Chapter 5: Experimental Work

5.1. Materials and methods

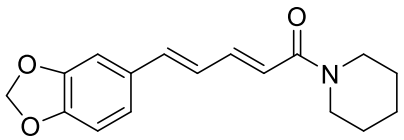
Prior to usage, solvent distillation procedures were used to dry all the solvents needed for the production of the compounds. All required chemicals were purchased from Alfa Aesar (Massachusetts), Sigma-Aldrich (Missouri, USA), and S. D. Chemicals & Avra Chemicals (India). Except where otherwise specified, reactions were carried out in an inert condition (N2). TLC was used to observe the reactions on precoated TLC silica gel G 60 F254 (Merck), and the results were seen under a UV light chamber, with iodine vapours, or after being treated with some stain, ninhydrin reagent. Utilizing silica gel with a mesh size of 60–120, column chromatographic purifications were carried out (CDH Laboratory Reagents, India). Tetramethyl silane (TMS) served as the internal standard as the Bruker Advance 500 MHz spectrometers measured the proton nuclear magnetic resonance (1H &13C NMR) spectra. Deuterated chloroform or Dimethyl-*d6* sulfoxide were the NMR solvents employed, as specified. "Coupling constants" (J) were expressed in Hz, and chemical shifts were quantified in ppm. Peak splitting patterns are denoted by the acronyms t = triplet, d = doublet, dd = doublet of the doublet, q = quartet, and m = multiplet. At the Department of Chemistry (BHU) HRMS was acquired using HRMS-6540-UHD equipment.

5.2. Extraction and isolation of piperine from black pepper:

5.2.1. Extraction

The plant material of black pepper (*Piper nigrum*) was obtained from the local market, and the voucher specimen number (JK-IIT 01) was deposited in the "Department of Pharmaceutical Engineering and Technology," IIT (BHU). Cleaned black pepper fruit was taken and dried below 50 ^{o}C in the oven, then reduced to powder form (80 #) using

an electric mixer and grinder. Dried *Piper nigrum* fruit powder (120 gm) was extracted with 95 % ethanol (500 ml) at room temperature through maceration for seven days. The extract obtained was filtered with Whatman paper, and the filtrate liquid was evaporated with a rotary evaporator to dryness below 50^{o}C. Finally, 10 gm. of crude dark greenish-brown extract powder was obtained.



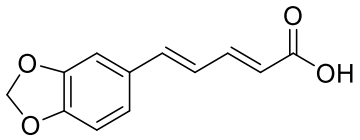
(2E,4E)-5-(benzo[d][1,3]dioxol-5-yl)-1-(piperidin-1-yl)penta-2,4-dien-1-one

^{1}H NMR (500 MHz, $CDCl_3$) δ 7.40 (dd, *J* = 15.2, 8.9 Hz, 1H), 6.98 (s, 1H), 6.89 (d, *J* = 8.1 Hz, 1H), 6.76 (dd, *J* = 18.3, 8.4 Hz, 3H), 6.44 (d, *J* = 14.7 Hz, 1H), 5.98 (d, *J* = 0.9 Hz, 2H), 3.59 (d, *J* = 54.3 Hz, 4H), 1.67 (dd, *J* = 10.6, 5.7 Hz, 2H), 1.64 – 1.56 (m, 4H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 165.44, 148.20, 148.12, 142.47, 138.21, 131.03, 125.38, 122.49, 120.09, 108.48, 105.68, 101.28, 46.91, 43.24, 26.73, 25.64, 24.67.

5.2.2. Isolation of piperine and conversion into Piperic acid

Piperine was isolated using an earlier proposed method with slight modification (15). The crude extract (10 gm) was dissolved in 50 ml of ethanol, and potassium hydroxide (9.0 gm.) was mixed until it completely solubilized. The mixture was filtered with the Whatman No. 1 filter and left overnight for evaporation at room temperature only to precipitate crystals of piperine. The crude product was washed thrice to remove water-soluble residues leaving crystals of piperine. The solid crystal obtained was recrystallized using isopropanol at 15 ^{o}C for three days. The yellow crystal (3.462 gm) obtained was analyzed using a solvent system (acetone: hexane, 3:2) in a TLC chamber, and a melting point of 129-131 ^{o}C was observed. Piperine obtained was identified depending on the

similarity with data in the literature (18, 19).The crystal obtained was analyzed and confirmed using ^{1}H NMR.



(2E,4E)-5-(benzo[d][1,3]dioxol-5-yl)penta-2,4-dienoic acid

Yellow solid ;^{1}H NMR (500 MHz, DMSO-*d6*) δ 12.16 (s, 1H), 7.31 (dd, *J* = 15.2, 7.3, 3.0 Hz, 1H), 7.24 (s, 1H), 7.05 – 6.91 (m, 4H), 6.06 (s, 2H), 5.93 (d, *J* = 15.2 Hz, 1H).^{13}C NMR (126 MHz, DMSO-*d6*) δ 168.14, 148.57, 148.45, 144.99, 140.19, 131.00, 125.33, 123.55, 121.73, 108.97, 106.16, 101.83.

A. Basic methodology for synthesis of intermediates substituted 2-chloro-N-phenyl acetamides (3a-3n)

The compounds **3a-3n** were prepared following our earlier procedure (14). Briefly, into the RBF substituted aniline **2a-2n (**1.0 equiv.) in DCM (CH_2Cl_2), followed by K_2CO_3 (1.5 equiv.) addition, and the mixture was shaken continuously at 0°C in RBF for 10 min. Finally, slow addition of chloroacetylchloride (1.0 equiv.) while maintaining 0°C. The mixture was again stirred for 2h at normal temperature. Reaction progress is monitored through the help of TLC till the completion of the reaction, followed by the addition of DCM and water. The Dichloromethane phase was evaporated through a rota evaporator under vacuum to compounds **3a-3n**. The NMR data are matching with our earlier publications(14).

B. Basic methodology for the synthesis of intermediate substituted 2-amino-N-phenyl acetamides (4a-4n)

The compounds **3a-3n** were prepared following our earlier procedure(14). Substituted phenyl acetamides, **3a-3n** (0.25 g, 1.0 equiv) in 1, 4-dioxane and added excess amount of

liquid ammonia (NH_3) (10 mL). Heated, the reaction mixture was refluxed at 60^o C for 6 h in RBF. The TLC indicated the completion of the reaction. The reaction mixture was mixed with 25 mL ethyl acetate. The organic layer was concentrated under vacuum, which gives the free amine group of compounds **4a-4n** to solid with a yield of 55-65 %. The NMR data are matching with our earlier publications.

C. Procedure for synthesis of final compounds 6a-6n

Piperic acid (0.3 g, 1.54 mmol) was dissolved in tetrahydrofuran (10 mL), EDCI.HCl (0.35 g, 2.31 mmol), N-hydroxy benzotriazole (HOBt) (0.52 g, 3.82 mmol), substituted 2-amino-N-phenyl acetamides (1.53 mmol), were added then reaction stirred at RT for 15 min followed by addition of DIPEA (0.49 g, 3.86 mmol). The reaction was allowed to get stirred for 12 hrs. at r.t. Upon reaction gets completed, the mixture was washed using water and ethyl acetate. The organic layer was washed and dried through sodium sulphate and concentrated under vacuum. Purification of compounds was carried out through column chromatography.

