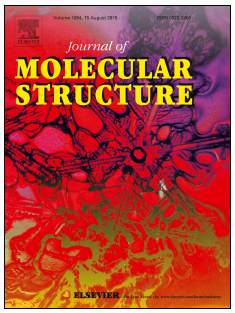


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## Credit Author Statement

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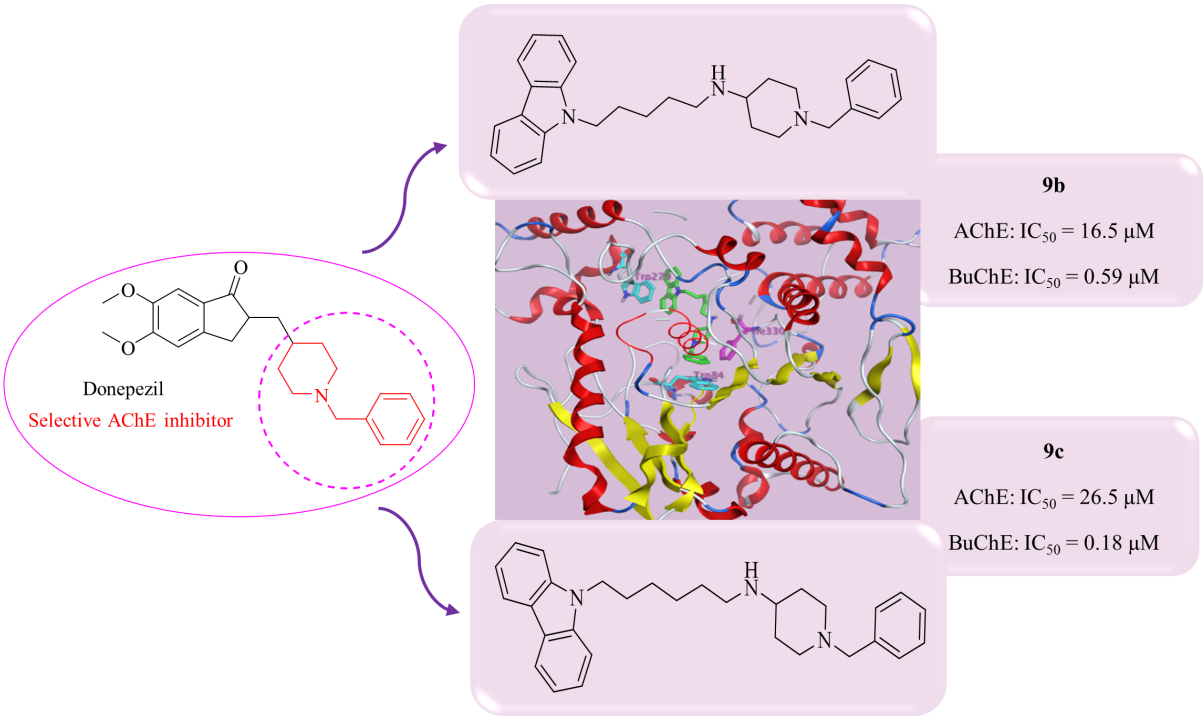

# Design, synthesis and biological activity evaluation of novel carbazole-benzylpiperidine hybrids as potential anti Alzheimer agents

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**Abstract**

Alzheimer's disease (AD) as the most common form of dementia in aged people, is an intricate neurodegenerative disease. Therefore, a novel strategy so-called multi-target-directed ligand has received much attention for the effective treatment of AD. In this study a series of novel carbazole-benzylpiperidine hybrids **9a-m** was designed, synthesized and evaluated as acetylcholinesterase and butyrylcholinesterase inhibitors. Moreover, some of these compounds evaluated for anti β-secretase (BACE1) activity and metal chelation properties. Among the synthesized compounds, compounds **9b** ($IC_{50}$ = 16.5 µM for AChE and $IC_{50}$ = 0.59 µM for BuChE) and **9c** ($IC_{50}$ = 26.5 µM for AChE and $IC_{50}$ = 0.18 µM for BuChE) showed the highest inhibitory activity against acetylcholinesterase and butyrylcholinesterase. Furthermore, these compounds (**9b** and **9c**) displayed interaction with $Zn^{2+}$ ion and compound **9c** showed moderate inhibitory activity against BACE1 (24.5% at 50 µM). Kinetic and docking studies exhibited that these compounds likely act as a non-competitive inhibitor able to interact with the catalytic active site (CAS) and peripheral anionic site (PAS) of acetylcholinesterase simultaneously.

*Keywords*: Alzheimer's disease, Carbazole, Benzylpiperidine, Acetylcholinesterase, Butyrylcholinesterase, Docking study.

## 1. Introduction

Alzheimer's disease (AD) as the most common form of dementia in aged people, is an intricate neurodegenerative disease characterized by the irreversible loss of cognitive ability, severe behavioral abnormalities, decreased language skills and ultimately death [1, 2]. According to the World Alzheimer Report 2018, around 50 million people are living with dementia in the world. This number is expected to raise to 152 million people in 2050 [3]. Huge financial and human resources annually are devoted to the care and treatment of these patients. The estimated worldwide funding devoted to the therapy of AD is US$818 billion, and expected to rise to one trillion by 2018 [4].

Although numerous research studies have been focused on understanding the pathophysiology of AD, its etiology is not comprehensively perceived. However, several factors including low levels of acetylcholine (ACh), amyloid-β (Aβ) deposits, τ-protein aggregation, oxidative stress and dyshomeostasis of biometals have been identified which involved in the pathogenesis of AD. According to these factors several hypotheses have been suggested to describe the mechanism of AD pathology [4-9].

The cholinergic hypothesis describes that memory impairments in AD are caused by the drastic decline of acetylcholine (ACh) in the brain [10, 11]. Thus, cholinesterase inhibitors (AChEIs) such as donepezil, tacrine, rivastigmine and galantamine have been major agents in AD therapy. However, these treatments are only a symptomatic treatment that is not able to prevent the progression of the disorder [12-15]. Therefore, further research is needed to develop more effective treatment strategies in AD.

ACh can be degraded by two types of cholinesterases termed acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE) [1]. Since the AChE is the major ChE in the normal brain, it has received much attention as a target in the treatment of AD [16]. But in recent years BuChE received much attention because of its key modulatory role in regulating the ACh level in AD patients [17]. Although BuChE only plays a secondary role in the healthy brains, some new evidence indicates that by decreasing AChE activity during disease progression, BuChE acts as a key degradation enzyme [10, 18]. It is reported that BuChE inhibition is not associated with notable adverse complications and may show efficacy without remarkable side effects. Therefore, BuChE can be considered as an ideal drug target for the treatment of AD [19, 20].

Consequently, simultaneous inhibition of AChE and BuChE can be an appropriate strategy in the treatment of AD.

Based on the crystal structure of AChE, two ligand-binding sites have been identified: 1) a catalytic active site (CAS) located at the bottom, and 2) a peripheral cationic site (PAS) located at the entrance. It is suggested that the interaction of AChE inhibitors to either of these sites (CAS and PAS) could inhibit the enzyme activity [13, 21, 22]. In addition, studies also show that AChE can promote the formation of Aβ fibrils and Aβ plaques through the interaction between Aβ and PAS site of the enzyme. Accordingly, the development of dual binding site inhibitors of both CAS and PAS could be a promising strategy for the treatment of AD [23].

Another hypothesis called the amyloid hypothesis implies that aggregation of plaques composed of β-amyloid peptides (Aβ) in the brain is the major factor in the pathogenesis of AD [24]. $A\beta_{1-40}$ and $A\beta_{1-42}$ are two major isoforms that derived from cleavage of APP (amyloid precursor protein) by two proteases named β-site amyloid precursor protein cleaving enzyme-1 (BACE1) and δ-secretase. $A\beta_{1-42}$ is the predominant form in senile plaques, having the high aggregative capability and stronger neurotoxicity than $A\beta_{1-40}$. BACE1 is considered as the rate-limiting enzyme for the production of Aβ. Therefore, BACE1 inhibitors can be considered as good candidates for preventing Aβ aggregation to treat and/or prevent AD [25, 26].

Furthermore, several pieces of evidence have indicated that metal ions such as zinc, iron and copper may be involved in the AD pathology. It is suggested that Aβ as a metalloprotein exhibits a high affinity for $Cu^{2+}$ and $Zn^{2+}$, which might cause its accumulation and toxicity. In addition, the binding of these metal ions to Aβ may also lead to the formation of ROS species that have neurodegenerative effects. Therefore, preventing metal ions from interacting with Aβ may be an effective strategy for Alzheimer's treatment [4, 27].

Due to the multifactorial nature of AD and the involvement of various enzymes and protein in its development, it is indicated that the classic ‘one molecule, one target’ approach may not have enough effectiveness for the treatment of AD [28, 29]. Therefore, a novel strategy so-called multi-target-directed ligand (MTDL) strategy has received much attention for the effective treatment of AD. In MTDL a single compound can simultaneously interact with multiple targets, thus may provide better therapeutic effects compared to those of single targeted drugs [22, 30]. Donepezil is the most effective AChE inhibitor currently used in clinical context for the

treatment of AD [31]. However, it can only reduce the symptoms of this disease and has very limited potential in treatment of moderate to severe AD [2, 32]. Hence, in recent years, rational modification of donepezil scaffold has attracted a lot of attention [2]. Accordingly, various donepezil derivatives have been designed and synthesized as MTDLs agents by replacements of the benzylpiperidine or indanone moieties of this drug [33]. For example, Donepezil-coumarin hybrids [34], donepezil-ebselen hybrids [35], donepezil-melatonin hybrids [13], donepezil-curcumin hybrids [5] and donepezil-indolyl hybrids [36] have been reported as MTDLs agents.

In this study, a series of novel carbazole-benzylpiperidine hybrids as multifunctional agents for the treatment of AD was designed and synthesized. To provide the novel hybrids with increased biological properties, the N-benzylpiperidine fragment of the donepezil as a recognized AChE inhibitor, attached to the carbazole fragment with various biological activities related to Alzheimer's disease, using various linkers. The *in vitro* activity of these compounds to inhibition of AChE, BuChE and BACE1, as well as metal chelation activity were evaluated. Finally, molecular modelling and kinetic studies of the most active compounds were performed to find the possible interaction mode with AChE and BuChE.

## 2. Experimental

### *2.1. General methods*

All reagents and analytical grade solvents were purchased from Sigma-Aldrich and Merck Chemical Company and were used without additional purification. The chemical reactions were routinely followed by thin-layer chromatography (TLC) on commercially available alumina plates coated with silica gel 60 $F_{254}$, and the spots were detected using a UV light. The purification of synthesized compounds was performed by recrystallization or column chromatography. The chemical structure of synthesized compounds was confirmed by $^{1}$H-NMR, $^{13}$C-NMR and Mass spectroscopy. $^{1}$H NMR and $^{13}$C NMR spectra were recorded in DMSO or $CDCl_3$ solutions using TMS as the internal standard on a Brucker Avance DPX 300, 400 or 500 MHz instrument. Chemical shift values were reported in ppm (δ). The coupling constants (J values) were expressed in hertz (Hz) and proton coupling patterns were expressed as s (singlet), d (doublet), t (triplet), dd (doublet of doublet), m (multiplet) and br (broad singlet). Mass spectra

(MS) were recorded on Agilent Technologies (HP) MS. Melting points were measured in capillary tubes with an electrothermal 9200 instrument. Intermediate compounds **2-8** were synthesized according to previously reported methods with some modification [30, 37].

