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DESIGN, SYNTHESIS AND EVALUATION OF *p*-AMINOBENZOIC ACID AND 4-AMINOPYRIDINE ANALOGUES AS CHOLINESTERASE INHIBITORS FOR MANAGEMENT OF ALZHEIMER'S DISEASES

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ABSTRACT

Some new Schiff bases of *p*-aminobenzoic acid and semicarbazones of 4-aminopyridine has been designed, synthesized, characterized by analytic methods such as UV, IR, NMR, elemental analysis and evaluated for cognition enhancing activities through the inhibition of acetylcholinesterase (AChE) by applying the molecular docking studies, performed enzyme kinetics study by Ellman's spectrophotometric method 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-**2** and **5** 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: *P*-Aminobenzoic Acid, 4-Aminopyridine, Acetylcholinesterase, Passive Avoidance Test, Rivastigmine.

INTRODUCTION

The progressive loss of cholinergic neurons, accumulation of β-amyloid proteins and formation of neurofibrillary tangles of hyperphosphorylated tau protein in the selective brain regions is the most common cause of Alzheimer's disease (AD),[1] which is devasting neurodegenerative disorder of central nervous system.[2] According to World Alzheimer Report, around 47 million people were reported to have dementia in 2016 with a global cost of $818 billion.[3] At present, it is considered to be the sixth largest cause of death in the United States. The U.S. Food and Drug Administration (FDA) have approved only five medicines for the treatment of AD till date, among which four are AChE inhibitors. The function of cholinergic nervous system to improve cholinergic neurotransmission achieved by preventing degradation of Ach at the specific areas of brain is the most promising approach to discover new cognitive enhancer, 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.[4,5] Assorted molecular docking and dynamic studies on AChE inhibitors revealed that modulation of AChE catalytic activity is possible through binding of ligands at the peripheral anionic site (PAS) constituted by amino acid residues Tyr-72, Tyr-124, Trp-286 and Tyr-341.[6,7] The hydrazone derivatives of 3-methylpyridinium, dihydropyridine and indolinones have been reported as potent anticholinesterase activity. Numerous Schiff bases of styrylpyridine have been synthesized and evaluated for their anticholinesterase activity.[8] Some 2-indolinone and 4-aminobutyric acid (GABA) derivatives andderivatives of 4AP have also been reported to possess antiamnesic activity.[9,10] Several amides and imides derivatives of *m*-aminobenzoic acid and *p*-aminobenzoic acid have been synthesized and evaluated for their anticholinesterase activity, which suggested that, para-substituted derivatives are prominently active than their meta and ortho-substituted derivatives[11,12] Numerous Schiff bases of styrylpyridine and carbamate analogues of 4AP and have been synthesized and evaluated for their anticholinesterase activity.[13,14] Some 2-indolinone analogues of 4AP and 4-aminobutyric acid (GABA) have been also reported to possess antiamnesic activity.[15] The hydrazone analogues of dihydropyridine and indolinones have also been reported to elicit potent anticholinesterase and antibutyrylcholinesterase activity.[16,17] Several N-benzylpiperidine–purine, 3-Methylpyridinium, Novel Oximes, Phenitidine Derivatives, Phenyl Benzamide Derivatives and 2-thionaphthol analogues of berberine have evaluated for AChE and BChE inhibitory activity.[18-22] AD is associated with aging and more prone to geriatric persons. Since, humans and rats exhibit similar age-related alterations[23-25] therefore, estimation of age-related cognitive impairment and spatial memory deficit in aged rats may

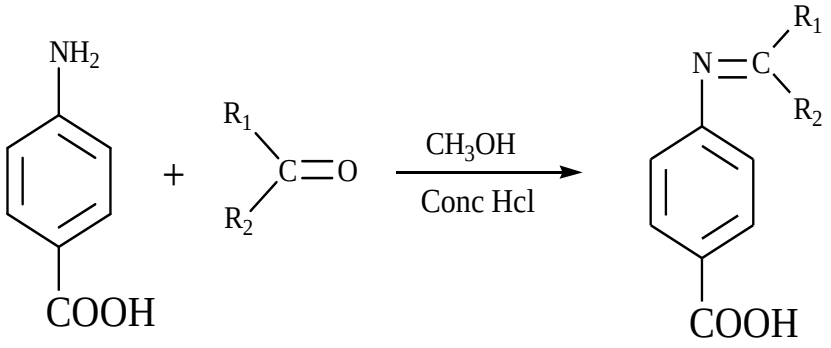
provide more beneficial information associated to humans.[26] Consider these facts, in the present study we synthesized some new Schiff bases of 4- aminobenzoic acid analogues and 4-aminopyridine (4AP) analogues are under intensive investigation due to their antiacetylcholinesterase activity which has shown promising effects in eliminating memory related dysfunctions.

MATERIALS AND METHODS

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 points of the compounds was determined on Veego 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) and chloroform:methanol (6:4) as the mobile phase on TLC silica gel 60 F254 aluminum sheets obtained from Merck company and activated at 110°C for 10 min. Iodine was used for the color visualization of the spots. UV spectral analysis was performed on JASCO (Model 7800) UV-VIS spectrophotometer. FTIR spectra were recorded on a Perkin Elmer Spectrum Version 10.03.08 spectrophotometer at the scanning range of 400–4000 cm^{-1}. ^{1}H and ^{13}C NMR spectra were recorded using a Bruker Avance II400MHz 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 Perkin–Elmer-2400 analyzer.

Syntheses

The syntheses of Schiff base analogues of *p*-aminobenzoic acid and semicarbazide of 4-aminopyridine analogues were carried out using the procedures as given in Scheme**1** and Scheme **2.**



Scheme. 1: Schiff bases of *p*-aminobenzoic acid.