2-(((2E, 4E)-5-(benzo[d][1, 3]dioxol-5-yl)penta-2,4-dien-1-yl)amino)-N-phenylacetamide (6a)

Yellow solid powder**,** 1H NMR (500 MHz, DMSO-*d6*) δ 10.03 (s, 1H), 8.44 (t, J = 5.9 Hz, 1H), 7.60 (d, J = 7.6 Hz, 2H), 7.34 – 7.27 (m, 3H), 7.19 (dd, J = 15.0, 10.6 Hz, 1H), 7.08 – 6.86 (m, 5H), 6.22 (d, J = 15.0 Hz, 1H), 6.05 (s, 2H), 3.99 (d, J = 5.9 Hz, 2H). ^{13}C NMR (126 MHz, DMSO-*d6*) δ 168.32, 166.13, 148.41, 148.23, 140.32, 139.40, 138.68, 131.31, 129.23, 125.69, 124.55, 123.69, 123.21, 119.56, 108.92, 106.13, 101.74, 43.30. HRMS $[M + H]^+$ Found 351.1325, calculated 351.1345 for $C_{20}H_{18}N_2O_4$.

2-(((2E,4E)-5-(benzo[d][1,3]dioxol-5-yl)penta-2,4-dien-1-yl)amino)-N-(o-tolyl)acetamide (6b)

Yellow solid powder,^{1}H NMR (500 MHz, DMSO-*d6*) δ 9.35 (s,1H),8.46(t,1H),7.43 (d, *J* = 7.9 Hz, 1H), 7.28 (s, 1H), 7.25 – 7.20 (m, 2H), 7.18 – 7.15(m, 1H), 7.08 (t, *J* = 7.4 Hz, 1H), 7.02 – 7.0 (m, 1H), 6.97 – 6.88 (m, 2H), 6.22 (d, *J* = 15.0 Hz, 2H), 6.05 (s, 2H), 4.02 (s, 2H), 2.20 (s, 3H). ^{13}C NMR (126 MHz, DMSO-*d6*) δ 168.37, 166.22, 148.42, 148.24, 140.36, 138.71, 136.56, 131.31, 130.77, 126.45, 125.69, 124.53, 123.22, 108.92, 106.13, 101.74, 43.18, 18.23. HRMS $[M + H]^+$ Found 365.1495, calculated 365.1501 for $C_{21}H_{20}N_2O_4$.

(2E,4E)-5-(benzo[d][1,3]dioxol-5-yl)-N-(2-oxo-2-(m-tolylamino)ethyl)penta-2,4-dienamide (6c)

Yellow solid powder**,**^{1}H NMR (500 MHz, DMSO-*d6*) δ 9.95 (s, 1H), 8.43 (t, 1H), 7.43 – 7.39 (m, 2H), 7.28 (s, 1H), 7.22 – 7.17 (m, 2H), 7.01 – 6.86 (m, 5H), 6.22 (d, *J* = 15.0 Hz, 1H), 6.05 (s, 2H), 3.98-3.97 (d, 2H), 2.28 (s, 3H).^{13}C NMR (126 MHz, DMSO-*d6*) δ 168.22, 166.12, 148.40, 148.22, 140.29, 138.66, 138.35, 131.29, 129.04, 125.67, 124.54, 124.38, 123.20, 120.09, 116.76, 108.90, 106.11, 101.73, 43.31, 21.66. HRMS $[M + H]^+$ found 365.1503 calculated 365.1501 for $C_{21}H_{20}N_2O_4$.

2-(((2E,4E)-5-(benzo[d][1,3]dioxol-5-yl)penta-2,4-dien-1-yl)amino)-N-(p-tolyl)acetamido (6d)

White solid powder,^{1}H NMR (500 MHz, DMSO-*d6*) δ 9.94 (s, 1H), 8.42 (t, *J* = 14.6 Hz, 1H), 7.49 – 7.45 (m, 2H), 7.28 (s, 1H), 7.19 (d, *J* = 25.6 Hz, 1H), 7.12 (d, *J* = 8.1 Hz, 2H), 7.01 – 6.84 (m, 4H), 6.21 (d, *J* = 15.0 Hz, 1H), 6.06 (d, *J* = 4.9 Hz, 2H), 3.97 (d, *J* = 5.8 Hz, 2H), 2.25 (s, 3H).^{13}C NMR (126 MHz, DMSO-*d6*) δ168.05, 166.14 , 148.23 , 143.30, 140.30, 129.59, 128.29, 127.81, 125.69, 124.98, 124.59, 123.18, 119.61, 110.07, 108.91,

106.15 , 101.73, 43.29, 20.89. HRMS $[M + H]^+$ found 365.1506, calculated 365.1501 for $C_{21}H_{20}N_2O_4$.

2-(((2E,4E)-5-(benzo[d][1,3]dioxol-5-yl)penta-2,4-dien-1-yl)amino)-N-(2-methoxyphenyl)acetamide (6e)

White solid powder,^{1}H NMR (500 MHz, DMSO-*d6*) δ 9.16 (s, 1H), 8.53 (t, *J* = 5.6 Hz, 1H), 8.01 (d, *J* = 7.9 Hz, 1H), 7.29 – 7.19 (m, 2H), 7.06 – 6.91 (m, 7H), 6.21 (d, *J* = 15.0 Hz, 1H), 6.05 (s, 2H), 4.03 (d, *J* = 5.8 Hz, 2H), 3.83 (s, 3H).^{13}C NMR (126 MHz, DMSO-*d6*) δ 168.38, 166.31, 149.53, 148.42, 148.27,140.61, 138.85, 131.29, 127.55, 125.65, 124.70,124.25, 123.24, 121.38, 120.83, 111.63, 108.92, 106.15, 101.75, 56.24, 43.69. HRMS $[M + H]^+$ found 381.1454, calculated 381.1450 for $C_{21}H_{20}N_2O_5$.

(2E,4E)-5-(benzo[d][1,3]dioxol-5-yl)-N-(2-((3-methoxyphenyl)amino)-2-oxoethyl)penta-2,4-dienamide (6f)

Yellow solid powder**,**^{1}H NMR (500 MHz, DMSO-*d6*) δ 10.03 (s, 1H), 8.45 - 8.42 (t, *J* = 5.9 Hz, 1H), 7.31-7.29 (dd, *J* = 10.0, 1.5 Hz, 2H), 7.23 (s, 1H), 7.20 (d, *J* = 2.0 Hz, 1H), 7.13 (d, *J* = 8.0 Hz, 1H), 7.0 - 7.01 (d, *J* = 8.2 Hz, 2H), 6.94 – 6.87 (m, 3H), 6.21 (d, *J* = 20.4 Hz, 1H), 6.05 (s, 2H), 3.98 (d, *J* = 5.8 Hz, 2H),3.73(s,3H).13C NMR (126 MHz, DMSO-*d6*) δ 168.36, 166.13, 159.98, 148.41, 148.24, 140.56, 140.34, 138.69, 131.30, 125.68, 124.52, 123.22, 111.84, 109.08,108.92,106.13,105.40,101.74,49.07,43.32. HRMS $[M + H]^+$ found 381.1447, calculated 381.1450 for $C_{21}H_{20}N_2O_5$.

