DESIGN, SYNTHESIS AND EVALUATION OF SOME NEW 4-AMINOBENZOIC ACID DERIVATIVES AS POTENTIAL COGNITION ENHANCER

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ABSTRACT

A series of new Schiff bases from p-amino benzoic acid has been designed, synthesized and evaluated for cognition enhancing activities through the inhibition of acetylcholinesterase (AChE) and by passive avoidance model. The results illustrated a significant cognition enhancing effect on passive avoidance test with a significant reversal of scopolamine-induced amnesia, which is comparable with standard drug rivastigmine. The in-vitro study of synthesized compounds showed maximum activity of compound-9 compared to standard drug rivastigmine, whereas its enzyme kinetic study revealed a non-competitive inhibition of acetylcholinesterase (AChE), which may be attributed to a possible interaction of compound with the peripheral anionic site (PAS) of AChE and was also confirmed by molecular docking studies.

KEYWORDS: 4-aminobenzoic acid, acetylcholinesterase, passive avoidance test, rivastigmine.

INTRODUCTION

Alzheimer’s disease (AD) is a slowly progressive devastating neurodegenerative disorder of central nervous system manifested by deterioration of memory, cognitive functions,
behaviour change and impairment in performing activities of daily life.\textsuperscript{[1]} AD is characterised by progressive loss of cholinergic neurons and accumulation of β-amyloid proteins in the selective brain regions such as cortex and hippocampus.\textsuperscript{[2]} According to World Alzheimer Report, around 47 million people were reported to have dementia in 2016 with a global cost of $818 billion.\textsuperscript{[3]} At present, it is considered to be the sixth largest cause of death in the United States. The most promising approach to discover new cognitive enhancer based on the function of cholinergic nervous system to improve cholinergic neurotransmission achieved by preventing biotransformation of Ach at the specific areas of brain, on the basis of this approach some acetylcholinesterase inhibitors which are capable of suppressing the normal degradation of Ach in the synoptic cleft, have been established to increase the concentration of Ach and used to treat the AD.\textsuperscript{[4, 5]} Collective interpretation of the various molecular docking and dynamic studies on different AChE inhibitors suggest that allosteric modulation of hAChE catalytic activity is possible through binding of some ligands at the peripheral anionic site (PAS) constituted by amino acid residues Tyr-72, Tyr-124, Glu-285, Trp-286, and Tyr-341.\textsuperscript{[6,7]} Several amides and imides derivatives of \textit{m}-aminobenzoic acid and \textit{p}-aminobenzoic acid have been synthesized and evaluated for their anticholinesterase activity. This activity has also been evaluated in some benzophenone derivatives.\textsuperscript{[8]} Numerous Schiff bases of styrylpyridine and carbamate analogues of 4AP and have been synthesized and evaluated for their anticholinesterase activity.\textsuperscript{[9, 10]} Some 4-aminobutyric acid (GABA) and 2-indolinone analogues of 4AP have been also reported to possess antiamnesic activity.\textsuperscript{[11, 12]} The hydrazone analogues of dihydropyridine and indolinones have also been reported to elicit potent anticholinesterase, antibutyrylcholinesterase and β-amyloid aggregation properties.\textsuperscript{[13]} Several N-benzylpiperidine–purine, 3-Methylpyridinium and 2-thionaphthol analogues of berberine have evaluated for AChE and BChE inhibitory activity.\textsuperscript{[14, 15]} Keeping these facts in considerations, we synthesized and evaluated some new Schiff bases of 4-aminobutyric acid analogues as potential antiamnesic and cognition enhancing agents.

**RESULTS AND DISCUSSION**

**Chemistry**

Synthesis of Schiff bases was achieved by reaction of \textit{p}-amino benzoic acid with various substituted aromatic aldehydes and ketones according to reaction scheme-1.
Scheme 1: Schiff bases of \( p \)-aminobenzoic acid.

