

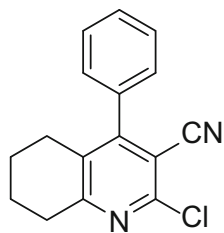
# Synthesis and biological evaluation of some tacrine analogs: study of the effect of the chloro substituent on the acetylcholinesterase inhibitory activity

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**Abstract** New tetrahydroquinoline derivatives were synthesized, characterized, and evaluated for their in vitro and in vivo acetylcholinesterase inhibitory activity as well as hepatotoxicity using tacrine as a reference standard. The obtained results revealed that most of the compounds comprising a chloro substituent displayed higher activity when compared with the other analogs. Among the newly synthesized compounds, four analogs displayed in vitro and in vivo acetylcholinesterase inhibitory activity comparable to or slightly higher than tacrine. Among these compounds, the 2-chlorotetrahydroquinoline derivative emerged with hepatotoxicity results comparable to saline.

*Graphical abstract*



**Keywords** Alzheimer's disease · Acetylcholinesterase inhibitory activity · Tacrine · Chlorinated analogs · Bioorganic synthesis

## Introduction

Alzheimer's disease (AD) is a progressive and neurodegenerative disorder which currently represents one of the biggest threats for the human kind [1–4]. The disease is characterized by progressive memory impairment related to a disordered cognitive function [5, 6]. Although the etiology of AD is still poorly understood, several factors are thought to play significant roles in the pathology of the disease such as accumulation of numerous amyloid plaques of  $\beta$ -amyloid peptide [7], neurofibrillary tangles, dramatic loss of synapses, shrinkage and death of neurons [8] in addition to inflammatory changes marked by accumulation of activated microglia [9]. Scientists have proposed several theories explaining the mechanism of AD development [10–14]. The oldest of the AD hypothesis is the cholinergic hypothesis [10, 11, 15] which has become the leading strategy for the development of cholinesterase inhibitors (ChEIs) aimed to increasing levels of acetylcholine (ACh) through inhibition of cholinesterases (ChEs) [16, 17]. Tacrine (THA, 9-amino-1,2,3,4-tetrahydroacridine) (**I**, Fig. 1) [18] was the first drug approved by the FDA for the treatment of cognitive impairment in AD [3, 19]. The acetylcholinesterase inhibitory (AChEI) activity of tacrine was thought to be mediated by its binding to a hydrophobic area close to the active site [20, 21].

Structure–activity relationship (SAR) studies of aminoacridine-related derivatives indicated that 4-aminopyridine (**II**, Fig. 1) and 4-aminoquinoline (**III**, Fig. 1) displayed a very weak AChEI activity, although their basicities were

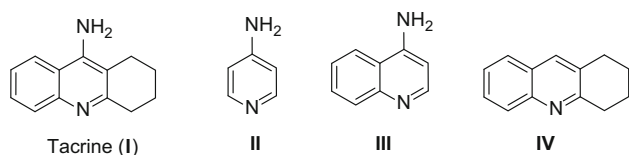
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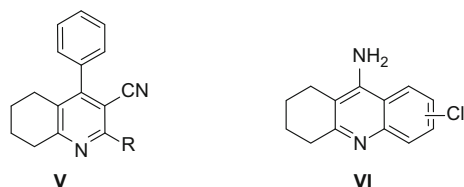
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**Fig. 1** Chemical structures of tacrine (I), 4-aminopyridine (II), 4-aminoquinoline (III), and tetrahydroacridine (IV)



**Fig. 2** Chemical structures of previously synthesized 3-cyano-2-substituted tetrahydroquinoline derivatives (V) and chlorotacrines (VI)

almost equal to that of tacrine [22]. On the other hand, the tetrahydroacridine derivative (IV, Fig. 1) was found to be almost as active as tacrine despite being much weaker base [23]. These findings indicated that the cyclohexyl ring is important for activity [22]. Confirmation was reached by examination of the X-ray crystal structure which revealed that the 4-aminoquinoline portion of the tacrine molecule was responsible for binding of the drug to AChE, while the cyclohexyl ring acted mostly as a block to the substrate at the active site [23]. However, tacrine was far from ideal due to its low bioavailability, short half-life and significant hepatotoxicity [24, 25]. Accordingly, several structural modifications were conducted on tacrine with the aim of producing potent compounds that are more selective for AChE inhibition and with minimum toxicity (if any), to provide clinically advantageous drugs [26]. In a trial to fulfill this target, a series of 3-cyano-2-substituted tetrahydroquinolines (V, Fig. 2) was designed and synthesized in our laboratory and their biological testing indicated their high AChEI activity [27]. SAR studies of these derivatives indicated that presence of a chloro group at the 2-position of the pyridyl ring (compound V; R = Cl) resulted in the most potent compound of the whole series [27]. Literature survey on other chlorinated tacrine analogs indicated that introduction of a chloro group to the phenyl ring of tacrine (VI, Fig. 2), resulted in potent AChEIs [28].

Correlating the results of both studies, it was clear that presence of a chloro group in these compounds, whether on the pyridyl or on the phenyl moieties had a positive influence on the AChEI activity. This was explained by the recent discovery that better binding of halogenated compounds to receptors was attributed to their ability to form halogen bonding with those receptors [29].

Based on the above-mentioned findings, we deemed it interesting to synthesize a new set of 3-cyano-2-substituted tetrahydroquinolines V in order to investigate whether the introduction of a chloro group to the phenyl ring at position 4 of the tetrahydroquinoline skeleton would have the same positive influence on activity as in case of chlorotacrines VI. This was accomplished by triggering the synthesis of two series of 3-cyano-2-substituted tetrahydroquinolines V; one comprising a 4-chloro group on the phenyl ring and the other lacking it. The synthesized compounds were evaluated for their acetylcholinesterase inhibitory activity and hepatotoxicity hoping to discover more potent and less hepatotoxic tacrine analogs.

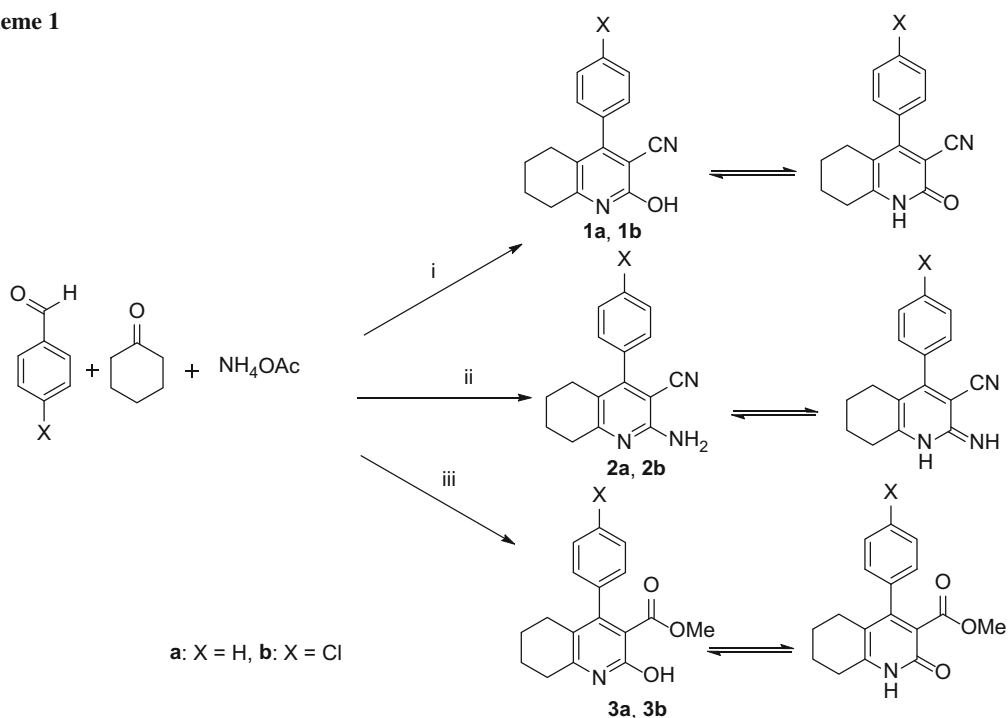
## Results and discussion

### Chemistry

The proposed synthetic routes adopted to obtain the target compounds are illustrated in Schemes 1 and 2. In Scheme 1, the 4-aryl-3-cyano-2-hydroxy-5,6,7,8-tetrahydroquinolines 1 were synthesized by multi-component one-pot reaction of cyclohexanone, the appropriate aldehyde and ethyl cyanoacetate in boiling ethanol in presence of excess ammonium acetate [30–32]. The IR spectra of these compounds revealed absorption bands characteristic for the cyano group in addition to absorption bands characteristic for OH, NH, and C=O functions, indicating existence of the products in a keto-enol tautomerism. This was further confirmed by investigation of the  $^1\text{H}$  NMR spectra which showed two  $\text{D}_2\text{O}$  exchangeable singlets, one integrated for 1/4 H corresponding to the NH and the other integrated for 3/4 H attributed to the OH. Synthesis of the 2-amino analog 2 was achieved by heating malononitrile in dry benzene utilizing a Dean-Stark head for water separation [33–35]. On the other hand, trials to synthesize the methyl carboxylate derivatives 3 according to a reported procedure described for the synthesis of analogous compounds [36] where the reaction was performed in presence of catalytic amount of zinc oxide using water as a solvent failed to give the required products. However, utilizing a procedure similar to that used for preparation of compounds 1 resulted in the corresponding methyl 4-aryl-2-hydroxy-5,6,7,8-tetrahydroquinoline-3-carboxylates 3. IR and  $^1\text{H}$  NMR spectra for these compounds again indicated their existence as keto-enol tautomers, in addition they revealed signals characteristic for the ester group.

