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Research Article

In vitro evaluation of the antioxidant activity of new pyrrole-based aroylhydrazones

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Abstract

Hydrazide-hydrazone derivatives of pyrrole possess different pharmacological properties, among them anti-inflammatory, analgesic, antiviral, antihyperlipidemic, antibacterial, antiseptic, antitumor, and anticonvulsant activities. Novel pyrrole-based aroylhydrazone derivatives (**9h–m**) were synthesized and evaluated for *in vitro* antioxidant properties by free-radical scavenging assays based on DPPH and ABTS methodology. The toxicity and potential antioxidant effects of the compounds were also studied in an *in vitro* oxidative stress model (H_2O_2 1 mM, 15 min) on the neuroblastoma cell line SH-SY5Y. To assess the safety profile of the newly synthesized compounds, their effects on cell viability were evaluated using an MTT assay. Results from DPPH and ABTS analyses showed that compound **9j** possesses higher antioxidant activity, most probably because of the presence of a phenolic hydroxyl group. *In vitro* toxicological evaluations demonstrated low cytotoxicity of the compounds. In a model of oxidative stress, the studied compounds showed a statistically significant neuroprotective effect in the neuronal cell line SH-SY5Y as follows: **9m > 9j > 9l, 9k**.

Keywords

Antioxidant activity, aroylhydrazones, cytotoxicity, oxidative stress, pyrrole

Introduction

Oxidative stress is defined as an imbalance between the production and accumulation of reactive oxygen species (ROS) in cells and tissues and the ability of the biological system to detoxify these reactive products, and it is a major contributor to the development and progression of many types of diseases, including cancers, metabolic disorders, cardiovascular disease, and neurodegenerative disorders (Pizzino et al. 2017). For instance, in the case of Alzheimer's disease, free radicals create the toxic beta-amyloid peptide, which is involved in the process of neurodegeneration (Pizzino et al. 2017). Similar pathogenic mechanisms have been shown in other neuronal pathologies. In the case of schizophrenia, oxidative stress has been correlated with reduced levels of antioxidants and increased levels of markers of cellular damage (Gunes et al. 2017; Solberg et al. 2019; Murray et al. 2021).

Antioxidants are an effective way to protect cells from oxidative stress through the donation of electrons to free radicals and the termination of chain reactions prior to damage to critical biomolecules (Amorati and Valgimigli 2015; Neha et al. 2019; Pinchuk et al. 2021). Many studies have demonstrated the efficacy of antioxidants in preventing and treating various diseases with different etiologies, including neurodegenerative pathologies.

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PENSOFT

Pyrrole molecules and their derivatives exhibit a broad spectrum of biological activities. Examples include anticancer, anti-inflammatory, analgesic, antidepressant, antibacterial, and antioxidant properties (Fan et al. 2008; Yurttaş et al. 2013; Zlatanova et al. 2018; Fatahala et al. 2019; Iqbal et al. 2020; Kundu and Pramanik 2020; Kuznietsova et al. 2020; Nocheva et al. 2023). The presence of the pyrrole ring is common to several naturally occurring biologically active molecules, such as porphyrins, bile pigments, and coenzymes (Fan et al. 2008; Kundu and Pramanik 2020).

Hydrazide-hydrazones containing an azomethine moiety represent one of the most commonly used frameworks for the synthesis of new active substances (Popiołek 2017; Krátký et al. 2021). These compounds exhibit significant antioxidant activity by scavenging free radicals and through the formation of hydrogen bonds with molecular targets to enhance their pharmacological activity (Zhang et al. 2012; Popiołek 2017; Krátký et al. 2021). Therefore, the synthesis of new pyrrole-based aroylhydrazones may provide a promising strategy for the discovery of new antioxidant compounds. The objective of this research is to develop a series of pyrrole aroylhydrazone derivatives and to test these compounds *in vitro* for antioxidant activity to determine their potential use as novel antioxidant agents. The toxicity and potential antioxidant properties of the compounds were studied in an *in vitro* model of H_2O_2-induced oxidative stress on the neuroblastoma cell line SH-SY5Y.

Materials and methods

Synthesis of pyrrole-based aroylhydrazones

The reagents and solvents used in the experiments were purchased from Sigma-Aldrich (St. Louis, MO, USA) and were utilized as received without further purification. The reaction progress was controlled by thin-layer chromatography (TLC), using TLC aluminum sheets coated with silica gel and a $CHCl_3$/CH_3CH_2OH mixture as the mobile phase. Melting points were determined on a Kruss M5000 apparatus (KRÜSS, Hamburg, Germany). The infrared (IR) spectra were recorded in the range 400–4000 cm^{-1} with a NicoletiS10 Fourier-transform infrared (FT-IR) spectrophotometer (Thermo Fisher Scientific, MA, USA), equipped with a Smart iTR accessory. Proton nuclear magnetic resonance (^{1}H-NMR) spectra were obtained on a Bruker Avance Neo 400 MHz spectrometer (Biospin GmbH, Baden-Württemberg, Germany) using tetramethylsilane (TMS) as the internal standard. Mass spectra were registered using an Agilent 6410 liquid chromatography–mass spectrometry (LC-MS) triple quadrupole mass spectrometer (Agilent Technologies, Santa Clara, CA, USA) with an electrospray ionization (ESI) interface.

