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Graphical Abstract

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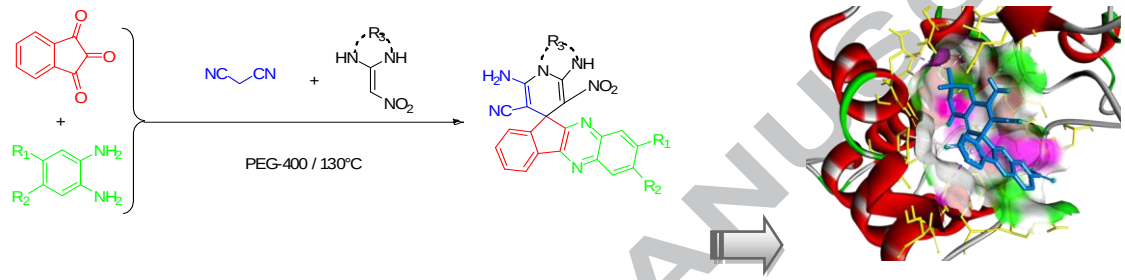

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ABSTRACT

A convenient synthesis of substituted spiroindenoquinoxalines at mild and green conditions was developed. Multicomponent reaction of substituted phenylene diamines, ninhydrin, malononitrile and *N,N'*-substituted-2-nitroethene-1,1-diamines produced the target compounds. Twelve new spiroindenoquinoxalines were obtained, and their ability in inhibition of acetyl and butyrylcholinesterases were investigated both *in-vitro* and *in-silico*. All compounds showed moderate level activity against both acetyl and butyrylcholinesterases.

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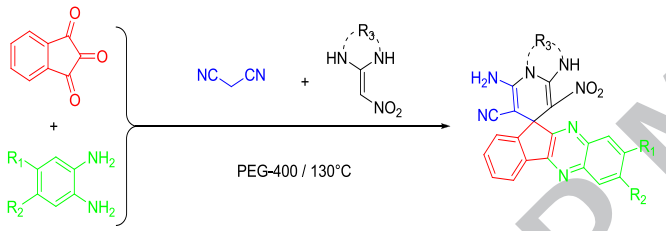
1. Introduction

The presence of cholinesterase (ChE) was predicted in 1914, before the actual discovery of such enzymes.[1,2] The existence of two major cholinesterases was discovered after 26 years and these were termed acetylcholinesterase (AChE) and butyrylcholinesterase (BChE).[3] In 1982, it was proposed that degeneration of cholinergic neurons by AChE and BChE in the basal forebrain and the associated loss of cholinergic neurotransmission, contributed significantly to the deterioration in cognitive function seen in patients with Alzheimer's disease (AD).[4,5] AD is characterized by memory loss, personality changes and deterioration of cognitive functions which finally leads to incapacitation of patient.[6,7]

The search for new compounds with potential effect to cure AD through inhibiting ChEs has attracted great attention in recent years.[8-10] The main purpose here is to enhance the acetylcholine level in the brain by inhibition of cholinesterases.[11] Acetylcholinesterase (AChE) is the key enzyme in hydrolysis of ACh that plays a key role not only in enhancing cholinergic transmission in the brain, but also in reducing the aggregation of amyloid‑beta (Aβ) peptide and the formation of the neurotoxic fibrils in AD.[12] Another enzyme, butyrylcholinesterase (BChE), expressed in selected areas of the central and peripheral nervous systems, is also capable of hydrolyzing ACh.[13] AChE and BChE sequences are similar up to 84% and hence, their responses to a definite therapy almost yield in similar results.[7]

Most heterocyclic systems have been investigated as a source to discover new compounds with cholinesterase inhibitory potential[14] and many of them such as spirooxindoles, quinoxalines and indenoquinoxalines have been applied *in-vitro* successfully.[15-17] In addition to indenoquinoxalines, some spiro-indenoquinoxalines derivatives based on biindeno[1,2-b]quinoxaline have been applied to inhibit the cholinesterase enzymes activity.[18]

In continuation of our works on green organic chemistry through catalyst-free and/or multi-component reactions[19-29] and according to the reports on biological application of spiro-compounds derived from ninhydrin, ketene aminals and substituted quinoxalines,[30-32] herein we report a simple and green method for the synthesis of spiro-indenoquinoxaline derivatives via a one-pot multicomponent condensation of indenoquinoxaline derivatives (1 equiv) and malononitrile (1 equiv) with ketene aminals (1 equiv) under catalyst-free conditions (Scheme 1). As far as our knowledge from the scientific databases, these compounds have not been synthesized nor studied for their cholinesterase inhibition activity.



Scheme 1- Overall reaction conditions (a: R^1,R^2=H, b: R^1,R^2=CH_3, c: R^1=NO_2, R^2= H ; R^3= -CH_2CH_2-, -$CH_2CH_2CH_2$-, -$CH_2(CH_3)_2CCH_2$-, 2CH_2Ph)