2.2. *General procedure for preparation of intermediates **2-4**, **5-6** and **7-8***

*2.2.1. General procedure for preparation of intermediates **2-4***

To a solution of 9H-carbazole **1a** (1 eq, 18 mmol) and NaH (3eq, 54 mmol) in dry DMF (30 mL), corresponding di-bromoalkane derivatives (3eq, 54 mmol) was added dropwise and the mixture was stirred in an ice bath for 30 min. Then, the mixture was extracted with dichloromethane (DCM) and water. The organic layer was dried over anhydrous sodium sulfate ($Na_2SO_4$) and evaporated. The obtained product was purified by column chromatography on silica gel using n-Hexane/Ethyl acetate (20/2) as eluent to provide the intermediates **2-4** as white crystals in 67-73 % yields.

*2.2.2. General procedure for preparation of intermediates **5-6***

A mixture of 9H-carbazole **1a** (1 eq, 24 mmol) and triethylamine (TEA) (1.2 eq, 28.8 mmol) was dissolved in acetonitrile. Then 3-chloropropionyl chloride or 4-chlorobutyryl chloride (3 eq, 72 mmol) was added dropwise to the stirred solution. The reaction mixture refluxed at 85 ˚C for 48 h. Then, the solvent was evaporated with a rotatory evaporator and extracted using dichloromethane (DCM) and water. The organic layer was dried on $Na_2SO_4$ and then the solvent was evaporated. The crude product was purified by column chromatography on silica gel using n-Hexane/Ethyl acetate (20/1) as eluent to give the intermediates **5** and **6** as white solids in 60-65 % yields.

*2.2.3. General procedure for preparation of intermediates **7-8***

In a round-bottomed flask (100mL), 9H-carbazole **1a** or 3,6-dibromocarbazole **1b** (1eq, 1.5 mmol) was treated with KOH (1.2 eq, 1.8 mmol) in dry DMF at room temperature for 1 h. Then epichlorohydrin (2.5 eq, 4 mmol) was added dropwise to the solution mixture. After stirring at room temperature for 24 h, the mixture was poured into crushed ice and the residue precipitate was purified by plate chromatography using n-Hexane/Ethyl acetate (20/4) as eluent to give intermediate **7** and **8** in 52-55 % yields.

*2.3. The general method for preparation of compounds **9a-m***

*2.3.1. The general method for preparation of compounds **9a-i***

A mixture of intermediates **2-6** (1 eq, 1 mmol), corresponding amines (4-amino-N-benzylpiperidine or 4-benzylpiperidine) (1 eq, 1 mmol) and TEA (1.2 eq, 1.2 mmol) were dissolved in 30 mL dry acetonitrile and refluxed at 85 ˚C for 24 h (compound **9a-f**) or stirred at room temperature for 1h (compound **9g-i**). Then, the resulting precipitated was filtered, washed with acetonitrile and dried at room temperature. The resulting product was purified by recrystallization or column chromatography on silica gel to afford pure compounds **9a-i**.

*2.3.2. The general method for preparation of compounds **9j-m***

In a round-bottomed flask (50 mL), a mixture of intermediate **7 or 8** (1eq, 1 mmol), appropriate amin such as 4-amino-N-benzylpiperidine or 4-benzylpiperidine (1 eq, 1 mmol) in EtOH (20 mL) was refluxed at 65 ˚C for 24 h. Then, the resulting products were purified by recrystallization with ethanol or column chromatography on silica gel to afford pure compounds **9j-m**.

*2.3.1.1. N-(4-(9H-carbazol-9-yl) butyl)-1-benzylpiperidin-4-amine (**9a**)*

According to the general method, a mixture of intermediate **2** (0.30 g, 1mmol), 4-amino-N-benzylpiperidine (0.19 g, 1 mmol) and TEA (0.12g, 1.2 mmol) were dissolved in 30 mL acetonitrile and refluxed at 85 ˚C for 24 h. After the reaction completed, the resulting precipitated was filtered, washed with acetonitrile and dried at room temperature. The resulting product was purified by recrystallization in n-hexane and DCM to afford compound **9a**. White solid; yield: 69.3 %, mp: 278-280 ˚C. $^{1}$H NMR (DMSO, 400 MHz): δ (ppm): 1.60-1.65 (m, 2H), 1.72-1.74 (m, 2H), 1.80-1.84 (m, 3H), 1.92-1.94 (br, 3H), 2.78-2.84 (m, 5H), 3.42 (s, 2H), 4.40 (t, J = 6.8 Hz, 2H), 7.18 (t, J = 7.6 Hz, 2H), 7.23-7.32 (m, 5H), 7.43 (t, J = 7.6 Hz, 2H), 7.62 (d, J = 8.0 Hz, 2H), 8.14 (d, J = 7.6 Hz, 2H), NH signal not detected. $^{13}$C NMR (DMSO, 400 MHz): δ (ppm): 139.84, 138.06, 128.75, 128.14, 126.93, 125.65, 122.00, 120.22, 118.67, 109.29, 61.54, 54.32, 50.99, 43.28, 41.80, 27.56, 25.80, 23.29. Formula: $C_{28}H_{33}N_3$; MS: m/z 411.4 [$M^+$] (5.93), 320.4 (9.70), 222.2 (7.50), 193.2 (4.22), 180.2 (29.56), 173.3 (100), 91.2 (68.27), 56.2 (8.09).

*2.3.1.2. N-(5-(9H-carbazol-9-yl) pentyl)-1-benzylpiperidin-4-amine (**9b**)*

According to the general method, a mixture of intermediate **3** (0.315g, 1mmol), 4-amino-N-benzylpiperidine (0.19 g, 1mmol,) and TEA (0.12g, 1.2 mmol) were dissolved in 30 mL acetonitrile and refluxed at 85 ˚C for 24 h. After the reaction completed, the resulting precipitated was filtered, washed with acetonitrile and dried at room temperature. The resulting product was purified by recrystallization in n-hexane and DCM to afford compound **9b**. White solid; yield: 72.9 %, mp: 158-160 ˚C. $^{1}$H NMR ($CDCl_3$, 400 MHz): δ ppm: 1.32-1.39 (m, 2H), 1.80-1.84 (m, 2H), 1.94-1.96 (br, 5H), 2.11-2.13 (br, 3H), 2.81 (t, J = 7.6 Hz, 2H), 2.92-3.01 (m, 3H), 3.51 (s, 2H), 4.22 (t, J = 6.8 Hz, 2H), 7.22 (t, J = 7.2 Hz, 2H), 7.25-7.31 (m, 5H + $CDCl_3$), 7.36 (d, J = 8.0 Hz, 2H), 7.45 (t, J=8.0 Hz, 2H), 8.09 (d, J = 8.0 Hz, 2H), NH signal not detected. $^{13}$C NMR ($CDCl_3$, 400 MHz): δ ppm: 139.92, 137.84, 128.87, 128.18, 127.06, 125.65, 122.01, 120.25, 118.64, 109.24, 61.39, 54.12, 50.77, 43.46, 41.99, 28.00, 27.71, 25.35, 23.51. Formula: $C_{29}H_{35}N_3$; MS: m/z 425.4 [$M^+$] (6.18), 334.3 (12.49), 279.3 (8.72), 180.2 (25.56), 173.3 (100), 146.2 (11.22), 91.2 (59.05), 56.2 (8.85).

*2.3.1.3. N-(6-(9H-carbazol-9-yl) hexyl)-1-benzylpiperidin-4-amine (**9c**)*

According to the general method, a mixture of intermediate **4** (0.330g, 1mmol), 4-amino-N-benzylpiperidine (0.19 g, 1mmol) and TEA (0.12g, 1.2 mmol) were dissolved in 30 mL acetonitrile and refluxed at 85 ˚C for 24 h. After the reaction completed, the resulting precipitated was filtered, washed with acetonitrile and dried at room temperature. The resulting product was purified by recrystallization in n-hexane and DCM to afford compound **9c**. White solid; yield: 75.9 %, mp: 205-208 ˚C. $^{1}$H NMR ($CDCl_3$,300 MHz) δ ppm: 0.84-0.91 (m, 2H), 1.83-2.13 (m, 12H), 2.79 (t, J = 7.2 Hz, 2H), 2.93-3.03 (m, 3H), 3.54 (s, 2H), 4.24 (t, J = 6.6 Hz, 2H), 7.21 (t, J = 7.5 Hz, 2H), 7.25-7.32 (m, 5H+ $CDCl_3$), 7.37 (d, J = 8.1 Hz, 2H), 7.46 (t, J = 7.8 Hz, 2H), 8.08 (d, J = 7.5 Hz, 2H), NH signal not detected. $^{13}$C NMR (DMSO, 100 MHz): δ ppm: 140.31, 139.91, 128.92, 128.04, 125.65, 125.55, 121.96, 120.21, 118.55, 109.21, 57.34, 53.20, 42.40, 41.95, 37.46, 31.70, 26.17, 23.73. Formula: $C_{30}H_{37}N_3$; MS: m/z 439.4 [$M^+$] (0.75), 291.1 (5.79), 236.3 (11.13), 188.2 (39.99), 167.1 (26.86), 127.0 (60.97), 91.1 (48.60), 74.1 (8.37), 57.1 (100).

*2.3.1.4. 9-(4-(4-benzylpiperidin-1-yl) butyl)-9H-carbazole (**9d**)*

According to the general method, a mixture of intermediate **2** (0.30 g, 1mmol), 4-benzylpiperidine (0.175 g, 1mmol) and TEA (0.12g, 1.2 mmol) were dissolved in 30 mL acetonitrile and refluxed for 24 h. After the completion of reaction, the mixture was dried with a rotatory evaporator and then extracted with dichloromethane (DCM) and water. The organic fraction was dried with anhydrous sodium sulfate ($Na_2SO_4$), evaporated and purified by column chromatography using n-Hexane/Ethyl acetate (20/1) as eluent to afford compound **9d**. White solid; yield: 78.6 %, mp: 71-73˚C.$^1$H NMR (DMSO, 400 MHz) δ ppm: 1.06-1.15 (m, 2H), 1.21-1.25 (m, 1H), 1.39-1.47 (m, 4H), 1.66-1.78 (m, 4H), 2.20 (t, J = 7.2 Hz, 2H), 2.44 (d, J = 6.8 Hz, 2H), 2.69-2.72 (br, 2H), 4.37 (t, J = 7.2 Hz, 2H), 7.12 (d, J = 7.2 Hz, 2H), 7.18 (t, J = 7.6 Hz, 3H), 7.25 (t, J = 7.6 Hz, 2H), 7.43 (t, J = 7.2 Hz, 2H), 7.60 (d, J = 8.0 Hz, 2H), 8.14 (d, J = 8.0 Hz, 2H). $^{13}$C NMR (DMSO, 400 MHz): δ ppm: 140.81, 140.40, 129.42, 128.54, 126.15, 126.05, 122.46, 120.71, 119.04, 109.71, 57.84, 53.70, 42.89, 42.45, 37.96, 32.20, 26.67, 24.23. Formula: $C_{28}H_{32}N_2$; MS: m/z 396.4 [$M^+$] (10.41), 222.2 (2.64), 188.3 (100), 180.2 (14.42), 91.2 (11.80), 57.2 (5.76).

