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Synthesis and investigation of radical scavenging activity, neurotoxicity and neuroprotection of new theophilline hydrazones

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The imbalance between generations and clearances of oxidants leads to oxidative stress which nowadays has become a major interest in point of basic science and clinical research. The current work is focused on synthesis and radical scavenging activity of new theophilline derivatives, comprising a hydrazone group in 7th position in the xanthine core. The structures of the new compounds were elucidated through IR, ^{1}H NMR and MS spectral data. The purity of the compounds was proven by the corresponding melting points and TLC characteristics. The obtained structures were evaluated for radical scavenging activity through DPPH and ABTS methodology. Two structures were outlined as most effective radical scavengers – **4** and **4b**. In addition the neroprotective and neurotoxic effects of the target hydrazones were also evaluated. The most neurotoxic molecules were determined to be compounds **4a** and **4d**. Highest neuroprotective properties were obtained for **4b**.

Key words: theophilline, DPPH, ABTS, 6-OHDA, neurotoxicity, neuroprotection

INTRODUCTION

Since oxidative stress has been considered as a major component of the pathophysiology of many conditions including neurodegenerative, metabolic diseases and cancer an increase in the search of new compounds that could act as antioxidants in order to achieve neuroprotection and improve the physiological defense mechanisms of the cells is observed.

Methylxanthines are natural compounds found in coffee, tea and cocoa. They have a long history of usage due to the various pharmacological effects they exhibit, including stimulation of the central nervous system, enhanced cognition, bronchodilation, diuretic and neuroprotective effects, etc. Later methylxanthines are considered as promising structures in the synthesis of new biologically active molecules in means of radical scavengers, based on the antioxidant properties of caffeine, theobromine and xanthine [1]. The semisynthetic xanthine derivative aminophylline is an antioxidant that antagonizes the effects of hydroxyl radicals, which are believed to take part in the pathophysiology of asthma [2]. Theophylline is widely used in the treatment of various pulmonary diseases. It has dual mechanism of action: inhibits phosphodiesterase and acts as an antagonist to the adenosine receptors. Many studies prove that it also has antioxidant [3, 4] and neuroprotective properties [5]. Theophylline also shows protective properties in animal models of Parkinson's disease [6].

Hydrazone derivatives exhibit a variety of pharmacological effects, some of which include antibacterial, anti-inflammatory, analgesic and cytotoxic activity [7]. Hydrazones are being studied extensively for the treatment of many neurological diseases – benziliden hydrazones with monoamine oxidase inhibitory activity for Parkinson's disease [8], methoxyacyl hydrazones inhibiting phosphodiesterase 10A [9], hydrazones with anticonvulsant [10] and antidepressant activity [11].

Based on the observed relationship on the effects expressed by the xanthine molecule, depending on the position of structural substituents [12] and the recent findings on compounds with hydrazone structure, their facile synthesis and important number of interesting biological effects, such as antioxidant [13], antitumor, antiinflammatory, anticonvulsive, analgesic, antimicrobial and antiviral [14] we decided to combine these two moieties in an attempt to identify new perspective antioxidative agents.

EXPERIMENTAL

Chemicals and reagents

The starting materials used for the synthesis of the target compounds were of commercially available synthetic grade chemicals procured from Acros organics, Belgium, used as received. Non-commercially available intermediates required for the synthesis of novel xanthine derivatives were

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prepared according to the procedure explained below.

The reagents: 2,2′-Diphenyl-1-picrylhydrazyl (DPPH), 2,2′-azinobis-(3-ethylbenzo thiazine-6-sulfonic acid) (ABTS), sulfanilamide, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), 2,4,6-tripyridyl-s-triazine (TPTZ), ferric chloride x $6H_2O$, sodium acetate and potassium persulphate were purchased from Sigma-Aldrich. All the other chemicals including the solvents were of analytical grade.

