

Design, Synthesis, Molecular Docking, and Biological Evaluation of Chalcones Bearing Phenothiazine Analogues as Anti-inflammatory Agents

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ABSTRACTS

Current Nonsteroidal Anti-Inflammatory Drugs (NSAIDs) are associated with considerable toxicities and the development of resistance, leading to the urgent need for safer alternatives. Phenothiazine serves as a "privileged scaffold" for the creation of innovative therapeutics. This research presents the rational design of new Phenothiazine-chalcone analogues aimed at inhibiting Cyclooxygenase-2 (COX-2). Eight novel Phenothiazine-chalcone analogues (PJ-1 to PJ-8) were synthesised and characterised using FTIR, ¹H NMR, and ¹³C NMR and mass spectroscopic techniques. A comprehensive evaluation was conducted, which included *in silico* ADMET screening (SwissADME/ProTox-III) against COX-2 (4PH9). The analogues were subsequently validated through *in vivo* assays of carrageenan-induced paw oedema. All analogues exhibited favourable *in silico* drug-likeness and high predicted gastrointestinal absorption. Molecular docking studies indicated strong COX-2 binding affinity, with PJ-3 (-8.8 kcal/mol) and PJ-8 (-8.9 kcal/mol) showing significant results. *In vivo* experiments demonstrated that PJ-3 and PJ-8 led to substantial (*p*<0.001) inhibition of paw oedema. This study identifies (PJ-3) (E)-2-((4-methoxybenzylidene) amino)-1-(10H-phenothiazin-10-yl) ethan-1-one and (PJ-8) (E)-2-((4-dimethylamino) benzylidene) amino)-1-(10H-phenothiazin-10-yl) ethan-1-one as highly promising lead compounds. The strong correlation between their potent *in silico* binding affinities and experimentally validated biological activities, along with favourable ADMET profiles, positions them as strong candidates for further preclinical development.

Keywords: Anti-inflammatory, *In silico* Technique, Molecular Docking, Phenothiazine.

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INTRODUCTION

Phenothiazine (PTZ) is an organic thiazine compound with the chemical formula S(C₆H₄)₂NH. It appears as a powder with colors ranging from light green to steel-blue, taking on a greenish-brown hue when exposed to sunlight. With a molecular weight of 199.27 g/mol, it has a melting point of 185°C and a boiling point of 371°C. The IUPAC name for this compound is 10H-dibenzo-1,4-thiazine. Its core structure features two benzene rings, connected by a tricyclic nucleus that includes a sulfur atom at the 5th position and a nitrogen atom at the 10th position (Mosnaim *et al.*, 2006). Heterocyclic compounds containing nitrogen and sulfur atoms exhibit a range of biological activities, as the presence of these functional groups enhances the reactivity and potency of these compounds (Saraswat *et al.*, 2016). Phenothiazine is classified within the thiazine group of

heterocyclic compounds, characterized by its structure, which consists of two benzene rings interconnected in a tricyclic arrangement, along with, a sulfur and a nitrogen atom (Meyer and Ryan, 2016). Various modifications to the phenothiazine nucleus have led to the development of numerous derivatives with promising therapeutic effects (Wolan *et al.*, 2018). The neuroleptic properties of phenothiazine derivatives represent one of their most fascinating pharmacological characteristics (Kalkman *et al.*, 1998). Phenothiazine offers a broad spectrum of medicinal advantages, including anti-inflammatory activity (Motohashi *et al.*, 2000), Bactericidal Properties (Motohashi *et al.*, 1999), anti-depressant effects (Andreani *et al.*, 1991), Anti-Psychotropic Capabilities (Motohashi *et al.*, 1997), Anti-Tumor Properties, anti-viral effects (Dhople, 1999), Anti-Cancer activities (Bansal, 1999). and anti-tubercular effects (Viveiros and Amaral, 2001). Phenothiazines are heterocyclic derivatives, typically defined by a tricyclic structure that includes two benzene rings along with a central ring that contains both sulfur and nitrogen (Dighe, 2025). Phenothiazine derivatives are a versatile class of heterocyclic compounds known for a broad spectrum of biological activities beyond their traditional use as antipsychotics, including anticancer, antimicrobial, antitubercular, antioxidant, anti-inflammatory,



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antiviral, anthelmintic activity and Multidrug Resistance (MDR) reversing properties (Jaszczyszyn *et al.*, 2012). Phenothiazine derivatives are a diverse class of heterocyclic compounds known for their significant therapeutic, pharmacological, and industrial applications. Beyond their well-established role as foundational psychiatric medications, these derivatives are recognized for their multi-targeting potential, which allows them to address complex, multi-factorial diseases, including cancer and infections, with potential for improved safety and lower costs (Edwards *et al.*, 2022). Phenothiazine derivatives possess some benefits that render them a useful scaffold in medicinal chemistry, particularly for CNS diseases. Their stable framework provides high stability and favorable receptor-binding orientation, which is beneficial for pharmacological activity. One of their benefits is that they easily cross the BBB because of their moderate to high lipophilicity (Log P 3.5-5.5) and, therefore, are ideal for psychiatric and neurological disorders (Mehrab *et al.*, 2023). Such diversity enables medicinal chemists to design the phenothiazine core for various therapeutic applications by varying substituents at the 10th position or on the aromatic rings. Their redox-active sulfur and nitrogen atoms are also responsible for novel interactions with biological macromolecules, which could improve efficacy in conditions involving oxidative stress (Mehrab *et al.*, 2023).

MATERIALS AND METHODS

All chemicals and reagents were of Laboratory Reagent (LR) grade and procured from Sigma-Aldrich, Merck, and a local supplier and were used as received without further purification. Reaction progress was monitored by Thin Layer Chromatography (TLC) using Merck 60 F254 silica gel pre-coated aluminum plates and visualized under a UV lamp at 254 nm. Melting Points (MP) were determined in open-glass capillaries using a Fisher digital melting point apparatus and are uncorrected. Fourier-Transform Infrared (FTIR) spectra were recorded as on an ATR accessory on a Bruker Alpha-II spectrometer. Spectral data is reported in wavenumbers. ¹H NMR and ¹³C NMR spectra were acquired on a Bruker Avance III 500 spectrometer, operating at 500 MHz for ¹H and 125 MHz for ¹³C nuclei.

