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Syntheses of some hydrazones derived from 2-(aryloyloxy) benzaldehydes and 2,4-dinitrophenylhydrazine and evaluation of their anticholinesterase and antioxidant activities

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Abstract: In this research, five novel hydrazone derivatives (**2a-e**) were obtained for the first time, characterized and investigated for their antioxidant properties, and acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) inhibitory activities. The target molecules were easily synthesized by the condensation reaction of 2,4-dinitrophenylhydrazine (2,4-DNPH) with aryl esters (**1a-e**) derived from salicylaldehyde as a starting material. These molecules were fully elucidated by some spectroscopic techniques and elemental analysis. Antioxidant activities of newly synthesized molecules were examined by CUPRAC reducing, ABTS and DPPH radical scavenging assays. The IC_{50} values of the screened molecules were determined in the range of 72.54-221.52 µM against AChE and in the range of 8.46-48.28 µM against BChE. Among the tested molecules, compound **2e** indicated the highest activity against both AChE and BChE. Also, the inhibitory capacities of all tested molecules were compared to the standard molecules galanthamine. On the other hand, In CUPRAC reducing assay, the target molecules exhibited antioxidant activities in the range of 30.29 and 59.43 µM. Among these compounds, compound **2b** (IC_{50}=30.29 µM) showed the closest activity to the standard compounds butylated hydroxytoluene (BHT) (IC_{50}=30.62 µM) and butylated hydroxyanisole (BHA) (IC_{50}=34.24 µM).

Keywords: Aryl ester, hydrazone, anti-cholinesterase activity, antioxidant activity.

1. Introduction

Antioxidants are molecules that have the ability to protect living cells and vital molecules such as DNA and protein against reactive oxygen species and different radical species[1] . These molecules are obtained from natural sources as well as synthetically. In recent years, various natural antioxidants have been used often in various fields such as health and food industries. On the other hand, there is a great increase in studies on the usability of synthetic antioxidants in these areas.[2] Today, synthetic and natural antioxidants are used in food technology for long-term preservation of foods.[3] In addition, these molecules are used as additives to reduce the negative effects of diseases in the treatment processes of cancer, neurodegenerative and chronic diseases in the health field.[4-7]

Hydrazones constitute a class of bioactive molecules notable for both their broad biological and broad pharmacological properties. Due to their minimum toxicity and maximum effects in the treatment of some diseases, these compounds have been reported to be synthesized as drug candidates and used in biological activities in many studies.[8,9] Hydrazones, carrying an imine (azomethine) group,[10,11] are

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characterized by showing strong antioxidant activities in scavenging free radicals.[12,13] However, hydrazone compounds have been reported in many studies where they are also used in inhibition studies of some metabolic enzymes.[14]

Enzyme inhibition theory is one of the important methods for synthesizing and using new drugs in the diagnosis process of some diseases. Studies on some metabolic diseases have reported Alzheimer's disease (AD) with acetylcholine (ACh) deficiency using this theory.[15] AD occurs due to the level differences of ACh neurotransmitter; and a decrease in ACh synthesis is observed in this disease. The reason for hydrazone this is defined as a decrease in the amount and function of the acetylcholine transferase enzyme.[16] AChE is an esterase enzyme that hydrolyzes the neurotransmitter ACh and belongs to the carboxylesterase enzyme family. On the other hand, BChE, which hydrolyzes choline ester species, is found in almost every part of the human body, such as blood serum and central nervous system.[17,18] BChE, which plays a supportive role in the brain, is involved in the hydrolysis of the neurotransmitter ACh, like AChE, and is shown to be a viable therapeutic target in AD, a disorder associated with cholinergic deficiency.[19] Therefore, the inhibitors of these enzymes work to prevent inflammation in brain synaptic cavities or muscle junctions and also to transmit neuronal signals. These days, some molecules such as donepezil and galanthamine are used as inhibitors for these enzymes.[20-22]

The investigation on searching for novel and more effective AChE and BChE inhibitors with antioxidant activity against AD is still of great interest. Therefore, five novel hydrazone derivatives (**2a-e**) in this research were successfully prepared and assessed for their inhibitory potentials on AChE and BChE. On the other hand, their antioxidant activities were determined by means of three different methods. The structures of the target molecules were precisely elucidated by three spectroscopic techniques and elemental analysis.

