https://doi.org/10.31925/farmacia.2022.5.12 **ORIGINAL ARTICLE**

EVALUATION OF THE *IN VITRO* NEUROTOXIC AND NEUROPROTECTIVE EFFECTS AT CELLULAR AND SUBCELLULAR LEVELS OF NEWLY SYNTHESIZED N-PYRROLYL HYDRAZONES

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Abstract

The study presents the safety, antioxidant activity and neuroprotective effects of a series of newly synthesized N-pyrrolyl hydrazide-hydrazones (**5**, **5a-g)** in two *in vitro* models: human neuronal cells SH-SY5Y and isolated rat brain synaptosomes. The performed *in vitro* toxicological evaluation in neuronal SH-SY5Y cell line models determined the lowest cytotoxicity and best safety profile for compound **5a**, followed by **5d** on both evaluated parameters. The protective effect of the newly synthesized hydrazone **5a** was found to be comparable to melatonin used as a standard. The obtained results indicate that the presence of pyrrole ring, containing multiple phenyl nuclei and hydrazide-hydrazone group in the side chain, leads to increase in the antioxidant effect.

Rezumat

Studiul demonstrează siguranța, activitatea antioxidantă și efectele neuroprotectoare ale unei serii de N-pirrolil hidrazide-hidrazone (**5, 5a-g**) nou sintetizate prin două modele *in vitro*: celule neuronale umane SH-SY5Y și sinaptosomi izolați din creier de șobolan. Evaluarea toxicologică *in vitro* efectuată în modelele de linii celulare neuronale SH-SY5Y a determinat cea mai mică citotoxicitate și cel mai bun profil de siguranță pentru compusul **5a**, urmat de **5d** la ambii parametri evaluați. Efectul protector al hidrazonei **5a** nou sintetizate s-a dovedit a fi comparabil cu cel al melatoninei utilizate ca standard. Rezultatele obținute indică faptul că prezența inelului de pirol, care conține mai multe nuclee fenilice și gruparea hidrazidă-hidrazonă în lanțul lateral, duce la creșterea efectului antioxidant.

Keywords: N-pyrrolyl hydrazones, SH-SY5Y, neuroprotection, antioxidant activity

Introduction

Reactive oxygen radicals can be formed during normal physiological processes in living organisms, but cells are able to balance these free radical processes. Oxidative stress occurs when there is an imbalance between pro-oxidant and antioxidant cellular mechanisms [1]. Oxidative stress leads to damage to biomolecules, such as nucleic acids, proteins, carbohydrates and lipids, and as a consequence of normal cellular functions' disruption [2]. Free radical mechanisms of damage play an important role in the pathogenesis of a number of serious diseases: Parkinson's disease, Alzheimer's disease, Lafora's disease, gene mutations and oncological diseases, diabetes mellitus, myocardial infarction, neurological disorders and many others [1-8]. The most common neurodegenerative disorders are Parkinson's, Alzheimer's, Friedreich's ataxia, Huntington's and amyotrophic lateral sclerosis [9, 10], with aging being one of the major risk factors contributing to neurodegeneration. During aging, mutations in mtDNA accumulate, cytosolic calcium dysregulates, and ETC function decreases [11]. In the brain tissue to a number of patients with neurodegenerative disorders are observed oxidized molecules of DNA, proteins and lipids which shows the role of oxidative stress in these diseases [12].
The relationship between oxidative stress and neuro-degeneration in various diseases opens perspectives in the development of new active molecules with

potent antioxidant and neuroprotective activity. Therefore, biologically active substances with antioxidant potential might be a useful tool for providing an effective neuroprotection [13].