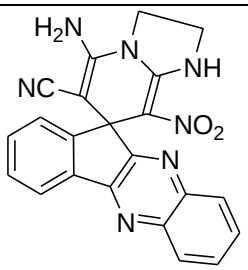
2. Results and discussion

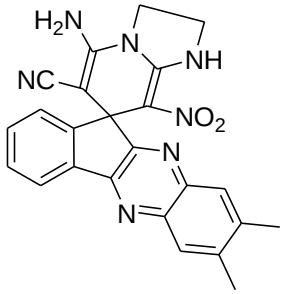
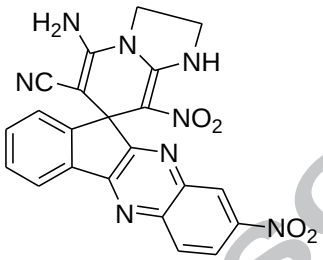
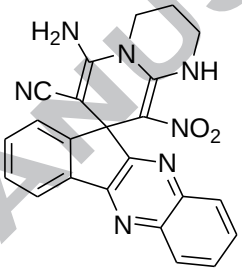
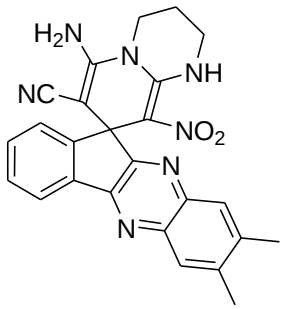
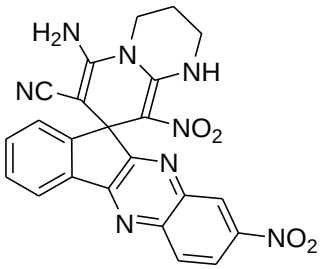
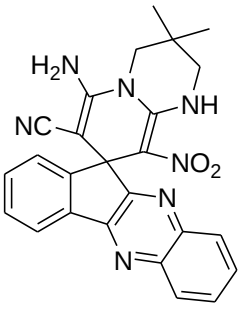
2.1. Synthesis

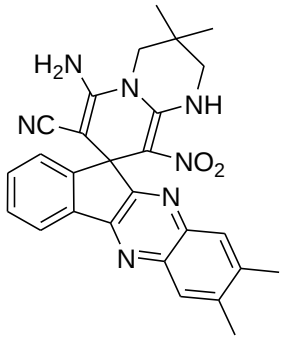
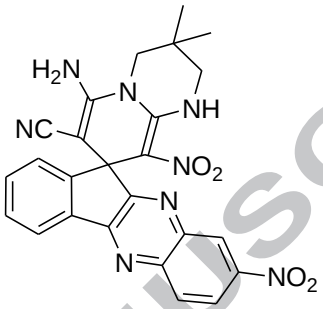
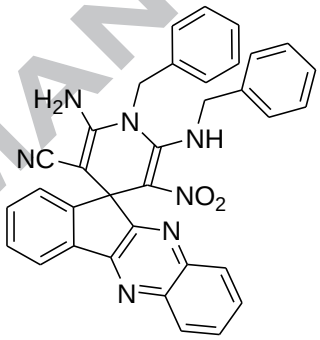
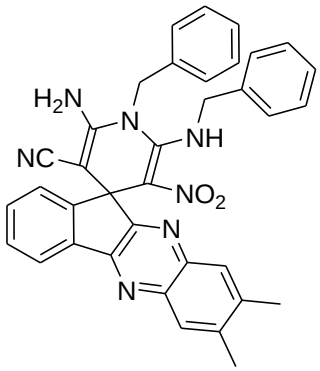
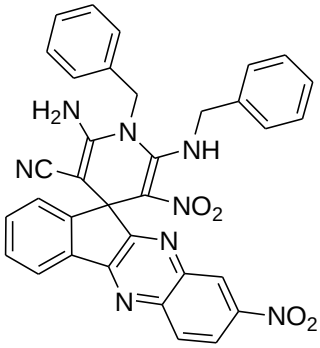
Optimization of the reaction conditions was done by using the condensation reaction of indenoquinoxaline (1 mmol) with malononitrile (1 mmol) and 2-(nitromethylene)imidazolidine (1 mmol) as a model reaction. For this purpose, various solvents at different temperatures (70, 100, and 130 °C) were used under catalyst-free conditions to obtain the best optimized conditions through carrying out the model reaction. The results revealed that PEG-400 is the best choice as a solvent and rising its temperature to 130°C resulted in higher yields and shorter reaction times. More increase in the reaction temperature did not improve the yield or the reaction time. Having identified the optimal conditions, indenoquinoxaline derivatives (1 mmol) were reacted with malononitrile (1 mmol) and various 2-nitroethene-1,1-diamines (1 mmol) (Scheme 1). The results are given in Table 1.

As shown in Table 1, indenoquinoxaline derivatives were successfully applied in this process to afford the corresponding spiro-indenoquinoxaline derivatives in good yields and short reaction times. These indenoquinoxaline derivatives were obtained from the reaction of ninhydrin with phenylenediamine derivatives in the same reaction pot. Moreover, a range of cyclic and acyclic 2-nitroethene-1,1-diamines were used to prepare the corresponding spiro-indenoquinoxaline.

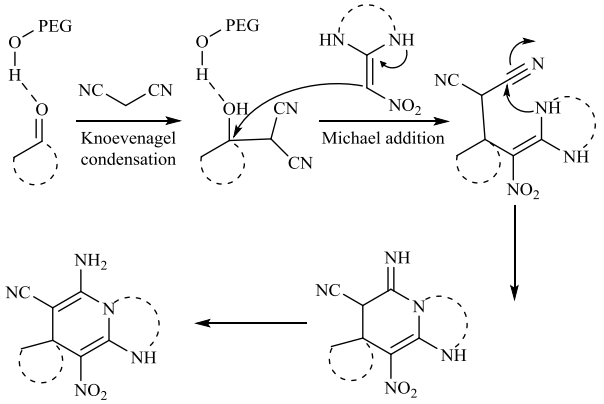
Table 1. The catalyst-free synthesis of spiro-indenoquinoxaline derivatives.

