biological potential, using blood samples obtained from clinically healthy individuals.

MATERIAL AND METHODS

Study Design and Setting

This was an experimental *ex vivo/in vitro* study designed to investigate the response of the antioxidant system to copper(II) coordination compounds with various thiosemicarbazones and their derivatives. The compounds were synthesized at the Advanced Materials Research Laboratory in Biopharmaceutics, Moldova State University (Tab. 1). All biochemical assays were performed using a Synergy H1 Hybrid Microplate Reader (BioTek Instruments, USA). Incubations were carried out in 24-well culture plates at 37 °C, 3.5 % CO_2 for 48 h.

Table 1. Newly Studied Copper(II) Coordination Compounds with Thiosemicarbazones and Their Derivatives (9).

No.	Cod	Chemical name of the substance
1	Control	0.1 mL of 0.9% saline solution + Dulbecco's modified eagle medium (DMEM)
2	DOXO	Doxorubicin
3	CMA-18	Chloro-{1-(1,2-benzothiazol-3-yl)-2-[1-(pyridin-2-yl)ethylidene]diazanido} copper
4	CMD-8	Chloro-{4-ethyl-2-[phenyl(pyridin-2-yl)methylidene]hydrazine-1-carbothioamido} copper
5	MG-22	Di-Chloro-{N'-(4-methoxyphenyl)-N,N-dimethylcarbamimidothioato} copper
6	CMC-34	Chloro-{N'-[phenyl(pyridin-2-yl)methylidene]-N-pyridin-2-ylcarbamohydrazonothioato} copper
7	CMJ-33	Chloro-{4-(3-methoxyphenyl)-2-[1-(pyridin-2-yl)ethylidene]hydrazine-1-carbothioamido} copper
8	CMT-67	Nitrato-{N-phenyl-N'-(pyridin-2-ylmethylidene)carbamohydrazonothioato} copper
9	CMG-41	Nitrato-{N'-[phenyl(pyridin-2-yl)methylidene]-N-prop-2-en-1-ylcarbamohydrazonothioato} copper
10	TIA-123	Di-Chloro-{N'-[phenyl(pyridin-2-yl)methylidene]-N-prop-2-en-1-ylcarbamohydrazonothioato} copper
11	TIA-160	Acetato-{2-({[(methylsulfanhyl)(prop-2-en-1-lamino)ethylidene]hydrazinylidene} methyl)enolato}copper

Note: *The chemical structures of the compounds are available in bibliographic reference no. 9.*

Study Population and Ethics

The study protocol was reviewed and approved by the Research Ethics Committee of "Nicolae Testemițanu" State University of Medicine and Pharmacy (Approval no. 5, Ref. no. 38, June 20, 2024).

Participants were enrolled only after signing written informed consent forms.

Control and Reference Groups

- Negative control (baseline): 0.1 mL of 0.9 % NaCl solution added to DMEM.
- Reference drug: Doxorubicin (DOXO) at final concentrations of 10.0 µmol/L and 1.0 µmol/L.

Investigational Compounds

Newly synthesized copper(II) coordination compounds tested at final concentrations of 10.0 µmol/L and 1.0 µmol/L, diluted in 0.1 mL of 0.9 % saline. Each dilution was tested in duplicate.

Table 1 lists the full names and codes of the compounds (CMA-18, CMD-8, MG-22, CMC-34, CMJ-33, CMT-67, CMG-41, TIA-123, TIA-160). Chemical structures are available in bibliographic reference 9.

Data Collection and Research Tools

Sample Collection and Processing

Blood samples were drawn in the morning, under fasting conditions, *via* venipuncture of the cubital vein (5 mL/each subject). The blood was transferred into flasks containing 20 mL of Dulbecco's modified eagle medium (DMEM) with heparin (2.5 IU/mL), gentamicin (100 µg/mL), and L-glutamine (0.6 mg/mL).

For the evaluation of the antioxidant system response to the copper(II) coordination compounds, 0.9 mL of this mixture was pipetted into each well of a 24-well culture plate. As a control (baseline values) 0.1 mL of 0.9% NaCl solution was added in parallel to 4 wells. The remaining wells received the tested compounds, diluted in 0.1 mL of physiological saline (final concentrations – 10.0 µmol/L and 1.0 µmol/L). All dilutions were tested in duplicate. The reference drug, Doxorubicin (DOXO), was added to the wells to the same final concentration (10.0 µmol/L and 1.0 µmol/L), previously being diluted with 0.9% NaCl solution.