2. Experimental

All chemicals, procured from Sigma-Aldrich or Merck companies, were employed without any additional purification. NMR spectra were recorded on a Bruker AVANCE III 400 MHz spectrometer. FT-IR spectra were recorded on a Cary 630 FT-IR spectrometer. Barnstead IA9100 Electrothermal Digital Melting Points Apparatus was employed to determine melting points of newly synthesized molecules. Thermo Scientific Flash 2000 Elemental Analyzer was used to carry out their elemental analysis.

*2.1. The preparation of the Target Molecules (**6-10**)*

The aryl esters **(1a-e)** were synthesized and characterized in a previous study.[23] These molecules were reacted with 2,4-DNPH to obtain the target compounds.[24] Briefly, a solution of a aryl ester (2 mmol) and 2,4-DNPH (2 mmol) and in ethanol (10 mL) was reflux for 4 h. Afterwards, the reaction mixture was cooled down to room temperature. The crude product was gathered by filtration, and then thoroughly rinsed with petroleum ether. Subsequently, the residue was recrystallized from ethanol to give hydrazone compound.

(*E*)-*2-((2-(2,4-Dinitrophenyl)hydrazono)methyl)phenyl benzoate (**2a**)*: Orange solid (80%); M.P.=266-267 °C. FT-IR (cm^{-1}) υ_{max}: 3369, 3120, 3063, 1723, 1614, 1581, 1328. ^{1}H NMR (DMSO-d_6): δ 11.78 (s, 1H), 8.88 (s, 1H), 8.84 (d, *J* = 2.7 Hz, 1H), 8.24 (d, *J* = 7.5 Hz, 2H), 8.22 – 8.19 (m, 1H), 8.11 (d, *J* = 7.8 Hz, 1H), 7.95 (d, *J* = 9.7 Hz, 1H), 7.82 (d, *J* = 7.4 Hz, 1H), 7.69 (t, *J* = 7.7 Hz, 2H), 7.58 (d, *J* = 8.2 Hz, 1H), 7.47 (s, 1H), 7.41 (d, *J* = 7.7 Hz, 1H) ppm. ^{13}C NMR (DMSO-d_6): δ 165.01, 149.76, 144.77, 144.57, 137.62, 134.78, 132.01, 130.65, 130.14, 129.82, 129.57, 129.13, 127.80, 127.18, 127.14, 124.14, 123.34, 117.27 ppm. Anal. Calcd. for $C_{20}H_{14}N_4O_6$: C, 59.12; H, 3.47; N, 13.79%. Found: C, 59.34; H, 4.68; N, 13.88%.

(*E*)-*2-((2-(2,4-Dinitrophenyl)hydrazono)methyl)phenyl 2-nitrobenzoate (**2b**)*:Yellow solid (75%); M.P.=208-209 °C. FT-IR (cm^{-1}) υ_{max}: 3271, 3097, 3070, 1735, 1614, 1532, 1331. ^{1}H NMR (DMSO-d_6): δ 11.80 (s, 1H), 8.86 (d, *J* = 2.7 Hz, 1H), 8.85 (s, 1H), 8.28 (s, 1H), 8.23 (s, 1H), 8.15 (d, *J* = 7.6 Hz, 1H), 8.06 – 7.97 (m, 4H), 7.63 (d, *J* = 7.4 Hz, 1H), 7.50 (s, 1H), 7.41 (d, *J* = 7.7 Hz, 1H) ppm. ^{13}C NMR (DMSO-d_6): δ 163.52, 149.08, 148.83, 144.81, 143.87, 137.78, 134.52, 134.19, 132.18, 131.45, 130.22, 130.00, 127.66, 127.60, 126.88, 124.87, 123.35, 123.22, 117.35 ppm. Anal. Calcd. for $C_{20}H_{13}N_5O_8$: C, 53.22; H, 2.90; N, 15.52%. Found: C, 53.16; H, 2.88; N, 15.59%.