Entry	R^1	R^2	R^3	Product	Time (h)	Yield[a] (%)
D1	H	H	-$(CH_2)_2$-		11	80

D2	CH_3	CH_3	$-(CH_2)_2-$		13	78
D3	NO_2	H	$-(CH_2)_2-$		11	80
D4	H	H	$-(CH_2)_3-$		12	81
D5	CH_3	CH_3	$-(CH_2)_3-$		13	76
D6	NO_2	H	$-(CH_2)_3-$		11	81
D7	H	H	$-CH_2C(CH_3)_2CH_2-$		12	74

D8	CH_3	CH_3	$-CH_2C(CH_3)_2CH_2-$		13	78
D9	NO_2	H	$-CH_2C(CH_3)_2CH_2-$		10	80
D10	H	H	$2PhCH_2-$		13	75
D11	CH_3	CH_3	$2PhCH_2-$		14	74
D12	NO_2	H	$2PhCH_2-$		11	79

[a]Isolated Yield



Scheme 2- Proposed mechanism for synthesis of spiro-indenoquinoxalines

^{1}H NMR spectra of **D1-D12** displayed the characteristic down-field shift of 1-H proton at about δ=10.02–10.22 ppm due to intra-molecular hydrogen bonding of NH proton and oxygen of the NO_2 group originated from the ketene aminal part. A broad singlet peak with integral of two protons, responsible for NH_2 group, appeared at δ=6.0–7.0. Aromatic protons of indenoquinoxaline backbone are seen at δ=7.41–8.54 ppm. Aromatic signals for phenyl ring in **D9-D12** also have been seen in the same region. The ^{13}C NMR spectra showed an agreement between the numbers of observed signals with the proposed structure. The characteristic signal due to the spiro carbon displayed at δ=50.4-54.2 ppm. The IR spectra displayed the characteristic vibrations for NH and NH_2 at 3190, CN at 2180 and NO_2 at 1340 cm^{-1} (these numbers varies in small ranges for different compounds).

A proposed reaction pathway to spiro-indenoquinoxalines is presented in Scheme 2. In the first step, a fast Knoevenagel condensation occurs between acidic protons of malononitrile and carbonyl group of indenoquinoxaline to afford 11-(propan-2-ylidene)-11H-indeno[1,2-b]quinoxaline derivatives. Michael addition of 2-nitroethene-1,1-diamine followed by cycloaddition of amine group to the cyano moiety provides the desired product spiro-indenoquinoxaline after successive rearrangements (Scheme 2).

2.2. Cholinesterase inhibition activity

Plenty of AChE and BChE inhibitors have been identified in recent decades which the concentrations for related activity fall in picomolar to micromolar range. Fasciculins, a class of toxic proteins primarily found in snake venoms, are the dominant examples for cholinesterase inhibition in picomolar concentrations,[33] while in nanomolar range there are more classes of compounds such as Tacrine, Donepzil, Rivastigmine and Xanthostigmine derivatives. Furthermore, Galanthamine, Coumarin and Flavonoid derivatives have been shown to impose cholinesterase inhibition activities in micromolar range.[34] As shown in table 2, compounds **D9**, **D6** and **D4** have shown the lowest IC_{50}s amongst spiroindenoquinoxaline derivatives which are comparable to some of Galanthamine, Coumarin and Flavonoid derivatives which could be regarded as moderate inhibition activities.

Furthermore, AChE inhibition activities were approximately two times greater than for BChE inhibition by the same compounds. Although amongst the synthesized compounds as a whole, there are tendency to have a preference for AChE over BChE inhibition, these differences in tendency are too small when compared to highly selective inhibitors like Donepezil which is 10,000-fold more potent toward AChE than BChE.

Table 2. Acetyl and butyrylcholinesterase inhibitory activity of spiro-indenoquinoxaline derivatives

compound	AChE inhibition IC_{50} (μM)[a]	BChE inhibition IC_{50} (μM)[a]	Selectivity[b]	
			AChE	BChE
Galantamine	0.6±0.1	1.2±0.2	1.875	0.533
D1	6.4±0.3	8.4±0.3	1.312	0.761
D2	6.6±0.2	7.3±0.2	1.106	0.904
D3	6.5±0.1	9.6±0.3	1.476	0.677
D4	4.2±0.1	10.2±0.3	2.428	0.411
D5	5.7±0.2	9.5±0.4	1.666	0.600
D6	3.9±0.1	9.7±0.3	2.487	0.402
D7	4.3±0.1	9.8±0.3	2.279	0.438
D8	4.0±0.2	8.8±0.2	2.200	0.454
D9	3.6±0.1	10.1±0.2	2.805	0.356
D10	5.3±0.2	8.6±0.3	1.622	0.616
D11	5.8±0.3	8.9±0.4	1.534	0.651
D12	4.4±0.1	9.1±0.2	2.068	0.483

[a] All experiments were performed in triplicate and the results have been rounded to the nearest 0.1 μM.
[b] Selectivity for AChE is defined as IC_{50} (BChE)/IC_{50} (AChE) and Selectivity for BChE is defined as IC_{50} (AChE)/IC_{50} (BChE).

2.3. Cytotoxicity

To determine the maximum non-toxic concentrations of synthesized spiroindenoquinoxalines, cytotoxicity test was assessed using MTT assay in the HepG2 cell model. HepG2 cells represent the most likely target tissue of antituberculotics-related toxicity.[35] The cytotoxicity results for the tested compounds are summarized in Table 3.

The IC_{50} values for the tested compounds were in the range of 161.2–193.9 μM. According to the located functional groups, nitro substituted indenoquinoxalines showed the lowest IC_{50} values (161.2–174.4μM), while unsubstituted analogues showed higher IC_{50} values (173.5–193.9μM). On the other hand, there is no direct correlation between the substitution at the diamine part and cytotoxicity of the target molecule.

