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Synthesis, characterization and investigation of cholinesterase enzyme inhibition and antioxidant activities of some 4-aryl-1,4-dihydropyridine derivatives

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ABSTRACT: The aim of this study is to synthesize and characterize 4-aryl-1,4-dihydropyridine derivatives and evaluate their antioxidant and cholinesterase inhibitory properties. Hantzsch reaction was used in the synthesis of compounds; the compounds were prepared by the reaction of methyl 3- acetoacetate, appropriate aromatic aldehyde, ammonia and catalyst. The reactions were carried out in the presence of copper sulfate (for Method A) and boric acid /acetic acid catalyst (for Method B). $CuSO_4$ was used as a catalyst for the Hantzsch reaction for the first time. The structure of the synthesized compounds were characterized by IR and ^{1}H-NMR spectral studies. Furthermore, the enzym (acetylcholinesterase and butyrylcholinesterase) inhibition activity of the synthesized compounds was evaluated using Ellman's spectrophotometrical method as a novel approach. Antioxidant studies of the synthesized compounds were performed by measuring the 1,1-diphenyl-2-picrylhydrazyl radical scavenging assay, phosphomolibdenum-reducing antioxidant power assay, and metal chelating activity test. Results showed that 4-bromo substituted derivative (**1b**) has the highest antioxidant activity compared to other tested compounds. Moreover, compound **1b** also has higher cholinesterase inhibitory effect (34.05 ± 2.23% and 24.93 ± 0.68% at 250 µM) than other tested compounds. In this study, eight 1,4-dihydropyridine derivatives, dimethyl 4-(phenyl/substituted phenyl)-2,6-dimethyl-1,4-dihydropyridine-3,5-dicarboxylate compounds were synthesized according to the Hantzsch reaction, using $CuSO_4$ as a catalyst, for the first time. Compared to the classical reaction conditions, the presence of catalyst has been offered several advantages such as excellent good yields and short reaction times.

KEYWORDS: 1,4-Dihydropyridine; Hantzsch reaction; copper sulfate catalyst; boric acid/acetic acid catalyst; antioxidant activity; cholinesterase inhibitory effect.

1. INTRODUCTION

In recent years, Hantzsch reaction has attracted significant attention [1,2]. Dihydropyridine synthesis was first performed by Hantzsch in 1882, by the reaction of ethyl acetoacetate, aldehyde and ammonia, and then various modifications were made on this method [3,4]. The resulting 4-aryl-1,4-dihydropyridine (1,4-DHP) derivatives are widely used in the treatment of cardiovascular diseases, such as hypertension, cardiac arrhythmia, angina, and have become interesting compounds because of their ability to stop calcium channels and α-1-adrenergic receptors [5-7].

Nifedipine and amlodipine are the precursors of 1,4-DHP derivatives and are suitable for many modifications in their chemical structure [8]. Isradipine was synthesized as an analog of these compounds and has been used as a calcium channel blocker antihypertensive agent with the most recent anticonvulsant activity [9-11]. Moreover, 1,4-DHP and its derivatives have been shown to possess antitumoral [12], anti-inflammatory [13], analgesic [14], and antioxidant activities [15,16]. However, to the best of our knowledge, no study has been conducted yet to investigate the enzyme inhibition activity for 1,4-DHP compounds in literature.

On the one hand, acetylcholinesterase (AChE; E.C. 3.1.1.7) enzyme, which is found in the central and peripheral tissues, plays a crucial role in the degradation of the neurotransmitter acetylcholine (ACh) into

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acetic acid and choline [17]. On the other hand, the butyrylcholinesterase (BuChE; E.C. 3.1.1.8) enzyme, which is found in the liver, involves hydrolysis or detoxification of many compounds such as succinylcholine [17]. Their higher expressions are correlated with Alzheimer's disease (AD), which is a progressive neurodegenerative disease that is characterized by defects in memory and cognitive functions [18]. Therefore, cholinesterase inhibitors are important for treating or stopping this disease. However, although cholinesterase inhibitors such as tacrine, donepezil, galantamine, and rivastigmine are important in the treatment of AD, they have unpleasant effects including hepatotoxicity and gastrointestinal problems [19].

