Chapter-3

Materials & Methods

3.1 Drugs, chemicals and antibodies

Following is the list of drugs, chemicals and antibodies used in this work along with their catalog number and source of procurement.

Table 3.1 List of drugs, chemicals and antibodies

S.NO	Drugs/chemical/antibodies	Source
1.	Betaine	Sigma-Aldrich (St. Louis, MO, USA)
2.	Morphine	Sir Sunderlal Hospital Pharmacy (Banaras Hindu University), Varanasi, India
3.	Gabapentin	Sigma-Aldrich (St. Louis, MO, USA)
4.	NR2B antibody (ab28373)	Abcam (USA)
5.	β-actin antibody (ACTN05 (C4)	Abcam (USA)
6.	KIF17 antibody (SC137040)	SantaCruz Biotechnology (U.S.A)
7.	NF-κβ antibody (ab16502)	Abcam (USA)
8.	IBA1 (SC32725)	SantaCruz Biotechnology (U.S.A)
9.	ICAM1 (SC8439)	SantaCruz Biotechnology (U.S.A)
10.	Anti-Mouse IgG H&L (#ab6728)	Abcam (USA)
11.	Goat anti-rabbit IgG H&L (HRP) (ab6721)	Abcam (USA)
12.	Formaldehyde	Sigma-Aldrich (St. Louis, MO, USA)
13.	TriZol	Thermo scientific (USA)
14.	Maxima SYBR Green/Fluorescein qPCR Master Mix (#K0241)	Thermo scientific (USA)
15.	RevertAid first-strand cDNA synthesis kit (#K1622)	Thermo scientific (USA)
16.	Nuclease Free Water	Sigma-Aldrich (St. Louis, MO, USA)
17.	Bovine Serum Albumin (BSA)	Sisco Research Laboratories Pvt Ltd (Mumbai)

18.	Bradford reagent	Sigma-Aldrich (St. Louis, MO, USA)
19.	Enhanced chemiluminescence reagent	Biorad (USA)
20.	(5,5'-dithio bis-(2-nitrobenzoic acid)	Sigma-Aldrich (St. Louis, MO, USA)
21.	Nitrocellulose membrane	Biorad (USA)
22.	Transfer buffer	Biorad (USA)
23.	Malondialdehyde	Sisco Research Laboratories Pvt Ltd (Mumbai)
24.	Griess reagent	Sisco Research Laboratories Pvt Ltd (Mumbai)
25.	Glutathione	Sigma-Aldrich (St. Louis, MO, USA)
26.	Povidone iodine solution	Win-Medicine (India)
27.	Acetone	Sisco Research Laboratories Pvt Ltd (Mumbai)
28.	Chloroform	Sisco Research Laboratories Pvt Ltd (Mumbai)
29.	Ethanol	Sigma-Aldrich (St. Louis, MO, USA)
30.	Tris-hydrochloric acid	Sisco Research Laboratories Pvt Ltd (Mumbai)
31.	EDTA	Sigma-Aldrich (St. Louis, MO, USA)
32.	Disodium hydrogen phosphate	Sigma-Aldrich (St. Louis, MO, USA)
33.	Potassium dihydrogen phosphate	Sigma-Aldrich (St. Louis, MO, USA)
34.	Potassium chloride	Sisco Research Laboratories Pvt Ltd (Mumbai)
35.	PMSF	Sigma-Aldrich (St. Louis, MO, USA)
36.	2- mercaptoethanol	Sigma-Aldrich (St. Louis, MO, USA)
37.	Bromophenol blue dye	Sigma-Aldrich (St. Louis, MO, USA)
38.	Sodium dodecyl sulphate (SDS)	Sisco Research Laboratories Pvt Ltd (Mumbai)
39.	Ammonium persulphate	Sisco Research Laboratories Pvt Ltd (Mumbai)
40.	Tetramethylethylenediamine (TEMED)	Sisco Research Laboratories Pvt Ltd (Mumbai)
41.	Glycerol	Sisco Research Laboratories Pvt Ltd (Mumbai)