In Scheme 2, chlorination of the 2-hydroxy derivatives 1 was readily achieved through reflux in a mixture of  $\text{POCl}_3/\text{PCl}_5$  adopting reaction conditions reported for synthesis of allied compounds [27]. The IR spectra of the chloro derivatives 4 showed the CN band at 2223 and 2230  $\text{cm}^{-1}$

Scheme 1



**Reagents and reaction conditions:** (i) ethyl cyanoacetate, ethanol, reflux; (ii) malononitrile, benzene, reflux; (iii) dimethyl malonate, ethanol, reflux

but lacked the OH, NH, and C = O absorption bands, whereas, the  $^1\text{H}$  NMR spectra displayed signals for the eight protons of the cyclohexyl ring in addition to the phenyl protons at their expected chemical shifts. Treatment of the chloro derivatives **4** with excess hydrazine hydrate 98 % in boiling ethanol yielded the corresponding 3-amino-tetrahydropyrazolo[3,4-*b*]quinolines **5**. The reaction smoothly proceeded forming at first a clear solution which gradually separated the product as a heavy yellow solid. Complete deposition of the product was achieved by leaving the reaction mixture for 24 h at room temperature. The reaction involved nucleophilic replacement of the chloro group by the hydrazine moiety giving the unstable intermediate 3-cyano-2-hydrazino-4-phenyl-5,6,7,8-tetrahydroquinolines, which underwent internal cycloaddition to form **5** (Fig. 3). The IR spectra proved this proposed mechanism since it lacked the absorption bands of the CN group, whereas the  $^1\text{H}$  NMR spectra provided further confirmation of the assigned structures.

As for the synthesis of derivatives **6–10**, a general nucleophilic substitution reaction was employed in which the chloro derivatives **4** were reacted with the appropriate amine or phenyl hydrazine in boiling dioxane for 20 h. The IR spectra of these compounds retained the absorption bands corresponding to the CN group, whereas the  $^1\text{H}$

NMR spectra showed additional signals for the newly introduced alkylamino or phenylhydrazino moieties.

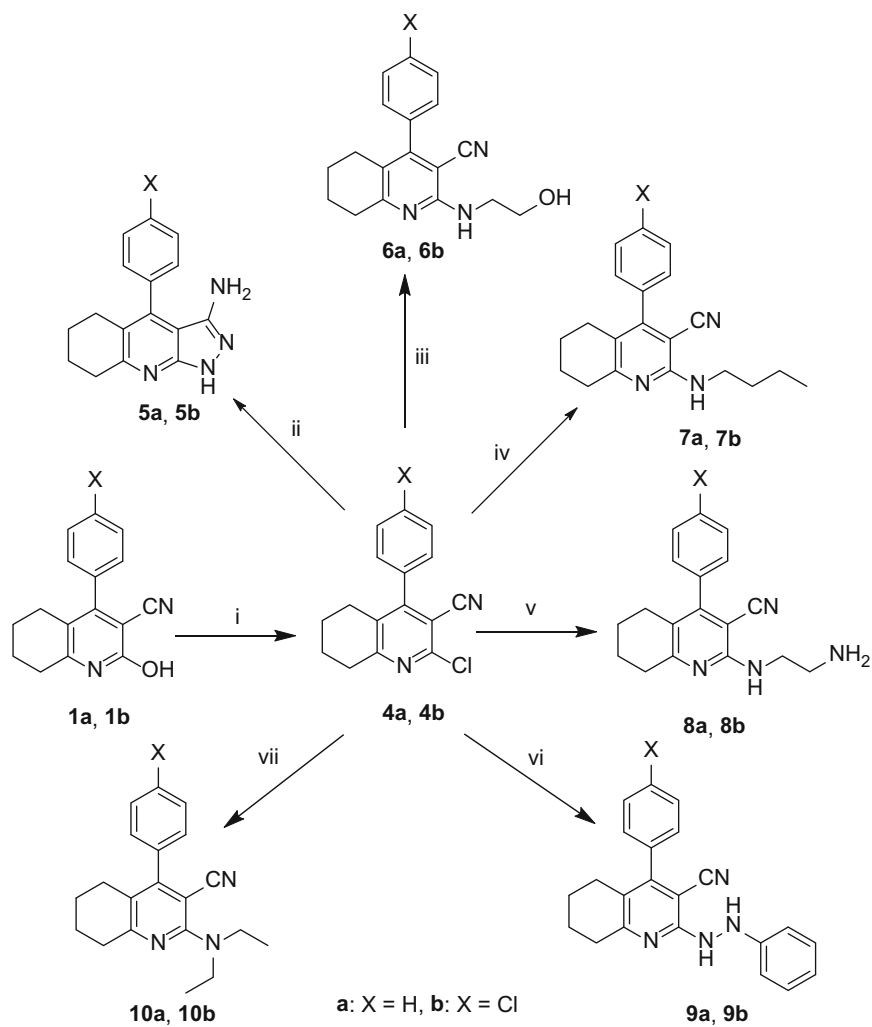
### Acetylcholinesterase inhibitory activity

The synthesized compounds were evaluated for their acetylcholinesterase inhibitory effects using in vitro pharmacological experiments. Compounds showing promising results were further investigated by in vivo pharmacological experiment.

### In vitro acetylcholinesterase inhibitory activity

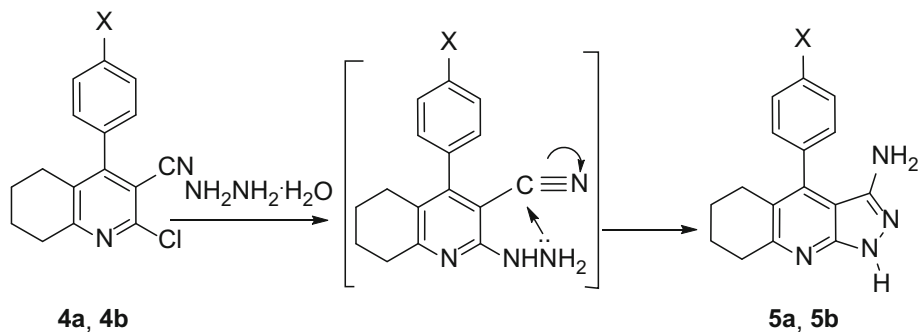
The in vitro biological activity of the synthesized compounds was evaluated through the measurement of the contraction of frog's Rectus abdominis [37], as an example of a skeletal muscle that is known to be rich in AChE [38], the enzyme responsible for the breakdown and deactivation of ACh. Such muscle contractions could be measured using force displacement transducer (FTO 3C) connected to a grass polyview 16 data acquisition and analysis system version 1 Grass technology WARWICK, RI, USA. Therefore, administration of a cholinesterase inhibitor before the addition of ACh to a muscle preparation leads to accumulation of unhydrolyzed ACh with resultant increase

Scheme 2



**Reagents and reaction conditions:** (i)  $\text{POCl}_3$ ,  $\text{PCl}_5$ , reflux; (ii)  $\text{NH}_2\text{NH}_2$  (98%), ethanol, reflux; (iii) ethanolamine, dioxane, reflux; (iv) butylamine, dioxane, reflux; (v) ethylene diamine, dioxane, reflux; (vi) phenylhydrazine, dioxane, reflux; (vii) diethylamine, dioxane, reflux

**Fig. 3** Proposed mechanism for the synthesis of compounds 5



in the height of ACh-induced contractions. The difference in response of the muscle to ACh before and after exposure to a suspected cholinesterase inhibitor indicates the extent

of its inhibitory effect on the enzyme. The percentage inhibition of ACh activity, as indicated by the percentage potentiation of ACh-induced contractions, was determined.

**Table 1** In vitro AChE inhibitory activity of the synthesized compounds on frog's rectus abdominus

Compound	X	In vitro Increase in contraction/%
Control		5.3 ± 1.7
Tacrine		160.0 ± 34.9*
<b>1a</b>	H	23.5 ± 2.1
<b>1b</b>	Cl	111.7 ± 11.8*
<b>2a</b>	H	76.5 ± 9.2*
<b>2b</b>	Cl	178.8 ± 58.9*
<b>3a</b>	H	17.5 ± 3.5
<b>3b</b>	Cl	161.7 ± 45.4*
<b>4a</b>	H	240.0 ± 42.7*
<b>4b</b>	Cl	188.7 ± 33.2*
<b>5a</b>	H	170.0 ± 7.1*
<b>5b</b>	Cl	131.7 ± 37.5*
<b>6a</b>	H	62.5 ± 10.6
<b>6b</b>	Cl	107.5 ± 17.7*
<b>7a</b>	H	66.0 ± 5.7
<b>7b</b>	Cl	227.5 ± 24.7*
<b>8a</b>	H	183.3 ± 47.3*
<b>8b</b>	Cl	233.3 ± 66.6*
<b>9a</b>	H	80.0 ± 28.3*
<b>9b</b>	Cl	183.3 ± 68.1*
<b>10a</b>	H	225.5 ± 21.9*
<b>10b</b>	Cl	81.5 ± 2.1*

The tested compounds were added at a concentration of 1 µg/cm<sup>3</sup>

Data are expressed as mean ± SD (*n* = 5–7) of at least three different experiments

Data were analyzed using One factor ANOVA followed by post hoc Tukey simultaneous comparison *t*-values (pairwise *t* tests)

\* Denotes significant difference from control value at *P* < 0.05

The values ± standard deviation (at confidence levels of 95 %) are recorded in Table 1 and presented in Fig. 4. Tacrine was used as a reference standard.