All the synthesized analogues were characterized by FTIR, \(^1\)HNMR, \(^{13}\)CNMR and elemental analysis in order to verify their purity. All the spectral characterization data were found to support the synthesis of Schiff bases. The synthesized compounds were characterised by FT-IR, \(^1\)H and \(^{13}\)C NMR. At the same time, the purity of compounds was checked further by...
TLC and combustion analysis. FTIR data proved the formation of Schiff bases as the C=N peak was observed at region of 1622-1630 cm\(^{-1}\). In \(^1\)H NMR spectra, compounds (1-5) showed peak at \(\delta\) 7.92-8.3 ppm, reflecting the presence of N=CH proton. In other compounds (6-10) where this single proton was substituted by different groups, resulted in the formation of N=C bond due to disappearance of peak at \(\delta\) 7.92-8.3 ppm which confirmed the substitution. The \(^{13}\)C NMR values of \(\delta\) 145 and 156-162 ppm confirmed the formation of N=CH and N=C bond respectively.

**Biological activity**

*Invitro AChE Inhibition*

AChE inhibitory activity of the all synthesized analogues was determined by using Ellman spectrophotometric method.\(^{[16]}\) The nature of AChE inhibition was elucidated by performing enzyme kinetics study of all synthesized analogues. The IC\(_{50}\) values of all synthesized analogues were calculated by using Graph Pad Prism. All the analogues exhibited moderate to excellent IC\(_{50}\) values. The IC\(_{50}\) values of analogues 9 & 5 are 7.49±0.16 \(\mu M\) & 7.92±0.94 \(\mu M\) respectively with respect to standard rivastigmine (6.15±0.57 \(\mu M\)). Further, enzyme kinetics study was also performed for all analogues to gain an insight on their nature of inhibition (Table 1).\(^{[17]}\) The most active compounds 9 and 5 demonstrated a non-competitive inhibition for AChE (Ki = 8.14± 0.65 and 12.45±0.57 respectively) enzyme (Table 1).

**Table 1: Cholinesterase activity and Enzyme kinetics study of synthesized derivatives and Rivastigmine.**

<table>
<thead>
<tr>
<th>Compound</th>
<th>AChE IC(_{50}) ((\mu M)) ± SEM</th>
<th>AChE Ki ((\mu M)) ± SEM</th>
<th>Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>10.42±0.42</td>
<td>21.52±1.48</td>
<td>c</td>
</tr>
<tr>
<td>2</td>
<td>11.46±0.60</td>
<td>18.14± 0.66</td>
<td>nc</td>
</tr>
<tr>
<td>3</td>
<td>8.84±0.57</td>
<td>15.62±0.42</td>
<td>c</td>
</tr>
<tr>
<td>4</td>
<td>26.52±0.84</td>
<td>54.16±1.26</td>
<td>c</td>
</tr>
<tr>
<td>5</td>
<td>7.92±0.94</td>
<td>12.45±0.57</td>
<td>nc</td>
</tr>
<tr>
<td>6</td>
<td>38.48±0.76</td>
<td>58.18±0.84</td>
<td>c</td>
</tr>
<tr>
<td>7</td>
<td>20.15±0.65</td>
<td>34.54±0.80</td>
<td>c</td>
</tr>
<tr>
<td>8</td>
<td>14.64±0.60</td>
<td>24.42±0.94</td>
<td>nc</td>
</tr>
<tr>
<td>9</td>
<td>7.49±0.16</td>
<td>8.14± 0.65</td>
<td>nc</td>
</tr>
<tr>
<td>10</td>
<td>9.25±0.80</td>
<td>14.62± 0.76</td>
<td>nc</td>
</tr>
<tr>
<td>Rivastigmine(^*)</td>
<td>6.15±0.57</td>
<td>130.9 ± 0.6</td>
<td>c</td>
</tr>
</tbody>
</table>

\(^{c}=\text{competitive, nc=noncompetitive.}\)
The non-competitive inhibition is attributed to a possible interaction of compound with the peripheral anionic site (PAS) of AChE and was also confirmed by docking studies.[18]

**Passive avoidance test**

The synthesized analogues were then evaluated for antiamnesic and cognition enhancing activities by passive avoidance test.[19] In this test, animals received punishment when it enters the dark room during the training session and thus remembers it in the session on the following day, unless their memory is impaired due to the amnesic drug. Pre-treatment with tested compounds resulted in elevate entry latency as compared to control group in significant and dose dependent manner, indicating facilitated learning process. A prolonged latency indicates that the animal remembers that it has been punished and therefore, does avoid the darken chamber. The effect of inhibitors compound 9 and compound 5 on changes in entry latency in scopolamine-induced amnesia showed significant differences [p < 0.05] among treated groups (Table 2). Post-hoc analysis revealed that scopolamine (1.5 mg/kg) significantly [p < 0.05] decreased entry latency as compared to control group indicating amnesia. The inhibitors compound 9, 5 and rivastigmine, dose dependently reversed scopolamine-induced decrease in entry latency.