The corresponding IUPAC names and structural characteristics of the new compounds are listed below.

9h: *(E)-ethyl-5-(4-bromophenyl)-1-(1-(2-(4-chlorobenzylidene)hydrazinyl)-4-methyl-1-oxopentan-2-yl)-2-methyl-1H-pyrrole-3-carboxylate*: Yield 84%, Melting point 78.1-79.2 °C, IR (vmax, cm^{-1}): 3200 (NH), 1670 (C=O), 1569 (C=N), 818 (p-substituted C6H4), 603 (C-Br); ^{1}H-NMR (400 MHz, $DMSOd_6$) δ, ppm: 0.82 (d, 3H, $CHCH_3$, J = 6.2 Hz), 0.86 (d, 3H, $CHCH_3$, J = 6.5 Hz), 1.30 (t, 3H, OCH_2CH_3, J = 7.2 Hz,), 1.58-1.62 (m, 1H, CH_2CH), 1.76-1.87 (m, 2H, CH_2CH), 2.46 (s, 3H, CH_3(2)), 4.22 (q, 2H, OCH_2CH_3, J = 7.2 Hz), 4.56-4.62 (m, 1H, $CHCH_2$), 6.76 (s, 1H, H(4)), 7.52 (d, 2H, H(3”), H(5”), J = 8.4 Hz) 7.56 (d, 2H, H(2”), H(6”), J = 8.2 Hz), 7.58 (d, 2H, H(2’), H(6’), J = 7.8 Hz), 7.68 (d, 2H, H(3’), H(5’), J = 7.6 Hz), 8.38 (s, 1H, CONH), 8.60 (s, 1H, CH=N); *m/z* (FTMS + pESI): 559.92

9i: *(E)-ethyl-5-(4-bromophenyl)-2-methyl-1-(4-methyl-1-(2-(4-nitrobenzylidene)hydrazinyl) -1-oxopentan-2-yl)-1H-pyrrole-3-carboxylate*: Yield 76%, Melting point 117.2-118.5 °C, IR (vmax, cm^{-1}): 3198 (NH), 1682 (C=O), 1570 (C=N), 819 (p-substituted C_6H_4), 594 (C-Br); ^{1}H-NMR (400 MHz, $DMSOd_6$) δ, ppm: 0.88 (d, 3H, $CHCH_3$, J = 6.4 Hz), 0.90 (d, 3H, $CHCH_3$, J = 6.5 Hz), 1.28 (t, 3H, OCH_2CH_3, J = 7.4 Hz,), 1.56-1.60 (m, 1H, CH_2CH), 1.82-1.89 (m, 2H, CH_2CH), 2.47 (s, 3H, CH_3(2)), 4.28 (q, 2H, OCH_2CH_3, J = 7.6 Hz,), 4.58-4.62 (m, 1H, $CHCH_2$), 6.76 (s, 1H, H(4)), 7.64 (d, 2H, H(2’), H(6’), J = 7.8 Hz), 7.76 (d, , 2H, H(3’), H(5’), J = 7.6 Hz), 7.86 (d, 2H, H(2”), H(6”), J = 8.4 Hz), 8.28 (d, 2H, H(3”), H(5”), J = 8.2 Hz), 8.36 (s, 1H, CONH), 8.58 (s, 1H, CH=N); *m/z* (FTMS + pESI): 570.44

9j: *(E)-ethyl-5-(4-bromophenyl)-1-(1-(2-(4-hydroxy-3,5-dimethoxybenzylidene)hydrazinyl)-4-methyl-1-oxopentan-2-yl)-2-methyl-1H-pyrrole-3-carboxylate*: Yield 93%, Melting point 121.7-122.1 °C, IR (vmax, cm^{-1}): 3303 (OH), 3214 (NH), 1674 (C=O), 1569 (C=N), 817 (p-substituted C_6H_4), 603 (C-Br); ^{1}H-NMR (400 MHz, $DMSOd_6$) δ, ppm: 0.90 (d, 3H, $CHCH_3$, J = 6.5 Hz), 0.92 (d, 3H, $CHCH_3$, J = 6.5 Hz), 1.29 (t, 3H, OCH_2CH_3, J = 7.5 Hz), 1.60-1.62 (m, 1H, CH_2CH), 1.88-1.96 (m, 2H, CH_2CH), 2.46 (s, 3H, CH_3(2)), 3.80 (s, 6H, OCH_3), 4.30 (q, 2H, OCH_2CH_3, J = 7.6 Hz), 4.56-4.60 (m, 1H, $CHCH_2$), 5.32 (s, 1H, OH), 6.77 (s, 1H, H(4)), 6.96 (d, 2H, H(2”), H(6”), J = 8.2 Hz), 7.64 (d, 2H, H(2’), H(6’), J = 7.8 Hz), 7.76 (d, 2H, H(3’), H(5’), J = 7.6 Hz), 8.34 (s, 1H, CONH), 8.56 (s, 1H, CH=N); *m/z* (FTMS + pESI): 601.50

