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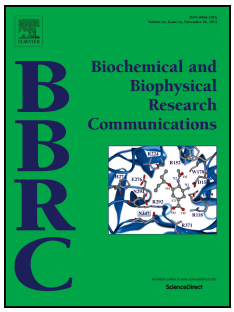

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Graphical Abstract

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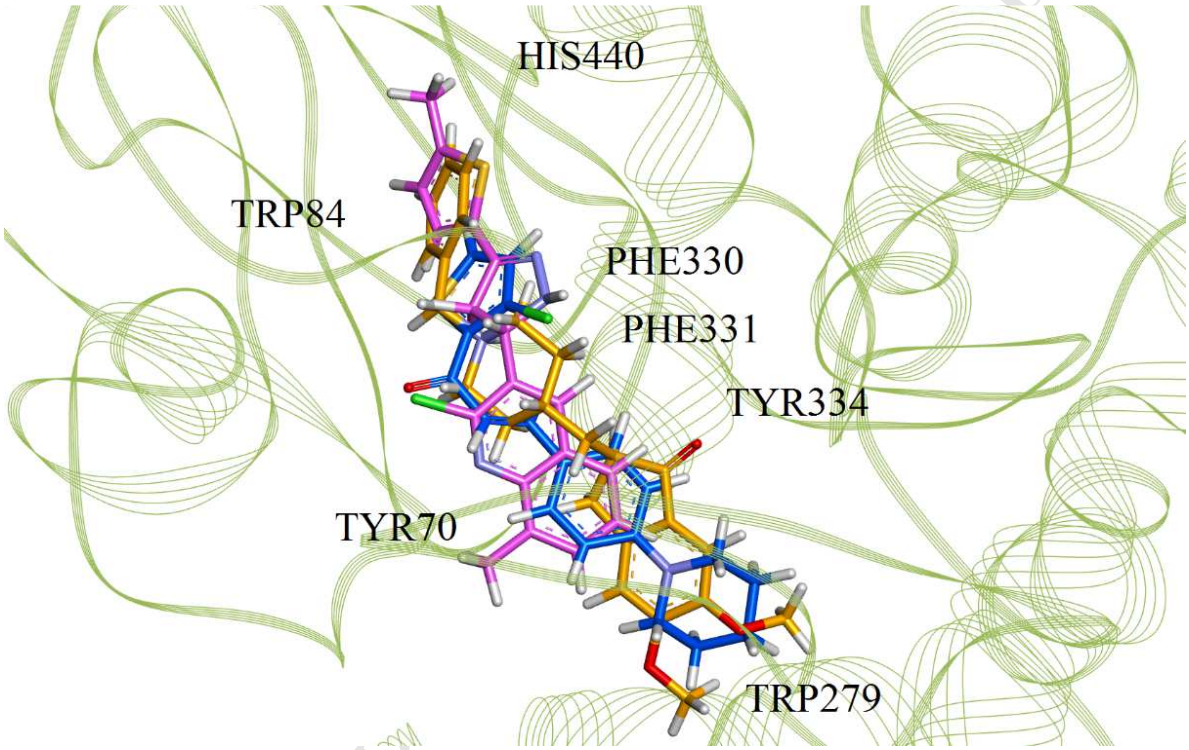

Binding orientation of active compounds and co-crystalized ligand within active site of target enzyme

Cholinesterases Inhibition and Molecular Modeling Studies of Piperidyl-thienyl and 2-pyrazoline Derivatives of Chalcones

Muhammad Shakil Shah[a,b], Shafi Ullah Khan[a], Syeda Abida Ejaz[a], Saifullah Afridi[a], Syed Umar Farooq Rizvi[c], Muhammad Najam-ul-Haq[b], Jamshed Iqbal[a]*

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Abstract

Super-activation of cholinesterases (acetylcholinesterase and butyrylcholinesterase) are linked to various neurological problems most precisely Alzheimer's disease (AD), which leads to senile dementia. Therefore, cholinesterases (AChE & BChE) inhibition are considered as a promising strategy for the treatment of Alzheimer's disease. FDA approved drugs for the treatment of AD, belong to a group of cholinesterase inhibitors. However, none of them is able to combat or completely abrogate the disease progression. Herein, we report a series of newly synthesized chalcone derivatives with anti-AD potential. For this purpose, a series of piperidyl-thienyl and 2-pyrazoline derivatives of chalcones were tested for their cholinesterases (AChE & BChE) inhibitory activity. All compounds were found as selective inhibitor of AChE. In piperidyl chalcones derivatives compound **1e** having IC_{50} of 0.16±0.008 µM and **2m** in 2-pyrazoline chalcones with IC_{50} of 0.13±0.006 µM, were found to be the most potent inhibitors of AChE, exhibiting ≈142 and ≈ 173-fold greater inhibitory potential compared to the reference inhibitor i.e., Neostigmine (IC_{50} ± SEM = 22.2 ± 3.2 µM). Molecular docking studies of most potent inhibitors were carried out to investigate the binding interactions inside the active site. Molecular docking study revealed that potent compounds and co-crystalized ligand had same binding orientation within the active site of target enzyme. Most of these compounds are selective inhibitors of AChE that can be to use against progressive neurodegenerative disorder and age related problems in near future.

Key words: Alzheimer's disease, cholinesterases inhibitors, piperidyl-thienyl derivatives, 2-pyrazoline derivatives of chalcones, molecular docking.