Table 3. Cytotoxicity results of synthesized compounds on HepG2 cells.

Compound	IC_{50} (μM)
D1	193.9
D2	176.4
D3	173.5
D4	174.4
D5	170.9
D6	161.2
D7	183.5
D8	180.2
D9	168.6
D10	189.7
D11	174.3
D12	167.1

2.4. Molecular docking

The inhibition potency of all the newly synthesized compounds were subjected for further docking studies to explore the binding pattern against AChE and BChE. In AChE, this binding site is formed by residues at the C-terminal end of each subunit in the cavity formed by residues Ala237, Ser240, Arg296, Thr311, Glu313, Gln369, Hsd405, Leu536 and Pro537. In BChE, this binding site is formed by residues Asn228, Pro320, Asp304, Tyr396, Glu404, Cys400, Pro401, Trp522, Thr523 and Pro527.

The affinity values resulted from docking procedure were summarized in Table 4 where all compounds had shown slightly greater selectivity toward AChE which confirms the practical results. In both *in-silico* and *in-vitro* investigations, among synthesized compounds, **D9** and **D2** have shown the greatest affinity toward AChE and BChE, respectively. The 3D and 2D bonding pattern between **D9** and AChE has been shown in figure 1 and bonding pattern between **D2** and BChE has been illustrated in figure 2.

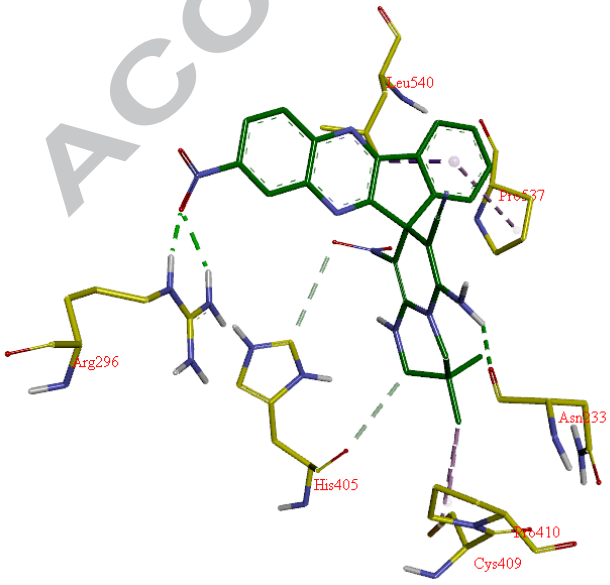
Table 4. Docking results of galantamine and synthesized compounds against acetyl and butyrylcholinesterases

Compound	Affinity (kcal.mol^{-1})		Selectivity[a]	
	AChE	BChE	AChE	BChE
Galantamine	-7.1	-5.9	1.203	0.830
D1	-8.9	-6.7	1.328	0.752
D2	-8.9	-7.2	1.236	0.808
D3	-8.8	-6.7	1.313	0.761
D4	-9.1	-6.5	1.400	0.714
D5	-8.9	-6.9	1.289	0.775
D6	-9.2	-6.6	1.393	0.717
D7	-8.9	-6.5	1.369	0.730
D8	-9.3	-7.1	1.309	0.763
D9	-9.3	-6.5	1.430	0.698
D10	-8.6	-7.0	1.228	0.813
D11	-8.6	-6.3	1.365	0.732
D12	-9.0	-7.2	1.251	0.801

[a] Selectivity for AChE is defined as affinity (BChE)/ affinity (AChE) and Selectivity for BChE is defined as affinity (AChE)/ affinity (BChE).

The molecular docking analysis for **D9** revealed that its binding interactions with AChE receptor were both hydrophobic and hydrophilic in nature. Hydrophobic interactions with His405, Cys409, Pro410, Pro537 and Leu540 at binding pocket as well as classical H-bond interactions with Asn233 and Arg296 are the dominant interactions of **D9** with the active site residues of AChE receptor. Furthermore, there are two non-classical H-bonds between His405 of receptor and the ligand (Fig. 1).

Regarding molecular docking simulation on BChE receptor, aromatic cores of indenoquinoxaline in **D2** displayed effective p-alkyl-stacking interaction with Pro335, Lys355, Ile356 and Pro359 of BChE enzyme, along with classical H-bonding interaction with the carbonyl group of Gly283 and non-classical H-bonding between nitro group of ligand and Ile356 of BChE (Fig. 2).



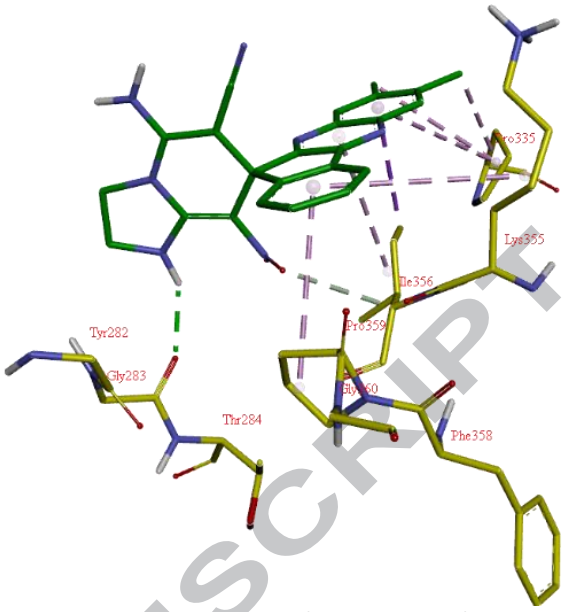


Figure 1- Spatial presentation of docking result for **D9** against acetylcholinesterase.