9k: *(E)-ethyl-5-(4-bromophenyl)-1-(1-(2-(2,4-dimethoxybenzylidene)hydrazinyl)-4-methyl-1-oxopentan-2-yl)-2-methyl-1H-pyrrole-3-carboxylate*: Yield 85%, Melting point 101.3-102.5 °C, IR (vmax, cm^{-1}): 3231 (NH), 1674 (C=O), 1569 (C=N), 819 (p-substituted C_6H_4), 603 (C-Br); ^{1}H-NMR (400 MHz, $DMSOd_6$) δ, ppm: 0.89 (d, 3H, $CHCH_3$, J = 6.4 Hz), 0.91 (d, 3H, $CHCH_3$, J = 6.4 Hz), 1.27 (t, 3H, OCH_2CH_3, J = 7.3 Hz), 1.56-1.61 (m, 1H, CH_2CH), 1.88-1.92 (m, 2H, CH_2CH), 2.49 (s, 3H, CH_3(2)), 3.82 (s, 6H, OCH_3), 4.29 (q, 2H, OCH_2CH_3, J = 7.8 Hz), 4.57-4.61 (m, 1H, $CHCH_2$), 6.48 (s, 1H, H(3”)), 6.60 (s, 1H, H(5”)), 6.78 (s, 1H, H(4)), 7.65 (d, 2H, H(2’), H(6’), J = 7.7 Hz), 7.77 (d, 2H, H(3’), H(5’), J = 7.5 Hz), 7.49 (s, 1H, H(6”)), 8.26 (s, 1H, CONH), 8.68 (s, 1H, CH=N); *m/z* (FTMS + pESI): 585.50

9l: *(E)-ethyl-1-(1-(2-benzylidenehydrazinyl)-4-methyl-1-oxopentan-2-yl)- 5-(4-bromophenyl)-2-methyl-1H-pyrrole-3-carboxylate*: Yield 91%, Melting point 92.6-93.3 °C, IR (vmax, cm^{-1}): 3217 (NH), 1674 (C=O), 1569 (C=N),

817 (p-substituted C_6H_4), 598 (C-Br); ^{1}H-NMR (400 MHz, $DMSOd_6$) δ, ppm: 0.90 (d, 3H, $CHCH_3$, J = 6.4 Hz), 0.92 (d, 3H, $CHCH_3$, J = 6.5 Hz), 1.27 (t, 3H, OCH_2CH_3, J = 7.4 Hz), 1.56-1.60 (m, 1H, CH_2CH), 1.88-1.95 (m, 2H, CH_2CH), 2.50 (s, 3H, CH_3(2)), 4.30 (q, 2H, OCH_2CH_3, J = 7.5 Hz), 4.54-4.62 (m, 1H, $CHCH_2$), 6.8 (s, 1H, H(4)), 7.46 – 7.52 (m, 2H, H(3"), H(4"), H(5")), 7.65 (d, 2H, H(2'), H(6'), J = 7.7 Hz), 7.76 (d, 2H, H(3'), H(5'), J = 7.6 Hz), 7.78 (d, 2H, H(2"), H(6"), J = 8.5 Hz), 8.20 (s, 1H, CONH), 8.48 (s, 1H, CH=N); *m/z* (FTMS + pESI): 525.45

9m: *(E)-ethyl-5-(4-bromophenyl)-1-(1-(2-(3-fluorobenzylidene)hydrazinyl)-4-methyl-1-oxopentan-2-yl)-2-methyl-1H-pyrrole-3-carboxylate*: Yield 76%, Melting point 95.4-96.5 °C, IR (vmax, cm^{-1}): 3220 (NH), 1682 (C=O), 1569 (C=N), 818 (p-substituted C_6H_4), 605 (C-Br); ^{1}H-NMR (400 MHz, $DMSOd_6$) δ, ppm: 0.89 (d, 3H, $CHCH_3$, J = 6.4 Hz), 0.93 (d, 3H, $CHCH_3$, J = 6.5 Hz), 1.30 (t, 3H, OCH_2CH_3, J = 7.2 Hz), 1.56-1.64 (m, 1H, CH_2CH), 1.90-1.95 (m, 2H, CH_2CH), 2.52 (s, 3H, CH_3(2)), 4.32 (q, 2H, OCH_2CH_3, J = 7.6 Hz), 4.54-4.60 (m, 1H, $CHCH_2$), 6.72 (s, 1H, H(4)), 7.28 (s, 1H, H(4")), 7.58 (s, 1H, H(6")), 7.62 (s, 1H, H(5")), 7.68 (d, 2H, H(2'), H(6'), J = 7.8 Hz), 7.74 (d, 2H, H(3'), H(5'), J = 7.6 Hz), 7.80 (s, 1H, H(2")), 8.22 (s, 1H, CONH), 8.52 (s, 1H, CH=N); *m/z* (FTMS + pESI): 543.44