**Table 2: Effect of synthesized derivative and Rivastigmine on cognition enhancing and scopolamine-induced amnesia on rat passive avoidance test.**

<table>
<thead>
<tr>
<th>Treatment [Dose(mg/kg)]</th>
<th>Entry latency (s)</th>
<th>Training trial</th>
<th>Retention trial</th>
<th>Δ</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>18.83±0.60</td>
<td>96.51±0.76</td>
<td>77.68</td>
<td></td>
</tr>
<tr>
<td>3 (3.0)</td>
<td>22.74±0.49</td>
<td>157.48±0.66a</td>
<td>134.74</td>
<td></td>
</tr>
<tr>
<td>3 (6.0)</td>
<td>23.61±0.57</td>
<td>163.62±0.47b</td>
<td>140.01</td>
<td></td>
</tr>
<tr>
<td>5 (3.0)</td>
<td>20.36±0.66</td>
<td>120.68±0.66a</td>
<td>100.32</td>
<td></td>
</tr>
<tr>
<td>5 (6.0)</td>
<td>21.62±0.60</td>
<td>135.42±0.60a</td>
<td>113.8</td>
<td></td>
</tr>
<tr>
<td>9 (3.0)</td>
<td>17.56±0.66</td>
<td>176.62±0.76a</td>
<td>159.06</td>
<td></td>
</tr>
<tr>
<td>9 (6.0)</td>
<td>18.48±0.98</td>
<td>208.44±0.66a</td>
<td>189.96</td>
<td></td>
</tr>
<tr>
<td>Riva (3.0)</td>
<td>14.68±0.60</td>
<td>184.56±0.57a</td>
<td>169.88</td>
<td></td>
</tr>
<tr>
<td>Riva (6.0)</td>
<td>14.46±0.76</td>
<td>200.84±0.66a</td>
<td>186.38</td>
<td></td>
</tr>
<tr>
<td>SCP (1.5)</td>
<td>20.12±0.63</td>
<td>35.17±0.60a</td>
<td>15.05</td>
<td></td>
</tr>
<tr>
<td>3 (3.0) +SCP (1.5)</td>
<td>20.84±0.60</td>
<td>98.46±0.60b</td>
<td>77.62</td>
<td></td>
</tr>
<tr>
<td>3 (6.0) +SCP</td>
<td>20.17±0.60</td>
<td>96.41±0.66b</td>
<td>76.24</td>
<td></td>
</tr>
<tr>
<td>5 (3.0) +SCP</td>
<td>21.00±0.63</td>
<td>94.84±0.60b</td>
<td>73.84</td>
<td></td>
</tr>
<tr>
<td>5 (6.0) +SCP</td>
<td>20.38±0.80</td>
<td>92.46±0.60b</td>
<td>72.08</td>
<td></td>
</tr>
<tr>
<td>9 (3.0) +SCP</td>
<td>19.51±0.76</td>
<td>86.14±0.60b</td>
<td>66.63</td>
<td></td>
</tr>
<tr>
<td>9 (6.0) +SCP</td>
<td>16.48±0.49</td>
<td>88.00±0.63b</td>
<td>71.52</td>
<td></td>
</tr>
<tr>
<td>Riva (3.0) +SCP</td>
<td>19.62±0.63</td>
<td>89.36±0.60b</td>
<td>69.74</td>
<td></td>
</tr>
<tr>
<td>Riva (6.0) +SCP</td>
<td>17.28±0.60</td>
<td>97.58±0.49b</td>
<td>80.3</td>
<td></td>
</tr>
</tbody>
</table>
Data are expressed as mean ± SEM (n = 6). Data were statistically analyzed by one way ANOVA. aSignificantly different from control p < 0.05. bSignificantally different from scopolamine treated group p < 0.05. SCP=Scopolamine
Δ=Difference between Retention trial and Training trial, Riva= Rivastigmine.

