Chapter-4

Experimental

4.1. Computational Studies

4.1.1. Molecular docking study

All *in silico* studies were performed using the Schrödinger Maestro 2018.1 glide XP software. The protein data bank (PDB) has been initially utilized to obtain the protein structures of COX-2 (PDB ID: 3LN1) and 5-LOX (PDB ID: 3V99/6N2W) for the purpose of the structure-based drug design approach. The protein error was addressed with the protein preparation wizard tool. Energy minimizations and structural refinements were conducted using the OPLS force field. Subsequently, grids were generated around the active sites of the co-crystallized ligands within the crystal structures using the protein grid generation module. Protein grid generation represents the active binding domain by maintaining its co-crystallized moiety in the central part of a rectangular-shaped box within the enzyme. A 20 Å grid space was described for co-crystallized ligand/moiety using the software's Glide grid module. The grids were validated by extracting and re-docking the co-crystallized ligands into the respective generated grids of COX-2 and 5-LOX. The LigPrep module is programmed to generate low-energy conformers of all ligands. Ligands were kept flexible, and the docking study was conducted using the 'Glide XP' module and the 'Glide XP visualizer' tool utilized to obtain docking results and discover ligand interactions with active site residues.

The virtual screening was conducted using Glide's structure-based virtual screening protocol. This method identified the compounds based on their interactions with the targeted active site. The screening process involves three hierarchical steps: high throughput virtual screening (HTVS), standard precision (SP) docking, and extra precision (XP) docking. During the screening, a 30% filter criterion was applied at each step of HTVS, SP, and XP docking to identify potential leads. The screened compound

poses were then analyzed using the docking post-processing and pose filtration protocols in Maestro.

4.1.2. Molecular dynamics simulation study

Using the Desmond module, molecular dynamics simulations validated the stability of the protein-ligand docked complex. The system builder tool was initially employed to build a system creating a virtual TI3P water environment and neutralizing it by the addition of counter atoms. The minimization tool was set at a convergence threshold of 1.0 kcal/mol/Å with a maximum of 2,000 iterations. Subsequently, the docked complexes underwent a 100-nanosecond MD simulation to capture their dynamic behaviour and assess stability under physiological-like conditions. During this simulation, the energy threshold was maintained at 9.6 to ensure stability, and the trajectory was recorded at 100ns intervals, yielding 1000 frames over the simulation period. These frames provided a detailed record of conformational changes and interactions. Post-simulation analysis, including RMSD, RMSF, hydrogen bond analysis, and secondary structure evaluation, enabled a comprehensive assessment of the complex's dynamic stability and interaction profile. This workflow ensured a robust and reliable evaluation of the protein-ligand complex's stability in a simulated environment.

4.1.3. Drug-likeliness determination by Qikprop module

The drug-likeliness for the most potent derivative and the conventional drugs were calculated using the Qikprop Software module of Schrödinger Maestro 2018.1. The prediction ensured that the compound abided by Lipinski's rule along with prediction of other important parameters including Log P estimation. The experimental Log P values in the range of ≤5 and other molecular properties predict if a novel compound with certain pharmacological potential would consider an orally active drug, vital in achieving a good bioavailability in humans.