*2.3.1.5. 9-(5-(4-benzylpiperidin-1-yl) pentyl)-9H-carbazole (**9e**)*

According to the general method, a mixture of intermediate **3** (0.315g, 1mmol), 4-benzylpiperidine (0.175 g, 1mmol) and TEA (0.12g, 1.2 mmol) were dissolved in 30 mL acetonitrile and refluxed at 85 ˚C for 24 h. After the reaction completed, the mixture was dried with a rotatory evaporator and then extracted with dichloromethane (DCM) and water. The organic fraction was dried with anhydrous sodium sulfate ($Na_2SO_4$), evaporated and purified by column chromatography using n-Hexane/Ethyl acetate (20/1) as eluent to afford **9e**. White solid; yield: 80.4 %, mp: 53-55˚C. $^1$H NMR (DMSO,400 MHz) δ ppm: 1.03-1.11 (m, 2H), 1.21-1.29 (m, 2H), 1.35-1.45 (m, 5H), 1.66 (t, J = 10.8 Hz, 2H), 1.71-1.79 (m, 2H), 2.09 (t, J = 7.2 Hz, 2H), 2.44 (d, J = 6.8 Hz, 2H), 2.68-2.71 (br, 2H), 4.36 (t, J = 7.2 Hz, 2H), 7.12 (d, J = 7.2 Hz, 2H), 7.18 (t, J = 7.6 Hz, 3H), 7.25 (t, J = 7.6 Hz, 2H), 7.43 (t, J = 8.0 Hz, 2H), 7.57 (d, J = 8.4 Hz, 2H), 8.14 (d, J = 7.6 Hz, 2H). $^{13}$C NMR (DMSO, 400 MHz): δ ppm: 140.36, 139.92, 128.91, 128.04, 125.64, 125.57, 121.97, 120.20, 118.54, 109.18, 58.04, 53.32, 42.39, 42.14, 37.49, 31.70, 28.33, 26.20, 24.46. Formula: $C_{29}H_{34}N_2$; MS: m/z 410.4 [$M^+$] (8.09), 319.3 (0.48), 244.3 (2.85), 230.3 (3.63), 217.2 (0.47), 203.2 (2.46), 188.3 (100), 180.2 (15.57), 167.2 (4.06), 152.2 (4.24), 91.2 (14.63), 69.2 (12.68).

*2.3.1.6. 9-(6-(4-benzylpiperidin-1-yl) hexyl)-9H-carbazole (**9f**)*

According to the general method, a mixture of intermediate **4** (0.330g, 1mmol), 4-benzylpiperidine (0.175 g, 1mmol,) and TEA (0.12g, 1.2 mmol) were dissolved in 30 mL acetonitrile and refluxed at 85 ˚C for 24 h. After the reaction completed, the resulting precipitated was filtered, washed with acetonitrile and dried at room temperature. The resulting product was purified by recrystallization in n-hexane and DCM to afford **9f**. White solid; yield: 83.8 %, mp: 82-84˚C. $^{1}$H NMR ($CDCl_3$, 500 MHz): δ ppm: 1.31-1.36 (m, 4H), 1.41-1.53 (m, 5H), 1.63-1.65 (br, 2H), 1.82 (t, J = 12.0 Hz, 2H), 1.87-1.93 (m, 2H), 2.26 (t, J = 7.5 Hz, 2H), 2.55 (d, J= 7.0 Hz, 2H), 2.87-2.89 (br, 2H), 4.32 (t, J = 7.0 Hz, 2H), 7.17 (d, J = 7.5 Hz, 2H), 7.21-7.28 (m, 3H), 7.30 (t, J = 8.0 Hz, 2H), 7.43 (d, J = 8.0 Hz, 2H) 7.49 (t, J = 7.5 Hz, 2H), 8.13 (d, J = 8.0 Hz, 2H).$^{13}$C NMR ($CDCl_3$, 300 MHz): δ ppm: 140.80, 140.43, 129.17, 128.18, 125.79, 125.61, 122.83, 120.37, 118.74, 108.68, 59.06, 54.02, 43.29, 43.02, 38.03, 32.23, 28.96, 27.52, 27.29, 26.99. Formula: $C_{30}H_{36}N_2$; MS: m/z 424.4 [$M^+$] (7.75), 331.2 (4.33), 244.3 (6.08), 188.3 (100), 180.2 (46.18), 91.2 (11.07).

*2.3.1.7. 3-((1-benzylpiperidin-4-yl) amino)-1-(9H-carbazol-9-yl) propan-1-one (**9g**)*

According to the general method, a mixture of intermediate **5** (0.257g, 1mmol), 4-amino-N-benzylpiperidine (0.190 g, 1mmol) and TEA (0.12g, 1.2 mmol) were dissolved in 30 mL acetonitrile and stirred at 0˚C for 1h. After the reaction completed, the resulting precipitated was filtered, washed with acetonitrile and dried at room temperature to afford compound **9g**. White solid; yield: 82.7 %, mp: 206-208˚C. $^{1}$H NMR (DMSO, 400 MHz) δ ppm: 1.64-1.67 (m, 2H), 2.01-2.08 (m, 4H), 2.88-2.90 (br, 2H), 3.10-3.15 (m, 1H), 3.41 (t, J = 6.4 Hz, 2H), 3.50 (s, 2H), 3.77 (t, J = 6.4 Hz, 2H), 7.26-7.34 (m, 5H), 7.46 (t, J = 7.2 Hz, 2H), 7.55 (t, J = 8.4 Hz, 2H), 8.23 (d, J = 7.2 Hz, 2H), 8.28 (d, J = 8.4 Hz, 2H), 9.11 (br, 0.98H), NH signal not detected. $^{13}$C NMR (DMSO, 400 MHz): δ ppm: 171.01, 138.27, 129.35, 128.68, 128.02, 127.53, 126.26, 124.38, 120.77, 116.91, 116.02, 61.94, 54.88, 51.36, 36.08, 31.15, 28.49. Formula: $C_{27}H_{29}N_3O$; MS: m/z 411 [$M^+$] (1.75), 320.3 (1.08), 221.2 (26.79), 188.2 (10.39), 167.2 (90.29), 91.1 (100), 55.1 (36.03).

*2.3.1.8. 3-(4-benzylpiperidin-1-yl)-1-(9H-carbazol-9-yl) propan-1-one (**9h**)*

According to the general method, a mixture of intermediate **5** (0.257g, 1mmol), 4-benzylpiperidine (0.175 g, 1 mmol) and TEA (0.12g, 1.2 mmol) were dissolved in 30 mL acetonitrile and stirred at 0˚C for 1 h. After the reaction completed, the resulting precipitated was filtered, washed with acetonitrile and dried at room temperature to afford **9h**. White solid; yield: 63.1 %, mp: 218-220˚C. $^{1}$H NMR (DMSO, 400 MHz) δ ppm: 1.51-1.65 (m, 2H), 1.76-1.84 (m, 3H), 2.56 (d, J = 6.8 Hz, 2H), 2.98-3.06 (m, 2H), 3.50-3.54 (m, 2H), 3.60-3.63 (br, 2H), 3.87 (t, J = 7.2 Hz, 2H), 7.22 (t, J = 7.2 Hz, 3H), 7.31 (t, J = 7.6 Hz ,2H), 7.46 (t, J = 7.6 Hz, 2H), 7.55 (t, J = 8.4 Hz, 2H), 8.23 (d, J = 7.2 Hz, 2H), 8.32 (d, J = 8.4 Hz, 2H), 10.24 (br, 0.89H). $^{13}$C NMR (DMSO, 400 MHz): δ ppm: 170.40, 139.45, 137.75, 128.99, 128.26, 127.57, 126.03, 125.77, 123.92, 120.24, 116.58, 52.21, 51.83, 41.46, 34.89, 33.31, 28.82. Formula: $C_{27}H_{28}N_2O$; MS: m/z 396.4 [$M^+$] (8.15), 221.2 (14.45), 188.3 (100), 167.2 (49.39), 140.1 (6.27), 91.2 (19.37), 55.2 (17.57).

*2.3.1.9. 4-(4-benzylpiperidin-1-yl)-1-(9H-carbazol-9-yl) butan-1-one (**9i**)*

According to the general method, a mixture of intermediate **6** (0.271g, 1mmol), 4-benzylpiperidine (0.175 g, 1mmol) and TEA (0.12g, 1.2 mmol) were dissolved in 30 mL acetonitrile and stirred at 80˚C for 24 h. After the reaction completed, the resulting precipitated was filtered, washed with acetonitrile and dried at room temperature and recrystallization in acetonitrile to afford **9i**. White solid; yield: 78.5 %, mp: 199-204˚C. $^{1}$H NMR (DMSO, 400 MHz): δ ppm: 1.60.1.66 (m, 2H), 1.72-1.80 (m, 3H), 2.22 (br, 2H), 2.53 (d, J = 6.4 Hz, 2H), 2.86-2.89 (br, 2H), 3.17 (br, 2H), 3.41-3.50 (m, 4H), 7.20 (t, J = 7.2 Hz, 3H), 7.31 (t, J = 6.8 Hz, 2H), 7.43 (t, J = 6.8 Hz, 2H), 7.52 (t, J = 7.2 Hz, 2H), 8.21 (d, J = 7.2 Hz, 2H), 8.34 (d, J = 8.4 Hz, 2H), 10.53 (br, 0.89H). $^{13}$C NMR (DMSO, 400 MHz): δ ppm: 172.48, 139.49, 137.89, 128.97, 128.27, 127.45, 126.02, 125.66, 123.64, 120.14, 116.65, 55.27, 51.66, 41.51, 35.71, 35.04, 28.63, 18.68. Formula: $C_{28}H_{30}N_2O$; MS: m/z 410.4 [$M^+$] (7.29), 244.3 (23.93), 215.3 (2.41), 202.3 (5.68), 188.3 (100), 167.2 (15.11), 91.2 (18.85), 70.2 (6.37).

*2.3.2.1. 1-((1-benzylpiperidin-4-yl) amino)-3-(9H-carbazol-9-yl) propan-2-ol (**9j**)*

According to the general method, in a round-bottomed flask (100 mL), a mixture of intermediate **7** (0.223 g, 1 mmol), appropriate 4-amino-N-benzylpiperidine (0.190 g, 1 mmol) in EtOH

(30mL), was refluxed at 65 ˚C for 24 h. Then, the resulting products were purified by plate chromatography to afford compound **9j**. yellow oil; yield: 56.7 %. $^{1}$H NMR (DMSO, 400 MHz): δ ppm: 1.21-1.32 (m, 2H), 1.72-1.77 (m, 2H), 1.89 (t, J = 11.2 Hz, 2H), 2.32-2.38 (m, 1H), 2.56-2.74 (m, 5H), 3.40 (s, 2H), 3.99-4.04 (m, 1H), 4.27-4.33 (dd, J = 14.8, 6.8 Hz, 1H), 4.44-4.49 (dd, J = 14.4, 5.2 Hz, 1H), 7.18 (t, J = 7.2 Hz, 2H ), 7.24-7.32 (m, 5H), 7.43 (t, J = 7.2 Hz, 2H),7.66 (d, J=8.4 Hz, 2H ), 8.13 (d, J = 8.0 Hz, 2H), NH signal not detected .$^{13}$C NMR (DMSO, 400 MHz): δ ppm: 140.55, 138.61, 128.69, 128.07, 126.75, 125.42, 121.97, 119.99, 118.58, 109.79, 68.75, 62.17, 55.98, 54.70, 51.81, 50.02, 47.03, 31.87, 18.54. Formula: $C_{27}H_{31}N_3O$.