The necessary tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 6-hydroxydopamine (6-OHDA), Percoll, 4-(2-hydroxyethyl)-1-piperazineethane-sulfonic acid (HEPES) and Sucrose were obtained from Sigma Aldrich, Germany. The NaCl, KCl, $CaCl_2$x2H2O, $MgCl_2$, $NaHPO_4$, D-glucose, trichloroacetic acid and 2,2'-dinitro-5,5'-dithiodibenzoic acid (DTNB) were supplied from Merck (Darmstadt, Germany).

Chemistry

Synthetic procedure for obtaining the sodium salt of theophylline. The necessary initial theophilline sodium salt was synthesized through a literary method, explained in Peikov et. al. [15]. The obtained product was included in the next step without isolation.

Synthetic procedure for obtaining ethyl 2-(1,3-dimethyl-2,6-dioxo-2,3-dihydro-1H-purin-7(6H)-yl)propanoate (3). A mixture of theophilline (**1**, 0.01 mol) and 30% solution of sodium ethylate (0.01 mol) in anhydrous ethanol (50 ml) was refluxed. After 1 hour 2-bromopropylethylate was added (0.01 mol) and the reaction mixture was refluxed for 38 hours. The progress of the reaction was monitored by TLC. Then the ethanol was evaporated to dryness and yellow-brown oil was isolated. The product was washed with small amounts of chloroform and filtered. The obtained yellow-brown filtrate was not isolated and was used in the next step of synthesis.

Synthetic procedure for obtaining the initial 2-(1,3-dimethyl-2,6-dioxo-2,3-dihydro-1H-purin-7(6H)-yl)propanehydrazide (4). The initial hydrazide **4** was prepared by classical hydrazinolysis of the relevant intermediate ethyl ester (**3**) by interaction of **3** (0.01 mol) in ethanol with excess of hydrazine hydrate (0.025 mol) under reflux. The reaction was monitored by TLC until exhaustion of initial ester. The obtained white precipitate was isolated and re-crystallized from ethanol.

2-(1,3-dimethyl-2,6-dioxo-2,3-dihydro-1H-purin-7(6H)-yl)propanehydrazide (4): Yield 67%. mp 168-175°C. ν_{max} (iATR)/cm^{-1} 3370 (NH_2), 3268 (NH), 1703 ($COOC_2H_5$, CO - Amide I), 1651 (Amide II), 1615 (C-O). δ_H (at 600 MHz, in $CDCl_3$): 9,08 (s, 1H, -NH-) 8,53 (d, 1H, -CH-, Th) 4,74 (m, 1H, -CH-) 4,22 (m, 2H, $-NH_2$); 3.35 (s, 3H, $-CH_3$, Th); 3,41 (s, 3H, $-CH_3$, Th); 1,79 (d, 3H, $-CH_3$); *m/z* (FTMS + pESI) = 267.12.

General synthetic procedure for obtaining of the target compounds 4a-d. The synthesis of the target hydrazones was performed by two general methods.

Method 1: Equimolar quantities of 0.001 mol of carbohydrazide **4** and any of the carbonyl partners **a, b, c or d** were dissolved in 2 ml glacial acetic acid in a round bottom flask and stirred at 100ºC for 30-270 min to complete the reaction under TLC-control. The products were isolated after adding water and recrystallized from ethanol.

Method 2: In a round bottom flask were mixed equimolar quantities of 0.001 mol of carbohydrazide **4** and any of the carbonyl partners **a, b, c or d** dissolved in 4 ml ethanol in the presence of few drops glacial acetic acid, used as a catalyst. The mixture was stirred at 100ºC for 10-180 min to complete the reaction under TLC-control. The products were isolated after adding water and recrystallized from ethanol.