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1. Introduction

Cholinesterases (ChEs) belong to a supper family of esterase/lipase enzyme that catalyze the hydrolysis of a neurotransmitter acetylcholine (ACh) into choline and acetic acid and thus terminate the cholinergic neurotransmission [1]. This catalytic reaction plays an important role to allow a cholinergic neuron to come back into its resting state after activation. Mostly present in cholinergic and non-cholinergic tissues and other body fluids including plasma [2]. On the basis of substrate specificity and inhibitors, two types of cholinesterase co-exist simultaneously throughout the body; acetylcholinesterase and butyrylcholinesterases [3]. Both these forms are highly homologous i.e., > 65% but are products of different genes on the chromosomes 7 and 3 in case of human, respectively [4]. Acetylcholinesterase (AChE; E.C. 3.1.1.7) is a membrane- bound enzyme found in many types of conducting tissues; nerve and muscle, cholinergic and non-cholinergic fibers, central and peripheral tissues, sensory and motor neuron fibers while butyrylcholinesterase (BChE; E.C.3.1.1.8), also called plasma cholinesterase or pseudo- cholinesterase is mainly distributed in the liver, intestine, heart and lungs [5,6]. The main biological role of AChE is the termination of impulse transmission by quick hydrolysis of the cationic neurotransmitter acetylcholine [7] while BChE more favorably catalyze butyrylcholine (BCh) but can also hydrolyzes ACh up to some extent [8,9]. On the basis of cholinergic hypothesis, memory impairment in the patients of Alzheimer's disease (AD) and dementia is due to selective and irreversible insufficiency in the cholinergic functions in the brain [10]. Dementia, rottenly also called senility, is a vast group of brain diseases that often cause long term and gradual decline in thinking ability of the person. It is great enough for affecting a person's daily life functions [11]. Other most common syndrome includes problem with language, emotional problem and a decrease in motivation [12]. Usually, a person's consciousness is not affected in dementia [13]. There are different types of dementia, most common is Alzheimer's disease which is responsible for 50-60 % cases of dementia are noticed in the adults of USA and Europe. Other common types include vascular dementia (25%), Lewy body dementia (15%) and frontotemporal dementia. Less common types include Parkinson's disease, syphilis, normal pressure hydrocephalus and Creutzfeldt-Jakob disease. In DSM-5, dementia was reclassified on the basis of various degree of severity as a neurocognitive disorder[14].

According to WHO report 2001, the number of dementia especially, AD cases in western countries will be doubled by every twenty years and will become tripled in China and India with twenty nine million peoples in 2020, mostly owed to increased human longevity [15]. Even though the unknown morphology of AD, levitation of ACh amount through the inhibition of AChE has been accepted as the most potent treatment scheme against AD [16]. Therefore, AChE and BChE inhibitors have become the curious option in the treatment of AD patients. However, existent drugs (donepezil, tacrin and rivastigmine) having AChE inhibitory are only convincing against the mild type of AD while there is no drug available that shows BChE activity to present, yet [17]. Consequently, a lot of pressure develop on researchers to

discover new drugs in order to conflict dementia and AD. Our group selected a series of chalcones derivatives to test the inhibitory activity of Cholinesterases because chalcones (natural and synthetic) illustrate a lot of biological activities like anti-fungal [18], anti-tuberculosis [19], analgesic [20], anti-oxidant [21], anti-leishmanial [22], anti-malarial [23], anti-viral [24,25], anti-inflammatory and molluscicidal [22], anti-amoebic [26], anti-depressant, anti-convulsant properties [27], and monoamine oxidase inhibitory (MAO) activity [28] etc. Keeping in-mind the aforementioned biological importance of chalcones derivatives, herein we explored a series of chalcones based pyrazoline derivatives as potent cholinesterase inhibitors along with their molecular docking, ADME properties and established SAR.

2. Materials and methods

2.1. Synthesis of chalcones derivatives

A systematic scheme for the synthesis of piperidyl-thienyl chalcones (**1a-1j**) and 2-pyrazoline derivatives of quinolyl thienyl chalcones (**2a-2ab**) has been already published in our previous papers [29,30].

2.2. Materials

All the chemicals and reagents including Electric eel AChE, quine serum BChE, acetythiocholine chloride, butyrylthiocholine chloride, 5,5′-Dithiobis[2-nitrobenzoic acid]] (DTNB) were purchased from Sigma Aldrich (St. Louis, MO, USA) and Merck (Darmstadt, Germany). All chemicals used were of analytical grade. Neostigmine and donepezil were used as standard drugs.

2.3. Cholinesterases (AChE and BChE) assay protocol

The cholinesterases (AChE& BChE) inhibition studies were determined by Ellmann's spectrophotometric method [31] with slight modifications using acetylthiocholine chloride and butyrylthiocholine chloride as substrates for AChE & BChE, respectively. Total reaction mixture was 100 µL that contain 60 µL phosphate buffer (50 mM, pH 7.7), 10 µL test compound (1% DMSO, final conc. of compound 0.1 mM well^{-1}) and 10 µL of AChE (0.015U/well, E.C.3.1.1.7, from electric eel) or 10 µL of BChE (0.01U/well, E.C.3.1.1.8, from equine serum). The contents of each well was mixed thoroughly followed by incubation at 37 $^{\circ}$C for 10 min and their absorbance was recorded at 405 nm as optical density. Then, 10 µL substrate (0.5 mM acetylthiocholine chloride) for AChE inhibition assay or (0.5 mM butyrylthiocholine chloride) for BChE inhibition assay was added followed by addition of 10 µL DTNB (0.5 mM well^{-1}). Then the mixture was further incubated at 37 $^{\circ}$C for 20 min. Finally, absorbance at 405 nm was recorded using 96-well plate reader (BioTek ELx800, Instruments, Inc. USA). All experiments were performed in triplicate with their respective control. Neostigmine (0.1 mM well^{-1}) was used as positive control. Percent inhibition was calculated by using the following formula