The presence of active hydrogen atom in pyrroles structure is prerequisite for performance of antioxidant activity [14-16], where the availability of substituents that remove or donate electrons in the ring may play an important role in its antioxidant activity [17]. There is evidence in the literature for the synthesis of pyrrole derivatives with pronounced antioxidant potential [18]. For example, Wang et al. reported the synthesis of a series of 1-alkyl pyrroles with different side chain sizes to investigate the relationship between structure and antioxidant activity. The antioxidant activity of pyrrole compounds was assessed by methods of binding to ABTS and DPPH radicals. The results of both tests (ABTS and DPPH) show that with increase in the size of the R-groups in the side chain, the antioxidant activity gradually decreases [19].

In another study, Kareem et al. designed and synthesized new hydrazones containing 3,4,5-trimethoxy-benzyloxy group, which were tested for antioxidant activity using DPPH and FRAP methods. The results demonstrated that compounds with hydroxyl group on para-position and electron-donor substitutes of the aldehyde component have high antioxidant activity, while electron-acceptor substituents reduce antioxidant activity [20].

Recently we have synthesized a new series of effective and safe compounds with antioxidant and neuroprotective properties containing a pyrrole ring, phenyl nuclei and a hydrazide-hydrazone group in the side chain [21]. The promising results determined the aim of the current study - to screen a series of newly synthesized N-pyrrolyl hydrazide-hydrazones for safety, antioxidant activity and neuroprotective effects in two neuronal *in vitro* models: human neuronal cells SH-SY5Y and isolated rat brain synaptosomes.

Materials and Methods

Materials

All chemicals and reagents for synthesis were purchased from Merck (Darmstadt, Germany). Commercially unavailable compounds were synthesized in our laboratory [21].

The SH-SY5Y cell line was purchased from the European Collection of Cell Cultures (ECACC, Salisbury, UK). The cell culture medium Roswell Park Memorial Institute (RPMI), heat inactivated foetal bovine serum (FBS), L-glutamine, (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), hydrogen peroxide (H_2O_2), dimethyl sulfoxide (DMSO) Percoll, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), sucrose, 2-thiobarbituric acid, Tris hydrochloride, dithiothreitol, phenylmethylsulfonyl fluoride, EDTA and anatoxin were obtained from Sigma Aldrich, Germany; NaCl, KCl, $CaCl_2 \times 2\ H_2O$, $MgCl_2 \times 2\ H_2O$, $NaHPO_4$, D-glucose, trichloroacetic acid, 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) and sulfuric acid were purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). The human neuroblastoma cell line SH-SY5Y was obtained from the European Collection of Cell Cultures (ECACC, Salisbury, UK). The neurobastoma cell line SH-SY5Y was cultured in the complete RPMI 1640 medium with L-glutamine (2 mM) and 10% heat inactivated foetal bovine serum and was maintained in 75 cm^2 flasks, in a humidified, 5% CO_2, 37°C incubator (Esco, CCl-170B-8, Elta 90, Singapure).

Synthesis of N-pyrrolyl hydrazide-hydrazones

The target compounds were obtained through a previously described in Tzankova *et al*. procedure [21].

Cell viability

The viability of SH-SYY cells was estimated by standard MTT test according to the method of Mosmann [22].

Model of H_2O_2 - induced oxidative stress in vitro

In the model of H_2O_2-induced oxidative stress**,** SH-SY5Y cells were plated at $3.5x10^4$/well density for 24 hours. Thereafter the cells were pretreated for 90 min with different concentrations of the tested compounds (0.1 - 10 μM) (**5, 5a-g**). The oxidative stress on the SH-SY5Y cells was induced by treatment of hydrogen peroxide (3 mM H_2O_2 in PBS) for 15 min, then the H_2O_2 content was changed with cell culture medium for 24 h. Afterwards the viable cells were evaluated by MTT assay. Negative controls (cells without hydrogen peroxide treatment) were considered as 100% protection and hydrogen peroxide treated cells as 0% protection.

Animals

For the synaptosomes' isolation, brains from old male Wistar rats (250 - 300 g) were used. The animals were taken from the National Breeding Centre, Sofia, Bulgaria. The vivarium, where the rats were housed, provided the standard conditions of temperature (20 ± 5°C). The animals were fed with standard pellet diet and free access to water. All the experimental procedures were approved by the Bulgarian Agency for Food Safety with Permission № 226, which is valid to 2023 year.