42.	Tris-base	Sisco Research Laboratories Pvt Ltd (Mumbai)
43.	Glycine	Sisco Research Laboratories Pvt Ltd (Mumbai)
44.	Prestained protein ladder	Genetex biotech (India)
45.	DEPC-treated water	Qiagen, Germany
46.	Sodium chloride	Loba Chime (India)
47.	Sodium fluoride	Sigma-Aldrich (St. Louis, MO, USA)
48.	Triton x100	Loba Chime (India)
49.	Sodium orthovendate	Loba Chime (India)
50.	Sodium deoxycholate	Loba Chime (India)
51.	Acrylamide	Sigma-Aldrich (St. Louis, MO, USA)
52.	Bis-acrylamide	Sigma-Aldrich (St. Louis, MO, USA)
53.	HCL	Sigma-Aldrich (St. Louis, MO, USA)
54.	Na OH	Sigma-Aldrich (St. Louis, MO, USA)
55.	2,2-diphenyl-1-picrylhydrazyl (DPPH)	Hi-Media
56.	standard gallic acid	Merck
57.	Aluminium trichloride	Finar
58.	Folin-ciocalteu reagent	Sigma-Aldrich (St. Louis, MO, USA)
59.	Standard Quercetin	Sigma-Aldrich (St. Louis, MO, USA)
60.	DCM	

3.2 Equipment and software

Following is the list of equipments and softwares along with their make, utilized in the present study:

Table 3.2 List of equipments and software

S. No	Equipment/software	Source
1.	Chemidoc	Biorad (USA)
2.	Gel electrophoresis assembly	Biorad (USA)
3.	Transblot	Biorad (USA)
4.	Nanodrop	Thermo scientific (USA)
5.	Rotor-Gene Q 2plex HRM real-time PCR System	Qiagen (Germany))
6.	CO2 Incubator	Eppendorf (Germany)
7.	Biosafety Cabinet	Clean Air (India)
8.	pH meter	Eutech (UK)
9.	Cold centrifuge	Eppendorf (Germany)
10.	Microplate reader SpectraMax M5	Molecular Devices (USA)
11.	Micropipettes	Eppendorf (Germany)
12.	Refrigerator (4°C)	Remi (India)
13.	Deep freezer (-40°C)	Remi (India)
14.	Deep freezer (-80°C)	Thermo scientific (USA)
15.	Millipore system	Merk-Sigma (USA)
16.	Tissue homogenizer (MT-30K)	MIULAB (China)
17.	Hargreaves apparatus	Ugo Basile (Italy)
18.	Von-Frey filaments	Anesthesio (USA)
19.	Conditioned Place Preference Apparatus	Rolex (India)
20.	Ranjour	Fine Science Tools (USA)
21.	Temperature sensor	Aptech Deals (Indian)
22.	Von-Frey mesh	Workshop IIT (BHU), Varanasi
23.	Dry Bath	Precious Instrument techno (Delhi, India)
24.	Rocker	Precious Instrument techno (Delhi, India)
25.	Vortex	Remi (India)
26.	Spinwin	Abdos (India)
27.	Weighing balance	Sartorius (Germany)
28.	Surgical tools	Bharat Surgicals (Varanasi, India)/Fine Science Tools (USA)

29.	Matlab software	MathWorks (USA)
30.	Microsoft word	Microsoft (USA)
31.	Microsoft power point	Microsoft (USA)
32.	Microsoft excel	Microsoft (USA)
33.	Gpower software	Heinrich Heine University (Germany)
34.	Chemdraw Software	PerkinElmer (USA)
35.	Image Lab software	Biorad (USA)
36.	Rotor-Gene Q Series software	Qiagen (Germany)
37.	GraphPad Prism 5.0	GraphPad (USA)
38.	Schodinger	Schodinger (USA)
39.	SIM alignment tool	Swiss Institute of Bioinformatics
40.	Mutalin Software	Florence Corpet
41.	HPTLC	CAMAG (Switzerland)
42.	LC-MS	
43.	HRMS	
44.	HPTLC plates	Merck, Darmstadt, Germany

3.3 Plant material and preparation of extract of roots of *Sida cordifolia*

3.3.1 Collection, identification and authentication of *Sida cordifolia* L. Roots: Roots of *Sida cordifolia* were procured from a botanist in Tirupati, India and were identified and authenticated at the Centre of Advance Study, Department of Botany, Institute of Science, Banaras Hindu University, Varanasi, India. A voucher specimen (Malva.2021/3) has been preserved in the departmental Herbarium.

3.3.2 Preparation of Extract Collected roots were cleaned of unwanted material manually, shade dried, chopped and powdered. 2 kg of powdered roots were soaked in DCM: Methanol (1:1) and set for cold maceration for 7 days with intermittent stirring.

Whole extract was filtered first with a muslin cloth and then with Whatman filter paper. Brown resinous DCM: Methanolic crude extract (SCE) thus obtained was evaporated using Rota evaporator. Completely dried extract was stored at 4 °C till further use.