*2.3.2.2. 1-((1-benzylpiperidin-4-yl) amino)-3-(3,6-dibromo-9H-carbazol-9-yl) propan-2-ol (**9k**)*

According to the general method, in a round-bottomed flask (100 mL), a mixture of intermediate **8** (0.381 g, 1 mmol), appropriate 4-amino-N-benzylpiperidine (0.190 g, 1 mmol) in EtOH (30mL) was refluxed at 65 ˚C for 24 h. Then, the resulting product was purified by recrystallization with ethanol to afford compounds **9k**. white solid; yield: 55.7 %, mp: 92-94˚C. $^{1}$H NMR (DMSO, 400 MHz): δ ppm: 1.20-1.28 (m, 2H), 1.73 (br, 2H), 1.91 (t, J = 10.8 Hz, 2H), 2.27-2.32 (m, 1H), 2.50-2.62 (m, 2H), 2.72 (d, J = 10.8 Hz, 2H), 3.41 (s, 2H+ DMSO), 3.90 (br, 1H), 4.23-4.28 (dd, J = 14.8, 7.2 Hz, 1H), 4.42-4.46 (dd, J = 14.8, 4.0 Hz, 1H), 5.04 (br, 1H), 7.21-7.32 (m, 5H), 7.57-7.64 (m, 4H), 8.45 (s, 2H), NH signal not detected. $^{13}$C NMR (DMSO, 400 MHz) δ ppm: 139.71, 138.68, 128.68, 128.54, 128.07, 126.74, 123.12, 122.86, 112.23, 111.14, 69.11, 62.23, 56.00, 54.70, 51.91, 50.13, 47.34, 32.34, 18.54. Formula: $C_{27}H_{29}Br_2N_3O$.

*2.3.2.3. 1-(4-benzylpiperidin-1-yl)-3-(9H-carbazol-9-yl) propan-2-ol (**9l**)*

According to the general method, in a round-bottomed flask (100 mL), a mixture of intermediate **7** (0.223 g, 1 mmol), appropriate 4-benzylpiperidine (0.175 g, 1 mmol) in EtOH (30mL) was refluxed at 65 ˚C for 24 h. Then, the resulting products were purified by column chromatography on silica gel to afford compounds **9l**. white solid; yield: 59.12 %, mp: 88-90˚C. $^{1}$H NMR (DMSO, 500 MHz): δ ppm: 1.21-1.33 (m, 2H), 1.51-1.52 (m, 1H), 1.62 (br, 2H), 1.83 (t, J = 11.5 Hz, 1H), 2.19 (t, J = 11.5 Hz, 1H), 2.38-2.47 (m, 2H), 2.53 (d, J = 7.0 Hz, 2H), 2.75 (d, J = 11.0 Hz, 1H), 2.90 (d, J = 10.5 Hz, 1H), 3.71-3.72 (m, 1H), 4.21-4.24 (m, 1H), 4.35-4.44 (m, 2H), 7.15 (d, J = 7.5 Hz, 2H), 7.22 (t, J = 7.0 Hz, 1H), 7.26-7.32 (m, 4H), 7.48-7.53 (m, 4H), 8.13 (d,

J = 7.5 Hz, 2H). $^{13}$C NMR (DMSO, 500 MHz): δ ppm: 141.08, 140.52, 129.12, 128.23, 125.89, 125.75, 123.00, 120.28, 119.12, 109.22, 66.28, 62.10, 55.58, 52.33, 47.30, 43.08, 37.73, 32.41, 32.12. Formula: $C_{27}H_{30}N_2O$.

*2.3.2.4. 1-(4-benzylpiperidin-1-yl)-3-(3,6-dibromo-9H-carbazol-9-yl) propan-2-ol (**9m**)*

According to the general method, in a round-bottomed flask (100 mL), a mixture of intermediate **8** (0.381, 1 mmol), appropriate 4-benzylpiperidine (0.175 g, 1 mmol) in EtOH (30mL) was refluxed at 65 ˚C for 24 h. After the reaction completed, the resulting products were purified by recrystallization with ethanol afford desired compounds **9m**. white solid; yield: 53.1 %, mp: 134-136 ˚C. $^1$H NMR ($CDCl_3$, 300 MHz): δ ppm: 1.16-1.34 (m, 2H), 1.49-1.64 (m, 3H), 1.87 (t, J = 11.4 Hz, 1H), 2.20 (t, J = 11.7 Hz, 1H), 2.36 (d, J = 6.6 Hz, 2H), 2.51 (d, J = 6.3 Hz, 2H), 2.73 (d, J = 10.5 Hz, 1H), 2.89 (d, J = 10.5 Hz, 1H), 4.15-4.17 (m, 1H), 4.29 (br, 2H), 7.13 (d, J = 7.2 Hz, 2H), 7.20 (t, J = 6.9 Hz, 1H), 7.28 (t, J= 8.1 Hz, 2H), 7.37 (d, J= 8.7 Hz, 2H), 7.56 (d, J = 8.4 Hz, 2H), 8.13 (s, 2H). $^{13}$C NMR ($CDCl_3$, 400 MHz): δ ppm: 140.35, 139.98, 129.12, 129.06, 128.22, 125.91, 123.58, 123.12, 112.33, 111.06, 66.22, 61.71, 55.56, 52.38, 47.37, 42.97, 37.61, 32.25, 31.95. Formula: $C_{27}H_{28}Br_2N_2O$.

*2.4. Biological activity*

*2.4.1. In vitro inhibition studies on AChE*

The inhibitory activity of compounds **9a-m** was investigated against AChE using the Ellman's method. Acetylcholinesterase (AChE, E.C. 3.1.1.7, from electric eel), 5, 5′-dithiobis-( 2-nitrobenzoic acid) (DTNB), acetylthiocholine iodide (ATCI) and donepezil were purchased from Sigma Aldrich company. For the assay, donepezil was used as the reference compound. The stock solutions of the synthesized compounds were prepared in DMSO (20 mM). The assay solution consisted of 2000 µl of phosphate buffer (0.1 M, pH = 8.0), 60 µl of DTNB (0.01 M), 20 µl of AChE (1ku prepared in phosphate buffer (0.1 M) and glycerine 25%, pH=8.0, 2 units/mL in 0.5 mL aliquots) and 30 µl of various concentrations of tested compounds were incubated 15 min at room temperature. Then, 20 µl of the substrate, acetylthiocholine iodide (0.15 M), was added and the changes in absorbance were measured at the wavelength of 412 nm in the 2 min intervals using a Synergy HTX Multi-Mode Reader-BioTek. The $IC_{50}$ values for compounds with percentage of inhibition higher than 50% at the screening concentration (20 mM) were calculated

using nonlinear regression analysis of the response–concentration (log) curve. Results are presented as the mean ± standard deviation (SD) at least three different experiments performed in triplicate.

*2.4.2. In vitro inhibition studies on BuChE*

The BuChE inhibition activity of compounds **9a-m** was performed using the Ellman's method in 96-well plates. Butyrylcholinesterase (BuChE, E.C. 3.1.1.8, from equine serum), 5, 5′-dithiobis-( 2-nitrobenzoic acid) (DTNB) and butylthiocholine iodide (BTCI) were obtained from Sigma Aldrich company. The stock solutions of testing compounds were prepared in DMSO (20 mM) and diluted in MeOH to obtain final concentrations. The assay solution consisted of 200 µl of phosphate buffer (0.1 M, pH = 7.4), 20 µl of DTNB (0.01 M), 20 µl of BuChE and 10 µl of various concentrations of tested compounds (0.1-30 µM) were incubated 15 min at room temperature. Then, 20 µl of the substrate, butylthiocholine iodide (0.15 M), was added and the changes in absorbance were measured at the wavelength of 412 nm in the 2 min intervals using a Synergy HTX Multi-Mode Reader-BioTek. The $IC_{50}$ values for compounds with percentage of inhibition higher than 50% at the screening concentration (20 mM) were calculated using nonlinear regression analysis of the response–concentration (log) curve. Results are presented as the mean ± standard deviation (SD) at least three different experiments performed in triplicate.

*2.4.3. Kinetic study of AChE inhibition*

The mechanism of AChE inhibition for compound **9g** was investigated at different concentrations, 14.1, 28.2, and 56.3 µM using Ellman's method. The reciprocal plot of 1/velocity versus 1/[substrate] was plotted at different concentrations of the substrate acetylthiocholine (0.47, 0.94 and 2.31 mM). The plots were assessed by a weighted least-squares analysis that assumed the variance of velocity (v) to be a constant percentage of v for the entire data set. Slopes of these reciprocal plots were then plotted against the concentration of **9g** in a weighted analysis, and the inhibition constant ($K_i$) was determined as the intercept on the negative x-axis. Data analysis was performed using Microsoft Excel 2016.

*2.4.4. Kinetic study of BuChE inhibition*

The mechanism of BuChE inhibition for compound **9c** was investigated at different concentrations, 0.18, and 0.36 µM using Ellman's method. The reciprocal plot of 1/velocity versus 1/[substrate] was plotted at different concentrations of the substrate butyrylcholinesterase (0.05, 0.17, 0.25, 0.37 and 0.56 mM). The plots were assessed by a weighted least-squares analysis that assumed the variance of velocity (v) to be a constant percentage of v for the entire data set. Slopes of these reciprocal plots were then plotted against the concentration of **9c** in a weighted analysis, and the inhibition constant (Ki) was determined as the intercept on the negative x-axis. Data analysis was performed using Microsoft Excel 2016.

*2.4.5. BACE1 assay*

BACE1 fluorescence resonance energy transfer (FRET) assay kit of PanVera was used following the supplied protocol with small modifications. The kit containing BACE1 enzyme (purified baculovirus-expressed BACE1), the substrate (Rh-EVNLDAEFK-quencher, based on the Swedish" mutant of APP), reaction buffer (50 mM sodium acetate, pH = 4.5) and stop solution. The test compounds stock solutions were prepared in DMSO and further diluted with assay buffer (50 mM sodium acetate; pH = 4.5) to prepare a 3X concentration. The final concentration of DMSO was 6% (v/v). 10 µL of 3X concentration BACE1 substrate was added to 10 µL of each test compound (3X concentration) in separate wells of a black 96-well microplate and gently mixed. Then 10 µL of 3X BACE1 enzyme was added to each well to start the reaction. The reaction mixtures were incubated at 25 °C for 90 min in the dark and to stop the reaction, 10 µL of the stop solution (2.5 M sodium acetate) was added. Finally, the fluorescent intensity of the enzymatic product was measured using a multiwell spectrofluorometer instrument (BMG LABTECH, Polar star, Germany) capable of measuring at 544 nm excitation and 590 nm emission wavelengths. Each experiment was repeated 3–5 times. The $IC_{50}$ values were determined using nonlinear regression (GraphPad Prism 5) by plotting the residual enzyme activities against the applied inhibitor concentration [38].