General procedure for the synthesis of Schiff base analogues (1-4)

p-aminobenzoic acid (0.05mol)was dissolved in 5ml of methanol in a 250-ml conical flask and was stirred at room temperature for15 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 upto12–18h 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.[27]

(Z)-4-(2, 4-Dihydroxybenzylidene amino) benzoic acid (compound-1)

Yield: 70.7%, m.p.: 141-143^{o}C, R_f 0.67, IR (KBr, υcm^{-1}): 3526 (OH, Phenolic), 3509 (OH, COOH), 3014(=CH, Aromatic), 1705 (C=O, COOH), 1625 (C=N), 1469 (C=C, Aromatic); ^{1}H NMR (DMSO-d_6) (δ ppm): 11.33 (s, 1H, COOH), 8.2 (s, 1H, N=CH), 6.73-8.36 (m, 7H, aromatic), 5.67 (s, 3H, Phenolic); ^{13}C NMR (δ ppm): 172.12 (COOH), 146.22 (N=CH), 164.16, 163.66, 143.84, 131.82, 125.77, 123.30, 111.1, 106.20, 96.69 (Aromatic); Anal. calcd (%) for $C_{14}H_{11}NO_4$: C 65.34., H 4.30, N 5.46; found (%)C 65.14., H 4.16, N 5.20.

(Z)-4-(2, 4, 6-Trihydroxybenzylidene amino) benzoic acid (compound-2)

Yield: 68.7%, m.p.: 142-144^{o}C, R_f 0.65, IR (KBr, υcm^{-1}): 3525 (OH, Phenolic), 3510 (OH, COOH), 3015(=CH, Aromatic), 1703 (C=O, COOH), 1628 (C=N), 1470 (C=C, Aromatic); ^{1}H NMR (DMSO-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); ^{13}C 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.

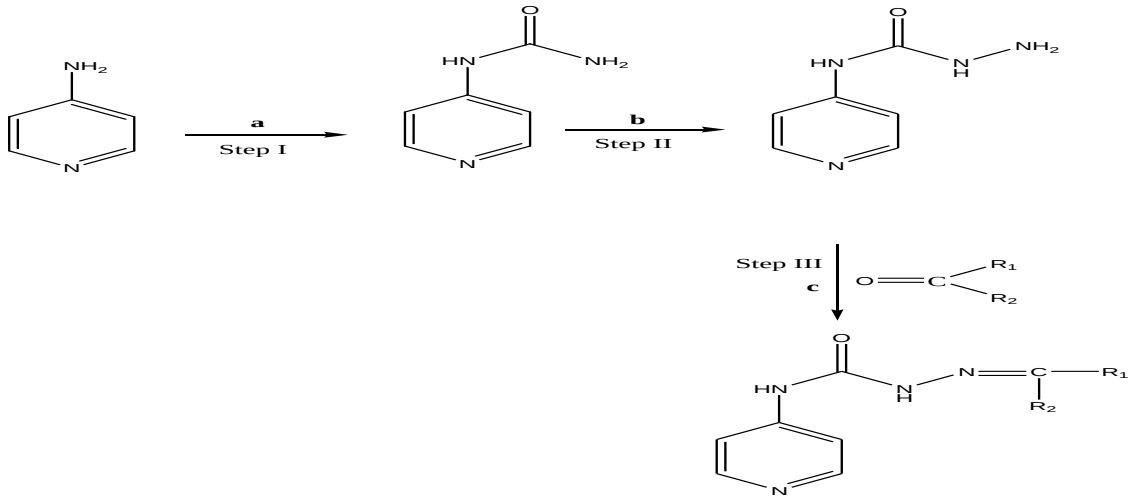
(Z)-4-(1-phenylpropylidene amino) benzoic acid (compound-3)

Yield: 65.2%, m.p.: 127-129^{o}C, R_f 0.41, IR (KBr, υcm^{-1}): 3510 (OH, COOH), 3015 (=CH, Aromatic), 1703 (C=O, COOH), 1623 (C=N), 1500, 1456 (C=C, Aromatic); ^{1}H NMR (DMSO-d_6) (δ ppm): 11.38 (s, 1H, COOH), 7.35-8.34 (m, 9H, aromatic), 1.34 (t, 3H, CH3), 0.93 (q, 2H, CH2); ^{13}C NMR (δ ppm): 171.62 (COOH), 162.62 (N=C), 163.62, 142.37, 132.13, 131.81, 128.29, 127.27, 126.13, 122.78, 115.13 (Aromatic), 19.13 (CH2), 8.66 (CH3); Anal. calcd (%) for $C_{16}H_{15}NO_2$: C 75.77, H 6.01, N 5.64; found (%)C 75.13, H 6.09, N 5.88.

(Z)-4-(1-(4-methoxyphenyl) ethylidene amino) benzoic acid (compound-4)

Yield: 69.7%, m.p.: 132-134°C, R_f 0.45, IR (KBr, υcm^{-1}): 3510 (OH, COOH), 3015 (=CH, Aromatic), 2905 (CH, CH3), 1703 (C=O, COOH), 1623 (C=N), 1500, 1456 (C=C, Aromatic); ^{1}H NMR (DMSO-d_6) (δ ppm): 11.38 (s, 1H, COOH), 7.35-8.34 (m, 8H, aromatic), 4.98 (s, 3H, OCH3), 1.34 (t, 3H, CH3); ^{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 (OCH3), 8.66 (CH3); Anal. calcd (%) for $C_{16}H_{15}NO_3$: C 71.07, H 5.05, N 5.94, O 17.67; found (%)C 72.10, H 5.10, N 5.88, O 17.63.