$$\% \text{ inhibition} = 100-(A_t/A_c)\ x100$$

Where "A_t" and "A_c" are absorbance obtained for respective enzyme (AChE & BChE) in the presence and absence of the inhibitors, after subtracting the respective background (pre read absorbance)

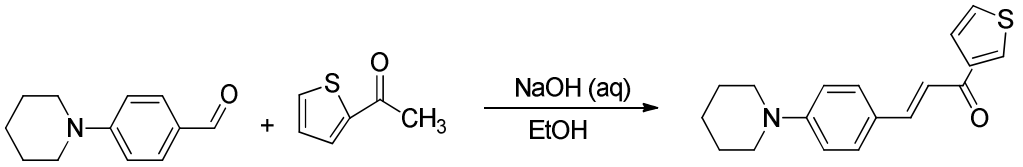
2.4. Molecular docking studies

Molecular docking studies of most potent compounds in both series were carried out against AChE using MOE [32]. Prior to docking of potent compounds inside the target enzyme, structure were drawn and protonated in the molecules sketcher tool of MOE. The required protonated 3D structures of these compounds were obtained using the three-dimensional tool of MOE. Subsequently, the energy minimization of generated molecules was carried out using the MMFF94x force field with the adjustment of hydrogen. Finally, the created database was used as input file for docking studies in MOE. For docking purpose X-ray structure of AChE (PDB ID 1EVE) was selected as template and downloaded from RSC Protein Data Bank [33]. Prior to docking process protonation of target structure was accomplished using MOE protonate 3D tools which was followed by energy minimization up to 0.05 Gradient using Amber99 force field. Prior to molecular docking, active site of receptor was selected around the co-crystallized ligands. Then the required ligands were docked into the active site of protein using Triangular Matching docking method and 30 conformations of each Ligand protein complex were generated with docking score. Each complex was analyzed for interactions and their respective 3D pose was visualized using discovery studio visualizer v4 [34]. Binding free energies were determined and tabulated in table 3. Those poses having lowest free binding energy values were considered as the most stable-one and selected for visualization of binding interactions with the target enzyme.

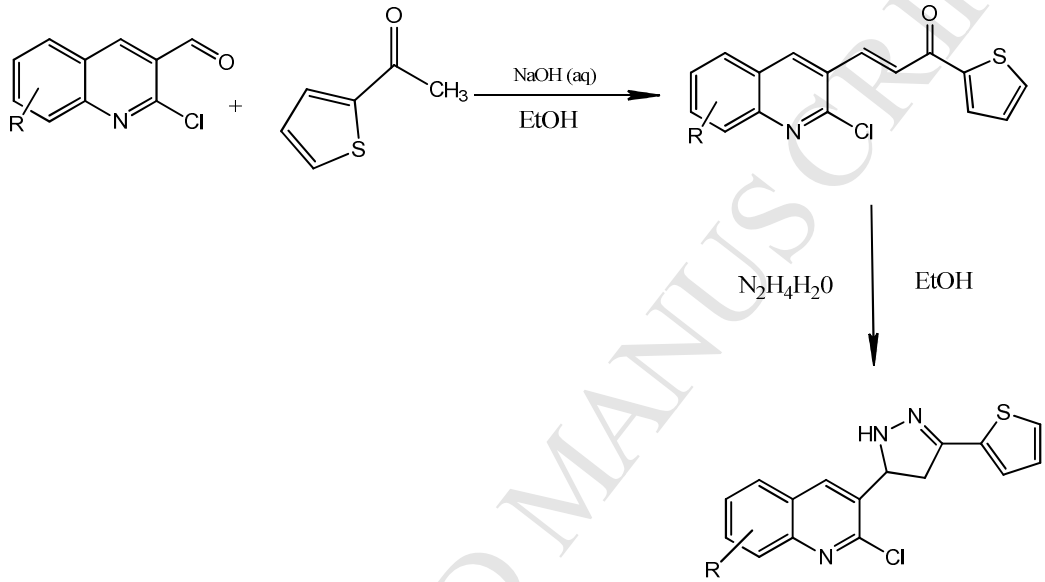
3. Results and discussions

3.1. Chemistry

4-(Piperidin-l-yl) benzaldehyde was prepared by N-arylation of piperidine with 4-flurobenzaldehyde in the presence of cetyltrimethylammonium bromide (CTAB) as catalyst. It was then condensed with wide range of different substituted acetylthiophenenes and acetylfurans for getting respective chalcones, scheme A [30]. Another Chalcones based quinolone series was prepared by reacting it with substituted 2-chloro-3-formylquinolines and was further reacted with hydrazine hydrate to get 2-pyrazoline derivatives, scheme B [29]. All these newly synthesized compounds were characterized by different analytical techniques. The relevant spectroscopic data and physicochemical properties of all these compounds (**1a-1j** and **2a-2ab**) have been already reported previously [29,30]



Scheme A. systematic layout for the synthesis of piperidyl chalcones [29]



Scheme B. systematic layout for the synthesis of 2-pyrazoline derivatives of chalcones [30]

3.2. In vitro cholinesterases inhibition studies

A series of piperdyl-thienyl and 2-pyrazoline derivatives of quinolyl-thienyl chalcones were screened against cholinesterases (AChE and BChE). For both enzymes, all compounds were initially tested at 100 µM concentration and their % inhibition was calculated. Compounds having > 50% inhibition were further diluted and tested at eight different concentrations to create dose response curves from which their IC_{50} values were calculated. Neostigmine and donepezil were used as standard inhibitors for both enzymes (Table 1-2).