*2.4.6. Metal chelating assay*

The study of metal chelation was performed in methanol using UV–vis spectrophotometer (Agilent 100, in a 1 cm quartz) with wavelength ranging from 200 to 600 nm. A fixed amount of compounds **9b** and **9c** (25 μM) were mixed with increasing amounts of $FeSO_4$ and $ZnCl_2$ (0–80 μM) for 30 min at room temperature [38]. The stoichiometry of the ligands : Zn(II) was determined by employing the mole ratio method, through titration of the methanol solution of the selected compounds with ascending concentrations of mentioned metal [39].

*2.4.7. Docking study method*

The co-crystal structures of AChE (PDB code: 1EVE) and BuChE (PDB code: 5K5E) were obtained from the RCSB Protein Data Bank (www.rcsb.org). Docking studies were carried out using the Autodock Vina (1.1.2) with the docking parameters set at default values. The docking procedure was validated by re-docking the original ligands into the AChE and BuChE proteins. For docking studies, the structure of compounds was drawn, optimized and saved in $mol_2$ format which then converted into the pdbqt format using Auto Dock Tools package (1.5.6). To prepare protein, cognate ligand and water molecules were removed and hydrogen atoms were subsequently added to the protein. Non-polar hydrogens were merged with their corresponding carbons and then the protein was converted into the required pdbqt format using Auto Dock Tools package (1.5.6). The size of grid box was set at 30× 30 × 30 and the center of grid box was placed at the co-crystalized ligand with coordinates x = 5.16, y = 66.91, z = 66.42 for 1EVE and x = 2.35, y = 12.53, z = 15.40 for 5K5E. The exhaustiveness was set to 100 and the other docking parameters were set as default. Finally, the conformations with the most favorable free energy of binding were selected for analyzing the enzyme-inhibitor interactions.

## 3. Results and discussion

*3.1. Design*

The purpose of this study was the design and synthesis of a novel series of carbazole-benzylpiperidine hybrid with increased biological properties, such as anti-BuChE, anti-BACE1 and metal chelating properties. Donepezil (**a**), an FDA-approved drug for AD treatment, is a potent and selective AChE inhibitor [30]. The main pharmacophoric part of this drug, N-benzylpiperidine moiety that interacts with the CAS of AChE [28], incorporated in the design of the final scaffold of target compounds. While the indanone fragment that interacts with PAS of AChE [28], replaced with carbazole group in order to provide novel carbazole-benzylpiperidine hybrids. Carbazole is a tricyclic structure with various biological activities related to Alzheimer's disease that previously some of its derivatives have been reported with anti-BACE1 (**b**), neuroprotective (**c**), anti-AChE (**d**), anti-Aβ-aggregation (**e**), anti BuChE and antioxidative activity [37, 40, 41] (Fig.1). Since, benzylpiperidine derivatives (4-amino-N-benzylpiperidine and 4-benzylpiperidine) attached to the carbazole moiety by various linkers to produce the target hybrids with cholinesterase and BACE1 inhibitory potential.

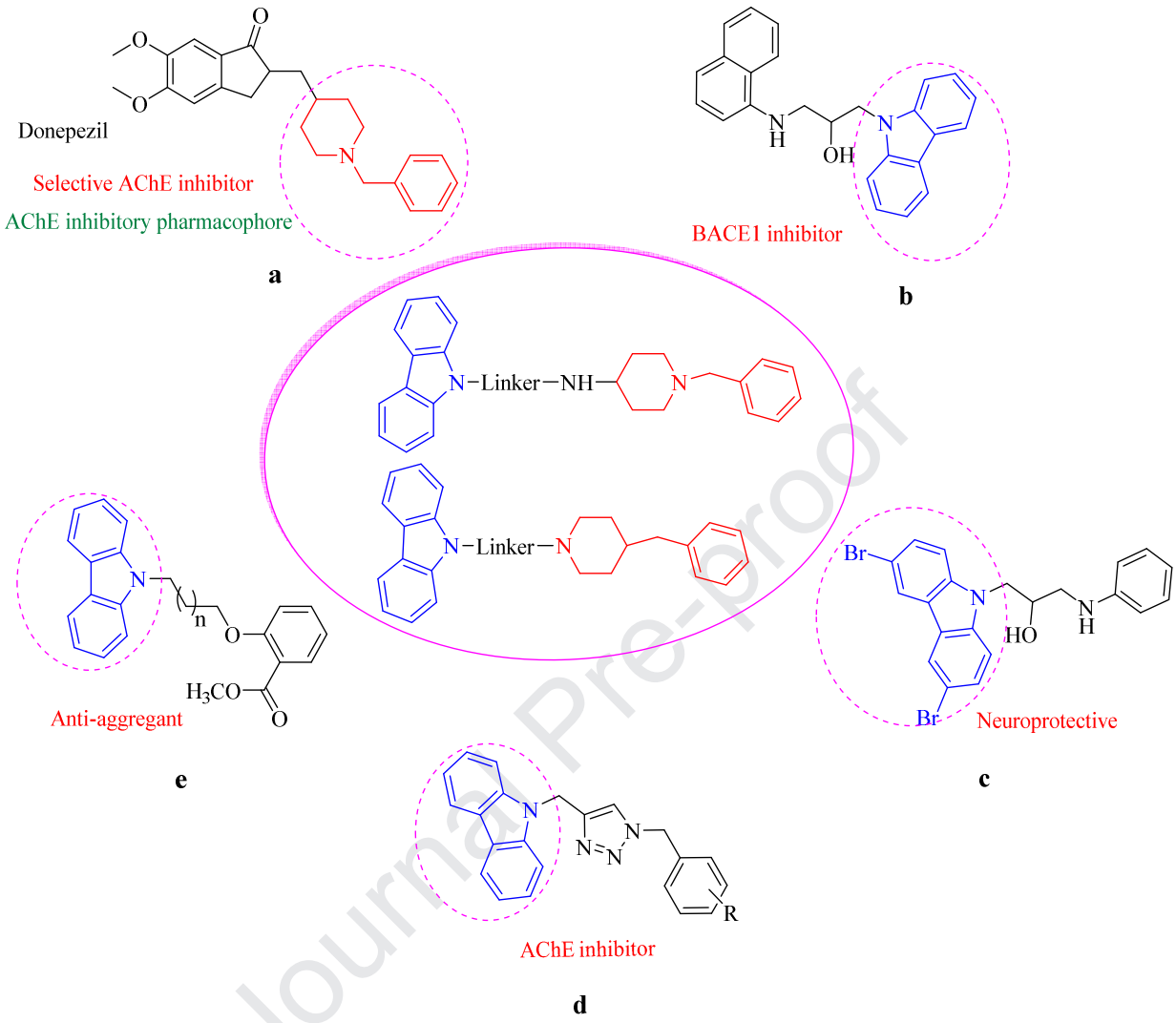
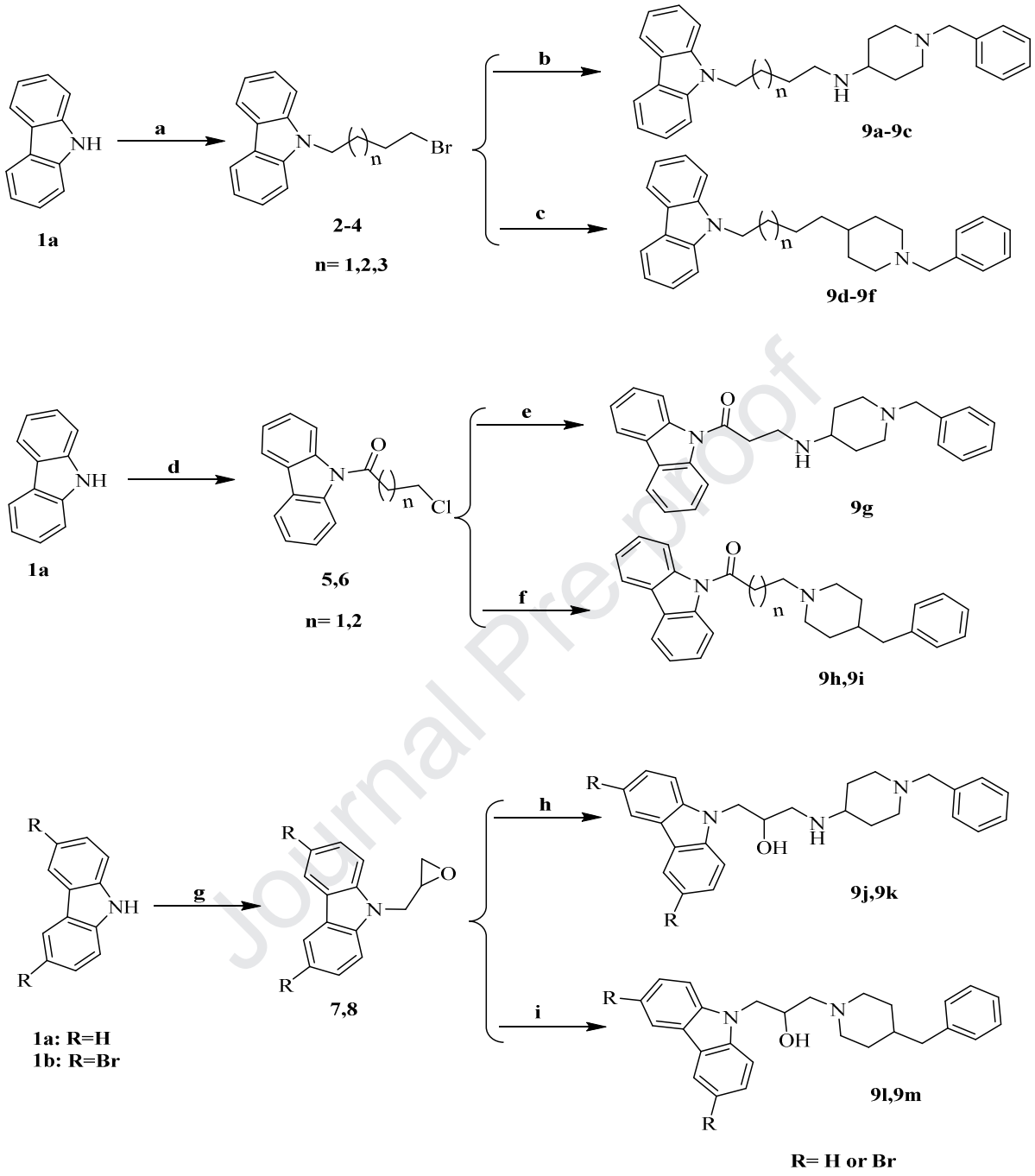



**Fig. 1.** The design strategy of novel carbazole-benzylpiperidine hybrids.

### *3.2. Chemistry*

The general method for the synthesis of the novel carbazole-benzylpiperidine hybrids **9a-m** is shown in Scheme 1. These novel hybrids were synthesized in a two-step route. The intermediates were synthesized according to the previous methods by some modifications [30, 37]. To prepare compounds **9a-f**, in the first step, intermediates **2-4** bearing different alkyl chains were synthesized via N-alkylation of carbazole (**1a**) using different di-bromoalkane derivatives in dry DMF and NaH as a base (67-73% yields). In the next step, the resulting intermediates (**2-4**)

reacted with corresponding amines in acetonitrile and triethylamine (TEA) in order to obtain desire compounds. Compounds **9g-i** were prepared by N-acylation of carbazole using different acyl-chlorides in acetonitrile and TEA under reflux conditions in order to afford compounds **5** and **6** (60-65% yields). Afterward, the further reaction of these intermediates (**5, 6**) with corresponding amines in acetonitrile and TEA resulted in compounds **9g-i**. Compounds **9j-m** were obtained via N-alkylation of carbazole (**1a**) or 3,6-dibromocarbazole (**1b)** with epichlorohydrin in dry DMF and KOH with 52-55% yields and subsequent reaction of these resulting epoxide intermediates (**7, 8**) with corresponding amines in EtOH. Different spectroscopic techniques, such as Mass, $^{1}$H NMR and $^{13}$C NMR were used to confirmed chemical structures of the synthesized compounds.