2-(1,3-dimethyl-2,6-dioxo-2,3-dihydro-1H-purin-7(6H)-yl)-N'-(3-fluorobenzylide-ne) propanehydrazide (4a): Reaction time **Method 2** 30 min. Yield: 99.9%, m.p. 240-242°C, Rf=0.44, IR: ν_{max} (iATR)/cm^{-1} 3125 (-NH-), 2995 (-CH-), 2920 ($-CH_3$ Th), 2889 ($-CH_3$ Th), 1706 (-C=O Th), 1667 (-C=O Th), 1591 (-C=O), ^{1}H NMR δ_H (at 600 MHz, in $CDCl_3$): 11.07 (m, 1H, -NH-), 8,53 (d, 1H, -CH=, Th), 8,47 (s, 1H, -CH=), 7,80 (d, 1H, -CH=, C_6H_6), 7,63, 7,53, 7,44 (m, 1H, -CH=, C_6H_6), 4,74 (m, 1H, -CH-), 3,41; 3,35 (s, 3H, $-CH_3$, Th), 1,79 (d, 3H, $-CH_3$), *m/z* (FTMS + pESI) = 373.14.

N'-(2,4-dimethoxybenzylidene)-2-(1,3-dimethyl-2,6-dioxo-2,3-dihydro-1H-purin-7(6H)-yl)propanehydrazide (4b). Reaction time **Method 2** 60 min. Yield: 84%,, m.p. 175-177°C, Rf=0.50, IR: ν_{max} (iATR)/cm^{-1} 3272 (-NH-), 2994 ($-CH_3$ Th), 2948 ($-CH_3$ Th), 2837 ($-OCH_3$); ^{1}H NMR δ_H (at 600 MHz, in $CDCl_3$): 11,07 (m, 1H, -NH-), 8,78 (d, 1H, -CH=), 8,53 (d, 1H, -CH=, Th), 7,51; 6,67; 6,65 (m, 1H, -CH=, C_6H_6), 4,74 (m,1H, -CH-), 3,84; 3,81 (s, 3H, $-OCH_3$), 3,41; 3,35 (s, 3H, $-CH_3$, Th), 1,79 (d, 3H, $-CH_3$), *m/z* (FTMS + pESI) = 413.26.

N'-(2,3-dimethoxybenzylidene)-2-(1,3-dimethyl-2,6-dioxo-2,3-dihydro-1H-purin-7(6H)-yl)propanehydrazide (4c). Reaction time **Method 2** 60 min. Yield: 66%, m.p. 260-262°C, Rf=0.53, IR: ν_{max} (iATR)/cm^{-1} 3454 (-OH), 3132 (-NH-), 2828 ($-OCH_3$); ^{1}H NMR δ_H (at 600 MHz, in

$CDCl_3$): 11,07 (m, 1H, -NH-), 8,78 (d, 1H, -CH=), 8,53 (d, 1H, -CH=, Th), 7,50; 7,21; 6,99 (m, 1H, -CH=, C_6H_6), 4,74 (m,1H, -CH-), 3,89; 3,85 (s, 3H, -OCH_3), 3,41; 3,35 (s, 3H, -CH_3, Th), 1,79 (d, 3H, -CH_3), *m/z* (FTMS + pESI) = 415.2.

N-(4-((2-(2-(1,3-dimethyl-2,6-dioxo-2,3-dihydro-1H-purin-7(6H)-yl)propanoyl) hydrazono)methyl)phenyl)acetamide(4d). Reaction time **Method 2** 180 min. Yield: 89.9%,, m.p. 279-281°C, Rf=0.63, IR: ν_{max} (iATR)/cm^{-1} 3174 (-HN-), 2918 (-CH_3 Th), 2849 (-CH_3 Th), 1667 (-C=O) Amide; ^{1}H NMR δ_H (at 600 MHz, in $CDCl_3$): 11,07 (m, 1H, -NH-), 10,16 (d, 1H, -NH-), 8,53 (d, 1H, -CH=, Th), 8,47 (d, 1H, -CH=), 7,78; 7,68 (d, 1H, -CH=, C_6H_6), 4,74 (m,1H, -CH-), 3,41; 3,35 (s, 3H, -CH_3, Th), 2,06 (s, 3H, -CH_3), 1,79 (d, 3H, -CH_3), *m/z* (FTMS + pESI) = 412.3.