Scheme 2: Semicarbazones of 4-aminopyridine.



Scheme: The synthetic pathway of **5-10**. (a) NaCNO, glacial acetic acid, 4 h. (b) $NH_2NH_2.H_2O$, C_2H_5OH, NaOH, 3 h. (c) C_2H_5OH, glacial acetic acid, 2 h.

Procedure for the synthesis of semicarbazide of 4-aminopyridine

1-(pyridin-4-yl) urea (intermediate-1)

4-Aminopyridine (0.01mol) was dissolved in mixture containing 5ml of glacial acetic acid and 25ml with distilled water. Equimolar (0.01mol) quantity of NaCNO in 25ml of warm water was added with continuous stirring, the reaction mixture was allowed to stand for 4 h and the product was obtained by filtration, washed with water, dried in an oven below melting point and recrystallized from ethanol to afford key intermediate-**1**[28], Yield: 86.0%, mp:212–214°C, R_f 0.65, IR (KBr, υcm-^{1}): 3431, (NH), 3325, 3122 (doublet NH_2),1678 (C=O), 1588, 1579 (C=N), 1474 (C=C, aromatic); ^{1}H NMR (DMSO-d_6) (δ ppm): 8.91 (bs, 1H, NH), 8.39 (d, 2H, pyridine), 6.78 (d, 2H, pyridine), 6.55 (s, 2H, NH_2); ^{13}C NMR (δ ppm): 169.43 (C=O), 154.27, 150.10, 109.48 (pyridine); Anal. calcd (%) for $C_6H_7N_3O$: C 52.55, H 5.14, N 30.64; found (%) C 52.35, H 5.23, N 30.57.

4-(pyridin-4-yl) semicarbazide ((intermediate-2)

Intermediate-1 (0.01mol) and hydrazine hydrate (0.01mol) were dissolved in 5ml of ethanol and refluxed for 3h in presence of sodium hydroxide (0.01mol). The precipitate was obtained by filtration, washed with water, dried in an oven below melting point and recrystallized from ethanol to afford key intermediate-**2**[29] Yield: 82.3%, mp: 230–232 °C , R_f 0.50, IR (KBr, υcm-^{1}): 3431, (NH), 3325, 3122 (doublet NH_2), 1678 (C=O), 1588, 1579 (C=N), 1474 (C=C, aromatic); ^{1}H NMR (DMSO-d_6) (δ ppm): 10.48, 9.31 (bs, 2H, NH), 8.36 (d, 2H, pyridine), 6.78 (d, 2H, pyridine), 6.55 (s, 2H, NH_2); ^{13}C NMR (δ ppm): 159.14 (C=O), 154.32, 150.66, 109.42 (pyridine); Anal. calcd (%) for $C_6H_8N_4O$: C 47.36, H 5.30, N 36.82; found (%) C 47.17, H 5.25, N 36.89.

General procedure for the synthesis semicarbazide of 4-aminopyridine (compounds 5-10)

Equal moles of intermediate-2 (0.456g, 0.003mol) in 5ml of ethanol mixed with equal moles of the different aldehyde or ketone was refluxed for 2hrs and glacial acetic acid was added to adjust the pH of the reaction between 5-6. The solid obtained after cooling was filtred, dried and crystallized from 95% ethanol to afford compounds (**5-10**).[30]

1-(2, 6-dihydroxybenzylidene)-4-(pyridin-4-yl) semicarbazide (compound-5)

Yield: 82.3%, mp:230–232 °C , R_f 0.50, IR (KBr, υcm-^{1}): 3515 (OH), 3432, 3325 (NH), 1677 (C=O), 1589, 1578 (C=N), 1473 (C=C, aromatic); ^{1}H NMR (DMSO-d_6) (δ ppm): 11.5 (s, 3H, OH), 10.16, 10.10 (bs, 2H, NH), 8.38 (d, 2H, pyridine), 7.99 (s, 1H, N=CH), 7.38 (m, 2H, aromatic), 6.56 (d, 2H, pyridine); ^{13}C NMR (δ ppm): 167.54 (C=O), 153.15 (N=CH), 155.31, 150.48, 109.17 (pyridine), 137.70, 130.68, 129.39, 128.10 (aromatic); Anal. calcd (%) for $C_{11}H_9N_4O_3$: C 53.88, H 3.70, N 22.85; found (%)C 53.57, H 3.74, N 22.80.

1-(2,4,6-trimethoxybenzylidene)-4-(pyridin-4-yl) semicarbazide (compound-6)

Yield: 81.3%, mp:230–231 °C , R_f 0.57, IR (KBr, υcm-^{1}): 3432, 3325 (NH), 3062 (CH, CH_3), 1677 (C=O), 1589, 1578 (C=N), 1473 (C=C, aromatic); ^{1}H NMR (DMSO-d_6) (δ ppm): 8.78, 8.54 (bs, 2H, NH), 8.44 (d, 2H, pyridine), 7.95 (s, 1H, N=CH), 6.99, 6.97 (m, 2H, aromatic), 8.44 (d, 2H, pyridine), 4.21 (s, 9H, CH_3); ^{13}C NMR (δ ppm): 169.41 (C=O), 153.23 (N=CH), 155.84, 150.78, 109.12 (pyridine), 137.54, 130.34, 129.12, 128.20 (aromatic), 55.86 (CH_3); Anal. calcd (%) for $C_{16}H_{18}N_4O_4$: C 58.17, H 5.49, N 16.96; found (%)C 58.20, H 5.35, N 17.10.