**Molecular docking**
Docking studies were carried out to provide a better interpretation of the biological profile of compound 9 and 5 toward AChE. It was observed that compound 9 and 5 were properly positioned into the enzyme gorge and showed interaction with the internal amino acid residue Phe-330, Phe-331 and Trp-84 by means of a π–π interaction. (Fig1&2)
Fig. 1: Molecular docking of compound 9 into the active sites of AChE (A-2D, B-3D). Ligand is in green colour, dotted show H-bond interaction.
Ligand is in green colour, dotted show H-bond interaction.

The study clearly demonstrated that both compounds were able to bind with the key peripheral anionic site (PAS) residue Trp-84, Tyr-334, Phe-330 and Phe-331. The hydroxyl group of both phenyl of compound 9 were involved in forming a hydrogen bond with Tyr-130 and Asp-72 and carboxylic oxygen of 5 was involved in forming a hydrogen bond with Phe-288 (determine substrate specificity). The hydroxyl group of compound 9 was involved in forming a hydrogen bond with Tyr-130 and Asp-72 suggested that the compounds might probably act via the AChE inhibition.

CONCLUSIONS
From the above study, it was concluded that the hydroxyl substituted compounds 9 and 5 demonstrated a comparable activity with that of rivastigmine. In docking studies, the hydroxyl group of one of the phenyl rings of these compounds was observed establishing H-bond with Tyr-130 which plays a dual role in the active centre: (a) its hydroxyl appears to maintain the functional orientation of Glu-202 by hydrogen bonding and (b) its aromatic moiety maintains the functional orientation of the subsite Phe-330 and Phe-331.
Experimental

Instrumentation and chemicals
All the chemicals used in the study were of analytical grade purity and were procured from Sigma-Aldrich (India). Rivastigmine was obtained as a gift sample from Sun Pharmaceutical Industries Ltd (Silvassa, India). Melting point of the compounds was determined in open capillary tubes using a BI 9300 Bumstead/Electrothermal Stuart (SMPIO) melting point apparatus and were uncorrected. The reaction progress was monitored by thin layer chromatography with Ethyl acetate: Pyridine: Acetic acid: water (100:18.5:2.5:5) as the mobile phase on TLC silica gel 60 F254 aluminium sheets obtained from Merck company and activated at 110°C for 10 min. Iodine was used for the colour visualization of the spots. UV spectral analysis was performed on JASCO (Model 7800) UV-VIS spectrophotometer. FTIR spectra were recorded on a Shimadzu FTIR 8400S spectrophotometer at the scanning range of 400–4000 cm⁻¹. ¹H and ¹³C NMR spectra were recorded on a JEOL AL 300 FT-NMR spectrophotometer in deuterated chloroform and deuterated dimethylsulfoxide as solvent and are recorded in parts per million (ppm) downfield from Tetramethylsilane (Me₄Si) as internal reference. Elemental analysis was performed using Exeter CE-440 elemental analyzer.

Syntheses
The syntheses of Schiff base analogues of p-aminobenzoic acid were carried out using the procedures as given in Scheme 1.

General procedure for the synthesis of compounds (1-10)
p-aminobenzoic acid (0.05mol) was dissolved in 5ml of methanol in a 250-ml conical flask and was stirred at room temperature for 15 min to get a clear solution. To this solution, equimolar quantity (0.05mol) of each substituted aryl aldehydes (in methanol) were added with few drops of concentrated hydrochloric acid (catalyst) and reaction mixture was refluxed with stirring upto 12–18h at 70°C on magnetic stirrer. The reaction progress was monitored by TLC using mobile phase as Ethyl acetate: Pyridine: Acetic acid: water (100:18.5:2.5:5) on completion of reaction, compounds were obtained by precipitation on addition of 10 ml ethylacetate and recrystallized by ethylacetate and absolute methanol.[²⁰]

4-(2, 4, 6-Trihydroxybenzylidene amino) benzoic acid (1)
Yield: 68.7%, m.p.: 142-144°C, Rf 0.65, IR (KBr, cm⁻¹): 3525 (OH, Phenolic), 3510 (OH, COOH), 3015(=CH, Aromatic), 1703 (C=O, COOH), 1628 (C=N), 1470 (C=C, Aromatic);
1H NMR (DMSO-\textit{d}_6) (δ ppm): 11.32 (s, 1H, COOH), 8.3 (s, 1H, N=CH), 6.72-8.37 (m, 6H, aromatic), 5.68 (s, 3H, Phenolic); 13C NMR (δ ppm): 171.24 (COOH), 145.24 (N=CH), 165.14, 164.65, 142.83, 130.83, 126.76, 122.32, 106.22, 97.66 (Aromatic); Anal. calcd (%) for C_{14}H_{11}NO_5: C 61.54, H 4.06, N 5.13; found (%)C 61.44, H 4.10, N 5.17.