The plates were incubated at 37°C, 48 hours, with 3.5% CO_2. After incubation, the contents of each well were transferred into 2.0 mL Eppendorf tubes and centrifuged for 5 minutes at 3000 rpm. The supernatants were stored at –40°C until analysis.

Outcome Measures

In the supernatant, the activity of SOD and CAT was assessed by spectrophotometry. SOD activity was determined according to the method described by Matyushin B.N. et al., and CAT activity according to the method of Korolyuk M.A. et al. (15, 16). SOD activity was expressed in conventional units (c.u.). One unit of SOD activity was defined as the amount of enzyme required to achieve 50% inhibition of the nitro blue tetrazolium reduction reaction. Enzyme activity was normalized to 1 mL of serum. CAT activity was expressed in micromoles of degraded H_2O_2 per liter of serum (µmol/L). All biochemical assays were carried out using methods adapted for Synergy H1 Hybrid Microplate Reader (BioTek Instruments, USA).

Data Analysis

The statistical analysis was conducted using the Statistical Package for the Social Sciences (SPSS), version 23 (SPSS Inc., Chicago, IL, USA). After checking the distribution and dispersion of the data, intergroup differences in the analyzed biochemical parameters were evaluated using one-way analysis of variance (ANOVA), followed by the Games-Howell post-hoc test for multiple comparisons. Statistical significance was defined as $p < 0.05$. Results are presented as median values with interquartile ranges (IQR).

RESULTS

The reference drug doxorubicin, tested *in vitro*, demonstrated a marked increase in SOD activity at 10.0 µmol/L by 61%, $p < 0.001$, and at 1.0 µmol/L by 27%, $p < 0.001$ compared to the control group, whereas CAT activity was significantly elevated only at 10.0 µmol/L, by 23% ($p < 0.001$).

Analyzing the research results of the impact of the copper(II) coordination compounds with thiosemicarbazone *in vitro* on SOD, a statistically significant increase was recorded for the compound CMA-18 at both concentrations (of 24% to 88%, $p < 0.001$). In comparison, the CMD-8 showed an increase at the concentration of 10.0 µmol/L by 48%, $p < 0.001$, while at the concentration of 1.0 µmol/L it increased by 17%, $p > 0.05$. The MG-22 at the concentration of 10.0 µmol/L increased by 47%, $p < 0.001$, and at the concentration of 1.0 µmol/L, an increase of 10%, $p > 0.05$, was observed compared to the control group (Tab. 2).

CMC-34, CMJ-33, and CMT-67 copper(II) coordination compounds with thiosemicarbazones *in vitro* at a concentration of 10.0 µmol/L significantly increase SOD by 41–80%, $p < 0.001$. Concurrently, at 1.0 µmol/L CMC-34 showed an increase of 18%, $p>0.05$, while CMJ-33 induced a decrease of 17%, $p>0.05$

and CMT-67 – a statistically significant decrease of 30%, p<0.001 compared to the control group.

The *in vitro* influence of the CMG-41, TIA-123 and TIA-160 copper(II) coordination compounds with thiosemicarbazones on SOD activity revealed a statistically significant increase for all compounds at concentration of 10.0 µmol/L (from 34% to 65%, p < 0.001). For CMG-41 at 1.0 µmol/L, a significant decrease was observed (24%, p < 0.05), while TIA-123 and TIA-160 induced an increase by 9% to 24%, p < 0.01.

Table 2. The Influence of Copper(II) Coordination Compounds with Thiosemicarbazones and Their Derivatives *in vitro* on Activity of the Antioxidant Enzymes.