The results revealed that, in general, presence of a 4-chloro group in the test compounds caused significant

**Table 2** In vivo inhibition of AChE activity by the synthesized compounds in rat brain using Ellman method

Compound	X	In vivo AchE activity/% inhibition
Control		0.00 ± 6.60
Tacrine		38.43 ± 6.39*
<b>1b</b>	Cl	34.43 ± 4.31*
<b>2b</b>	Cl	12.41 ± 2.72
<b>3b</b>	Cl	10.27 ± 9.96
<b>4a</b>	H	43.63 ± 1.27*
<b>4b</b>	Cl	11.17 ± 9.82
<b>5a</b>	H	6.39 ± 3.06
<b>5b</b>	Cl	8.50 ± 2.82
<b>6b</b>	Cl	9.01 ± 3.28
<b>7b</b>	Cl	54.00 ± 5.05*
<b>8a</b>	H	7.43 ± 9.86
<b>8b</b>	Cl	2.25 ± 7.57
<b>9a</b>	H	15.75 ± 1.99*
<b>9b</b>	Cl	20.23 ± 1.58*
<b>10a</b>	H	14.44 ± 0.04*
<b>10b</b>	Cl	42.55 ± 5.35*

The tested compounds were administered at a dose of 10 mg/kg body weight

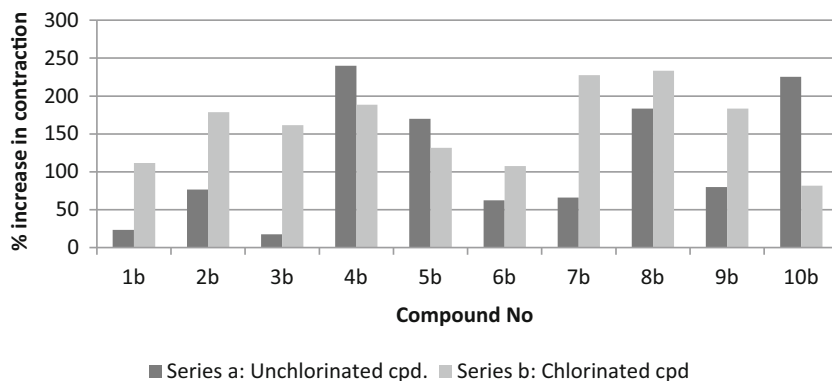
Data are expressed as mean ± SD (*n* = 5–7) of at least three different experiments

Data were analyzed using One factor ANOVA followed by post hoc Tukey simultaneous comparison *t*-values (pairwise *t* tests)

\* Denotes significant difference from control value at *P* < 0.05

potentiation of the contractions induced by Ach (2–9 times) compared to chloro free analogs except for compounds **4**, **5**, and **10**. Results of compounds **1** and **3** were highly consistent with the assumed rationale where the chlorinated analogs **1b** and **3b** showed almost 5- and 9-fold inhibition of the cholinesterase activity, respectively. However, presence of two chloro groups (compound **4b**) unexpectedly showed lower inhibition than the monochlorinated analog **4a**. This might probably

**Fig. 4** Comparison between the in vitro percentage acetylcholinesterase inhibitory activity of the two synthesized series (chlorinated versus the unchlorinated compounds) at a dose of 1 µg/cm<sup>3</sup>. Values are expressed as % increase in Ach-induced contraction in the isolated rectus abdominis



be due to the introduction of a sort of competition between the two halogens for the binding site of the receptor. In addition, compounds **5** and **10** showed an opposite behavior to the proposed rationale where the non-chlorinated compounds showed higher in vitro activity than the chlorinated ones.

**Table 3** Biochemical estimation of the liver enzyme SGPT level in rats after administration of the tested compounds

Compound	X	SGPT/IU cm <sup>-3</sup>
Control (DMF)		204.3 ± 26.5
Tacrine (in DMF)		304.7 ± 30.9*
Saline		47.25 ± 4.5*
<b>1b</b>	Cl	145.5 ± 6.3*
<b>2b</b>	Cl	56.8 ± 8.5*
<b>3b</b>	Cl	116 ± 10.0*
<b>4a</b>	H	63.2 ± 25.7*
<b>4b</b>	Cl	43.0 ± 8.9*
<b>5a</b>	H	301.5 ± 10.2
<b>5b</b>	Cl	236.4 ± 10.6
<b>6b</b>	Cl	216.4 ± 19.6
<b>7b</b>	Cl	139.1 ± 16.7*
<b>8a</b>	H	158.5 ± 9.8*
<b>8b</b>	Cl	39.1 ± 3.7*
<b>9a</b>	H	136.9 ± 17.2*
<b>9b</b>	Cl	40.7 ± 2.5*
<b>10a</b>	H	253.15 ± 16.7
<b>10b</b>	Cl	211.7 ± 11.7

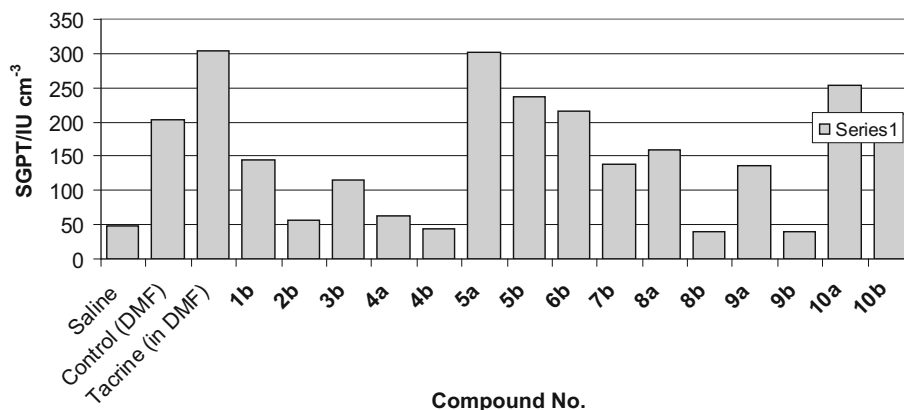
The tested compounds were administered at a dose of 7 mg/kg body weight

Data are expressed as mean ± SD ( $n = 5-7$ ) of at least three different experiments

Data were analyzed using One factor ANOVA followed by post hoc Tukey simultaneous comparison  $t$ -values (pairwise  $t$  tests)

\* Denotes significant difference from control (DMF) value at  $P < 0.05$

**Fig. 5** Comparison between the SGPT levels in rats after administration of the two synthesized series (chlorinated versus the unchlorinated compounds) at a dose of 7 mg/kg body weight



### In vivo acetylcholinesterase inhibitory activity

Compounds showing in vitro cholinesterase inhibitory activity that was significantly different from control values ( $P < 0.05$ ) were further investigated by quantitative in vivo anti-cholinesterase assay according to the method developed by Ellman et al. [39]. The principle of the assay is based on the fact that active enzyme hydrolyzes acetyl thiocholine into free thiocholine which reacts with Ellman reagent (DTNB; 5,5'-dithiobis-(2-nitrobenzoic acid)) to form a yellow colored disulfide complex. The activity of AchE is therefore, measured by the intensity of the yellow color produced due to the amount of hydrolyzed thiocholine. In other words, a decrease in the amount of produced thiocholine than in the control would show a decreased AchE activity and indicate a successful cholinesterase inhibitory effect [40]. The results ± standard deviation (at confidence levels of 95 %) are recorded in Table 2.

The results revealed that most of the in vivo tested compounds showed moderate to high cholinesterase inhibitory activity and four of the synthesized compounds (**1b**, **4a**, **7b**, and **10b**) showed the same or significantly higher activity compared to tacrine.

### Hepatotoxicity testing

The hepatotoxicity of compounds showing in vitro and/or in vivo cholinesterase inhibitory activity that was significantly different from control values at  $P < 0.05$  was evaluated through determination of serum glutamic pyruvic transaminase (SGPT) levels using Ultraviolet (UV) kinetic method in which SGPT was assayed based in enzyme coupled system, where the enzyme is involved in the transfer of an amino group from alanine to an  $\alpha$ -ketoacid (2-oxoglutarate). The formed pyruvate reacts in a system using NADH which is oxidized to NAD and the rate of oxidation is measured by determining the absorbance at 340 nm at 0, 1, 2, 3 min.

The higher the values obtained for SGPT, the higher the hepatotoxicity caused and vice versa [41, 42]. The results  $\pm$  standard deviation (at confidence levels of 95 %) are recorded in Table 3 and presented in Fig. 5.