4-(2-Methoxybenzylidene amino) benzoic acid (2)

Yield: 67.8%, m.p.: 163-165°C, R_f 0.54, IR (KBr, υ cm^{-1}): 3510 (OH, COOH), 3015 (=CH, Aromatic), 2905 (CH, CH_3), 1703 (C=O, COOH), 1630 (C=N), 1456 (C=C, Aromatic); 1H NMR (DMSO-\textit{d}_6) (δ ppm): 11.33 (s, 1H, COOH), 7.95 (s, 1H, N=CH), 7.10-8.39 (m, 8H, aromatic), 4.93 (s, 3H, OCH_3); 13C NMR (δ ppm): 171.62 (COOH), 145.42 (N=CH), 162.26, 142.36, 132.78, 131.52, 130.78, 126.72, 122.35, 117.36, 115.00 (Aromatic), 58.66 (OCH_3); Anal. calcd (%) for C_{15}H_{13}NO_3: C 70.58, H 5.13, N 5.49; found (%)C 70.62, H 5.10, N 5.44.

4-(2, 4-Dimethoxybenzylidene amino) benzoic acid (3)

Yield: 68.6%, m.p.: 155-157°C, R_f 0.58, IR (KBr, υ cm^{-1}): 3510 (OH, COOH), 3015 (=CH, Aromatic), 2905 (CH, CH_3), 1703 (C=O, COOH), 1625 (C=N), 1456 (C=C, Aromatic); 1H NMR (DMSO-\textit{d}_6) (δ ppm): 11.36 (s, 1H, COOH), 7.96 (s, 1H, N=CH), 7.16-8.38 (m, 7H, aromatic), 4.96 (s, 6H, OCH_3); 13C NMR (δ ppm): 171.54 (COOH), 145.44 (N=CH), 165.25, 162.24, 142.66, 132.62, 131.82, 111.36, 108.26, 105.02, 126.68, 122.32, (Aromatic), 58.26 (OCH_3); Anal. calcd (%) for C_{16}H_{15}NO_4: C 67.36, H 5.30, N 4.91; found (%)C 67.41, H 5.33, N 4.87.

4-(2, 4, 6-Trimethoxybenzylidene amino) benzoic acid (4)

Yield: 64.5%, m.p.: 148-150°C, R_f 0.68, IR (KBr, υ cm^{-1}): 3510 (OH, COOH), 3015 (=CH, Aromatic), 2905 (CH, CH_3), 1703 (C=O, COOH), 1622 (C=N), 1456 (C=C, Aromatic); 1H NMR (DMSO-\textit{d}_6) (δ ppm): 11.31 (s, 1H, COOH), 7.94 (s, 1H, N=CH), 7.16-8.37 (m, 6H, aromatic), 4.96 (s, 9H, OCH_3); 13C NMR (δ ppm): 171.54 (COOH), 145.42 (N=CH), 165.34, 162.64, 142.36, 131.42, 103.33, 93.66, 126.73, 122.26, (Aromatic), 58.36 (OCH_3); Anal. calcd (%) for C_{17}H_{17}NO_5: C 64.75, H 5.43, N 4.44; found (%)C 64.66, H 5.38, N 4.48.

4-(4-Hydroxy-3, 5-dimethoxybenzylidene amino) benzoic acid (5)

Yield: 67.4%, m.p.: 168-170°C, R_f 0.57, IR (KBr, υ cm^{-1}): 3530(OH, Phenolic), 3510 (OH, COOH), 3015 (=CH, Aromatic), 2905 (CH, CH_3), 1703 (C=O, COOH), 1627 (C=N), 1456 (C=C, Aromatic); 1H NMR (DMSO-\textit{d}_6) (δ ppm): 11.37 (s, 1H, COOH), 7.92 (s, 1H, N=CH), 7.15-8.37 (m, 6H, aromatic), 5.65 (s, 1H, Phenolic), 4.92 (s, 6H, OCH_3); 13C NMR (δ ppm):
171.29 (COOH), 145.42 (N=CH), 153.26, 135.82, 142.36, 131.72, 129.32, 127.35, 126.55, 122.36, (Aromatic), 58.65 (OCH3); Anal. calcd (%) for C_{16}H_{15}NO_{5}: C 63.78, H 5.02, N 4.65; found (%)C 63.75, H 5.09, N 4.70.