The classical synthesis process of the compounds derived from 1,4-DHP using the Hantzsch reaction involves the use of catalysts that have prolonged reaction time and limited efficiency. Numerous studies using different catalysts were recorded in the literature. Examples of these catalysts are $HClO_4$–SiO_2 under solvent-free conditions [20], iodine [21,22], trimethylsilyl iodide in acetonitrile [23], ceric ammonium nitrate [24], triphenylphosphine [25], and cellulose sulfuric acid [26]. Moreover, there are several alternate chemical processes involving other catalysts such as ionic liquids, zinc chloride, indium(III)chloride, SiO_2/$NaHSO_4$, tetrabutylammonium hydrogen sulfate, metal triflates, iron(III)fluoride, and tetrabutylammonium hexatungstate [27-34].

In this study, a series of 1,4-DHP derivatives, dimethyl 4-(phenyl/substituted phenyl)-2,6-dimethyl-1,4-dihydropyridine-3,5-dicarboxylate compounds, were synthesized according to the Hantzsch reaction [35-38] using benzaldehyde/substituted benzaldehyde, methyl acetoacetate, and ammonia with copper sulfate or boric/acetic acid as the catalyst (Figure 1). The structure of the synthesized compounds was elucidated spectroscopically using IR and ^{1}H NMR analyses.

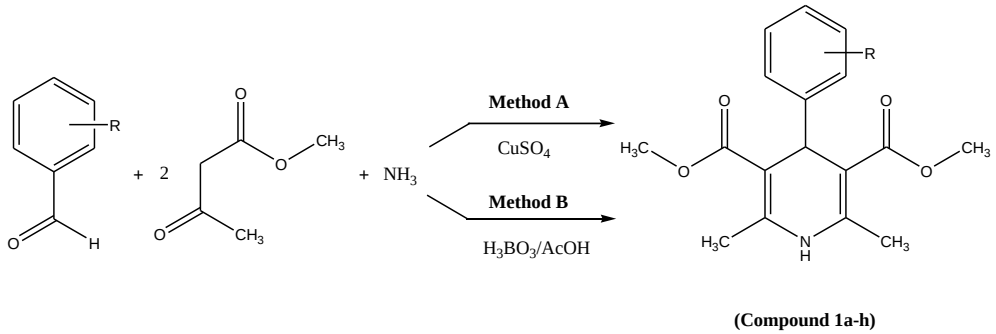
Antioxidant activities of these compounds were investigated via 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging, phosphomolibdenum-reducing antioxidant power (PRAP), and metal chelating activity assays [39]. In addition, AChE and BuChE inhibitory effects of the compounds were determined using Ellman's spectrophotometrical methods [40,41].

A novelty reaction method was performed using $CuSO_4$ as the catalyst. Moreover, a new approach was used for the determination of antioxidant and cholinesterase inhibition effects of the 1,4-DHP derivatives.

2. RESULTS AND DISCUSSION

2.1. Chemistry

The synthesis pathway for compounds **1a–h** is shown in Figure 1.



R: 1a-H, **b-**4-Br, **c-**3-Br, **d-**3-Cl, **e-**4-CH_3, **f-**3-CH_3, **g-**4-OCH_3, **h-**3-OCH_3.

Figure 1. Synthesis of 1,4-DHP derivative compounds **1a–h** (Method **A**: Compound **1a-d**, Method **B**: Compound **1e-h**).

Compounds **1a–h** were synthesized by the reaction of benzaldehyde/substituted benzaldehyde, methyl acetoacetate, and ammonia with copper sulfate or boric/acetic acid as catalyst according to the Hantzsch reaction [35]. Their molecular structures were confirmed by IR and ^{1}H NMR spectral data.