Antioxidant assays

DPPH radical scavenging activity. Free radical scavenging activity was measured using DPPH method [16]. Percent of the DPPH radicals scavenged by the studied concentration was calculated according to equation:

$$DPPH\ radical\ scavenging\ activity\ (\%) = \frac{Abs_{control} - Abs_{sample}}{Abs_{control}} \text{x} 100 \qquad (1)$$

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

IC_{50} value (concentration of sample where absorbance of DPPH decreases 50% with respect to absorbance of blank) of the sample was determined. Butylated hydroxytoluene (BHT) was used as positive control. All determinations were performed in triplicate.

ABTS radical scavenging assay. For ABTS assay, the procedure followed the method of Arnao et al. [17] with some modifications [16]. The capability to scavenge the ABTS radical was compared with that of BHT, used as positive control, and was calculated using the following equation:

$$ABTS\ radical\ scavenging\ activity\ (\%) = \frac{Abs_{control} - Abs_{sample}}{Abs_{control}} \text{x}\ 100 \qquad (2)$$

where – $Abs_{control}$ is the absorbance of ABTS radical in methanol; Abs_{sample} is the absorbance of an ABTS radical solution mixed with sample.

IC_{50} value (concentration of sample where absorbance of ABTS decreases 50% with respect to absorbance of blank) of the sample was determined. All determinations were performed in triplicate.

Isolation and incubation of rat brain synaptosomes. The isolation of the rat brain synaptosomes was performed by multiple, subcellular fractionation using a Percoll gradient, according to a procedure described in [18].

After incubation, a MTT-test was performed to determine synaptosomal vitality by method described by Mungarro-Menchaca et al. [19], based on the proportionality of the quantity of the obtained formazan to the cellular vitality, as described in [20].

Determination of reduced glutathione (GSH). The level of reduced glutathione is determined by measuring the non-protein SH-groups after precipitation of the proteins with trichloroacetic acid.

After incubation, synaptosomes were centrifuged at 400 x g for 3 minutes. The pellet was treated with 5 % trichloroacetic acid and left for 10 minutes on ice. Samples were centrifuged at 8000 x g for 10 minutes (2^{o}C). The supernatant was removed to determine the level of GSH and it can be stored at -20°C. Immediately before the measurement, the samples were neutralized with 5 N NaOH.

The presence of thiols in the supernatant is determined using Elmman reagent. The resulting yellow color is measured spectrophotometrically (λ = 412 nm) [21].

Model of 6-OHDA-induced neurotoxicity. This *in vitro* model resembles the neurodegenerative processes occurring in PD. Dopamine metabolism and oxidation lead to the formation of reactive oxygen species (ROS) and reactive quinones. They induce dopamine neurotoxicity and neurodegeneration [22]. The synaptosomes were incubated with 150 μM 6-OHDA and the test substance for 1 hour.

