6. Development of piperine derivatives

6.1. Experimental section

6.1.1. Chemistry

6.1.1.1. General method

The commercially available chemicals and solvents were used for all the experiments. The synthetic reactions were carried out under an N_2 environment, and all glassware was dried in an oven before conducting any experiments. The silica gel plates (silica gel 60 F_{254} Merck KGaA, Germany) were used to monitor all the reactions and TLC plates were derivatized using Dragendorff's reagent, iodine chamber, and UV light. Compounds structures were confirmed through ^{1}H NMR (500 MHz) and ^{13}C NMR (126 MHz) (Brucker bio spin international AG, Germany). Further HRMS (SCIEX. X500R QTOF, US) analysis was also performed at Banaras Hindu University, Varanasi, to confirm the mass of the molecules. The waters HPLC (Waters 1500, Germany) was used to estimate the peak purity of compounds. Before injection of sample into HPLC system, compounds were filtered through a 0.45 µm membrane filter. Additional chromatographic parameters conditions were set as follows, injection volume 10 µl, column Lichro CART 250-4 C_{18}, sample concentration 10 µg, mobile phase methanol: water (9:1), flow rate 0.8 mL/min, PDA detector at 345 nm, and waters breeze software was used for calculation of peak purity in specified run time.

6.1.1.2. Extraction and isolation of piperine from *Piper nigrum*

The dried black pepper fruits were purchased from Yuvika Herbs (Batch no. YUVI0413), India. Further, cold maceration technique was used to isolate piperine. The dehydrated course powdered fruits were soaked in 3 L of methanol. The obtained extract was filtered every 72 h, and methanolic extract was concentrated using a rotary evaporator under reduced pressure. The acquired crude extract was then dissolved into 10 % KOH solution and kept a side for 30 min.

Further, piperine residue was filtered and dried at room temperature. The piperine was purified using column chromatography under the conditions of mobile phase: ethyl acetate and hexane (20:80), stationary phase: silica gel (60–120 mesh).

Black pepper seeds were purchased from Yuvika Herbs (Batch no. YUVI0413), India

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Powder of seeds was soaked into 3 L of methanol

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Filtered, every 72 h, and concentrated using a rotary evaporator

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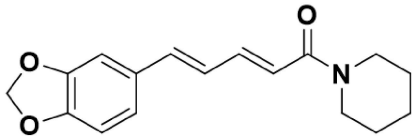
10% KOH solution and kept a side for 30 min

↓

Piperine residue filtered

↓

Column chromatography, ethyl acetate and hexane (20:80), silica gel (60–120 mesh)



Yield, 4.3% w/w; HPLC peak purity 97.4%

(2E,4E)-5-(benzo[d][1,3]dioxol-5-yl)-1-(piperidin-1-yl)penta-2,4-dien-1-one (PIP): yellow solid, yield- 4.3 % w/w, M. P- 130-134°C, ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.44 – 7.39 (m, 1H), 7.00 (d, *J* = 1.6 Hz, 1H), 6.91 (dd, *J* = 8.1, 1.7 Hz, 1H), 6.81 – 6.71 (m, 3H), 6.46 (d, *J* = 14.6 Hz, 1H), 5.99 (s, 2H), 3.65 – 3.50 (m, 4H), 1.72 – 1.65 (m, 2H), 1.63 – 1.59 (m, 4H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 165.46, 148.21, 148.13, 142.48, 138.23, 131.05, 125.38, 122.50, 120.09, 108.50, 105.69, 101.28, 46.94, 43.27, 26.76, 25.64, 24.68. HRMS $[M + H]^+$: m/z was found 286.1400, and calculated 286.1443 for $C_{17}H_{20}NO_3$. HPLC: peak purity - 97.99 %, retention time – 5.726 min.

6.1.1.3. Synthesis of piperic acid (PC) from piperine (PIP)

Piperine (1.0 *eq*) was transferred into 50 ml of RBF, and 20 % potassium hydroxide solution in methanol was added. Then, refluxed at 80°C for 18 h. The reaction was monitored by TLC with a mobile phase of DCM: MeOH (9:1). Further, reaction mixture was cooled to room temperature, followed by HCl (1.0 M) added to reach an acidic pH range of 3–4. The compound PC was extracted with dichloromethane (DCM) using separating funnel. The compound PC was concentrated using a rotary evaporator under reduced pressure.

(2E,4E)-5-(benzo[d][1,3]dioxol-5-yl)penta-2,4-dienoic acid (PC): yellow solid, yield- 98.5% w/w, M. P- 120-124°C, ^{1}H NMR (500 MHz, DMSO) δ 12.19 (s, 1H), 7.33 – 7.28 (m, 1H), 7.24 (d, *J* = 1.4 Hz, 1H), 7.02 – 6.99 (m, 1H), 6.99 – 6.94 (m, 2H), 6.93 (t, *J* = 5.7 Hz, 1H), 6.06 (d, *J* = 6.7 Hz, 2H), 5.93 (d, *J* = 15.2 Hz, 1H). ^{13}C NMR (126 MHz, DMSO) δ 168.09, 148.58, 148.46, 145.08, 140.26, 130.99, 125.32, 123.56, 121.59, 108.98, 106.17, 101.84. HPLC: purity – 98.14 %, retention time – 3.644 min.

6.1.1.4. General procedure for synthesis of compounds PC01-PC10 and PD01-PD25

Carboxylic acids (1.0 *eq*) and secondary amine compounds (1.1 *eq*) were transferred into 50 ml RBF and 10 ml of acetonitrile was added. The reaction was stirred at room temperature followed by DIPEA (2.5 *eq*) was added. Further, HOBT (2.5 *eq*) and EDC.HCl (1.5 *eq*) was added. The reaction was kept at room temperature for 12 h. and was monitored by TLC using DCM: MeOH (9:1). The TLC plates were observed under UV light, iodine chamber, and Dragendorff's reagent. The reaction was stopped by quenching ice-cold water and product was extracted with DCM. The DCM layer was concentrated under reduced pressure. The obtained compounds were purified by column chromatography using silica gel (60-120 mesh), and mobile phase ethyl acetate: hexane (40:60).

(2E,4E)-5-(benzo[d][1,3]dioxol-5-yl)-1-(pyrrolidin-1-yl)penta-2,4-dien-1-one (PC01): white solid, yield- 97.6 % w/w, M. P- 126-130°C, ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.45 (dd, *J* = 14.7, 10.1 Hz, 1H), 7.00 (s, 1H), 6.91 (d, *J* = 7.8 Hz, 1H), 6.84 – 6.71 (m, 3H), 6.27 (d, *J* = 14.7 Hz, 1H), 5.99 (s, 2H), 3.57 (dd, *J* = 12.5, 6.3 Hz, 4H), 2.02 – 1.97 (m, 2H), 1.92 – 1.87 (m, 2H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 164.98, 148.21, 148.18, 141.81, 138.72, 131.01, 125.22, 122.58, 121.40, 108.50, 105.71, 101.29, 46.50, 45.94, 26.14, 24.37. HRMS $[M + H]^+$: m/z was found 372.1276, calculated 372.1287 for $C_{16}H_{18}NO_2$. HPLC: purity - 97.88 %, retention time - 4.535 min.

(2E,4E)-5-(benzo[d][1,3]dioxol-5-yl)-1-(piperidin-1-yl)penta-2,4-dien-1-one (PC02): yellow solid, yield- 97.7 % w/w, M. P- 130-134°C, ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.44 – 7.39 (m, 1H), 7.00 (d, *J* = 1.6 Hz, 1H), 6.91 (dd, *J* = 8.1, 1.7 Hz, 1H), 6.82 – 6.71 (m, 3H), 6.46 (d, *J* = 14.6 Hz, 1H), 5.99 (s, 2H), 3.65 – 3.55 (m, 4H), 1.71 – 1.67 (m, 2H), 1.63 – 1.59 (m, 4H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 165.46, 148.21, 148.13, 142.48, 138.23, 131.05, 125.38, 122.50, 120.09, 108.50, 105.69, 101.28, 46.94, 43.27, 26.76, 25.64, 24.68. HRMS $[M + H]^+$: m/z was found 286.1400, calculated 286.1443 for $C_{17}H_{20}NO_3$. HPLC: purity - 97.44 %, retention time - 4.528 min.

(2E,4E)-5-(benzo[d][1,3]dioxol-5-yl)-1-morpholinopenta-2,4-dien-1-one (PC03): pale yellow solid, yield- 98.7 % w/w, M. P- 137-140°C, ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.47 (dd, *J* = 14.6, 10.6 Hz, 1H), 7.00 (d, *J* = 1.4 Hz, 1H), 6.92 (dd, *J* = 8.0, 1.4 Hz, 1H), 6.83 – 6.71 (m, 3H), 6.38 (d, *J* = 14.6 Hz, 1H), 6.00 (s, 2H), 3.72 – 3.62 (m, 8H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 165.73, 148.33, 148.25, 143.49, 139.13, 130.83, 125.00, 122.72, 118.75, 108.53, 105.72, 101.34, 66.86, 46.14, 42.37, 29.69. HRMS $[M + H]^+$: m/z was found 288.1202, calculated 288.1236 for $C_{16}H_{18}NO_4$. HPLC: purity - 97.88 %, retention time – 5.841 min.

(2E,4E)-5-(benzo[d][1,3]dioxol-5-yl)-1-(piperazin-1-yl)penta-2,4-dien-1-one (PC04): pale orange solid, yield- 98.9 % w/w, M. P- 136-139°C, ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.45 (dd, *J* = 14.6, 10.3 Hz, 1H), 7.00 (s, 1H), 6.92 (d, *J* = 8.0 Hz, 1H), 6.83 – 6.71 (m, 3H), 6.42 (d, *J* = 14.6 Hz, 1H), 6.00 (s, 2H), 3.71 – 3.61 (m, 4H), 2.92 (s, 4H), 1.96 (s, 1H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 165.66, 148.25 (2C), 143.11, 138.79, 130.92, 125.15, 122.64, 119.29, 108.53, 105.71, 101.31, 46.36 (2C), 43.02 (2C). HRMS $[M + H]^+$: m/z was found 287.1352, calculated 287.1396 for $C_{16}H_{19}N_2O_3$. HPLC: purity - 97.55, retention time - 4.529 min.

(2E,4E)-5-(benzo[d][1,3]dioxol-5-yl)-1-(4-methylpiperazin-1-yl)penta-2,4-dien-1-one (PC05): pale orange solid, yield- 99.1 % w/w, M. P- 126-129°C, ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.51 – 7.39 (m, 1H), 7.00 (s, 1H), 6.91 (d, *J* = 7.4 Hz, 1H), 6.81 – 6.74 (m, 3H), 6.41 (d, *J* = 14.5 Hz, 1H), 5.99 (s, 2H), 3.75 – 3.64 (m, 3H), 2.46 – 2.34 (m, 8H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 165.61, 148.26, 148.23, 143.18, 138.85, 130.90, 125.12, 122.65, 119.25, 108.52, 105.72, 101.31, 54.67 (2C), 45.93, 45.53, 41.92. HPLC: purity – 96.46, retention time - 4.019 min.

(2E,4E)-5-(benzo[d][1,3]dioxol-5-yl)-1-(4-ethylpiperazin-1-yl)penta-2,4-dien-1-one (PC06): yellow to orange solid, yield- 97.4 % w/w, M. P- 125-128°C, ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.44 (dd, *J* = 14.6, 10.1 Hz, 1H), 6.99 (d, *J* = 1.1 Hz, 1H), 6.93 – 6.88 (m, 1H), 6.81 – 6.71 (m, 3H), 6.42 (d, *J* = 14.6 Hz, 1H), 5.99 (s, 2H), 3.75 – 3.63 (m, 4H), 2.50 – 2.43 (m, 6H), 1.12 (t, *J* = 7.2 Hz, 3H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 165.51, 148.23 (2C), 143.02, 138.72, 130.93, 125.17, 122.61, 119.38, 108.51, 105.71, 101.30, 53.12, 52.45, 52.23, 45.69, 42.03, 11.90. HPLC: purity – 98.31 %, retention time – 3.666 min.

(2E,4E)-1-(4-acetylpiperazin-1-yl)-5-(benzo[d][1,3]dioxol-5-yl)penta-2,4-dien-1-one (PC07): pale yellow solid, yield- 97.4 % w/w, M. P- 132-135°C, ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.47 (dd, *J* = 14.5, 10.6 Hz, 1H), 7.00 (s, 1H), 6.92 (d, *J* = 7.7 Hz, 1H), 6.86 – 6.70 (m, 3H),

6.40 (d, *J* = 14.5 Hz, 1H), 6.00 (s, 2H), 3.73 – 3.52 (m, 8H), 2.15 (s, 3H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 169.28, 165.86, 148.41, 148.27, 143.92, 139.48, 130.75, 124.87, 122.80, 118.56, 108.55, 105.73, 101.36, 46.13 (2C), 41.42 (2C), 21.37. HPLC: purity – 98.09 %, retention time – 3.641 min.

tert-butyl 4-((2E,4E)-5-(benzo[d][1,3]dioxol-5-yl)penta-2,4-dienoyl)piperazine-1-carboxylate (PC08): pale green to yellow solid, yield- 97.8 % w/w, M. P- 133-136°C, ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.46 (dd, *J* = 14.6, 10.5 Hz, 1H), 7.00 (s, 1H), 6.91 (dd, *J* = 8.0, 1.1 Hz, 1H), 6.83 – 6.77 (m, 3H), 6.40 (d, *J* = 14.6 Hz, 1H), 6.00 (s, 2H), 3.68 – 3.59 (m, 4H), 3.48 (s, 4H), 1.49 (s, 9H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 165.78, 154.60, 148.34, 148.25, 143.57, 139.18, 130.82, 124.99, 122.72, 118.92, 108.53, 105.73, 101.33, 80.32, 45.58 (2C), 41.91 (2C), 28.39 (3C). HRMS $[M + H]^+$: m/z was found 387.1902, calculated 387.1920 for $C_{21}H_{27}N_2O_5$. HPLC: purity – 97.78 %, retention time – 3.672 min.

(2E,4E)-5-(benzo[d][1,3]dioxol-5-yl)-1-(4-(pyridin-2-yl)piperazin-1-yl)penta-2,4-dien-1-one (PC09): pale green, yield- 97.3 % w/w, M. P- 138-140°C, ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.54 (s, 1H), 8.05 (s, 1H), 7.85 – 7.81 (m, 2H), 7.59 (s, 1H), 7.29 (s, 1H), 7.20 (d, *J* = 15.1 Hz, 1H), 7.08 – 6.86 (m, 4H), 6.05 (s, 2H), 5.32 (s, 1H), 3.51 (s, 1H), 1.73 (s, 6H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 168.79, 149.32 (2C), 144.09 (2C), 132.81 (2C), 126.72 (2C), 123.97, 116.79, 116.47 (2C), 115.53, 108.71, 106.22, 101.61, 64.34 (2C), 45.78 (2C). HRMS $[M + H]^+$: m/z was found 364.1652, calculated 364.1661 for $C_{21}H_{22}N_3O_3$. HPLC: purity – 98.47 %, retention time – 3.620 min.