(*E*)-*2-((2-(2,4-Dinitrophenyl)hydrazono)methyl)phenyl 3-nitrobenzoate (**2c**)*: Light orange solid (77%); M.P.=246-247 °C. FT-IR (cm^{-1}) υ_{max}: 3265, 3105, 3089, 1729, 1617, 1532, 1336. ^{1}H NMR ($CDCl_3$): δ 11.30 (s, 1H), 9.14 (d, *J* = 2.2 Hz, 2H), 8.63 (d, *J* = 1.7 Hz, 1H), 8.61 (d, *J* = 1.8 Hz, 1H), 8.27 – 8.24 (m, 1H), 8.21 (s, 1H), 8.13 (d, *J* = 6.5 Hz, 1H), 7.95 (d, *J* = 9.5 Hz, 1H), 7.86 (d, *J* = 8.2 Hz, 1H), 7.59 (d, *J* = 8.1 Hz, 1H), 7.48 (s, 1H), 7.31 (s, 1H) ppm. ^{13}C NMR (DMSO-d_6): δ 163.48, 149.50, 148.50, 144.77, 144.27, 137.72, 136.70, 132.12, 131.55, 130.78, 130.13, 129.97, 129.09, 127.56, 127.52, 127.03, 124.92, 123.97, 123.33, 117.28 ppm. Anal. Calcd. for $C_{20}H_{13}N_5O_8$: C, 53.22; H, 2.90; N, 15.52%. Found: C, 53.27; H, 2.99; N, 15.61%.

(*E*)-*2-((2-(2,4-Dinitrophenyl)hydrazono)methyl)phenyl 4-nitrobenzoate (**2d**)*: Dark yellow solid (79%); M.P.=275-276 °C. FT-IR (cm^{-1}) υ_{max}: 3284, 3092, 3028, 1741, 1611, 1539, 1325. ^{1}H NMR (DMSO-d_6): δ 11.68 (s, 1H), 9.18 – 9.17 (m, 4H), 8.89 (s, 1H), 8.84 (d, *J* = 2.6 Hz, 1H), 8.34 – 8.30 (m, 1H), 8.18 (d, *J* = 7.5 Hz, 1H), 8.03 (d, *J* = 9.6 Hz, 1H), 7.64 (s, 1H), 7.52 (d, *J* = 7.6 Hz, 2H) ppm. Anal. Calcd. for $C_{20}H_{13}N_5O_8$: C, 53.22; H, 2.90; N, 15.52%. Found: C, 53.29; H, 2.88; N, 15.42%.

(*E*)-*2-((2-(2,4-Dinitrophenyl)hydrazono)methyl)phenyl 3,5-dinitrobenzoate (**2e**)*: Light orange solid (82%); M.P.=273-274 °C. FT-IR (cm^{-1}) υ_{max}: 3286, 3096, 3071, 1743, 1613, 1516, 1328. ^{1}H NMR (DMSO-d_6): δ 11.73 (s, 1H), 8.88 (s, 1H), 8.84 (d, *J* = 2.6 Hz, 1H), 8.48 (s, 4H), 8.27 – 8.24 (m, 1H), 8.14 (d, *J* = 7.8 Hz, 1H), 7.97 (d, *J* = 9.6 Hz, 1H), 7.60 (d, *J* = 7.6 Hz, 1H), 7.49 (d, *J* = 4.7 Hz, 1H) ppm. ^{13}C NMR (DMSO-d_6): δ 163.66, 151.19, 149.49, 144.75, 144.28, 137.71, 134.60, 132.16, 132.10, 130.13, 129.92, 127.63, 127.50, 126.97, 124.54, 123.96, 123.32, 117.26 ppm. Anal. Calcd. for $C_{20}H_{12}N_6O_{10}$: C, 48.40; H, 2.44; N, 16.93%. Found: C, 48.42; H, 2.49; N, 16.97%.