3.2.1. Structure- activity relationships (SAR)

Screening of piperidyl-thienyl chalcones derivatives **(1a-1j)** against cholinesterases (AChE & BChE) revealed that all these compounds exhibited good inhibitory potential against acetylcholinesterase while exhibited relatively less inhibitory potential against butyrylcholinesterase enzyme (Table 1). Experimental results showed that compounds with no halogen or methyl group attached to thiophene-2-yl or thiophene-3-yl, **(1a**&**1b)** showed relatively low activity against AChE inhibition. While addition of halogen/methyl

group at thiophene ring increased the inhibition potency up to several folds. However, when tested on AChE, compounds having 3-chlorothiophen-2-yl, **(1e)** and 3-bromothiophen-2-yl **(1h)** moiety at piperidene chalcones, were found to be the most potent inhibitors having IC_{50} values 0.16±0.008 µM and 0.19±0.009 µM, respectively. These compounds exhibited up to 142 and 120 fold higher inhibitory potential as compared to the reference inhibitor, Neostigmine (IC_{50} ± SEM= 22.2± 3.2 µM). Higher inhibitory potency of **1e** and **1h** might be due to the presence of electron withdrawing group, chlorine and bromine, respectively at 3-position of thiophene ring. On the other side, substituting the same group at 5-position decreased their inhibitory potency **(1f** & **1i)**. Di-substitution in thiophine ring **(1g)** with chlorine at position 3 and 5 exhibited no significant change in inhibitory activity due to their cancellation effects while, inhibition potential seemed to be increased when methyl group was attached at 4-position in thiophene ring **(1c)** as compared to the attachment of methyl group at 5-position **(1d)**. Against BChE, compound having 5-iodiothiophen-2-yl moiety at piperidene chalcones **(1j),** was found to be the most potent inhibitor with 46.9% inhibition. It may be due to the presence of iodo-group.

Similar to piperidyl-thienyl derivatives, screening of 2-pyrazoline derivatives of chalcone compounds **(2a-2ab)** for the inhibition of AChE & BChE revealed, that all these compounds showed promising inhibitory potential against acetylcholinesterase. The behavior of the 2-pyrazoline derivatives for different groups attached at thiophene ring almost remained same as it was seen in piperidyl-thienyl derivatives. Out of all investigated compounds, compound **2m**, **2y** and **2w** exhibited most potent inhibitory potential against AChE with an IC_{50} of 0.13±0.006, 0.15±0.008 and 0.20±0.009 µM, respectively. It has been noticed that by changing position of different substituents at quinolone ring also potentially effect the inhibitory activities. From comparative analysis of inhibitory potential of compounds **2h**, **2p** and **2n**, revealed that compound **2p** exhibited high inhibitory activity while compound **2n** showed less inhibitory activities. All of the three compounds have 5-Cl at thiophene ring while **2h** contained 6-methyl, **2p** had 8-methyl and **2n** had 7-methyl, respectively at 2-pyrazoline ring. All the 2-pyrazoline derivatives showed relatively less (<50%) inhibitory potential against butyrylcholinesterase (Table 2).

3.2.2. Kinetic study

Kinetic studies of potent compounds **1e** and **2m** were performed to investigate the mechanism of inhibition. Based on the obtained data, compound **1e** inhibit the enzyme competitively (Fig. 1), while compound **2m** showed mix type of inhibition (Fig. 2). Briefly, the initial velocities of the reactions were measured at different concentrations of inhibitors (0.05-0.2 µM) and substrate acetylthiocholine chloride concentrations (0.25-1.5 mM). A double reciprocal plot of the inhibition kinetics of AChE by inhibitors **1e** was measured by using PRISM 5.0 (Graphpad, San Diego, California,USA). Results showed that value of V_{max} remained almost same in the presence or absence of inhibitor, represent a competitive type of inhibitory mechanism. Similarly, Linweaver-Burk double reciprocal plot for **2m** was also generated (Fig.

2). The interception of the lines in the Lineweaver-Burk plot above the x-axis with both increased slope and intercepts at increasing concentrations of the inhibitor proved a mixed type of inhibition. Compound **2k** might be capable to interact with both the catalytic active site (CAS) and peripheral anionic site (PAS) of acetylcholinesterase.