Figure 2- Spatial presentation of docking result for **D2** against butyrylcholinesterase.

All in all, in this study, twelve original derivatives of spiro-indenoquinoxaline were designed and synthesized successfully in PEG-400 under catalyst-free conditions. The synthetic pathway was green and effective. All the compounds were evaluated both *in-silico* and *in-vitro* for their ability to inhibit AChE and BChE and the results were comparable to those of galantamine as positive control standard.

3. Experimental

All chemicals were purchased from Merck or Fluka Chemical Companies. The ^{1}H NMR (300 MHz) and ^{13}C NMR (75 MHz) were run on a Bruker Avance 300. Nicolet IR-200 FT-NMR spectrometer (δ in ppm) was used to obtain infra-red spectra. Microanalyses were performed on a Leco Analyzer 932. Melting points were recorded on a Büchi B-545 apparatus in open capillary tubes.

3.1. General procedure for the preparation of spiro-indenoquinoxalines

A mixture of ninhydrine (1 mmol) and phenylenediamine derivative (1 mmol) were mixed in PEG-400 (3 mL) at 130 ^{o}C for about 5 min to obtain the desired indenoquinoxaline derivative. Then malononitrile (1 mmol) and keten aminal compound (1 mmol) were added to the reaction mixture and progress of the reaction was monitored by thin layer chromatography. After completion of the reaction, water (15 ml) was added into reaction mixture to precipitate the product, which filtered and washed by hot ethanol (10 mL) and then dried to obtain the pure product.

3.2. Cholinesterase inhibition activity

Ellman kinetic method, modified by Worek et al., was used for evaluation of inhibitory properties of samples against cholinesterase enzymes.[36,37] In this procedure, reduction of

dithiobisnitrobenzoic acid (DTNB) to thionitro benzoate (TNB) by thiocholine is measured. Hydrolysis of acetylthiocholine in presence of acetylcholinesterase or butyrylcholinesterase produces TNB.[38] Galantamine was selected as positive control. DMSO was used to prepare solutions of test samples and galantamine at initial concentration of 1 mg/mL (10 ml). The concentration of DMSO in final reaction mixture was 1 %. No inhibitory effect has been detected for DMSO at this concentration.[39]

Samples were diluted in 0.1 M phosphate buffer (pH: 7.4) and incubated with 10 mM of DTNB and acetyl or butyrylcholinesterase enzyme (25 µl) for 20 minutes at 37 °C prior to addition of acetylthiocholine. The change in the absorbance of DTNB was measured at 436 nm. The AChE activity was calculated using an absorption coefficient of TNB at 436nm (ε=10.6 $mM^{-1}cm^{-1}$). Absorbance of the test samples was corrected by subtracting the absorbance of their respective blank. For samples showing 50% or more inhibition activity against AChE or BChE enzyme at final concentration of 10 µg/mL in 1% of DMSO, a set of five concentrations (0.5, 1.0, 2.0, 4.0 and 8.0 µg/mL) were used to estimate their 50 % inhibitory concentration (IC_{50}). Then, IC_{50} were estimated from interpolating the obtained values for percentage inhibition (P.I).

Percentage of inhibition (P.I) was calculated using the following formula:

$$P.I = \frac{(absorbance\ of\ control - absorbance\ of\ sample)}{absorbance\ of\ control} * 100$$

3.3. Cytotoxicity assay

HepG2 cells were maintained in RPMI 1640 medium. All media were supplemented with newborn calf serum (10%). Cultures were incubated at 37 °C in a humidified atmosphere of 5% CO_2:95% air. All cultures were daily passaged. Assays of cytotoxicity were conducted in 96-well, flat-bottomed microtitre plates. The supplemented culture medium (50 µL) with cells (1 × 105 cells mL−1) was added to the wells. The test compounds were dissolved in DMSO and diluted in the culture medium with final concentrations of 1–1000 µM. Fifty microliters of the prepared solutions were added to each well. The microtitre plates were incubated at 37 °C in a humidified atmosphere of 5%CO_2:95% air for 48 h. All the assays were run in parallel with a 10% DMSO and untreated medium as positive and negative controls, respectively. The evaluation of cytotoxicity was carried out using a modified method of MTT assay. About 10 µL of MTT solution with a concentration of 4 mg mL^{-1} was added into each well. After 2h incubation at 37°C in a humidified atmosphere of 5% CO_2:95% air, a water solution containing 10% w/v of SDS and 50% v:v of DMF was added into each well to lyse the cells and to solubilize the formed formazan complex.[40] After 20 h of incubation, the optical density (OD) value was measured using a microtitre plate reader at 570 nm and the percentages of cell survival were determined. The cytotoxicity was evaluated based on the percentage cell survival in a dose-dependent manner relative to the negative control.

3.4. Molecular docking

Compounds **D1**-**D12** and Galantamine (Figure 3) were constructed and energy-minimized with the Gaussian09 program using DFT method and b3lyp/6-311g basis set.[41] The resulting structures were used for the docking study against AChE (PDB code: 4BDT) and BChE (PDB code: 4TPK) using Autodock Vina software and the results were visualized by Discovery Studio program.[42,43]

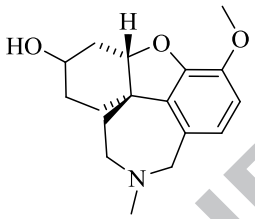


Figure 3- Galantamine

3.5. Spectral data

D1

Yellow powder; mp>290 °C; yield: 80%

IR (KBr): 3425, 3360, and 3190 (NH_2 and NH), 2180 (CN), 1665 (C=C), 1470 (CH_2), 1340 cm^{-1} (NO_2).