1-(1-phenylethylidene)-4-(pyridin-4-yl) semicarbazide (compound-7)

Yield: 78.35%, mp:234–236°C , R_f 0.54, IR (KBr, υcm-^{1}): 3432, 3325 (NH), 3063 (CH, CH_3), 1677 (C=O), 1589, 1578 (C=N), 1473 (C=C, aromatic); ^{1}H NMR (DMSO-d_6) (δ ppm): 8.87, 8.54 (bs, 2H, NH), 8.36 (d, 2H, pyridine), 7.66-7.38 (m, 6H, aromatic), 6.59 (d, 2H, pyridine), 1.11 (s, 3H, CH_3); ^{13}C NMR (δ ppm): 168.12 (C=O), 157.77 (N=C), 154.17, 150.42, 109.57 (pyridine), 139.27, 130.54, 129.15, 128.32 (aromatic), 24.80 (CH_3); Anal. calcd (%) for $C_{14}H_{14}N_4O_1$: C 66.13, H 5.55, N 20.03; found (%)C 66.18, H 5.61, N 20.13.

1-(4-hydroxy-3-methoxyphenyl) ethylidene)-4-(pyridin-4-yl) semicarbazide (compound-8)

Yield: 75.8%, mp:227–229 °C , R_f 0.53, IR (KBr, υcm-^{1}): 3516 (OH), 3433, 3324 (NH), 3063 (CH, CH_3), 1677 (C=O), 1589, 1578 (C=N), 1473 (C=C, aromatic); ^{1}H NMR (DMSO-d_6) (δ ppm): 8.86, 8.58 (bs, 2H, NH), 8.35 (d, 2H, pyridine), 7.64-7.50 (m, 3H, aromatic), 6.78 (d, 2H, pyridine), 1.16, 4.32 (s, 6H, CH_3); ^{13}C NMR (δ ppm): 169.28 (C=O), 155.14 (N=C), 154.60, 150.31, 109.44 (pyridine), 139.78, 130.40, 129.19, 128.00 (aromatic), 24.32, 55.64 (CH_3); Anal. calcd (%) for $C_{15}H_{16}N_4O_3$: C 59.99, H 5.37, N 18.66; found (%)C 59.56, H 5.34, N 18.70.

1-(1-phenylpropylidene)-4-(pyridin-4-yl) semicarbazide (compound-9)

Yield: 76.6%, mp:230–232 °C , R_f 0.50, IR (KBr, υcm-^{1}): 3432, 3325 (NH), 3063 (CH, CH_3), 1677 (C=O), 1589, 1578 (C=N), 1473 (C=C, aromatic); ^{1}H NMR (DMSO-d_6) (δ ppm): 8.55, 8.86 (bs, 2H, NH), 8.38 (d, 2H, pyridine), 7.66-7.36 (m, 6H, aromatic), 6.57 (d, 2H, pyridine), 1.10 (s, 3H, CH_3), 1.60 (s, 2H, CH_2); ^{13}C NMR (δ ppm): 168.14 (C=O), 157.68 (N=C), 154.19, 150.40, 109.54 (pyridine), 139.25, 130.52, 129.17, 128.20 (aromatic), 24.60 (CH_2), 8.10 (CH_3); Anal. calcd (%) for $C_{15}H_{16}N_4O$: C 67.15, H 6.01, N 20.88; found (%) C 67.13, H 6.09, N 20.92.

1-(4-hydroxy-3-methoxyphenyl)propylidene)-4-(pyridin-4-yl)semicarbazide (compound-10)

Yield: 79.5%, mp:234–236 °C , R_f 0.59, IR (KBr, υcm-^{1}): 3517 (OH), 3432, 3325 (NH), 3063, 2940 (CH, CH_3), 1677 (C=O), 1589, 1578 (C=N), 1473 (C=C, aromatic); ^{1}H NMR (DMSO-d_6) (δ ppm): 8.71 (s, 1H, OH), 8.86, 8.57 (bs, 2H, NH), 8.33 (d, 2H, pyridine), 7.58-7.35 (m, 3H, aromatic), 6.59 (d, 2H, pyridine), 1.14, 4.33 (s, 6H, CH_3), 1.59 (s, 2H, CH_2); ^{13}C NMR (δ ppm): 169.27 (C=O), 155.11 (N=C), 154.36, 150.14, 109.11 (pyridine), 139.44,

130.78, 129.58, 128.60 (aromatic), 24.51 (CH_2), 8.40, 56.20 (CH_3); Anal. calcd (%) for $C_{16}H_{18}N_4O_3$: C 61.13, H 5.77, N 17.82; found (%) C 61.03, H 5.71, N 17.76.

Biological studies

Estimation of cholinesterase activity (*in-vitro*): The ability of all tested compounds was determined by Ellman's spectrophotometric analysis[31] to inhibit acetylcholinesterase from electric eel (E.C. 3.1.1.7) could be conclusive through their IC_{50} values,by recording the rate of increase in the absorbance at 412 nm for 5 min. Dissolved AChE in 0.1 M phosphate buffer (pH 8.0) to obtain stock solution of AChE. 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) is the final solution for assay. Various concentrations of test compounds (inhibitors) between 20% and 80%) were taken in order to obtain the enzymatic inhibitory activity. From the aliquots (50 µL), increasing concentrations of the inhibitors were added to the assay solution and were pre-incubated for 20 min at 37 ^oC with the enzyme followed by the addition of substrate. Blank used in the assays consisted of all the components except AChE in order to account for the non-enzymatic reaction. The percent inhibition due to the presence of increasing concentrations of inhibitor was calculated after comparing the reaction rates which was analyzed in triplicate and IC_{50} values were determined graphically from log concentration percent inhibition curves.[32, 33]