Methodology

This study employed an integrated approach combining computational modeling, chemical synthesis, and biological evaluation to assess the therapeutic potential of analogues. All chemicals and reagents were LR grade.

Chemical Synthesis

The synthesis of the target Phenothiazine analogues (PJ-1 to PJ-8) was performed in a three-step process as depicted in the designed scheme Figure 1.

Step I: synthesis of 10H-phenothiazine (PJ-A)

The phenothiazine nucleus was synthesized of 11 g of diphenylamine, 4.1 g of sulphur and 1.6 g of anhydrous aluminium chloride in 250 mL of round bottom flask fitted with reflux condenser (Silberg *et al.*, 2006). The reaction sets 140-150°C for 30-40 min (Silberg *et al.*, 2006). Heat the reaction and then this reaction mixture was cooled and dissolved in hot water. The formed yellow precipitate and it filtered (Silberg *et al.*, 2006). This product is crystallized from hot ethanol. Product collected by filtration and purified by recrystallization (Silberg *et al.*, 2006). The progress of reactions monitored by TLC.

10H-Phenothiazine (PJ-A)

Yellow Powder; MP: 182 - 188°C; **Yield:** 85.2 %; **Mass :** 199.27 g/mol **FTIR (ATR, λ cm⁻¹):** 3370.52 (N-H Stretching), 3043.75 (C-H Stretch, aromatic), 1595.80 (C=C Stretch, aromatic), 1235.30 (C-N stretch), 1031.71 (C-S Stretch). **¹H NMR (500 MHz, Chloroform-d, δ, ppm):** ¹H NMR (500 MHz, Chloroform-d) δ 7.19 - 7.11 (m, 3H), 7.13 - 7.05 (m, 3H), 6.90 (ddd, J = 13.6, 7.3, 1.3 Hz, 2H), 5.93 (s, 1H). **¹³C NMR (125 MHz, Common NMR Solvents) δ** 142.31, 126.98, 126.92, 126.87, 121.85, 117.10, 117.07, 114.26, 114.20.

Step II. Synthesis of 2-amino-1-(10H-phenothiazin-10-yl) ethan-1-one (PJ-B)

The key intermediate (PJ-B) was synthesized by dissolving phenothiazine in DMSO as a solvent, adding glycine and anhydrous K₂CO₃ (Potassium Carbonate) as a base under continuous stirring. Stir the reaction mixture at 60-70°C for 5-6 hr. After completion of reaction, cool the mixture and pour into ice-cold water. Filter the solid product, wash with water, and dry. The crude product was purified by recrystallization from methanol to yield pure 2-amino-1-(10H-phenothiazin-10-yl) ethan-1-one.

2-amino-1-(10H-phenothiazin-10-yl)ethan-1-one (PJ-B): Light Yellow Powder; MP: 120-125°C; Yield: 68.42%; Mass : 256.32 g/mol ; FTIR (ATR, λ cm⁻¹): 3392.51 (N-H stretching), 1665.12 (Amide C=O Stretching), 1318.71 (Aromatic C=C Stretching), 3100.70 (Aromatic C-H Stretching), 2870.50 (Aromatic C-H Stretching), 1318.71 (Aryl amine C-N stretching), 1007.35 (Sulfide C-S Stretching); ¹H NMR (500 MHz, Chloroform-d) δ 7.63 (ddd, J = 6.9, 2.2, 1.4 Hz, 2H), 7.48 (dd, J = 7.2, 1.8 Hz, 2H), 7.41 (ddd, J = 7.9, 6.8, 1.8 Hz, 2H), 7.32 - 7.25 (m, 2H), 4.23 (t, J = 6.6 Hz, 2H), 3.79 (t, J = 6.7 Hz, 2H). ¹³C NMR (125 MHz, Common NMR Solvents) δ 167.84, 138.81, 138.57, 128.58, 127.83, 127.81, 125.78, 125.69, 124.81, 122.01, 121.98, 43.52.

Step III. Synthesis of Phenothiazine analogues (PJ-1 to PJ-8)

The final analogues were synthesized via aldol condensation reaction. Equimolar amounts of the intermediate PJ-B and

various substituted aldehydes were dissolved in ethanol. Dissolve PJ-B and aldehyde in 20 mL absolute ethanol in a round-bottom flask. Add 2.0 g anhydrous K₂CO₃ to absorb water. Add 2-3 drops glacial acetic acid as catalyst. Stir at room temperature for 2-3 hr. then heat to reflux on 60-80°C. Filter to remove drying agent, evaporate solvent under reduced pressure. Recrystallize crude from hot ethanol. Dry under vacuum or suction. Reaction progress monitored by TLC, and final structures confirmed by melting point determination and spectroscopic Analysis Analogues Shown in Figure 2.

(E)-2-((4-hydroxy-3-methoxybenzylidene) amino)-1-(10H-phenothiazin-10-yl) ethan-1-one (PJ-1)

Yellow Powder; MP: 125-145°C. Yield: 40%. Mass : 390.45g/mol; FTIR (ATR, λ cm⁻¹): 3402.52 (Phenol O-H Stretch), 3088.84 (Aromatic ring C-H Stretch), 2910.09 (Aliphatic Chain C-H Stretch), 1688.52 (Amide C=O Stretch), 1620.28 (Imine C=N Stretch), 1522.94 (C=C Stretch), 1166.82 (Ether (Methoxy) C-O Stretch), 1065.80 (C-S Stretch) ¹H NMR (500 MHz, Chloroform-*d*) δ 8.51 (t, *J* = 1.5 Hz, 1H), 7.69 (s, 1H), 7.62 (ddd, *J* = 6.9, 3.4, 1.5 Hz, 2H), 7.48 (dd, *J* = 7.2, 1.8 Hz, 2H), 7.41 (ddd, *J* = 7.9, 6.8, 1.8 Hz, 2H), 7.32 - 7.26 (m, 2H), 7.29 - 7.21 (m, 2H), 6.87 (d, *J* = 8.7 Hz, 1H), 4.56 (d, *J* = 1.3 Hz, 2H), 3.86 (s, 2H). ¹³C NMR (125 MHz, Common NMR Solvents) δ 167.11, 163.35, 151.53, 148.79, 138.13, 138.03, 128.61, 127.86, 125.78, 125.70, 124.81, 123.52, 122.18, 122.14, 114.61, 111.66, 61.54, 56.26.