Antioxidant activity

Two methods for assessing radical-scavenging activity were applied to determine the probable antioxidant effect of the new pyrrole aroylhydrazone derivatives. While performing 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) and DPPH antioxidant activity tests, UV-Vis absorbance measurements were performed using a Jenway 6715 spectrophotometer, Cole-Parmer Instrument Company Ltd., Vernon Hills, IL, USA.

DPPH radical scavenging assay

The DPPH radical scavenging assay was carried out according to the methodology published by Brand-Williams et al. (1995). To different concentrations (0.031–0.250 μmol/L; 1 mL) of the newly synthesized compounds in methanol, 1 mL of a methanolic solution of DPPH (2,2-diphenyl-1-picrylhydrazyl; 1 mmol/L) was added. The mixtures were incubated for 30 min in the dark at room temperature, after which their absorbance was read at 517 nm against a control sample containing a methanolic solution of DPPH. Trolox was used as a positive control. All determinations were performed in triplicate. The percentage of DPPH radicals scavenged at the tested concentration was calculated according to the equation:

$$DPPH_{radical\ scavenging\ activity}\% = \frac{Abs_{control} - Abs_{sample}}{Abs_{control}} \times 100$$

where $Abs_{control}$ is the absorbance of the DPPH radical in methanol and Abs_{sample} is the absorbance of the DPPH radical solution mixed with the sample.

ABTS radical scavenging assay

The ABTS radical scavenging assay for the determination of the antioxidant activity of the pyrrole-based aroylhydrazones was performed by the method described by Arnao et al. (1996). The stock solution was prepared by mixing an equal volume of 7 mmol/L ABTS solution with 2.45 mmol/L potassium persulfate solution. The resulting solution was stored in the dark at room temperature for 16 h. To 1 mL of the solutions of the test substance at different concentrations, 1 mL of the preincubated radical was added. The samples were incubated for 7 min in the dark, after which the absorbance at 734 nm was measured. Trolox was used as a positive control. All determinations were performed in triplicate. The percentage of antioxidant activity was calculated using the same equation as in the DPPH method.

Cells and culturing

The human neuroblastoma cell line SH-SY5Y was obtained from the European Collection of Cell Cultures (ECACC, Salisbury, UK) and used as an *in vitro* model system. The cells were cultured in RPMI 1640 medium supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS), 2 mM L-glutamine, and 1% (v/v) antibiotic solution containing penicillin and streptomycin to prevent bacterial contamination.

Cell cultures were maintained under standard humidified conditions at 37 °C in an atmosphere containing 5% CO_2. The culture medium was routinely replaced every 2–3 days to ensure optimal nutrient supply and removal of metabolic waste products. Cells were monitored regularly using an inverted microscope to assess morphology and confluency and were subcultured upon reaching approximately 70–80% confluence using standard trypsinization procedures.

Cell viability assay (MTT test)

SH-SY5Y cells were seeded in 96-well plates at a density of 2×10^4 cells per well and allowed to adhere and reach appropriate confluence over 24 h under standard incubation conditions (37 °C, 5% CO_2). Following this period, the cells were exposed to the tested compounds at concentrations ranging from 1 to 500 μM.

Cell viability was assessed using the MTT assay, which evaluates mitochondrial metabolic activity. In viable cells, mitochondrial succinate dehydrogenase reduces the yellow, water-soluble tetrazolium salt MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) to insoluble purple formazan crystals. After 24 h of treatment, MTT solution (10 mg/mL in phosphate-buffered saline (PBS)) was added to each well, followed by incubation for 3 h at 37 °C. Subsequently, the supernatant was carefully removed, and the formed formazan crystals were solubilized in 100 μL of dimethyl sulfoxide (DMSO). The absorbance was measured at 570 nm with a reference wavelength of 690 nm using a Synergy 2 microplate reader (BioTek Instruments, Inc., Winooski, VT, USA), and cell viability was expressed relative to untreated controls (Mosmann 1983).

H_2O_2-induced oxidative stress model

For the H_2O_2-induced oxidative stress model, SH-SY5Y cells were seeded in 96-well plates at a density of 3.5×10^4 cells per well in 100 µL culture medium and allowed to attach for 24 h under standard incubation conditions. Following this initial period, the culture medium was removed, and the cells were pretreated with the investigated compounds at concentrations of 0.1, 1, 5, 10, 20, and 50 µM for 90 min.