(2E,4E)-5-(benzo[d][1,3]dioxol-5-yl)-1-(4-(4-methoxyphenyl)piperazin-1-yl)penta-2,4-dien-1-one (PC10): orange solid, yield- 98.3 % w/w, M. P- 135-138°C, ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.48 (dd, *J* = 14.6, 10.5 Hz, 1H), 7.28 – 7.23 (m, 2H), 7.01 (d, *J* = 0.8 Hz, 1H), 6.92 (dd, *J* = 7.5, 4.2 Hz, 3H), 6.84 – 6.73 (m, 3H), 6.43 (d, *J* = 14.6 Hz, 1H), 6.00 (s, 2H), 4.00 (s, 4H),

3.81 (s, 3H), 3.24 (s, 4H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 165.72, 148.42, 148.28, 144.01, 139.51, 130.76, 124.87, 122.82, 120.21, 118.47 (2C), 114.91 (2C), 108.56 (2C), 105.75 (2C), 101.36, 55.62, 52.72, 51.75, 41.19, 41.13. HPLC: purity – 95.33 %, retention time – 3.680 min.

(2E,4E)-5-(benzo[d][1,3]dioxol-5-yl)-1-(4-benzoylpiperazin-1-yl)penta-2,4-dien-1-one (PD01): light orange solid, yield- 98.6 % w/w, M. P- 130-133°C, ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.52 – 7.41 (m, 6H), 7.00 (s, 1H), 6.92 (d, *J* = 7.8 Hz, 1H), 6.87 – 6.71 (m, 3H), 6.41 (s, 1H), 6.00 (s, 2H), 3.86 – 3.48 (m, 8H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 170.68, 165.87, 148.42, 148.27, 143.98, 139.51, 135.18, 130.75, 130.12, 128.67 (2C), 127.12 (2C), 124.86, 122.81, 118.52, 108.55, 105.74, 101.36, 47.64 (2C), 42.38, 42.03. HRMS $[M + H]^+$: m/z was found 391.1624, calculated 391.1658 for $C_{23}H_{23}N_2O_4$. HPLC: purity – 97.93 %, retention time – 5.841 min.

(2E,4E)-5-(benzo[d][1,3]dioxol-5-yl)-1-(4-(2-fluorobenzoyl)piperazin-1-yl)penta-2,4-dien-1-one (PD02): light orange solid, yield- 99.1 % w/w, M. P- 132-135°C, ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.51 – 7.40 (m, 3H), 7.29 – 7.24 (m, 1H), 7.14 (t, *J* = 8.9 Hz, 1H), 7.00 (s, 1H), 6.92 (d, *J* = 8.0 Hz, 1H), 6.85 – 6.71 (m, 3H), 6.42 (s, 1H), 6.00 (s, 2H), 3.86 – 3.72 (m, 6H), 3.39 (s, 2H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 165.83, 159.08, 148.42, 143.99, 139.50, 131.79, 131.72, 130.74, 129.33, 124.94, 124.91, 124.86, 122.81, 118.51, 115.96, 115.79, 108.55, 105.74, 101.36, 46.91 (2C), 42.25 (2C). HPLC: purity – 98.55 %, retention time – 6.476 min.

(2E,4E)-5-(benzo[d][1,3]dioxol-5-yl)-1-(4-(3-fluorobenzoyl)piperazin-1-yl)penta-2,4-dien-1-one (PD03): light brown to white solid, yield- 98.7 % w/w, M. P- 132-135°C, ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.51 – 7.44 (m, 2H), 7.28 – 7.17 (m, 3H), 7.00 (s, 1H), 6.92 (d, *J* = 6.6 Hz, 1H), 6.83 (d, *J* = 14.8 Hz, 3H), 6.41 (s, 1H), 6.00 (s, 2H), 3.71 – 3.50 (m, 8H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 169.16, 165.88, 148.45, 148.28, 144.10, 139.61, 137.23, 130.55, 124.82,

122.84, 122.75, 118.39, 117.27, 117.11, 114.57, 114.39, 108.56, 105.74, 101.37, 47.86 (2C), 42.38, 42.04. HPLC: purity – 98.73 %, retention time – 7.871 min.

(2E,4E)-5-(benzo[d][1,3]dioxol-5-yl)-1-(4-(4-fluorobenzoyl)piperazin-1-yl)penta-2,4-dien-1-one (PD04): light brown to white solid, yield- 98.9 % w/w, M. P- 133-136°C, ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.54 – 7.42 (m, 3H), 7.14 (t, *J* = 7.9 Hz, 2H), 7.00 (s, 1H), 6.92 (d, *J* = 7.4 Hz, 1H), 6.84 – 6.73 (m, 3H), 6.40 (d, *J* = 11.4 Hz, 1H), 6.00 (s, 2H), 3.70 (s, 8H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 169.74, 165.86, 164.63, 162.64, 148.43, 148.27, 144.04, 139.57, 131.16, 130.71, 129.54, 124.82, 122.82, 118.42, 115.88, 115.70, 108.55, 105.73, 101.36, 45.43 (2C), 42.38, 42.02. HPLC: purity – 96.87 %, retention time – 5.957 min.

(2E,4E)-5-(benzo[d][1,3]dioxol-5-yl)-1-(4-(2-chlorobenzoyl)piperazin-1-yl)penta-2,4-dien-1-one (PD05): light brown to white solid, yield- 98.6 % w/w, M. P- 132-135°C, ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.51 – 7.43 (m, 2H), 7.39 – 7.28 (m, 3H), 7.00 (s, 1H), 6.92 (d, *J* = 7.1 Hz, 1H), 6.82 (d, *J* = 13.8 Hz, 3H), 6.42 (s, 1H), 6.00 (s, 2H), 3.97 – 3.76 (m, 6H), 3.50 – 3.26 (m, 2H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 165.89, 148.44, 148.27, 144.08, 139.59, 130.72, 130.56, 130.31 (2C), 129.80, 127.82, 127.40 (2C), 124.81, 122.83, 118.40, 108.55, 105.75, 101.36, 46.53 (2C), 41.86 (2C). HPLC: purity – 99.43 %, retention time – 5.726 min.

(2E,4E)-5-(benzo[d][1,3]dioxol-5-yl)-1-(4-(3-chlorobenzoyl)piperazin-1-yl)penta-2,4-dien-1-one (PD06): light brown to white solid, yield- 98.8 % w/w, M. P- 132-135°C, ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.53 – 7.37 (m, 4H), 7.33 – 7.28 (m, 1H), 7.00 (s, 1H), 6.92 (d, *J* = 7.1 Hz, 1H), 6.87 – 6.72 (m, 3H), 6.41 (s, 1H), 6.00 (s, 2H), 3.71 – 3.50 (m, 8H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 169.05, 165.87, 148.45, 148.28, 144.10, 139.61, 136.89, 134.83, 130.72, 130.27, 130.05, 127.36, 125.16, 124.82, 122.84, 118.39, 108.56, 105.74, 101.37, 47.27, 45.43, 42.48, 41.75. HPLC: purity – 99.16 %, retention time – 6.301 min.

(2E,4E)-5-(benzo[d][1,3]dioxol-5-yl)-1-(4-(4-chlorobenzoyl)piperazin-1-yl)penta-2,4-dien-1-one (PD07): light brown to white solid, yield- 97.9 % w/w, M. P- 134-136°C, ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.50 – 7.40 (m, 5H), 7.00 (s, 1H), 6.92 (d, *J* = 6.6 Hz, 1H), 6.84 – 6.75 (m, 3H), 6.41 (s, 1H), 6.00 (s, 2H), 3.70 – 3.52 (m, 8H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 169.64, 165.89, 148.45, 148.28, 144.12, 139.64, 136.32, 133.45, 130.71, 128.98 (2C), 128.70 (2C), 124.80, 122.85, 118.37, 108.56, 105.74, 101.37, 45.78 (2C), 42.03 (2C). HRMS $[M + H]^+$: m/z was found 425.1247, calculated 425.1268 for $C_{23}H_{22}ClN_2O_4$. HPLC: purity – 99.24, retention time - 4.615 min.

(2E,4E)-5-(benzo[d][1,3]dioxol-5-yl)-1-(4-(2-bromobenzoyl)piperazin-1-yl)penta-2,4-dien-1-one (PD08): light brown to white solid, yield- 99.1 % w/w, M. P- 130-133°C, ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.63 (d, *J* = 7.9 Hz, 1H), 7.50 – 7.40 (m, 1H), 7.35 – 7.26 (m, 3H), 7.01 (s, 1H), 6.93 (d, *J* = 7.7 Hz, 1H), 6.90 – 6.61 (m, 3H), 6.43 (s, 1H), 6.01 (s, 2H), 3.97 – 3.76 (m, 6H), 3.39 – 3.21 (m, 2H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 165.86, 165.50, 148.28, 144.02, 139.57, 132.97, 132.97, 130.65, 127.92, 124.83, 122.83, 118.43, 118.43, 108.56, 108.56, 105.74, 105.74, 101.37, 101.37, 53.43 (4C). HPLC: purity – 97.89 %, retention time – 5.940 min.

(2E,4E)-5-(benzo[d][1,3]dioxol-5-yl)-1-(4-(3-bromobenzoyl)piperazin-1-yl)penta-2,4-dien-1-one (PD09): light brown to white solid, yield- 97.8 % w/w, M. P- 131-133°C, ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.59 (s, 2H), 7.53 – 7.44 (m, 1H), 7.34 (d, *J* = 10.1 Hz, 2H), 7.00 (s, 1H), 6.92 (d, *J* = 6.3 Hz, 1H), 6.83 (d, *J* = 14.8 Hz, 3H), 6.40 (s, 1H), 6.00 (s, 2H), 3.71 – 3.49 (m, 8H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 168.92, 165.86, 148.45, 148.28, 144.10, 139.61, 137.12, 133.20, 130.72, 130.28, 130.21, 125.61, 124.82, 122.85 (2C), 118.39, 108.56, 105.74, 101.37, 42.50 (4C). HPLC: purity – 97.61, retention time - 4.816 min.

(2E,4E)-5-(benzo[d][1,3]dioxol-5-yl)-1-(4-(4-bromobenzoyl)piperazin-1-yl)penta-2,4-dien-1-one (PD10): light brown to white solid, yield- 99.2 % w/w, M. P- 134-137°C, ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.60 (d, *J* = 7.6 Hz, 2H), 7.53 – 7.44 (m, 1H), 7.36 – 7.28 (m, 2H), 7.00 (s, 1H), 6.92 (d, *J* = 7.3 Hz, 1H), 6.87 – 6.70 (m, 3H), 6.41 (s, 1H), 6.00 (s, 2H), 3.70 – 3.51 (m, 8H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 169.65, 165.88, 148.45, 148.28, 144.12, 139.63, 133.94, 131.94 (2C), 130.71, 128.87 (2C), 124.81, 124.55, 122.85, 118.37, 108.56, 105.74, 101.37, 42.18 (4C). HPLC: purity – 95.30, retention time - 4.864 min.

(2E,4E)-5-(benzo[d][1,3]dioxol-5-yl)-1-(4-(2-iodobenzoyl)piperazin-1-yl)penta-2,4-dien-1-one (PD11): orange solid, yield- 98.3 % w/w, M. P- 133-136°C, ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.87 (d, *J* = 7.8 Hz, 1H), 7.50 – 7.40 (m, 2H), 7.23 (dd, *J* = 7.5, 1.3 Hz, 1H), 7.13 (td, *J* = 7.8, 1.5 Hz, 1H), 7.00 (s, 1H), 6.92 (d, *J* = 7.9 Hz, 1H), 6.87 – 6.69 (m, 3H), 6.42 (s, 1H), 6.00 (s, 2H), 3.96 – 3.77 (m, 5H), 3.50 (dd, *J* = 14.0, 7.0 Hz, 1H), 3.35 (dd, *J* = 10.3, 3.3 Hz, 1H), 3.27 – 3.18 (m, 1H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 169.45, 165.93, 148.44, 148.27, 144.13, 139.63, 139.38 (2C), 130.72, 130.58, 128.59, 127.05, 124.81, 122.85, 118.38, 108.56, 105.75, 101.37, 92.40, 65.86 (2C), 41.76 (2C). HPLC: purity – 95.99 %, retention time – 5.794 min.

3-(4-((2E,4E)-5-(benzo[d][1,3]dioxol-5-yl)penta-2,4-dienoyl)piperazine-1-carbonyl)benzonitrile (PD12): yellow to white solid, yield- 98.8 % w/w, M. P- 132-135°C, ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.83 – 7.72 (m, 2H), 7.68 (d, *J* = 7.8 Hz, 1H), 7.60 (t, *J* = 7.7 Hz, 1H), 7.49 (dd, *J* = 14.5, 10.7 Hz, 1H), 7.00 (s, 1H), 6.92 (d, *J* = 8.0 Hz, 1H), 6.88 – 6.70 (m, 3H), 6.41 (s, 1H), 6.00 (s, 2H), 3.72 – 3.47 (m, 8H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 168.20, 165.87, 148.48, 148.29, 144.25, 139.74, 136.51, 133.57, 131.38, 130.83, 130.68, 129.71, 124.76, 122.88, 118.24, 117.82, 113.22, 108.57, 105.74, 101.38, 45.09 (2C), 42.17 (2C). HPLC: purity – 98.17 %, retention time – 4.601 min.

(2E,4E)-5-(benzo[d][1,3]dioxol-5-yl)-1-(4-(2-nitrobenzoyl)piperazin-1-yl)penta-2,4-dien-1-one (PD13): light yellow solid, yield- 98.6 % w/w, M. P- 138-141°C, ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.24 (d, *J* = 7.8 Hz, 1H), 7.76 (t, *J* = 6.9 Hz, 1H), 7.64 (d, *J* = 7.4 Hz, 1H), 7.54 – 7.38 (m, 2H), 7.00 (s, 1H), 6.92 (d, *J* = 6.9 Hz, 1H), 6.82 (d, *J* = 14.0 Hz, 3H), 6.43 (s, 1H), 6.00 (s, 2H), 4.09 – 3.61 (m, 6H), 3.29 (s, 2H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 165.89 (2C), 148.44, 148.27, 145.48, 144.11, 139.61, 134.64, 132.34, 130.73, 130.19, 127.98, 124.99, 124.82, 122.84, 118.39, 108.56, 105.75, 101.37, 42.07 (4C). HPLC: purity – 97.69 %, retention time – 4.816 min.