In the literature, the synthesis of our 1,4-dihydropyridine derivative compounds by Hantzsch method is recorded [38, 42-45]. In order to obtain the same compounds, in this study, boric acid/acetic acid and copper sulfate were used as catalysts in order to shorten the reaction times and increase their yields. As a catalyst, copper sulfate as a catalyst that has never been used in 1,4-DHP Hantzsch synthesis in the literature. Compound **1a-d** (H, 4/3-Br, 3-Cl) copper sulfate was used as catalyst in Method **A**; Compound **1e-h** (4/3-CH_3, 4/3-OCH_3) was obtained according to Method **B** where boric acid/acetic acid was used as catalyst. The two

methods were compared in terms of reaction time and the results are given in Table 1. Comparing reaction times of all methods, the use of catalysts significantly reduced the reaction times. The reaction time varies between 36-72 hours when the catalyst was not used, and 12-24 hours when the catalyst was used. The yields vary between 62-86%.

Table 1. Comparison of reaction time and yields of catalytic methods in the synthesis of 1,4-DHP derivatives with classical Hantzsch conditions.

		Method A[a]		Method B[b]		
Compound	**R**	**Time**[c]	**Yield** (%)	**Time**[c]	**Yield** (%)	**Melting Point** (°C)
1a [42]	H	21	62.4	-	-	189-91
1b [43]	4-Br	18	79.8	-	-	186-87
1c [44]	3-Br	12	65.8	-	-	185-86
1d [42]	3-Cl	14	69.4	-	-	184-85
1e [42]	4-CH_3	-	-	13	75.1	170-71
1f [42]	3-CH_3	-	-	14	81.4	172-73
1g [45]	4-OCH_3	-	-	16	78.4	181-82
1h [45]	3-OCH_3	-	-	18	86.3	182-83

[a] Catalyst: $CuSO_4$; [b] Catalyst: H_3BO_3/AcOH; [c] Reaction times were indicated in hours.

2.2. Biological activities

2.2.1. Antioxidant activity

The DPPH radical scavenging, PRAP, and metal chelating activities of the compounds at 250 µM are presented in Table 2. Gallic acid (GA) was used as a positive control. Compound **1b** had the highest DPPH radical scavenging activity with 23.15 ± 1.33% because of the presence of bromine at the fourth position on the phenyl ring. The DPPH radical scavenging activities of compounds **1g** and **1h** were found to be 16.94 ± 1.08% and 11.42 ± 0.93%, respectively, at 250 µM. These results indicated that the presence of methoxy group in the aromatic ring increased the scavenging activities because of the electron releasing group. At 250 µM concentration, compounds **1b** and **1g** demonstrated the highest phosphomolibdenum-reducing activities with an absorbance of 0.180 ± 0.009 and 0.167 ± 0.004, respectively. In addition, the metal chelating activities for compounds **1b** and **1c** were found to be 27.07 ± 0.84% and 15.92 ± 0.18%, respectively. Compound **1a** had the lowest antioxidant effects because of the absence of any electron releasing groups. Anwar et al. [15] synthesized dihydropyridine analogs and determined their DPPH radical scavenging activities. The IC_{50} values of the compounds were found to range from 127.40 ± 3.50 µM to 284.50 ± 0.66 µM. Moreover, Dhinakaran et al. [16] reported that 1,4-dihydropyridines demonstrated better DPPH radical scavenging activity with IC_{50} values of 13.82–29.20 µg/mL. These results showed that the compounds with bromine and methoxy groups have higher antioxidant activity compared with other derivatives despite their antioxidant activity being lower than that of the positive control GA (Table 2).