2-(((2E,4E)-5-(benzo[d][1,3]dioxol-5-yl)penta-2,4-dien-1-yl)amino)-N-(4-methoxyphenyl)acetamide (6g)

Yellow solid powder**,**^{1}H NMR (500 MHz, DMSO-*d6*) δ 9.89 (s, 1H), 8.42 (t, 1H), 7.50 (d, *J* = 8.0 Hz, 2H), 7.28 (s, 1H), 7.19 (t, *J* = 12.4 Hz, 1H), 7.01 – 6.88 (m, 6H), 6.21 (d, *J* = 15.2 Hz, 1H), 6.05 (s, 2H), 3.96 (d, *J* = 3.9 Hz, 2H), 3.72 (s, 3H). ^{13}C NMR (126 MHz, DMSO-*d6*) δ 168.38, 166.31, 149.53, 148.42, 148.27, 140.61, 138.85, 131.29,

127.55, 125.65, 124.70, 124.25, 123.24, 121.38, 120.83, 111.63, 108.92, 106.15, 101.75, 56.24, 43.69. HRMS $[M + H]^+$ found 381.1439, calculated 381.1450 for $C_{21}H_{20}N_2O_5$.

2-(((2E,4E)-5-(benzo[d][1,3]dioxol-5-yl)penta-2,4-dien-1-yl)amino)-N-(2-fluorophenyl)acetamide (6h)

Yellow solid powder**,** ^{1}H NMR (500 MHz, DMSO-*d6*) δ 9.82 (s, 1H), 8.45 (t, 1H), 7.88 (d, *J* = 9.3 Hz, 1H), 7.28 – 7.25 (m, 2H), 7.23 – 7.16 (m, 3H), 7.02 -7.0 (dd, *J* = 8.1, 1.5 Hz, 1H), 6.97 (d, *J* = 4.9 Hz, 1H), 6.94 – 6.88 (dd, *J* = 13.0, 12.0 Hz, 2H), 6.21 (d, *J* = 15.0 Hz, 1H), 6.05 (s, 2H), 4.07 (d, 2H).^{13}C NMR (126 MHz, DMSO-*d6*) δ 168.82, 166.18, 148.41, 148.24, 140.42, 138.73, 131.30, 125.67, 124.86, 124.45, 123.22, 116.05, 115.89, 108.92, 106.13, 101.74, 43.11. HRMS $[M + H]^+$ found 369.1247, calculated 369.1251 for $C_{20}H_{17}FN_2O_4$.

2-(((2E,4E)-5-(benzo[d][1,3]dioxol-5-yl)penta-2,4-dien-1-yl)amino)-N-(3-fluorophenyl)acetamide (6i)

Yellow solid powder**,** ^{1}H NMR (500 MHz, DMSO-*d6*) δ 10.27 (s, 1H), 8.46 (t, *J* = 5.8 Hz, 1H), 7.64 – 7.57 (m, 1H), 7.40 – 7.26 (m, 3H), 7.19 (dd, *J* = 15.0, 10.7 Hz, 1H), 7.04 – 6.86 (m, 5H), 6.21 (d, *J* = 15.0 Hz, 1H), 6.05 (s, 2H), 4.00 (d, *J* = 5.9 Hz, 2H). ^{13}C NMR (126 MHz, DMSO-*d6*) δ 168.76, 166.18, 163.56, 161.65, 148.41, 148.24, 141.08, 140.40, 138.73, 131.30, 130.93, 125.66, 124.44, 123.23, 115.29, 110.23, 110.07, 108.92, 106.42, 106.13, 101.74, 43.35**.** HRMS $[M + H]^+$ found 369.1258, calculated 369.1251 for $C_{20}H_{17}FN_2O_4$.

2-(((2E,4E)-5-(benzo[d][1,3]dioxol-5-yl)penta-2,4-dien-1-yl)amino)-N-(4-fluorophenyl)acetamide (6j)

Yellow solid powder**,** ^{1}H NMR (500 MHz, DMSO-*d6*) δ 10.11 (s, 1H), 8.46 (t, *J* = 5.8 Hz, 1H), 7.62 (dd, *J* = 9.1, 5.0 Hz, 2H), 7.29 (d, *J* = 1.3 Hz, 1H), 7.22– 7.14(m, 3H), 7.01 – 6.87 (m, 4H), 6.21 (d, *J* = 15.0 Hz, 1H), 6.05 (s, 2H), 3.98 (d, *J* = 5.9 Hz, 2H).^{13}C NMR

(126 MHz, DMSO-*d6*) δ 168.26, 166.14, 159.36, 157.46,148.41, 148.23, 140.35, 138.70, 135.78, 131.30, 125.67, 124.51,123.23, 121.32,121.26, 115.88, 115.70, 108.92, 106.12, 101.74, 43.23. HRMS $[M + H]^+$ found 369.1240, calculated 369.1251 for $C_{20}H_{17}FN_2O_4$.

2-(((2E,4E)-5-(benzo[d][1,3]dioxol-5-yl)penta-2,4-dien-1-yl)amino)-N-(2-chlorophenyl)acetamide (6k)

Yellow solid powder,^{1}H NMR (500 MHz, DMSO-*d6*) δ 9.54 (s, 1H), 8.54 (t, *J* = 5.8 Hz, 1H), 7.82 (d, *J* = 8.0 Hz, 1H), 7.51 (dd, *J* = 8.0, 1.3 Hz, 1H), 7.37 – 7.33 (m, 1H), 7.28 (d, *J* = 1.5 Hz, 1H), 7.25 -7.17 (dd, *J* = 15.4, 12.3, 6.0 Hz, 2H), 7.02 – 6.89 (m, 4H), 6.21 (d, *J* = 15.0 Hz, 1H), 6.05 (s, 2H), 4.06 (d, *J* = 5.8 Hz, 2H).^{13}C NMR (126 MHz, DMSO-*d6*) δ 168.81, 166.34, 148.42,148.27, 140.62, 138.87, 135.10, 131.28, 129.95, 128.59,128.02,126.57, 125.64, 124.25, 123.26, 108.92, 106.14, 101.75, 43.32. HRMS $[M + H]^+$ found 385.0955, calculated 385.0955 for $C_{20}H_{17}ClN_2O_4$.

(2E,4E)-5-(benzo[d][1,3]dioxol-5-yl)-N-(2-((3-chlorophenyl)amino)-2-oxoethyl)penta-2,4-dienamide (6l)

Yellow solid powder**,**^{1}H NMR (500 MHz, DMSO-*d6*) δ 10.23 (s, 1H), 8.48-8-46 (t, *J* = 5.9 Hz, 1H), 7.81 (t, *J* = 2.0 Hz, 1H), 7.47 (d, *J* = 8.2 Hz, 1H), 7.35 (t, *J* = 8.1 Hz, 1H), 7.29 (d, *J* = 1.5 Hz, 1H), 7.19 (dd, *J* = 15.0, 10.6 Hz, 1H), 7.12 (dd, *J* = 7.6, 1.6 Hz, 1H), 7.02 – 6.88 (m, 4H), 6.21 (d, *J* = 15.0 Hz, 1H), 6.05 (s, 2H), 3.99 (d, *J* = 5.9 Hz, 2H). ^{13}C NMR (126 MHz, DMSO-*d6*) δ 168.79,166.19, 148.41, 148.25, 140.83, 138.74, 133.53, 131.30, 130.97,125.66, 124.43,123.42,123.23,119.02,117.94,108.92,106.13, 101.74, 43.36. HRMS $[M + H]^+$ found 385.0951, calculated 385.0955 for $C_{20}H_{17}ClN_2O_4$.