The results indicated that DMF showed considerably high hepatotoxicity itself. However, most of the synthesized compounds, unlike tacrine, were able to reduce this hepatotoxic effect. In other words, the compounds showed

**Fig. 6** **a** Binding mode of the inhibitor in the binding site of *hAChE* (PDB ID: 1B41) using MOE software. **b** Binding mode of compound **4a** docked and minimized in the *hAChE* binding site using MOE software. **c** Binding mode of compound **4b** docked and minimized in the *hAChE* binding site using MOE software

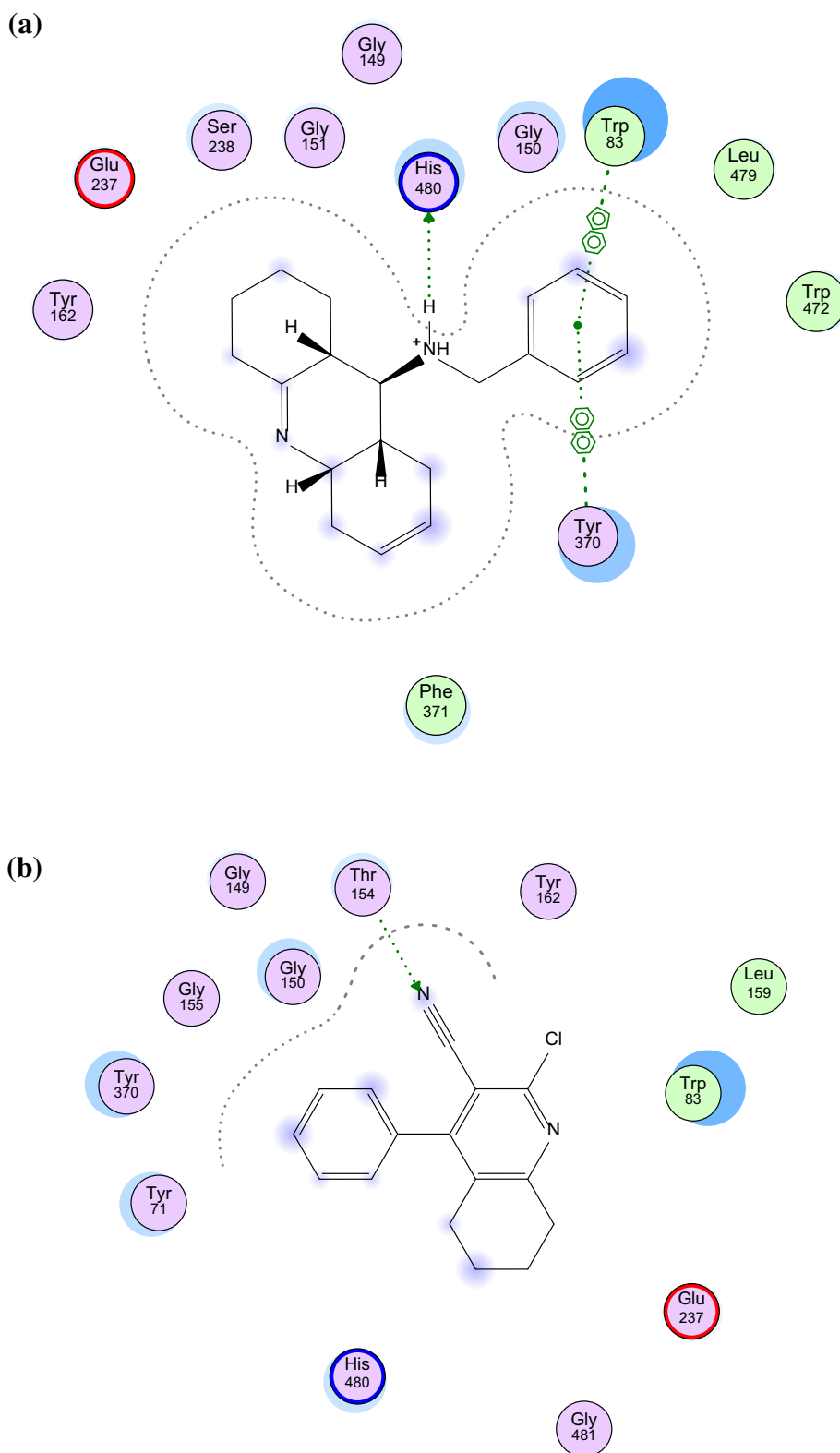
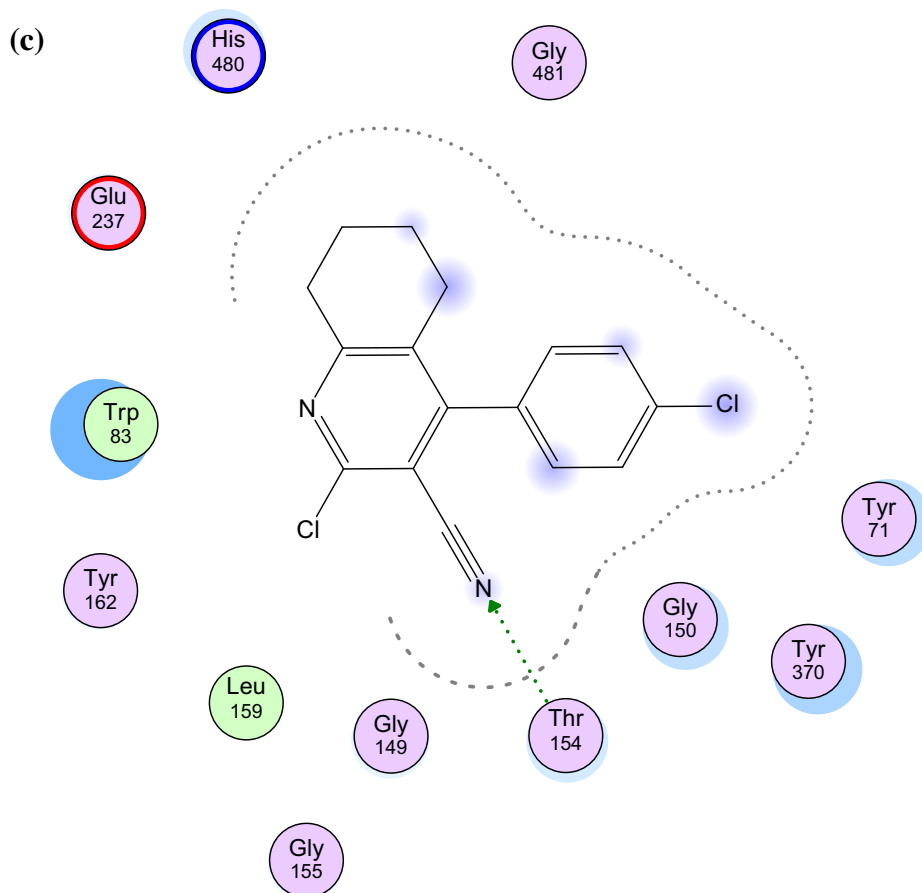


Fig. 6 continued



to some extent a hepatoprotective effect. Compounds **2b**, **4a**, **4b**, **8b**, and **9b** almost brought SGPT level back to its normal value despite the presence of DMF. However five compounds (**5a**, **5b**, **6b**, **10a**, and **10b**) showed high SGPT levels which were comparable to that of tacrine.

### Docking studies

Docking study was carried out using the enzyme parameters obtained from the crystallographic structure of the complex between AChE (PDB ID: 1DX4) with the co-crystallized ligand tacrine derivative 9-(3-phenylmethylamino)-1,2,3,4-tetrahydroacridine [43]. The docking simulation for the ligand was carried out using molecular operating environment (MOE) software supplied by the Chemical Computing Group, Inc., Montréal, QC [44] (Fig. 6a). The X-ray crystal structure revealed that the NH group in the ligand is hydrogen-bonded to His 480, while the phenyl ring displayed arene–arene interactions with Trp 83 and Tyr 370 residues. Besides, it showed hydrophobic interactions with Trp 83, Gly 149, Gly 150, Tyr 162, Glu 237, Tyr 370, and His 480. Figure 6b and c shows the binding mode of compounds **4a** and **4b** where a hydrogen bond is observed between the nitrogen of the

CN group and Thr 154 for both compounds. In addition, both compounds displayed hydrophobic interactions with the same amino acid residues as those found in the X-ray crystal structure of AChE (Trp 83, Gly 149, Gly 150, Tyr 162, Glu 237, Tyr 370, and His 480). Therefore, it could be concluded that the docking pattern of the test compounds revealed different hydrogen bonding interaction from that of the ligand whereas they showed nearly identical hydrophobic binding pattern to that observed with the inhibitor.

### Conclusion

The present study reports the synthesis and biological investigation of acetylcholinesterase inhibitory activity of chlorinated versus non chlorinated tetrahydroquinolines. The obtained results clearly revealed that introduction of a chloro group at the 4-position of the phenyl ring potentiated the acetylcholinesterase inhibitory activity relative to chloro free analogs. This was obvious on comparing in vitro results for both chlorinated and non chlorinated analogs of compounds **1**, **2**, **3**, **6**, **7**, **8**, and **9**. The in vivo results of the promising compounds indicated that most of



the compounds retained their cholinesterase inhibitory activity. Furthermore, most of these compounds showed reduced hepatotoxicity compared to tacrine. Among the synthesized compounds, compound **4a** displayed high cholinesterase inhibitory activity both in vitro and in vivo, together with low hepatotoxicity. Collectively, the above findings could establish a molecular basis for the development of new tetrahydroquinolines with higher cholinesterase inhibitory activity and lower hepatotoxicity compared to tacrine. MOE-based molecular docking results suggested that compounds **4a** and **4b** showed a binding pattern close to the pattern observed for the ligand regarding the hydrophobic binding, whereas, the hydrogen bonding interaction was different from that of the ligand. Although compounds **4a** and **4b** showed similar binding pattern, they differ in their biological activity. This discrepancy in activity of compounds **4a** and **4b** might be attributed to difference in halogen bonding capabilities of both compounds to the target enzyme.