Study Groups	SOD		CAT	
	u/c	% vs. control	µmol/L	% vs. control
Control	47.07 ; IQR 5.65	100%	14.20 ; IQR 1.29	100%
DOXO – 10 µmol/L	75.61 ; IQR 5.65 ***	161%	17.53 ; IQR 1.46 ***	123%
DOXO – 1 µmol/L	59.74 ; IQR 2.73 ***	127%	14.80 ; IQR 0.89	104%
CMA-18 – 10 µmol/L	88.53 ; IQR 1.62 ***	188%	15.08 ; IQR 0.36	106%
CMA-18 – 1 µmol/L	58.48 ; IQR 1.55 **	124%	15.05 ; IQR 0.73	106%
CMD-8 – 10 µmol/L	69.58 ; IQR 2.00 ***	148%	15.83 ; IQR 1.23 *	111%
CMD-8 – 1 µmol/L	54.88 ; IQR 5.57	117%	14.64 ; IQR 0.73	103%
MG-22 – 10 µmol/L	69.32 ; IQR 5.63 ***	147%	17.20 ; IQR 1.79 **	121%
MG-22 – 1 µmol/L	52.03 ; IQR 4.87	110%	15.10 ; IQR 2.49	106%
CMC-34 – 10 µmol/L	84.80 ; IQR 2.38 ***	180%	16.08 ; IQR 1.38 *	113%
CMC-34 – 1 µmol/L	55.92 ; IQR 10.34	118%	15.15 ; IQR 1.54	107%
CMJ-33 – 10 µmol/L	71.32 ; IQR 2.80 ***	151%	15.53 ; IQR 3.14	109%
CMJ-33 – 1 µmol/L	43.82 ; IQR 1.07	93%	14.00 ; IQR 0.39	99%
CMT-67 – 10 µmol/L	66.65 ; IQR 1.57 ***	141%	14.60 ; IQR 0.73	103%
CMT-67 – 1 µmol/L	32.95 ; IQR 2.10 ***	70%	13.85 ; 0.37	97%
CMG-41 – 10 µmol/L	77.72 ; IQR 1.60 ***	165%	16.18 ; 2.11 *	114%
CMG-41 – 1 µmol/L	40.50 ; IQR 1.98 *	86%	14.45 ; 0.77	102%
TIA-123 – 10 µmol/L	62.92 ; IQR 5.27 ***	134%	14.05 ; 0.86	99%
TIA-123-1 µmol/L	51.37 ; IQR 2.89 **	109%	13.98 ; 0.98	98%
TIA-160-10 µmol/L	68.33 ; IQR 5.22 ***	145%	13.98 ; 0.41	98%
TIA-160-1 µmol/L	58.43 ; IQR 4.49 **	124%	13,85 ; 0.41	97%

Note: *Statistical significance compared to the control group: * – p < 0.05; ** – p < 0.01; *** – p < 0.001. SOD – Superoxide Dismutase; CAT – Catalase;*

CAT activity was increased by CMA-18 by 6%, $p > 0.05$ at concentrations of 10.0 µmol/L and 1.0 µmol/L. At the same time, the compound CMD-8 at the concentration of 10.0 µmol/L showed an increase of 11%, $p < 0.05$, and at 1.0 µmol/L, a rise of 3%, $p > 0.05$ was noted. The coordination compound MG-22 indicated an increase at the concentration of 10.0 µmol/L by 21%, $p < 0.01$, while at 1.0 µmol/L, an increase of 6%, $p > 0.05$ was observed compared to the control group.

Analysis of the impact of the copper(II) coordination compounds on CAT highlighted CMC-34 at 10.0 µmol/L with an increase of 13%, $p < 0.05$, and at 1.0 µmol/L – of 7%, $p>0.05$. For the compounds CMJ-33 and CMT-67, a non-significant increase was observed at 10.0 µmol/L by 3% to 9%, $p > 0.05$, while at 1.0 µmol/L, no changes were noted compared to the control group (Tab. 2).

CAT activity showed a statistically significant increase of 14% with CMG-41 at 10.0 µmol/L ($p < 0.05$) compared to the control. In contrast, the other compounds caused non-significant decreases ranging from 1% to 3% ($p > 0.05$) relative to the control group (Tab. 2).

DISCUSSIONS

In this study, the activity of the antioxidant system was analyzed in the supernatant derived from peripheral blood exposed to copper(II) coordination compounds with thiosemicarbazones. Copper(II) coordination compounds with thiosemicarbazones have attracted significant interest among chemists and biologists due to their wide range of pharmacological effects. These compounds have demonstrated highly effective antitumor properties across various cancer types, including leukemia, pancreatic cancer, breast cancer, lung cancer, cervical cancer, prostate cancer, and bladder cancer (9,12,14).

To enhance their biological activity, several copper(II) coordination compounds with thiosemicarbazones series have been synthesized with modifications targeting the heteroaromatic system. Antineoplastic activity increased significantly when the carbonyl group of the side chain was attached at the α-position to the nitrogen atom in the ring, while attachment at the β- or γ-position rendered the compounds inactive (17). The tested copper(II) coordination compounds with thiosemicarbazones showed selective effects on antioxidant system indices *in vitro*, which may contribute to their strong antiproliferative and cytotoxic effects on tumor cells while sparing healthy cells. Elucidating their molecular mechanisms expands the theoretical understanding of these compounds and offers new prospects for developing effective drugs.