Isolation and incubation of rat brain synaptosomes

Synaptosomes were isolated by multiple centrifugation [23] by using Percoll gradient. After isolation, they were incubated with the test compounds (50 μM) and melatonin in a 5% CO_2 + 95% atmospheric O_2 for 60 min. The synaptosomes' protein content was measured by the method of Lowry *et al*., using serum albumin as a standard [24]. The synaptosomal viability was evaluated by MTT analysis and the

level of reduced glutathione (GSH) was measured using Ellman's reagent, which forms stained complexes with -SH at pH = 8 with maximum absorption at 412 nm [25]. The resulting yellow colour was detected spectrophotometrically (λ = 412 nm).

Model of 6-hydroxydopamine-induced neurotoxicity in isolated rat brain synaptosomes

The neurotoxin 6-hydroxydopamine (6-OHDA) is a common method for modelling neurotoxicity and neurodegeneration by inducing generation of toxic metabolite para-quinone and the free radicals: H_2O_2, superoxide and hydroxyl radicals [26]. First, the rat brain synaptosomes were pre-incubated with the tested compounds (10 µM) (**5, 5a-g**) for 30 min and after that were treated with 6 hydroxydopamine (150 mmol/L, 1 h) for neurotoxicity induction. The synaptosomal viability and the level of GSH were evaluated.

Statistical analysis

All analysed data points were normalized to the mean values of different control groups. Statistical analysis was performed using GraphPad Prism 6 Software. Different groups were compared using one-way Anova and Dunnet's multiple comparisons post-test. Results are expressed as mean ± SD (n = 6) for 3 experiments. Values of $p < 0.05$, $p < 0.01$ and $p < 0.001$ are considered statistically significant.

Results and Discussion

Synthesis of N-pyrrolyl hydrazide-hydrazones

The final N-pyrrolyl hydrazide-hydrazones were synthesized via classical Paal-Knorr cyclization as presented on Figure 1 and described in Tzankova *et al*. [21]. The structures of the used carbonyl compounds are outlined on Figure 2.