(2E,4E)-5-(benzo[d][1,3]dioxol-5-yl)-1-(4-(3-nitrobenzoyl)piperazin-1-yl)penta-2,4-dien-1-one (PD14): light yellow solid, yield- 98.7 % w/w, M. P- 138-141°C, ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.33 (d, *J* = 12.5 Hz, 2H), 7.79 (d, *J* = 5.9 Hz, 1H), 7.68 (d, *J* = 7.1 Hz, 1H), 7.57 – 7.43 (m, 1H), 7.00 (s, 1H), 6.92 (d, *J* = 6.7 Hz, 1H), 6.85 – 6.81 (m, 3H), 6.41 (s, 1H), 6.00 (s, 2H), 3.74 – 3.50 (m, 8H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 167.98, 165.90, 148.48, 148.29, 148.18, 144.27, 139.76, 136.76, 133.11, 130.68, 130.02, 124.92, 124.75, 122.88, 122.40, 118.22, 108.56, 105.73, 101.38, 42.52 (4C). HPLC: purity – 96.38 %, retention time – 4.862 min.

(2E,4E)-5-(benzo[d][1,3]dioxol-5-yl)-1-(4-(3-methoxy benzoyl)piperazin-1-yl)penta-2,4-dien-1-one (PD15): light brown to white solid, yield- 97.6 % w/w, M. P- 129-131°C, ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.52 – 7.44 (m, 1H), 7.35 (s, 1H), 6.99 (d, *J* = 11.4 Hz, 4H), 6.92 (d, *J* = 5.8 Hz, 1H), 6.81 (s, 3H), 6.41 (s, 1H), 6.00 (s, 2H), 3.85 – 3.50 (m, 11H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 170.40, 165.84, 159.77, 148.40, 148.26, 143.96, 139.49, 136.47, 130.73, 129.77, 124.85, 122.80, 119.06, 118.51, 115.86, 112.56, 108.54, 105.73, 101.35, 55.39, 42.34 (4C). HPLC: purity – 99.29 %, retention time – 4.615 min.

(2E,4E)-5-(benzo[d][1,3]dioxol-5-yl)-1-(4-(4-methoxybenzoyl)piperazin-1-yl)penta-2,4-dien-1-one (PD16): light orange solid, yield- 97.9 % w/w, M. P- 120-133°C, ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.55 – 7.38 (m, 3H), 7.00 – 6.91 (m, 4H), 6.88 – 6.72 (m, 3H), 6.41 (d, *J* = 13.3 Hz, 1H), 6.00 (s, 2H), 3.86 (s, 3H), 3.70 (s, 8H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 170.64, 165.84, 161.10, 148.39, 148.26, 143.89, 139.44, 130.75, 129.27 (2C), 127.15, 124.88, 122.79, 118.58, 113.88 (2C), 108.54, 105.73, 101.35, 55.39, 42.21 (4C). HPLC: purity – 97.55 %, retention time – 4.529 min.

(2E,4E)-5-(benzo[d][1,3]dioxol-5-yl)-1-(4-(4-(trifluoromethyl)benzoyl)piperazin-1-yl)penta-2,4-dien-1-one (PD17): orange solid, yield- 98.3 % w/w, M. P- 134-137°C, ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.73 (d, *J* = 7.7 Hz, 2H), 7.56 (d, *J* = 7.7 Hz, 2H), 7.49 (dd, *J* = 14.2, 10.9 Hz, 1H), 7.00 (s, 1H), 6.93 (d, *J* = 7.7 Hz, 1H), 6.87 – 6.72 (m, 3H), 6.41 (s, 1H), 6.00 (s, 2H), 3.88 – 3.42 (m, 8H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 169.16, 165.88, 148.47, 148.29, 144.20, 139.70, 138.72, 132.23, 130.69, 127.51 (2C), 125.82, 125.79, 124.77, 122.86, 118.28, 108.57, 105.73, 101.38, 47.54, 42.30 (4C). HPLC: purity – 97.44 %, retention time – 4.528 min.

(2E,4E)-5-(benzo[d][1,3]dioxol-5-yl)-1-(4-(4-(trifluoromethoxy)benzoyl)piperazin-1-yl)penta-2,4-dien-1-one (PD18): light orange solid, yield- 98.7 % w/w, M. P- 135-138°C, ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.49 (t, *J* = 11.6 Hz, 3H), 7.33 – 7.28 (m, 2H), 7.00 (s, 1H), 6.92 (d, *J* = 7.5 Hz, 1H), 6.87 – 6.71 (m, 3H), 6.41 (s, 1H), 6.00 (s, 2H), 3.72 – 3.52 (m, 8H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 169.34, 165.87, 150.33, 148.46, 148.28, 144.13, 139.64, 133.68, 130.71, 129.05 (2C), 124.80, 122.85, 121.04 (2C), 118.36, 108.56, 105.73, 101.38, 42.42 (5C). HPLC: purity – 97.88 %, retention time – 4.535 min.

(2E,4E)-1-(4-(4-aminobenzoyl)piperazin-1-yl)-5-(benzo[d][1,3]dioxol-5-yl)penta-2,4-dien-1-one (PD19): light yellow solid, yield- 99.1 % w/w, M. P- 132-135°C, ^{1}H NMR (500 MHz, DMSO) δ 7.28 (dd, *J* = 14.3, 10.1 Hz, 1H), 7.22 – 7.13 (m, 3H), 6.95 (dt, *J* = 15.3, 7.3 Hz,

4H), 6.67 (d, *J* = 14.5 Hz, 1H), 6.57 (d, *J* = 8.0 Hz, 2H), 6.05 (s, 2H), 5.55 (s, 2H), 3.60 – 3.35 (m, 8H). ^{13}C NMR (126 MHz, DMSO) δ 170.58, 165.10, 151.16, 148.43, 148.33, 142.90, 138.79, 131.22, 129.85 (2C), 125.91, 123.11, 121.97, 120.58, 113.14 (2C), 109.01, 106.01, 101.78, 45.55 (2C), 42.05 (2C). HPLC: purity – 99.29 %, retention time – 4.574 min.

(2E,4E)-5-(benzo[d][1,3]dioxol-5-yl)-1-(4-(2,4-dichlorobenzoyl)piperazin-1-yl)penta-2,4-dien-1-one (PD20): light yellow solid, yield- 97.3 % w/w, M. P- 135-138°C, ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.53 – 7.43 (m, 2H), 7.36 (dd, *J* = 8.2, 1.9 Hz, 1H), 7.28 (d, *J* = 3.6 Hz, 1H), 7.00 (s, 1H), 6.92 (d, *J* = 8.0 Hz, 1H), 6.85 – 6.71 (m, 3H), 6.41 (s, 1H), 6.00 (s, 2H), 3.99 – 3.92 (m, 1H), 3.76 (d, *J* = 10.8 Hz, 5H), 3.38 – 3.30 (m, 1H), 3.25 (dd, *J* = 11.9, 6.5 Hz, 1H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 166.28, 165.81, 148.45, 148.28, 144.13, 139.63, 135.98, 133.74, 131.28, 130.71, 129.75, 128.81, 127.87, 124.80, 122.85, 118.34, 108.56, 105.73, 101.38, 46.67 (2C), 41.96 (2C). HPLC: purity – 98.12 %, retention time – 4.589 min.

(2E,4E)-5-(benzo[d][1,3]dioxol-5-yl)-1-(4-(3,5-dimethoxybenzoyl)piperazin-1-yl)penta-2,4-dien-1-one (PD21): white solid, yield- 97.9 % w/w, M. P- 132-135°C, ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.48 (dd, *J* = 14.4, 10.7 Hz, 1H), 7.06 – 6.98 (m, 3H), 6.91 (t, *J* = 9.4 Hz, 2H), 6.84 – 6.73 (m, 3H), 6.41 (d, *J* = 14.3 Hz, 1H), 6.00 (s, 2H), 3.93 (d, *J* = 5.8 Hz, 6H), 3.70 (s, 8H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 170.58, 165.85, 150.67, 149.14, 148.42, 148.28, 143.93, 139.48, 130.74, 127.35, 124.87, 122.81, 120.25, 118.56, 110.99, 110.55, 108.55, 105.73, 101.36, 56.03 (2C), 42.17 (4C). HPLC: purity – 98.09 %, retention time – 3.641 min.

(2E,4E)-5-(benzo[d][1,3]dioxol-5-yl)-1-(4-(3,5-dinitrobenzoyl)piperazin-1-yl)penta-2,4-dien-1-one (PD22): orange solid, yield- 99.2 % w/w, M. P- 140-143°C, ^{1}H NMR (500 MHz, DMSO) δ 8.89 (s, 1H), 8.69 (s, 2H), 7.33 – 7.14 (m, 2H), 6.99 – 6.94 (m, 4H), 6.71 (s, 1H), 6.05 (s, 2H), 3.71 – 3.34 (m, 8H). ^{13}C NMR (126 MHz, DMSO) δ 165.54, 165.20, 148.66, 148.43 (2C), 148.35, 143.03, 139.23, 138.90, 131.19, 128.11 (2C), 125.87, 123.13, 120.46,

119.70, 109.02, 106.01, 101.79, 47.22 (2C), 45.21 (2C). HPLC: purity – 98.14 %, retention time – 3.644 min.

(2E,4E)-5-(benzo[d][1,3]dioxol-5-yl)-1-(4-(5-chloro-2-methoxybenzoyl)piperazin-1-yl)penta-2,4-dien-1-one (PD23): light orange solid, yield- 98.6 % w/w, M. P- 137-140°C, ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.47 (dd, *J* = 14.2, 10.7 Hz, 1H), 7.35 (d, *J* = 7.0 Hz, 1H), 7.27 (d, *J* = 9.0 Hz, 1H), 7.00 (s, 1H), 6.93 – 6.87 (m, 2H), 6.81 (t, *J* = 10.4 Hz, 3H), 6.42 (s, 1H), 6.00 (s, 2H), 3.93 – 3.62 (m, 9H), 3.34 – 3.27 (m, 2H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 166.50, 165.83, 153.89, 148.42, 148.27, 143.95, 139.48 (2C), 130.74, 130.52, 128.14, 126.24, 124.86, 122.81, 118.55, 112.32, 108.55, 105.73, 101.37, 55.98, 46.68 (2C), 41.96 (2C). HPLC: purity – 97.47 %, retention time – 4.572 min.

(2E,4E)-5-(benzo[d][1,3]dioxol-5-yl)-1-(4-(benzo[d][1,3]dioxole-5-carbonyl)piperazin-1-yl)penta-2,4-dien-1-one (PD24): light orange solid, yield- 97.2 % w/w, M. P- 131-134°C, ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.56 – 7.42 (m, 1H), 7.05 – 6.90 (m, 4H), 6.87 – 6.76 (m, 4H), 6.41 (d, *J* = 13.1 Hz, 1H), 6.03 – 6.00 (m, 4H), 3.69 – 3.50 (m, 8H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 170.19, 165.84, 149.20, 148.41, 148.27, 147.79, 143.95, 139.48, 130.75, 128.71, 124.87, 122.81, 121.78, 118.54, 108.55, 108.33, 108.11, 105.74, 101.56, 101.36, 45.46 (2C), 42.16 (2C). HRMS $[M + H]^+$: m/z was found 435.1540, calculated 435.1556 for $C_{24}H_{23}N_2O_6$. HPLC: purity – 96.45 %, retention time - 4.018 min.

(2E,4E)-5-(benzo[d][1,3]dioxol-5-yl)-1-(4-cinnamoylpiperazin-1-yl)penta-2,4-dien-1-one (PD25): light orange solid, yield- 98.4 % w/w, M. P- 130-133°C, ^{1}H NMR (500 MHz, DMSO) δ 7.74 (d, *J* = 6.9 Hz, 2H), 7.54 (d, *J* = 15.3 Hz, 1H), 7.45 – 7.37 (m, 3H), 7.34 – 7.25 (m, 2H), 7.20 (s, 1H), 7.01 – 6.91 (m, 4H), 6.71 (d, *J* = 14.5 Hz, 1H), 6.06 (s, 2H), 3.76 – 3.63 (m, 8H). ^{13}C NMR (126 MHz, DMSO) δ 165.12, 165.09, 148.44, 148.34, 142.93, 142.28, 138.78, 135.57, 131.23, 130.07, 129.23 (2C), 128.55 (2C), 125.96, 123.10, 120.61, 118.56, 109.02,

106.01, 101.79, 45.35 (2C), 42.44 (2C). HRMS $[M + H]^+$: m/z was found 417.1809, calculated 417.1814 for $C_{25}H_{25}N_2O_4$. HPLC: purity – 98.90 %, retention time – 5.918 min.

6.1.1.5. Preparation of compound PC04

The Boc-piperazine (PC08) 1.0 *eq* was dissolved into DCM 10 ml and cooled to 0° C, then trifluoroacetic acid (TFA) (5.0 *eq*) was added dropwise and reaction mixture was stirred at room temperature for 12 h. Further, reaction mixture was washed with water, then treated with $NaHCO_2$ solution. The compound was extracted with DCM and evaporated under reduced pressure to get orange colour powder.

6.1.1.7. Preparation of compound PD26

The compound PC04 (1.0 *eq*) and diisopropylethylamine (DIPEA) (2.5 *eq*) in DMF (5 ml), aryl halide (1.1 *eq*) was added at 10°C to RBF. The reaction mixture was stirred at 90°C for 4 h. After completion of reaction, it was cooled to room temperature and treated with ice-cold water. Then, compound was extracted with DCM. The separated DCM layer was treated with sodium sulfate and concentrated under reduced pressure. Further, compound PD26 was purified using column chromatography using ethyl acetate: hexane and silica gel (60 – 120 mesh) to get compound of interest.

(2E,4E)-5-(benzo[d][1,3]dioxol-5-yl)-1-(4-benzylpiperazin-1-yl)penta-2,4-dien-1-one (PD26): light yellow solid, yield- 97.2 % w/w, M. P- 128-131°C, ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.44 (dd, *J* = 14.6, 10.2 Hz, 1H), 7.38 – 7.28 (m, 5H), 7.00 (s, 1H), 6.91 (d, *J* = 8.0 Hz, 1H), 6.84 – 6.70 (m, 3H), 6.41 (d, *J* = 14.6 Hz, 1H), 5.99 (s, 2H), 3.78 – 3.53 (m, 6H), 2.48 (s, 4H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 165.52, 148.23, 143.00, 138.71, 137.66, 130.94, 129.14 (2C), 128.35 (3C), 127.29, 125.19, 122.61, 119.41, 108.52, 105.72, 101.31, 62.89, 53.26, 52.82,

45.74, 42.10. HRMS $[M + H]^+$: m/z was found 377.1842, calculated 377.1865 for $C_{23}H_{25}N_2O_3$. HPLC: purity – 98.84 %, retention time – 5.927 min.