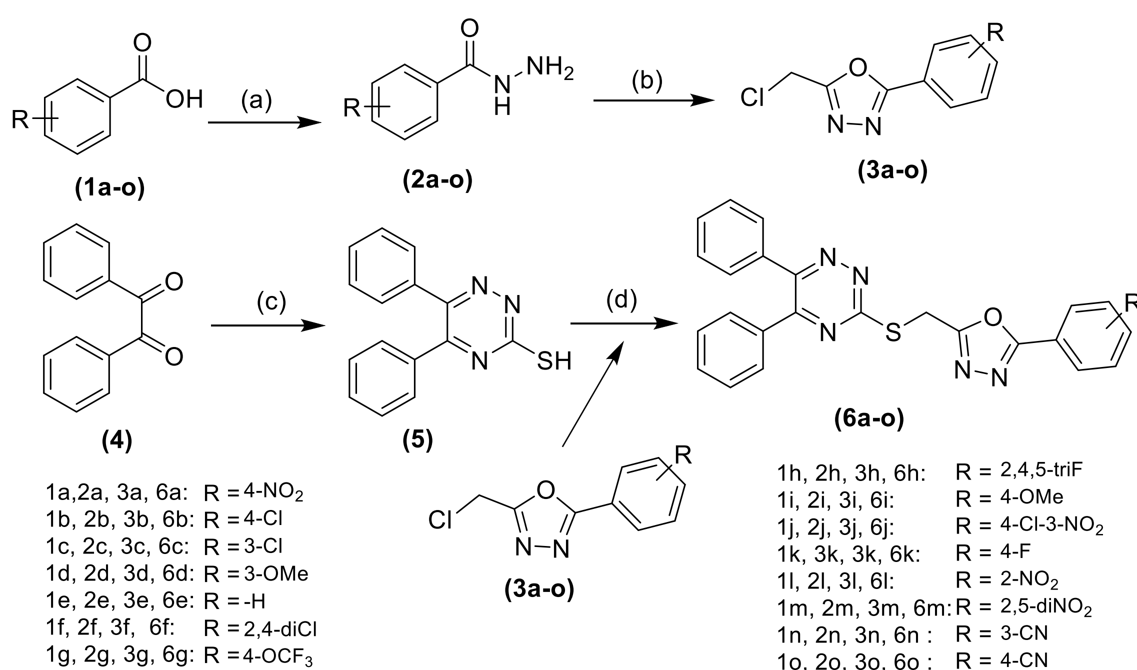
4.2. Synthesis of Novel Compounds

4.2.1. Chemicals and reagents

All chemicals and reagents used in the study were sourced from reliable suppliers to ensure consistency and quality. These included Sigma-Aldrich (India), Cayman Chemicals (USA), and Avra Synthesis (India), each renowned for providing high-purity products suitable for research applications. Unless explicitly stated otherwise, the chemicals and reagents were used as received without any additional purification.

4.2.2. Synthesis of novel compounds series I

The designed novel series of 5,6-diphenyl-1,2,4-triazine-3-thiol derivatives **6a-o** were synthesized in sequential steps mentioned in Scheme 1.



Scheme 1. Reagents and conditions (a) HOBT, EDC, Acetonitrile (ACN), hydrazine hydrate, stir for 2-4 h, 0-5°C to RT, 75-80%; (b) Chloroacetic acid, $POCl_3$, reflux, 4-6 h, 85-85%; (c) Glacial acetic acid, thiosemicarbazide, reflux, 5-6 h, 80-85% (d) DMF, KOH, RT, stirring, 4 h, 69-78%

4.2.2.1. General procedure of the synthesis of Intermediates (2, 3, 5)

Synthesis of benzhydrazides derivative (2a-o)

Benzoic acid derivatives (1 Mol. Eq.) were taken and dissolved in acetonitrile at room temperature. Then HOBt (1.2 Mol. Eq.) was added to the above mixture, followed the adding of the small amounts of EDC.HCl (1.2 Mol. Eq.) with stirring. The reaction lasted 2 hours at room temperature.TLC validated the progression of every synthesis using hexane/ethyl acetate as a mobile phase in a 7:3 approximate ratio. After the reaction was completed, it was kept as such (reaction mixture 1), and in another RBF hydrazine hydrate (2 Mol. Eq.) dissolved in a small amount of acetonitrile & maintained its internal temperature at 0-10°C (reaction mixture 2). Then reaction mixture 1 was dropwise introduced into reaction solution 2 in cold conditions. The precipitate was dissolved in solvent ethyl acetate before being treated with a sodium bicarbonate solution. Pure solid compounds were collected after the evaporation of excessive ethyl acetate in a rotary evaporator.

2-(chloromethyl)-5-phenyl-1,3,4-oxadiazole (3a-o)

The mixture of aryl hydrazide (2a-2n) (1 Mol. Eq.), chloroacetic acid (1.2 Mol. Eq.), and $POCl_3$ (6-7ml) was heated at reflux for around 2-3 h. Under reduced pressure, The remaining POCl3 was extracted, and the residue left was poured over crushed ice. The resulting product was collected, and processed with a solution of saturated sodium bicarbonate and water to collect 2-(chloromethyl)-5-phenyl-1,3,4-oxadiazole (Sharma, Tripathi et al. 2019).