3.4. Bioactivity guided fractionation

Crude extract of roots (SCE) thus obtained was further fractioned using separation funnel method as described previously (Abubakar and Haque 2020). Briefly, 60 g of crude extract (SCE) was dissolved in 300 ml of distilled water and transferred to a separating funnel. Extract was successively fractioned using equal volumes of hexane, chloroform, and ethyl acetate as solvents. Resulting four fractions hexane fraction, (SHF), chloroform fraction (SCF), ethyl acetate fraction (SEF) and residual aqueous fraction (SAF) were evaporated to dryness using Rota evaporator and stored at 4°C until further use.

3.5. Phytochemical analysis

3.5.1. Flavonoid Content Estimation: Total flavonoid content of the extract was measured using the aluminium chloride colorimetric method (Patel et al., 2022). Briefly, 50 µl of 1mg/ml solution of crude extract (SCE) was diluted to 1 ml in methanol. 4 ml of distilled water and 0.3 ml of 5% NaNO2 solution was added to methanolic solution After 5 minutes 0.3 ml of 10% AlCl3 solution were added incubated for 6 min followed by addition of 2 ml of 1 mol/l NaOH solution. Final volume was made up to 10 ml with double distilled water. Absorbance was measured at 510 nm after 15 minutes of incubation. A standard curve was plotted using quercetin as standard and the total flavonoid content was calculated from the standard curve. The values were expressed as mg quercetin equivalent per gram of extract.

3.5.2 Phenolic Content Estimation: The Folin–Ciocalteu method was performed for assessment of phenolic components using gallic acid as the standard (Nugroho et al. 2014). Crude extract of DCM: Me (SCE) at a concentration 1mg/ml was taken in the 25 ml of volumetric flask. To this 10 ml of distilled water and 1.5 ml of Folin Ciocaltue reagent was added. After 5 mins 4 ml of 20% Na2CO3 solution was added to the mixture. Volume was made up to 25 ml with water and after 30 minutes' absorbance was taken at 765 nm using UV spectrophotometer. A standard curve was plotted, and the total phenolic content was calculated. Results were expressed as mg of gallic acid equivalent per gram of extract.

3.5.3. Assessment of Antioxidant activity: Antioxidant potential of DCM: Me extract of *Sida cordifolia* roots was assessed by identifying it free radical scavenging effect of stable 1, 1-diphenyl-2-picrylhydrazyl (DPPH) free radical (Saini et al. 2014). Gallic acid was used as standard in this test. A 0.2 mM DPPH radical solution was prepared in methanol, 3 mL of this solution was mixed with 3 mL of the sample solution in different concentrations. Resulting mixture was kept in the dark for 30 minutes and optical density was measured at 517 nm using a UV-Vis spectrophotometer against methanol as blank.

Percentage inhibition was calculated by using the following equation:

% of inhibition of DPPH activity = (A–B/A) × 100

Where, A is optical density of the control and B is optical density of the sample. Anti-oxidant capacity of the sample was evaluated by comparison of inhibition concentration at 50% and 0.5 of absorbance value with standard gallic acid.

3.5.4. Characterization of crude extract of *Sida cordifolia* (SCE) and its aqueous fraction (SAF) by LC-MS/TOF and HRAMS respectively

LC-MS was used for the determination of bioactive phytoconstituents in crude extract (SCE) and was analyzed using LC-MS Q-TOF system consisted of Agilent LC-MS/TOF (G6550A) (SAIF-IIT, Bombay). Samples were analyzed using ZORBX Eclipse Plus C18, Narrow Bore 2.1 x 150mm,5-micron Column at a column temperature of 40°C. Mobile phase used was MilliQ water (solvent A) and acetonitrile (solvent B) and the flow rate was set at 500 µL/min.10 µL volume of sample was injected using auto sample and the scans were made for a run time of 30 minutes. While SAF was analyzed for bioactive components using Thermo Fisher Scientific High resolution accurate mass spectrometry system (Q Exactive Plus) With UHPLC (Dionex Ultimate 3000 RS Series) (SATHI, BHU, Varanasi) The mobile phase consisted of water (solvent A), acetonitrile (solvent B) and methanol (Solvent C). Scans were made at both positive and negative mode with a run time of 15 minutes.