2-(((2E,4E)-5-(benzo[d][1,3]dioxol-5-yl)penta-2,4-dien-1-yl)amino)-N-(4-chlorophenyl)acetamide (6m)

Yellow solid powder**,** ^{1}H NMR (500 MHz, DMSO-*d6*) δ 10.24 (s, 1H), 8.47 (t, *J* = 5.6 Hz, 1H), 7.81 (s, 1H), 7.47 (d, *J* = 7.9 Hz, 1H), 7.35 (t, *J* = 8.1 Hz, 1H), 7.28 (s, 1H), 7.19

(dd, *J* = 14.9, 10.7 Hz, 1H), 7.12 (d, *J* = 7.7 Hz, 1H), 7.02 – 6.88 (dd, *J* = 25.7, 21.2, 12.3 Hz, 4H), 6.21 (d, *J* = 15.0 Hz, 1H), 6.05 (s, 2H), 3.99 (d, *J* = 5.7 Hz, 2H).^{13}C NMR (126 MHz, DMSO-*d6*) δ 168.79,166.19, 160.65,148.42, 148.25, 140.83, 140.40, 138.74, 133.54, 130.96, 125.66, 124.43, 123.42, 123.23, 119.02, 117.94, 108.92, 106.13, 101.74, 43.37**.** HRMS $[M + H]^+$ found 385.0960, calculated 385.0955 for $C_{20}H_{17}ClN_2O_4$.

2-(((2E,4E)-5-(benzo[d][1,3]dioxol-5-yl)penta-2,4-dien-1-yl)amino)-N-(2-(trifluoromethyl)phenyl) acetamide (6n)

Yellow solid powder**,**^{1}H NMR (500 MHz, DMSO-*d6*) δ 9.57 (s, 1H), 8.52 (t, 1H), 7.74 (d, *J* = 7.8 Hz, 1H), 7.70 (t, *J* = 7.6 Hz, 1H), 7.60 (d, *J* = 7.9 Hz, 1H), 7.44 (s, 1H), 7.28 (s, 1H), 7.24 – 7.19 (dd, *J* = 14.9, 10.5 Hz, 1H), 7.01 (dd, *J* = 8.1 Hz, 1H), 6.97 – 6.88 (m, 3H), 6.21 (d, *J* = 15.0 Hz, 1H), 6.05 (s, 2H), 4.02 (s, 2H). ^{13}C NMR (126 MHz, DMSO-*d6*) δ 169.39, 166.28, 148.42, 148.26, 140.56, 138.83, 135.59, 133.58, 131.28, 126.94, 126.82, 126.78, 125.65, 124.27, 123.26, 108.92, 106.13, 101.74, 42.99. HRMS $[M + H]^+$ found 419.1248, calculated 419.1219 for $C_{21}H_{17}F_3N_2O_4$.

D. General procedure for the synthesis of intermediates 8a-8m

The procedure described in our publication involves addition of a substituted benzyl halide (1.0 equiv, 1 mmol) to a solution of ethanol (20 mL). To this mixture, K_2CO_3 (3.0 equiv, 3.0 mmol) and anhydrous piperazine (4.0 equiv) were added in a round-bottom flask (RBF). After the addition of the components, the reaction mixture was subjected to reflux for a period of 4-6 h. The progress of the reaction was monitored using thin-layer chromatography (TLC). Once the reaction was complete, the ethanol was removed by evaporation under the reduced pressure. The resulting reaction mixture was then diluted with ethyl acetate (25 mL) and subsequently washed with ice-cold water. The aqueous layer was subjected to extraction using ethyl acetate (3 × 25 mL). The organic phase was then dried using sodium sulfate to eliminate any residual moisture and concentrated. The

resulting concentrated solution, containing compounds 2a-2n, was used in the subsequent step without undergoing any purification steps. The synthesized intermediates were characterized using ^{1}H-NMR spectroscopy. The obtained NMR spectra of the intermediates showed a close match with the reported values, confirming their identity and structural integrity.(Singh et al., 2021)

E. General procedure for the synthesis of target compounds 9a-9m

In a round-bottom flask (RBF), piperic acid (PA) was added (1.0 equiv.), along with EDCI.HCl (1.5 equiv.) and HOBt (1.5 equiv.) in 10 ml of THF, and the resulting reaction mixture was stirred for 10 mints. Next, a 1:1 equimolar mixture of compounds 2a-2n was added to the flask followed by triethylamine (0.33 g, 2.5 equiv.), and the reaction mixture was stirred at room temperature. After the completion of the reaction, the mixture was subjected to washing with water and ethyl acetate. The organic layer was then washed and dried using sodium sulfate, followed by concentration under vacuum. The purification of the compounds was performed using column chromatography.(Singh et al., 2021)

(2E,4E)-5-(benzo[d][1,3]dioxol-5-yl)-1-(4-benzylpiperazin-1-yl)penta-2,4-dien-1-one (9a)

Yellow powder, ^{1}H NMR (600 MHz, $CDCl_3$) 7.33 (dd, *J* = 14.6, 10.3 Hz, 1H), 7.25 – 7.24 (m, 4H), 7.20 – 7.18 (m, 1H), 6.89 (s, 1H), 6.80 (d, *J* = 8.0 Hz, 1H), 6.71 – 6.61 (m, 3 H), 6.30 (d, *J* = 14.6 Hz, 1H), 5.87 (s, 2H), 3.57 (d, *J* = 82.1 Hz, 4H), 3.45 (s, 2H), 2.38 (s, 4H).^{13}C NMR (151 MHz, $CDCl_3$) δ 164.46, 147.18, 142.01, 137.69, 136.39, 129.86, 128.15, 127.32, 126.30, 124.12, 121.59, 118.31, 107.46, 104.66, 100.26, 76.28, 76.07, 75.86, 61.78, 52.18, 51.70, 44.62, 40.97, 28.66. HRMS $[M + H]^{+}$ Found 377.1850, calculated 377.1860 for $C_{23}H_{24}N_2O_3$.

(2E,4E)-5-(benzo[d][1,3]dioxol-5-yl)-1-(4-(2-methylbenzyl)piperazin-1-yl)penta-2,4-dien-1-one (9b)

White solid powder, ^{1}H NMR (500 MHz, DMSO-d_6) δ 7.27 – 7.22 (m, 2H), 7.18 – 7.13 (m, 4H), 7.00 – 6.88 (m, 4H), 6.66 (d, *J* = 14.6 Hz, 1H), 6.05 (s, 2H), 3.54 (s, 4H), 3.44 (s, 2H), 2.37 (s, 4H), 2.33 (s, 3H).^{13}C NMR (126 MHz, DMSO-d_6) δ 164.83, 148.35, 142.62, 138.50, 137.60, 136.43, 131.26, 130.59, 130.10, 127.51, 125.95, 123.03, 120.77, 109.00, 105.98, 101.77, 60.38, 53.69 52.99, 45.60, 42.08, 19.32. HRMS $[M + H]^+$ Found 391.2015, calculated 391.2016 for $C_{24}H_{26}N_2O_3$.

(2E,4E)-5-(benzo[d][1,3]dioxol-5-yl)-1-(4-(3-methylbenzyl)piperazin-1-yl)penta-2,4-dien-1-one (9c)

Yellow solid powder ,^{1}H NMR (600 MHz, $CDCl_3$) δ 7.44 – 7.39 (m, 1H), 7.21 (t,1H), 7.13 – 7.08 (m, 3H), 6.97 (s, 1H), 6.88 (d, *J* = 7.8 Hz, 1H), 6.77 – 6.69 (m, 3H), 6.38 (d, *J* = 14.6 Hz, 1H), 5.96 (s, 2H), 3.65 (d, *J* = 81.7 Hz, 4H), 3.49 (s, 2H), 2.46 (s, 4H), 2.35 (s, 3H).^{13}C NMR (151 MHz, $CDCl_3$) δ 164.47, 147.18, 142.00, 137.69, 136.93, 136.29, 129.87, 128.91, 127.18, 127.04, 125.26, 124.12, 121.59, 118.32, 107.46, 104.66, 100.26, 76.28, 76.07, 75.87, 61.82, 52.21, 51.74, 44.63, 40.99, 28.66, 20.37. HRMS $[M + H]^+$ Found 391.2006, calculated 391.2016 for $C_{24}H_{26}N_2O_3$.