## Experimental

All reagents and solvents were purchased from commercial suppliers and were dried and purified when necessary by standard techniques. Melting points were determined in open glass capillaries using Thomas-Hoover melting point apparatus. Infrared spectra (IR) were recorded for KBr discs on Nicolet-800 FT-IR infrared spectrophotometer. NMR spectra were scanned on a Bruker 300 ultrashield spectrometer using tetramethylsilane (TMS) as internal standard and DMSO- $d_6$  as solvent (chemical shifts are given in  $\delta$ /ppm). Splitting patterns were designated as follows: s: singlet, br s: broad singlet, d: doublet, t: triplet, and m: multiplet. Microanalyses were performed at the regional Center for Mycology and Biotechnology, El Azhar University and the found values were within  $\pm 0.3$  % of the theoretical values. Follow up of the reactions and checking the purity of the compounds was made by thin layer chromatography (TLC) on silica gel-precoated aluminum sheets (Type 60 GF254; Merck; Germany) and the spots were detected by exposure to UV lamp at  $\lambda = 254$  nm for few seconds.

### 4-Aryl-2-hydroxy-5,6,7,8-tetrahydroquinoline-3-carbonitriles **1a**, **1b**

Equimolar amounts of the appropriate aldehyde (45 mmol), 4.41 g cyclohexanone (45 mmol, 4.66 cm<sup>3</sup>), 5.08 g ethyl cyanoacetate (45 mmol, 4.78 cm<sup>3</sup>), and 6.93 g ammonium acetate (90 mmol) were heated under reflux in 25 cm<sup>3</sup> ethanol for 15 min. The mixture was then stirred at room temperature for 3 h. Most of the solvent was removed

by concentration under vacuum and the residue was left to stand for 24 h to separate yellow crystals. These were filtered, washed with ethanol, dried, and recrystallized from ethanol.

### 5,6,7,8-Tetrahydro-2-hydroxy-4-phenylquinoline-3-carbonitrile (**1a**) [30, 31]

Yield 35.7 %; m.p.: 260–262 °C (Ref. [26, 27] 267–268 °C).

### 4-(4-Chlorophenyl)-2-hydroxy-5,6,7,8-tetrahydroquinoline-3-carbonitrile (**1b**)

Yield 29.6 %; m.p.: 296 °C; <sup>1</sup>H NMR (300 MHz, DMSO- $d_6$ ):  $\delta = 1.66$ – $1.70$  (m, 2H, C<sub>6</sub>-H<sub>2</sub>),  $2.02$ – $2.07$  (m, 2H, C<sub>7</sub>-H<sub>2</sub>),  $2.60$ – $2.64$  (m, 2H, C<sub>5</sub>-H<sub>2</sub>),  $2.80$ – $3.11$  (m, 2H, C<sub>8</sub>-H<sub>2</sub>),  $7.31$ ,  $7.59$  (2d,  $J = 7.8$  Hz, each 2H, chlorophenyl-H),  $8.14$  (s, 1/4H, NH, D<sub>2</sub>O-exchangeable),  $11.75$  (s, 3/4H, OH, D<sub>2</sub>O-exchangeable) ppm; <sup>13</sup>C NMR (75 MHz, DMSO- $d_6$ ):  $\delta = 24.4$ ,  $26.3$ ,  $29.9$ ,  $31.1$  (cyclohexyl-C),  $116.8$  (CN),  $119.5$ ,  $121.6$ ,  $127.1$ ,  $129.6$ ,  $133.5$ ,  $135.6$ ,  $151.9$ ,  $157.5$ ,  $164.2$  (Ar-C) ppm; IR (KBr):  $\bar{\nu} = 3400$ ,  $3220$  (NH and OH),  $2250$  (CN),  $1695$  (C = O),  $1600$ ,  $1576$ ,  $1544$ ,  $1480$  (C=N and C=C) cm<sup>-1</sup>.

### 2-Amino-4-aryl-5,6,7,8-tetrahydroquinoline-3-carbonitriles **2a**, **2b**

A mixture of equimolar amounts of the appropriate aldehyde (45 mmol), 4.41 g cyclohexanone (45 mmol), 2.97 g malononitrile (45 mmol), and 6.93 g ammonium acetate (90 mmol) in 25 cm<sup>3</sup> dry benzene was heated under reflux while stirring, using Dean Stark head for 5 h. The solvent was distilled off and the residue was boiled in 15 cm<sup>3</sup> ethanol. The clear solution was left to stand for 24 h to separate yellow crystals. These were filtered, washed with ethanol, dried, and recrystallized from ethanol.

### 2-Amino-4-phenyl-5,6,7,8-tetrahydroquinoline-3-carbonitrile (**2a**) [33]

Yield 24.3 %; m.p.: 233–235 °C (Ref. [33] 234–236 °C).

### 2-Amino-4-(4-chlorophenyl)-5,6,7,8-tetrahydroquinoline-3-carbonitrile (**2b**) [34, 35]

Yield 33.7 %; m.p.: 263–266 °C (Ref. [34] 278–280 °C and Ref. [35] 258–259 °C).

### Methyl 4-aryl-2-hydroxy-5,6,7,8-tetrahydroquinoline-3-carboxylates **3a**, **3b**

Equimolar amounts of the appropriate aldehyde (45 mmol), 4.41 g cyclohexanone (45 mmol, 4.66 cm<sup>3</sup>), 5.94 g dimethyl malonate (45 mmol, 5.15 cm<sup>3</sup>), and 6.93 g ammonium acetate (90 mmol) were heated under reflux in 25 cm<sup>3</sup> ethanol for 3 h. Most of the solvent was removed by evaporation under vacuum and the residue was left to stand for 24 h to separate white crystals that were filtered, washed with ethanol, dried, and recrystallized from ethanol.

*Methyl 2-hydroxy-4-phenyl-5,6,7,8-tetrahydroquinoline-3-carboxylate (3a, C<sub>17</sub>H<sub>17</sub>NO<sub>3</sub>)*

Yield 45.9 %; m.p.: 167–168 °C; <sup>1</sup>H NMR (300 MHz, DMSO-*d*<sub>6</sub>): δ = 1.63–1.68 (m, 2H, C<sub>6</sub>-H<sub>2</sub>), 1.76–1.81 (m, 2H, C<sub>7</sub>-H<sub>2</sub>), 2.61–2.66 (m, 2H, C<sub>5</sub>-H<sub>2</sub>), 2.77–2.82 (m, 2H, C<sub>8</sub>-H<sub>2</sub>), 3.59 (s, 3H, OCH<sub>3</sub>), 7.32–7.45 (m, 5H, phenyl-H), 7.81 (s, 1/4H, NH, D<sub>2</sub>O-exchangeable), 11.21 (s, 3/4H, OH, D<sub>2</sub>O-exchangeable) ppm; <sup>13</sup>C NMR (75 MHz, DMSO-*d*<sub>6</sub>): δ = 22.4, 27.3, 31.2, 38.4 (cyclohexyl-C), 56.1 (OCH<sub>3</sub>), 119.4, 121.0, 126.4, 128.7, 132.6, 133.8, 152.1, 157.0, 161.8 (Ar-C), 164.3 (ester C = O) ppm; IR (KBr):  $\bar{\nu}$  = 3405 (NH or OH), 1743 (ester C = O), 1653 (amide C = O), 1599, 1519, 1498 (C=N and Ar-C=C) cm<sup>-1</sup>.

*Methyl 4-(4-chlorophenyl)-2-hydroxy-5,6,7,8-tetrahydroquinoline-3-carboxylate (3b, C<sub>17</sub>H<sub>16</sub>ClNO<sub>3</sub>)*

Yield 42.3 %; m.p.: 171 °C; <sup>1</sup>H NMR (300 MHz, DMSO-*d*<sub>6</sub>): δ = 1.64–1.68 (m, 2H, C<sub>6</sub>-H<sub>2</sub>), 1.80–1.84 (m, 2H, C<sub>7</sub>-H<sub>2</sub>), 2.62–2.67 (m, 2H, C<sub>5</sub>-H<sub>2</sub>), 2.76–2.81 (m, 2H, C<sub>8</sub>-H<sub>2</sub>), 3.32 (s, 3H, OCH<sub>3</sub>), 7.51, 7.59 (2d, *J* = 8.0 Hz, each 2H, chlorophenyl-H), 7.89 (s, 1/4H, NH, D<sub>2</sub>O-exchangeable), 11.43 (s, 3/4H, OH, D<sub>2</sub>O-exchangeable) ppm; <sup>13</sup>C NMR (75 MHz, DMSO-*d*<sub>6</sub>): δ = 25.37, 26.29, 31.12, 38.22 (cyclohexyl-C), 56.5 (OCH<sub>3</sub>), 119.2, 120.6, 127.1, 128.6, 132.5, 132.9, 152.8, 157.0, 165.3 (Ar-C), 165.8 (ester C = O) ppm; IR (KBr)  $\bar{\nu}$  = 3420 (NH or OH), 1745 (C = O ester), 1659 (C = O amide), 1604, 1510, 1500 (C=N and Ar-C=C) cm<sup>-1</sup>.

*2-Chloro-4-phenyl-5,6,7,8-tetrahydroquinoline-3-carbonitrile (4a) [27]*

A solution of 5.0 g 5,6,7,8-tetrahydro-2-hydroxyquinoline **1a** (20 mmol) in 15 cm<sup>3</sup> phosphorus oxychloride was heated under reflux for 20 h in presence of catalytic amount of phosphorus pentachloride. The reaction mixture was cooled and poured onto crushed ice to give a yellowish orange precipitate, which was filtered, dried, and crystallized from ethanol. Yield 73 %; m.p.: 200–202 °C (Ref. [27] 201–202 °C); IR (KBr):  $\bar{\nu}$  = 2230 (CN), 1559, 1532, 1493 (C=N and Ar-C=C) cm<sup>-1</sup>.