Statistical methods

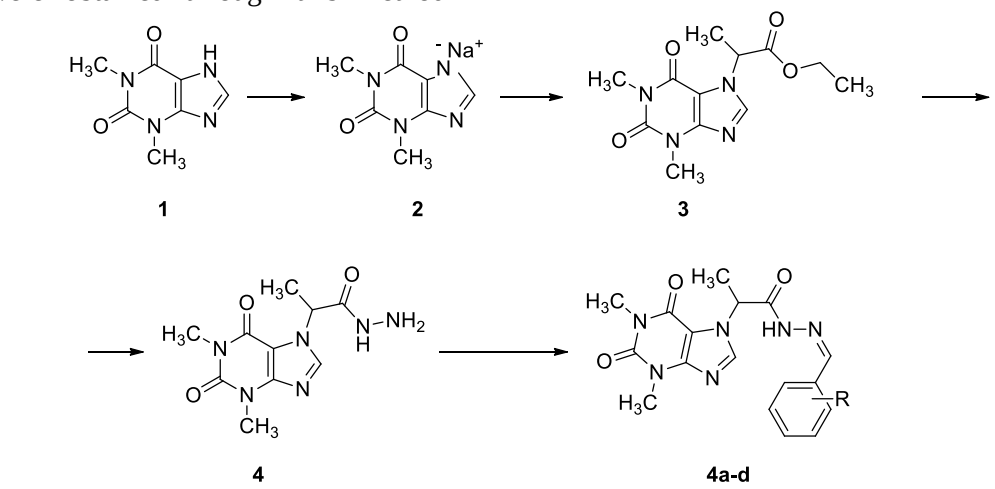
Statistical analysis was performed using statistical programme "MEDCALC". Results are expressed as mean ± SEM for 6 experiments. The significance of the data was assessed using the nonparametric Mann-Whitney test (synaptosomes). Values of $p \leq 0,05$; $p \leq 0,01$ and $p \leq 0,001$ were considered statistically significant.

RESULTS AND DISCUSSION

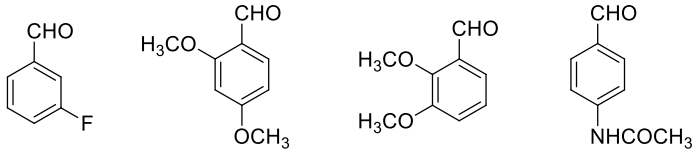
Based on the role of oxidative stress in many diseases, we synthesized a series of new theophylline hydrazones.

The synthesis was based on condensation of previously prepared 2-(1,3-dimethyl-2,6-dioxo-2,3-dihydro-1H-purin-7(6H)-yl)propanehydrazide (**4**) and with a series of substituted aryl aldehydes. The condensation was performed by two general methods, as explained in the Experimental part, where for the purp ose of the investigation as more

appropriate was selected **Method 2**, since it gave higher yields and shorter reaction times. All target hydrazones were obtained through this method according to the procedure demonstrated on **Scheme 1**:



where, the used carbonyl compounds are as follows:



Scheme 1. Synthesis of target compounds 4a-d.

The synthesized compounds were found to be freely soluble in chloroform, dichloromethane, DMF and DMSO. Most of the structures were insoluble in non-polar solvents.

The compounds were purified and the structures of the pure products were confirmed by IR, ^{1}H NMR and MS spectral analysis. The results were consistent with the assigned structures. The purity was determined through the corresponding TLC characteristics and melting points.

Antioxidant assays

The free radicals scavenging activity was determined using DPPH and ABTS methods, with slight modifications [23]. The inhibitory effect of the compounds on DPPH and ABTS was measured and the corresponding graphical dependency of the two most active compounds and the BHT was drawn and presented on **Fig. 1** and **2**, as follows:

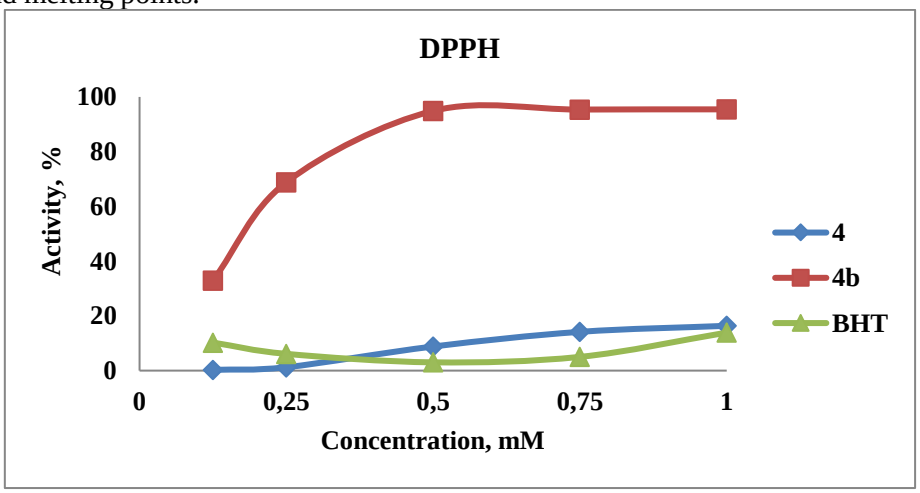


Fig. 1. DPPH radical scavenging activity of most active compounds and BHT.