After pretreatment, the cells were gently washed with PBS and subsequently exposed to oxidative stress by incubation with 1 mM H_2O_2 prepared in PBS for 15 min. The oxidative solution was then removed and replaced with fresh culture medium, and the cells were further incubated for 24 h.

Cell viability was subsequently determined using the MTT assay, reflecting the number of metabolically active, adherent cells. Untreated cells not exposed to hydrogen peroxide were used as a negative control and defined as 100% protection, whereas cells treated with H_2O_2 alone were considered 0% protection.

Statistical analysis

Statistical analysis was performed using GraphPad Prism 8 software. All experiments were conducted in triplicate, and the results are presented as the mean ± standard deviation (SD) (n = 6). Differences between groups were evaluated using one-way analysis of variance (ANOVA), followed by Dunnett's multiple comparisons *post hoc* test. Statistical significance was assessed to determine the reliability of the observed differences, with thresholds set at $p < 0.05$, $p < 0.01$, and $p < 0.001$.

Results and discussion

Synthesis of pyrrole-based aroylhydrazones

The starting hydrazide ethyl 5-(4-bromophenyl)-1-(1-hydrazinyl-4-methyl-1-oxopentan-2-yl)-2-methyl-1H-pyrrole-3-carboxylate **5** was obtained according to a previously reported procedure by Tzankova et al. (2019). A series of pyrrole-based aroylhydrazones (Fig. 1) were synthesized by condensation of equimolar amounts of ethyl 5-(4-bromophenyl)-1-(1-hydrazinyl-4-methyl-1-oxopentan-2-yl)-2-methyl-1H-pyrrole-3-carboxylate **5** and the corresponding aldehydes (**h–m**) (Fig. 2) in 1 mL of glacial acetic acid. The reaction mixtures were heated at 100 °C with continuous stirring for 40–60 min until complete consumption of the starting materials, as monitored by TLC. The products were isolated after adding water and recrystallized from ethanol.

The synthesis of the new aroyl derivatives was performed in good yields (76–93%). The structures of the obtained molecules were elucidated through appropriate spectral methods, and the corresponding characteristics were in agreement with the assigned structures. The purity of the compounds was proven by the corresponding melting point determinations and TLC characteristics. All the results are presented in the Materials and methods section.

Antioxidant activity

DPPH radical scavenging assay

The antioxidant activity of the newly synthesized pyrrole-based aroylhydrazones was evaluated by the DPPH method, which is based on the characteristic property of the stable free radical 2,2-diphenyl-1-picrylhydrazyl to absorb light at 517 nm. The method is based on a decrease in absorption as a result of electron acceptance by the free radical, which leads to visual discoloration of the violet solution.

The results of the analysis are presented in Fig. 3, where it is clearly shown that Trolox effectively binds the DPPH radical at the applied concentrations.

Of the tested compounds, the highest radical-scavenging activity was observed with compound **9j**, with its effect at the tested concentrations being weaker than that of Trolox. The remaining molecules showed low or no antioxidant activity with this method.

ABTS radical scavenging assay

Screening for antioxidant activity of the newly synthesized compounds was also performed using the ABTS test, based on the ability of the studied compounds to bind the cationic radical ABTS•+. The results of the analysis for radical-binding activity are presented in Fig. 4.

The data obtained show that three of the newly synthesized compounds possess ABTS radical-binding properties. These are compounds **9j**, **9m**, and **9l**. Of these, **9j** has the best expressed ABTS radical-binding potential, and its activity at the applied concentrations is comparable to that of the Trolox standard.

The remaining compounds **9h**, **9i**, and **9k** show low antioxidant activity with this method.

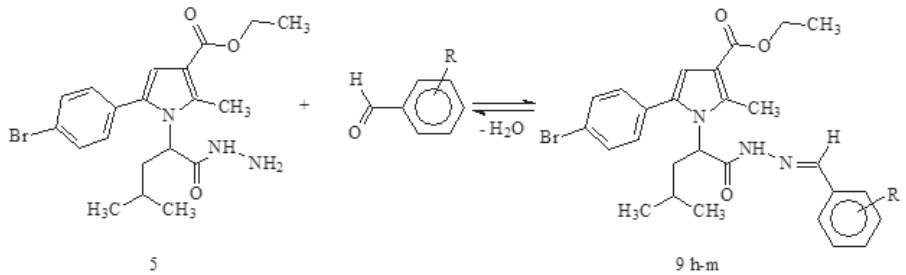

Figure 1. Synthesis of pyrrole-based aroylhydrazones (**9h–m**).