(E)-2-((3-hydroxy-4-methoxybenzylidene) amino)-1-(10H-phenothiazin-10-yl) ethan-1-one PJ-2:

Yellow Powder; MP: 121-130°C. Yield: 52.63%. Mass : 390.45g/mol; FTIR (ATR, λ cm⁻¹): 3498.90 (Phenol O-H Stretch), 3089.76 (Aromatic ring C-H Stretch), 2857.20 (Aliphatic Chain C-H Stretch (CH₂, CH₃)), 1695.94 (Amide C=O Stretch), 1612.21 (Imine C=N Stretch), 1571.23 (C=C Stretch), 1078.98 (Ether (Methoxy) C-O Stretch), 1045.24 (C-S Stretch), 1235.98 (C-N Stretch): ¹H NMR (500 MHz, Chloroform-*d*) δ 8.48 (t, *J* = 1.3 Hz, 0H), 7.62 (ddd, *J* = 6.9, 3.3, 1.5 Hz, 1H), 7.48 (dd, *J* = 7.2, 1.8 Hz, 1H), 7.41 (ddd, *J* = 7.9, 6.8, 1.8 Hz, 1H), 7.32 - 7.23 (m, 2H), 6.97 (d, *J* = 8.4 Hz, 1H), 5.57 (s, 0H), 4.56 (d, *J* = 1.3 Hz, 1H), 3.83 (s, 1H). ¹³C NMR (125 MHz, Common NMR Solvents) δ 167.11, 163.18, 150.45, 147.92, 138.13, 138.03, 130.88, 128.61, 127.86, 125.78, 125.70, 124.81, 122.18, 122.14, 121.78, 114.66, 112.89, 61.54, 56.21.

(E)-2-((4-methoxybenzylidene) amino)-1-(10H-phenothiazin-10-yl) ethan-1-one PJ-3:

Yellow Powder; MP: 130-145°C. Yield: 34.24%. Mass : 374.46g/mol; FTIR (ATR, λ cm⁻¹): 3090.24 (Aromatic ring C-H Stretch), 2977.73 (Aliphatic Chain C-H Stretch (OCH₃)), 1646.87 (Amide C=O Stretch), 1621.12 (Imine C=N Stretch), 1480.25 (Aromatic C=C Stretch), 1249.50 (Ether C-O Stretch), 1346.20 (C-N Stretch), 1071.09 (C-S Stretch) ¹H NMR (500 MHz, Chloroform-*d*)

δ 8.53 (t, *J* = 1.2 Hz, 0H), 7.61 (ddt, *J* = 9.0, 4.4, 1.6 Hz, 2H), 7.48 (dd, *J* = 7.2, 1.8 Hz, 1H), 7.41 (ddd, *J* = 7.9, 6.8, 1.8 Hz, 1H), 7.32 - 7.25 (m, 1H), 6.95 - 6.89 (m, 1H), 4.56 (d, *J* = 1.3 Hz, 1H), 3.80 (s, 1H). ¹³C NMR (125 MHz, Common NMR Solvents) δ 167.11, 163.30, 161.46, 138.13, 138.03, 130.34, 128.61, 128.01, 127.86, 125.78, 125.70, 124.81, 122.18, 122.14, 113.79, 61.47, 55.35.

(E)-2-((2-chlorobenzylidene) amino)-1-(10H-phenothiazin-10-yl) ethan-1-one PJ-4:

Yellow Powder; MP: 118-121°C. Yield: 40.81%. Mass : 378.87g/mol; FTIR (ATR, λ cm⁻¹): 3090.21 (Aromatic Ring C-H Stretch), 2937.23 (Aliphatic Chain C-H Stretch), 1656.76 (Amide C=O Stretch), 1630.21 (Imine C=N stretch), 1583.20 (Aromatic ring C=C Stretch), 1270.03 (Aromatic C-N Stretch), 1053.92 (C-S Stretch), 739.42 (Aromatic C-Cl Stretch); ¹H NMR (500 MHz, Chloroform-*d*) δ 8.62 (t, *J* = 1.3 Hz, 1H), 7.69 - 7.63 (m, 1H), 7.62 (ddd, *J* = 6.9, 3.3, 1.5 Hz, 2H), 7.48 (dd, *J* = 7.2, 1.8 Hz, 2H), 7.45 - 7.38 (m, 4H), 7.37 - 7.25 (m, 3H), 4.57 (d, *J* = 1.3 Hz, 2H). ¹³C NMR (125 MHz, Common NMR Solvents) δ 167.11, 163.06, 138.13, 138.03, 136.32, 134.18, 132.14, 129.82, 129.40, 128.80, 128.61, 127.86, 125.78, 125.70, 124.81, 122.18, 122.14, 61.48.

(E)-2-(benzylideneamino)-1-(10H-phenothiazin-10-yl) ethan-1-one PJ-5:

Yellow Powder; MP: 120-140°C. Yield: 61.53%. Mass : 344.43g/mol; FTIR (ATR, λ cm⁻¹): 3054.30 (Aromatic Ring C-H Stretch), 2979.99 (Aliphatic Chain C-H Stretch), 1510.22 (Amide C=O Stretch), 1650.66 (Imine C=N stretch), 1599.96 (Aromatic ring C=C Stretch), 1324.43 (Aromatic C-N Stretch), 1077.66 (C-S Stretch); ¹H NMR (500 MHz, Chloroform-*d*) δ 8.49 (t, *J* = 1.4 Hz, 0H), 7.77 - 7.70 (m, 1H), 7.62 (ddd, *J* = 6.9, 3.3, 1.5 Hz, 1H), 7.48 (dd, *J* = 7.2, 1.8 Hz, 1H), 7.45 - 7.35 (m, 3H), 7.31 - 7.25 (m, 1H), 4.56 (d, *J* = 1.3 Hz, 1H). ¹³C NMR (125 MHz, Common NMR Solvents) δ 167.11, 163.19, 138.13, 138.03, 134.03, 130.66, 128.71, 128.64, 128.61, 127.86, 125.78, 125.70, 124.81, 122.18, 122.14, 61.48.