3.6 Assessment of in-vitro anti-inflammatory activity by egg albumin protein denaturation assay

Whole crude extract (SCE) as well as fractions obtained after fractionation (SHF, SCF, SEF and SAF) were analysed for respective anti-inflammatory potential using egg albumin (Sangeetha et al., 2016, Dharmadeva et al., 2018). 100 mg sample from each extract (SCE, SHF, SCF, SEF and SAF) was dissolved in 100 ml of double distilled water and was used for serial dilutions. 100 mg of diclofenac sodium used as reference drug was mixed in 100 ml of phosphate buffer saline and was further used for serial dilutions. Reaction mixture was prepared adding 0.2 ml of Egg albumin in 2.8 ml of

phosphate buffer saline pH (6.4). To this 2 ml of extract from different concentration were mixed gently with reaction mixtures. A similar procedure was used for reference drug. Reaction mixtures were incubated at 37°C ± 2°C in a biological oxygen demand incubator for 15 min followed by heating at 70°C for 5 min. After cooling, individual absorbance was measured at 660 nm (UV- Vis Spectrophotometer), using distilled water as blank. All the samples were run in triplicate. **The percentage inhibition of protein denaturation was calculated by using the following equation:**

% inhibition = 100 × ([Vt / Vc] −1).

Where, Vt = absorbance of test sample, Vc = absorbance of control

3.7 High performance thin layer chromatography

HPTLC Profiling CAMAG HPTLC equipment with automatic TLC sampler 4 (ATS4),TLC plate heater III, visualizer and TLC scanner coupled to vision CATS version 2.1 software was used for quantitative estimation of betaine in SCE and SAF. The reference standard solutions of Betaine were prepared at concentration of 100 mg/ml in methanol. Dragendroff's reagent was prepared according to Bregoff–Delwiche for quaternary nitrogen compounds (Mehta et al. 2011).

200 mg of solid samples of both SCE (Crude Extract) and SAF (Aqueous fraction) were weighed individually followed by addition of 5 ml of methanol. The resultant mixture was shaken for 10 min at 300 rpm and centrifuged at 5000 rpm for 5 min. Supernatant was transferred into individual vials, and used for further HPTLC analysis. Chromatography was performed on 10 cm × 10 cm aluminium foil HPTLC plates coated with 0.25 mm layers of silica gel 60 F254 (E. Merck, Darmstadt,

Germany). Serial dilutions of Betaine as standard and extracts SCE and SAF were applied on precoated plates using spray-on technique with a CAMAG (Switzerland) Linomat 5 sample applicator fitted with a 100-µL Hamilton syringe.6 mm bands were applied under nitrogen at 4 bar. Number of tracks in plate were 8. After application, the plate was allowed to dry in air for 15 min then developed with methanol–ammonia 3:1 (v/v) in a CAMAG vertical twin-trough chamber (10 cm × 10 cm with stainless-steel lid). Development chamber was allowed to saturate with 10 ml of mobile phase for 30 minutes. After development, plate was dried and inspected under short and long-wavelength UV light in a UV cabinet. Derivatisation of plates was performed by dipping in modified Dragendroff's reagent and dried in air for 15 minutes and heated at 120°C for 20 mins on the TLC plate heater. The plate was photographed using CAMAG documentation system DigiStore2. Densitograms were obtained using CAMAG TLC scanner equipped with winCATS planar chromatography manager and were recorded at 540 nm.

3.8 *In-silico* studies

3.8.1 Homology Modeling Using NCBI Database and Sequence Retrieval

NCBI Database and Sequence Retrieval the NCBI database to evaluate the already existing information on our target protein KIF-17 using the URL https://www.ncbi.nlm.nih.gov was used .The 3-dimensional structure of KIF-17 protein for the rats is yet to be explored, so, to extract the information related to the amino acid sequencing using UniProt Database on www.uniprot.org was used. Only two KIF17 protein sequences were predicted. For further analysis, UniProt ID (UniProt KB Database) D3ZYC9 was used for the final selected long isoform protein

sequence of KIF-17. The retrieved information was in form of a FASTA sequence. Sequence Alignment Amino acid sequences were compared using the BLASTp tool of NCBI database www.blast.ncbi.nlm.nih.gov and UniProt database www.uniprot.org. However, to monitor the similarity index, a correlation with the OSM-3 Protein Data Bank https://www.rcsb.org/structure/7A3Z was performed. To isolate the information of the available sequence, the first BLAST optimization was conducted on the non-reducing database. The second BLAST was then performed to assess similarity among the sequences in the protein data bank www.rcsb.org. The percentage similarity index was calculated according to the methods reported for further analysis (Akhilesh et al. 2022).

3.8.2 Structure Prediction and Homology Modeling of KIF-17

In this study the comparative modeling and threading methods because for the docking-based drug design in the virtual screening simulation was performed, the 3D structure of KIF-17 is required which is yet to be obtained from the NMR and/or X-ray crystallography. Further, to obtain the protein structure against PDB, BLASTp by retrieving relevant templates was used.