6.1.2. *In vitro* studies

6.1.2.1. Cholinesterase inhibition assay

The cholinesterase enzymes hAChE, eeAChE, and eqBuChE were purchased from Sigma Aldrich (USA). The spectrometric analysis method was followed to evaluate the ChEs inhibitory activity of compounds [217]. The test compound stock solutions were prepared by dissolving compounds into biological grade DMSO (1 mg/mL). Further, compounds final concentrations (0.01, 0.1, 1.0, 10, 20, and 40 µM) were made in phosphate buffer (pH 7.4). Test compounds (10 µl) were added to a 96-well plate followed by AChE/BuChE (0.022 U, 50 µL) enzymes added and incubated at room temperature for 30 min. Then, ATCI (1.5 mM, 30 µL) or BTCI (1.5 mM, 30 µL) substrates were added with respective enzymes AChE or BuChE, and incubated for another 30 min. Then, multimode microplate reader (SpectraMax M5, USA) was used to measure the absorbance at 415 nm after the addition of DTNB (0.15 mM, 160 µL). Blank sample absorbance was measured using 10 µL of DMSO instead of test samples. The donepezil served as a positive control in this study.

6.1.2.2. BACE1 inhibitory activity

β-secretase (BACE1) activity detection kit (Sigma Aldrich, US) was used for the evaluation of compounds potency. The calibration curve was prepared using standard solution as per protocol mentioned in catalog. Further, compounds IC_{50} results were calculated in graph pad prism 5.

6.1.2.3. PAMPA-BBB assay

The drugs which act on CNS have the ideal lipophilicity, which is advantageous to cross the blood-brain barrier (BBB). To evaluate compounds lipophilicity Parrel artificial membrane permeability assay (PAMPA) was performed [217]. Biological grade DMSO was used to make the test compound stock solutions, then further diluted with phosphate buffer pH 7.4. Then, 200 µL of each test sample was transferred into donor plates which contain a pore size of 0.45 mm, where the acceptor was fixed before the donor plates were incubated for 18 h. After incubation, test sample absorbance was measured at 345 nm using a microplate reader (SpectraMax M5, USA).

6.1.2.4. Propidium iodide displacement assay

The compound PD07 binding specificity at the AChE PAS site was evaluated through propidium iodide displacement assay [196,218]. Various concentrations of test compound PD07 (5, 10, and 20 M; 150 µL) were incubated with AChE 5.0 U ml^{-1} for 6 h at 25°C. Then, propidium iodide solution (1.0 M; 50 µL) was added and incubated for another 10 min. The multimode microplate reader (SpectraMax M5, USA) was used to measure the fluorescence intensity at excitation (535 nm) and emission (595 nm) wavelengths. The donepezil was used as a positive control, and DMSO was used instead of test samples to measure blank readings.

6.1.2.5. Free-radical scavenging assay

The DPPH method was followed to evaluate the compounds antioxidant activity [218]. The various concentrations (200, 100, 50, 30, 20, and 10 µM) of compounds were prepared in methanol. Further, test samples (75 µL) were added to 96-well plate. Then, 75 µL of DPPH solution (100 µM) was added and incubated for 25 min at 37°C. The absorbance was measured

using a multimode microplate reader (SpectraMax M5, US) at 525 nm. Ascorbic acid (AA) served as a reference compound in this study.

6.1.2.6. $A\beta_{1-42}$ aggregation inhibition assay

Thioflavin T (ThT) was used in this study to estimate the compound PD07 activity on self-induced $A\beta_{1-42}$ and AChE-induced $A\beta_{1-42}$ aggregation inhibition [198,218]. The $A\beta_{1-42}$ was dissolved in 80 µL of NH_4OH and then diluted to 1.0 ml in PBS buffer 7.4 to prepare a stock solution. The test compound PD07 (5, 10, and 20 µM) was prepared in DMSO, then they were diluted in PBS buffer 7.4 to get final concentrations.

Self-induced $A\beta_{1-42}$ assay, a solution of $A\beta_{1-42}$ was incubated with test compound PD07 (5 mM, 10 mM, and 20 mM; 2 µL) or without test compound for 48 h at 37°C. ThT solution (20 M, 178 µL) was added to the well plate after the incubation time, and fluorescence intensity was measured using a microplate reader (SpectraMax M5, USA) at 450 nm (excited) and 485 nm (emission) wavelengths. The same method was used for AChE-induced $A\beta_{1-42}$ aggregation assay, additionally, AChE (230 µM, 16 µL) was used to promote Aβ aggregation. Furthermore, fluorescence images were taken, using confocal microscope (Carl Zeiss microscopy GMBH, LSM 900). The test samples used for confocal images in this study were previously incubated for 15 days. The fluoresce filter cubes are set to excitation wavelength (495 nm) and an emission wavelength (519 nm). The detector type is set to Multialkali-PMT and dye is set to FITC. Then, images were captured at 20X magnification.

6.1.2.7. Evaluation of neuroprotection activity

The compound PD07 neuroprotective action was evaluated through MTT assay using neuroblastoma cell line SH-SY5Y [199]. The cells (1×10^5 cells/well) were added to the 96-well plate, they were incubated before at 37°C for 24 h in a humid environment with 5 % CO_2.

Further cells were treated with $A\beta_{1-42}$ (10 μM) and incubated for an additional 24 h. Then, various concentrations of compound PD07 (5, 10, and 20 μM) were added, and incubated for another 72 h at 37°C. Finally, 20 μL of MTT reagent was added and incubated for an additional 24 h. The obtained formed formazan crystals were dissolved into 100 μL of DMSO. Then, absorbance was measured at 570 nm, and percentage of cell viability was calculated.

6.1.3. *In silico* studies

6.1.3.1. Molecular docking studies

The co-crystallized structure of protein recombinant human acetylcholinesterase complexed with ligand donepezil (PDB: 4ey7) [186], recombinant human butyrylcholinesterase complexed with ligand tacrine (PDB: 4bds) [187], and beta-secretase (BACE1) complexed with ligand BSD (N-{(1S,2R)-1-benzyl-2-hydroxy-3-[(3-methoxybenzyl)amino] propyl}-5-[methyl(methylsulfonyl)amino-N'-[(1R)-1phenylethyl]benzene-1,3-dicarboxamine)) (PDB: 2vkm) [219] were retrieved from protein data bank (PDB) (https://www.rcsb.org/) [188]. Further, proteins were prepared by deleting unwanted chains, water molecules, hetero atoms, and ligand groups using discovery studio visualizer 2021 [189]. Furthermore, quality of proteins was evaluated by saves v6.0 (https://saves.mbi.ucla.edu/) by analyzing the Ramachandran plots. The missing fragments and broken amino acid chains were rebuilt by using the Swiss model server (https://swissmodel.expasy.org/) in the user template mode. The structure of ligands was drawn using Chem3D 20.1.1 software and energies were minimized to optimize the bond angle and lengths. After completion of above sequential steps, protein preparation was carried out by AutoDock tools (MGL tools 1.5.6) [190]. The docking confirmations were examined and documented using the Discovery Studio Visualizer 2021 [189].

6.1.3.2. Molecular dynamic simulations

Desmond (Desmond maestro 2019.1) software was used to carry out molecular dynamic studies [191]. The protein preparation wizard process was used to preprocess the protein. The solvent model TIP3P was used for system builder. The boundary conditions were set to cubic, excluding ions and salt replacement within 4 A°, and the system was neutralized with Na+ and Cl^{-} at a salt concentration of 0.15 M. The simulation time 300 (PS) was used to complete minimization process [218]. Molecular dynamics (MD) was performed using an NTP-enabled system and simulation times 100 ns, approximate number of frames was set to 1000. Further, pressure was given to 1 bar, and temperature was fixed to 310 K. The results were analyzed by observing the ligand RMSF and protein RMSF, and ligand RMSD and protein RMSD.

6.1.3.3. Drug-likeness, ADME, and toxicity predictions

The compound PD07 properties were analyzed by preADMET and swissADME (http://www.swissadme.ch/) online servers [192]. The compound PD07 drug-likeness (https://preadmet.webservice.bmdrc.org/druglikeness/) was analyzed by Lipinski rule of five [193]. Further absorption, distribution, metabolism, and eliminations (https://preadmet.webservice.bmdrc.org/adme/) were predicted [193]. Moreover, compound PD07 toxicity profile (https://preadmet.webservice.bmdrc.org/toxicity/) was also evaluated [193].

6.1.4. *In vivo* studies

6.1.4.1. Animals, housing, and ethical approval

The healthy Wistar rats (200 ± 10 g) were used for *in vivo* experiments. Animals were kept under 12 h light/12 h dark conditions, with a temperature of 25 ± 2°C and relative humidity of 55 ± 10 %. They were allowed to acclimatize for seven days with proper diet and water. All

the experimental protocols were approved (approval no. IIT(BHU)/IAEC/2022/022) by the Institute Animal Ethical Committee (IAEC), Indian Institute of Technology, Banaras Hindu University, Varanasi.

6.1.4.2. Acute oral toxicity studies

OECD 423 guidelines were followed to evaluate compound PD07 safety profile [220]. The male Wistar rats were divided into two groups (n = 6), group 1 received vehicle alone (control group), and group 2 received single dose of compound PD07 (300 mg/kg, p.o.). The vehicle alone (0.5 % carboxyl methyl cellulose) was administered orally to the control group. Rats were observed for up to 14 days for toxic symptoms, body wight, food consumption, behavioral changes, and mortality. Then, rats were sacrificed on the final day (after 14 days) and major organs (brain, heart, kidney, and liver) were isolated. The organ transverse sections were prepared using a cryostat (Slee Mev, Germany). Then, sections were stained with hematoxylin and eosin stains and were observed under a 10X microscope. Rats toxic reactions were also evaluated through biochemical tests such as the creatine kinase myocardial band (Ck-MD), creatinine, alkaline phosphatase (ALP), alkaline transaminase (ALT), and aspartate transaminase (AST).

6.1.4.3. Rotarod test

The motor coordination activity of rats was assessed using Rotarod test, before and after treatment with PD07 (10 and 50 mg/kg). The rats were trained for 120 sec at 5 rpm on day one in the Rotarod apparatus. Then, rats performance was assessed at speed of 25 rpm to evaluate motor coordination before and after drug administration.

6.1.4.4. Scopolamine induced amnesia model

The scopolamine-induced amnesia (Y maze test) model is most commonly used to evaluate spatial working memory in rats [217,218]. The rats were divided into six groups (n=6), with group 1 being the vehicle alone (control group), group 2 being scopolamine 0.5 mg/kg, p.o. (disease control), group 3 being PD07 2.5 mg/kg, p.o., group 4 being PD07 5 mg/kg, p.o., group 5 being PD07 10 mg/kg, p.o., and group 6 being DPZ 5 mg/kg, p.o. Groups 3 to 6 received compound PD07/DPZ orally (p.o.) for seven days as stated above doses. Whereas, groups 1 and 2 received vehicle alone (0.5 % CMC). On the seventh day, scopolamine (0.5 mg/kg, i.p.) was dissolved in normal saline and given to all rodent groups through intraperitoneal injection (i.p.) except group 1 (control group). Then, rats spatial working memory was tested using Y maze apparatus after 30 min of scopolamine injection. The rat was placed at the center of Y maze apparatus and given five minutes to explore. Each rat's spontaneous alternations were noted manually. The percentage of spontaneous alternations was calculated using the following formula: [number of alternations/ (total arm entries) – 2 × 100].

6.1.4.5. AChE and ACh analysis

After completion of behavioral studies, all animals were sacrificed and brain was isolated. Then, whole brains were washed with normal saline and homogenized individually using PBS 7.4 buffer. The supernatants were centrifuged for 15 min at 4°C and 4350 g. The Ellman method was followed to assess AChE activity [204]. The 100 µl of supernatant and 100 µL of 15 mM ATCI was added to 96 well plates and were incubated at 37°C for 5 min. Then, microplate reader (SpectraMax M5, USA) was used to measure the absorbance at 415 nm after addition of 100 µL of DTNB (1.5 mM). The amount of substrate hydrolyzed per minute per mg of protein was used to determine the rate of hydrolysis. Further, ACh levels were determined using brain homogenate by rat ACh ELISA kit (Krishgen biosciences, India).

6.1.5. Statistical analysis

The statistical data were presented as mean ± S.D., and graphs were prepared using graph pad Prism 5. One-way ANOVA followed by Tuckey's post hoc analysis was performed to calculate all the findings. The P value less than 0.05 was considered statistically significant.

6.2. Results and discussion

6.2.1. Extraction and isolation of piperine

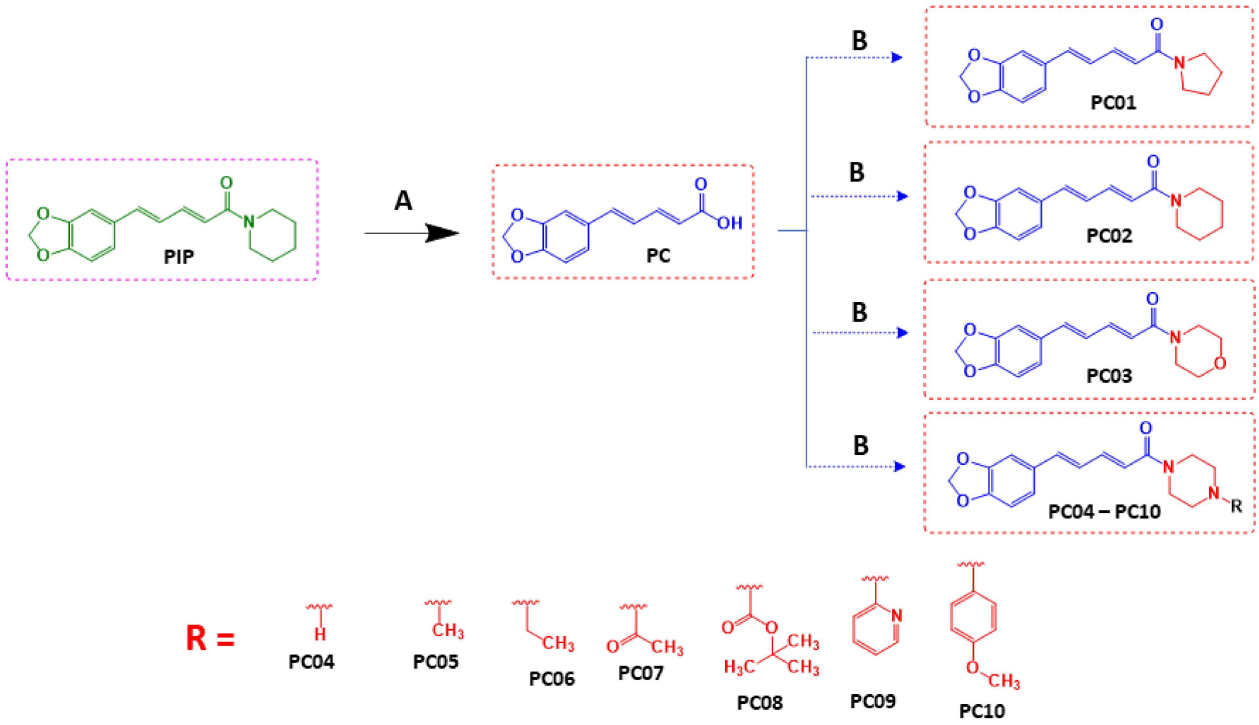
The cold maceration technique was used to prepare ethanolic extract of *Piper longum* fruits, and extractive yield was found to be 12.51 % w/w. Further, concentrated crude ethanolic extract of *Piper longum* was used to isolate the piperine through column chromatography, and it was found in faction numbers 89 – 106 under conditions of hexane: ethyl acetate (40:60). The percentage of piperine was determined to be 4.35 % w/w in *Piper nigrum* dried fruits.