(2E,4E)-5-(benzo[d][1,3]dioxol-5-yl)-1-(4-(4-methylbenzyl)piperazin-1-yl)penta-2,4-dien-1-one (9d)

Yellow solid powder,^{1}H NMR (500 MHz, $CDCl_3$) ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.43(dd, *J* = 14.6, 10.0 Hz, 1H), 7.28 (s, 1H), 7.22 (d ,*J* = 7.8 Hz, 1H), 7.15 (d, *J* = 7.8 Hz, 2H), 6.99 (s, 1H), 6.90 (d, *J* = 7.8 Hz, 1H), 6.80 – 6.71 (m, 3H), 6.40 (d, *J* = 14.6 Hz, 1H), 5.98 (s, 2H), 3.73 (s, 2H), 3.59 (s, 2H), 3.51 (s, 2H), 2.47 (s, 4H), 2.36 (s, 3H).^{13}C NMR (126 MHz, $CDCl_3$) δ 165.49, 148.23, 142.97, 138.67, 136.92, 134.47, 130.95, 129.09, 125.21,

122.60, 119.45, 108.51, 105.72, 101.30, 77.35, 77.10, 76.85, 62.59, 52.97, 45.73, 42.09, 29.70, 21.12.HRMS $[M + H]^+$ Found 391.1991, calculated 391.2016 for $C_{24}H_{26}N_2O_3$.

(2E,4E)-5-(benzo[d][1,3]dioxol-5-yl)-1-(4-(4-(tert-butyl)benzyl)piperazin-1-yl)penta-2,4-dien-1-one (9e)

White solid powder, ^{1}H NMR (500 MHz, DMSO-*d6*) δ δ 7.35 (d, *J* = 8.3 Hz, 2H), 7.26 - 7.21 (m, 3H), 7.18 (d, *J* = 1.4 Hz, 1H), 6.99 – 6.88 (m, 4H), 6.65 (d, *J* = 14.6 Hz, 1H), 6.05 (s, 2H), 3.55 (s, 4H), 3.45 (s, 2H), 2.35 (s, 4H), 1.28 (s, 9H). ^{13}C NMR (126 MHz, DMSO-*d6*) δ 164.82, 149.80, 148.41,148.28, 142.63, 138.52, 135.25, 131.25, 129.10, 125.99, 125.41, 123.05, 120.74, 109.01, 105.97, 101.77, 61.98, 34.65, 31.66. HRMS $[M + H]^+$ Found 433.2481, calculated 433.2486 for $C_{27}H_{32}N_2O_3$.

(2E,4E)-5-(benzo[d][1,3]dioxol-5-yl)-1-(4-(2-fluorobenzyl)piperazin-1-yl)penta-2,4-dien-1-one (9f)

Yellow solid powder,^{1}H NMR (500 MHz, $CDCl_3$) δ 7.43 (dd, *J* = 14.6, 10.2 Hz, 1H), 7.36 (s, 1H), 7.28 (s, 1H), 7.27-7.26 (m,, 1H), 7.23-7.21 (m,, 1H), 6.99 (d, *J* = 1.6 Hz, 1H), 6.91 (dd, *J* = 8.1, 1.6 Hz, 1H), 6.81 – 6.71 (m, 3H), 6.40 (d, *J* = 14.6 Hz, 1H), 5.99 (s, 2H), 3.67 (d, d, *J* = 62.8 Hz 4H),, 3.51 (s, 2H), 2.47 (s, 4H).^{13}C NMR (126 MHz, $CDCl_3$) δ 165.54, 148.25 ,143.09 ,139.92, 138.78, 134.31, 130.93, 129.61, 129.00, 127.48, 127.12, 125.16 , 122.61 , 119.32 , 108.51 , 105.73 , 101.30 , 62.20, 53.23 , 52.84 , 45.69 , 42.03. HRMS $[M + H]^+$ Found 395.1756, calculated 395.1765 for $C_{23}H_{23}FN_2O_3$.

(2E,4E)-5-(benzo[d][1,3]dioxol-5-yl)-1-(4-(3-fluorobenzyl)piperazin-1-yl)penta-2,4-dien-1-one (9g)

Yellow solid powder ,^{1}H NMR (500 MHz, DMSO-*d6*) δ 7.37 (dd, *J* = 14.1, 7.9 Hz, 1H), 7.24 (dd, *J* = 14.6, 10.3 Hz, 1H), 7.17 – 7.13 (m, 3H), 7.10-7.06 (m, 1H),6.97 (dd, *J* = 14.6, 6.6 Hz, 2H), 6.93 – 6.88 (m, 2H), 6.65 (d, *J* = 14.6 Hz, 1H), 6.05 (s, 2H), 3.57 (s, 4H), 3.52 (s, 2H), 2.37 (s, 4H).^{13}C NMR (126 MHz, *DMSO-d6*) δ 164.86, 163.68, 161.74,

148.33, 142.64, 141.52, 138.51, 131.24, 130.56, 125.97, 125.22, 123.02, 120.71, 115.78, 115.61, 114.33, 114.17, 108.98, 106.15, 105.98, 101.76, 65.38, 61.48, 53.44, 52.78 , 45.47, 41.98 . HRMS $[M + H]^+$ Found 395.1744, calculated 395.1765 for $C_{23}H_{23}FN_2O_3$.

(2E,4E)-5-(benzo[d][1,3]dioxol-5-yl)-1-(4-(4-fluorobenzyl)piperazin-1-yl)penta-2,4-dien-1-one (9h)

Yellow solid powder ,^{1}H NMR (600 MHz, $CDCl_3$) δ 7.34 (dd, *J* = 14.4, 10.6 Hz, 1H), 7.22 – 7.19 (m, 2H), 6.93 (t, *J* = 8.3 Hz, 2H), 6.89 (s, 1H), 6.81 (d, *J* = 7.9 Hz, 1H), 6.70 – 6.61 (m, 3H), 6.30 (d, *J* = 14.6 Hz, 1H), 5.89 (s, 2H), 3.57 (d, *J* = 77.6 Hz, 4H), 3.42 (s, 2H), 2.37 (s, 4H).. ^{13}C NMR (151 MHz, $CDCl_3$) δ 165.55, 162.95, 161.33, 148.24, 143.15, 138.83, 133.15, 130.88, 130.66, 125.12, 122.65, 119.25, 115.25, 115.11, 108.51, 105.69, 101.32, 77.32, 77.18, 76.87, 61.97, 52.89, 52.62, 45.63, 41.99, 29.70. HRMS $[M + H]^+$ Found 395.1759, calculated 395.1765 for $C_{23}H_{23}FN_2O_3$.