Enzyme kinetics study: The Ellman's spectrophotometric analysis was used to elucidate the mechanism of inhibition in this study. Acetylthiocholine iodide was used as a substrate in various concentrations, both below and above, near to K_m in a phosphate buffer at pH 8, keeping a fixed concentration of cholinesterase in the absence or presence of different inhibitors. The concentration of the inhibitor was kept close to one which corresponds to 50% inhibition of the enzyme activity (IC_{50}) and their inhibitory kinetics were evaluated by the Lineweaver and Burk method.[34]

Animals: Charles foster rats of albino strain (4 to 5 months old and 150 to 200 g in weight) of both sexes were purchased from Animal House, Faculty of Pharmacy, Pacific academy of higher education & research university Udaipur (Registration No. 1622/PO/a/12/CPCSEA). five rats were housed per cage with food with occasional supply of green vegetables (salad leaves) and water available *ad-libitum* atconstant temperature (25 ºC ± 1º C) and 45-55%

relative humidity under twelve hours of light and dark cycles was strictly followed in a fully ventilated room.

Acute toxicity evaluation

Acute toxicity studies for all the Synthesized Schiff base analogues were performed as per OECD-425 guidelines.[35] In this evaluation, nulliparous, non-pregnant, healthy female albino rats weighing between 150-200 gm were fasted overnight with water *ad-libitum* prior to test. On the day of experiment, analogues were administered at graded dose up to 100 mg/kg p.o in 0.3% carboxymethyl cellulose as vehicle. The animals were 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

Standard rivastigmine and the synthesized Schiff base analogues were suspended in 0.3% carboxymethyl cellulose and a dose of 3 mg/kg and 6 mg/kg was administered orally and the same volume of 0.3% carboxymethyl cellulose was administered to all animals of control groups.

Passive avoidance task: The test apparatus consists of two identical light chambers connected to dark chamber via a guillotine door. Both the chambers with grid floors which can be electrified separately, the light chamber is illuminated with a 7 W/12 V bulb. During the training trial, each rat was placed in the light compartment and after 10 s, the door was raised. Immediately after the animal enters the dark compartment, the door is shut automatically and an unavoidable foot shock (0.02 mA/10 g body weight lasting 2 sec was delivered. The time elapsed by the rat being placed in light and entering the dark chamber 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 chamber was measured. The synthesized Schiff base analogues 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.[36, 37]

Molecular docking

Preparation of the small molecules: All the synthesized Schiff base analogues 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 A^o, Average R-value 0.234).[38] 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.

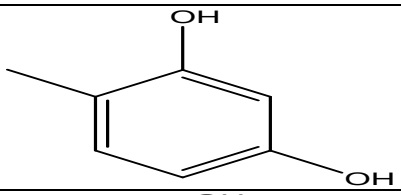
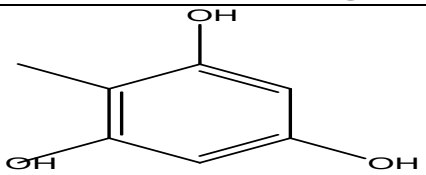
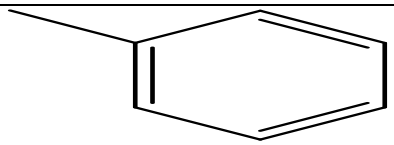
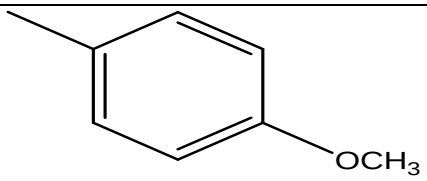
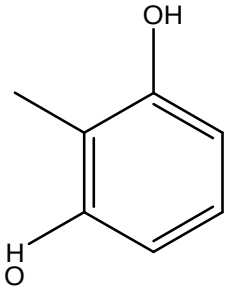
Molecular docking: For the receptor structure, crystallographic and trajectory water molecules, ions and ligand compounds were removed. Proteins were prepared using Schrodinger software, Maestro 9.3 and Glide 5.8. The Glide XP algorithm was employed using a grid box volume of 10_10_10 Å. All the structures were fitted in binding pocket and the lowest energy pose for each docking run was retained.[39]

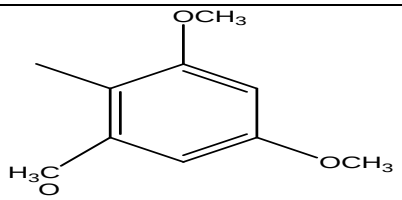
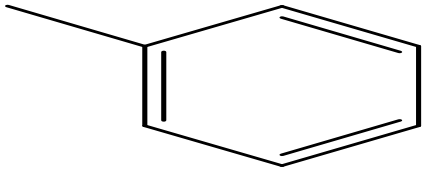
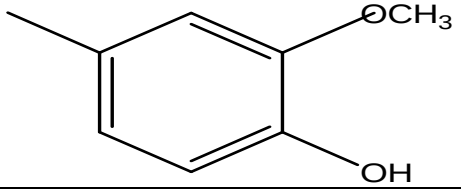
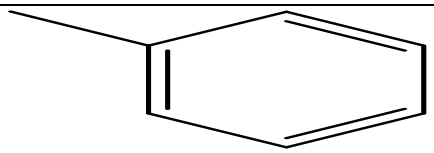
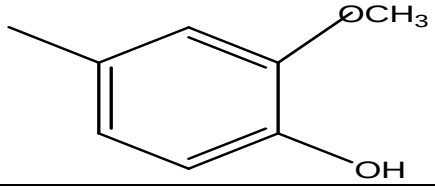
RESULTS AND DISCUSSION