(E)-2-((2-hydroxybenzylidene) amino)-1-(10H-phenothiazin-10-yl) ethan-1-one PJ-6:

Yellow Powder; MP: 115-120°C. Yield: 46.60%. Mass : 360.43 g/mol; FTIR (ATR, λ cm⁻¹): 3390.35 (Phenol O-H Stretch), 3078.89 (Aromatic ring C-H Stretch), 2887.80 (Aliphatic C-H Stretch), 1615.20 (Imine C=N Stretch), 1636.93 (Amide C=O Stretch), 1476.35 (Aromatic Ring C=C Stretch), 1277.73 (Aliphatic C-N Stretch), 1077.11 (C-S Stretch); ¹H NMR (500 MHz, Chloroform-*d*) δ 8.59 (t, *J* = 1.3 Hz, 1H), 7.62 (ddd, *J* = 6.9, 3.3, 1.5 Hz, 1H), 7.48 (dd, *J* = 7.2, 1.8 Hz, 1H), 7.41 (ddd, *J* = 7.9, 6.8, 1.8 Hz, 1H), 7.33 - 7.25 (m, 2H), 6.85 (dt, *J* = 8.1, 1.0 Hz, 1H), 6.73 (td, *J* = 7.4, 1.5 Hz, 1H), 4.57 (d, *J* = 1.3 Hz, 1H). ¹³C NMR (125 MHz, Common NMR Solvents) δ 167.10, 163.78, 161.96, 138.13, 138.03, 132.75, 131.64, 128.61, 127.86, 125.78, 125.70, 124.81, 122.18, 122.14, 119.22, 118.82, 117.23, 61.20.

(E)-2-((2-nitrobenzylidene) amino)-1-(10H-phenothiazin-10-yl) ethan-1-one PJ-7:

Yellow Powder; MP: 155-160°C. Yield: 46.05%. Mass : 389.43g/mol ; FTIR (ATR, λ cm⁻¹):3078.89 (Aromatic ring C-H Stretch), 2950.29(Aliphatic C-H Stretch), 1655.21(Imine C=N Stretch), 1635.45(Amide C=O Stretch),1480.84(Aromatic Ring C=C Stretch), 1370.99(Nitro group NO₂), 1294.95(Aliphatic C-N Stretch), 1066.34(C-S Stretch); ¹H NMR (500 MHz, Chloroform-*d*) δ 9.07 - 9.03 (m, 1H), 8.05 (dd, *J* = 8.7, 1.5 Hz, 1H), 7.88 (dd, *J* = 6.9, 1.6 Hz, 1H), 7.71 (ddd, *J* = 8.2, 6.8, 1.5 Hz, 1H), 7.68 - 7.59 (m, 2H), 7.48 (dd, *J* = 7.2, 1.8 Hz, 1H), 7.41 (ddd, *J* = 7.9, 6.8, 1.8 Hz, 1H), 7.32 - 7.25 (m, 1H), 4.57 (d, *J* = 1.3 Hz, 1H). ¹³C NMR (125 MHz, Common NMR Solvents) δ 167.18, 161.54, 148.98, 138.13, 138.03, 132.14, 130.81, 130.41, 128.79, 128.61, 127.86, 125.78, 125.70, 124.81, 124.74, 122.18, 122.14, 60.84.

(E)-2-((4-(dimethylamino) benzylidene) amino)-1-(10H-phenothiazin-10-yl) ethan-1-one PJ-8:

Yellow Powder; MP: 130-145°C. Yield: 60%. Mass : 387.53 g/mol ; FTIR (ATR, λ cm⁻¹):3029.76 (Aromatic ring C-H Stretch), 2955.61(Aliphatic C-H Stretch), 1655.21(Imine C=N Stretch), 1660.45(Amide C=O Stretch), 1452.35(Aromatic Ring C=C Stretch), 2955.61(Aliphatic Alkane C-H Stretch (CH₃)), 1270.12(Aliphatic C-N Stretch), 1069.33(C-S Stretch), 744.05(C-H Stretch (out of plane)); ¹H NMR (500 MHz, Chloroform-*d*) δ 8.53 (t, *J* = 1.3 Hz, 0H), 7.65 - 7.54 (m, 2H), 7.48 (dd, *J* = 7.2, 1.8 Hz, 1H), 7.41 (ddd, *J* = 7.9, 6.8, 1.8 Hz, 1H), 7.32 - 7.25 (m, 1H), 6.75 - 6.69 (m, 1H), 4.56 (d, *J* = 1.3 Hz, 1H), 2.92 (s, 2H). ¹³C NMR (125 MHz, Common NMR Solvents) δ 167.11, 163.40, 152.08, 138.13, 138.03, 129.73, 128.61, 127.86, 126.63, 125.78, 125.70, 124.81, 122.18, 122.14, 111.34, 61.48, 40.31.

Biological Evaluation

Carragenan-induced paw oedema method

Wistar albino rats (150-180 g) were procured from National Lacsmi BioFarm, Aundh Pune CCSEA Reg. Number: 1277/ PO/ RcBt/S/09/CCSEA and maintained under standard laboratory conditions. All animal procedures were performed in accordance with the Institutional Animal Ethics Committee (IAEC) (CCSEA registration 1942/PO/Re/S/17/CCSEA) guidelines, under protocol number PRCOP/CCSEA/IAEC/2025-26/01F.

Six groups (*n*=6) of Wistar albino rats (150-180 g), including vehicle control (0.1% DMSO), standard (Diclofenac Sodium, 25 mg/kg), and test groups receiving the synthesized analogues (Morris, 2003). Inflammation was induced by a subplantar inj. of 0.1 mL of 1% carrageenan solution into right paw after 1 hr of administration of analogues (Morris, 2003). Paw swelling was measured using a Mitutoyo Digital Vernier Caliper 0-150 mm 500-196 at 0, 30, 60, 90, 120, and 150 min (Morris, 2003). The percentage inhibition of edema was calculated relative to the control group.

$$\text{Percentage Inhibition} = \frac{V_c - V_t}{V_c} \times 100$$

Where:

V_c = increase in inflammation of the control.