*2-Chloro-4-(4-chlorophenyl)-5,6,7,8-tetrahydroquinoline-3-carbonitrile (4b, C<sub>16</sub>H<sub>12</sub>Cl<sub>2</sub>N<sub>2</sub>)*

A solution of 5.69 g 5,6,7,8-tetrahydro-2-hydroxyquinoline **1b** (20 mmol) in 15 cm<sup>3</sup> phosphorus oxychloride was heated under reflux for 20 h in presence of catalytic amount of phosphorus pentachloride. The reaction mixture was cooled and poured onto crushed ice to give a yellowish orange oil which was extracted using chloroform. The chloroform extract was dried over anhydrous Na<sub>2</sub>SO<sub>4</sub> and evaporated to dryness. The produced oil was purified using column chromatography (chloroform: methanol 9:1). Yield 87 %; m.p.: 117 °C; <sup>1</sup>H NMR (300 MHz, DMSO-*d*<sub>6</sub>):

δ = 1.61–1.66 (m, 2H, C<sub>6</sub>-H<sub>2</sub>), 1.73–1.78 (m, 2H, C<sub>7</sub>-H<sub>2</sub>), 2.25–2.31 (m, 2H, C<sub>5</sub>-H<sub>2</sub>), 2.81–2.86 (m, 2H, C<sub>8</sub>-H<sub>2</sub>), 7.22, 7.56 (2d, *J* = 7.8 Hz, each 2H, chlorophenyl-H) ppm; <sup>13</sup>C NMR (75 MHz, DMSO-*d*<sub>6</sub>): δ = 21.5, 26.4, 30.6, 36.3 (cyclohexyl-C), 112.02 (CN), 104.5, 128.1, 130.3, 132.7, 134.4, 135.9, 150.4, 152.7, 160.9 (Ar-C) ppm; IR (KBr): 2223 (CN), 1578, 1545, 1498 (C=N and Ar C=C) cm<sup>-1</sup>.

*3-Amino-4-aryl-5,6,7,8-tetrahydro-1H-pyrazolo[3,4-b]-quinolines 5a, 5b*

A mixture of the 2-chloro-5,6,7,8-tetrahydroquinolines **4** (3.7 mmol) and 0.93 g hydrazine hydrate (98 %, 18.6 mmol, 0.91 cm<sup>3</sup>) in 5 cm<sup>3</sup> absolute ethanol was heated under reflux for 4 h. The mixture became clear at first, and then a heavy yellow solid gradually separated out. The reaction mixture was left to stand at room temperature for 24 h for complete deposition of the product which was then filtered, washed with ethanol, dried, and crystallized from ethanol.

*3-Amino-4-phenyl-5,6,7,8-tetrahydro-1H-pyrazolo[3,4-b]-quinoline (5a) [27]*

Yield 96 %; m.p.: 264–266 °C (Ref. [27] 264–266 °C).

*3-Amino-4-(4-chlorophenyl)-5,6,7,8-tetrahydro-1H-pyrazolo[3,4-b]quinoline (5b, C<sub>16</sub>H<sub>15</sub>ClN<sub>4</sub>)*

Yield 94.5 %; m.p.: 254 °C; <sup>1</sup>H NMR (300 MHz, DMSO-*d*<sub>6</sub>): δ = 1.63–1.68 (m, 2H, C<sub>6</sub>-H<sub>2</sub>), 1.85–1.90 (m, 2H, C<sub>7</sub>-H<sub>2</sub>), 2.46–2.52 (m, 2H, C<sub>5</sub>-H<sub>2</sub>), 2.75–2.81 (m, 2H, C<sub>8</sub>-H<sub>2</sub>), 5.62 (s, 2H, NH<sub>2</sub>, D<sub>2</sub>O-exchangeable), 7.22, 7.47 (2d, *J* = 8.2 Hz, each 2H, chlorophenyl-H), 11.60 (s, 1H, NH, D<sub>2</sub>O-exchangeable) ppm; <sup>13</sup>C NMR (75 MHz, DMSO-*d*<sub>6</sub>): δ = 22.4, 27.7, 29.7, 35.9 (cyclohexyl-C), 91.7, 128.6, 129.4, 132.9, 133.3, 135.9, 146.5, 150.7, 152.3, 157.6 (Ar-C) ppm; IR (KBr):  $\bar{\nu}$  = 3450–3178 (NH), 1599, 1560, 1528, 1490 (C=N and Ar-C=C) cm<sup>-1</sup>.

*General procedure for 4-aryl-2-(substituted amino)-5,6,7,8-tetrahydroquinoline-3-carbonitriles 6-8, 10 and 4-aryl-2-(2-phenylhydrazinyl)-5,6,7,8-tetrahydroquinoline-3-carbonitriles 9a, 9b*

A mixture of the 2-chlorotetrahydroquinolines **4** (3.7 mmol) and the appropriate amine or phenyl hydrazine (7.4 mmol) in 10 cm<sup>3</sup> dioxane was heated under reflux for 20 h. The reaction mixture was left to attain room temperature, then poured onto crushed ice to give a brownish precipitate, which was filtered, dried, and purified using column chromatography.

*4-Aryl-2-(2-hydroxyethylamino)-5,6,7,8-tetrahydroquinoline-3-carbonitriles 6a, 6b*

Purified using column chromatography (methylene chloride: methanol: ammonium hydroxide, 5:1:0.1) as a yellowish solid.

**2-(2-Hydroxyethylamino)-4-phenyl-5,6,7,8-tetrahydroquinoline-3-carbonitrile (6a, C<sub>18</sub>H<sub>19</sub>N<sub>3</sub>O)**

Yield 46 %; m.p.: 164 °C; <sup>1</sup>H NMR (300 MHz, DMSO-*d*<sub>6</sub>): δ = 1.66–1.70 (m, 2H, C<sub>6</sub>-H<sub>2</sub>), 1.76–1.81 (m, 2H, C<sub>7</sub>-H<sub>2</sub>), 2.10–2.16 (m, 2H, C<sub>5</sub>-H<sub>2</sub>), 2.87–2.92 (m, 2H, C<sub>8</sub>-H<sub>2</sub>), 3.37 (t, *J* = 6.9 Hz, 2H, NCH<sub>2</sub>), 3.46 (t, *J* = 6.9 Hz, 2H, OCH<sub>2</sub>), 4.55 (s, 1H, OH, D<sub>2</sub>O-exchangeable), 6.43 (s, 1H, NH, D<sub>2</sub>O-exchangeable), 7.18–7.53 (m, 5H, phenyl-H) ppm; <sup>13</sup>C NMR (75 MHz, DMSO-*d*<sub>6</sub>): δ = 21.3, 25.2, 29.8, 36.1 (cyclohexyl-C), 45.3 (NCH<sub>2</sub>), 59.2 (OCH<sub>2</sub>), 95.4 (pyridine-C<sub>3</sub>), 115.3 (CN), 125.7, 126.8, 128.1, 128.6, 137.2, 150.6, 159.3, 161.2 (Ar-C) ppm; IR (KBr):  $\bar{\nu}$  = 3458 (OH), 3289 (NH), 2230 (CN), 1593, 1552, 1543, 1478 (C=N and Ar-C=C) cm<sup>-1</sup>.

**4-(4-Chlorophenyl)-2-(2-hydroxyethylamino)-5,6,7,8-tetrahydroquinoline-3-carbonitrile (6b, C<sub>18</sub>H<sub>18</sub>ClN<sub>3</sub>O)**

Yield 46 %; m.p.: 143 °C; <sup>1</sup>H NMR (300 MHz, DMSO-*d*<sub>6</sub>): δ = 1.65–1.70 (m, 2H, C<sub>6</sub>-H<sub>2</sub>), 1.79–1.84 (m, 2H, C<sub>7</sub>-H<sub>2</sub>), 2.19–2.24 (m, 2H, C<sub>5</sub>-H<sub>2</sub>), 2.51–2.56 (m, 2H, C<sub>8</sub>-H<sub>2</sub>), 3.41 (t, *J* = 6.9 Hz, 2H, NCH<sub>2</sub>), 3.47 (t, *J* = 6.9 Hz, 2H, OCH<sub>2</sub>), 4.46 (s, 1H, OH, D<sub>2</sub>O-exchangeable), 6.43 (s, 1H, NH, D<sub>2</sub>O-exchangeable), 7.30, 7.59 (2d, *J* = 7.8 Hz, each 2H, chlorophenyl-H) ppm; <sup>13</sup>C NMR (75 MHz, DMSO-*d*<sub>6</sub>): δ = 21.9, 25.1, 29.7, 35.9 (cyclohexyl-C), 45.7 (NCH<sub>2</sub>), 59.3 (OCH<sub>2</sub>), 95.7 (pyridine-C<sub>3</sub>), 115.7 (CN), 126.1, 128.4, 129.5, 132.4, 134.7, 151.3, 159.6, 161.5 (Ar-C) ppm; IR (KBr):  $\bar{\nu}$  = 3423 (OH), 3254 (NH), 2245 (CN), 1584, 1551, 1524, 1485 (C=N and Ar-C=C) cm<sup>-1</sup>.

**4-Aryl-2-(butylamino)-5,6,7,8-tetrahydroquinoline-3-carbonitriles 7a, 7b**

Purified using column chromatography (chloroform: methanol: ammonium hydroxide, 9:1:0.1) as a pale yellow solid.