**Scheme 1.** Synthesis of carbazole-benzylpiperidine hybrids **9a-m**. Reagents and conditions: a) di-bromoalkanes, NaH, DMF, r.t., 30 min; b) 4-amino-N-benzylpiperidine, TEA, acetonitrile, reflux 24 h; c) 4-benzylpiperidine, TEA, acetonitrile, reflux 24 h; d) 3-chloropropionyl chloride or 4-chlorobutyryl chloride, TEA, acetonitrile, reflux 48 h; e) 4-amino-N-benzylpiperidine, TEA, acetonitrile, r.t., 1 h; f) 4-benzylpiperidine, TEA, acetonitrile, r.t., 1 h; g) epichlorohydrin, KOH, DMF, r.t., 24 h; h) 4-amino-N-benzylpiperidine, EtOH, 65 ˚C, 24 h; i) 4-benzylpiperidine, EtOH, 65 ˚C, 24 h.

*3.3. Biological activity*

*3.3.1. AChE and BuChE inhibitory activity*

The AChE (from electric eel) and BuChE (from equine serum) inhibitory activities of the synthesized compounds **9a-m** were evaluated by Ellman's assay [42]. In the first step, the inhibition percentage of synthesized compounds was determined at 20 mM for eelAChE and eqBuChE. In the following, the $IC_{50}$ values of the synthesized compounds that displayed inhibitory potency higher than 50% were determined in comparison with donepezil as a control. The results of the biological evaluation are shown in Table 1.

According to the results, synthesized compounds exhibited moderate eelAChE inhibitory activity with $IC_{50}$ values in the micromolar range. Among synthesized derivatives, the most active compounds against eelAChE, **9b**, **9c**, **9g** and **9i** demonstrated $IC_{50}$ values of 16.5, 26.5, 13.4 and 26.9 µM respectively.

Investigation of the results on eqBuChE showed that all the synthesized compounds except **9k**, **9m** and **9h** were potent for inhibition of eqBuChE with $IC_{50}$ values of 0.18-12.63 µM (Table 1). The most active derivative for inhibition of eqBuChE was compound **9c** bearing C6 alkyl linker with $IC_{50}$ value of 0.18 µM.

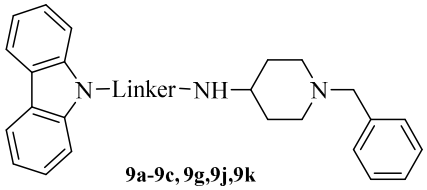
*3.3.2. Structure activity relationship (SAR)*

According to the biological results, N-alkyl-carbazole derivatives that attached to the 4-amino-N-benzylpiperidine fragment with C5 and C6 alkyl linkers (**9b** with $IC_{50}$ = 16.5 ± 0.21 µM and **9c** with $IC_{50}$ = 26.5 ± 0.17 µM), as well as the N-acyl-carbazole derivative with C3 and C4 spacers that attached to the 4-amino-N-benzylpiperidine and 4-benzylpiperidine respectively (**9g** with $IC_{50}$ = 13.4 ± 0.08 µM and **9i** with $IC_{50}$ = 26.9 ± 0.26 µM), were active for inhibition of eelAChE. These results indicated that the inhibitory activity against eelAChE significantly affected by the length and nature of the linker between carbazole and benzylpiperidine fragment. In general, derivatives containing alkyl linker were more potent than derivatives containing acyl and 2-hydroxypropyl linkers. Among the compounds with alkyl linkers (**9a-f**), 4-amino-N-benzylpiperidine derivatives were more active than 4-benzylpiperidine derivatives that indicated the nitrogen atom in the linker may form an effective hydrogen bond with the active site of eelAChE. In addition, for 4-amino-N-benzylpiperidine derivatives, the inhibitory potency against

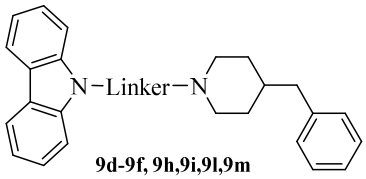
eelAChE increased in the order of C5 > C6 > C4 linker length. Unexpectedly, compound **9g** ($IC_{50}$ = 13.4 ± 0.08 µM) containing acyl linker with C3 spacer, displayed higher activity than other compounds. This higher activity might be due to the more favorable orientation of compound **9g** inside the active site of eelAChE.

According to the results, the modification of the linker between carbazole and benzylpiperidine fragment significantly influenced the eqBuChE inhibitory activity. Among synthesized compounds, **9a-f** derivatives that in which N-benzylpiperidine moiety were attached to the carbazole fragment through four-, five-, and six-membered alkyl spacers, respectively, displayed superior inhibitory potential against eqBuChE compared to other derivatives. It was also observed that replacement of 4-amino-N-benzylpiperidine fragment with 4-benzylpiperidine has slightly influence on eqBuChE inhibitory activity (**9b** with $IC_{50}$ = 0.59 ± 0.17 µM vs **9e** with 1.39 ± 0.51 µM and **9c** with $IC_{50}$ = 0.18 ± 0.08µM vs **9f** with 0.81 ± 0.17 µM). Results showed that replacement of 9H-carbazole moiety with 3,6-dibromocarbazole in 4-benzylpiperidine derivatives that bearing 2-hydroxypropyl linker resulted in reduced inhibitory potential against eqBuChE, **9l** with 2.4 ± 0.85 µM vs **9m** (inactive) (Table 1).

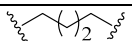
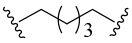
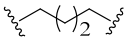
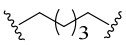
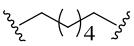
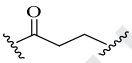
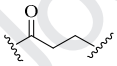
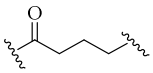
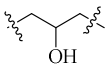
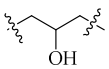
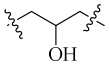
**Table 1.** In vitro inhibition of eelAChE and eqBuChE of compounds **9a-m** and donepezil.

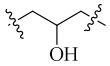



**9a-9c, 9g,9j,9k**




**9d-9f, 9h,9i,9l,9m**

| Compounds | Linker | R | eelAChE $IC_{50}$ (μM) [a], %inhibition[b] | eqBuChE $IC_{50}$ (μM)[a], %inhibition[b] |
|---|---|---|---|---|
| **9a** |  | H | 33.18% | 5.4 ± 0.70 |
| **9b** |  | H | 16.5 ± 0.21 | 0.59 ± 0.17 |
| **9c** |  | H | 26.5 ± 0.17 | 0.18 ± 0.08 |
| **9d** |  | H | 45.02% | 0.90 ± 0.24 |
| **9e** |  | H | 46.18% | 1.39 ± 0.51 |
| **9f** |  | H | 47.79% | 0.81 ± 0.17 |
| **9g** |  | H | 13.4 ± 0.08 | 12.6 ± 0.30 |
| **9h** |  | H | 36.56% | 35.59% |
| **9i** |  | H | 26.9 ± 0.26 | 10.3 ± 0.12 |
| **9j** |  | H | 23.70% | 6.4 ± 0.47 |
| **9k** |  | Br | 21.94% | 39.26 % |
| **9l** |  | H | 16.35% | 2.4 ± 0.85 |
| **9m** | | Br | 4.9% | Inactive[c] |

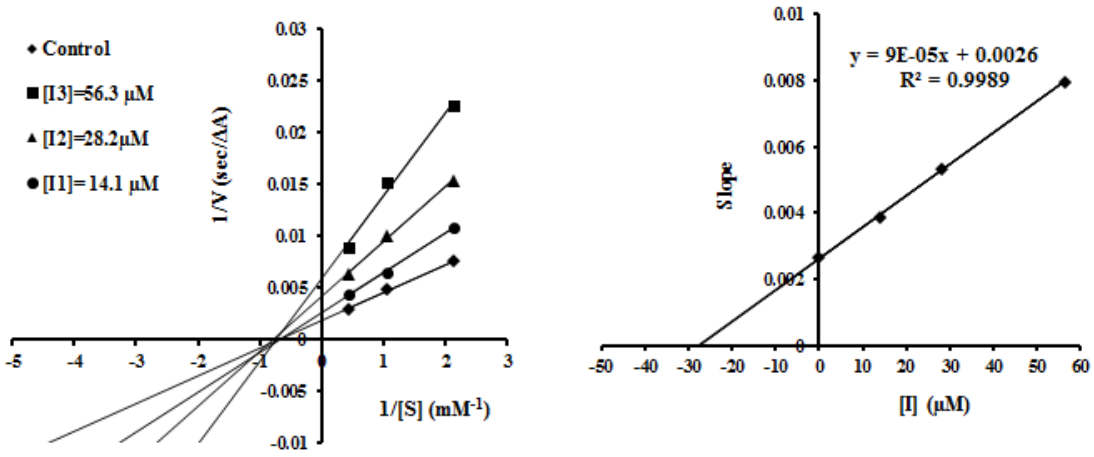



| | | | | |
|---|---|---|---|---|
| **donepezil** | - | - | 0.027 ± 0.01 | 10.6 ± 2.1 |

[a] $IC_{50}$: 50% inhibitory concentration (mean ± SD of three independent experiments).
[b] %inhibition at 20mM concentration.
[c] inactive at 20mM concentration.

*3.3.3. Kinetic study of AChE inhibition*

The kinetic studies for the most active inhibitor of eelAChE (**9g)**, was performed to elucidate the involved mechanism of eelAChE inhibition. For this purpose, three fixed inhibitory concentrations of test compound **9g** (14.1 µM, 28.2 µM and 56.3 µM) were used and for each concentration, the initial velocity (V) of the substrate hydrolysis was measured in the range of 0.47-2.31 mM. The data was collected and used for drawing Lineweaver-Burk double reciprocal plots (Fig. 2) which showed increased slopes (decreased $V_{max}$) and preserved intercepts (unchanged $K_m$) at higher concentrations for compound **9g** as an inhibitor. This pattern demonstrated a linear non-competitive inhibitory effect of **9g** on the enzyme, which showed compound **9g** might be able to interact with the peripheral anionic site (PAS) rather than the catalytic active site (CAS) of eelAChE. The inhibition constant (Ki) of 27.57 µM was estimated using the slopes of double reciprocal plots versus the different concentrations of compound **9g** (Fig. 2).