(2E,4E)-5-(benzo[d][1,3]dioxol-5-yl)-1-(4-(3-chlorobenzyl)piperazin-1-yl)penta-2,4-dien-1-one (9i)

Yellow solid powder,^{1}H NMR (600 MHz, $CDCl_3$) δ 7.34 (dd, *J* = 13.8, 11.1 Hz, 1H), 7.16 (t, *J* = 20.0 Hz, 4H), 6.89 (s, 1H), 6.81 (d, *J* = 8.0 Hz, 1H), 6.70 – 6.62 (m, 3H), 6.31 (d, *J* = 14.6 Hz, 1H), 5.89 (s, 2H), 3.58 (d, *J* = 79.9 Hz, 4H), 3.41 (s, 2H), 2.37 (s, 4H).^{13}C NMR (151 MHz, $CDCl_3$) δ 164.48, 147.20, 142.08, 138.88, 137.75, 133.26, 129.86, 128.59, 127.97, 126.45, 126.11, 124.11, 121.61, 118.26, 107.48, 104.67, 100.28, 61.17, 52.24, 51.99, 44.64, 41.00, 28.67. HRMS $[M + H]^+$ Found 411.1479, calculated 411.1470 for $C_{23}H_{23}ClN_2O_3$.

(2E,4E)-5-(benzo[d][1,3]dioxol-5-yl)-1-(4-(4-chlorobenzyl)piperazin-1-yl)penta-2,4-dien-1-one (9j)

Yellow solid powder ,^{1}H NMR (500 MHz, $CDCl_3$) δ 7.43 (dd, *J* = 14.6, 10.3 Hz, 1H), 7.32 (d, *J* = 8.5 Hz, 2H), 7.27 (s, 1H), 6.99 (s, 1H), 6.91 (d, *J* = 8.1 Hz, 1H), 6.81 – 6.71

(m,4H), 6.40 (d, *J* = 14.6 Hz, 1H), 5.99 (s, 2H), 3.76 (s, 4H), 3.60 (s, 2H), 2.46 (s, 4H).^{13}C NMR (126 MHz, $CDCl_3$) δ 165.54, 148.25, 143.10, 138.78, 136.27, 133.01, 130.92, 130.34, 128.50, 125.14, 122.62, 119.30, 108.51, 105.71, 101.30, 67.96, 62.07, 45.66, 29.69, 25.61. HRMS $[M + H]^+$ Found 411.1465, calculated 411.1470 for $C_{23}H_{23}ClN_2O_3$.

(2E,4E)-5-(benzo[d][1,3]dioxol-5-yl)-1-(4-(3-nitrobenzyl)piperazin-1-yl)penta-2,4-dien-1-one (9k)

Brown solid powder ,^{1}H NMR (500 MHz, , $CDCl_3$) δ 8.20 (s, 1H), 8.11 (d, *J* = 8.6 Hz, 1H), 7.67 (d, *J* = 7.3 Hz, 1H), 7.49 (t, *J* = 8.0 Hz, 1H), 7.40 (dd, *J* = 14.5, 10.1 Hz, 1H), 6.95 (s, 1H), 6.87 (d, *J* = 8.0 Hz, 1H), 6.82 – 6.65 (m, 3H), 6.36 (d, *J* = 14.5 Hz, 1H), 5.94 (d, *J* = 4.1 Hz, 2H), 3.71 (s, 2H), 3.61 (s, 4H), 2.47 (s, 4H). ^{13}C NMR (126 MHz,) δ 165.63, 148.66, 143.36, 139.01, 136.21, 135.09, 130.91, 129.43, 126.81, 125.12, 123.73, 123.62, 122.66, 122.01, 119.16, 109.39, 108.52, 105.75, 101.32, 61.86, 53.07, 45.65, 41.98, 29.76, 1.08. HRMS $[M + H]^+$ Found 422.1710, calculated 422.1710 for $C_{23}H_{23}N_3O_5$.

2-((4-((2E,4E)-5-(benzo[d][1,3]dioxol-5-yl)penta-2,4-dienoyl)piperazin-1-yl)methyl)benzonitrile (9l)

Yellow solid powder, ^{1}H NMR (500 MHz, , $CDCl_3$) δ 7.65 (d, *J* = 7.7 Hz, 1H), 7.567 – 7.50(m, 2H), 7.43 – 7.35 (m, 2H), 6.96 (s, 1H), 6.88 (d, *J* = 7.8 Hz, 1H), 6.80 – 6.69 (m, 3H), 6.38 (d, *J* = 14.6 Hz, 1H), 5.95 (s, *J* = 5.4 Hz, 2H), 3.70 (s, 4H), 3.59 (s, 2H), 2.51 (s, 4H). ^{13}C NMR (126 MHz,) δ 165.60, 148.31, 143.23, 141.89, 138.89, 133.20, 132.68, 130.98, 130.13, 127.92, 125.20, 122.72, 119.31, 117.80, 108.59, 105.78, 101.38, 60.44, 32.00, 29.78, 14.20, 1.09. HRMS $[M + H]^+$ Found 402.1805, calculated 402.1812 for $C_{24}H_{23}N_3O_3$.

3-((4-((2E,4E)-5-(benzo[d][1,3]dioxol-5-yl)penta-2,4-dienoyl)piperazin-1-yl)methyl)benzonitrile (9m)

Yellow solid powder, ^{1}H NMR (500 MHz, $CDCl_3$) 7.66 (s, 1H), 7.56 (d, *J* = 7.7 Hz, 2H), 7.45 – 7.40(m, 2H), 6.97 (s, 1H), 6.89 (d, *J* = 7.9 Hz, 1H), 6.79 – 6.69 (m, 3H), 6.38 (d, *J* = 14.6 Hz, 1H), 5.97 (s, 2H), 3.66 (d, *J* = 58.4 Hz, 4H), 3.54 (s, 2H), 2.45 (s, 4H)..^{13}C NMR (126 MHz, $CDCl_3$)) δ 165.62, 148.32, 143.27, 139.65, 138.94, 133.33 , 132.42, 131.03 , 129.25, 125.17, 122.70, 119.26, 118.88, 112.66, 108.58, 105.78, 101.37, 61.94, 52.98 , 45.76 , 42.08. HRMS $[M + H]^+$ Found 402.1810, calculated 402.1812 for $C_{24}H_{23}N_3O_3$.

4-((4-((2E,4E)-5-(benzo[d][1,3]dioxol-5-yl)penta-2,4-dienoyl)piperazin-1-yl)methyl)benzonitrile (9n)

Yellow solid powder ,^{1}H NMR (500 MHz, $CDCl_3$) δ 7.65 (d, *J* = 8.0 Hz, 2H), 7.49 (d, *J* = 8.2 Hz, 2H), 7.46 – 7.41 (m, 1H), 7.00 (s, 1H), 6.92 (d, *J* = 8.1 Hz, 1H), 6.84 – 6.71 (m, 3H), 6.41 (d, *J* = 14.6 Hz, 1H), 6.00 (s, 2H), 3.75 (s, 4H), 3.60 (s, 2H), 2.48 (s, 4H).^{13}C NMR (126 MHz, $CDCl_3$) δ 165.56, 148.25, 148.37, 143.26, 140.18 ,138.92, 132.25, 130.87, 129.46, 125.08, 122.68, 119.14, 111.19 , 108.54, 105.70, 101.33, 62.27, 53.36 , 52.96,45.69,45.64. HRMS $[M + H]^+$ Found 402.1818, calculated 402.1812 for $C_{24}H_{23}N_3O_3$.