6.2.2. Design and synthesis of piperine derivatives

The pathophysiology of AD involves multiple pathophysiologic factors which contribute to the onset and progression of AD. To MTDLs, piperine was used as a precursor compound for the development of novel series of compounds (PC01-PC10 and PD01-PD26). The crucial intermediate compound piperic acid (PC) was synthesized by hydrolyzing piperine (PIP) under the rection conditions of 20 % KOH ethanolic solution for 20 h of reflux. Further, piperic acid (PC) was reacted with various substituted secondary amines (1–10) to get compounds of interest (Scheme 6.1). The compounds PC04 to PC10 exhibited the most selective cholinesterase inhibition activity as compared to compounds PC01 to PC03, it might be due to substituted piperazine ring. Previous reports also suggest that substitution of piperazine ring is advantageous for ChEs inhibition and neuroprotective action [221].

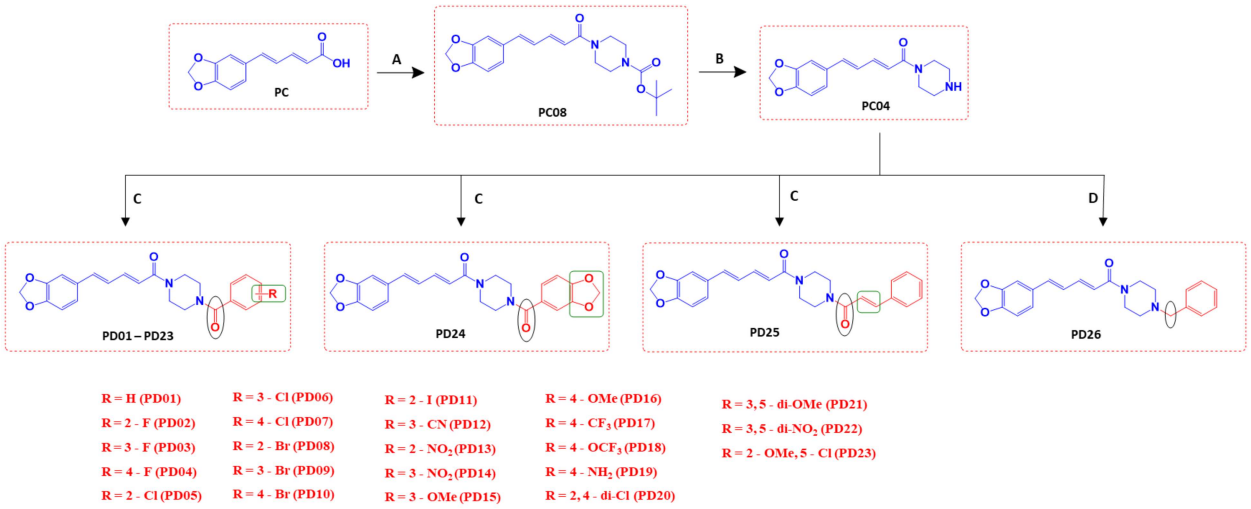
In Scheme 6.2, key intermediate compound piperic acid (PC) was coupled with BOC-piperazine. Then, BOC group was removed from piperazine ring (PC08) using trifluoroacetic acid (TFA) leading to compound PC04. Further, compound PD04 was reacted with various substituted carboxylic acids to synthesize compounds of interest (PD01-PD25). The compound PD26 was synthesized by coupling compound PC04 with benzyl bromide under basic conditions.

Scheme 6.1. Synthesis of piperine derivatives (PC01 – PC10) [a]



[a] Reagents and conditions: (A) Piperine (PIP), 20 % KOH, MeOH, r.t., reflux, 20h. (B) Piperic acid (PC) 1.0 *eq*, secondary amines (1-10) 1.1 *eq*, diisopropylethylamine (DIPEA) 2.5 *eq*, 1-hydroxy benzotriazole (HOBT) 2.5 *eq*, 1-(3-dimethyl aminopropyl)-3-ethyl carbodiimide hydrochloride (EDC.HCl) 1.5 *eq*, acetonitrile, r.t., 8h.

Scheme 6.2. Synthesis of piperine derivatives (PD01 – PD26) [b]



[b] Reagents and conditions: (A) Piperic acid (PC) 1.0 *eq*, BOC-piperazine 1.1 *eq*, DIPEA 2.5 *eq*, HOBT 2.5 *eq*, EDC.HCl 1.5 *eq*, acetonitrile, r.t., 8h. (B) Compound PC08 1.0 *eq*, trifluoroacetic acid 5.0 *eq*, DCM, r.t., 3h. (C) Compound PC04 1.0 *eq*, carboxylic acids 1.1 *eq*, DIPEA 2.5 *eq*, HOBT 2.5 *eq*, EDC.HCl 1.5 *eq*, acetonitrile, r.t., 8h. (D) Compound PC04 1.0 *eq*, benzyl bromide 1.1 *eq*, DIPEA 2.5 *eq*, DMF, r.t., reflux, 10h.

6.2.3. *In vitro* studies

6.2.3.1. Cholinesterase inhibitory activity and SAR studies

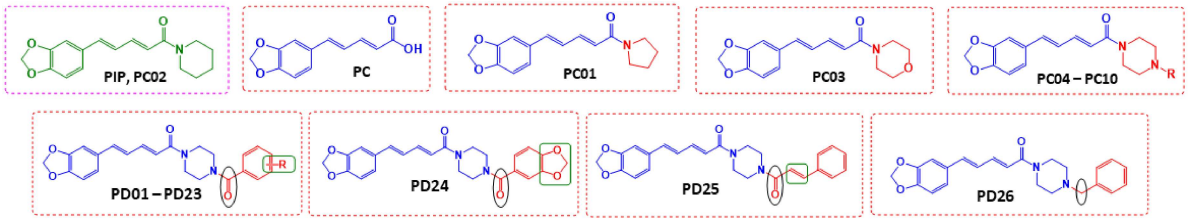
AChE is essential for the hydrolysis of ACh in cholinergic synapses, and BuChE exhibits secondary action after AChE. Generally, ACh levels decrease in Alzheimer's disease people as compared to healthy individuals. Therefore, increasing ACh levels and inhibiting ChEs may reduce the complexity of AD [207]. The synthetic piperine derivatives effect on ChEs was evaluated and compared with the reference compound Donepezil (Table 6.1).

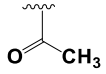
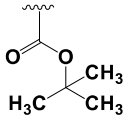
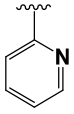
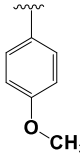
In SAR studies, pyrrolidine substitution (PC01) exhibited less inhibitory potential as compared to piperidine substitution (PC02). Next, compound PC03 in which piperidine is replaced by morpholine displayed a superior inhibition as compared to pyrrolidine (PC01) and piperidine (PC02) substitutions. Further, piperazine-substituted compounds (PC04-PC10) displayed

greater inhibition and mores selectivity towards ChEs. Furthermore, acetyl substitution (PC07) at piperazine ring enhanced compound potency as compared to methyl (PC05) and ethyl (PC06) substitutions. The methoxybenzyl substitution at piperazine ring (PC10) displayed highest ChEs inhibition (Table 6.1) as compared to all other compounds from Scheme 6.1.

Considering the activity of piperazine substituted compounds (Scheme 6.1) towards ChEs selectivity and previous reports on piperazine neuroprotective potency, we have designed Scheme 6.2 compounds (PD01-PD26). Further, cholinesterase inhibition studies were carried out on Scheme 6.2 compounds, the results suggested that substitution at para position is favorable for ChEs selectivity as compared to meta and ortho positions. The substitution of electron-donating groups displayed more ChEs inhibitory activity as compared to electron-withdrawing groups substitution (Table 6.1). The potency and specificity of ChEs inhibition are decreased in nitro-substituted compounds (PD13 and PD14). The chlorine substituted (para position) compound PD07 showed significant ChEs activity (hAChE IC_{50}: 0.29 ± 0.007 μM; eeAChE IC_{50}: 0.31 ± 0.009 μM; eqBuChE IC_{50}: 0.82 ± 0.021 μM). Further, substitution of 1,3 dioxole ring (PD24) showed no improvement in compound potency. In compound PD25, increasing carbon chain after the acetyl group did not improved the ChEs inhibition. The removal of C=O group between the piperazine ring and aromatic ring (PD26) demonstrated least ChEs selectivity. The disrupted aryl rings (PD23) exhibited least activity than electron-donating groups (Table 6.1). The ChEs inhibition results suggested that substitution of chlorine at para position (PD07) is advantageous for ChEs inhibition.

Table 6.1. Effect of piperine derivatives on Cholinesterase (AChE and BuChE), BACE1, and PAMPA



S. No.	Comp.	R	eeAChE (IC_{50}) µM [a]	hAChE (IC_{50}) µM [a]	eqBuChE (IC_{50}) µM [a]	BACE1 (IC_{50}) µM [a]	P_e (10^{-6} cm s^{-1}) [a, b]	CNS (±)
1	PIP	-	285.4 ± 5.8	280.4 ± 7.2	230.5 ± 5.9	63.51 ± 1.58	7.80 ± 0.21	+
2	PC	-	650.8 ± 13.6	nd	560.7 ± 15.6	nd	6.58 ± 0.19	+
3	PC01	-	300.2 ± 6.6	nd	270.6 ± 6.21	70.79 ± 1.84	6.31 ± 0.15	+
4	PC02	-	283.6 ± 6.40	nd	233.6 ± 6.53	nd	7.79 ± 0.17	+
5	PC03	-	200.3 ± 4.3	196.32 ± 5.1	236.4 ± 6.84	60.42 ± 1.38	7.24 ± 0.18	+
6	PC04		142.5 ± 2.9	138.64 ± 3.3	186.7 ± 4.65	52.62 ± 1.46	8.36 ± 0.24	+
7	PC05		133.4 ± 2.6	nd	153.9 ± 3.97	50.45 ± 1.26	8.61 ± 0.23	+
8	PC06		121.3 ± 2.4	nd	142.4 ± 3.26	49.78 ± 1.27	8.87 ± 0.26	+
9	PC07		93.81 ± 2.2	89.67 ± 2.34	109.8 ± 2.72	40.55 ± 0.93	9.16 ± 0.28	+
10	PC08		80.9 ± 1.30	nd	96.53 ± 2.20	nd	6.93 ± 0.21	+
11	PC09		24.5 ± 0.53	nd	37.21 ± 1.04	35.19 ± 0.87	8.14 ± 0.18	+
12	PC10		19.8 ± 0.47	17.92 ± 0.41	26.74 ± 0.54	28.93 ± 0.66	9.86 ± 0.31	+

13	PD01	H	4.83 ± 0.081	4.33 ± 0.116	7.71 ± 0.19	20.66 ± 0.54	9.97 ± 0.19	+
14	PD02	2-F	2.32 ± 0.047	nd	5.94 ± 0.15	nd	8.53 ± 0.28	+
15	PD03	3-F	1.36 ± 0.036	nd	3.81 ± 0.091	nd	8.89 ± 0.24	+
16	PD04	4-F	0.92 ± 0.027	0.89 ± 0.020	1.95 ± 0.043	17.53 ± 0.42	10.12 ± 0.31	+
17	PD05	2-Cl	1.22 ± 0.029	nd	2.41 ± 0.054	nd	9.72 ± 0.33	+
18	PD06	3-Cl	0.92 ± 0.027	nd	1.86 ± 0.047	nd	10.03 ± 0.29	+
19	PD07	4-Cl	0.31 ± 0.009	0.29 ± 0.007	0.82 ± 0.021	13.42 ± 0.36	10.96 ± 0.34	+
20	PD08	2-Br	1.34 ± 0.036	nd	2.58 ± 0.056	nd	8.79 ± 0.24	+
21	PD09	3-Br	1.08 ± 0.026	nd	1.49 ± 0.029	nd	8.99 ± 0.18	+
22	PD10	4-Br	0.59 ± 0.014	0.56 ± 0.012	1.06 ± 0.023	15.65 ± 0.43	9.74 ± 0.23	+
23	PD11	2-I	2.03 ± 0.054	nd	3.56 ± 0.087	18.97 ± 0.45	8.21 ± 0.27	+
24	PD12	3-CN	1.88 ± 0.041	nd	2.48 ± 0.069	18.12 ± 0.17	9.56 ± 0.24	+
25	PD13	2-NO_2	3.89 ± 0.106	nd	5.63 ± 0.156	nd	10.46 ± 0.19	+
26	PD14	3-NO_2	3.62 ± 0.091	nd	5.07 ± 0.115	22.51 ± 0.63	10.29 ± 0.29	+
27	PD15	3-OMe	1.69 ± 0.044	1.66 ± 0.034	2.73 ± 0.062	nd	8.67 ± 0.16	+
28	PD16	4-OMe	1.21 ± 0.031	1.23 ± 0.028	2.55 ± 0.066	17.46 ± 0.40	9.71 ± 0.18	+
29	PD17	4-CF_3	1.29 ± 0.032	1.31 ± 0.035	2.84 ± 0.078	18.13 ± 0.45	9.45 ± 0.20	+
30	PD18	4-OCF_3	1.32 ± 0.039	1.28 ± 0.032	2.33 ± 0.056	17.34 ± 0.39	10.64 ± 0.26	+
31	PD19	4-NH_2	1.19 ± 0.026	1.17 ± 0.026	3.81 ± 0.087	19.18 ± 0.51	8.61 ± 0.15	+

32	PD20	2,4-di-Cl	1.06 ± 0.025	nd	1.29 ± 0.032	nd	10.13 ± 0.31	+
33	PD21	3,5-di-OMe	1.23 ± 0.028	nd	2.06 ± 0.045	nd	9.44 ± 0.26	+
34	PD22	3,5-di-NO_2	3.61 ± 0.082	nd	5.44 ± 1.41	nd	10.23 ± 0.29	+
35	PD23	2-OMe, 5-Cl	2.46 ± 0.064	nd	4.38 ± 0.122	nd	8.72 ± 0.30	+
36	PD24	-	1.76 ± 0.039	1.72 ± 0.043	2.64 ± 0.071	17.69 ± 0.49	8.58 ± 0.16	+
37	PD25	-	1.63 ± 0.046	1.58 ± 0.036	2.39 ± 0.062	19.54 ± 0.51	8.95 ± 0.18	+
38	PD26	-	12.83 ± 0.32	12.54 ± 0.32	18.67 ± 0.46	25.13 ± 0.65	9.75 ± 0.24	+
39	DPZ	-	0.046 ± 0.0013	0.043 ± 0.0012	1.12 ± 0.025	1.89 ± 0.045	8.09 ± 0.21	nd

[a] Results were expressed as means ± SD. [b] Compounds with $P_e > 4.32 \times 10^{-6}$ cm s^{-1} could cross BBB (CNS^+), compounds with $P_e < 1.84 \times 10^{-6}$ cm s^{-1} could not cross BBB (CNS^-), compounds P_e between 1.84×10^{-6} cm s^{-1} and 4.32×10^{-6} cm s^{-1} uncertain BBB permeation ($CNS^{\pm}$). nd= not determined.