3.3. Molecular docking

Molecular docking studies of all tested compounds were carried out to calculate the free binding energy using MOE (Tab. 3). To identify the plausible binding modes, detailed molecular docking analysis of potent compounds in both series against AChE were also performed. The binding orientation of potent compounds **1e, 2m** and co-crystal ligand within active site of target enzyme is shown in (fig.3). The putative binding mode of **1e** (most potent inhibitor in piperidyl chalcone series) and **2m** (most potent inhibitor in 2-pyrazoline chalcone series) within active site of AChE (fig.4). Analyzing the key interactions of both **1e** and **2m** in active site of AChE revealed that both ligands were surrounded by aromatic ring containing amino acid residue that was Tyr70, Asp72, Trp84, Tyr279, Phe330, Try334 and His440 (Fig. 4). Further analysis of docking results showed that both ligands are stacked well in the grove between Trp279 and Trp84 amino acid residues. Both potent compounds had orientation along the active-site gorge just like reference compound donepezil, extending from active site amino acid residue Trp84, to the peripheral site amino acid residue Trp279 (Fig. 3). Piperidine ring in compound **1e** formed only one π–π stacking interaction with the six-membered ring of the amino acid residue Phe330, contrary to the interaction of compound **2m** in which 2,3-dihydropyridine moiety formed two π–π stacking interaction with Trp84 and Phe330. Benzene ring adjacent to piperidine ring in compound **1e** formed two π–π T-shaped interaction with amino acid residue Tyr121 and Phe331. Carbonyl group adjacent to pyrazole ring in compound **1e** formed two hydrogen bond with Phe288 and Arg289, while NH group of pyrazole ring in **2m** formed hydrogen bond with Asp72. Thiophene ring in both potent compounds formed π–π interaction with Trp29.

3.3.1. ADME profile of piperidyl-thienyl and 2-pyrazoline derivatives of chalcones

To evaluate the drug like properties of all investigated molecules, a series of computational filters, including filters for clogP and predicted solubility were used to select the right compounds from their library. In general, compounds adhered to Lipinski's rules 5 (i.e., molecular mass <500, H-bond donors <5, H-bond acceptors <10, and logP < 5) were proceed for their catalytic potentials. *In-silico* evaluation of ADME profile for all these compounds were done along with calculation of free binding energies and other different ADME properties. The targeted properties, i.e., logP(o/w) (octanol-water partition coefficient), HBDH (number of hydrogen bond donor atom), HBAH, (number of hydrogen bond acceptor atom and TPSA (topological polar surface area) were successfully evaluated (Table 3). Among all these 38 tested compounds only 9 compounds **1a, 1b, 1c, 1d, 2a, 2b, 2i, 2l** and **2u** has logP < 5, which is a

measure of lipophilicity, while all the remaining compounds have logP > 5. Similarly, other ADME properties of all tested compounds are rightly in an agreement with the Lipinski's rules 5. On the other side most important aspect of these compounds are TPSA values which often used as a model for assessment of ability of molecules to cross the blood–brain (BBB) was < 40, indicating its potential to reach brain quickly, bypassing the BBB cut off filter TPSA < 60.

Conflict of interest

The authors confirm that this article content has no conflict of interest.

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[34] D. Studio, I. Insight, Accelrys Software Inc, San Diego, CA 92121 (2009).

Figures Legends

Fig.1. Double reciprocal plot of the inhibition kinetics of AChE by compound **1e**, indication of competitive inhibition.

Fig.2. Double reciprocal plot of the inhibition kinetics of AChE by compound **2m**, indication of mixed type inhibition (competitive & noncompetitive).

Fig. 3. Binding orientation of compound **1e**, **2m** and **co-crystalized** ligand within active site of target enzyme

Fig. 4. Putative binding mode of compound **1e** (left sided in blue colored) and **2m** compound (most potent inhibitor right side, pink colored) in active site of AChE (green colored)

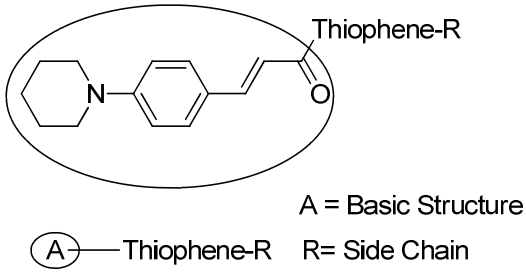
Tables Legends

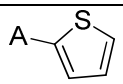
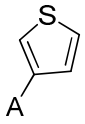
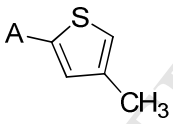
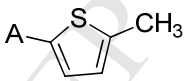
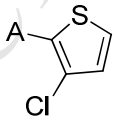
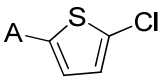
Table1. Cholinesterases (AChE & BChE) inhibitory activity of piperidyl- thienyl chalcones

Table 2. Cholinesterases (AChE & BChE) inhibitory activity of 2-pyrazoline derivatives of chalcones

Table 3. Free binding energy and ADME profile of all tested compounds

Table 1.