5.3. 5.3 Biological evaluation

Determination of IC_{50} values

The enzymes human acetylcholinesterase (*h*AChE) and equine butyrylcholinesterase *(eq*BuChE), Ellman's reagent (DTNB), acetylthiocholine iodide (ATCI), and butyrylthiocholine iodide (BTCI) were procured from Sigma Aldrich (U.S.) For the experiment, donepezil hydrochloride served as the standard control. The cholinergic activity of all synthesized compounds was evaluated using the Ellman colorimetric

method, and our previous publications with slight modification. The 50 mM Tris-HCl buffer with a pH of 8 was used in the experiment. Briefly, 50 µL of the enzyme (AChE or BChE) and 10 µL compound were incubated for 30 min in 96 well plates at rt. followed by the addition of either ATCI or BTCI and kept at rt. for 30 min. Then add 160 µL of Ellman reagent (DTNB, 0.15 mM), and absorption spectra was measured at 415 nm using a microplate reader. (Ellman et al., 1961; Singh et al., 2020)

Kinetic Analysis and Mode of AChE & BChE Inhibition

In order to assess the inhibitory effect of 6j and **9m** on ChEs, a standard reciprocal plot of 1/[V] versus 1/[S] was developed using the Ellman Method, with varying concentrations of ATCI or BTCI at 2.5, 2.0, 1.5, 1.0, and 0.5 mM for *h*AChE or *eq*BChE. In brief, the experimental procedure involved pre-incubating 50 µL of *h*AChE (0.022 U/mL), or *eq* BChE (50 µL of 0.06 U/mL), and 10 µL of (6j and **9m**) at different concentrations for (0.1,0.5 and 1 µM for BChE and 1,5,10 µM for AChE) for 30 min at room temperature. Then, substrate (30 µL) ATCI or BTCI at varying concentrations (2.5, 2.0, 1.5, 1.0, 0.5 mM for of hAChE or *eq*BChE) was added. Subsequently, 160 µL of 0.15 mM concentration of DTNB was added, and the resultant absorbance profile was monitored at 415 nm after 30 seconds. This experiment was conducted in accordance with our previous publication.(Kumar et al., 2023; Singh et al., 2021; Singh et al., 2020)

Propidium iodide displacement assay

The Propidium iodide displacement assay was conducted to investigate the binding affinity of compounds 6j and **9m** **3m** *h*AChE enzyme. Tris buffer with a pH of 8.0 and a concentration of 0.1 mM was prepared as the optimal reaction medium. The *h*AChE enzyme was diluted to a concentration of 5.0 U/mL using the Tris buffer. Various concentrations of compounds 6j and **9m** (5, 10, 20, and 50 µM) were prepared for the

assay. The enzyme and compound 6j and **9m** were mixed in a 1:1 ratio and incubated for 6 h at 25 °C. This incubation period allowed the enzyme and compound to interact and reach equilibrium. Propidium iodide (PI) was added to a final concentration of 20 μM to the enzyme-compound mixture and incubated for 20 min. PI, a fluorescent dye that binds to nucleic acids, served as an indicator of displacement from the enzyme-compound complex. The fluorescence pattern of the mixture was observed on a microplate reader with excitation and emission wavelengths of 535 nm and 595 nm, respectively. This analysis provided insights into the displacement of PI and, consequently, the binding affinity of compounds 6j and **9m** to hAChE. The assay outcome evaluates compounds 6j and **9m** potential as an inhibitor of *h*AChE activity. (Singh et al., 2021; Singh et al., 2020)

DPPH radical-scavenging potency

DPPH assay measures antioxidant activity by measuring a compound's ability to donate hydrogen atoms and diminish the stable free radical DPPH, which turns violet to yellow. Thus, the assay measures the compound's antioxidant activity by quantifying its hydrogen atom donation strength. Methanol was used in experiments for stock solution preparation as well as for further dilutions. In summary, the 96-well plate included 75 μL **9m** and Piperic acid at 200, 160, 80, 40, and 20 μM concentrations. Subsequently, 75 μL of 100 μM DPPH reagent was added and subjected to incubation for a duration of 25 min at 37 ºC with moderate stirring on a thermo mixer. An alteration in the light absorption profile was observed at a specific wavelength of 517 nm, utilizing a microplate reader. The reduction of absorbance is considered an effective capability of compounds to scavenge free radicals. “The calculation of the reducing percentage (RP) of the DPPH involved the use of the formula RP = [((absorbance of control-absorbance of the test)/ absorbance of control] X 100”. Each trial was conducted in duplicate or triplicate.(Singh et al., 2021; Singh et al., 2020)

In-vitro metal chelating assay

Metal chelating studies were conducted using a 96-well micro-plate reader with a wavelength range of 200 to 700 nm. The compounds 6j and **9m** was solubilized in methanol to achieve a concentration of 600 µM, and the pH was determined utilizing a digital pH meter. Subsequently, the 6j and **9m** compound was diluted to 300 µM conc. The resultant solution was analyzed using a UV-vis spectrophotometer within the wavelength range of 200 to 700 nm. Finally, a solution devoid of color was synthesized through the dissolution of $FeCl_3$ (600 µM) in methanol, resulting in a stock solution. A mixture comprising of equimolar amounts of the compounds 6j and **9m** (600 µM) and $FeCl_3$ (600 µM) was prepared and subjected to pH monitoring. In order to maintain the pH of the system at 7.4, a solution of Diisopropylethylamine (100 µL DIPEA + 900 µL methanol) was employed, followed by a 30-min. incubation at 25 °C, after which UV scanning was conducted. This was carried out in accordance with our previous publication. (Kumar et al., 2023)

Molecular modelling studies

Molecular docking

To investigate the molecular interactions between the compounds and the BChE protein (PDB ID: 4bds), a molecular docking approach was utilized using Auto-Dock tools software (version 20).(Morris et al., 2009) The BChE protein target was prepared with Kollman charges, and docking interactions were performed using the Lamarckian genetic algorithm with 10 GA (Genetic Algorithm) runs. The grid box dimensions on the protein were defined as - 138.426 × 123.269 × 38.163 in xyz coordinates, with a spacing of 0.375 Å.

Molecular dynamics

Following previously described procedures, classical molecular dynamics simulations were conducted using GROMACS2020 and the CHARMM 36m force field. The highest-scoring complex from molecular docking served as input for system generation through the CHARMM-GUI Web server. The complex was solvated with the TIP3P water model in a cubic box ($10 \times 8 \times 8$ nm^3) and neutralized with Na+ and Cl− ions using the Monte Carlo ion placing method. The prepared systems underwent energy minimization, followed by a 2 ns equilibration step with Nose−Hoover temperature coupling at 303.15 K. A 100 ns isobaric−isothermal (NPT) production simulation run was performed under periodic boundary conditions using Parrinello−Rahman pressure coupling at 1 atm pressure. The simulation trajectory was quantitatively analyzed for root mean square deviation (RMSD), root-mean-square fluctuation (RMSF), and using GROMACS functionalities.(Abraham et al., 2015; Kumar et al., 2023)

DFT calculation

The atomic, energetic, and electrostatic properties of the hit compounds were estimated through Density-functional theory (DFT) analysis, as described in the works of Alesawy et al. (2021). To perform these calculations, Gaussian 16 W software was used. Before the analysis, all compounds underwent energy optimization using GaussView 6.1.1 software. The total energies, dipole moment, HOMO and LUMO energy levels, and band gap energies of the compound was calculated. Additionally Molecular Electrostatic Potential (MEP) maps was generated for compound. (Frisch et al., 2016; Thomas et al., 2023)

PAMPA assay for in-vitro analysis of permeability

The brain's permeability is primary requirement for any compounds to serve as anti-Alzheimer medicines. Corticosterone and hydrocortisone which serves as positive and

negative controls, respectively. The PAMPA kit purchased from "Bioassays systems Pvt. Ltd." includes the donor plate (PVDF membrane with 0.45 mm pore size) and the acceptor plate. Avanti Polar Lipids and Avra Synthesis supplied PBL and n-dodecane, respectively. All experiments were done in pH 7.4 PBS. Test and standard control compounds were dissolved in DMSO or MeOH (for low-solubility compounds) to make a 10 mM stock solution. Diluting 25 µL of stock solution with 475 µL of PBS (pH 7.4) produced 500 µL of 6j and **9m** and 500 M standard control concentration. Diluting 80 µL of 6j and **9m** and 120 µL of PBS yielded 200 µL equilibrium standards for the test and control. 5 µL of DMSO in 245 µL of PBS created a blank control. Di et al.'s method and previous publication were followed to perform this experiment. (Di et al., 2003; Kumar et al., 2023; Singh et al., 2020).