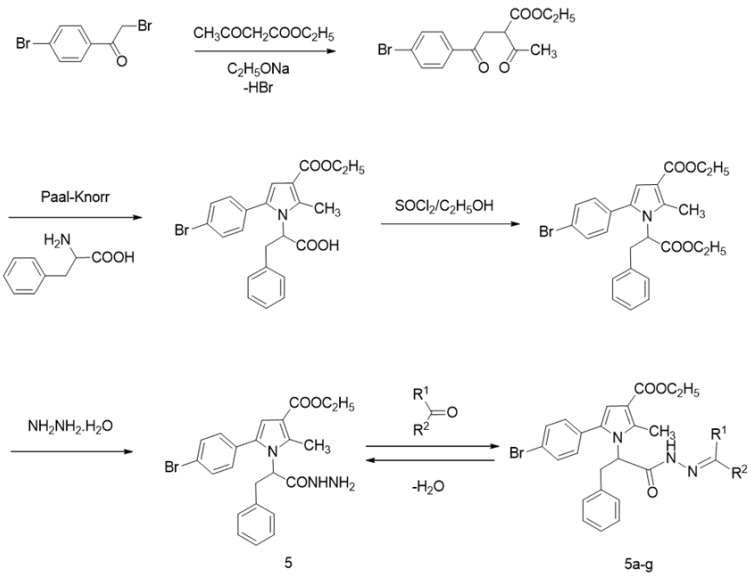

Figure 1.
Synthesis of N-pyrrolyl hydrazide-hydrazones

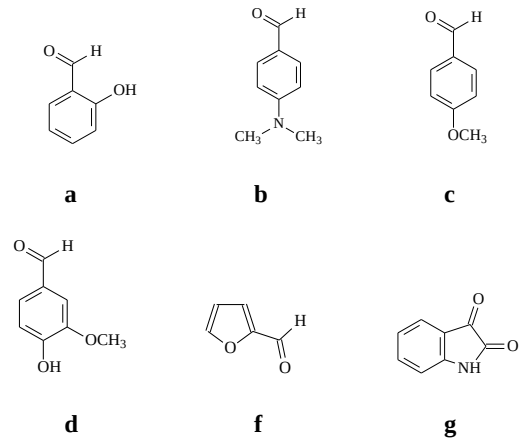

Figure 2.
Structures of the used carbonyl compounds

The results from our previous investigations of the target compounds on inhibition of DPPH and ABTS radicals identified that the presence of an amide and/or hydrazide-hydrazone group in the side chain leads to an increase in the antioxidant activity. The highest antioxidant activity observed for the hydrazide derivative is considered to be due to the free NH-NH_2 group. Additionally, promising results are observed for molecules containing OH and methoxy groups in the phenyl nucleus in the side chain of the pyrrole-based hydrazide [21].

These promising results pointed our attention to enrich our investigation in determination of the *in vitro* neurotoxicity and neuroprotective effects of the target hydrazones.

In vitro toxicity assessment in neuronal cell lines and subcellular models

In vitro cytotoxicity evaluation on SH-SY5Y cells

The effects of the newly synthesized N-pyrrolyl hydrazide-hydrazones was evaluated on cellular and subcellular *in vitro* models. Thereafter the potential neuroprotective effects were studied in a model of H_2O_2-induced oxidative stress on SH-SY5Y cells and on 6-OHDA-induced oxidative stress in isolated rat brain synaptosomes.

Effects on cell viability

SH-SY5Y cells were treated with test substances at concentrations of 1 - 500 μM for 24 h, and the corresponding IC50 values were calculated (Table I). The determined IC50 values of the studied hydrazones are in the micromolar concentration range. Compound **5a** (59.43 μM) showed the lowest *in vitro* cytotoxicity and best safety profile, respectively, followed by **5d** (43.71 μM) and **5g** (44.16 μM); the remaining compounds of series **5**, **5b**, **5c**, **5f** showed weak cytotoxicity on the SH-SY5Y neuronal cell line.

Table I

IC50 values of newly synthesized hydrazones in the human neuronal cell line SH-SY5Y

Compounds	IC50 24 h	IC50 (95% Confidence interval)
5	38.69	35.19 ÷ 42.98
5a	59.43	55.07 ÷ 64.12
5b	31.06	27.25÷35.41
5c	29.97	26.55÷33.84
5d	43.71	40.78÷46.86
5f	35.49	31.01 ÷ 40.62
5g	44.16	37.66 ÷ 51.78

Effects in isolated rat brain synaptosomes

Synaptosomes are an important source of information about neurotransmitters, cell membrane polarization and ion exchange. This largely allows their use as an *in vitro* system for assessing the degree and rate of release of neurotransmitters in the CNS. In addition, they are a commonly used approach to drug evaluation at the subcellular level [27].

To determine the toxicity of the newly obtained hydrazones at the subcellular level, two approaches were applied: assessment of synaptosomal viability by MTT test and determination of reduced glutathione levels.

Effects on synaptosomal viability (MTT test)

Figure 3 shows the results of the effects of newly synthesized substances on the viability of isolated brain synaptosomes administered alone at a concentration of 50 μM. The effects were compared to untreated synaptosomes (controls) and melatonin. Melatonin is a well-known antioxidant that effectively reduces the level of lipid peroxidation, caused by oxidative stress. The main process in the oxidation of melatonin is the release of the pyrrole ring from the indole system, as the formed free radical stimulates the release of OH-radicals, superoxide anions and plays the role of an oxidative stress inducer [28]. The use of melatonin (control substance) in the conducted studies is based mainly on its well-defined antioxidant properties. Its direct and indirect protective effects against very toxic substances have been established [29].