Compound Code	A-- Thiophene-R	Acetylcholinesterase IC_{50} ± SEM (µM)	Butyrylcholinesterase % inhibition
1a		2.92 ±0.12	25.6
1b		2.20 ±0.10	38.5
1c		1.29±0.06	29.4
1d		1.59±0.07	15.0
1e		0.16±0.008	39.4
1f		1.70±0.07	43.7

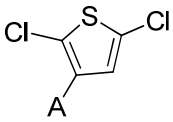
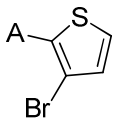
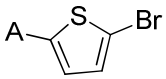
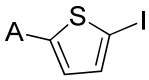
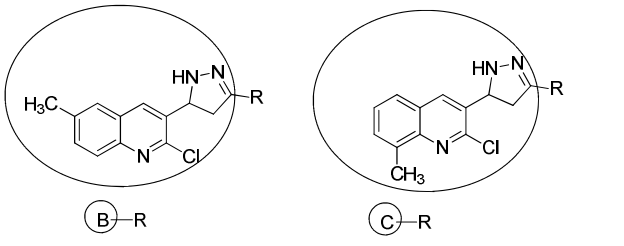
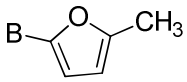
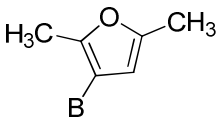
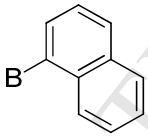
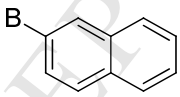
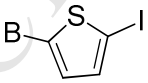
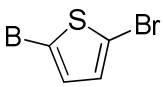
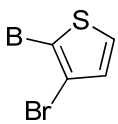
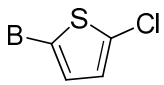
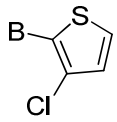
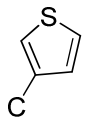
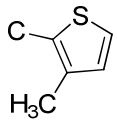
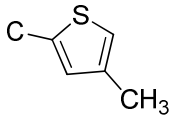
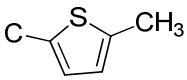
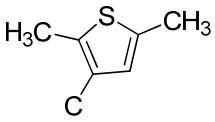
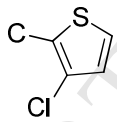
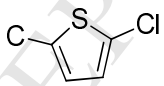
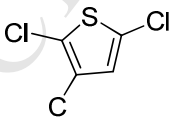
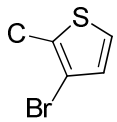
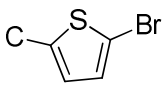
1g		0.26±0.01	19.0
1h		0.19±0.009	30.9
1i		0.40±0.02	19.7
1j		1.31±0.06	46.9

Table 2.



B, C, D = Basic Structure R= Side Chain

Compound Code	B---R C---R D---R	Acetylcholinesterase IC_{50} ± SEM (µM)	Butyrylcholinesterase % inhibition
2a		2.79±0.12	20.3
2b		0.86±0.04	41.1
2c		0.67±0.05	29.4
2d		0.79±0.05	25.0
2e		0.95±0.05	39.1
2f		1.20±0.06	26.7
2g		0.48±0.02	29.0

2h		0.75 ±0.04	33.4
2i		0.99±0.05	39.7
2j		0.96±0.05	21.9
2k		1.01 ±0.05	25.5
2l		1.50±0.07	28.5
2m		0.13±0.006	29.4
2n		0.94±0.04	25.0
2o		1.60±0.06	39.4
2p		0.29 ±0.02	32.7
2q		1.50±0.06	29.0
2r		2.80±0.13	37.9
2s		2.40±0.12	29.7

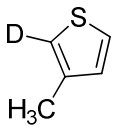
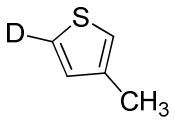
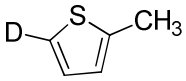
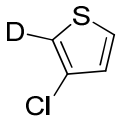
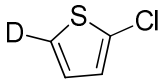
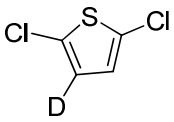
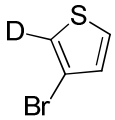
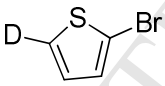
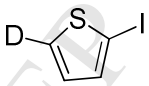
2t		1.20±0.12	41.4
2u		0.34±0.02	20.4
2v		3.80±0.18	25.0
2w		0.20±0.009	29.4
2x		2.80±0.13	40.7
2y		0.15±0.008	28.9
2z		3.82±0.18	40.9
2aa		0.40±0.03	29.7
2ab		0.90±0.06	42.9
Positive control	**Neostigmine**	22.2 ± 3.2	-

Table 3.

Compound Code	Free binding Energy	logP	M. wt	HBAH	HBDH	TPSA
1a	-12.7258	4.484	297.422	1	0	20.31
1b	-12.8723	4.484	297.422	1	0	20.31
1c	-12.2148	4.818	311.449	1	0	20.31
1d	-12.5155	4.661	311.449	1	0	20.31
1e	-13.2676	5.074	331.867	1	0	20.31
1f	-12.3297	5.288	331.867	1	0	20.31
1g	-13.0677	6.248	366.312	1	0	20.31
1h	-13.2028	5.280	376.318	1	0	20.31
1i	-12.2910	5.494	376.318	1	0	20.31
1j	-11.9044	5.886	423.318	1	0	20.31
2a	-12.6916	4.183	325.799	2	1	50.42
2b	-13.6930	4.516	339.826	2	1	50.42
2c	-14.6531	6.511	371.871	2	1	37.28
2d	-12.4782	6.223	453.735	2	1	37.28
2e	-13.6872	5.831	406.735	2	1	37.28
2f	-13.6625	5.617	406.735	2	1	37.28
2g	-13.7014	5.625	362.284	2	1	37.28
2h	-13.5584	5.411	362.284	2	1	37.28
2i	-13.4378	4.902	327.839	2	1	37.28
2j	-12.6852	5.078	341.866	2	1	37.28
2k	-12.4904	5.116	341.866	2	1	37.28
2l	-12.9312	4.959	341.866	2	1	37.28
2m	-13.7514	5.292	355.893	2	1	37.28
2n	-13.7103	5.372	362.284	2	1	37.28
2o	-12.5622	5.586	362.284	2	1	37.28
2p	-13.5187	6.546	396.729	2	1	37.28
2q	-13.4250	5.578	406.735	2	1	37.28
2r	-13.5699	5.792	406.735	2	1	37.28
2s	-13.1794	5.117	341.866	2	1	37.28
2t	-13.3148	5.155	341.866	2	1	37.28
2u	-13.7570	4.998	341.866	2	1	37.28
2v	-13.2814	5.411	362.284	2	1	37.28
2w	-14.6108	5.625	362.284	2	1	37.28
2x	-13.1510	6.585	396.729	2	1	37.28
2y	-14.6335	5.155	341.866	2	1	37.28
2z	-13.7570	5.831	406.735	2	1	37.28
2aa	-13.4612	6.223	453.735	2	1	37.28
2ab	-13.1807	1.311	223.296	1	0	29.54