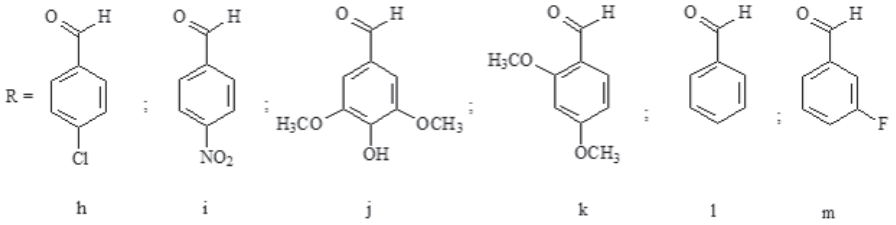

Figure 2. Aldehydes used in the synthesis (**h–m**).

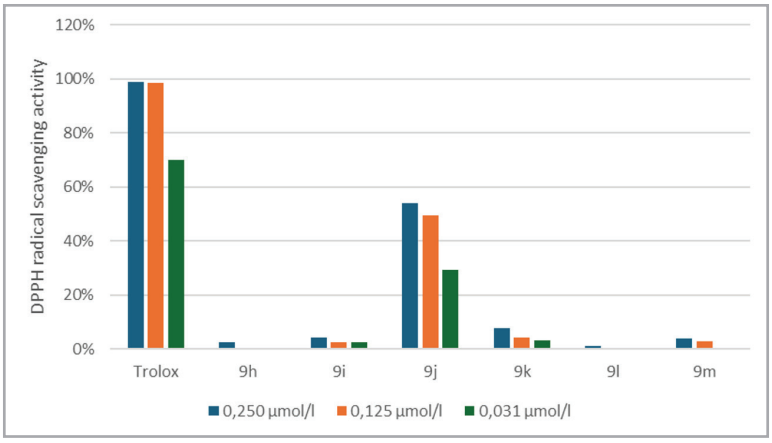

Figure 3. DPPH radical scavenging assay of compounds **9h–m** and Trolox. Standard deviation (SD) (n = 3).

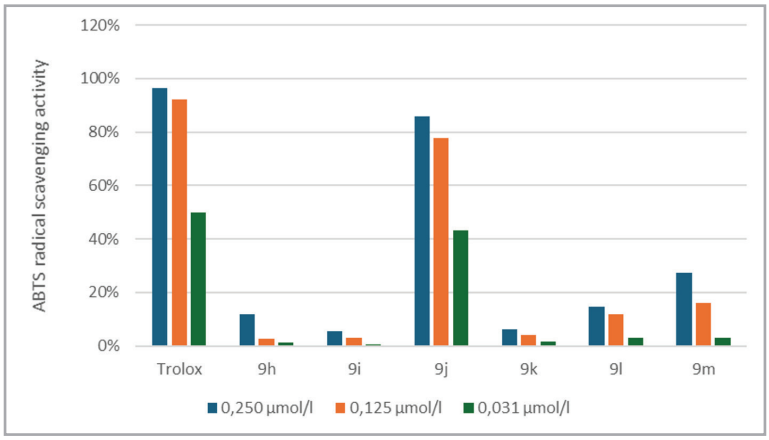

Figure 4. ABTS radical scavenging assay of compounds **9h–m** and Trolox. Standard deviation (SD) (n = 3).

The observed high antioxidant activity of hydrazone **9j** is expected since the substance contains a free hydroxyl OH group (Yamauchi et al. 2024). This makes compound **9j** the most suitable for further testing among all the newly synthesized compounds.

Cytotoxicity evaluation of pyrrole derivatives on neuroblastoma SH-SY5Y cell line

The potential cytotoxic effects of pyrrole derivatives on the human neuroblastoma cell line SH-SY5Y were evaluated by the MTT assay. The cells were treated with the newly synthesized compounds at concentrations ranging from 0.1 to 500 µM (Table 1). IC_{50} values of the compounds were calculated following 24 h treatment. Melatonin was used as a reference compound.

In the neuroblastoma cell line SH-SY5Y, compound **9l** showed the lowest cytotoxicity, with an IC_{50} value > 500 µM, followed by compounds **9k**, **9i**, **9m**, and **9j**, with calculated IC_{50} values of 360.2 µM, 195.60 µM, 186.04 µM, and 127.78 µM, respectively. Compound **9h** demonstrated the highest cytotoxicity among the tested compounds in the series (IC_{50} = 59.84 µM).

Table 1. Effects of a newly synthesized series of pyrrole derivatives (1–500 µM) on cell viability (MTT assay, 24 h treatment).