4-(1-(4-methoxyphenyl)propyldene amino) benzoic acid (6)
Yield: 69.7%, m.p.: 132-134°C, R\textsubscript{f} 0.45, IR (KBr, \textit{υ}\textsubscript{cm}^{-1}): 3510 (OH, COOH), 3015 (=CH, Aromatic), 2905 (CH, CH\textsubscript{3}), 1703 (C=O, COOH), 1623 (C=N), 1500, 1456 (C=C, Aromatic); \textsuperscript{1}H NMR (DMSO-\textit{d}_6) (δ ppm): 11.38 (s, 1H, COOH), 7.35-8.34 (m, 8H, aromatic), 4.98 (s, 3H, OCH\textsubscript{3}), 1.34 (t, 3H, CH\textsubscript{3}), 0.93 (q, 2H, CH\textsubscript{2}); \textsuperscript{13}C NMR (δ ppm): 171.62 (COOH), 162.62 (N=C), 163.62, 142.37, 132.13, 131.81, 127.27, 126.13, 122.78, 115.13 (Aromatic), 58.27 (OCH\textsubscript{3}), 19.13 (CH\textsubscript{2}); Anal. calcd (%) for C\textsubscript{17}H\textsubscript{17}NO\textsubscript{3}: C 72.07, H 6.05, N 4.94; found (%)C 72.13, H 6.10, N 4.88.

4-(1-(4-hydroxy-3-methoxyphenyl amino) benzoic acid (7)
Yield: 667%, m.p.: 148-150°C, R\textsubscript{f} 0.66, IR (KBr, \textit{υ}\textsubscript{cm}^{-1}): 3525 (OH, Phenolic), 3510 (OH, COOH), 3015 (=CH, Aromatic), 2905 (CH, CH\textsubscript{3}), 1703 (C=O, COOH), 1628 (C=N), 1500, 1456 (C=C, Aromatic); \textsuperscript{1}H NMR (DMSO-\textit{d}_6) (δ ppm): 11.28 (s, 1H, COOH), 7.32-8.35 (m, 7H, aromatic), 5.64 (s, 3H, Phenolic), 4.93 (s, 3H, OCH\textsubscript{3}), 1.32 (t, 3H, CH\textsubscript{3}), 0.90 (q, 2H, CH\textsubscript{2}); \textsuperscript{13}C NMR (δ ppm): 171.52 (COOH), 162.25 (N=C), 152.26, 148.42, 142.36, 131.48, 129.71, 128.29, 127.72, 126.72, 122.32, 117.34, 115.11 (Aromatic), 57.29 (OCH\textsubscript{3}), 19.29 (CH\textsubscript{3}); Anal. calcd (%) for C\textsubscript{17}H\textsubscript{17}NO\textsubscript{4}: C 68.21, H 5.72, N 4.68; found (%)C 68.26, H 5.66, N 4.56.

4-((4-hydroxyphenyl)(phenyl)methylene amino) benzoic acid (8)
Yield: 76.2%, m.p.: 167-169°C, R\textsubscript{f} 0.42, IR (KBr, \textit{υ}\textsubscript{cm}^{-1}): 3525 (OH, Phenolic), 3510 (OH, COOH), 3015 (=CH, Aromatic), 1703 (C=O, COOH), 1630 (C=N), 1500, 1456 (C=C, Aromatic); \textsuperscript{1}H NMR (DMSO-\textit{d}_6) (δ ppm): 11.44 (s, 1H, COOH), 6.85-8.34 (m, 13H, aromatic), 5.65 (s, 1H, Phenolic OH); \textsuperscript{13}C NMR (δ ppm): 171.24 (COOH), 156.71 (N=C), 162.46, 142.31, 133.36, 131.71, 130.01, 129.31, 128.77, 126.31, 125.15, 122.33, 116.02 (Aromatic); Anal. calcd (%) for C\textsubscript{20}H\textsubscript{15}NO\textsubscript{3}: C 75.70, H 4.76, N 4.41; found (%)C 75.70, H 4.76, N 4.41.