2.2.2. Cholinesterase inhibitory effects

The AChE and BuChE inhibitory effects of the compounds are shown in Table 3. Galantamine was used as the positive control. The addition of bromine group at the fourth position on the ring caused compound **1b** to have the highest AChE inhibitory effect (34.05 ± 2.23%) at 250 µM, which is consistent with its antioxidant activities. AChE inhibition for compounds **1g** and **1h** were 25.93 ± 0.82% and 16.46 ± 0.29%, respectively. By contrast, compounds **1a** and **1d** did not show any AChE inhibitory effects at 250 µM. As for the BuChE activity, compounds **1b**, **1c**, and **1g** were found to have the highest effect (24.93 ± 0.68%, 21.26 ± 0.16%, and 16.83 ± 1.59%, respectively) at 250 µM, whereas other compounds did not exhibit an effect. Leon et al. reported that the compounds they synthesized had IC_{50} values ranging from 0.22 µM to 0.60 µM, showing a higher inhibitory effect than our compounds [41].

Table 2. Antioxidant activities of compounds at 250 µM.

	DPPH (%) ± SD	PRAP (Absorbance) ± SD	Metal Chelating Activities (%) ± SD
1a	4.10 ± 0.06	nd	nd
1b	23.15 ± 1.33	0.180 ± 0.009	27.07 ± 0.84
1c	18.81 ± 0.49	0.159 ± 0.004	15.92 ± 0.18
1d	3.93 ± 0.42	nd	5.62 ± 0.07
1e	8.54 ± 0.25	0.126 ± 0.002	11.35 ± 0.03
1f	5.63 ± 0.15	nd	nd
1g	16.94 ± 1.08	0.167 ± 0.004	8.35 ± 0.32
1h	11.42 ± 0.93	0.140 ± 0.005	nd
GA	88.09 ± 0.03	1.042 ± 0.004	43.92 ± 0.08

*nd: not detected *GA: Gallic acid *SD: Standard deviation

Table 3. Cholinesterase inhibitory effects of compounds at 250 µM.

	AChE (%) ± SD	BuChE (%) ± SD
1a	nd	nd
1b	34.05 ± 2.23	24.93 ± 0.68
1c	24.95 ± 1.57	21.26 ± 0.16
1d	nd	nd
1e	15.39 ± 1.50	nd
1f	11.84 ± 0.62	nd
1g	25.93 ± 0.82	16.83 ± 1.59
1h	16.46 ± 0.29	nd
Galantamine	94.03 ± 0.35	86.50 ± 0.07

*nd: not z *SD: Standard deviation

3. CONCLUSION

Hantzsch reaction has been modified using anhydrous copper sulfate (Method **A**) and boric acid/acetic acid (Method **B**) as catalysts in the synthesis of the compounds to increase reaction yields and shorten the reaction time. Although a number of Lewis acids (barium chloride, etc.) were used as catalyst, the anhydrous copper sulfate is the first Lewis acid catalyst that was tested for this type of synthesis reactions.

Spectral (IR and ^{1}H NMR) data of the synthesized compounds **1a–h** were consistent with the literature spectral data [46,47]. In the IR spectrum, the vibration bands of the C=O group found at 1699-1647; the N-H group was found at 3355-3334 cm^{-1}. According to the results of IR spectral analysis in the literature, the carbonyl groups of the compounds appear at 1715-1635 and N-H group at 3317-3359 cm^{-1}. The findings obtained from the ^{1}H NMR spectra of compounds which we have synthesized confirm the structures of the compounds. ^{1}H NMR peaks in the aliphatic field were observed at 2.23–4.88 ppm and in the aromatic field at 6.67–8.98 ppm in accordance with chemical shift laws. Also, in the literature, the results of ^{1}H NMR spectral analysis the aliphatic groups of the compounds appear at 2.24–4.86 ppm and the aromatic groups of the compounds appear at 7.07–8.87 ppm.