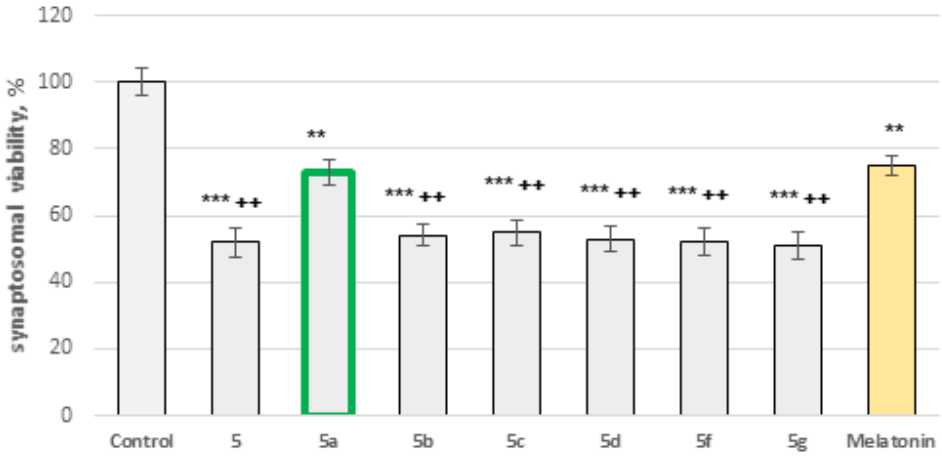

Figure 3.

Effects of a series of newly synthesized substances **5** and **5a-g** and melatonin on synaptosomal viability

The results show that only **5a** and melatonin, used as a positive control show low toxicity compared to untreated controls. **5a** decreases the synaptosomal viability by 27% and melatonin by 25%, compared to untreated controls ($p < 0.01$).

The remaining substances in the series show a pronounced statistically significant toxic effect.

Effects on reduced glutathione (GSH) levels

In the synaptosomal fraction, one of the main parameters giving information about the presence of toxicity, is the determination of the reduced glutathione level. Thus, this parameter was also investigated for the newly synthesized pyrrole containing hydrazones.

The results for the effects of the compounds on the GSH level in isolated rat synaptosomes are presented in Figure 4.

Substances **5a** and melatonin show the least toxic effect on GSH levels compared to controls. **5a** and melatonin decreased the level of GSH statistically significant by 30% and 20%, respectively, compared to the control ($p < 0.05$). The other compounds in the series showed a more pronounced toxic effect compared to the untreated control group ($p < 0.001$).

The results from the performed *in vitro* toxicological evaluation in neuronal models showed that in the SH-SY5Y cell line, the lowest cytotoxicity and best safety profile was shown by compound **5a**, followed by **5d** on all evaluated parameters.

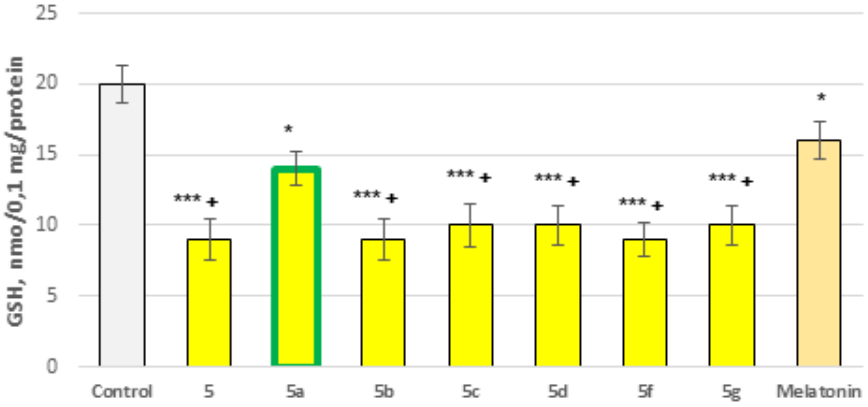

Figure 4.