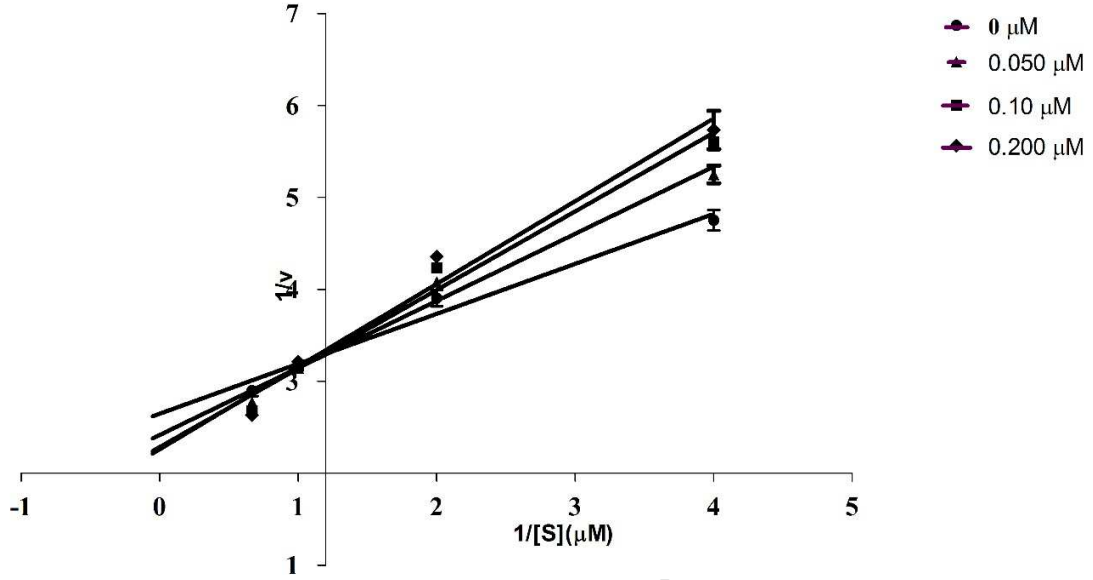

7
6
5
4
3
1
1/v
-1
0
1
2
3
4
5
1/[S](μM)
0 μM
0.050 μM
0.10 μM
0.200 μM

Fig. 1.

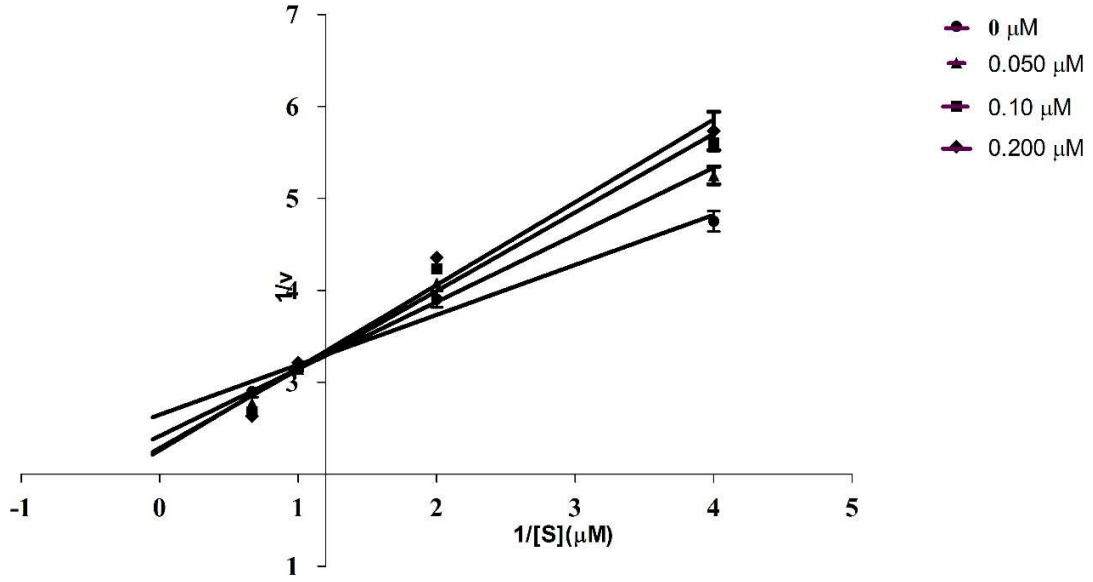

7
6
5
4
3
1
1/v
-1
0
1
2
3
4
5
1/[S](μM)
0 μM
0.050 μM
0.10 μM
0.200 μM

Fig. 2.