^{1}H NMR (300 MHz, $CDCl_3$): δ=3.89 (2 H, t, CH_2NH), 4.13 (2 H, t, CH_2N), 6.61 (2 H, s, NH_2), 7.66 (1 H, t, Ar), 7.76-7.89 (3 H, m, Ar), 8.02-8.19 (2 H, m, Ar), 8.26 (1 H, d, Ar), 8.51 (1 H, d, Ar), 10.28 (1 H, s, NH).

^{13}C NMR (75 MHz, $CDCl_3$): δ=43.8, 44.4, 51.2, 104.8, 110.7, 118.8, 123.4, 127.5, 130.3, 131.4, 132.8, 134.2, 136.8, 139.4, 140.3, 141.7, 142.5, 144.3, 146.5, 149.1, 150.3, 152.5.

Anal. Calcd. for $C_{22}H_{15}N_7O_2$: C, 64.54; H, 3.69; N, 23.95%. Found: C, 64.59; H, 3.64; N, 23.93%.

D2

Cream powder; mp>290 °C; yield: 78%

IR (KBr): 3420, 3357, and 3193 (NH_2 and NH), 2176 (CN), 1662 (C=C), 1467 (CH_2), 1338 cm^{-1} (NO_2).

^{1}H NMR (300 MHz, $CDCl_3$): δ=2.31 (3 H, s, CH_3), 2.35 (3 H, s, CH_3), 3.92 (2 H, t, CH_2NH), 4.11 (2 H, t, CH_2N), 6.69 (2 H, s, NH_2), 7.57-7.61 (1 H, m, Ar), 7.71-7.75 (1 H, m, Ar), 7.86 (1 H, s, Ar), 7.91 (1 H, s, Ar), 8.08 (1 H, d, Ar), 8.31 (1 H, d, Ar), 10.20 (1 H, s, NH).

^{13}C NMR (75 MHz, $CDCl_3$): δ=22.4, 23.1, 43.6, 45.1, 52.3, 108.4, 111.0, 119.4, 124.2, 128.0, 129.6, 131.0, 131.9, 133.8, 136.2, 136.9, 139.1, 141.5, 142.3, 144.6, 148.2, 151.4, 153.1.

Anal. Calcd. for $C_{24}H_{19}N_7O_2$: C, 65.89; H, 4.38; N, 22.41%. Found: C, 65.86; H, 4.36; N, 22.45%.

D3

White powder; mp>290 °C; yield: 80%

IR (KBr): 3422 and 3191 (NH_2 and NH), 2180 (CN), 1660 (C=C), 1469 (CH_2), 1525 and 1341 cm^{-1} (NO_2).

^{1}H NMR (300 MHz, $CDCl_3$): δ= 3.87 (2 H, t, CH_2NH), 4.08 (2 H, t, CH_2N), 6.72 (2 H, s, NH_2), 7.56-7.62 (2 H, m, Ar), 8.00 (1 H, d, Ar), 8.08 (1 H, d, Ar), 8.14 (1 H, d, Ar), 8.21 (1 H, s, Ar), 8.54 (1 H, d, Ar), 10.14 (1 H, s, NH).

^{13}C NMR (75 MHz, $CDCl_3$): δ=43.2, 44.4, 51.6, 109.2, 110.3, 118.8, 124.0, 126.8, 131.0, 131.4, 134.6, 139.3, 141.4, 143.2, 146.7, 149.1, 149.8, 150.2, 151.6, 152.1, 152.9, 154.1.

Anal. Calcd. for $C_{22}H_{14}N_8O_4$: C, 58.15; H, 3.11; N, 24.66%. Found: C, 58.17; H, 3.09; N, 24.69%.

D4

Cream powder; mp>290 °C; yield: 81%

IR (KBr): 3430, 3355, and 3195 (NH_2 and NH), 2175 (CN), 1660 (C=C), 1464 (CH_2), 1337 cm^{-1} (NO_2).

^{1}H NMR (300 MHz, $CDCl_3$): δ=1.98-2.05 (2 H, m, $NCH_2CH_2CH_2NH$), 3.41-3.45 (2 H, m, $NCH_2CH_2CH_2NH$), 3.88-

3.91 (2 H, m, N $CH_2CH_2CH_2$NH), 6.57 (2 H, s, NH_2), 7.58 (1 H, t, Ar), 7.66-7.79 (3 H, m, Ar), 7.81-7.89 (2 H, m, Ar), 8.14 (1 H, d, Ar), 8.34 (1 H, d, Ar), 10.24 (1 H, s, NH).
^{13}C NMR (75 MHz, $CDCl_3$): δ=22.3, 38.2, 42.1, 51.3, 61.4, 107.6, 119.8, 124.2, 128.6, 130.2, 131.7, 133.9, 135.4, 136.6, 139.2, 140.3, 140.9, 141.6, 143.9, 148.4, 150.3, 151.2, 153.0.
Anal. Calcd. for $C_{23}H_{17}N_7O_2$: C, 65.24; H, 4.05; N, 23.16%. Found: C, 65.28; H, 4.03; N, 23.12%.