**2-(Butylamino)-4-phenyl-5,6,7,8-tetrahydroquinoline-3-carbonitrile (7a, C<sub>20</sub>H<sub>23</sub>N<sub>3</sub>)**

Yield 41 %; m.p.: 209–210 °C; <sup>1</sup>H NMR (300 MHz, DMSO-*d*<sub>6</sub>): δ = 0.91 (t, *J* = 6.9 Hz, 3H, CH<sub>3</sub>), 1.25–1.31 (m, 2H, CH<sub>2</sub>CH<sub>3</sub>), 1.47–1.52 (m, 2H, CH<sub>2</sub>CH<sub>2</sub>CH<sub>3</sub>), 1.54–1.60 (m, 2H, C<sub>6</sub>-H<sub>2</sub>), 1.63–1.68 (m, 2H, C<sub>7</sub>-H<sub>2</sub>), 2.07–2.13 (m, 2H, C<sub>5</sub>-H<sub>2</sub>), 2.69–2.74 (m, 2H, C<sub>8</sub>-H<sub>2</sub>), 3.43 (t, *J* = 6.9 Hz, 2H, NCH<sub>2</sub>), 6.45 (s, 1H, NH, D<sub>2</sub>O-exchangeable), 7.38–7.61 (m, 5H, phenyl-H) ppm; <sup>13</sup>C NMR (75 MHz, DMSO-*d*<sub>6</sub>): δ = 13.50 (CH<sub>3</sub>), 19.20 (CH<sub>2</sub>CH<sub>3</sub>), 21.3, 21.9, 29.9, 30.5, 35.6 (cyclohexyl-C and NH-CH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>CH<sub>3</sub>), 40.31 (NH-CH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>CH<sub>3</sub>), 95.4 (pyridine-C<sub>3</sub>), 115.3 (CN), 124.1, 127.7, 129.1, 129.5, 137.2, 150.0, 158.7, 162.8 (Ar-C) ppm; IR (KBr):  $\bar{\nu}$  = 3325 (NH), 2240 (CN), 1585, 1474, 1455 (C=N and Ar-C=C) cm<sup>-1</sup>.

**2-(Butylamino)-4-(4-chlorophenyl)-5,6,7,8-tetrahydroquinoline-3-carbonitrile (7b, C<sub>20</sub>H<sub>22</sub>ClN<sub>3</sub>)**

Yield 39 %; m.p.: 163 °C; <sup>1</sup>H NMR (300 MHz, DMSO-*d*<sub>6</sub>): δ = 0.89 (t, *J* = 6.9 Hz, 3H, CH<sub>3</sub>), 1.30–1.36 (m, 2H, CH<sub>2</sub>CH<sub>3</sub>), 1.44–1.50 (m, 2H, CH<sub>2</sub>CH<sub>2</sub>CH<sub>3</sub>), 1.56–1.62 (m, 2H, C<sub>6</sub>-H<sub>2</sub>), 1.64–1.69 (m, 2H, C<sub>7</sub>-H<sub>2</sub>), 2.16–2.21 (m, 2H, C<sub>5</sub>-H<sub>2</sub>), 2.81–2.87 (m, 2H, C<sub>8</sub>-H<sub>2</sub>), 3.36 (t, *J* = 6.9 Hz, 2H, NCH<sub>2</sub>), 6.51 (s, 1H, NH, D<sub>2</sub>O-exchangeable), 7.41, 7.67 (2d, *J* = 8.2 Hz, each 2H, chlorophenyl-H) ppm; <sup>13</sup>C NMR (75 MHz, DMSO-*d*<sub>6</sub>): δ = 13.7 (CH<sub>3</sub>), 19.5 (NH-CH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>CH<sub>3</sub>), 21.5, 21.9, 29.8, 30.7, 35.1 (cyclohexyl-C and NH-CH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>CH<sub>3</sub>), 41.2 (NH-CH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>CH<sub>3</sub>), 95.6 (pyridine-C<sub>3</sub>), 115.67 (CN), 125.9, 128.4, 129.6, 133.3, 137.2, 150.6, 159.1, 162.9 (Ar-C) ppm; IR (KBr):  $\bar{\nu}$  = 3319 (NH), 2235 (CN), 1590, 1486, 1466 (C=N and Ar-C=C) cm<sup>-1</sup>.

**2-(2-Aminoethylamino)-4-aryl-5,6,7,8-tetrahydroquinoline-3-carbonitriles 8a, 8b**

Purified using column chromatography (chloroform: methanol: ammonium hydroxide, 9:1:0.1) as a yellow solid.

**2-(2-Aminoethylamino)-4-phenyl-5,6,7,8-tetrahydroquinoline-3-carbonitrile (8a, C<sub>18</sub>H<sub>20</sub>N<sub>4</sub>)**

Yield 43 %; m.p.: 130 °C; <sup>1</sup>H NMR (300 MHz, DMSO-*d*<sub>6</sub>): δ = 1.60–1.66 (m, 2H, C<sub>6</sub>-H<sub>2</sub>), 1.74–1.80 (m, 2H, C<sub>7</sub>-H<sub>2</sub>), 2.20–2.26 (m, 2H, C<sub>5</sub>-H<sub>2</sub>), 2.74 (t, *J* = 6.2 Hz, 2H, NCH<sub>2</sub>), 2.90–2.96 (m, 2H, C<sub>8</sub>-H<sub>2</sub>), 3.38 (t, *J* = 6.2 Hz, 2H, NCH<sub>2</sub>), 6.30 (br s, 2H, NH<sub>2</sub>, D<sub>2</sub>O-exchangeable), 6.52 (br s, 1H, NH, D<sub>2</sub>O-exchangeable), 7.23–7.56 (m, 5H, Ar-H) ppm; <sup>13</sup>C NMR (75 MHz, DMSO-*d*<sub>6</sub>): δ = 21.4, 22.1, 30.7, 36.4 (cyclohexyl-C), 45.7, 49.5 (NCH<sub>2</sub>), 90.7 (pyridine-C<sub>3</sub>), 116.4 (CN), 125.8, 127.8, 128.3, 128.6, 136.1, 153.9, 159.1, 160.3 (Ar-C) ppm; IR (KBr):  $\bar{\nu}$  = 3394, 3294 (NH), 2208 (CN), 1595, 1586, 1489, 1465 (C=N and Ar-C=C) cm<sup>-1</sup>.

**2-(2-Aminoethylamino)-4-(4-chlorophenyl)-5,6,7,8-tetrahydroquinoline-3-carbonitrile (8b, C<sub>18</sub>H<sub>19</sub>ClN<sub>4</sub>)**

Yield 41 %; m.p.: 152–155 °C; <sup>1</sup>H NMR (300 MHz, DMSO-*d*<sub>6</sub>): δ = 1.60–1.64 (m, 2H, C<sub>6</sub>-H<sub>2</sub>), 1.70–1.76 (m, 2H, C<sub>7</sub>-H<sub>2</sub>), 2.29–2.35 (m, 2H, C<sub>5</sub>-H<sub>2</sub>), 2.79 (t, *J* = 6.2 Hz, 2H, NCH<sub>2</sub>), 2.98–3.14 (m, 2H, C<sub>8</sub>-H<sub>2</sub>), 3.37 (t, *J* = 6.2 Hz, 2H, NCH<sub>2</sub>), 6.34 (br s, 2H, NH<sub>2</sub>, D<sub>2</sub>O-exchangeable), 6.50 (br s, 1H, NH, D<sub>2</sub>O-exchangeable), 7.33, 7.59 (2d, *J* = 8.2 Hz, each 2H, chlorophenyl-H) ppm; <sup>13</sup>C NMR (75 MHz, DMSO-*d*<sub>6</sub>): δ = 21.5, 22.4, 30.8, 36.5 (cyclohexyl-C), 45.3, 51.8 (NCH<sub>2</sub>), 88.8 (pyridine-C<sub>3</sub>), 117.6 (CN), 125.9, 128.7, 129.2, 134.6, 136.7, 155.4, 158.6, 160.45 (Ar-C) ppm; IR (KBr):  $\bar{\nu}$  = 3389, 3302 (NH), 2228 (CN), 1590, 1579, 1492, 1460 (C=N and Ar-C=C) cm<sup>-1</sup>.

4-Aryl-2-(2-phenylhydrazinyl)-5,6,7,8-tetrahydroquinoline-3-carbonitriles **9a**, **9b**

Purified using column chromatography (methylene chloride:methanol:petroleum ether, 12:1:0.5) as a light beige solid.

2-(2-Phenylhydrazinyl)-4-phenyl-5,6,7,8-tetrahydroquinoline-3-carbonitrile (**9a**, C<sub>22</sub>H<sub>20</sub>N<sub>4</sub>)

Yield 65 %; m.p.: 134–136 °C; <sup>1</sup>H NMR (300 MHz, DMSO-*d*<sub>6</sub>): δ = 1.64–1.69 (m, 2H, C<sub>6</sub>-H<sub>2</sub>), 1.72–1.77 (m, 2H, C<sub>7</sub>-H<sub>2</sub>), 2.38–2.43 (m, 2H, C<sub>5</sub>-H<sub>2</sub>), 2.85–2.90 (m, 2H, C<sub>8</sub>-H<sub>2</sub>), 7.15–7.55 (m, 12H, Ar-H and NH) ppm; <sup>13</sup>C NMR (75 MHz, DMSO-*d*<sub>6</sub>): δ = 21.5, 23.2, 30.6, 36.3 (cyclohexyl-C), 88.8 (pyridine-C<sub>3</sub>), 112.02 (CN), 116.3, 119.6, 127.4, 128.7, 129.4, 129.8, 130.3, 136.2, 151.4, 152.6, 156.5, 158.8 (Ar-C) ppm; IR (KBr):  $\bar{\nu}$  = 3210, 3163 (NH), 2211 (CN), 1580, 1500, 1488 (C=N and Ar-C=C) cm<sup>-1</sup>.