Compound	IC_{50} [µM]	95% Confidential interval
9h	59.84	36.55–95.62
9i	195.60	161.0–236.8
9j	127.78	122.90–132.51
9k	360.2	309.1–423.5
9l	> 500	-
9m	186.04	175.09–199.93
Melatonin	> 500	-

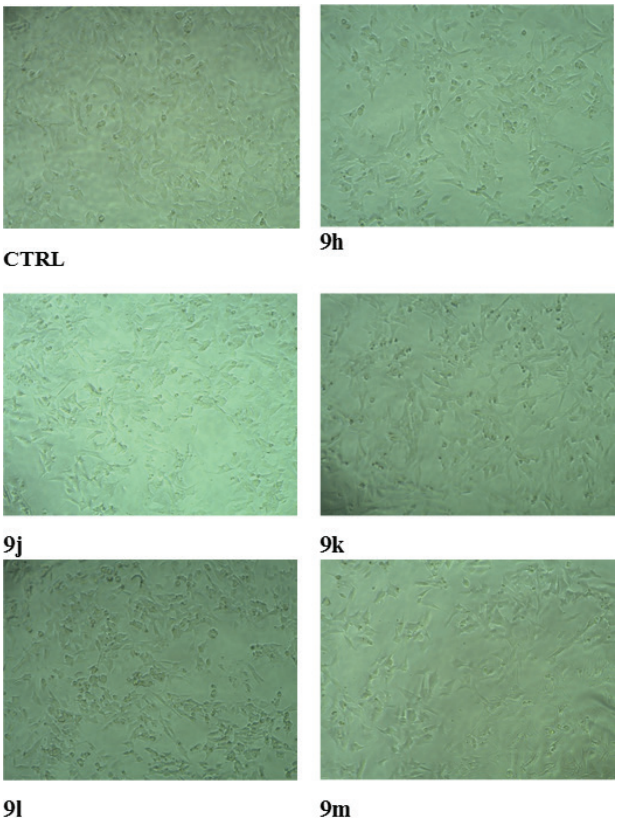

Figure 5. Photomicrographs of SH-SY5Y cells treated for 24 h with the newly synthesized compounds **9h**, **9j**, **9k**, **9l**, and **9m** (100 μM).

Effects of pyrrole derivatives on cell morphology

The 24 h treatment with the newly synthesized pyrrole derivatives at concentrations of 100 μM did not show noticeable differences in cell morphology compared to the control group (untreated cells) (Fig. 5). No increase in the number of rounded cells was detected, which usually accompanies impaired functions of the cytoskeleton of damaged cells. The analysis of SH-SY5Y cell morphology confirms the results from the MTT cytotoxicity assay.

In vitro neuroprotective effects evaluation of newly synthesized pyrrole derivatives in a model of H_2O_2-induced oxidative stress in the neuronal SH-SY5Y cell line

The potential neuroprotective effects of the newly synthesized hydrazide-hydrazones were investigated *in vitro* in a model of H_2O_2-induced oxidative stress in SH-SY5Y cells (Fig. 6). The cells were pretreated with the test compounds for 90 min at concentrations of 0.1, 1, 5, 10, 20, and 50 μM. Thereafter, the cells were treated with H_2O_2 (1 mM, 15 min). The protective effects were compared with those of melatonin, used as a reference compound because of its well-established antioxidant neuroprotective effect. Preincubation with melatonin improved cell viability in a concentration-dependent manner