4-(bis(4-hydroxyphenyl)methylene amino) benzoic acid (9)
Yield: 70.6%, m.p.: 156-158°C, R\textsubscript{f} 0.67, IR (KBr, \textit{υ}\textsubscript{cm}^{-1}): 3525 (OH, Phenolic), 3510 (OH, COOH), 3015 (=CH, Aromatic), 1703 (C=O, COOH), 1640 (C=N), 1500, 1456 (C=C, Aromatic), 171.29 (COOH), 145.42 (N=CH), 153.26, 135.82, 142.36, 131.72, 129.32, 127.35, 126.55, 122.36, (Aromatic), 58.65 (OCH3); Anal. calcd (%) for C_{16}H_{15}NO_{5}: C 63.78, H 5.02, N 4.65; found (%)C 63.75, H 5.09, N 4.70.
Aromatic; $^1$H NMR (DMSO-$d_6$) ($\delta$ ppm): 11.22 (s, 1H, COOH), 6.80-8.32 (m, 12H, aromatic), 5.68 (s, 2H, Phenolic OH); $^{13}$C NMR ($\delta$ ppm): 171.86 (COOH), 156.28 (N=C), 161.26, 142.36, 131.85, 130.82, 126.26, 125.36, 122.64, 116.56 (Aromatic); Anal. calcd (%) for C$_{26}$H$_{15}$NO$_4$: C 72.06, H 4.54, N 4.20; found (%)C 72.12, H 4.60, N 4.17.

4-((2, 4-dimethoxyphenyl) (4-hydroxyphenyl)methylene amino) benzoic acid (10)
Yield: 67.4%, m.p.: 184-186°C, R$_f$ 0.57, IR (KBr, $\nu$cm$^{-1}$): 3525 (OH, Phenolic), 3510 (OH, COOH), 3015 (=CH, Aromatic), 2904 (CH, CH$_3$), 1703 (C=O, COOH), 1629 (C=N), 1500, 1456 (C=C, Aromatic); $^1$H NMR (DMSO-$d_6$) ($\delta$ ppm): 11.36 (s, 1H, COOH), 6.82-8.31 (m, 11H, aromatic), 5.65 (s, 1H, Phenolic OH), 4.95 (s, 6H, OCH$_3$); $^{13}$C NMR ($\delta$ ppm): 171.24 (COOH), 156.24 (N=C), 165.43, 162.40, 161.59, 142.35, 132.43, 131.82,126.74, 125.25, 122.43, 116.30, 109.43, 107.02 102.32, (Aromatic), 61.16 (OCH$_3$); Anal. calcd (%) for C$_{22}$H$_{19}$NO$_5$: C 70.02, H 5.07, N 3.71; found (%)C 70.08, H 5.04, N 3.77.

**Biological studies**

**Estimation of cholinesterase activity (in-vitro)**
The effectiveness of tested compounds to inhibit acetylcholinesterase from electric eel (E.C. 3.1.1.7) could be conclusive through their IC$_{50}$ values. Ellman’s spectrophotometric analysis was used to determine IC$_{50}$ values, which was performed by recording the rate of increase in the absorbance at 412 nm for 5 min.$^{[16]}$ Stock solution of AChE was prepared by dissolving AChE in 0.1 M phosphate buffer (pH 8.0). The final solution for assay consisted of 0.1 M phosphate buffer (pH 8.0) with the addition of 340 mM 5, 5’-dithio-bis (2-nitrobenzoic acid) (DTNB), 0.02 unit/mL of AChE and 550 mM of substrate (acetylthiocholine iodide, ATChI). Different concentrations of test compounds (inhibitors) between 20% and 80% were selected in order to obtain inhibition of the enzymatic activity. From the aliquots (50 $\mu$L), increasing concentrations of the inhibitors were added to the assay solution and were pre-incubated for 20 min at 37 °C with the enzyme followed by the addition of substrate. For this assay blank consisted of all the components except AChE in order to account for the non-enzymatic reaction. Further, reaction rates of the assay were compared and the percent inhibition due to the presence of increasing concentrations of inhibitor was calculated. The concentration of each test compound was recorded in triplicate, and their IC$_{50}$ values were determined graphically from log concentration percent inhibition curves.$^{[21]}$
Enzyme kinetics study
The Ellman’s spectrophotometric analysis was used to identify the type of inhibition in this study. acetylthiocholine iodide was used as a substrate in different concentrations, both below and above, near to Km in a phosphate buffer at pH 8, with a fixed concentration of cholinesterase in the absence or presence of different inhibitors. The concentration of the inhibitors was kept close to one that corresponds to IC\textsubscript{50} value of enzyme inhibition. Further, their inhibitory kinetics was evaluated by the Lineweaver and Burk method.\cite{17}