D5
Yellow powder; mp=254 °C; yield: 76%
IR (KBr): 3422, 3364, and 3184 (NH_2 and NH), 2170 (CN), 1662 (C=C), 1460 (CH_2), 1345 cm^{-1} (NO_2).
^{1}H NMR (300 MHz, $CDCl_3$): δ=2.08-2.14 (2 H, m, $NCH_2CH_2CH_2$NH), 2.39 (3 H, s, CH_3), 2.45 (3 H, s, CH_3),3.37-3.42 (2 H, m, $NCH_2CH_2CH_2$NH), 3.76-3.84 (2 H, m, $NCH_2CH_2CH_2$NH), 6.62 (2 H, s, NH_2), 7.60 (1 H, t, Ar), 7.63-7.68 (1 H, t, Ar), 7.76 (1 H, s, Ar), 7.84 (1 H, s, Ar), 7.92 (1 H, d, Ar), 8.13 (1 H, d, Ar), 10.07 (1 H, s, NH).
^{13}C NMR (75 MHz, $CDCl_3$): δ=20.1, 20.7, 22.6, 41.3, 43.5, 52.1, 62.3, 108.1, 119.2, 124.8, 128.9, 129.4, 130.6, 132.4, 133.1, 135.3, 137.8, 139.1, 140.6, 143.2, 144.9, 149.0, 150.2, 152.6.
Anal. Calcd. for $C_{25}H_{21}N_7O_2$: C, 66.51; H, 4.69; N, 21.72%. Found: C, 66.53; H, 4.64; N, 21.76%.

D6
White powder; mp=268 °C; yield: 81%
IR (KBr): 3433 and 3200 (NH_2 and NH), 2174 (CN), 1664 (C=C), 1463 (CH_2), 1520 and 1347 cm^{-1} (NO_2).
^{1}H NMR (300 MHz, $CDCl_3$): δ=2.12-2.18 (2 H, m, $NCH_2CH_2CH_2$NH), 3.41-3.47 (2 H, m, $NCH_2CH_2CH_2$NH), 3.72-3.80 (2 H, m, $NCH_2CH_2CH_2$NH), 6.47 (2 H, s, NH_2), 7.41-7.47 (2 H, m, Ar), 7.88 (1 H, d, Ar), 8.01 (1 H, d, Ar), 8.14 (1 H, d, Ar), 8.25 (1 H, s, Ar), 8.48 (1 H, d, Ar), 10.06 (1 H, s, NH).
^{13}C NMR (75 MHz, $CDCl_3$): δ=19.2, 38.3, 42.4, 52.9, 63.0, 105.1, 118.8, 123.0, 127.2, 131.0, 131.7, 135.1, 138.7, 140.4, 145.3, 147.9, 149.5, 150.1, 150.7, 151.5, 152.0, 153.6, 154.8.
Anal. Calcd. for $C_{23}H_{16}N_8O_4$: C, 58.97; H, 3.44; N, 23.92%. Found: C, 58.94; H, 3.46; N, 23.93%.

D7
Cream powder; mp>290 °C; yield: 74%
IR (KBr): 3428, 3351, and 3181 (NH_2 and NH), 2174 (CN), 1661 (C=C), 1463 (CH_2), 1336 cm^{-1} (NO_2).
^{1}H NMR (300 MHz, $CDCl_3$): δ=1.05 (3 H, s, Me), 1.12 (3 H, s, Me), 3.38 (2 H, s, CH_2NH), 3.54 (2 H, m, NCH_2), 6.51 (2 H, s, NH_2), 7.62-7.79 (4 H, m, Ar), 7.86-7.91 (2 H, m, Ar), 8.14 (1 H, d, Ar), 8.28 (1 H, d, Ar), 10.18 (1 H, s, NH).
^{13}C NMR (75 MHz, $CDCl_3$): δ=21.9, 23.0, 26.8, 48.3, 51.2, 52.7, 60.8, 107.4, 118.9, 124.1, 126.8, 129.5, 131.0, 131.8, 133.7, 135.9, 138.4, 140.0, 141.5, 141.9, 143.2, 145.8, 149.3, 152.4, 154.7.
Anal. Calcd. for $C_{25}H_{21}N_7O_2$: C, 66.51; H, 4.69; N, 21.72%. Found: C, 66.55; H, 4.64; N, 21.74%.

D8
Cream powder; mp>290 °C; yield: 78%
IR (KBr): 3430, 3371, and 3194 (NH_2 and NH), 2175 (CN), 1659 (C=C), 1458 (CH_2), 1340 cm^{-1} (NO_2).
^{1}H NMR (300 MHz, $CDCl_3$): δ=1.08 (3 H, s, Me), 1.16 (3 H, s, Me), 2.29 (3 H, s, CH_3), 2.37 (3 H, s, CH_3), 3.27 (2 H, s, CH_2NH), 3.44 (2 H, m, NCH_2), 6.44 (2 H, s, NH_2), 7.63 (1 H, t, Ar), 7.68 (1 H, t, Ar), 7.74 (1 H, s, Ar), 7.86 (1 H, s, Ar), 7.90 (1 H, d, Ar), 8.01 (1 H, d, Ar), 10.17 (1 H, s, NH).
^{13}C NMR (75 MHz, $CDCl_3$): δ=20.1, 21.4, 22.5, 23.8, 27.4, 49.2, 52.0, 53.8, 60.7, 107.8, 119.1, 124.5, 127.6, 128.2, 131.1, 133.0, 133.7, 135.3, 137.1, 139.3, 142.1, 142.8, 145.6, 147.8, 149.3, 152.4.
Anal. Calcd. for $C_{27}H_{25}N_7O_2$: C, 67.63; H, 5.25; N, 20.45%. Found: C, 67.61; H, 5.27; N, 20.48%.