Homology modeling techniques and molecular docking were used to predict the binding mode of the protein KIF-17 and for the construction and molecular recognition of the same. The similarity between the designed 3D structures were validated using BLASTp, Robetta, RaptorX, and SWISS model (Akhilesh et al. 2022), hence confirming the suitability of designed templates for further analysis. Thereafter, to analyze the confirmation of the same protein, secondary structure matching (SSM) was performed. SWISS MODEL (https://swissmodel.expasy.org) a structural

bioinformatics tool was used to evaluate the selected model of KIF-17 in terms of quality and accuracy. The consistency of the predicted structure was determined by estimating the degree angles of all residues in the most recommended regions of the Ramachandran (RC) Plot. In the present work, the predicted structures were quantified by disclosing the exemption of residues using RC plots. The distribution of torsional angles such as ϕ and ψ for non-glycine and non-proline residues along with differentiation of unfavorable and favorable regions was also predicted (Mahrosh and Mustafa 2021). The residues falling outside the favored zone were considered to be unfavorable outliers. Later on the outliers in the generated model were used to analyze the confirmations of the confidence scores. Visualizer tool UCSF chimera 1.13.1 was used to predict and modify the 3D structures of the protein KIF-17 (Pettersen et al. 2004). Finally the accuracy and performance of designed model by using ERRAT and ProCheck was assessed.

3.8.3 Structure-based virtual screening

Structure-based virtual screening was performed to investigate the interaction of betaine with KIF-17. AutoDock Vina tools was used to carried out docking studies subsequently docking score and binding affinity of the betaine to KIF-17 were recorded. The obtained molecular docking findings were quantified using PyMol to investigate the detailed ligand-protein interactions.

3.8.4 Molecular dynamics simulation

To investigate the functional and structural interactions between the ligand and targeted protein, a mature technology has been developed from all-atom classical molecular dynamic simulation employing GORMACS 2020 using the CHARMM36

force field. Further, a complex was obtained by docking of betaine at the active site of KIF-17. Briefly, inside the dodecahedron box (1 nm per side) the protein-ligand complex was placed. Following the ionization with Na+ and Cl– ions, the final simulation system was solvated and later diminished by steepest descent algorithm. After the energy minimization runs, a position restricted equilibration run was performed for 1 ns using the constant number, volume, and temperature (NVT) and isothermal-isobaric (NPT) ensemble to prevent any damage to the system during simulation. To maintain the system pressure of 1 bar and temperature at 300 K a coupling constant of 2 picoseconds for pressure and 0.1 picoseconds for temperature was applied until coupling was done (Parrinello and Rahman 1981). Using particle mesh Ewald (PME) method, Van der Walls interactions and long-range electrostatic interactions were calculated keeping cut-off for the short-range Van der Walls to 1.2 nm. Moving on, to constrain all the bonds LINCS algorithm was used and for the simulation time step was set to 0.002 ps. Finally, the production simulation of 100 ns run was carried out.

3.9 *In-vivo* studies

3.9.1 Experimental animals

Adult male Sprague Dawley rats weighting between 200- 250 gm were housed in standard cages in temperature-controlled environment maintained at 21 ± 2°C. 12-h light/dark cycle was maintained and animals were fed with standard laboratory diet and sterile water *ad libitum.* Animals were divided into different experimental groups with n=8 rats/group. The experimental protocol was approved by the Institutional Animal Ethics Committee (IAEC) of Indian Institute of Technology (Banaras Hindu University), Varanasi, India. All the studies were carried out in accordance with the

guidelines of Committee for the Control and Supervision of Experiments on Animals (CCSEA), Government of India, New Delhi and guidelines for laboratory animal use and care, National Institute of Health (NIH), U.S.A

3.9.2 Ethical committee approval

The experimental protocol was approved by the Institutional Animal Ethics Committee (IAEC) of Indian Institute of Technology (Banaras Hindu University), Varanasi, India. All the studies were carried out in accordance with the guidelines of Committee for the Control and Supervision of Experiments on Animals (CCSEA), Government of India, New Delhi and guidelines for laboratory animal use and care, National Institute of Health (NIH), U.S.A.

3.9.3 Animal model of neuropathic pain and experimental design

Neuropathic pain was induced in animals of all group except healthy control by performing chronic constriction injury in left sciatic nerve of rats. Procedure for CCI was performed in accordance with descried in literature (Li et al. 2019; Uniyal et al. 2021a). Rats were injected with ketamine (80 mg/kg i.p.) and xylazine (10 mg/kg i.p.) to induce anesthesia and the same was confirmed by pinching their toes with blunt forcep for any sign of pain. After application of povidone iodine, a blunt incision was made at bicep femoris muscle of left hind paw to expose the sciatic nerve. Approximately 10 mm of the sciatic nerve was freed from connective tissues and three silk ligatures (4.0) at a distance of 1mm apart were tied proximal to the trifurcation of the sciatic nerve, causing brief twitch of the muscle. Finally, the muscle layer was sutured with 6-0 silk (Teleflex Medical, USA). To prevent the wound from any kind of infection povidone-iodine solution 10% w/v was applied at the site of incision.