Chemistry: Synthesis of Schiff base analogues was achieved by the well-known nucleophilic addition reaction of *p*-amino benzoic acid with various substituted aromatic aldehydes and ketones according to reaction scheme-1and the ligands are enlisted in Table1. 4-aminopyridine reacts with sodium cyanate in the presence of glacial acetic acid formed 4-aminopyridineurea (intermediate-**1**), which on condensed with hydrazine hydrate in the presence of sodium hydroxide yielded the 4-aminopyridine semicarbazide (intermediate-**2**). The Semicarbazones (**1-6**) were synthesized by treating with the various aldehydes or ketones with 4-aminopyridine semicarbazide as comprised in Table1. In order to verify their purity, all the synthesized analogues were confirmed by TLC and characterized by FTIR, ^{1}HNMR, ^{13}CNMR and elemental analysis. The FTIR spectra of the synthesized Schiff base analogues showed strong vibration bands at region of 1623-1628 cm^{-1} due to presence of C=N functional group. The ^{1}H NMR spectrum of the synthesized Schiff base analogues **1** and **2** showed peak at δ 8.2-8.3 ppm towards the presence of the N=CH proton. The ^{1}H NMR spectrum of the synthesized Schiff base analogues **3** and **4** revealed that disappearance of peak at 7.92-8.3 ppm corresponding to the substitution of single proton by different groups and the formation of N=CH and N=C bond also confirmed by ^{13}C NMR spectrum of the

synthesized Schiff base analogues between δ 156-162 ppm. The IR spectra of the synthesized 4-Aminopyridine analogues showed peak of C=N and NH stretching vibrations was observed at 1589 cm^{-1} and 3433-3324 cm^{-1} respectively. In ^{1}H NMR spectra, intermediate-**1** and intermediate-**2** showed peak at δ 6.55 ppm due to the presence of $-NH_2$ proton. Compounds **5** and **6** showed peak at δ 7.98 and 7.95 ppm, reflecting the presence of N=CH proton, While a total disappearance of peak at δ 7.98-7.95 ppm in compounds (**7-10**) where this single proton was substituted by different groups, resulted in the formation of N=C bond due to which confirmed the substitution. The ^{13}C NMR values of δ 153-157 ppm also confirmed the formation of N=CH and N=C bond.

Table. 1: Synthesized Schiff base analogues of *p*-aminobenzoic acid and semicarbazide of 4-aminopyridine analoguesof Scheme.

Compounds	R^1	R^2
1	—H	
2	—H	
3	—C_2H_5	
4	—CH_3	
5	—H	

6	—H	
7	—CH_3	
8	—CH_3	
9	—C_2H_5	
10	—C_2H_5	

Biological activity

***Invitro* AChE Inhibition:** Determination of AChE inhibitory activity of the all synthesized analogues was done by Ellman spectrophotometric method.[31] Graph Pad Prism was used to calculate the IC_{50} values of all synthesized Schiff base analogues and observed that all the analogues exhibited moderate to excellent IC_{50} values. The IC_{50} values of analogues **2** & **5** are 7.93±0.96 *µM* & 7.48±0.14 *µM* respectively with respect to standard rivastigmine (6.14± 0.59*µM*). The enzyme kinetic study[34] of all synthesized analogues was performed to elucidate the mechanism of AChE inhibition. The most active compounds **2** and **5** demonstrated a non-competitive inhibition for AChE (Ki = 12.47±0.54 and 8.16± 0.68 respectively) enzyme (Table 2). The results of IC_{50} values and Ki of the most active analogues **2** & **5** are 7.93±0.96 *µM* & 7.48±0.14 *µM* and Ki = Ki = 12.47±0.54 and 8.16± 0.68 respectively (a non-competitive inhibition for AChE enzyme) and structure activity relationship (SAR) revealed that the activity increases to increase the number of hydroxyl groups as compare to methoxy fuctionals on the phenyl ring. The non-competitive inhibition[22] is approved to a possible interaction of compound with the peripheral anionic site (PAS) of AChE and was also confirmed by molecular docking studies.

Table. 2: Cholinesterase activity and Enzyme kinetics study of synthesized derivatives and Rivastigmine.

Compound	*AChE*	*AChE*	*Inhibition*
	IC_{50} (µM) ± SEM	*Ki (µM) ± SEM*	
1	10.3±1.77	18.29±1.479	c
2	7.93±0.96	12.47±0.54	nc
3	10.35±1.48	11.26±0.88	nc
4	9.95±1.55	30.25±1.12	c
5	7.48±0.14	8.16± 0.68	nc
6	32.76±0.78	41.90±0.94	nc
7	22.4± 0.61	25.81± 0.85	c
8	11.9± 0.60	20.38± 0.91	nc
9	12.54±0.013	6.47± 0.61	nc
10	11.89±0.74	9.46± 0.96	nc
Rivastigmine*	6.14± 0.59	131.0± 0.9	c

c=competitive, nc=noncompetitive.

Passive avoidance test

The passive avoidance test was conducted for antiamnesic and cognition enhancing activities of all the synthesized analogues.[40] 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 dependant manner, indicating facilitated learning process and the Effect of synthesized analogues and Rivastigmine on cognition enhancing and scopolamine-induced amnesia on rat passive avoidance test reported in Table 3. A prolonged latency indicates that the animal remembers that it has been punished and therefore, does avoid the darken chamber. The effect of inhibitors analogue **2** and analogue **5** on changes in entry latency in scopolamine-induced amnesia showed significant differences [$p < 0.05$] among treated groups (Table 3). 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 **2, 5** and rivastigmine, dose dependently reversed scopolamine-induced decrease in entry latency.