General procedure of the synthesis of 5,6-diphenyl-1,2,4-triazine-3-thiol (5)

5,6-diphenyl-1,2,4-triazine-3-thiol **(5)** was prepared with minor variations to the previously described procedure (Khan, Obaid et al. 2015). Benzil (1 Mol. Eq.) was dissolved in glacial acetic acid and then added to a hot water mixture of thiosemicarbazide

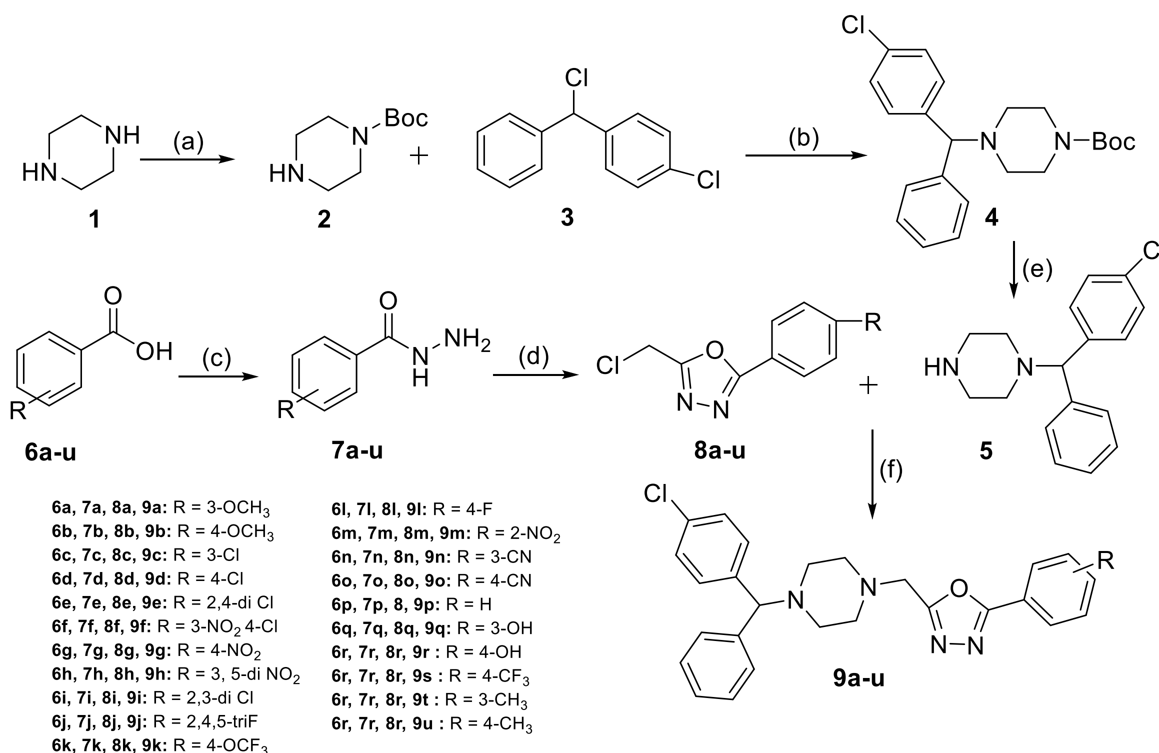
(2.2 Mol. Eq) and further reflux for 4 hours. The precipitate obtained was recrystallized with ethanol to obtain orange-yellow color crystals of the desired compound.