6.2.3.2. BACE1 inhibitory activity

The development of AD involves deposition of Aβ plaques in the brain. The β-secretase (BACE1) breaks amyloid precursor protein (APP), it results production of amyloid-beta (Aβ) peptide [222]. Previous knockout experiments on transgenic mice deficient in BACE1 do not produced the Aβ plaques [223]. The studies suggested that therapeutic BACE1 inhibition might be possible without causing side effects. The β-secretase (BACE1) inhibitory activity of piperine derivatives was evaluated using the β-secretase (BACE1) activity detection kit (Table 6.1). The BACE1 experimental data suggested that compound PD07 (IC_{50}: 13.42 ± 0.36 μM) showed significant BACE1 inhibitory activity as compared to donepezil.

6.2.3.3. BBB-PAMPA assay

The compounds lipophilicity was assessed through parallel artificial membrane assay (PAMPA) [218]. The piperine derivatives exhibited significant permeation properties (Table 6.1) which suggests compounds may penetrate through BBB [209].

6.2.3.4. Free-radical scavenging assay

The compounds antioxidant potential was evaluated by DPPH method [218]. In Alzheimer's disease, oxidative stress increases in the brain due to reactive oxygen species. This oxidative stress affects cells regulation process and it may cause dementia. Compound PD07 demonstrated the most antioxidant activity as compared to other tested compounds (Table 6.2). The compound PD07 (IC_{50}: 26.46 ± 0.47 µM) exhibited significant DPPH inhibitory activity ($P < 0.005$) as compared to AA (Table 2).

Table 6.2. Free-radical scavenging assay of piperine derivatives

S. No.	Compound	DPPH assay (IC_{50} µM) [a]
1	Piperine	60.41 ± 1.02
2	PC01	42.35 ± 0.93
3	PC03	38.36 ± 0.79
4	PC04	35.18 ± 0.68
5	PC08	34.31 ± 0.62
6	PC10	31.67 ± 0.56
7	PD01	30.41 ± 0.51
8	PD07	26.46 ± 0.47
9	PD24	28.72 ± 0.44
10	PD25	29.55 ± 0.53
11	PD26	30.57 ± 0.61
12	AA	25.38 ± 0.76

[a] Data presented as mean ± SD (n = 3), AA = ascorbic acid

6.2.3.5. Propidium iodide displacement assay

The propidium iodide displacement assay was performed to assess the PD07 selectivity towards AChE PAS site [208]. Propidium iodide fluorescence intensity was decreased in PD07 treated samples in a concentration-dependent manner (Figure 6.1). It indicates substance PD07 effectively replaced the propidium iodide at AChE PAS site. The study suggested that compound PD07 (20 µM) effectively displaced the propidium iodide as compared to DPZ (20 µM) ($P < 0.0001$).

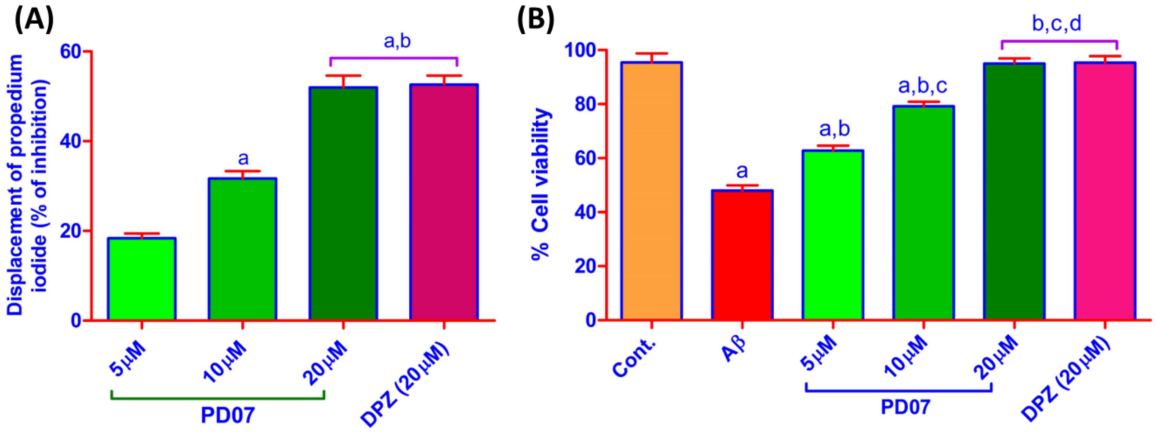


Figure 6.1. (A) Shows displacement of propidium iodide at AChE PAS site, [a]P<0.05 compared to PD07 (5 µM), [b]P<0.05 compared to PD07 (10 µM), (B) shows percentage of cell viability of PD07 on SH-SY5Y cell line, [a]P<0.05 compared to control, [b]P<0.05 compared to $A\beta_{1-42}$, [c]P<0.05 compared to PD07 (5 µM), [d]P<0.05 compared to PD07 (10 µM). One-way ANOVA followed by Tuckey's post hoc test, n=3.

6.2.3.6. $A\beta_{1-42}$ aggregation inhibition assay

Thioflavin T (ThT) assay was used to evaluate the compound PD07 effect on self-induced $A\beta_{1-42}$ aggregation (Figure 6.2) and AChE induced $A\beta_{1-42}$ aggregation (Figure 6.2) [218]. Cholinesterase inhibitors bind to the PAS site of AChE and prevent Aβ aggregation and deposition [198]. At lower concentrations (5 and 10 µM), compound PD07 displayed a moderate aggregation inhibitory activity. Further, higher concentration (20 µM) of PD07

significantly inhibited the self-induced $A\beta_{1-42}$ and AChE-induced $A\beta_{1-42}$ aggregation as compared to DPZ ($P < 0.0001$) (Figure 2A, 2B). The Aβ aggregation was also observed under confocal microscope at 20X magnification. The confocal image results suggested that $A\beta_{1-42}$ aggregate size was decreased in PD07 treated samples as compared to non-treated samples (Figure 6.2). The PD07 treatment at higher concentrations (20 µM) showed less $A\beta_{1-42}$ aggregation. The PD07 (20 µM) results were significant to DPZ in both self and AChE-induced studies. The aggregation studies revealed that compound PD07 (20 µM) effectively inhibited the Aβ-aggregation development.

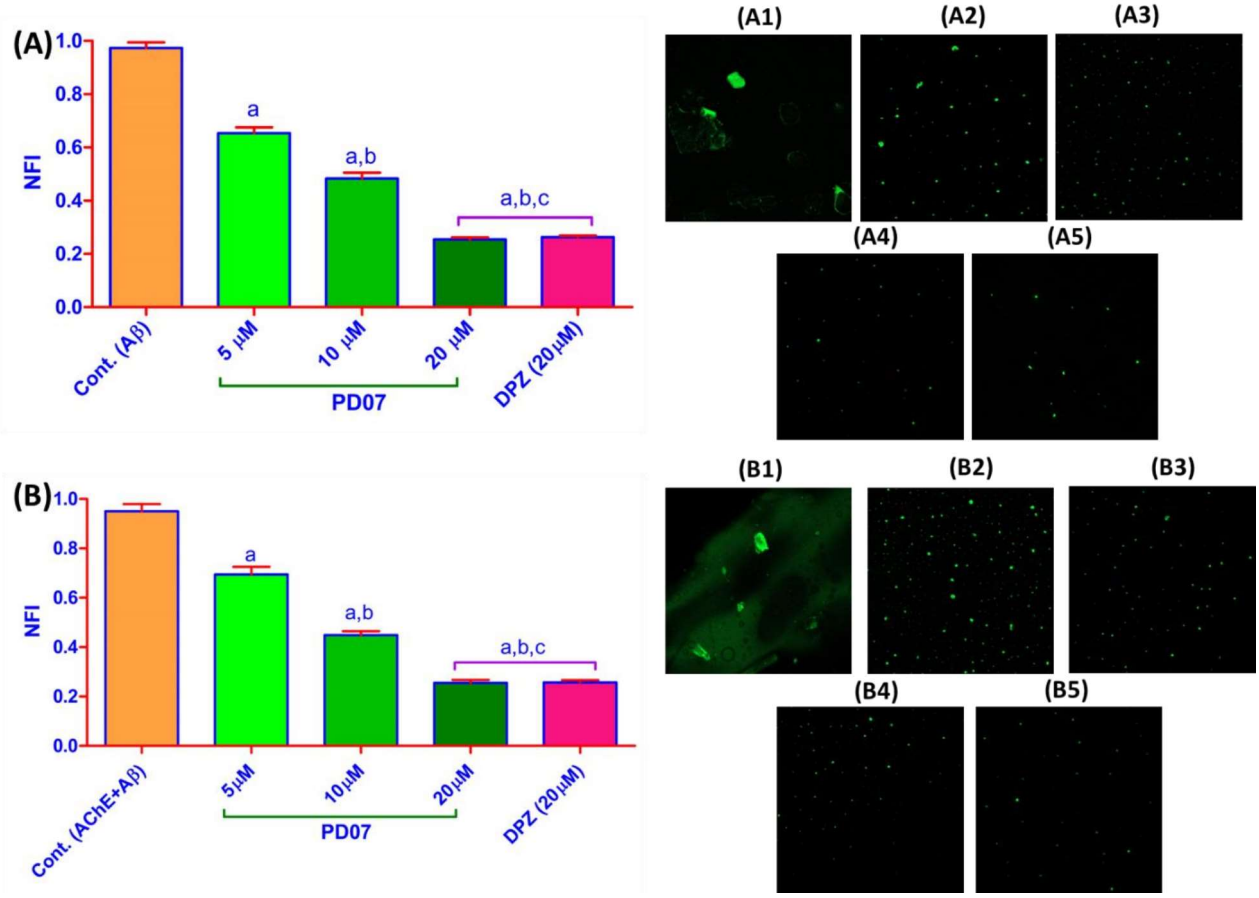


Figure 6.2. (A) and (B) effect of compound PD07 on self-induced $A\beta_{1-42}$ aggregation and AChE induced $A\beta_{1-42}$ aggregation, (A1) and (B1) shows $A\beta_{1-42}$ aggregation in the absence of inhibitor PD07, (A2) and (B2) shows $A\beta_{1-42}$ aggregation in the presence of PD07 (5 µM), (A3) and (B3) shows $A\beta_{1-42}$ aggregation in the presence of PD07 (10 µM), (A4) and (B4) shows $A\beta_{1-42}$ aggregation in the presence of PD07 (20 µM), (A5) and (B5) shows $A\beta_{1-42}$ aggregation in the

presence of DPZ (20 μM). [a]P<0.05 compared to control, [b]P<0.05 compared to PD07 (5 μM), [c]P<0.05 compared to PD07 (10 μM). One-way ANOVA followed by Tuckey's post hoc test, n=3.

6.2.3.7. Neuroprotection activity

The $A\beta_{1-42}$ treated SH-SY5Y neuroblastoma cell line was used to assess the neuroprotective effect of hit compound PD07 through MTT assay [210]. The neurotoxic isoform of $A\beta_{1-42}$ peptide was used to create toxicity in SH-SY5Y cell line. The study findings indicated that compound PD07 (20 μM) inhibited $A\beta_{1-42}$ aggregation and showed no toxic effects on the SH-SY5Y cell line (Figure 1B). The compound PD07 (20 μM) significantly increased the cell viability as compared to DPZ (Figure 6.1).

6.2.4. *In silico* studies

6.2.4.1. Molecular docking studies on AChE, BuChE, and BACE1

The protein-ligand interactions studies were performed on all synthesized compounds (PC01-PC10 and PD01-PD26) to assess key interaction profile and binding energy of compounds at target protein sites AChE, BuChE, and BACE1 (Table 6.3). The binding energy of the lead compound PD07 exhibited -12.74, -9.53, and -11.13 Kcal/mol at active sites of AChE, BuChE, and BACE1 respectively (Table 6.3). The compound PD07 interaction profile on target proteins (AChE, BuChE, and BACE1) was presented in Figure 6.3. The molecular docking studies suggested that compound PD07 formed similar key interaction profile as compared to reference compounds (donepezil, tacrine, BSD) at AChE, BuChE, and BACE1 active sites (Figure 6.3).