Effects of a series of newly synthesized substances **5** and **5a-g** and melatonin on the level of GSH

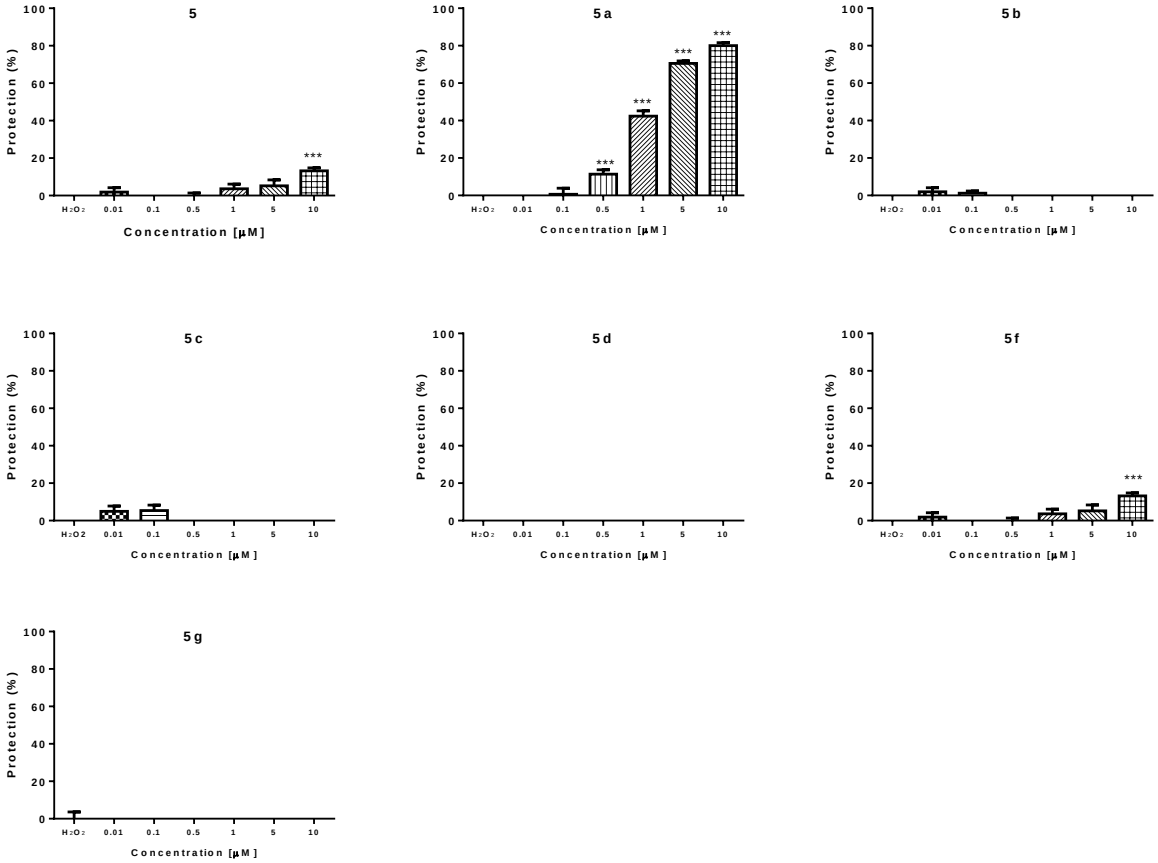

Figure 5.

Protective effects of newly synthesized compounds **5** and **5a-g** (concentration 0.01 - 10 µM) in a model of H_2O_2 - induced oxidative stress in the neuroblastoma cell line SH-SY5Y. Cell viability was calculated from the untreated control assumed to be 100%. Values ± SD (n = 3) are presented. ***$p < 0.001$ relative to H_2O_2

Protective effects in neuronal models of induced oxidative stress

The neuronal protective activity of the target compounds was studied in various models of oxidative damage in SH-SY5Y neuronal cells and at subcellular level in brain synaptosomes.