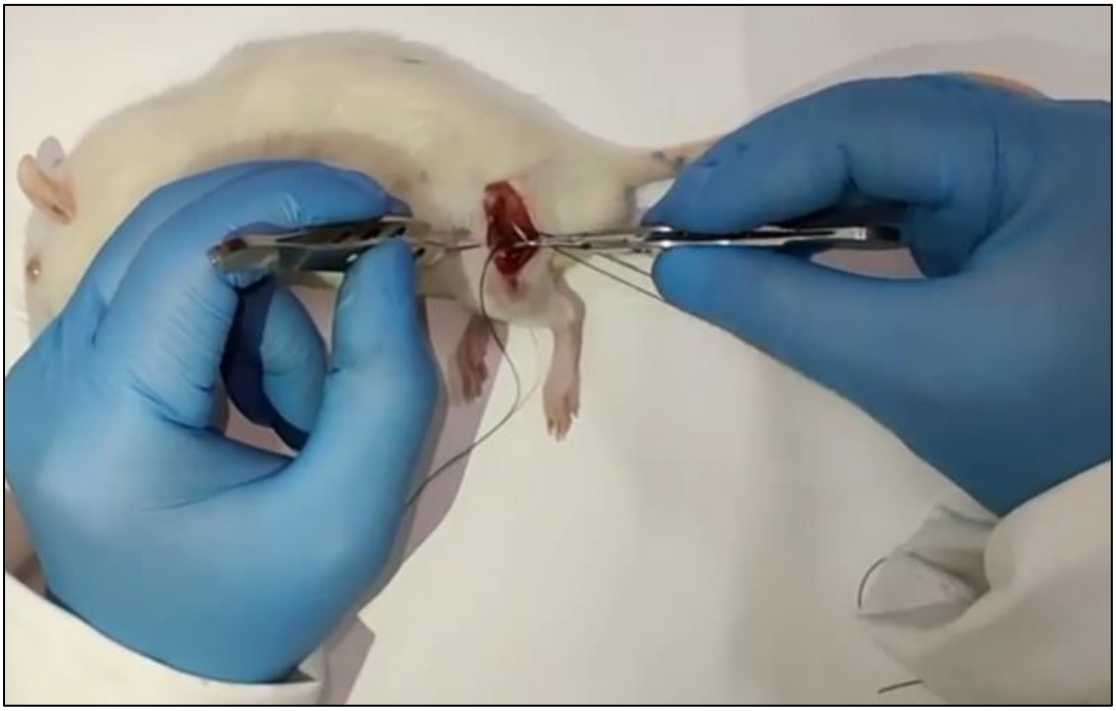

Figure 3.1 CCI model being performed at IIT (BHU) Varanasi

The animals were continuously monitoring until they fully emerged from anesthesia, after which they were carefully returned to their designated cages. To mitigate the risk of infection resulting from the surgical procedure, postoperative care included routine application of povidone-iodine solution to the incision sites until complete skin recovery. Pain behavior assessments were conducted both before and after sciatic nerve ligation to validate the onset and progression of the neuropathic pain phenotype in the rats. The dosage selection for *Sida cordifolia* crude extract (SCE) and aqueous fraction SAF (200, 400, and 800 mg/kg p.o.) and betaine (25, 50,100 mg/kg i.p.) was based on our preliminary investigation and literature survey.