In this study, the synthesis of eight 1,4-DHP derivative compounds, dimethyl 4-(phenyl/substituted phenyl)-2,6-dimethyl-1,4-dihydropyridine-3,5-dicarboxylate derivative compounds (**1a–h**), were accomplished using a new catalyst method, in which the electron withdrawing substituent moieties (R: –H, –Cl, –Br) were carried out using Method **A**, and the electron donating substituent moieties (R: –CH_3, –OCH_3) were carried out using Method **B**.

The results of these experiments showed that compound **1b** had higher antioxidant activity and cholinesterase inhibitory effect than the other compounds.

These results indicate that the methods used in this study have a strong potential for the synthesis of biologically active compounds in the future.

4. MATERIALS AND METHODS

4.1. Chemistry

The general synthesis of compounds **1a–h** is summarized in the Figure. All of the solvents and reagents were obtained from commercial suppliers. Furthermore, all reactions were monitored using a thin-layer chromatography on silica gel pre-coated F_{254} Merck plates, and the TLC plates were examined under 254 nm UV light. The melting points were recorded using the electrothermal digital melting point apparatus without correction. ^{1}H NMR spectrum was recorded on Varian Mercury 400 (400MHz) digital FT-NMR instrument with tetramethylsilane as the internal standard. Chemical shifts (δ) were given in parts per million (ppm). The significant ^{1}H NMR data are reported in the following order: multiplicity (s, singlet; d, doublet; dd, doublet of doublet; t, triplet; and m, multiplet) and number of protons. IR spectra were recorded on Perkin Elmer Spectrum FT-IR spectrophotometer using attenuated total reflectance (ATR) FT-IR method.

4.1.1. Synthesis of compounds

General procedure for the synthesis of dimethyl 4-(phenyl/substitutedphenyl)-2,6-dimethyl-1,4-dihydropyridine-3,5-dicarboxylate derivatives (Compounds **1a–h**)

The compounds were synthesized in 50 ml ethanol with 28 mmol of benzaldehyde/substituted benzaldehyde, 56 mmol of methyl 3-acetoacetate, and 28 mmol of 25% ammonia solution in accordance with the Hantzsch method [35]. The reaction mixture was treated with 14 mmol catalyst and then refluxed for an appropriate period. We used two different catalysts; in Method **A** (for compounds **1a–d**) anhydrous copper sulfate was catalyst and boric acid/acetic acid in Method **B** (for compounds **1e–h**) as catalyst. The crude product which was obtained by cooling was filtered and washed with 50% ethanol. Then it was recrystallized from appropriate solvent. The obtained products are shown in the Figure.

Dimethyl 2,6-dimethyl-4-phenyl-1,4-dihydropyridin-3,5-dicarboxylate (1a) [CASRN: 70677-78-0] [42] was synthesized according to method A. (Yield 62.4%); M.p. 189-191˚C; FT-IR (vmax, cm^{-1}) 3342 (N-H), 3028 (Aromatic C-H), 2951 (Aliphatic C-H), 1647 (C=O ester), 1215, 1118, 1099, 1051 (C-O, C-N), 840 (mono substituted benzene); ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm: 2.48 (s, 6H, Ar-CH_3), 3.31 (s, 6H, -OCH_3), 4.86 (s, 1H, H-4), 7.07-7.19 (m, 5H, C_6H_5), 8.87 (s, 1H, N_1H).

Dimethyl 2,6-dimethyl-4-(4-bromophenyl)-1,4-dihydropyridin-3,5-dicarboxylate (1b) [CASRN: 94889-60-0] [43] was synthesized according to method A. (Yield 79.8%); M.p. 186-187 ˚C; FT-IR (vmax, cm^{-1}) 3334 (N-H), 3008 (Aromatic C-H), 2947 (Aliphatic C-H), 1697 (C=O ester), 1209, 1182, 1141, 1097 (C-O, C-N), 839 (1,4-disubstituted benzene); ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm: 2.36 (s, 6H, Ar-CH_3), 3.36 (s, 6H, -OCH_3), 4.84 (s,1H, H-4), 7.08-7.41 (m, 4H, 4-Br-C_6H_4), 8.97 (s, 1H, N_1H).