V_t = increase in inflammation of the treated group.

In silico analysis

Drug-likeness and ADMET prediction

Physicochemical properties, and pharmacokinetic parameters (ADME) were predicted using the SwissADME tool (www.swissadme.ch) (Bakchi *et al.*, 2022). Compliance with Lipinski's and Veber's rules was assessed to evaluate oral bioavailability potential.

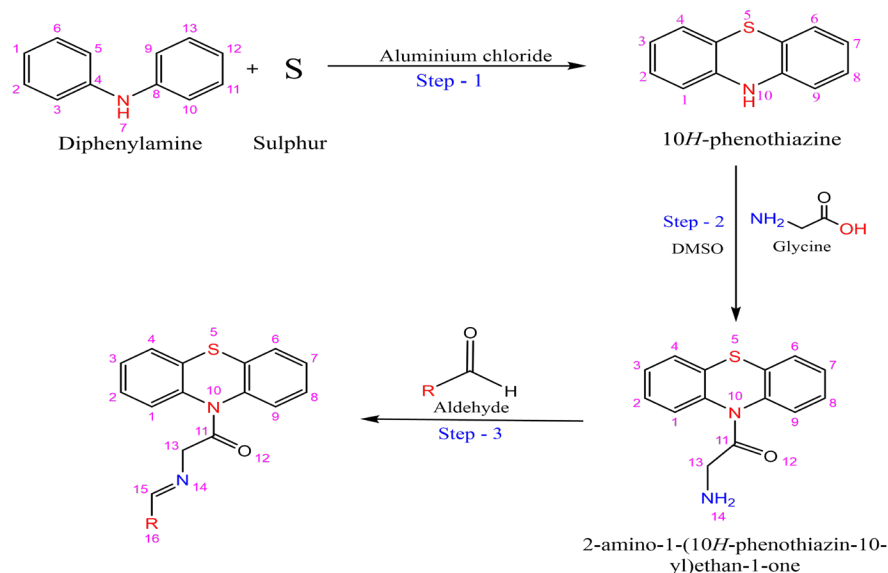


Figure 1: Scheme for synthesis of Analogues PJ-1 to PJ-8.

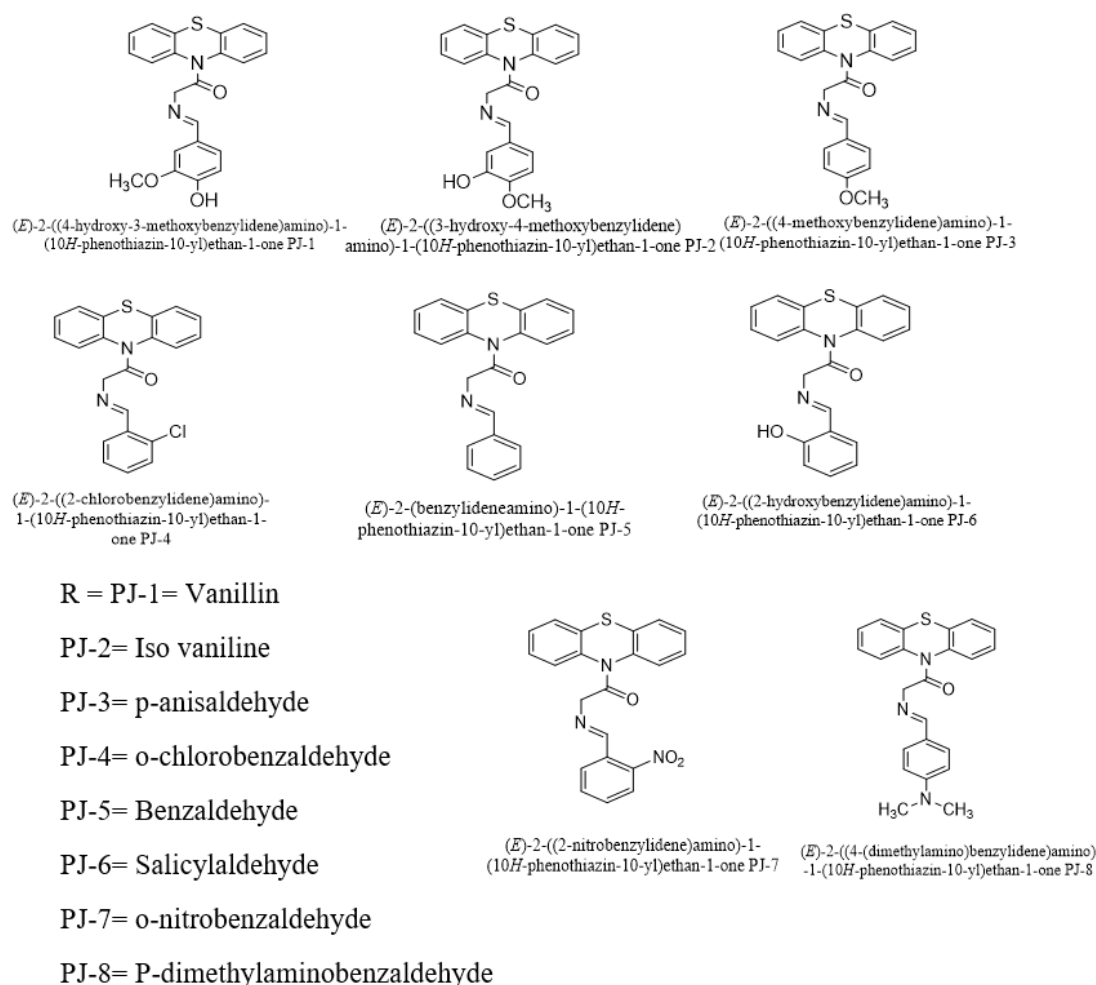


Figure 2: Analogues of Phenothiazine.

Table 1: Molecular Docking Finding of Analogues on 4PH9.