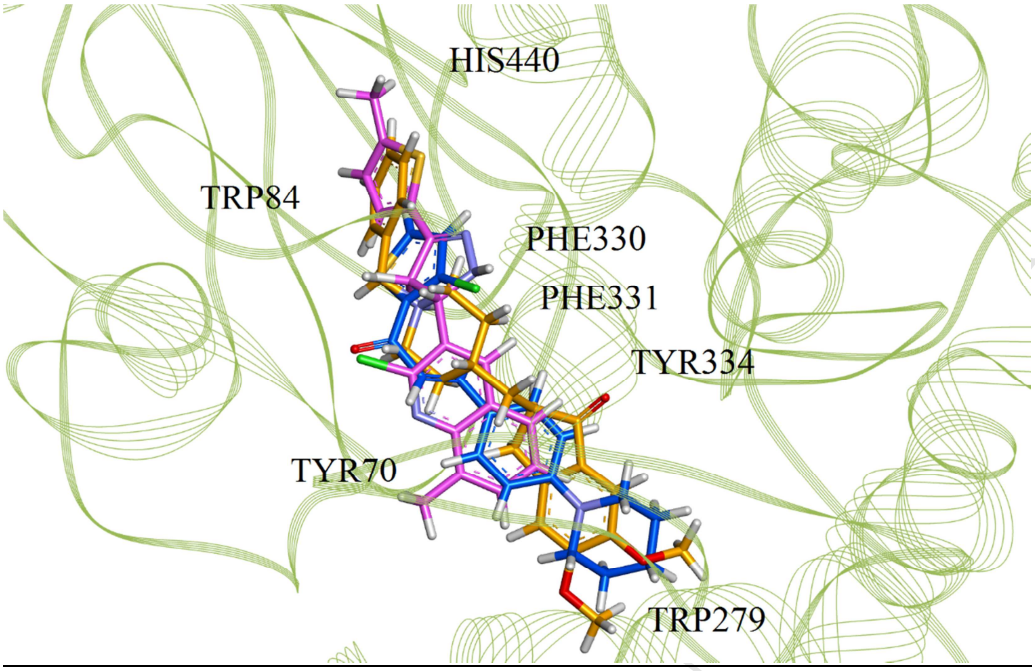

HIS440
TRP84
PHE330
PHE331
TYR334
TYR70
TRP279

Fig. 3.

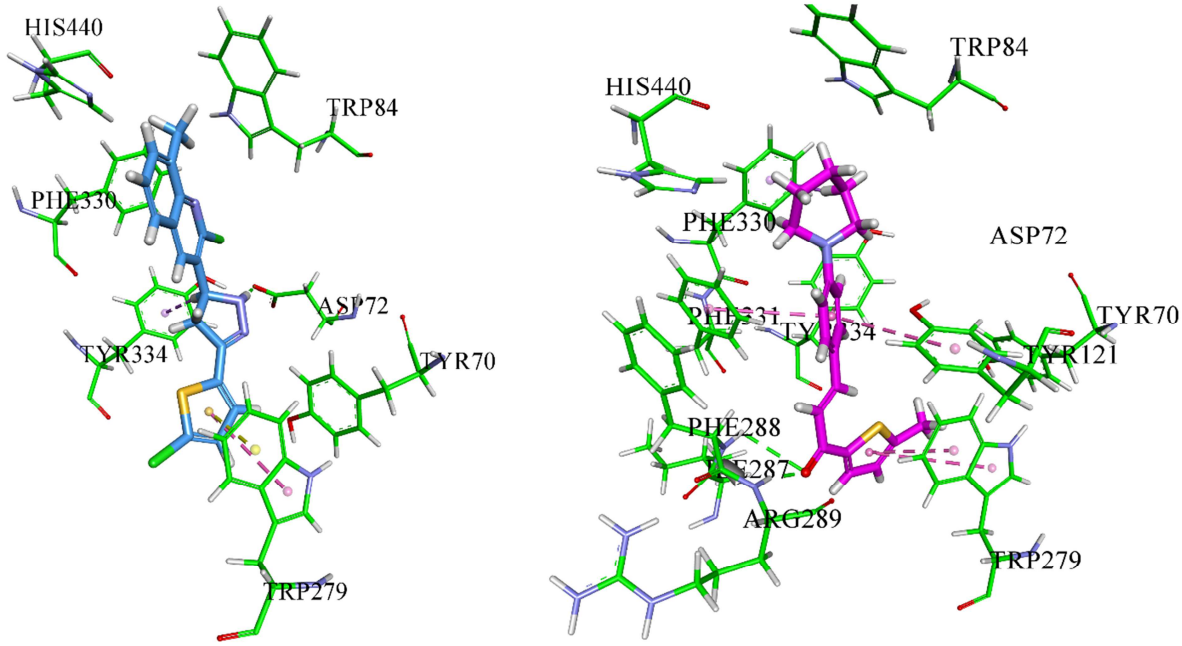

HIS440
TRP84
PHE330
ASP72
TYR334
TYR70
TRP279
TRP84
HIS440
PHE330
ASP72
TYR70
TYR121
PHE288
ARG289
TRP279

Fig. 4.