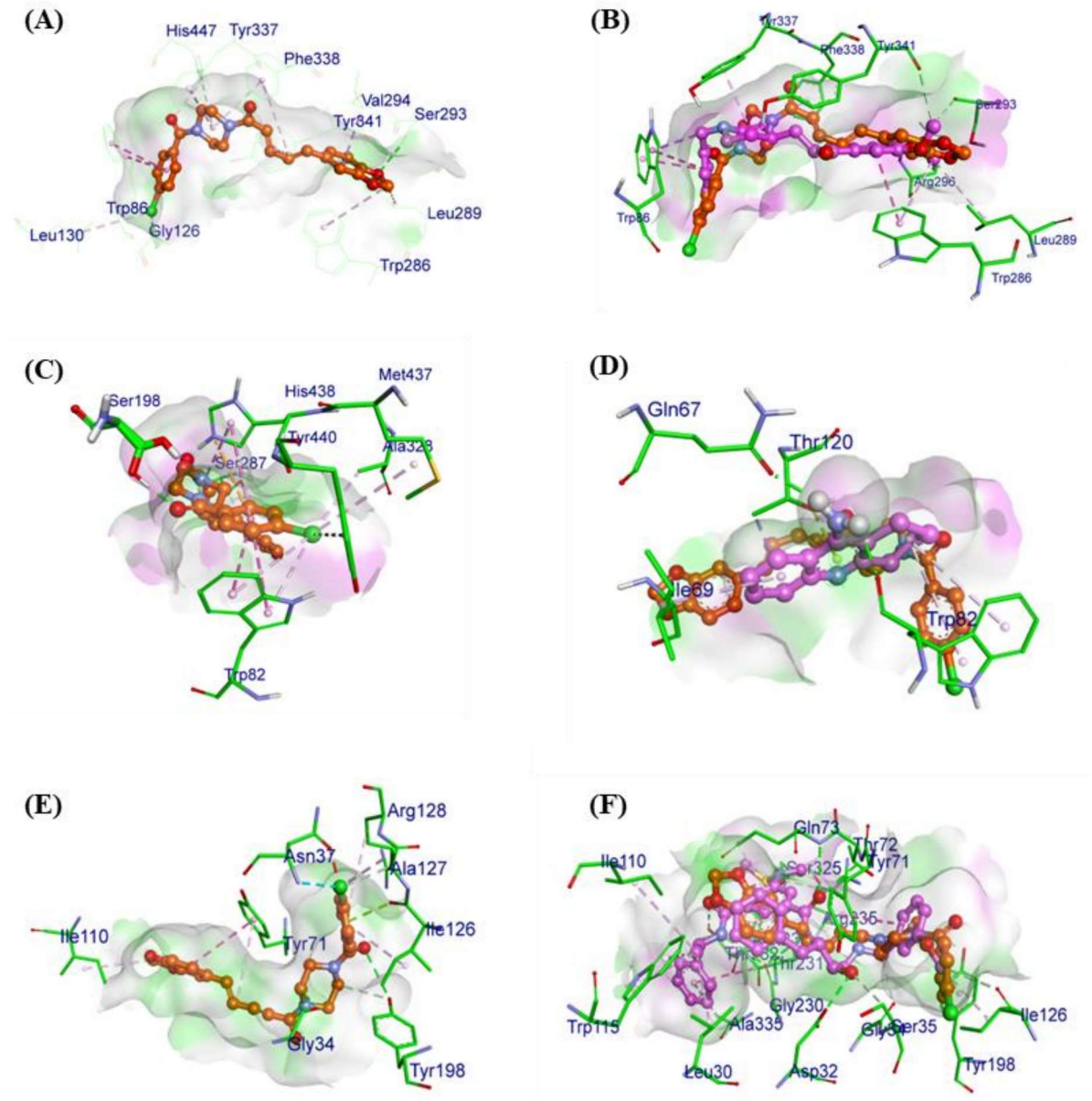
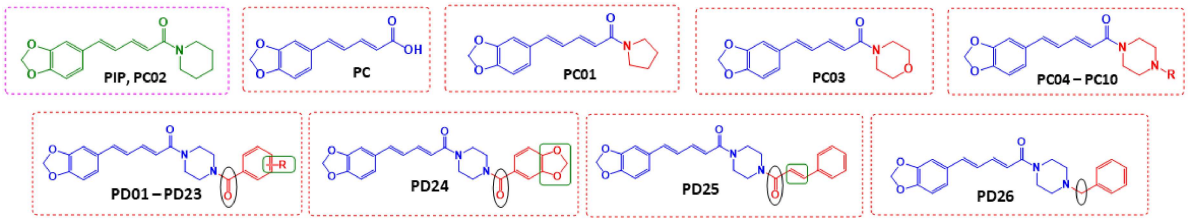
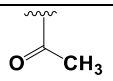
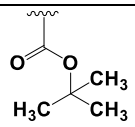
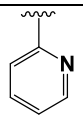
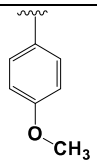


Figure 6.3. Shows PD07 interaction at active sites of AChE, BuChE, and BACE1 proteins. (A) depict the interaction profile of compound PD07 at AChE site, (B) The docking confirmation of compound PD07 was placed over reference compound donepezil, (C) interaction profile of the compound PD07 at the BuChE active site, (D) position of compound PD07 at the active site of BuChE overlaid over reference compound tacrine, (E) interaction profiles of the compound PD07 at the BACE1 active site, and (F) position of compound PD07 at the BACE1 active site placed over reference compound BSD.

Table 6.3. Molecular docking studies of piperine derivatives on AChE, BuChE, and BACE1 proteins



S. No.	Comp.	R	AChE		BuChE		BACE1	
			binding energy (Kcal/mol)	ligand efficiency	binding energy (Kcal/mol)	ligand efficiency	binding energy (Kcal/mol)	ligand efficiency
1	PIP	-	-9.80	-0.47	-8.93	-0.38	-8.36	-0.40
2	PC	-	-6.15	-0.38	-5.78	-0.36	-5.72	-0.36
3	PC01	-	-9.18	-0.46	-8.41	-0.42	-8.06	-0.40
4	PC02	-	-9.90	-0.47	-8.86	-0.42	-8.44	-0.40
5	PC03	-	-9.37	-0.45	-8.19	-0.39	-8.10	-0.39
6	PC04		-10.49	-0.50	-8.15	-0.39	-9.29	-0.44
7	PC05		-10.71	-0.49	-7.38	-0.34	-9.40	-0.43
8	PC06		-10.92	-0.47	-7.29	-0.32	-9.75	-0.42
9	PC07		-10.45	-0.44	-7.64	-0.32	-8.74	-0.36
10	PC08		-11.89	-0.42	-7.61	-0.27	-9.83	-0.35
11	PC09		-11.86	-0.44	-7.90	-0.29	-9.92	-0.37
12	PC10		-12.15	-0.42	-8.42	-0.29	-10.45	-0.36
13	PD01	H	-12.43	-0.43	-8.79	-0.30	-11.07	-0.38
14	PD02	2-F	-12.26	-0.41	-8.80	-0.29	-11.17	-0.37
15	PD03	3-F	-12.54	-0.42	-8.76	-0.29	-10.93	-0.36
16	PD04	4-F	-12.35	-0.41	-8.71	-0.29	-10.72	-0.36
17	PD05	2-Cl	-12.74	-0.42	-8.97	-0.30	-11.85	-0.39

18	PD06	3-Cl	-13.03	-0.43	-9.12	-0.30	-11.09	-0.37
19	PD07	4-Cl	-12.74	-0.42	-9.53	-0.32	-11.13	-0.37
20	PD08	2-Br	-12.92	-0.43	-9.15	-0.31	-11.98	-0.40
21	PD09	3-Br	-13.26	-0.44	-9.36	-0.31	-11.16	-0.37
22	PD10	4-Br	-13.02	-0.43	-9.58	-0.32	-11.39	-0.38
23	PD11	2-I	-13.27	-0.44	-9.42	-0.31	-11.66	-0.39
24	PD12	3-CN	-13.28	-0.43	-9.28	-0.30	-11.28	-0.36
25	PD13	2-NO_2	-11.29	-0.35	-8.13	-0.32	-11.24	-0.38
26	PD14	3-NO_2	-11.92	-0.37	-8.48	-0.27	-11.11	-0.35
27	PD15	3-OMe	-12.8	-0.41	-8.79	-0.28	-10.93	-0.35
28	PD16	4-OMe	-12.44	-0.40	-8.78	-0.29	-10.93	-0.35
29	PD17	4-CF_3	-12.42	-0.38	-8.93	-0.27	-10.86	-0.33
30	PD18	4-OCF_3	-12.61	-0.37	-8.93	-0.26	-10.87	-0.32
31	PD19	4-NH_2	-12.48	-0.42	-8.56	-0.29	-11.42	-0.38
32	PD20	2,4-di-Cl	-13.51	-0.44	-9.19	-0.30	-11.44	-0.37
33	PD21	3,5-di-OMe	-12.92	-0.39	-8.35	-0.25	-10.84	-0.33
34	PD22	3,5-di-NO_2	-11.12	-0.32	-7.70	-0.22	-10.73	-0.31
35	PD23	2-OMe, 5-Cl	-13.05	-0.41	-8.94	-0.28	-10.79	-0.34
36	PD24	-	-12.82	-0.40	-8.87	-0.28	-11.25	-0.35
37	PD25	-	-12.52	-0.40	-7.82	-0.25	-11.34	-0.37
38	PD26	-	-12.47	-0.45	-9.13	-0.33	-10.93	-0.39
39	DPZ	-	-11.80	-0.42	-	-	-	-
40	Tacrine	-	-	-	-6.79	-0.45	-	-
41	BSD	-	-	-	-	-	-9.50	-0.20

6.2.4.2. Molecular dynamic simulation on AChE and BACE1

The interaction between proteins (AChE and BACE1) and ligand PD07 was further evaluated through molecular dynamic simulation studies. Hydrogen bonding, ionic interactions, hydrophobic interactions, and water-formed bridges were analyzed to evaluate binding interactions at the AChE and BACE1 active sites individually. The findings of RMSF and RMSD (Figure 6.4) indicated that compound PD07 interactions are stable up to 100 ns at the AChE site. Then, BACE1 and PD07 complex also showed an ideal interaction profile up to

100 ns (Figure 6.4). These simulation studies suggested that compound PD07 formed various key interactions and well resided at active sites of AChE and BACE1.

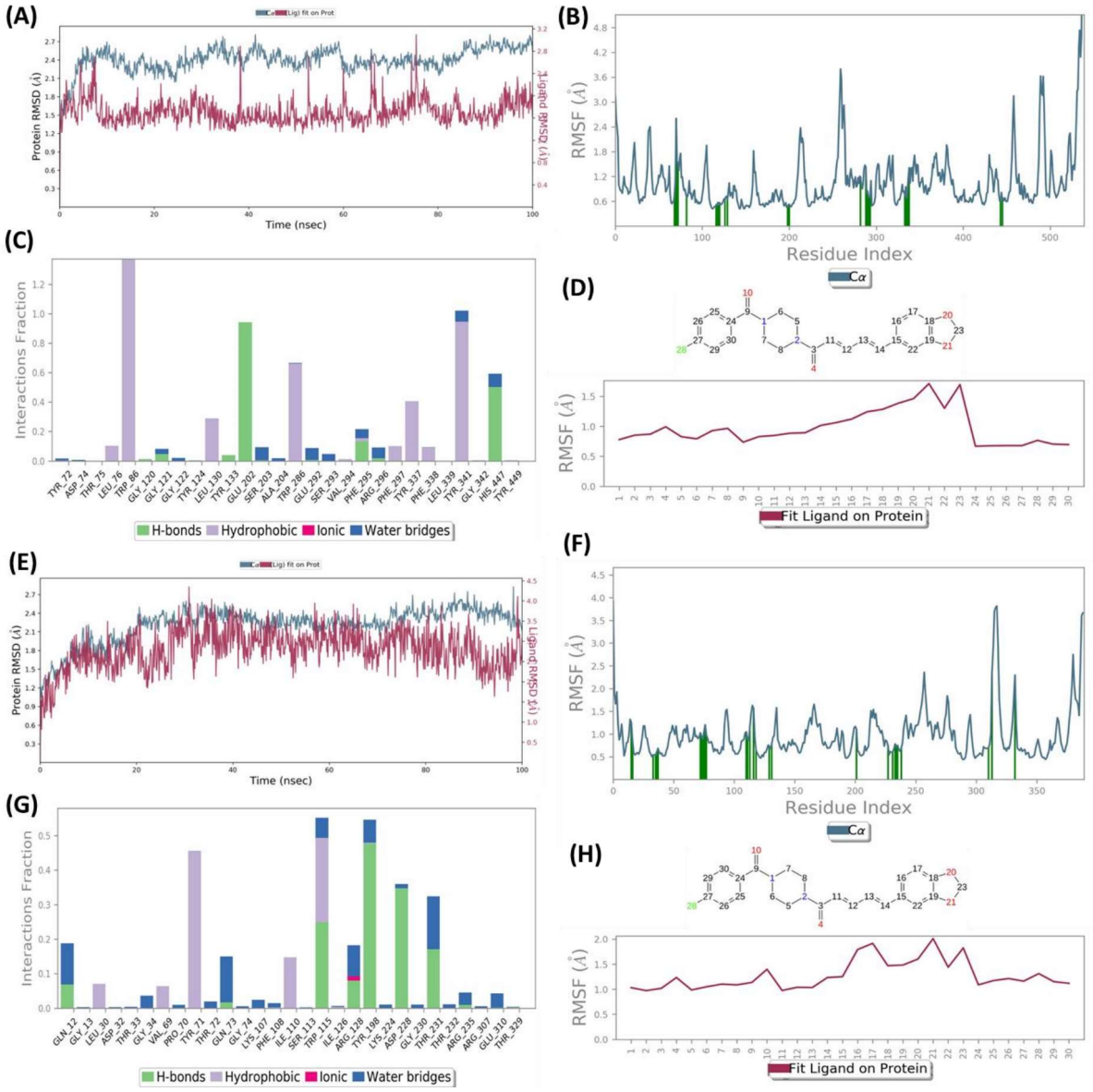


Figure 6.4. Molecular dynamic simulation studies of PD07 on AChE and BACE1. (A) shows the protein RMSD (left Y axis) and ligand RMSD (right Y axis) indicating the suitability of ligand with respect to protein AChE, (B) shows RMSF plot of the protein AChE during simulation, (C) bar graph shows percentage of simulation time with specific amino acid at site of AChE with ligand, (D) shows ligand RMSF and fit ligand on protein AChE, (E) shows the protein BACE1 RMSD (left Y axis) and ligand RMSD, (F) shows RMSF plot of the protein BACE1 during simulation, (G) bar graph shows percentage of simulation time with specific

amino acid at BACE1 site with ligand, and (H) shows ligand RMSF and fit ligand on protein BACE1.

6.2.4.3. Drug-likeness, ADME, and toxicity predictions

The physical properties, drug-likeness, ADME, and toxicity profile of compound PD07 were evaluated through swissADME and preADMET servers (Table 6.4). The compound PD07 showed druglike properties according to Lipinski rule. Further, compound showed human intestinal absorption, MDCK, and Caco2 permeability which indicates, drug may be given through the oral route. Furthermore, compound showed no toxic results in rodents. The *in silico* predicted results were presented in the supplementary information (Table 6.4).