4.2.2.2. General procedure for the synthesis of compounds (6a-o)

2-(((5,6-diphenyl-1,2,4-triazin-3-yl)thio)methyl)-5-phenyl-1,3,4-oxadiazoles *(6a-o)* were synthesized by dissolving *compound 5a-o (1 Mol. Eq.)* in minimum amount of Dimethylformamide (DMF), followed by addition of potassium hydroxide *(1.2 Mol. Eq.)*. Compounds 3a-o were added to the above mixture, and the reaction was continued for 2 hours at room temperature with stirring. The progress of the reaction was monitored through TLC. Upon completion of the reaction, the reaction mixture was added to ice cold water with stirring to get precipitate. Then the obtained precipitate was washed further with water and purified through column chromatography using n-hexane/EtOAc (90:10 v/v) mixture as a mobile phase to obtain pure compounds (6a-o). The final compounds were purified by recrystallization using ethanol as the solvent. This process ensured the removal of impurities, yielding high-purity crystalline products suitable for further analysis and characterization.

4.2.3. Synthesis of novel compound series II

The series of designed novel molecular hybrids (**9a-u**) were synthesized by connecting benzhydrylpiperazine and substituted phenyl oxadiazoles with –CH_2 linker as depicted in Scheme 2.



Scheme 2. Reagents and conditions (a) DCM, Boc_2O, stirring 0-5°C 1-2 h (b) Toluene, stirring 80-90°C; 6-8 h (c) HOBT, EDC, ACN 2-4 h stirring; hydrazine hydrate, 0-5°C to RT, (d) $POCl_3$, chloroacetic acid 4-6 h reflux (e) DCM, CF_3CO_2H, 0°C to RT (f) DMF, KOH, RT, stirring, 4 h

4.2.3.1. General procedure for synthesis of compounds 4

The initial conversion of piperazine to Boc-piperazine occurred in the presence of DCM, with the dropwise addition of di-tert-butyl decarbonate at a temperature maintained between 0 and 5°C. Then, Boc-piperazine was reacted with 1-chloro-4-(chloro(phenyl)methyl)benzene in the presence of toluene (q.s.) at 60-80°C for 6-8 h. After completion of the reaction, the reaction mixture was allowed to cool down and then 50ml of water was added to the solution and the organic layer was separated in workup with EtOAc. The organic layer was washed with a mixture of conc. HCl and water (2×100ml); neutralized with 20% NaOH solution.

4.2.3.2. General procedure for synthesis of compound 8a-u

The respective benzoic acids (**6a-u**) were dissolved in ACN, followed by the addition of 1.2 equivalents of HOBT and EDC. The reaction mixture was stirred for 2 hours at room temperature. The ester formation was confirmed by TLC using EtOAc:Hexane (50:50 v/v). This reaction mixture was then added dropwise into the solution of hydrazine hydrate in ACN at 0-5°C. After the completion of the reaction, the reaction mixture was quenched with about 15-20ml of water and extracted in a workup with EtOAc. The pure compounds (**7a-u**) were obtained by concentrating the organic layer in a vacuum. Compounds (**8a-u**) were obtained by dissolving corresponding benzhydrazides (**7a-u**) and 1.2 equivalent chloroacetic acid in $POCl_3$ (3-4ml). The reaction was monitored by TLC in EtOAc:Hexane (30:70 v/v). The reaction mixture was carefully added to the crushed ice and the precipitate was collected as pure compounds after washing, filtering, and drying.

4.2.3.3. General procedure for synthesis of compound 9a-u

1-((4-chlorophenyl)(phenyl)methyl)piperazine (5) was dissolved in DMF and 1.5 equivalent of potassium hydroxide was added to it. The mixture was stirred under a nitrogen environment for around 30 minutes, followed by the addition of 2 equivalents of respective 2-(chloromethyl)-5-phenyl-1,3,4-oxadiazole derivatives (**8a-u**). The reaction mixture was stirred continuously for 2-4 h at room temperature and the reaction progress was tracked using TLC. After the completion of the reaction, the resulting mixture was carefully poured into the crushed ice. The obtained precipitate was collected and purified by column chromatography (n-hexane/EtOAc). The final products were recrystallized using ethanol.

4.3. Characterization of synthesized derivatives

4.3.1. Melting Point

The melting point indicates the association between a compound's structure and its properties, with various compounds having different melting points. This value is a key measure of compound purity. Melting points were determined using a Stuart Melting Point Apparatus (SMP10, Barloworld Scientific Ltd., UK) using capillary tubes and are presented without correction.