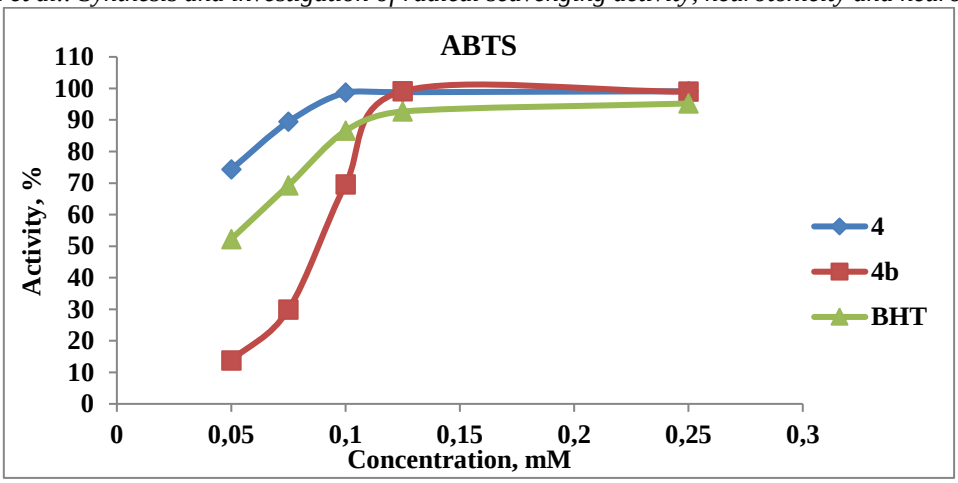


Fig. 2. ABTS radical scavenging activity of most active compounds and BHT.

The activity of compounds **4a-d** was evaluated as corresponding IC_{50} values (the concentration where the absorbance of DPPH and ABTS decreases by 50% with respect to absorbance of blank) from the absorbance of the reaction mixtures at 517 and 734 nm, respectively. The obtained results are outlined in Table 1.

Table 1. IC_{50} values for the DPPH and ABTS radical scavenging activity of the most active hydrazones.

ID	IC_{50} (mM)	
	DPPH	ABTS
4	0,089	0,348
4b	2,608	0,089
BHT	12,125	0,044

As a positive control for evaluation of the antioxidant activities of the compounds against DPPH and ABTS was used butylated hydroxytoluene (BHT).

Among the analyzed structures only compounds **4** and **4b** expressed DPPH radical scavenging activity with IC_{50} of 0.089 and 2.608 mM, respectively. As visible the evaluated hydrazone expressed about 6 times higher activity than the positive control BHT (IC_{50} 12.125 mM). In this case compound **4** (the initial hydraizde), is underlined with highest radical scavenging activity, probably due to the free NH-NH_2 group.

Among the analyzed structures again compounds **4** and **4b** expressed high ABTS radical scavenging activity with IC_{50} of 0.348 and 0.089 mM, respectively. The applied as positive control BHT has higher ABTS activity (IC_{50} value of 0.044 mM).