The antioxidant mechanisms include inhibition or scavenging of reactive species, metal reduction and chelation, and inhibition of oxidative enzymes (17). Although endogenous antioxidants play a vital role in protecting the body against OS, the intake of exogenous antioxidants through diet is considered to provide significant additional health benefits, contributing to the reduction of risk for chronic diseases. The role of antioxidants in maintaining health has been extensively studied over the past decades, particularly in the context of the free radical theory, which suggests that oxidative cellular damage is a central mechanism in the pathogenesis of chronic diseases and aging. This theory has been rigorously investigated through observational, clinical, and biochemical studies, which have highlighted the role of oxidative stress in numerous pathologies, including cardiovascular, neurodegenerative diseases, and cancer (18).

Although experimental *in vitro* studies using supplementation with isolated bioactive antioxidants have not consistently demonstrated significant benefits, scientific literature suggests that foods rich in natural antioxidants may positively contribute to health maintenance. This disparity in results may reflect the complexity of interactions between nutrients and the human physiological environment, emphasizing that the protective effects of antioxidants are more evident in the context of a balanced diet than through isolated supplement intake (19). Antioxidants have garnered increasing interest due to their protective roles in food and pharmaceutical products against oxidative degradation, as well as in the body against pathophysiological processes mediated by oxidative stress (18).

In this study, functional biomarkers within the antioxidant system were identified, quantified, and selected to assess OS levels in the supernatant derived from *in vitro* exposure of peripheral blood from healthy donors to locally administered copper(II) coordination compounds with thiosemicarbazones. These biomarkers can be used to determine the efficacy of new local pharmaceutical formulations.

Recent studies have increasingly provided evidence supporting the fundamental importance of copper in the formation and function of several enzymes and proteins, such as Cu/Zn SOD and cytochrome C oxidase. These molecules are involved in processes such as the neutralization of superoxide radicals, tissue respiration, energy metabolism, and DNA synthesis (20). Copper(II) coordination compounds have proven to be promising antitumor therapeutic agents, acting through multiple mechanisms (21). Aiming to protect the body from certain harmful prooxidants, this study evaluated a complex system of enzymatic and non-enzymatic antioxidants. The evaluation included the measurement of SOD activity, total antioxidant capacity, and CAT activity (22).

Catalase plays a crucial role in the decomposition of hydrogen peroxide into water and oxygen, thereby contributing to the efficient progression of cellular processes. H_2O_2 has been identified as a central redox metabolite, actively involved in detection, signaling, and regulation of redox homeostasis and has been recognized as the principal redox mediator in these fundamental cellular mechanisms. H_2O_2 is acknowledged as one of the leading non-transcriptional signaling molecules, alongside Ca^{2+} and ATP. As a signaling molecule, H_2O_2 diffuses through cells and tissues to initiate immediate cellular responses, such as changes in cell morphology, initiation of proliferation, and recruitment of immune cells. It is now clear that H_2O_2 plays fundamental regulatory roles in metabolic processes, going beyond its traditional function as a marker of oxidative stress or cellular damage and serving as a key regulator of metabolic homeostasis (23).

The function attributed to catalase is critical for protecting cells against oxidative damage caused by H_2O_2. Hydrogen peroxide is not only toxic due to its capacity to form other ROS, such as the hydroxyl radical *via* the Fenton reaction, but it also acts as a secondary messenger, being involved in multiple physiological and pathophysiological processes (24).

CONCLUSIONS

1. Copper(II) coordination compounds with thiosemicarbazones represent promising agents in biomedical research due to their redox properties and their antitumor, antimicrobial, and antioxidant activities. Regarding the antioxidant system, these compounds exhibit a complex action, influencing the balance between the production of ROS and the cellular antioxidant defense.
2. *In vitro* experimental studies have demonstrated that certain thiosemicarbazones modulate the activity of key antioxidant enzymes, such as SOD and CAT, thereby influencing the cellular capacity to neutralize ROS. These interactions suggest therapeutic potential in reducing oxidative stress involved in various degenerative and inflammatory conditions, including multifactorial diseases.
3. Such findings provide a valuable perspective on how copper(II) coordination compounds with thiosemicarbazones can modulate cellular antioxidant responses and open new directions for the development of treatment strategies based on targeted antioxidant activity. They also support the exploration of controlled oxidative stress as a therapeutic mechanism in the design of future therapeutic approaches.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ETHICAL APPROVAL

The study protocol was approved by the Research Ethics Committee of the "Nicolae Testemițanu" State University of Medicine and Pharmacy of the Republic of Moldova (approval no. 5, ref. no. 38, on June 20, 2024). Participants were included in the study only after signing informed consent forms.

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