4.3.2. TLC (R_f value)

TLC is an efficient method for qualitatively monitoring the progression of a reaction, as well as determining the purity of the substance. The R_f values were calculated using precoated Merck silica gel 60F254 aluminium sheets (Merck, Germany). TLC plates were visualised using UV light, iodine vapours, and Dragendorff reagent. The Rf value has been determined using the following equation:

$$R_f = \frac{\text{Distance travelled by solute}}{\text{Distance travelled by solvent}}$$

4.3.3. Percentage purity determination using HPLC

The compounds' purity was evaluated using high-performance liquid chromatography (Shimadzu, USA) with a 1 ml/min flowrate of ACN: H2O: TFA (95:5:0.1%) as mobile phase in the C18 column.

4.3.4. FT-IR

The FT-IR spectra of the compounds were captured as percentage Transmittance Vs. Wavenumber (cm-1) at a scanning range of 4000-667 cm−1 on the Alpha ECO-ATR Spectrophotometer (Bruker, USA).

4.3.5. ^1H and ^{13}C NMR

The ^{1}H NMR (500MHz) and ^{13}C NMR (125MHz) spectra of synthesised compounds were obtained on a Bruker (Avance III HD 500 MHz) spectrophotometer, using tetramethylsilane (TMS) as a reference standard in DMSO-d_6 or $CDCl_3$. The spectra were analysed with MestReNova 6.0.2. The splitting patterns were represented as singlet (s), doublet (d), triplet (t), quartet (q), doublet of doublets (dd), triplet of doublets (td), doublet of doublets of doublets (ddd), and multiplet (m).

4.3.6. Mass spectra

The HRMS spectra of the molecules were obtained using an AB Sciex X500R QTOF mass spectrometer integrated with high-performance liquid chromatography (Shimadzu, USA).

4.4. Pharmacological/biological assays

4.4.1. In vitro biological activities

4.4.1.1. COX-1 and COX-2 inhibition activity

The colorimetric inhibitor screening assay method has been employed to identify the COX-1/COX-2 inhibition efficiency of the synthesized derivatives *(Lee, Son et al. 2004, Fretz, Valdenaire et al. 2013)*. All the reagents and enzymes were purchased from Cayman Chemicals, USA. Initially, assay buffer (Item No. 760114) was used to prepare final concentrations of hemin (Item No. 760116) and COX enzymes (Item No. 760110 and 760108). The enzymes COX-1 (400 units/ml) and COX-2 (400 units/ml) were activated and kept on ice when thawed. The substrate arachidonic acid (Item No. 760113) was prepared in a final concentration of 1.1 mM. While performing the assay, 160 µl of assay buffer and 10 µl of hemin were added to the background wells, and 100% initial activity wells were added with 150 µl assay buffer, 10 µl of hemin, and 10 µl either COX-1 or COX-2. The synthesized inhibitors (10 µl) were added in the inhibitors well along with assay buffer, hemin, and enzyme COX-1 or COX-2. All the assay wells were

activated by the addition of 20 µl colorimetric substrate N,N,N´,N´,-tetramethylbenzene-1,4-diamine (TMPD) and 20 µl arachidonic acid to initiate the reaction. The UV-spectrometer was used to measure absorbance at 590 nm. The percent inhibition graphs were plotted to determine the IC_{50} values.

4.4.1.2. 5-LOX inhibition activity

The 5-LOX inhibitor screening assay kit developed by Cayman's Chemical, (catalog # 760700) was utilized to confirm the 5-LOX inhibition ability of the synthesized derivatives (Hsu, HuangFu et al. 2020). The compounds were tested in duplicates as per the manufacturer's protocol. The blank well contained 100 µl assay buffer, while 90 µl enzyme and 10 µl were added to the positive control wells. The inhibitor wells were added with 90 µl enzyme and 10 µl test inhibitor. After 5 minutes of incubation at room temperature, 10 µl AA substrate was added to each well, and the plate was shaken for 10 minutes. Finally, after adding 100 µl of chromogen to each well, the absorbance was determined with a UV spectrophotometer at 490 nm. The IC_{50} values were calculated by plotting percent inhibition graphs.