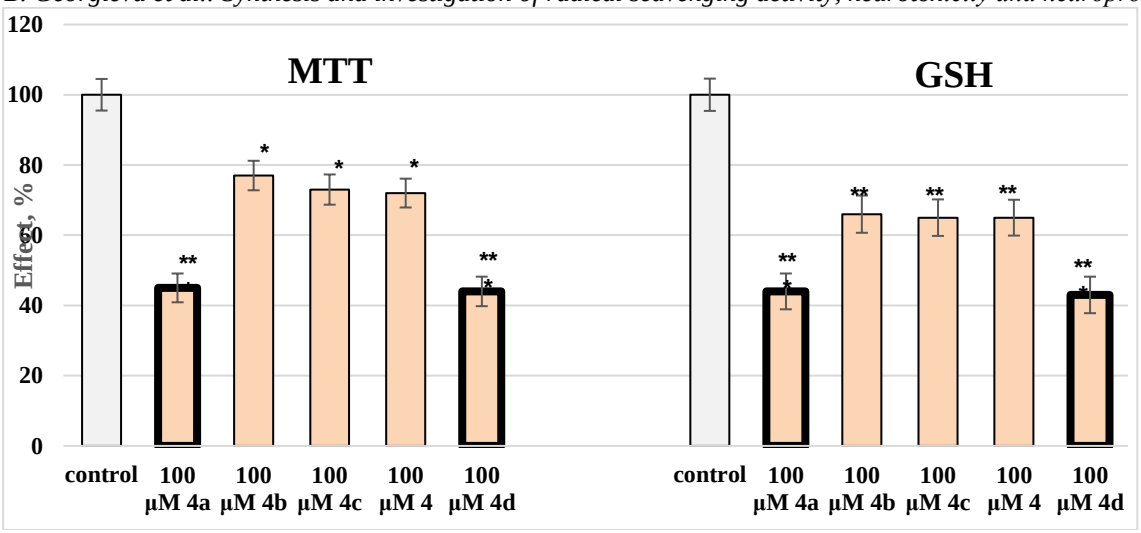
Comparing the calculated values for the evaluated parameters of both methods and taking into account the solubility of DPPH only in polar matrices, we consider this as a reason for slower reaction of targeted hydrazones with DPPH radical, in compare to ABTS. Thus it can be assumed that the ABTS test is more appropriate method for determining of radical scavenging activity of this group of compounds.

Neurotoxicity and neuroprotection

Since the hypothesis that oxidative stress is pathogenic in neurodegenerative disease through ROS mediates neurotoxicity, one strategy in disease control has been focused on development of antioxidants as preventive and therapeutic molecules [24].

Glutathione reduces disulfide bonds formed within cytoplasmic proteins to cysteines by serving as an electron donor. In the process, glutathione is converted to its oxidized form, glutathione disulfide (GSSG) [25]. Once oxidized, glutathione can be reduced back by glutathione reductase, using NADPH as an electron donor. The ratio of reduced glutathione to oxidized glutathione within cells is often used as a measure of cellular oxidative stress [26].

We applied this system as a model for evaluation of the neurotoxic properties of the newly synthesized derivatives **4** and **4a-d**. The results are demonstrated on **Fig. 3**.



* P < 0.05, ** P < 0.01, *** P < 0.001 compared to the control (not-treated synaptosomes).