Dimethyl 2,6-dimethyl-4-(3-bromophenyl)-1,4-dihydropyridin-3,5-dicarboxylate (1c) [CASRN: 93515-16-3] [44] was synthesized according to method A. (Yield 65.8%); M.p. 185-186 ˚C; FT-IR (vmax, cm^{-1}) 3349 (N-H), 3048 (Aromatic C-H), 2903 (Aliphatic C-H), 1690 (C=O ester), 1211, 1178, 1130, 1097 (C-O, C-N), 842 (1,3-disubstituted benzene); ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm: 2.28 (s, 6H, Ar-CH_3), 3.36 (s, 6H, -OCH_3), 4.88 (s,1H, H-4), 7.14-7.32 (m, 4H, 3-Br-C_6H_4), 8.98 (s, 1H, N_1H).

Dimethyl 2,6-dimethyl-4-(3-chlorophenyl)-1,4-dihydropyridin-3,5-dicarboxylate (1d) [CASRN: 73257-45-1] [42] was synthesized according to method A. (Yield 69.4%); M.p. 184-185 ˚C; FT-IR (vmax, cm^{-1}) 3345 (N-H), 3019 (Aromatic C-H), 2940 (Aliphatic C-H), 1699 (C=O ester), 1219, 1184, 1112, 1097 (C-O, C-N), 838 (1,3-disubstituted benzene); ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm: 2.27 (s, 6H, Ar-CH_3), 3.39 (s, 6H, -OCH_3), 4.87 (s,1H, H-4), 7.14-7.28 (m, 4H, 3-Cl-C_6H_4), 8.96 (s, 1H, N_1H).

Dimethyl 2,6-dimethyl-4-(4-methylphenyl)-1,4-dihydropyridin-3,5-dicarboxylate (1e) [CASRN: 73257-48-4] [42] was synthesized according to method B. (Yield 75.1%); M.p. 170-171 ˚C; FT-IR (vmax, cm^{-1}) 3344 (N-H), 3028 (Aromatic C-H), 2947 (Aliphatic C-H), 1693 (C=O ester), 1211, 1186, 1118, 1093 (C-O, C-N), 844 (1,4-disubstituted benzene); ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm: 2.23 (s, 3H, -CH_3), 2.48 (s, 6H, Ar-CH_3), 3.52 (s, 6H, -OCH_3), 4.79 (s,1H, H-4), 6.73-7.02 (m, 4H, 4-CH_3-C_6H_4), 8.84 (s, 1H, N_1H).

Dimethyl 2,6-dimethyl-4-(3-methylphenyl)-1,4-dihydropyridin-3,5-dicarboxylate (1f) [CASRN: 80307-08-0] [42] was synthesized according to method B. (Yield 81.4%); M.p. 172-173 ˚C; FT-IR (vmax, cm^{-1}) 3355 (N-H),

3028 (Aromatic C-H), 2909 (Aliphatic C-H), 1696 (C=O ester), 1206, 1176, 1126, 1091 (C-O, C-N), 848 (1,4-disubstituted benzene); ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm: 2.27 (s, 3H, -CH_3), 2.38 (s, 6H, Ar-CH_3), 3.40 (s, 6H, -OCH_3), 4.88 (s,1H, H-4), 6.67-7.15 (m, 4H, 3-CH_3-C_6H_4), 8.92 (s, 1H, N_1H).