Table. 3: Effect of synthesized derivative and Rivastigmine on cognition enhancing and scopolamine-induced amnesia on rat passive avoidance test.

Treatment [Dose(mg/kg)]	Entry latency (s)		
	Training trial	Retention trial	Δ
Control	17.81±0.70	95.49±0.66	77.67
2 (3.0)	20.70±0.47	152.59±0.65[a]	132.9
2 (6.0)	21.52±0.54	161.80±0.44[a]	140.3
2 (3.0)	18.31±0.64	118.65±0.68[a]	100.35
4 (6.0)	18.45±0.62	136.39±0.59[a]	117.95
5 (3.0)	16.61±0.58	175.60±0.74[a]	159.02
5 (6.0)	15.63±0.94	205.80±0.61[a]	190.19
Riva (3.0)	14.62±0.61	184.53±0.58[a]	169.93
Riva (6.0)	14.94±0.70	200.88±0.69[a]	185.88
SCP (1.5)	20.01±0.61	35.19±0.61[a]	15.17
2 (3.0) +SCP (1.5)	20.82±0.59	82.15±0.60[b]	61.34
2 (6.0) +SCP	20.19±0.62	86.38±0.68[b]	66.16
4 (3.0) +SCP	21.22±0.69	89.93±0.66[b]	68.83
4 (6.0) +SCP	20.38±0.88	84.73±0.70[b]	64.5
5 (3.0) +SCP	19.51±0.77	90.19±0.63[b]	70.67
5 (6.0) +SCP	16.65±0.50	98.06±0.68[b]	81.33
Riva (3.0) +SCP	19.08±0.65	89.89±0.63[b]	70.83
Riva (6.0) +SCP	17.19±0.62	97.66±0.50[b]	80.5

Data are expressed as mean ± SEM (n = 6). Data were statistically analyzed by one way ANOVA. [a]Significantally different from control $p < 0.05$. [b]Significantally 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 all the synthesized analogues toward AChE. Fig1&2 showed π–π interaction with the internal amino acid residue and synthesized analogues. It was observed that analogue **2** and **5** were properly positioned into the enzyme gorge and showed interaction with the internal amino acid residue Phe-331, Phe-333, Phe-290 and Trp-84 by means of a π–π interaction. The study showed that analogues were able to bind with the key peripheral anionic site (PAS). In order to determine substrate specificity the hydroxyl oxygen and amine hydrogen were involved in formation of hydrogen bond with Ser-122, Asp=72 and Try-121 respectively. The hydroxyl oxygen of Schiff base analogue **2** and amine hydrogen analogues **5** were involved in forming a hydrogen bond with Ser-122, Asp=72 and Try-121 respectively (determine substrate specificity). The hydroxyl oxygen of analogue **2** was involved in forming a hydrogen bond with Asp-72 suggested that the compounds might probably act via the AChE inhibition (Fig1&2).

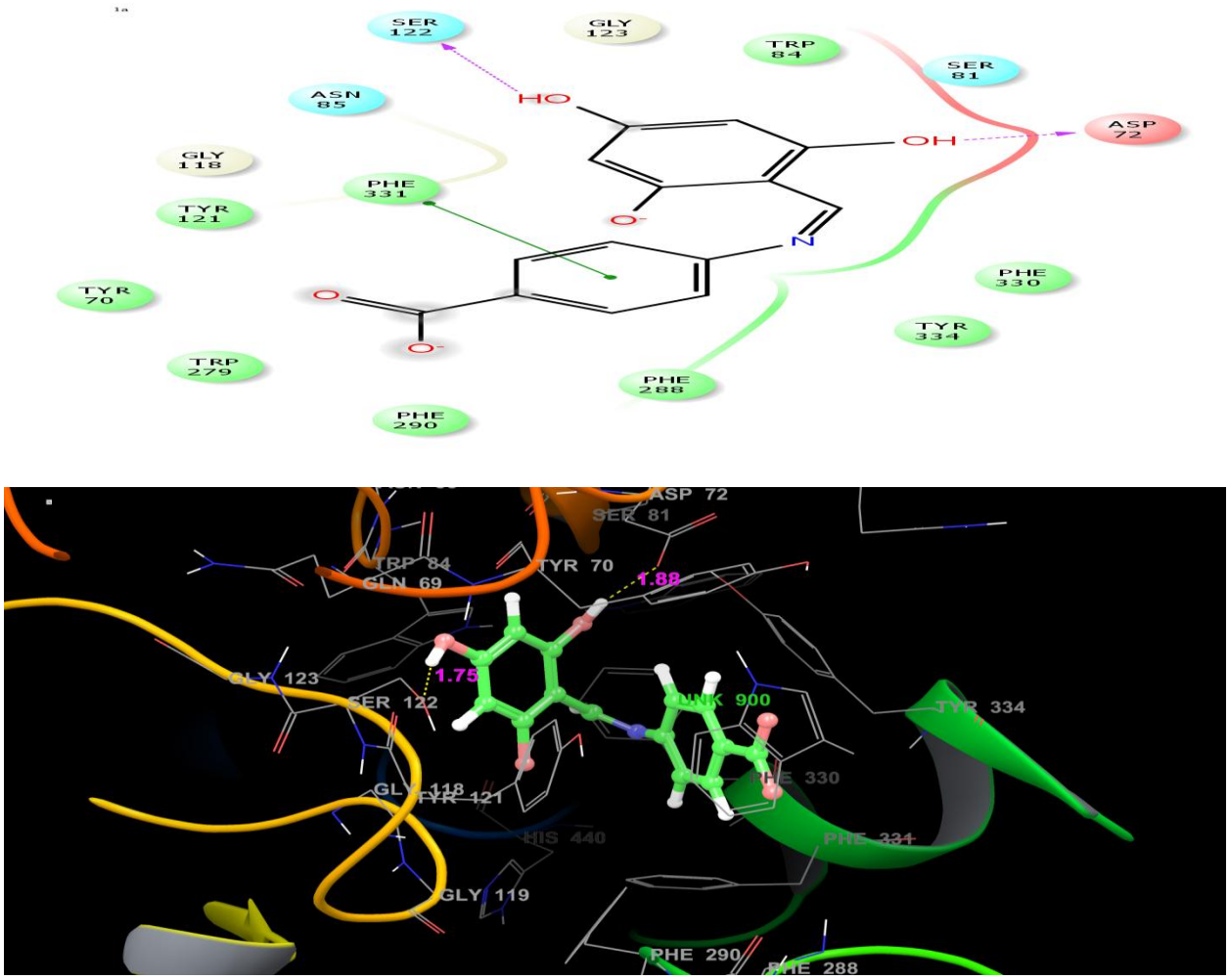
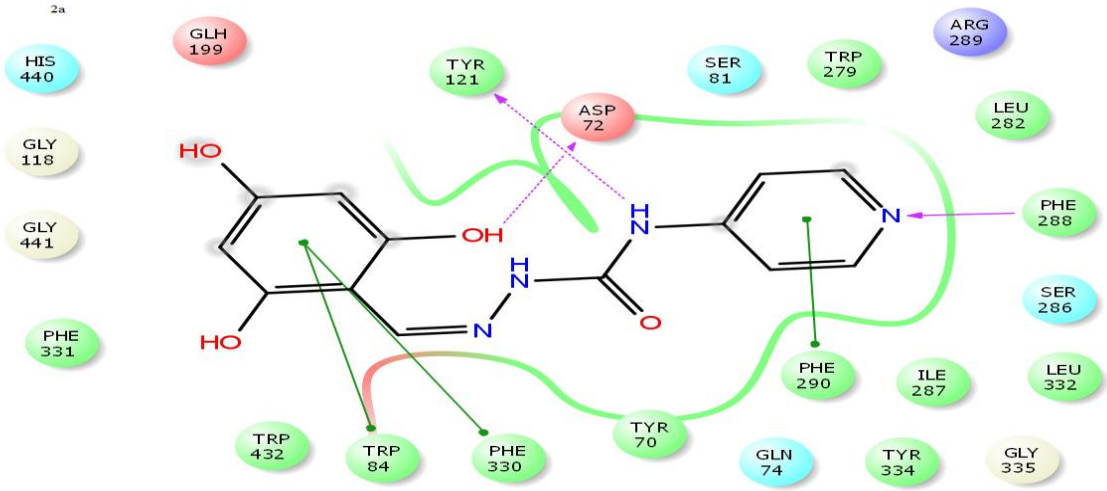