Cell culture

The experiments were performed using rat pheochromocytoma PC12 cells procured from the National Centre for Cell Science (NCCS) in Pune, India which were from passages 3 to 12. The cells were cultured in Ham's F-12K (Kaighn's) Medium, supplemented with 10% fetal bovine serum and 1% Gibco™ Antibiotic-Antimycotic solution which contains 10,000 units/mL of penicillin, 10,000 µg/mL of streptomycin, and 25 µg/mL of Gibco Amphotericin B. Undifferentiated PC12 cells were differentiated into sympathetic-like neurons, a well-established in vitro neuronal model. This was done by culturing the cells in nerve growth factor (NGF) at a concentration of 100ng/mL in Dulbecco's Modified Eagle Medium (DMEM), supplemented with 10% fetal bovine serum and 1% Gibco™ Antibiotic-Antimycotic. These reagents were acquired from GIBCO (ThermoFisher). Throughout the study, all experiments were conducted using the differentiated PC12 cells. Dimethyl sulfoxide (DMSO), Thiazolyl Blue Tetrazolium Bromide (MTT, CAS No. 298-

93-1) were purchased from Sigma-Aldrich and H_2O_2 from Himedia. Subsequently, the cells incubated at 37 °C with 5 % CO_2.

Evaluation of cytotoxicity and neuroprotection by MTT assay

The MTT assay (a tetrazolium salt, reduction assay) was performed to evaluate cell viability. Cells (PC-12) were seeded at a density of 1×10^4 cells per well in 96-well plates and were allowed to attach and recover for at least 24 h. Subsequently, the 6j and **9m** was added at various concentrations: 20, 15, 10, 7, 5, 2.5, 1, and 0.1 µM and incubated for 24 h at 37 °C with 5% CO_2. Following the incubation period, cells were incubated with MTT reagent (final concentration- 0.5mg/ml) for approximately 3 h at 37 °C with 5% CO_2. After discarding the medium, 100 µl of DMSO was then added to dissolve the formazan crystals, and the absorbance was measured at a 562 nm using a Spectramax-i3x instrument. Cell viability was calculated as a percentage, with untreated cells serving as the baseline negative control set at 100%. The following formula was used to calculate the percentage of cell viability: Percentage of viability = [Mean OD value of the experimental sample (treated)] / [Mean OD value of the experimental control (untreated)] × 100. The data was graphically represented by plotting mean values with their corresponding standard errors (Mean ± SEM). All assays were conducted in quadruplicate. (Cory et al., 1991).

The neuroprotection ability of 9m was carried out following our earlier publication.(Singh et al., 2022) Briefly, the PC-12 cells were pretreated with different concentrations of 3m followed by co-incubation with 600 µM H2O2 for an additional 24 h and MTT assay.

Animal studies

Animals: The Swiss albino mice, aged 6 weeks old and weighing 25-30 grams, were procured from "Central Animal Facility." The mice were acclimatized for one week in

the departmental animal house at I.I.T. (B.H.U.). During this period, the mice were subjected to a 12:12 hour dark/light cycle, mimicking natural day-night patterns. The temperature was maintained at 25 ± 2 °C to provide a stable environment for the mice. The mice were fed commercially supplied food pellets and provided with tap water. It is assumed that this diet was the standard nutrition for laboratory mice unless specified otherwise in the experimental procedures. The experimental procedures conducted on the mice received approval from the "Department of Pharmaceutical Engineering and Technology, IIT, Banaras Hindu University, Varanasi, India." The approval number for the techniques is "IIT (BHU)/ IAEC/2021/006." The date of approval is April 12, 2021.

Acute toxicity test

Compound 6j and **9m** was evaluated for acute oral toxicity following the OECD- **425** guidelines. The test involved administering 500 mg/kg of compound 6j and **9m** to healthy Swiss albino mice. The mice were then closely monitored for a 24-hour period following the dose administration to observe any behavioral changes such as seizures or diarrhea and body weight was monitored.

Y- Maze experiment for scopolamine induce impairment on spatial memory

Drugs and chemicals

In the Y-maze experiment, high-grade chemicals and reagents were used. Specifically, scopolamine hydrochloride and donepezil hydrochloride were obtained from Sigma Aldrich, a well-known supplier of high-quality chemicals and reagents.

Drug preparation and treatment protocol

The experiment was conducted over a six-day period, and on the seventh day, scopolamine was introduced to specific groups of mice after 30 min of receiving standard and test compounds. 15 min after the administration of either a vehicle or scopolamine,

the mice underwent a Y-maze test. The animals were divided in to following groups. 1) Vehicle control group: Mice received a suspension of the vehicle (TWEEN 80, 0.5% v/v) and distilled water. This group serves as a control to compare the effects of the compounds. 2) Control group: Mice received no treatment and serve as a baseline for comparison. 3) DPZ (Reference compound) group: mice received a dose of 5 mg/kg of DPZ. 4) Scopolamine group: Mice received a dose of 3 mg/kg of scopolamine hydrochloride. 5) Compound 6j and **9m**, 10 mg/kg group: mice received a dose of 10 mg/kg of compounds 6j and **9m**. 6) Compound 6j and **9m**, 20 mg/kg group: Mice received a dose of 20 mg/kg of compounds 6j and **9m**. 7) PA (Positive compound) group: Mice received a dose of 10 mg/kg of PA.

After the six-day experiment, on the seventh day, the mice in groups 3, 5 and 6 received an additional administration of scopolamine. The scopolamine was administered 30 min after the mice received their standard and test compounds. Finally, 15 min after the administration of the vehicle or scopolamine, the mice underwent a Y-maze test. The purpose of this experiment is to evaluate the effects of the compounds and scopolamine on the mice's performance in the Y-maze test. The results can help determine if the compounds have any potential therapeutic effects or if they can mitigate the cognitive impairments induced by scopolamine.

Y Maze test

The study was conducted with the aim of assessing the exploratory behavior and spatial memory of mice. The Y maze has three distinct arms that are angularly separated from each other by 120° angles. These arms are designated with the labels A, B, and C. During the experiment, the mice were placed at a single end of the Y-maze and allowed unrestricted movement throughout the arm for a duration of eight min. The number of complete entries was recorded via video camera. Once the experiment was complete, the

data was thoroughly analyzed and the spontaneous alteration was computed. The entry of mice into distinct arms results in the occurrence of spontaneous alternations, which may include the sequences CBA, ABC, BAC, BCA, ACB, CAB, and ABC.

The "% of spontaneous alteration computed as follows: % spontaneous alternation (SA) = [(number of alternations/total arm entries) - 2] X 100".

The % SA metric serves as an indicator of selective memory. (Chen et al., 2018; Kumar et al., 2023; Wang et al., 2018).