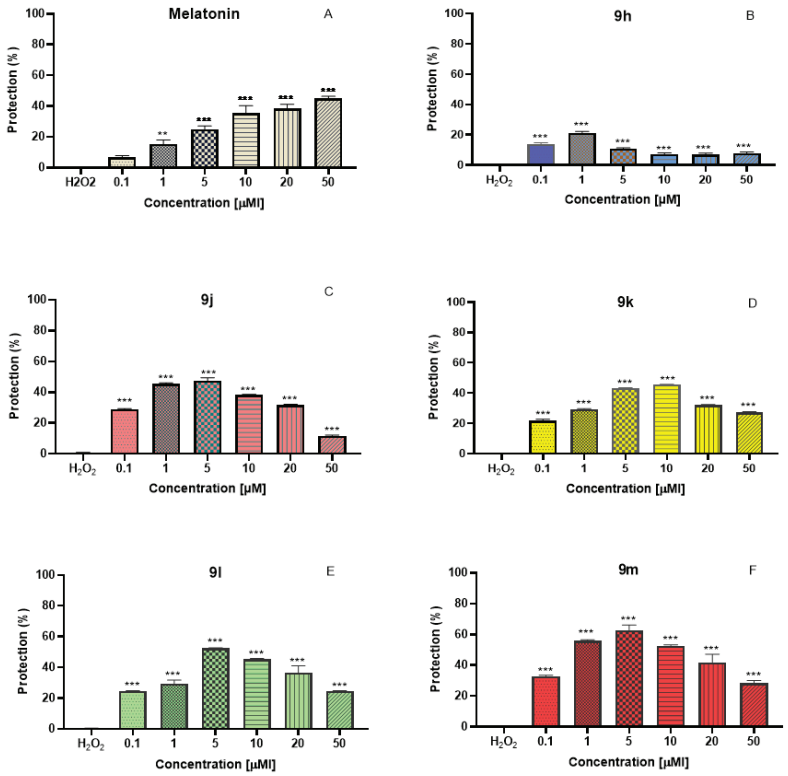

Figure 6. Protective effects of newly synthesized pyrrole derivatives (0.1, 1, 5, 10, 20, and 50 μM) in a model of H_2O_2-induced oxidative stress in the neuronal SH-SY5Y cell line. Melatonin (0.1–50 μM) was used as a reference substance. Data from three independent experiments are presented, with mean values ± SD ($n = 6$). $^{**}p < 0.01$; $^{***}p < 0.001$, compared to H_2O_2-challenge (one-way ANOVA, with Dunnett's post-test).

(Fig. 6A). Protection by 15% at 1 μM (**$p < 0.01$), by 25% at 5 μM, by 35% at 10 μM, by 39% at 20 μM, and by 45% at 50 μM (***$p < 0.001$), respectively, compared to H_2O_2-treatment, was determined.

Compound **9h** (the most toxic from the cell viability assay) shows weak neuroprotective effects *in vitro*. The highest neuroprotective effect was observed at a concentration of 1 μM, showing preservation of cell viability by 22% (***$p < 0.001$) (Fig. 6B). Compound **9j** (Fig. 6C) reduced H_2O_2-induced cell damage at concentrations of 0.1, 1, and 5 μM. Statistically significant protection of SH-SY5Y cell viability was observed at these concentrations by 29%, 46%, and 48%, respectively (***$p < 0.001$), compared to H_2O_2 treatment. However, at the higher tested concentrations of 10, 20, and 50 μM, the protection decreased to 38%, 32%, and 12% (***$p < 0.001$), respectively, indicating that the lower concentrations of the compound were most effective. Compound **9k** significantly reduced H_2O_2-induced cell damage at all tested concentrations (Fig. 6D). The strongest and statistically significant protection at concentrations of 5 and 10 μM was determined by 43% and 46%, respectively (***$p < 0.001$). The substance **9l** showed the highest and statistically significant protection at 5 and 10 μM, respectively, by 52% and 45% (***$p < 0.001$), vs. H_2O_2 treatment (Fig. 6E). Compound **9m** (Fig. 6F) also showed significant preservation of cell viability in the H_2O_2-induced oxidative stress model. The highest and statistically significant protection was achieved at concentrations of 1, 5, and 10 μM, respectively, by 56%, 63%, and 53% (***$p < 0.001$), compared to H_2O_2-induced damage.

Compound **9i** did not demonstrate neuroprotective effects in the neuroblastoma cell line SH-SY5Y (results are not presented).

Conclusion

Pyrrole-based aroylhydrazones (**9h–m**) were synthesized and evaluated for antioxidant properties by two different methods (DPPH and ABTS assays). Most of the compounds demonstrated low to moderate activity as free radical scavengers. In these assays, compound **9j** demonstrated the best activity, which is attributed to the presence of a phenolic hydroxyl group. These results are consistent with the idea that the structure of the molecule influences its antioxidant capacity. The *in vitro* cytotoxicity study on the neuroblastoma cell line SH-SY5Y showed that compound **9l** was the least toxic (IC_{50} > 500 μM), followed by compounds **9k** > **9i** > **9m** > **9j**. The cell morphological evaluation confirmed the lack of toxicity and the good safety profile of the compounds.

The neuroprotective effects of pyrrole derivatives were shown in a model of H_2O_2-induced oxidative stress in neuroblastoma SH-SY5Y cells. Compounds **9m** and **9l** demonstrated good protection in the entire concentration range. Compound **9j** demonstrated statistically significant protection at lower concentrations of 0.1 mM, 1 mM, and 5 mM. The protective effect shown by compound **9k** was also promising. In conclusion, the newly synthesized compounds have low toxicity and exhibit good antioxidant effects *in vitro*. They are promising candidates for further investigation of their neuroprotective potential *in vitro* and *in vivo* models of neurodegeneration.

Additional information

Conflict of interest

The authors have declared that no competing interests exist.

Ethical statements

The authors declared that no clinical trials were used in the present study.

The authors declared that no experiments on humans or human tissues were performed for the present study.

The authors declared that no informed consent was obtained from the humans, donors or donors' representatives participating in the study.

Experiments on animals: Permission No. 190, Bulgarian Food Safety Agency (approved on February 6, 2020; valid until 2026).

Use of commercially available immortalised human and animal cell lines: SH-SY5Y human neuroblastoma cell line (Sigma-Aldrich, ECACC, catalogue No. 94030304).

Artificial Intelligence (AI) use

The authors accept full responsibility for the content of the manuscript, including the disclosure of any use of AI.
No AI tools were used in the preparation of this manuscript.

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Data availability

All of the data that support the findings of this study are available in the main text.

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