Fig. 1: Molecular docking of compound 2 into the active sites of AChE (1a-2D, 1b-3D). Ligand is in green colour, dotted show H-bond interaction.



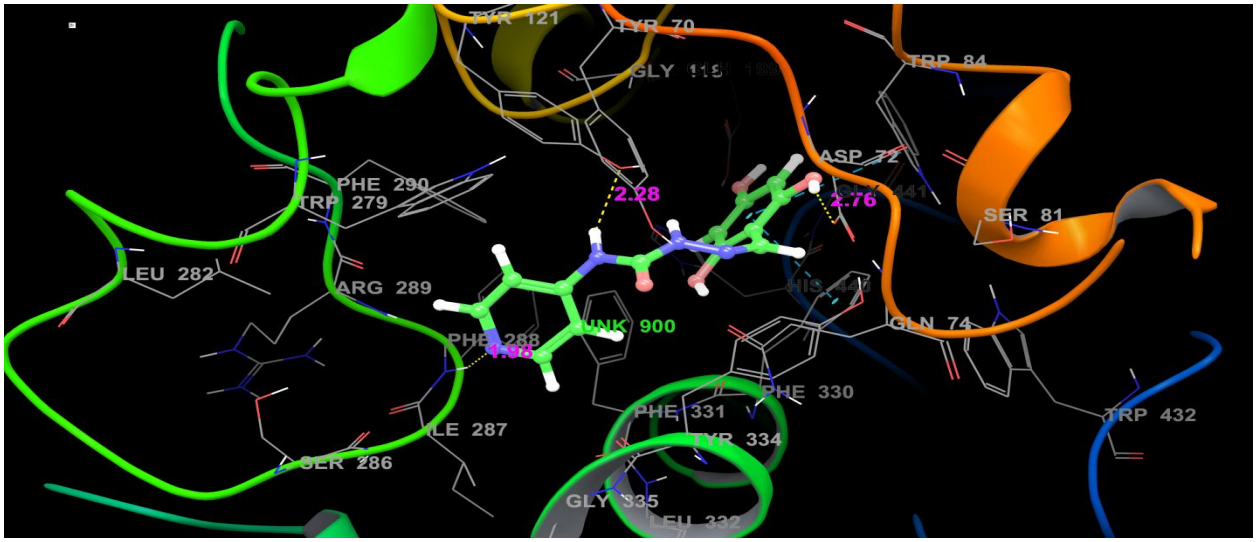


Fig. 2: Molecular docking of compound 5 into the active sites of AChE (1a-2D, 1b-3D). Ligand is in green color, dotted show H-bond interaction.

CONCLUSIONS

In the present study, it was concluded that the hydroxyl substituted analogues **2** 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 Tyr-121, Asp-72 and Phe-288 by hydrogen bonding and (b) its aromatic moiety maintains the functional orientation of the subsite Phe-330, Phe-331 and Trp-84.

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