Molecules	Mol. Docking Score
Standard	-7.0
PJ-1	-8.4
PJ-2	-8.5
PJ-3	-8.8
PJ-4	-8.0
PJ-5	-8.6
PJ-6	-8.7
PJ-7	-7.9
PJ-8	-8.9

The ProTox-III (<https://tox.charit e.de/protox3>) web server was utilized to predict acute oral toxicity (LD_{50}), hepatotoxicity, and carcinogenicity of the synthesized analogues (Kumar and Priya, 2020).

Molecular Docking Studies

The docking studies were generated using AutoDeck PyRx and Bio-via Discovery Studio 2021 client for our studies. The target

X-ray structure of COX-2 as receptors (PDB ID: 4PH9) (Khan *et al.*, 2024) respectively were taken from Protein Data Bank (PDB) website (<https://www.rcsb.or g/>). The ligand and the protein convert to the PDBQT format by using PyRx, the water molecules and the heteroatoms were removed. The docking was carried out by using Biovia Discovery Studio 2021 client software and we found specific and non-specific interactions through the link. Binding energy (kcal/mol), bond length and type of interactions between ligand and protein. All prepared compounds were studied for their molecular docking using the PyRx and Biovia Discovery Studio 2021 client) program against targets (Abbas *et al.*, 2026). Molecular docking revealed strong binding affinities for COX-2, with PJ-3 and PJ-8 showing the highest anti-inflammatory potential (-8.8 and -8.9 docking score). Cyclooxygenase-2 SER530, VAL523, MET522, PHE518, TYR385, ALA527, LEU359, ARG120 and TYR355, were considered the most accurate active region. Site map was carried out to find out best possible binding site. The pose of lowest binding energy and zero Å root-mean-square deviation RMSD) was considered the fittest and most complex with the receptor for analysis. The molecular interactions and binding modes of the top poses were visually examined using BIOVIA Discovery Studio 2024 (Version

2024, The 3DEXPERIENCE Company). The resulting poses analyzed for binding affinity (docking score) and key molecular interactions (Table 2 and Figures 3-6; Yadav *et al.*, 2021).

RESULTS

Pharmacological Evaluations

In vivo Anti-inflammatory Activity

DISCUSSION

This study successfully designed, synthesized, and evaluated eight novel Phenothiazine analogues (PJ-1 to PJ-8) as potential anti-inflammatory. The results underscore the versatility of the Phenothiazine scaffold and its capacity to yield derivatives with promising therapeutic profiles, as evidenced by their compliance with drug-likeness criteria, favorable ADMET profiles, and

significant biological activities. The Phenothiazine analogues were synthesized in a three-step sequence of cyclization, N-acylation, and condensation to provide compounds with varied substituents. The synthetic yields were from % 40% (PJ-1) to 60% (PJ-8), reflecting strong feasibility in synthesis, while the melting points and TLC established purity and compound identity. Spectroscopic studies (FTIR, ^1H NMR, and ^{13}C NMR) confirmed the structural integrity of every analogue (Salakolusu *et al.*, 2024), with diagnostic peaks like N-H stretching ($3340\text{--}3400\text{ cm}^{-1}$) and aromatic C-H stretching ($3000\text{--}3100\text{ cm}^{-1}$) establishing the Phenothiazine core, whereas substituent-specific peaks. These results corroborate earlier research on Phenothiazine derivatives that highlights *in silico* analyses demonstrated that all analogues complied with Lipinski's and Veber's rules, indicating excellent oral bioavailability potential (Jia *et al.* 2020). Molecular weights (344.43 g/mol – 390.45 g/mol), consensus LogP values ($3.40\text{--}4.73$),

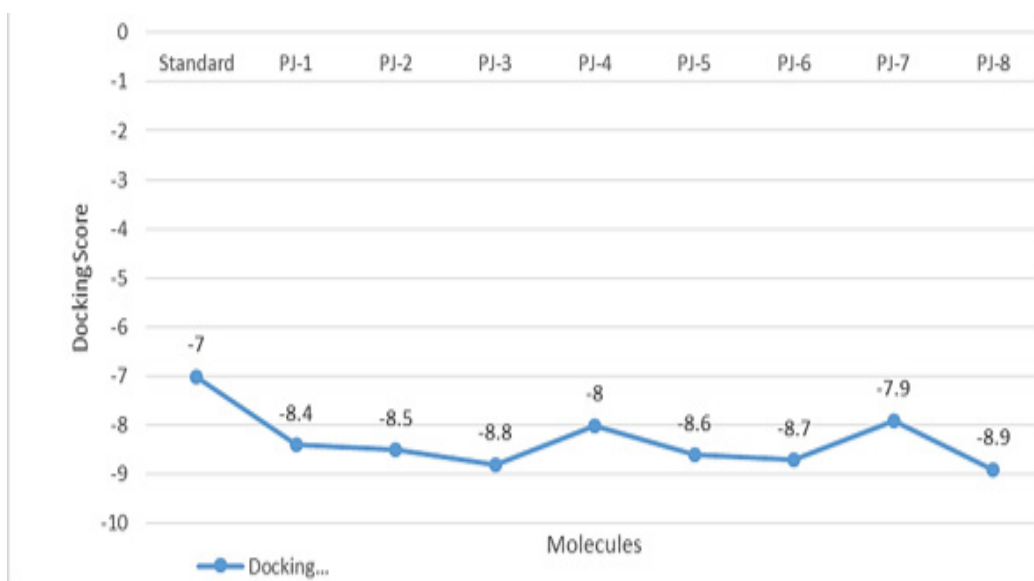


Figure 3: *In silico* Molecular Docking on 4PH9.