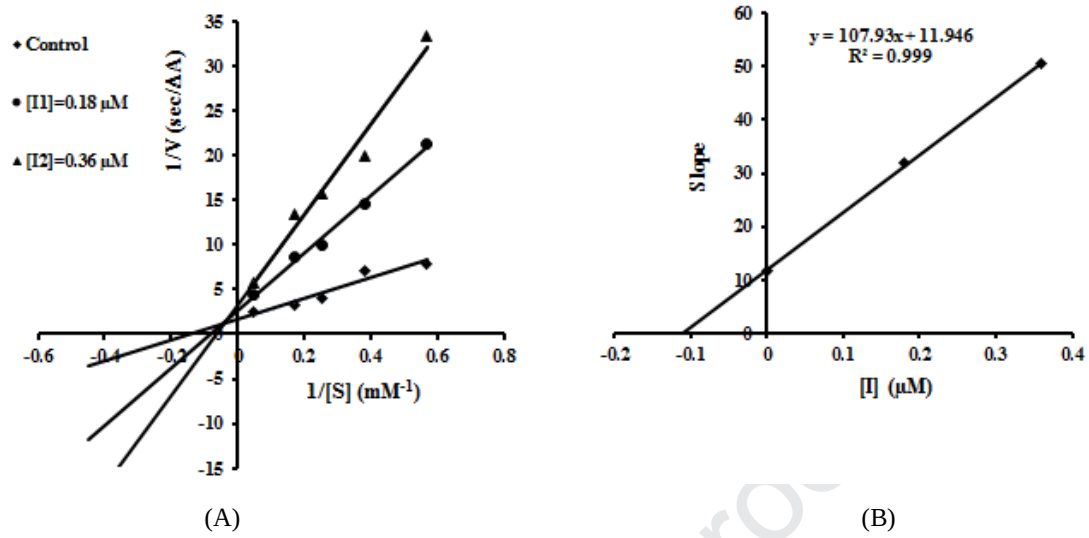

(A) (B)

**Fig. 2.** Kinetic study of eelAChE inhibition by compounds **9g**. A) Lineweaver-Burk double reciprocal plot of eelAChE initial velocity at increasing substrate concentration (0.47–2.31 mM) in the absence and presence of **9g**, B) Steady-state inhibition constant (Ki) of compound **9g**.

It has been reported that AChE could promote the generation of Aβ fibrils and Aβ plaques. This process was assumed to be mediated by an interaction between Aβ and peripheral anionic site of the enzyme (PAS) [43-45]. That finding provided a new opportunity for the development of AChE inhibitors that capable to block both CAS and the PAS site of the enzyme. This strategy may provide a better therapeutic effect for the treatment of AD, since it enables simultaneous inhibition of AChE activity and Aβ plaques generation.

*3.3.4. Kinetic study of BuChE inhibition*

To obtain insight into the eqBuChE inhibition mechanism, kinetic studies were performed at three concentrations of compound **9c**. As shown in Fig. 3 graphical analysis of a Lineweaver-Burk reciprocal plot demonstrated both increasing slopes (decreasing $V_{max}$) and intercepts ($K_m$) at increasing concentration of inhibitor **9c**. This pattern revealed a mixed-type inhibitory effect of compound **9c** on eqBuChE, which is indicated that compound **9c** may interact with both sites of the enzyme (CAS and PAS). The Ki= 0.11 µM was calculated from the secondary plots of the slope versus the concentration of inhibitors **9c** (Fig. 3).




(A) (B)

**Fig. 3.** Kinetic study of eqBuChE inhibition by compounds **9c**. A) Lineweaver-Burk double reciprocal plot of eqBuChE initial velocity at increasing substrate concentration (0.05-0.56 mM) in the absence and presence of **9c**, B) Steady-state inhibition constant (Ki) of compound **9c**.

*3.3.5. Molecular modelling study on AChE*

In order to find the possible interaction mode of the most active derivative **9g** with AChE the docking study was carried out. For this purpose, the co-crystal structure of AChE was obtained from the Protein Data Bank. Due to the structural similarity of synthesized compounds to donepezil, PDB code 1EVE which AChE complexed with donepezil was selected. Docking studies were done using Auto Dock Vina (1.1.2) program [46]. In the first step, re-docking of ligand E20 (original ligand) into the active site of AChE was done to confirm the reliability of the docking protocol (RMSD = 0.80). Then the most active derivative **9g** was docked using optimized parameters. The binding mode of compound **9g** into AChE active site is shown in Fig. 4. The docking results revealed that the benzyl fragment interacted with Trp84 in the CAS of the enzyme through π-π stacking interaction. The protonated piperidine ring interacted with Asp 72. The protonated nitrogen atom in linker forms cation-π interactions with Phe331 and hydrogen interaction with Phe330 in the middle of the active gorge. The carbazole fragment interacted with Trp279 in the PAS of the enzyme through π-π stacking interaction. The docking results showed that compound **9g** accommodated in the gorge of the enzyme and occupied the cavity between CAS and PAS. The interactions of ligand **9g** were similar to other ligands, but these interactions were more effective. According to the results of the docking study, this compound

interacted with CAS and PAS of AChE simultaneously. It seems that π−π stacking, cation-π and hydrogen bond interactions play an important role in the stability of complex which lead to the lower binding free energy (ΔG) and consequently, better biological effect of compound **9g**.

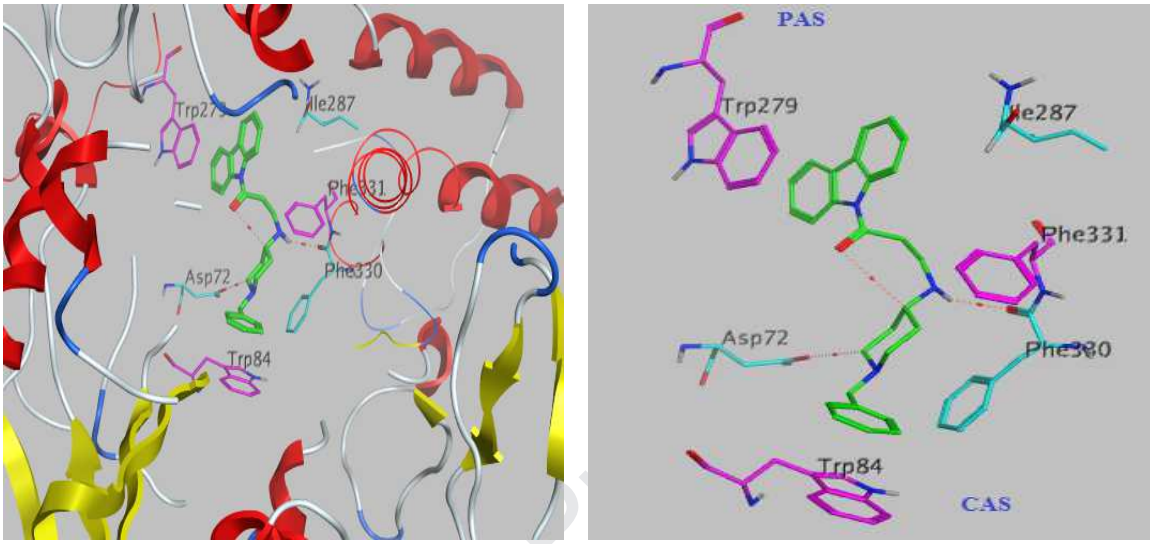



**Fig. 4.** Binding mode of compound **9g** in the active site of AChE, PDB code: 1EVE.

*3.3.6. Molecular modelling study on BuChE*

Due to the potency of synthesized compounds to inhibit the BuChE, the molecular modelling study was also performed on BuChE. The most potent derivative **9c** was selected to find the possible interaction mode (PDB ID: 5K5E). Docking study was done according to the same protocol used for AChE (RMSD = 0.96 Aº). The binding mode of compound **9c** into the BuChE active site is shown in Fig. 5. The docking results revealed that the ligand **9c** bound to the active site of BuChE with a folded conformation. The carbazole fragment interacted with Trp82 in the CAS of the enzyme through π-π stacking interaction. The benzyl fragment interacted with Trp231 in the PAS of the enzyme through H-π interaction. The protonated piperidine ring interacted with Pro285 through hydrogen interaction. According to the results, this compound accommodated in CAS and PAS of BuChE enzyme simultaneously.

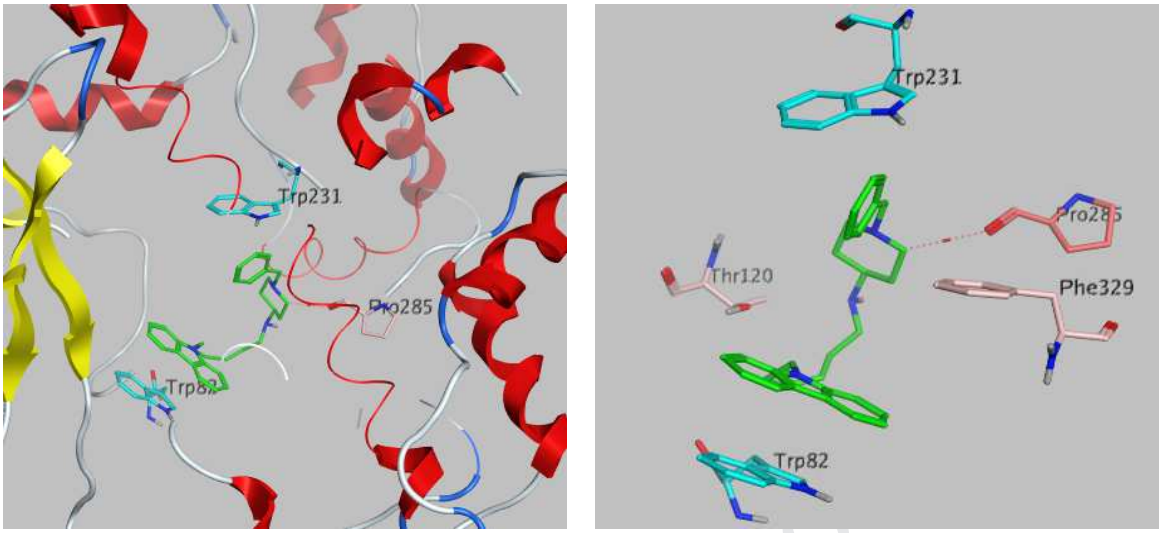



**Fig. 5.** Binding mode of compound **9c** in the active site of BuChE, PDB ID: 5K5E.

*3.3.7. BACE1 inhibitory activity*

Since our study was aimed at design and synthesis of MTDLs compounds, the potency of compound **9c**, as a potent AChE and BuChE inhibitor, to inhibit BACE1 was investigated by fluorescence resonance energy transfer (FRET) assay. OM99-2 was used as the standard compound in this assay. For each concentration (10, 50 and 100 µM), experiments were repeated three times and mean percentage inhibition was calculated. The results are shown in Table 2. According to the obtained results compound **9c** showed moderate activity against BACE1 at the concentration of 50 µM.

.