4-(4-Chlorophenyl)-2-(2-phenylhydrazinyl)-5,6,7,8-tetrahydroquinoline-3-carbonitrile (**9b**, C<sub>22</sub>H<sub>19</sub>ClN<sub>4</sub>)

Yield 60 %; m.p.: 179–181 °C; <sup>1</sup>H NMR (300 MHz, DMSO-*d*<sub>6</sub>): δ = 1.65–1.70 (m, 2H, C<sub>6</sub>-H<sub>2</sub>), 1.74–1.79 (m, 2H, C<sub>7</sub>-H<sub>2</sub>), 2.39–2.45 (m, 2H, C<sub>5</sub>-H<sub>2</sub>), 2.88–2.94 (m, 2H, C<sub>8</sub>-H<sub>2</sub>), 7.18–7.60 (m, 11H, Ar-H and NH) ppm; <sup>13</sup>C NMR (75 MHz, DMSO-*d*<sub>6</sub>): δ = 22.1, 26.9, 30.9, 36.2 (cyclohexyl-C), 89.6 (pyridine-C<sub>3</sub>), 114.7 (CN), 118.6, 120.2, 128.8, 129.3, 129.7, 130.5, 133.8, 137.4, 150.8, 151.6, 155.4, 157.8 (Ar-C) ppm; IR (KBr):  $\bar{\nu}$  = 3219, 3180 (NH), 2226 (CN), 1585, 1515, 1491 (C=N and Ar-C=C) cm<sup>-1</sup>.

4-Aryl-2-(diethylamino)-5,6,7,8-tetrahydroquinoline-3-carbonitriles **10a**, **10b**

Purified using column chromatography (chloroform:methanol: ammonium hydroxide, 9:1:0.1) as a white solid.

2-(Diethylamino)-4-phenyl-5,6,7,8-tetrahydroquinoline-3-carbonitrile (**10a**, C<sub>20</sub>H<sub>23</sub>N<sub>3</sub>)

Yield 46 %; m.p.: 167–169 °C; <sup>1</sup>H NMR (300 MHz, DMSO-*d*<sub>6</sub>): δ = 1.24 (t, *J* = 6.9 Hz, 6H, 2CH<sub>3</sub>), 1.67–1.72 (m, 2H, C<sub>6</sub>-H<sub>2</sub>), 1.75–1.81 (m, 2H, C<sub>7</sub>-H<sub>2</sub>), 2.31–2.36 (m, 2H, C<sub>5</sub>-H<sub>2</sub>), 2.95–3.01 (m, 2H, C<sub>8</sub>-H<sub>2</sub>), 3.62 (q, *J* = 6.9 Hz, 4H, 2NCH<sub>2</sub>), 7.38–7.56 (m, 5H, Ar-H) ppm; <sup>13</sup>C NMR (75 MHz, DMSO-*d*<sub>6</sub>): δ = 12.5 (CH<sub>3</sub>), 21.8, 26.7, 30.7, 35.9 (cyclohexyl-C), 43.8 (NCH<sub>2</sub>), 88.9 (pyridine-C<sub>3</sub>), 115.3 (CN), 125.2, 127.7, 128.5, 129.6, 137.2, 151.7, 160.0, 162.8 (Ar-C) ppm; IR (KBr):  $\bar{\nu}$  = 2223 (CN), 1597, 1568, 1492, 1460 (C=N and Ar-C=C) cm<sup>-1</sup>.

4-(4-Chlorophenyl)-2-(diethylamino)-5,6,7,8-tetrahydroquinoline-3-carbonitrile (**10b**, C<sub>20</sub>H<sub>22</sub>ClN<sub>3</sub>)

Yield 42 %; m.p.: 159–160 °C; <sup>1</sup>H NMR (300 MHz, DMSO-*d*<sub>6</sub>): δ = 1.23 (t, *J* = 6.9 Hz, 6H, 2 CH<sub>3</sub>), 1.69–1.75 (m, 2H, C<sub>6</sub>-H<sub>2</sub>), 1.78–1.84 (m, 2H, C<sub>7</sub>-H<sub>2</sub>), 2.35–3.01

(m, 2H, C<sub>5</sub>-H<sub>2</sub>), 2.94–3.00 (m, 2H, C<sub>8</sub>-H<sub>2</sub>), 3.67 (q, *J* = 6.9 Hz, 4H, 2NCH<sub>2</sub>), 7.37, 7.58 (2d, *J* = 7.8 Hz, each 2H, chlorophenyl-H) ppm; <sup>13</sup>C NMR (75 MHz, DMSO-*d*<sub>6</sub>): δ = 13.0(CH<sub>3</sub>), 22.3, 26.9, 30.7, 36.1 (cyclohexyl-C), 44.3 (NCH<sub>2</sub>), 92.4 (pyridine-C<sub>3</sub>), 115.7 (CN), 124.5, 127.9, 128.8, 132.7, 135.9, 150.2, 161.8, 163.6 (Ar-C) ppm; IR (KBr):  $\bar{\nu}$  = 2230 (CN), 1595, 1578, 1489, 1476 (C=N and Ar-C=C) cm<sup>-1</sup>.

## Animals

Frogs for the in vitro acetylcholinesterase testing were obtained from a local supplier, used within 2 days of their purchase and kept hydrated throughout the 2 days. Male albino rats (weighing 250–330 g) for the in vivo acetylcholinesterase and hepatotoxicity testing were obtained from the animal house of Faculty of Pharmacy, Beirut Arab University. The animals were housed under standard conditions as per the rules and regulations of the Faculty animal ethics committee (IRB) (approval code. 2014A-002-P-R-0004).

## In vitro acetylcholinesterase inhibitory activity [37]

All synthesized compounds were evaluated for their in vitro cholinesterase inhibitory effects on acetylcholine-induced contractions of the frog rectus abdominis. The triangular muscle of the frog was isolated and suspended in an organ bath of 10 cm<sup>3</sup> capacity containing aerated Ringer's solution which has been kept at room temperature. The contractions induced by acetylcholine (65 μg/cm<sup>3</sup>) were recorded by a force–displacement transducer connected to a Kymograph recorder. A submaximal dose of acetylcholine was determined using a contact time of 1.5 min in 3 min cycles. The solution of the tested compounds in dimethylformamide (1 μg/cm<sup>3</sup>) was added 10 min before adding the submaximal dose of acetylcholine. Five to seven observations were recorded for each compound. The percentage inhibition of acetylcholinesterase activity, as indicated by the percentage potentiation of acetylcholine-induced contractions, and the standard deviation were calculated as recorded in Table 1.

## In vivo acetylcholinesterase inhibitory activity

The rats were fasted overnight, and then given the solutions of the tested compounds at a dose of 10 mg/kg body weight, via intraperitoneal route. One hour after drug administration, the rats were sacrificed by decapitation. The whole brain was removed, immediately homogenized in 9 volumes of ice-cold 0.1 M sodium–potassium phosphate buffer (pH 8.0). The homogenate was centrifuged at

5000 rpm for 30 min and the supernatant was used for estimation of cholinesterase activity spectrophotometrically as reported by Ellman et al. [39].

### Measurement of acetylcholinesterase activity

Acetyl thiocholine reaction was initiated by mixing 0.1 cm<sup>3</sup> of the brain homogenate supernatant with 0.5 mM acetyl thiocholine and 0.33 mM 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) in phosphate buffer (pH 8.0). The produced thiocholine reacted with the DTNB to form a colored disulfide product whose intensity was measured kinetically over 10 min at 412 nm, using a Perkin Elmer type (Lambda 2S) spectrophotometer. Five to seven readings were recorded for each compound. The percent inhibition of acetyl cholinesterase activity and standard deviation were recorded in Table 2.

### Hepatotoxicity testing

The rats were given the solutions of the tested compounds in dimethylformamide, in a concentration of 7 mg/kg body weight, via intraperitoneal route. Control animals were given dimethylformamide. 24 h after drug administration, blood was collected in sterilized dry centrifuge tubes and allowed to coagulate for 10 min at 37 °C. The clear serum was separated at 6000 rpm for 10 min and subjected to biochemical investigation of serum glutamic pyruvated transaminase (SGPT). The kit used was obtained from Medilab in Beirut, Lebanon and contains L-alanine, buffer pH7.8, NADH, LDH, and oxoglutarate.

The reaction was affected by mixing 0.1 cm<sup>3</sup> of the serum sample with 1 cm<sup>3</sup> of the reconstituted kit, resulting in a colored product whose intensity was measured at 340 nm, using a Perkin Elmer type (Lambda 2S) spectrophotometer. Five to seven readings were recorded for each compound. The values are recorded in Table 3.

### Docking study

Computer-assisted simulated docking experiments were carried out under an MMFF94X force field using Molecular Operating Environment (MOE Dock 2009) software, Chemical Computing Group, Montreal, QC.

### Docking protocol

The coordinates from the X-ray crystal structure of AChE used in this simulation were obtained from the Protein Data Bank (PDB ID: 1DX4), where the active site is bound to the inhibitor tacrine derivative 9-(3-phenylmethylamino)-

1,2,3,4-tetrahydroacridine. The ligand molecules were constructed using the builder module and were energy minimized. The active site of AChE was generated using the MOE-Alpha Site Finder, and then ligands were docked within this active site using the MOE Dock. The lowest energy conformation was selected, and the ligand interactions (hydrogen bonding and hydrophobic interaction) with AChE were recorded.

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