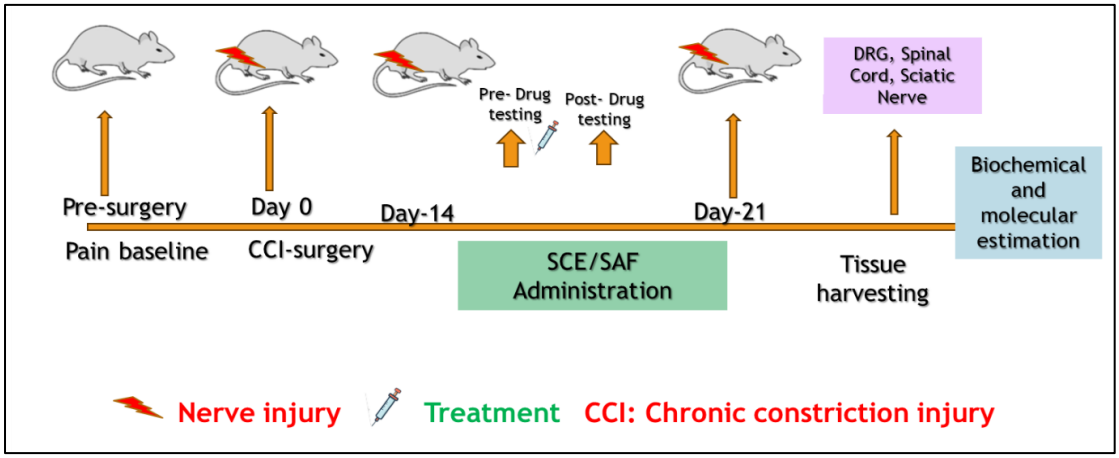

Figure 3.2 Experimental timeline. Reprinted with permission by American Chemical Society from own source reference.

3.9.4 Evoked Pain Behavior Assay

3.9.4.1 von-Frey hair test: Static allodynia: von-Frey hair test was employed in the nerve injured rats for the evaluation of static mechanical allodynia by evaluating the paw withdrawal threshold in right and left hind paw of the rats. von-Frey filaments of various strengths ranging from 0.40 gm to 13 gm were utilized to examine the mechanical hypersensitivity, as per the previously described procedure (Chaplan et al. 1994; Griffiths et al. 2018). Animals were allowed to accommodate to the environment 15 minutes prior to the experiment by briefly enclosing them in a plexi glass chambers placed on top of a metal mesh floor. von-Frey filaments were then carefully applied onto the sub-plantar region of the both left and right hind paw of rat perpendicularly. Mechanical sensitivity in rats was evaluated using the up-down method in the animals before and after the nerve injury. The following responses were considered to be positive to von-Frey filament, Paw withdrawal, shaking and licking of the paw. Forces of different strengths were applied, starting from a lower force of 1.8 gm and if no response was obtained, a filament with a higher force was applied. In case of a positive response, the filament with a lesser force was tested. This test was repeated until the attainment of maximum and minimum withdrawal threshold at greater and lower

intensities respectively. The assessments were done in all experimental groups for both right and left hind paws before and after the administration of drug/vehicle.

3.9.4.2 Cotton swab test: Dynamic mechanical test:

For the assessment of dynamic mechanical allodynia in rat's cotton swab test was used (Field et al. 1999). Rats were placed on elevated mesh apparatus inside plexi glass chambers and were allowed to habituate to the environment for 15 minutes. Further, the test was performed using a cotton swab by gently stroking it on the hind paws of rats and recording the paw withdrawal latencies before and at different time points after drug treatment. The cut-off time of 15 secs was used while recording the paw withdrawal latency of rats. Caution was taken to avoid the consideration of normal movements as pain response in the rats.

3.9.4.3 Assessment of thermal hyperalgesia: Hargreave's test

Hargreave's test (Ugo Basile, Italy) was used for the assessment of heat hyperalgesia in rodents (Deuis et al. 2017). Rats were placed in an elevated plexi glass cage and habituated on Hargreaves apparatus maintained at 30 °C for 1 hour. Paw withdrawal latency was evaluated using a movable radiant heat source with a cut-off period of 20 seconds to avoid tissue damage in rats. Thermal radiation was targeted on plantar surface of right and left hind paw to calculate the paw withdrawal latency in response to noxious thermal stimuli. The experiment was conducted in triplicate for both the right and left hind paw and the average paw withdrawal latency was calculated before and after chronic constriction injury and before and after treatment with different extracts obtained from *Sida cordifolia*. Decreased paw withdrawal latency in rats indicates increase thermal hyperalgesia in rats.

3.9.4.4 Evaluation of cold hyperalgesia: Cold plate test

Cold hyperalgesia in rats was assessed by using stainless steel plate maintained at 1°C ± 2°C with an ice floor at the bottom. The temperature fluctuations were monitored by using a temperature sensor mounted on the surface of a plexi glass chamber (Lim et al. 2022). Pain behavior was examined by placing the animal on cold plate and recording the test video for 50 seconds. Number of paw lifts and paw licking duration before and after drug treatment were measured as outcome measures of pain-like behavior in rats.