Table 6.4. Physicochemical properties, drug-likeness, ADME, and toxicity profile of compound PD07

S. No	Parameters	Compound PD07
Physicochemical Properties/Drug likeness		
1	Formula	$C_{23}H_{21}ClN_2O_4$
2	Molecular weight	424.88 g/mol
3	No. heavy atoms	30
4	No. rotatable bonds	6
5	No. H-bond acceptors	4
6	No. H-bond donors	0
7	TPSA	59.08 Å²
8	Log Po/w	3.48
9	BBB permeant	Yes
ADME		
10	BBB	0.037022
11	Buffer solubility (mg/L)	168.566
12	*In vitro* Caco-2 cell permeability (nm/sec)	44.7586
13	CYP_2D6_inhibition	Inhibitor
14	CYP_3A4_inhibition	Inhibitor
15	CYP_3A4_substrate	Weakly
16	Human intestinal absorption (HIA %)	97.420735
17	*In vitro* MDCK cell permeability (nm/sec)	0.11831
18	*In vitro* P-glycoprotein inhibition	Inhibitor
19	*In vitro* plasma protein binding (%)	91.695555
20	*In vitro* skin permeability (log Kp, cm/hour)	-3.22978
Toxicity		

21	Acute algae toxicity	0.025388
22	Ames test	Mutagen
23	Carcinogenicity (Mouse)	Positive
24	Carcinogenicity (Rat)	Negative
25	Acute daphina toxicity	0.0202623
26	*In vitro* hERG inhibition	Medium risk
27	Acute fish toxicity (medaka)	0.00100007
28	Acute fish toxicity (minnow)	0.00876062

6.2.5. *In vivo* studies

6.2.5.1. Acute oral toxicity studies

The acute oral toxicity studies were performed on healthy female Wistar rats to assess compound PD07 safety profile [220]. Test compound PD07 treated rats did not showed any sign of toxicity such as mortality, body color changes, weight loss, behavior, etc. (Table 6.5). The normal control group and PD07 treated rats showed significant results in biochemical parameters such as creatinine, creatine kinase myocardial band (Ck-MD), alkaline phosphatase (ALP), aspartate transaminase (AST), alanine transaminase (ALP) (Table 6.6). The results suggested that compound PD07 did not cause toxic effects on rats up to 300 mg/kg, p.o. Further, major organs like the brain, liver, kidney, and heart tissue sections were also observed, it indicated that PD07 therapy at doses up to 300 mg/kg, p.o. did not adverse reactions in any of these organs (Figure 6.5). The organ sections were presented in 10X magnification (Figure 6.5).

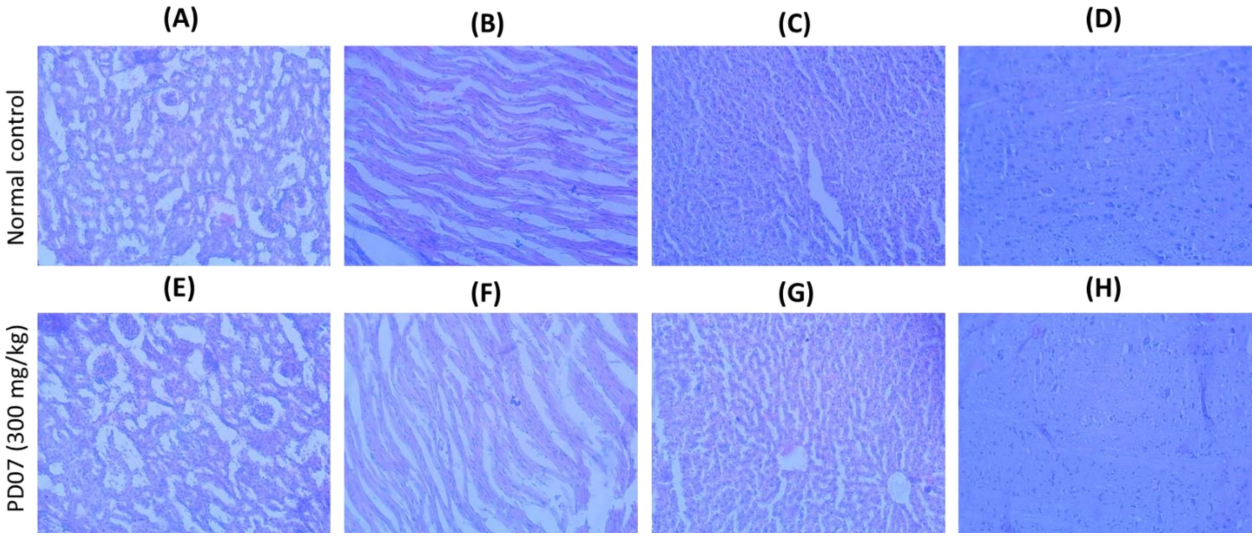


Figure 6.5. Effect of compound PD07 (300 mg/kg, p.o.) on acute oral toxicity test, (A), (B), (C), and (D) shows effect of normal control group on kidney, heart, liver, and brain respectively. (E), (F), (G), and (H) shows effect of PD07 (300 mg/kg, p.o.) treatment on kidney, heart, liver, and brain respectively.

Table 6.5. Effect of single-dose oral administration of PD07 on body weight of rats

No. of days	Normal control [a] (Body weight in grams)	Compound PD07 (300 mg/kg, p.o.) [a] (Body weight in grams)
0^{th} day	202.67 ± 10.52	205.09 ± 10.29
7^{th} day	206.84 ± 9.59	209.16 ± 10.16
14^{th} day	213.36 ± 11.64	215.44 ± 11.43

[a] All values are expressed as mean±SD (n=6)

Table 6.6. Effect of oral administration of compound PD07 on AST, ALT, ALP, creatinine, and Ck-MD

S. No.	Biochemical parameters	Normal control [a]	Compound PD07 (300 mg/kg, p.o.) [a]
1	ALP (U/L)	67.32 ± 2.36	68.47 ± 2.67
2	AST (U/L)	126.51 ± 5.86	130.63 ± 6.11
3	ALT (U/L)	46.37 ± 2.12	51.38 ± 2.34
4	Creatinine (mg/dl)	0.63 ± 0.03	0.66 ± 0.029
5	Ck-MD (U/L)	650.77 ± 16.12	660.91 ± 18.44

[a] All values are expressed as mean±SD (n=6), alanine transaminase (ALT), aspartate transaminase (AST), alkaline phosphatase (ALP), creatinine, and creatine kinase- myocardial band (Ck-MD).

6.2.5.2. Rotarod test

The motor coordination was evaluated by Rotarod test to assess the compound PD07 effect on CNS at a dose of 10 and 50 mg/kg, p.o. As it results, compound PD07 did not showed motor incoordination or latency to fall off the rod as compared to the control group (Figure 6.6). These findings suggested that compound PD07 did not cause any CNS toxic effects up to 50 mg/kg, p.o.

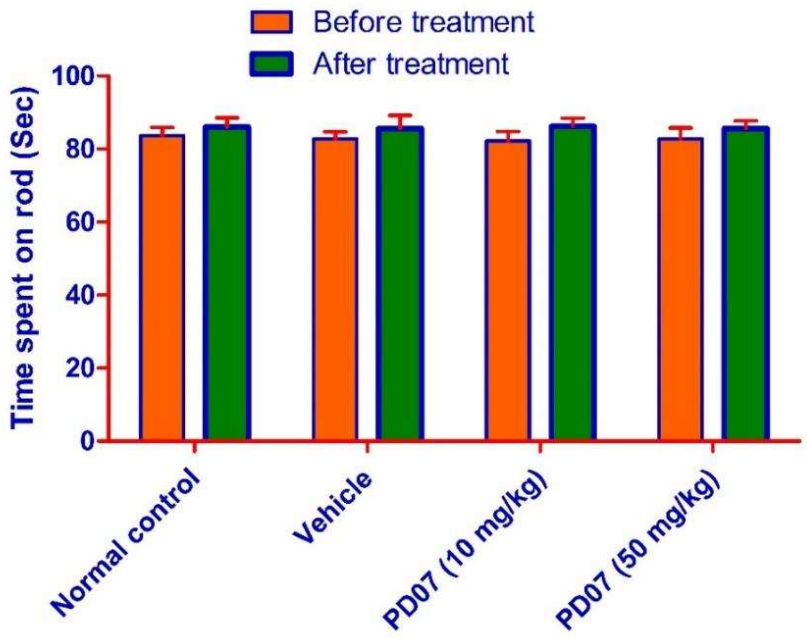


Figure 6.6. Shows the effect of PD07 on motor function before and after treatment

6.2.5.3. Scopolamine induced amnesia model

Scopolamine is widely used for the induction of amnesia in rodents, which blocks cholinergic system [212]. The compound PD07 potency towards memory and cognition was assessed through Y maze test by analyzing spontaneous alternations (Figure 6.7) [211]. The disease control group (scopolamine-treated) displayed least spontaneous alternations as compared to

normal control group. The test compound PD07 treated rats showed significant spontaneous alternations as compared to DPZ group in a dose-dependent manner (Figure 6.7) (P<0.0001). The Y maze test indicated that compound PD07 (10 mg/kg, p.o.) enhanced cognition and memory in rats.

Further, AChE activity and ACh levels were estimated in the brain homogenate (Figure 6.7). ACh binds to nicotinic and muscarinic receptors, which are involved in memory and learning [213]. Treatment with PD07 significantly lowered the AChE activity in rats in a dose-dependent manner as compared to DPZ. In addition, ACh levels were increased in the PD07 therapy groups, which was significant to DPZ (P<0.0001) group. According to these findings, compound PD07 effectively increased the ACh levels by reducing AChE activity (Figure 6.7).

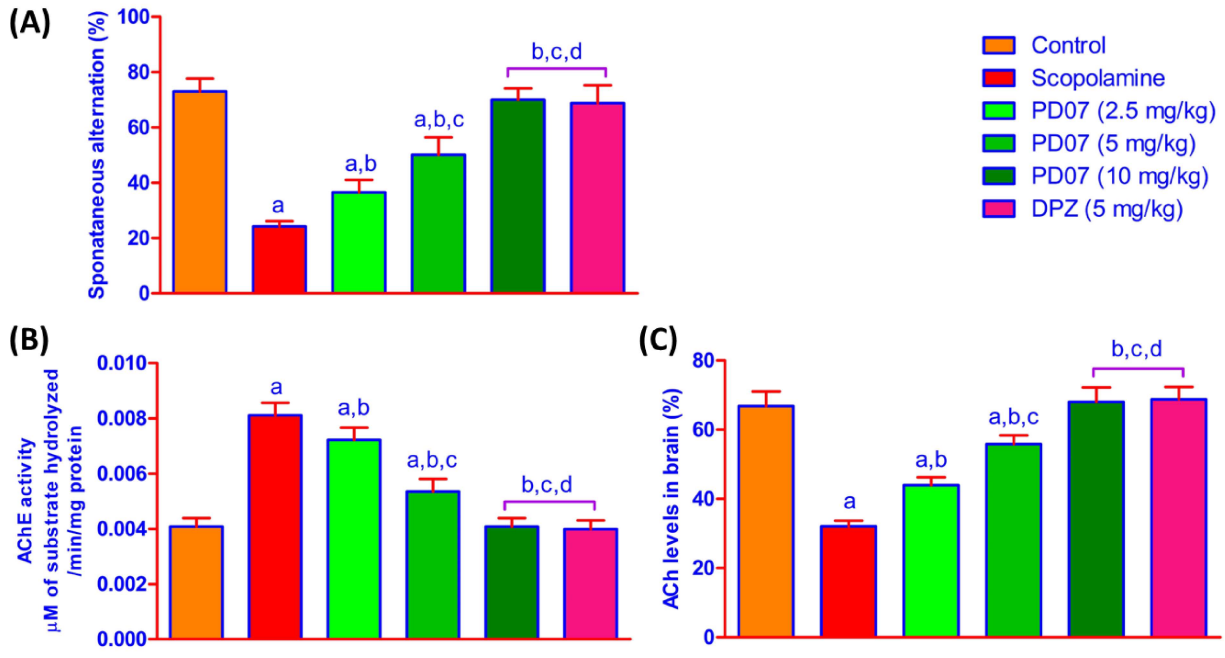


Figure 6.7. Effect of PD07 (2.5, 5, and 10 mg/kg, p.o.) seven days treatment on scopolamine-induced amnesia rats, (A) effect of PD07 on spontaneous alternations (%), (B) effect of PD07 on AChE activity in the brain, (C) effect of PD07 on ACh levels in brain. [a]P<0.05 vs. control, [b]P<0.05 vs. scopolamine, [c]P<0.05 vs. PD07 (2.5 mg/kg, p.o.), [d]P<0.05 vs. PD07 (5 mg/kg, p.o.). One-way ANOVA followed by Tuckey's post hoc test, n=6.

6.3. Conclusion

A semisynthetic approach was used to synthesize multifunctional piperine derivatives (PC01–PC10 and PD01–PD26) using alkaloid piperine as a precursor molecule. The compound PD07 exhibited most significant results in *in vitro*, *in silico*, and *in vivo* studies among all tested compounds. In *in vitro* experiments, compound PD07 showed lipophilic nature and displayed inhibitory activity on eeAChE, hAChE, eqBuChE, $A\beta_{1-42}$ aggregation, BACE1, and DPPH assay. The compound PD07 effectively displaced the propidium iodide at the AChE PAS site. Additionally, PD07 also showed neuroprotective action on $A\beta_{1-42}$ treated SH-SY5Y cell line. The compound PD07 showed drug-likeness, ideal ADME profile, and no toxic properties in *in silico* predictions. Further, DFT studies were performed to assess the structural properties of the compound PD07. Furthermore, molecular docking and molecular dynamic simulation suggested that PD07 formed most stable interactions and was well-resided at the active sites of the proteins AChE, BuChE, and BACE1. These *in vitro* and *in silico* data suggest compound PD07 have drug-like properties and acts through multiple targets i.e., inhibition of cholinesterases and amyloid beta aggregation by binding to AChE PAS region and binding to BACE1 active site, probably showing antioxidant activity through free radical scavenging activity by transferring hydrogen atoms to their substrates, and neuroprotective action. Moreover, acute oral toxicity and CNS toxicity tests suggests compound PD07 is safe in rats. The scopolamine-induced amnesia experiments indicated that PD07 treatment enhanced rats cognition and memory. Ex vivo experiments reveled that compound PD07 increased ACh levels by inhibiting the AChE activity in the brain. The *in vitro*, *in silico*, and *in vivo* suggested that synthesized piperine derivative PD07 is a potent molecule among all synthesized compounds and it is acting through multiple targets in AD.