2.3. Biological Activity Assays

The inhibition potentials of all tested molecules were assessed by determining anti-cholinesterase inhibitory activities. On the other hand, the antioxidant potentials of the same molecules were evaluated by CUPRAC reducing, ABTS and DPPH radical scavenging assays.

2.3.1. The inhibitory Assays

The inhibitory capacities of all synthesized hydrazone compounds (**2a-e)** on AChE and BChE were determined by utilizing the modified spectrophotometric method of Ellman et al.[25] In this modified method, the absorbance of each molecule was measured at 412 nm. Galanthamine was employed as a reference drug in this procedure.

2.3.2. Antioxidant Activity Assays

In the current research, to determine the antioxidant activities of the synthesized molecules, three different methods such as DPPH radical scavenging[26], ABTS radical scavenging [27] and CUPRAC reducing[28] assays were employed. The IC_{50} values of tested molecules were calculated from a concentration inhibition graph.[29]

2.3.3. Statistical Analysis

Antioxidant and anticholinesterase activity results of the tested molecules were reported as the mean ± standard deviation (SD) of three parallel measurements. The statistical significance was forecasted by using a Student's t-test, where $p < 0.05$ was considered significant.

3. Results and Discussion

3.1. Chemistry

General procedures for the synthesis of the target molecules (**2a-e**) are described in Scheme 1. Salicylaldehyde was first reacted with some benzoyl chloride derivatives in pyridine medium to give the corresponding esters. These esters were then reacted with 2,4-DNPH in ethanol medium. As a result, five new hydrazone derivatives were synthesized for the first time. The structures of all target molecules were fully elucidated by FT-IR, ^{1}H NMR, ^{13}C NMR and elemental analysis.