H_2O_2 - induced oxidative stress in SH-SY5Y cells (MTT-test)

The potential protective effects of the newly synthesized compounds were evaluated on the cell viability in a model of H_2O_2 - induced oxidative stress in the human neuroblastoma cell line SH-SY5Y. Cells were treated with compounds in a concentration range of 0.01 to 10 µM. Cell viability was determined by MTT assay as a marker of mitochondrial dysfunction. Treatment with 3 mM H_2O_2 (15 min) caused a significant reduction in cell viability. Pre-incubation of SH-SY5Y cells with compound **5a** was concentration-dependent (at concentrations 0.5 - 10 µM) and statistically significantly increased cell survival compared to the H_2O_2-treated group. At the highest concentration (10 µM) compound **5a** showed protection by 80 % ($p < 0.001$) (Figure 5). The remaining substances showed no protective effects in this model of oxidative damage.

6-OHDA-induced oxidative stress in isolated rat synaptosomes

The protective effects of the evaluated compounds were also determined under 6-OHDA-induced oxidative stress in isolated rat synaptosomes on two parameters: synaptosomal viability and reduced glutathione levels.

Protective effects on synaptosomal viability (MTT test)

This model is based on the metabolism of 6-OHDA, leading to the production of reactive quinones (p-quinone) and reactive oxygen species (ROS), which cause pre- and post-synaptic membrane damage and lead to neuronal cell damage [26]. Synaptosomes were incubated with 6-OHDA (150 µM) for 1 h, after which their viability was measured.

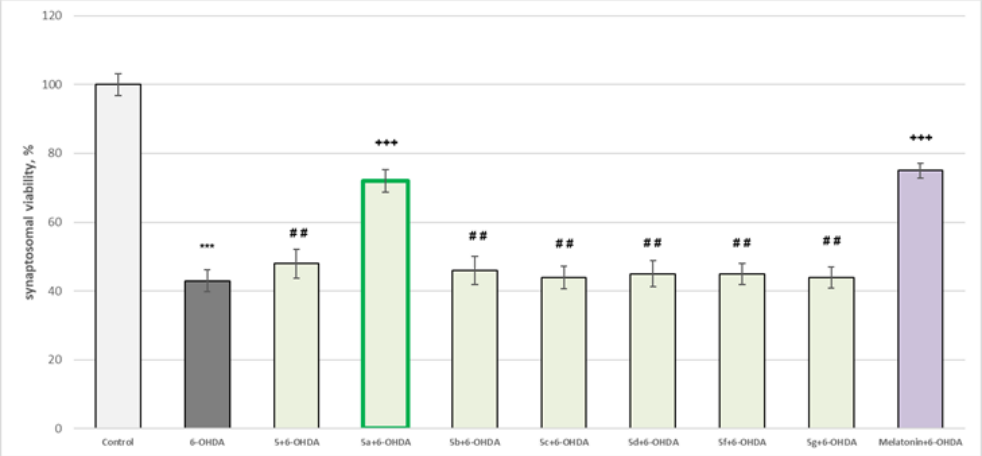

Figure 6.
Protective effects of newly synthesized substances in a model of 6-OHDA-induced oxidative stress: determination of synaptosomal viability

In the current experiments, the 6-OHDA induced neuronal toxicity is shown by the obtained statistically significant reduction in the synaptosomal viability by 57% compared to untreated control. On this parameter, only **5a** showed a statistically significant protective effect against 6-OHDA-induced oxidative stress, where its effects are comparable to those of the natural antioxidant and neuroprotector melatonin. The results show that **5a** retains synaptosomal viability by 67%, relative to the neurotoxic agent. In comparison, melatonin protects synaptosomes by 74%, relative to the toxic agent (Figure 6).