Animals
Charles foster rats of albino strain (4 to 5 months old and 150 to 200 g in weight) of either sex were procured from Central Animal House, Faculty of Pharmacy, Pacific academy of higher education & research university Udaipur (Registration No. 1622/PO/a/12/CPCSEA). They had free access to water \textit{ad-libitum} and were fed with semi synthetic balanced diet, with occasional supply of green vegetables (salad leaves). Six rats were housed per cage at temperature (22 °C ± 3° C) and 45-55% relative humidity. Twelve hours of light and dark cycles was strictly followed in a fully ventilated room.

Acute toxicity evaluation
The guidelines proposed by OECD (425) was used for determining acute toxicity studies of the analogues.\cite{22} In this study, nulliparous, non-pregnant, healthy female albino rats weighing between 150-200 gm were fasted overnight with water \textit{ad-libitum} prior to test. On the experimental day, analogues were administered at graded dose up to 100 mg/kg p.o in 0.3% carboxymethyl cellulose as vehicle. The animals were further monitored continuously for 30 min, 2 h and up to 48 h to detect any changes in the autonomic or behavioural responses and also for tremors, convulsions, salivation, diarrhoea, sleep, lacrimation, heart rate, pulse rate, blood pressure and feeding behaviour as a sign of acute toxicity.

Drug treatment
The synthesized compounds and standard rivastigmine were suspended in 0.3% carboxymethyl cellulose and was administered orally at a dose of 3 mg/kg and 6 mg/kg. All animals of control groups were treated with 0.3% carboxymethyl cellulose equal to volume of experimental drugs.
Passive avoidance task
The apparatus consists of two identical light and dark compartment with grid floors which can be electrified separately, where a guillotine door connects the two compartments. During the training trial, each rat was placed in the light compartment and after 10 s, the door was raised. As soon as the animal was placed with all four paws in the dark compartment, the door gets automatically closed and an electrical foot shock (0.02 mA/10 g body weight) lasting 2 s was delivered. The time elapsed by the rat being placed in light and entering the dark compartment was recorded as training trial entry latency time. Retention trial was performed 24 h after the training trial, following the similar procedure except that, the electric shock was not given and entry into the dark compartment was measured. The synthesized compounds and rivastigmine were suspended in 0.3% carboxymethyl cellulose and were administered orally 90 min before the training session, while amnesic drug was injected immediately after termination of the training session. The maximum entry latency allowed in the retention session was 120 s.\(^{[23,24]}\)

Molecular docking
Preparation of the small molecules
All the molecules with their inhibition activities were taken and 3D structures were sketched using Maestro 9.3 and geometrically minimized with Macromodel 9.9 based on OPLS-2005 force field.

Preparation of the protein
The crystal structure of AChE was retrieved from the protein data bank, pdb code: 1B41 (Resolution 2.8 Å, Average R-value 0.234).\(^{[25]}\) The structure was prepared by protein preparation wizard in Maestro 9.3, including adding hydrogens, assigning partial charges using the OPLS-2005 force field and assigning protonation states, restrained, partial energy minimization and the resulting structure was used as the receptor model in the following studies.

Molecular docking
For the receptor structure, crystallographic and trajectory water molecules, ions and ligand compounds were removed. Proteins were prepared using Schrodinger software, Maestro 9.3 and Glide 5.8. The Glide XP algorithm was employed using a grid box volume of 10_10_10 Å. All the structures were fitted in binding pocket and the lowest energy pose for each docking run was retained.\(^{[26]}\)
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REFERENCES