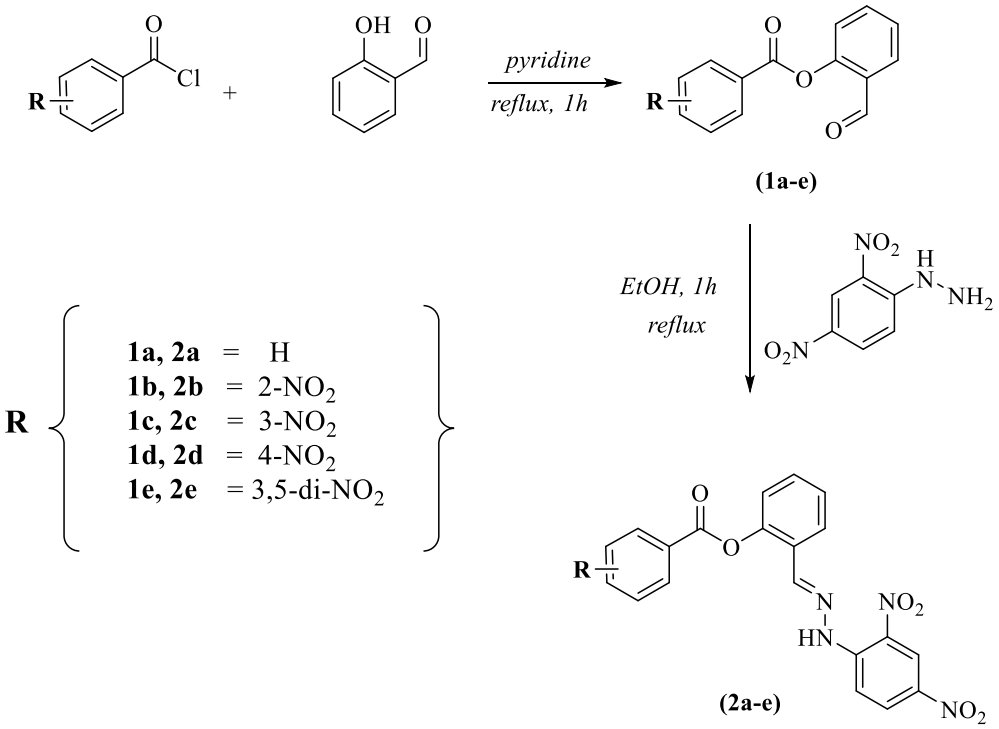

Figure 1. Synthetic route for the preparation of hydrazone derivatives

In IR spectra of hydrazone compounds (**2a-e**), the N–H stretching bands and the C=N stretching bands were at 3369-3265 cm^{-1} and 1617-1613 cm^{-1}, respectively.[30,31] The C=O stretching bands of **2a-e** were observed at 1743-1723 cm^{-1}.[32] In IR spectra of **2a-e**, asymmetric and symmetric NO_2 stretching bands were observed at 1581-1516 cm^{-1} and 1328-1336 cm^{-1}, respectively.[33] The aromatic C–H stretching bands were determined at 3120-3063 cm^{-1}. In ^{1}H NMR spectra of the compounds (**2a-e**), the signal owing to the CH=N appeared in the region 8.89-8.63 ppm.[34] The signal due to the -NH– protons was determined in the region 11.30-11.78 ppm. The signal due to the aromatic protons attached to phenyl ring was observed in the region 9.18-7.31 ppm. In their ^{13}C NMR spectra, the signal due to the C=O appeared at 165.01-163.48 ppm. The signal owing to the CH=N was detected at 149.50-149.08 ppm. The signal owing to the aromatic carbon attached to phenyl ring was determined in the region 151.19-117.26 ppm. The results of the spectroscopic analyses were found to be compatible with the literature.

3.2. Biological Activity

AChE inhibitors are the first choice for the therapy of AD, but they have several defects, such as dose limitation and unsatisfactory long-term therapy influences. Recent researches have exhibited that BChE inhibitors or both AChE and BChE inhibitors together have better therapeutic influences on AD; and the side influences are lower than those of specific AChE inhibitors.[35-42] In this research, the inhibitory activities of novel hydrazone compounds against AChE and BChE were determined. In addition, antioxidant activities of the same compounds were determined. The enzyme inhibition and antioxidant activities of all screened molecules and standard compounds are given in Table 1 and 2. In antioxidant activity assays, IC_{50} (μM) values for DPPH radical scavenging and ABTS radical scavenging assays were given. Moreover, $A_{0.5}$ values for CUPRAC reducing activity were given.

3.3. AChE and BChE Inhibitory Activiies

In AChE assay, the target molecules demonstrated the inhibitory activities with IC_{50} values ranging from 72.54 to 221.52 μM against AChE (Table 1). When the results in Table 1 were examined, it was determined that these molecules displayed the inhibitory activities at much lower concentrations than galanthamine (IC_{50} = 2.01 μM). Amongst the tested molecules, compound **2c** (IC_{50} = 221.52 μM) exhibited the weakest inhibitory activity against AChE. It was determined that compound **2e** (IC_{50} = 72.54 μM), with two electron withdrawing nitro groups at position 3 and 5 of the aromatic ring, was the most active molecule against this enzyme.

Table 1. AChE and BChE inhibitory results*

	IC_{50} (μM)		
Inhibitors	**AChE**	**BChE**	**Selectivity index[b]**
2a	150.69±1.62	28.89±0.48	5.22
2b	112.4 ± 1.3	48.28±1.61	2.33
2c	118.7 ± 1.1	9.68±0.27	12.26
2d	135.2 ± 1.4	11.06±0.43	12.22
2e	38.1 ± 0.4	8.46±0.49	4.50
Galanthamine[a]	6.07 ± 0.150	11.67±0.55	1.92

*Values are means of three parallel measurements ± Standard deviation, n = 3.
[a]Standard drug for AChE and BChE.
[b]Selectivity Index: IC_{50} of BChE / IC_{50} of AChE.