4.4.1.3. In vitro enzyme kinetics study

The enzyme kinetics assay was performed to determine the type of COX-2 inhibition by compound **9d**. The kinetic parameters K_m and V_{max} were calculated at a fixed concentration of enzyme against six varied concentrations of substrate (5-500 µM). The compound **9d** was examined at three different concentrations (0.03, 0.15, and 0.30 µM), and inhibitor concentration was tested against six varied concentrations of substrate. The experiment was carried out in triplicate. The Lineweaver-Burk technique was executed to determine inhibition kinetics. Furthermore, the relevant Ki value was obtained from a Dixon plot (Waiker, Verma et al. 2023).

4.4.2. In vivo experiments

4.4.2.1. Animals

The healthy Wistar rats of either sex weighing 200-250g were purchased from the Central Animal Facility, Banaras Hindu University. The rats were housed in cages and had unrestricted water and commercial food access. The rats were housed in a climate with controlled humidity and temperature (45-60% RH and 25 ± 2°C) with a 12-hour light/dark cycle. All of the research experiments followed CCSEA specifications and the institutional animal ethical committee authorized the research procedures (Approval no. IIT(BHU)/IAEC/2023/019).

4.4.2.2. Acute oral toxicity

The study was authorized by the Institutional Animal Ethical Committee (IAEC) with approval number, IIT(BHU)/IAEC/2023/019. The acute oral toxicity of the top two compounds from respective series of test compounds was studied in female Wistar rats. The animals had free water access and fasted for the entire night before the study. The drugs were given in gradually increasing dosages varying between 100 to 500 mg/kg after dissolving in 0.3% w/v Na-carboxymethylcellulose (CMC). The animals were kept under observation for any unusual signs and indications such as seizures, lethargy, diarrhea, salivation, drowsiness, etc. for 24 hours and then every day for 14 days. Animals were sacrificed for histopathological evaluation of possible GI, hepatic, cardiovascular, and renal damage (Manju 2020).

4.4.2.3. Carrageenan-induced paw edema model

Acute inflammation was triggered in rats by sub-plantar injection of 0.1 ml of a 1% w/v carrageenan solution in the left hind paw. The control group received the vehicle, while the standard group was given indomethacin in 10mg/kg, p.o. dose, in 0.3 % Na CMC. The test groups of animals were administered with test compounds orally in doses 5mg,

10mg, and 20mg/kg in 0.3 % Na CMC, 1 hour before the administration of carrageenan. The paw volume was measured every hour for up to six hours after the injection of carrageenan, employing a digital vernier caliper (Shrivastava, Srivastava et al. 2017).

4.4.2.4. Effect on prostaglandin E_2 (PGE_2)

The novel compounds were tested for their effect on PGE_2. Rat paw tissues were collected from different groups of carrageenan-induced paw edema model and rinsed in PBS (pH 7.4) to remove excess blood (Abdelrahman, Youssif et al. 2017). The tissues were minced, homogenized, filtered, and centrifuged at 2000rpm for 20 minutes at 4°C. The respective supernatant was collected carefully and analysis was performed as per the protocol mentioned in the ELISA kit (KLR0504) obtained from Krishgen Biosystems, USA.

4.4.2.5.Effects on cytokines level

Rat paw tissue homogenates were prepared from different rat groups in the carrageenan-induced rat paw edema model. These homogenates were filtered and centrifuged for 20 minutes at 2000rpm at 4°C. The supernatant was collected, and IL-6, TNF-α, and IL-10 levels were measured using ELISA kits (Krishgen Biosystems, India) according to the manufacturer's instructions.

4.4.2.6. Arachidonic acid-induced paw edema model

A sub-plantar injection of 0.1ml of 0.5% w/v arachidonic acid in 0.2 M carbonate buffer (pH 8.4) into the hind paw of rats caused significant edema within 5 minutes, which hit a peak in 1h. The control group received 0.3% CMC, 10ml/kg, p.o., while the standard group animals were given zileuton (10mg/kg, p.o.). The other groups administered the test compounds in doses 5mg, 10mg, and 20mg/kg in 0.3 % Na CMC. The digital vernier caliper was used to measure the swelling thickness (mm) 1h after the arachidonic acid injection (DiMartino, Campbell et al. 1987).