Dimethyl 2,6-dimethyl-4-(4-methoxyphenyl)-1,4-dihydropyridin-3,5-dicarboxylate (1g) [CASRN: 73257-47-3] [45] was synthesized according to method B. (Yield 78.4%); M.p. 181-182 ˚C; FT-IR (vmax, cm^{-1}) 3344 (N-H), 3018 (Aromatic C-H), 2949 (Aliphatic C-H), 1691 (C=O ester), 1209, 1172, 1132, 1091 (C-O, C-N), 854 (1,4-disubstituted benzene); ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm: 2.23 (s, 3H, -OCH_3), 2.48 (s, 6H, Ar-CH_3), 3.51 (s, 6H, -OCH_3), 4.81 (s,1H, H-4), 6.98-7.35 (m, 4H, 4-OCH_3-C_6H_4), 8.83 (s, 1H, N_1H).

Dimethyl 2,6-dimethyl-4-(3-methoxyphenyl)-1,4-dihydropyridin-3,5-dicarboxylate (1h) [CASRN: 43114-34-7] [45] was synthesized according to method B. (Yield 86.3%); M.p. 182-183 ˚C; FT-IR (vmax, cm^{-1}) 3342 (N-H), 3038 (Aromatic C-H), 2908 (Aliphatic C-H), 1698 (C=O ester), 1215, 1178, 1135, 1091 (C-O, C-N), 847 (1,4-disubstituted benzene); ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm: 2.23 (s, 3H, -OCH_3), 2.48 (s, 6H, Ar-CH_3), 3.38 (s, 6H, -OCH_3), 4.88 (s,1H, H-4), 6.91-7.11 (m, 4H, 3-OCH_3-C_6H_4), 8.86 (s, 1H, N_1H).

4.2. Biological activity

4.2.1. Antioxidant activity

DPPH radical scavenging assay

The DPPH radical scavenging activities of the synthesized compounds were analyzed following the descriptions from the previous study [39]. Gallic acid (GA) was used as the positive control, whereas the assay mixture was used as the negative control. Briefly, the DPPH solution (0.4 mM) and the compounds at 250 µM were mixed and incubated for 30 min at 25 ^{o}C in the dark. The absorbance of the solution was then measured at 517 nm using a spectrophotometer and calculated using the following formula (Eq. 1):

$$\text{Inhibition (\%)} = \left[\frac{(A_{control} - A_{compound})}{A_{control}}\right] \times 100 \quad \text{(Eq. 1)}$$

The scavenging percentages (%) of DPPH values are shown in Table 2.

PRAP assay

The PRAP assay of the compounds was determined using the method described previously [40]. GA was used as a positive control. Moreover, the phosphomolybdic acid solution in ethanol (10%) at various concentrations were mixed and incubated at 80 ^{o}C for 30 min. Subsequently, the absorbance of the solution was measured at 600 nm using a spectrophotometer. The PRAP values of the compounds at 250 µM are presented in Table 2.

Metal chelating assay

The metal chelating properties of compounds were investigated as described in previous reports [41]. GA was used as a positive control. The compounds were mixed with 2 mM $FeCl_2$ solution. The reaction was initiated by the addition of 5 mM ferrozine into the mixture and incubated at 25 ^{o}C for 10 min. The absorbance of the reaction mixture was measured at 562 nm. The metal chelating effects of the compounds at 250 µM were calculated using the above formula and are shown in Table 2.

4.2.2. Cholinesterase inhibitory effects

The AChE/BuChE inhibitory effects of the compounds were investigated using the previous method with slight modifications [40]. Galantamine was used as a positive control. Tris-HCl buffer (pH 8.00), AChE/BuChE, 5,5-dithio-bis(2-nitrobenzoic)acid (in buffer), and the compounds at 250 µM were mixed in a microplate. The mixtures were incubated for 15 min at room temperature. Afterward, acetylthiocholine/butyrylthiocholine iodide was added in a microplate and incubated for 5 more min. The absorbance was measured at 412 nm using a microplate reader. Finally, AChE/BuChE inhibitory effects of the compounds were calculated using the above formula and are presented in Table 3.

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Conflict of interest statement: The authors declared no conflict of interest.

Appendix A. Supplementary Material

Supplementary material related to this article can be accessed at http://doi.org/10.12991/jrp.2019.168.

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