In BChE assay, the target molecules displayed significant inhibitory activities at concentrations between 8.46 and 48.28 μM on BChE (Table 1). Among the tested molecules, compounds **2c** (IC_{50} = 9.68 μM), **2d** (IC_{50} = 11.06 μM) and **2e** (IC_{50} = 8.46 μM) were determined to have higher inhibitory activities compared to galanthamine (IC_{50} = 11.67 μM). Among the tested molecules, compound **2e** was the most potent inhibitor for BChE. As seen in Table 1, these molecules were determined to inhibit BChE more strongly than AChE. On the other hand, the selectivity index values of compounds (**2a-e)** were calculated. When the results given in Table 1 were examined, it was determined that compounds **2e**, **2c** and **2d** showed strong BChE inhibition effect. We determined that compounds **2c** and **2d** were the molecules with the best selectivity indexes. Compounds **2c-e** showed a stronger inhibition effect against BChE compared to reference molecule. The results showed that all compounds were more selective to BChE than AChE. This shows that the tested molecules, especially **2c** and **2d** with the best selectivity indexes, have the potential to be used in the treatment of neurodegenerative diseases.

3.4. The Antioxidant Activity

In this research, antioxidant potentials of the target molecules were determined by three assays. When the antioxidant activity results given in Table 2 were examined, these molecules showed lower activities than BHA and BHT in DPPH and ABTS radical scavenging assays. In contrast to these results, the same molecules indicated better results in CUPRAC reducing assay. In this assay, the target molecules exhibited antioxidant activities with IC_{50} values ranging from 30.29 to 59.43 µM. Compound **2b** (IC_{50} = 30.29 µM), with an electron withdrawing nitro group in the *ortho* position of the aromatic ring, demonstrated higher activity compared to BHA (IC_{50} = 34.24 µM) and BHT (IC_{50} = 30.62 µM). Except for this compound, the remaining compounds **2a** (IC_{50} = 45.95 µM), **2c** (IC_{50} = 43.96 µM), **2d** (IC_{50} = 51.00 µM) and **2e** (IC_{50} = 59.43 µM) exhibited antioxidant activities close to those standard compounds.

Table 2. Antioxidant activity results*

	IC_{50} (µM)		$A_{0.5}$ (µM)
Compounds	**DPPH**	**ABTS**	**CUPRAC**
2a	>1000	>1000	45.95±2.76
2b	>1000	>1000	30.29±1.81
2c	>1000	>1000	43.96±0.28
2d	>1000	>1000	51.00±1.23
2e	>1000	>1000	59.43±2.13
BHA	71.38±0.34	8.53±0.24	34.24±0.57
BHT	270.42±1.52	11.69±0.17	30.62±0.60

*Values are means of three parallel measurements ± Standard deviation, n=3

4. Conclusion

Nowadays, due to the fact that the existing cholinesterase inhibitors in clinical use are not effective enough and have some side effects, this study was carried out to find out novel cholinesterase inhibitor candidates with antioxidant activities. In this research, a series of new hydrazone derivatives was synthesized for the first time and tested for their antioxidant activities, anti-AChE and BChE activities. The structure of the target molecules was elucidated by elemental analysis and three spectroscopic methods. In antioxidant activity tests, newly synthesized molecules showed higher activity in the CUPRAC reducing assay compared to other methods. In this assay, the value of compound **2b** showed good activity close to the value of BHT. However, compound **2b** showed better activity than BHA. On the other hand, target molecules on AChE showed lower activity than galanthamine. Whereas compounds, **2c**, **2d** and **2e** in BChE assay, showed higher inhibition activities compared to galanthamine. As a result, it can be said that some hydrazone compounds may be employed as potential BChE inhibitor in future studies.

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Supporting Information

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