4.4.2.7. Ulcerogenic risk assessment

The ulcerogenic liability experiment was carried out on fasting rats, that were randomly divided into different groups, each group having n=6 rats. Rats in the control group received distilled water containing 0.3% CMC, while the rats in the standard group were given 10mg/kg indomethacin. Test compounds have been administered to the test groups in doses 5mg, 10mg, and 20mg/kg in 0.3 % Na CMC. All groups of animals were dosed via oral gavage once daily for three consecutive days and rats under anesthesia were sacrificed on the 3rd day after 6h of dosing. The stomachs were quickly taken out, and washed with saline to remove the excess material, stomach mucosa was examined using the hand-held lens. The percentage of stomach ulceration, ulcer index, and ulcer score were estimated following the previously reported scoring method (Marzouk, Taher et al. 2021). Some stomachs were randomly chosen from each group and preserved in 10% formalin solution. The slides have been stained with hematoxylin and eosin dyes for histological inspection.

4.4.2.8.Biochemical analysis

The levels of oxidative stress biomarkers were determined in random stomachs from each animal group. The stomach tissues were homogenized in cold phosphate buffer saline (pH 7.4), and centrifuged at 1000rpm for 10 minutes at 4°C. The respective supernatant was collected and their protein content was estimated following the standard Bradford assay. The levels of GSH (glutathione) and LPO (lipid peroxide) in the supernatants were determined for scheme 1 derivatives and the levels of GSH, SOD, and nitrate were measured for scheme 2 derivatives to evaluate the antioxidant activity of the test substances as per the reported procedures (Tripathi, Choubey et al. 2020, Waiker, Verma et al. 2023).

4.4.2.9. Assessment of liver and kidney functions

The blood sample from different rat groups was collected from the retro-orbital plexus and allowed to clot at room temperature. The serum was separated by centrifugation at 2000rpm for 20 minutes at 4°C. The serum biomarkers ALT, AST, creatinine, and urea were determined using commercial assay kits (Merck, Germany) according to the described protocol.

4.4.2.10. Effect on platelet aggregation

Platelet-rich plasma (PRP) was extracted from blood samples obtained from various rat groups during cardiotoxicity evaluation activities. The Tyrodes buffer was used to adjust the platelet count to $4X10^8$ cells/ml and the anti-platelet aggregation activity of test compounds was evaluated using 96-well plate aggregometry. Initially, absorbance was recorded at 570 nm on a UV-spectrophotometer. Afterward, 10 µl of ADP and 5 µg/ml of collagen were added to induce platelet aggregation. The plate was incubated for 5 min at 37°C with shaking. Again, the absorbance was measured at 570 nm to observe the change in value. The platelet aggregation percent was calculated as per the previously reported method (Ramakrishna and Krishnamurthy 2022).

4.4.2.11. Assessment of cardiotoxic liability

The rats were allocated into five different groups (n=6 per group). Group I consisted of control animals receiving 0.3% CMC solution as a vehicle, group II received ISO (100 mg/kg, s.c.), group III animals were administered with ISO (100 mg/kg, s.c.), followed by the treatment with celecoxib in 0.3% CMC solution. Group IV and group V animals received the treatment with the oral administration of test compounds, post ISO (100 mg/kg, s.c.) administration. The blood samples were taken from the retro-orbital plexus and serum was separated by centrifugation at 4000 rpm at 4°C for 10 minutes. The levels of LDH, CKMB, and cTn-1 were determined using the commercially available kits. For

the estimation of LDH and CKMB, the Span diagnostic kit, India, was utilized in accordance with the manufacturer's protocol, and the rat cTn-I ELISA kit from Krishgen Biosystems, India, was used for the cTn-I estimation (Kumar, Kasala et al. 2017, Leung, Galano et al. 2021).

4.4.2.12. Assessment of analgesic activity (Ugbogu, Okoro et al. 2024)

The rats were randomly divided into four different groups (n=6 per group). The control group received vehicle (0.3% CMC in distilled water), and other groups received standard indomethacin, compound **9d,** and **9g** respectively. After 30 minutes of vehicle and drug administration, 0.6% v/v of acetic acid was injected subcutaneously into all groups. The writhing activity was observed and the numbers of stretching were carefully counted for the next 30 minutes. When compared to the control group, a reduction in the number of writhing responses demonstrated the pain-inhibitory response in both the standard and test groups.