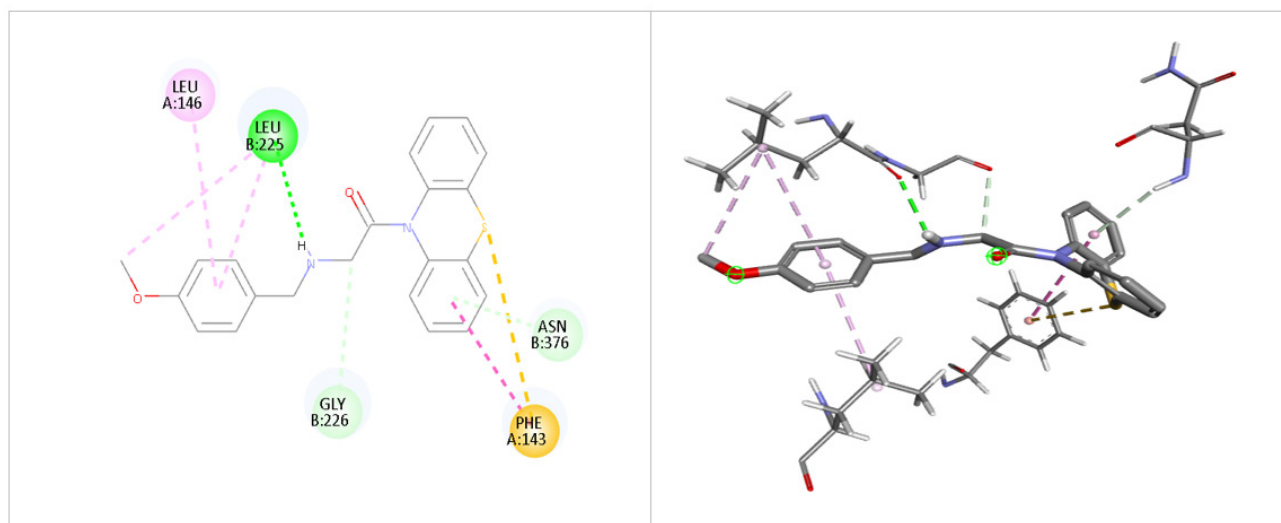


Figure 4: Binding surface and ligand interaction diagram of PJ-3 on 4PH9.

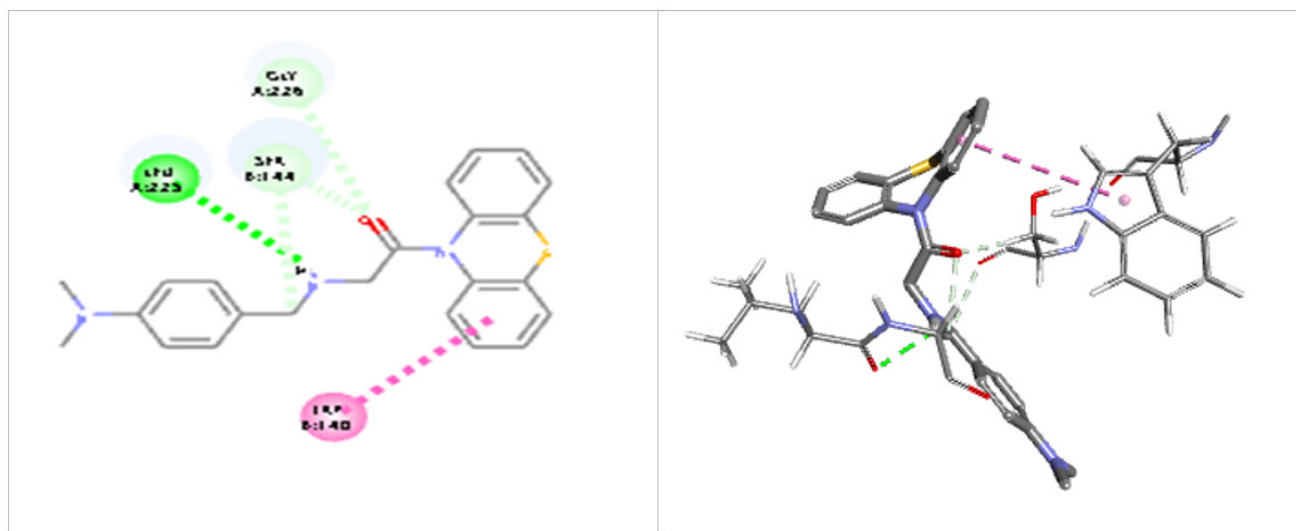


Figure 5: Binding surface and ligand interaction diagram of PJ-8 on 4PH9.

Table 2: Effect of analogues (PJ-1 to PJ-8) standard (Diclofenac) on inflammation.

Interval (min)	Control	Standard	PJ1	PJ2	PJ3
0	5.68±0.03	5.73±0.10	5.44±0.06	5.50±0.05	5.70±0.08
30	6.11±0.13	6.18±0.11	5.77±0.00	5.73±0.06	5.81±0.08
60	6.38±0.20	6.55±0.05	6.00±0.00	6.00±0.09	6.11±0.14
90	6.76±0.21	6.61±0.12	6.30±0.00	6.33±0.11	6.41±0.07
120	7.00±0.22	6.28±0.16**	6.50±0.00	6.15±0.08***	6.48±0.11
150	7.41±0.19	5.98±0.08***	6.30±0.00*	5.70±0.08***	6.26±0.20***
PJ4	PJ5	PJ6	PJ7	PJ8	
5.55±0.07	5.78±0.13	5.63±0.03	5.60±0.05	5.48±0.04	
5.85±0.09	6.15±0.16	5.91±0.01	5.88±0.07	5.78±0.07	
6.16±0.14	6.38±0.15	6.40±0.01	6.20±0.10	6.15±0.09	
6.45±0.14	6.71±0.10	6.60±0.09	6.58±0.09	6.60±0.07	
6.15±0.08***	6.30±0.16**	6.35±0.20**	6.21±0.08***	6.23±0.12***	
5.70±0.08***	5.91±0.15***	5.86±0.14***	5.71±0.07***	5.61±0.11***	

and low hydrogen bond donor/acceptor counts suggest favorable pharmacokinetic properties. The ADMET profiles further revealed high gastrointestinal absorption and BBB permeability, suggesting potential applications in systemic and neuroinflammatory conditions (Jia *et al.* 2020). Notably, the lack of CYP2D6 and CYP3A4 inhibition minimizes the risk of drug-drug interactions, a significant advantage over existing NSAIDs. However, the predicted hepatotoxicity for P J-1 and PJ-8 warrants further experimental validation, as computational prediction The synthesized Phenothiazine analogues demonstrated high-affinity interactions with the COX-2 active site, with docking scores ranging from -7.9 to -8.9. Notably, binding parameters compared to the standard reference compound. Molecular interactions were characterized by conserved hydrogen bonding with SER530 and VAL523, alongside extensive hydrophobic contacts including Pi-Pi Stacked, Conventional Hydrogen Bond, Pi-Alkyl involving VAL370, CYS437, ALA306. The frequent occurrence of Pi-Sulfur

and hydrophobic stabilization across analogues indicates favorable spatial orientation and potential for selective COX-2 inhibition. Docking scores are summarized in Table 1.