**Table 2**. BACE1 inhibitory activity of compound **9c**.

| Compound | Inhibition at 10 µM (%) | Inhibition at 50µM (%) | Inhibition at 100µM (%) | $IC_{50}$ (µM) |
|---|---|---|---|---|
| **9c** | 18.47 | 24.59 | 59.89 | 86.2 ± 2.97 |
| **OM99-2** | - | - | - | 0.014 ± 0.003 |

*3.3.8. Metal chelating activity*

$Fe^{2+}$ and $Zn^{2+}$ as a transition metal ions have the ability to gaining or losing electrons and produced free radicals. Besides, mentioned metal ions increased toxic Aβ oligomers generation through binding to Aβ peptides and inducing the Aβ accumulation. As a result, the formation of toxic Aβ aggregation and reactive oxygen species can be prevented by the chelation of metal ions with ligands. In order to evaluate the metal chelation properties of the most potent compounds, UV/Vis spectroscopic method was applied [7]. The results for compounds **9b** and **9c** are shown in Fig. 6 and Fig. 7, respectively. As depicted in Fig. 6, the maximum peak in the absorption spectrum of compound **9b** is at 235 nm. When $ZnCl_2$ was added, a noticeable hypochromic shift at 234 nm produced. However, the addition of $FeSO_4$ had a nearly small effect on the absorbance spectrum which just indicates small interaction with $Fe^{2+}$. Similarly, besides addition $FeSO_4$ to the solution of compound **9c,** no significant changes in the absorbance were observed. However, changes in the absorbance at 235 nm upon addition of $ZnCl_2$ suggest the binding of $Zn^{+2}$ to compound **9c.**

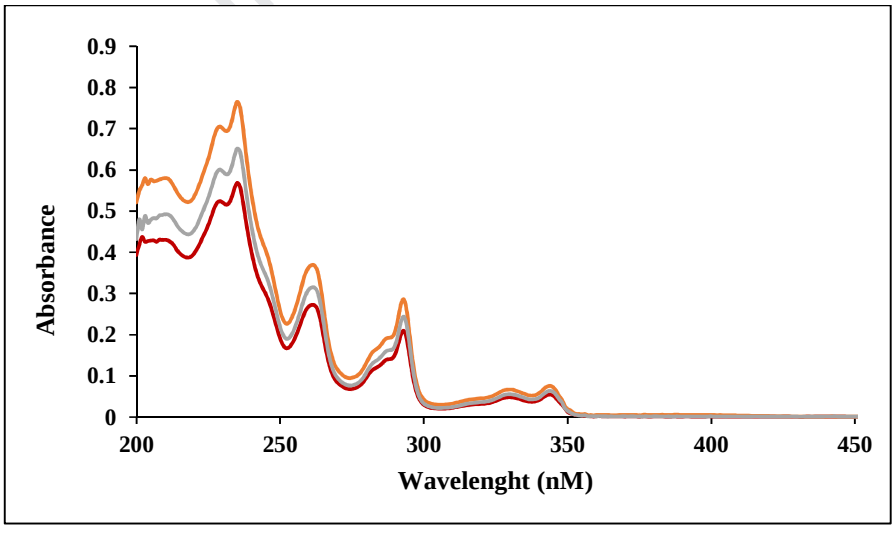



**Fig. 6.** The metal chelation ability of compound **9b** was evaluated by employing UV spectroscopy. Red colour represents the compound alone spectrum whereas orange and grey represent the spectrum in the presence of $ZnCl_2$ and $FeSO_4$, respectively.

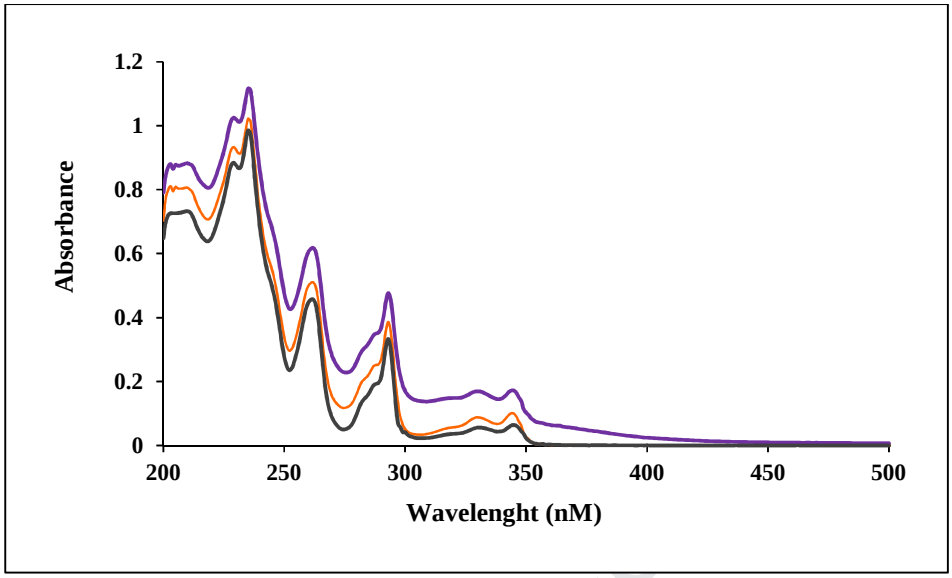



**Fig. 7.** The metal chelation ability of compound **9c** was evaluated by employing UV spectroscopy. Black colour represents the compound alone spectrum whereas purple and orange represent the spectrum in the presence of $ZnCl_2$ and $FeSO_4$, respectively.

In order to investigate the molar interaction between compounds **9b** and **9c** and the $Zn^{2+}$ biometal, the mole ratio's method was applied to determine the stoichiometry of the formed complexes. In the mole ratio's plot of compound **9b** (Fig. 8.), the maximum absorbance was reached 2(**9b**):1($Zn^{2+}$) molar fraction of 0.7, revealing 2:1 complex stoichiometry. The stoichiometry of the **9c**-Zn(II) complex was also determined by titration of the methanolic solution of compound **9c** with ascending amounts of $ZnCl_2$. The two straight lines crossed at a mole fraction of 0.6, indicating a 2:3 stoichiometry for the complex (Fig. 9.).

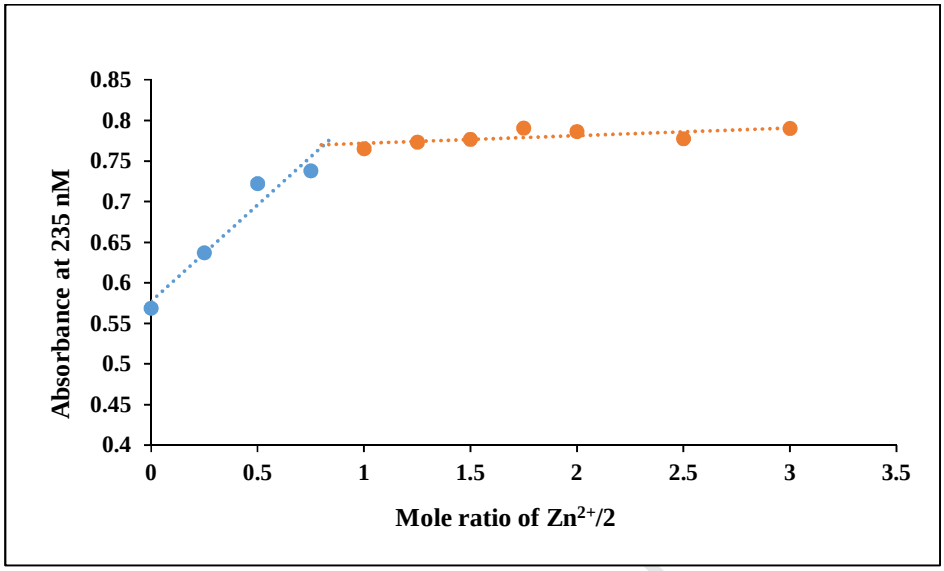



**Fig. 8.** Mole ratio's plot of compound **9b** with $ZnCl_2$ in methanolic solution.

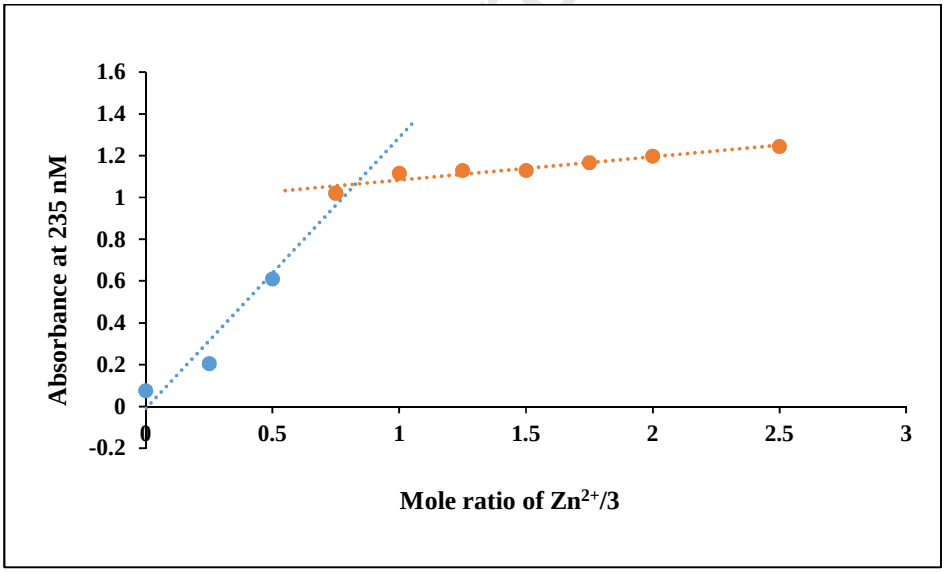



**Fig. 9.** Mole ratio's plot of compound **9c** with $ZnCl_2$ in methanolic solution.

## 4. Conclusion

In conclusion, a series of novel carbazole-benzylpiperidine hybrids as multifunctional agents for the treatment of AD was synthesized. In the synthesized compounds, N-benzylpiperidine moiety was attached to the carbazole fragment by various linkers. The activity of these hybrids against

AChE, BuChE and BACE1, as well as metal chelation activity was evaluated. Our results indicated that the synthesized compounds had a moderate eelAChE inhibitory activity and most of the synthesized compounds (except **9k**, **9m** and **9h**) were potent for inhibition of eqBuChE. Generally, among these compounds, compounds **9b** ($IC_{50}$ = 16.5 µM for AChE and $IC_{50}$ = 0.59 µM for BuChE) and **9c** ($IC_{50}$ = 26.5 µM for AChE and $IC_{50}$ = 0.18 µM for BuChE) by five- and six carbon spacers respectively, indicated the highest inhibitory activity against eelAChE and eqBuChE, as well as compound **9c** showed moderate inhibitory activity against BACE1 (24.5% at 50 µM). Moreover, the metal chelating study demonstrated that compounds **9b** and **9c** could interact with the $Zn^{+2}$ ion. Docking studies indicated that these compounds could interact simultaneously with the CAS and PAS of AChE. The results suggest that these synthesized compounds could be candidates for the development as multi-functional drugs in the treatment of Alzheimer's disease.

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## Highlights:

1. Synthesis of novel carbazole-benzylpiperidine hybrids.

2. Some of synthesized compounds were active as acetylcholinesterase (AChE), butyrylcholinesterase (BuChE) and β-secretase (BACE1) inhibitors.

3. Kinetic and docking studies exhibited that these compounds likely act as a non-competitive inhibitor able to interact with the catalytic active site (CAS) and peripheral anionic site (PAS) of AChE simultaneously.

4. Study of metal chelation properties of the synthesized compounds showed that they could interact with $Zn^{2+}$ Ion.

5. These synthesized compounds can be considered as multi-target ligand in the treatment of Alzheimer disease.

# Design, Synthesis and Biological Activity Evaluation of Novel Carbazole-benzylpiperidine Hybrids as Potential anti Alzheimer Agents

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The authors declare no conflict of interest.