The obtained results indicate that the presence of a pyrrole ring, containing multiple phenyl nuclei and a hydrazide-hydrazone group in the side chain, leads to an increase in the antioxidant effect, thus pointing these structures as possible neuroprotective agents.

Protective effects on reduced glutathione levels

The neurotoxic substance 6-OHDA (self-administered) reduced the statistically significant level of GSH by 55%, compared to untreated controls. The results show that only **5a** showed a statistically significant protective effect against 6-OHDA-induced oxidative stress in isolated synaptosomes. **5a** maintains the GSH level by 67%, relative to the neurotoxicant 6-OHDA. In comparison, melatonin used as a positive control maintained the GSH level by 89%, relative to the toxic agent (Figure 7). In conclusion, these data show that the protective effect of the newly synthesized substance **5a** is comparable to that of the melatonin used for the standard.

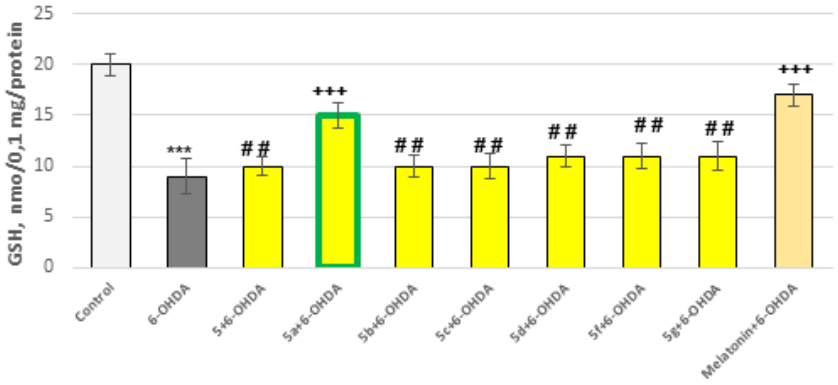

Figure 7.
Monitoring the effects of newly synthesized substances on GSH levels in isolated brain synaptosomes

In vitro toxicity screening is a rapid and effective tool for initial evaluation of newly synthetized compounds for a number of toxicological endpoints. The screening gives a useful information about the possible mechanisms of toxicity at the cellular and subcellular level. It allows preliminarily safety evaluation of new molecules and as early as possible exclusion of the chemical structures with the highest toxicological risk. The performed *in vitro* toxicological evaluation in cellular neuronal models, such as human SH-SY5Y cells, showed that compound **5a** exhibited the lowest cytotoxicity and the best safety profile, followed by **5d**. Among all tested newly synthetized compounds, **5a** showed better safety profile based on complex toxicity evaluation. It exhibited a statistically significant lower neurotoxic effect on rat brain synaptosomal viability and GSH levels, compared to the control (untreated synaptosomes). Thus, preliminary toxicological data show that the new hydrazones exhibit low or no toxicity *in vitro*, which gives a good perspective for further studies on the pharmacological profile and biological activity.

Depletion of GSH, disturbances in cell metabolism and cell death are known to be important mechanisms associated with oxidative stress and free radical damage in neuronal tissue. The application of exogenous inducers of oxidative stress, such as H_2O_2 or 6-OHDA, makes it possible to model oxidative damage and to study protective effects of substances with antioxidant activity. Therefore, the antioxidant and protective activity of the newly synthesized compounds was studied. The *in vitro* studies, performed in two different neuronal models of oxidative stress (H_2O_2 –induced oxidative damage in SH-SY5Y cells and 6-OHDA induced stress on isolated rat brain synaptosomes) showed that the best statistically significant antioxidant protection was shown by compound **5a**, whose protection was higher, compared to melatonin (a reference compound).

Conclusions

In conclusion, from all of the newly synthesized compounds, only hydrazone **5a** showed better safety profile and higher antioxidant activity, therefore appears to be the most promising for further studies of beneficial pharmacological effects.

Acknowledgement

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Conflict of interest

The authors declare no conflict of interest.

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