In vivo anti-inflammatory evaluations, PJ8 displayed a pharmacodynamic profile comparable to the standard (Diclofenac) at early intervals, suggesting a rapid onset of action (Azim *et al.*, 2021) PJ4 and PJ6 showed limited efficacy, potentially due to suboptimal structural features affecting target interaction (Azim *et al.*, 2021). These findings underscore the influence of molecular modifications on biological response and highlight PJ3 and PJ8 as promising candidates. This aligns with docking predictions, as compounds with higher COX-2 binding affinities exhibited superior anti-inflammatory effects, validating the computational approach.²⁶ The absence of adverse effects or mortality in acute toxicity studies ($LD_{50} > 2000$ mg/kg) further supports the safety profile of these analogues, addressing a key

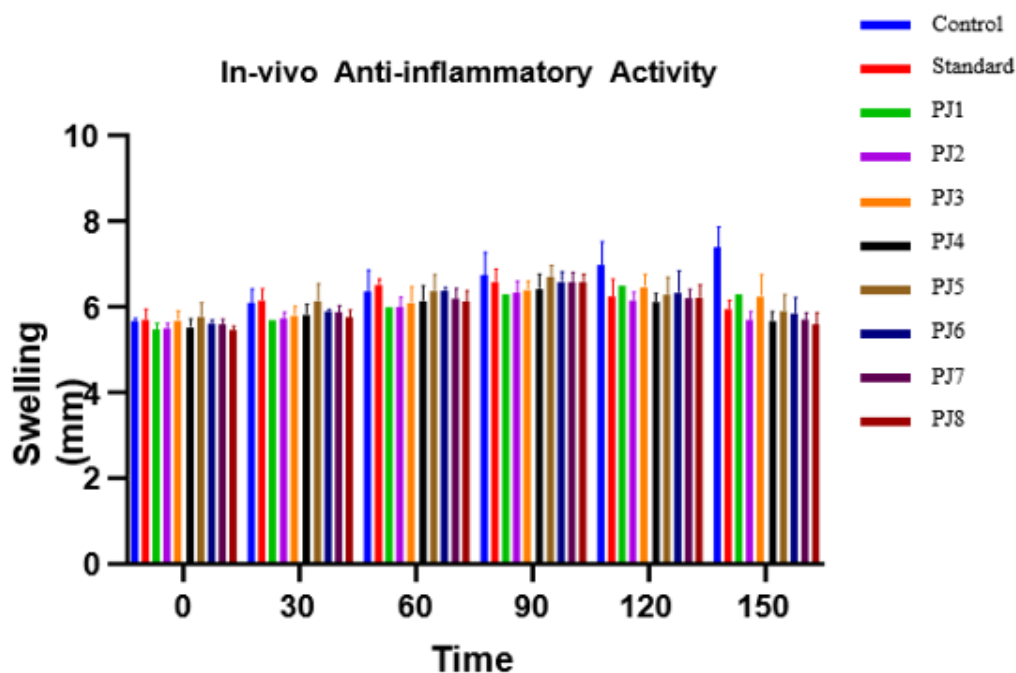


Figure 6: *In vivo* anti-inflammatory activity PJ-1 to PJ-8 Compared to Standard.

limitation of NSAIDs, which are associated with gastrointestinal and cardiovascular toxicities (Azim *et al.*, 2021).

While a comprehensive Structure-Activity Relationship (SAR) analysis is precluded by the limited number of analogues in this initial series, preliminary structure-activity observations can be noted. Appear to be favorable for biological activity, potentially by facilitating the hydrogen bonding and hydrophobic inter-actions observed in the docking models (Lea *et al.*, 2022).

CONCLUSION

This research successfully synthesized and evaluated eight novel Phenothiazine analogues, identifying promising action lead compounds: (E)-2-((4-methoxybenzylidene)amino)-1-(10H-phenothiazin-10-yl) ethan-1-one (PJ-3) and (E)-2-((4-(dimethylamino)benzylidene)amino)-1-(10H-phenothiazin-10-yl)ethan-1-one (PJ-8). Key findings confirmed their potential: 1. Anti-inflammatory Activity: Both PJ-3 and PJ-8 exhibited superior *in silico* binding to COX-2 (Docking scores: -8.8 and -8.9, respectively) than the standard drug Diclofenac (-7.0). This was validated *in vivo*, where both compounds produced significant ($p < 0.001$) inhibition of carrageenan-induced paw edema, comparable to the standard. 2. Drug-Likeness: All synthesized analogues showed favorable *in silico* ADMET profiles and complied with Lipinski's rules, indicating high potential for oral bioavailability.

ABBREVIATIONS

PTZ: Phenothiazine; **NSAIDs:** Non-Steroidal Anti-Inflammatory Drugs; **COX-2:** Cyclooxygenase-2; **FTIR:** Fourier-Transform Infrared Spectroscopy; **NMR:** Nuclear Magnetic Resonance; **ADMET:** Absorption, Distribution, Metabolism, Excretion, and Toxicity; **TLC:** Thin-Layer Chromatography; **MP:** Melting Point; **IAEC:** Institutional Animal Ethics Committee; **CCSEA:** Committee for Control and Supervision of Experiments on Animals; **SAR:** Structure-Activity Relationship; **BBB:** Blood-Brain Barrier; **LD₅₀:** Median Lethal Dose; **DMSO:** Dimethyl Sulfoxide; **RMSD:** Root-Mean-Square Deviation; **PDB:** Protein Data Bank.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ETHICAL APPROVAL

All animal procedures were performed in accordance with the Institutional Animal Ethics Committee (IAEC) (CCSEA registration 1942/PO/Re/S/17/CCSEA) guidelines, under protocol number PRCOP/CCSEA/IAEC/2025-26/01F).

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