D9
Yellow powder; mp>290 °C; yield: 80%
IR (KBr): 3418 and 3198 (NH_2 and NH), 2174 (CN), 1657 (C=C), 1461 (CH_2), 1519 and 1336 cm^{-1} (NO_2).
^{1}H NMR (300 MHz, $CDCl_3$): δ=1.03 (3 H, s, Me), 1.09 (3 H, s, Me), 3.34 (2 H, s, CH_2NH), 3.65 (2 H, m, NCH_2), 6.56 (2 H, s, NH_2), 7.56-7.62 (2 H, m, Ar), 7.82 (1 H, d, Ar), 8.11 (1 H, d, Ar), 8.21 (1 H, d, Ar), 8.29 (1 H, s, Ar), 8.51 (1 H, d, Ar), 10.21 (1 H, s, NH).
^{13}C NMR (75 MHz, $CDCl_3$): δ=21.8, 23.6, 26.7, 46.5, 54.2, 55.0, 62.1, 108.1, 119.6, 122.9, 127.0, 132.4, 133.0, 134.5, 139.2, 140.4, 143.2, 146.5, 147.6, 149.3, 150.1, 150.7, 151.5, 154.0, 156.3.
Anal. Calcd. for $C_{25}H_{20}N_8O_4$: C, 60.48; H, 4.06; N, 22.57%. Found: C, 60.49; H, 4.03; N, 22.54%.

D10
Yellow powder; mp>290 °C; yield: 75%
IR (KBr): 3433, 3370, and 3181 (NH_2 and NH), 2175 (CN), 1670 (C=C), 1469 (CH2), 1338 cm^{-1} (NO_2).
^{1}H NMR (300 MHz, $CDCl_3$): δ=5.33 (2H, s, CH_2), 5.42 (2H, s, CH_2), 6.71 (2 H, s, NH_2), 7.52-7.60 (7 H, m, Ar), 7.66-7.79 (7 H, m, Ar), 7.90-7.95 (2 H, m, Ar), 8.21 (1 H, d, Ar), 8.31 (1 H, d, Ar), 10.05 (1 H, s, NH).
^{13}C NMR (75 MHz, $CDCl_3$): δ=45.3, 47.2, 50.8, 62.1, 108.2, 118.5, 122.9, 126.1, 126.6, 127.0, 127.6, 128.1, 128.7, 129.2, 129.7, 130.1, 130.6, 130.9, 131.1, 131.6, 132.9, 133.8, 134.5, 135.8, 136.4, 140.2, 141.0, 141.6, 143.0, 144.2, 146.6, 149.4, 152.3, 155.1.
Anal. Calcd. for $C_{34}H_{25}N_7O_2$: C, 72.46; H, 4.47; N, 17.40%. Found: C, 72.49; H, 4.44; N, 17.43%.

D11
White powder; mp=264 °C; yield: 74%
IR (KBr): 3422 and 3186 (NH_2 and NH), 2168 (CN), 1662 (C=C), 1460 (CH_2), 1334 cm^{-1} (NO_2).
^{1}H NMR (300 MHz, $CDCl_3$): δ=2.34 (3 H, s, CH_3), 2.41 (3 H, s, CH_3), 5.21 (2H, s, CH_2), 5.29 (2H, s, CH_2), 6.56 (2 H, s, NH_2), 7.55-7.63 (6 H, m, Ar), 7.68-7.79 (6 H, m, Ar), 7.84 (1 H, s, Ar), 7.88 (1 H, s, Ar), 7.94 (1 H, d, Ar), 8.12 (1 H, d, Ar), 10.22 (1 H, s, NH).
^{13}C NMR (75 MHz, $CDCl_3$): δ=20.7, 21.5, 47.1, 48.3, 52.1, 61.8, 107.3, 118.1, 124.6, 125.9, 126.4, 127.0, 127.8, 128.2, 128.9, 129.4, 129.9, 130.0, 130.4, 130.9, 131.3, 131.8, 132.3, 132.9, 133.4, 134.2, 136.0, 136.7, 139.4, 142.0, 142.6, 144.4, 148.3, 150.4, 153.7.
Anal. Calcd. for $C_{36}H_{29}N_7O_2$: C, 73.08; H, 4.94; N, 16.57%. Found: C, 73.03; H, 4.92; N, 16.54%.

D12
Cream powder; mp>290 °C; yield: 79%
IR (KBr): 3430, 3361 and 3198 (NH_2 and NH), 2161 (CN), 1660 (C=C), 1470 (CH_2), 1347 cm^{-1} (NO_2).
^{1}H NMR (300 MHz, $CDCl_3$): δ=5.47 (2H, s, CH_2), 5.56 (2H, s, CH_2), 6.64 (2 H, s, NH_2),7.55-7.63 (6 H, m, Ar), 7.68-7.79 (6 H, m, Ar), 7.85 (1 H, d, Ar), 8.07 (1 H, d, Ar), 8.14 (1 H, d, Ar), 8.19 (1 H, s, Ar), 8.37 (1 H, d, Ar), 10.02 (1 H, s, NH).
^{13}C NMR (75 MHz, $CDCl_3$): δ=47.2, 51.4, 60.8, 107.4, 117.2, 123.1, 126.0, 126.6, 127.0, 127.4, 127.9, 128.6, 129.2, 129.8,

130.0, 130.5, 131.0, 131.4, 131.9, 132.4, 134.1, 134.8, 139.6, 141.0, 144.1, 146.3, 147.2, 149.0, 150.2, 150.6, 151.4, 154.0, 156.2.

Anal. Calcd. for $C_{34}H_{24}N_8O_4$: C, 67.10; H, 3.97; N, 18.41%. Found: C, 67.08; H, 3.95; N, 18.46%.

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