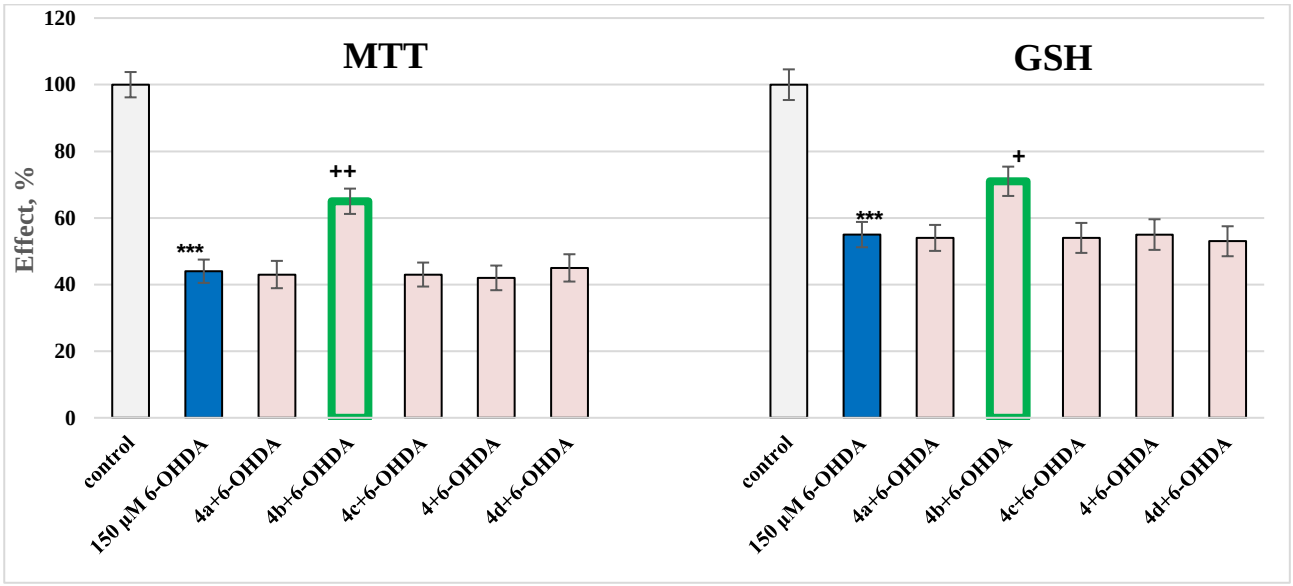
Fig. 3. Effect of tested compounds applied alone (in concentration 100 µM) on the synaptosomal viability and reduced glutathione level (GSH).

When applied alone the tested compounds perform statistically significant neurotoxic effect on the isolated synaptosomes. All evaluated hydrazones decrease the synaptosomal viability and the reduced glutathione level, where the highest toxic effect is expressed by **4a** and **4d**.

Compound **4a** decreases the cell viability (determined through MTT-test) by 55 %, and the GSH level – by 56 %, respectively, against the control.

Compound **4d** decreases the cell viability (determined through MTT-test) by 56 %, and the GSH level – by 57 %, respectively, against the control.

In addition we attempted to evaluate the neuroprotective properties of the newly synthesized derivatives **4** and **4a-d** in conditions of 6-OHDA induced oxidative stress. The results are demonstrated on **Fig. 4**.



*** P < 0.001 compared to the control (not-treated synaptosomes);+ P < 0.05, ++ P < 0.01 compared to 6-OHDA

Fig. 4. Effect of tested compounds (in concentration 100 µM) on the synaptosomal viability and reduced glutathione level (GSH) in conditions of 6-OHDA-induced oxidative stress.

In conditions of 6-hydroxydopamine (6-OHDA)-induced oxidative stress only compound **4b** performs statistically significant neuroprotective effect on isolated synaptosomes, against the toxic agent. **4b** stores the cell viability (determined through MTT-test) by 21 %, and the GSH level – by 16 %, respectively, against the toxic agent.

CONCLUSION

One new hydrazide and four new theophylline based hydrazones were synthesized and evaluated for *in vitro* radical scavenging activity against DPPH and ABTS radicals. The structures of the new compounds were elucidated by IR, ^{1}H and MS

spectral analysis. The purity of the obtained substances was proven by TLC characteristics and melting points. The activity against DPPH and ABTS radicals was evaluated for all new compounds. Two of the tested structures were indicated with high free radical scavenging activity, which is a prerequisite for this group of structures to be considered as a starting platform for synthesis of highly effective antioxidants. Two structures were outlined as most effective radical scavengers – **4** and **4b**.

The neroprotective and neurotoxic effects of the target hydrazones were also evaluated through identification of the activity on 6-hydroxydopamine (6-OHDA)-induced oxidative stress, underlining as most neurotoxic molecules compounds **4a** and **4d** and as highest neuroprotective structure **4b**, which makes it suitable for further evaluations.

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