4.4.3. In vitro anti-cancer activity

The cell lines were grown in an appropriate medium containing 10% fetal bovine serum and 2 mM L-glutamine. At the time of the experiment, four 10-fold serial dilutions (10^{-7} to 10^{-4}) were made using the complete medium. Aliquots of 10 µl different drug dilutions were added to the appropriate microtiter wells already containing 90 µl of medium, resulting in the required final drug concentrations. After compound addition, plates were incubated at standard conditions for 48 hours and the addition of cold TCA terminated the assay. The supernatant was discarded; the plates were washed with tap water and air-dried five times. Sulforhodamine B (SRB) solution (50 µl) at 0.4 % (w/v) in 1 % acetic acid was added to each of the wells, and plates were incubated for 20 minutes at room temperature. The absorbance was read on an ELISA plate reader at a wavelength of 540

nm with 690 nm reference wavelength (Vichai and Kirtikara 2006, Kode, Kovvuri et al. 2020).

Percent Growth was expressed as the ratio of the average absorbance of the test well to the average absorbance of the control wells * 100. The images of the sample tested were taken using Ti-S Inverted Research Microscope- Nikon with a magnification of ×20, with Eclipse Image processing software NIS-Elements.

4.4.4. Anti-cancer activity in in vivo model system

The *Drosophila* stocks of Oregon R^+ (wild-type) and oncogenic overexpressing *UAS-Ras*^{V12} fly stocks were obtained from Bloomington stock center, USA, and grown in a biological oxygen demand (BOD) incubator at 24 ± 1°C using standard food media. A population of 10 virgin female and male wild-type flies were crossed in food treated with different concentrations of compound **9d** (1µM, 10µM, 50µM, 100µM, and 200µM) separately. The F1 generation was observed for 15 days to determine the LD50 of compound **9d**. For the assessment of the anti-inflammatory efficacy of compound **9d**, Wild-type (Oregon R^+) flies were considered as the control group, while *UAS-Ras*^{V12} expressing flies were utilized for the cancer disease model. The flies were cultured at 24 ± 1 °C on standard corn food media treated with different concentrations of compound **9d** at 10 µM and 50 µM (Mukhopadhyay, Vander Heiden et al. 2021).

Throughout the investigation, developmental advances in treated flies were tracked and compared to untreated controls to determine the compound's potential consequences. Parameters such as larval growth, pupation rate, and the emergence of adult flies were observed to determine whether compound 9d could mitigate the developmental abnormalities associated with the UAS-RasV12 model.

4.4.5. Pharmacokinetic studies

The pharmacokinetic characteristics were determined using healthy male Wistar rats (200-250g, n=5). After an overnight fast, the animals received 10 mg/kg p.o. of compound **9d** in 0.3% sodium CMC. The blood sample was collected after anesthetizing animals and samples were taken at different time intervals as follows: 0 (pre-dose), 0.5, 1, 2, 4, 8, 16, and 24 in heparinized tubes. The samples were centrifuged at 6797×g for 6 min. at 4°C to separate the plasma. To extract compound **9d**, 200 µl of MeOH was added to 100 µl of collected plasma, and centrifuged at 6797×g for 6 min. at 4°C. The organic layer was separated, and vacuum-dried, the collected residue was dissolved in a mobile phase. The 10 µl of the above sample was injected at the flow rate of 1ml/min into Agilent Infinity II 1260 HPLC using MeOH: H_2O (85:15) as the mobile phase. The standard calibration curve was used to determine the plasma drug concentration in each sample at a detection wavelength of λ=254 nm. The pharmacokinetic parameters, including T_{max}, C_{max}, (AUC)0-24, $t_{1/2}$, and MRT, were determined using an extravascular non-compartmental model in Kinetica 5.0 (Thermo Scientific Kinetica, USA) (Tripathi, Choubey et al. 2019).