3.9.4.5 Assessment of cold allodynia: Acetone evaporation test

Cold allodynia was assessed using the acetone evaporation test. For this test, rats were first acclimatized in plexi glass chamber on von Frey mesh for 20 minutes. The test was then carried on by spraying 100 microliters of acetone through a 1 mL syringe to the dorsal surface of hind paw. The test video was recorded for a time period of 60 seconds and the aversive responses triggered by the evaporating cooling effect were graded by a blind observer. The scoring of these responses were done on a scale of four: 0, no response; 1, grooming and flicking of the paw; 2, repetitive flicking of the paw; 3, repetitive paw flicking with licking, 4, continuous flinching and guarding of hind paw; (Yoon et al. 1994). A total of three trials were conducted in both left and right hind paws for a period of five-minute.

3.9.4.6 Acute nociceptive stimuli: Pinprick test

Mechanical hyperalgesia was evaluated in the rats by the pin prick test. On a von-Frey hair, a 22- gauge needle was glued (Chung et al. 2012), which was then used to apply force on the hind paw of rats. Extreme caution was taken to avoid puncturing of skin while applying the pin on the skin. Ten alternate trials of pin-prick was performed for each paw and the frequency of paw withdrawal was recorded.

3.9.5 Spontaneous ongoing pain assessment: Conditioned place preference assay

Spontaneous ongoing pain was evaluated using conditioned place preference (CPP), a three chambered (A,B,C) apparatus (Tiwari et al. 2018). The chambers A and B had distinct tactile and visual cues for the rats the C chamber was the central corridor section providing entries to the adjacent chambers. Any Maze software (Version 7.0 Stoelting, USA) was used to record the video and simultaneously track the duration of time spent in each chamber by the rats. Rats were acclimatized in the CPP apparatus for 15 minutes, then preconditioning baselines were performed on the next day for each rat to assess the preference of the rats for the respective compartment for the pre-drug condition by allowing the rats to freely explore the environment. The time spent by rats in each chamber was measured by video tracking its movement for 15 minutes. Exclusion of the rats that showed preferences less than 20% and more than 80% for any of the chambers was done from the test. Following two days conditioning was performed involving two sessions. After administering the vehicle, the rats were confined for a duration of 30 minutes in the paired chamber in the first session. After four hours in the second session, the drug was administered in the rats and then confined for 30 minutes in the paired chamber. Post-conditioning was done on the trial day by recording the video for 15 minutes where the animals were gently placed in the corridor to allow easy access to both the compartments and the time spent in both chambers by each rats was measured.

3.9.6 Behavioral neurotoxicity assays

3.9.6.1 Rota-rod test : The motor-coordination activity of rats was assessed using rota-rod test prior and after the drug administration. The training of the rats was done for 2 minutes at the speed of 5 r.p.m. On the day of experiment, the speed was increased to 25 r.p.m. and the rat's performance was evaluated for 2minutes prior and later of drug

administration. The performance was evaluated by the time spent of the accelerating rod.

3.9.6.2 Open Field Test: Open field test was done to assess the locomotor activity on the rats and the effect of drug on the same. Rats were properly habituated in the open field apparatus (45x45x75cm) one day prior to the experiment. On the day of the experiment, rats were kept in the apparatus for 10 minutes and the video was recorded using Any Maze software (Version 7.0 Stoelting, USA) (Tiwari et al. 2018). Outcome measures in this test were average speed and total distance travelled by the rats after vehicle and drug treatment.

3.9.7 Tissue harvesting and storage

Euthanasia was performed after the completion of the behavioral studies and the animals were sacrificed humanely using deep isoflurane anesthesia followed by cervical decapitation. Ipsilateral and contralateral lumber L4-L5 dorsal root ganglion were dissected out by cutting through the muscles and pulling out the transverse processes of the spinal cord. Further, the deep cut was made through the skin above the spinal cord. The vertebral column was removed using a rongeur and Dumont forceps and the spinal cord was exposed fully. The L4-L5 lumbar region was identified and cut from the middle section to divide the ipsilateral and contralateral spinal cord into two equal halves. The sciatic nerve was also harvested by dissecting through the bicep femoris muscle. All tissues were stored at -80°C for further biochemical and molecular analysis.

3.9.8 Biochemical assays

The samples of sciatic nerves were homogenized on ice to prevent degradation of proteins and further processed to conduct biochemical estimations by using the radio

immunoprecipitation assay buffer (RIPA buffer) pH 7.4. The lysate was then centrifuged at 12000 rpm for 20 min, at 4°C and supernatant was collected. Finally, by performing colorimetric assays various markers of oxidative stress such as MDA, Nitrite, GSH and SOD were then evaluated.

3.9.8.1 Estimations of lipid peroxidation (LPO)

Malondialdehyde (MDA) is the end product of lipid peroxidation which was estimated using the thiobarbituric acid reactive substance (TBARS) assay (Fernandes et al. 2018). This assay is based on the principle that MDA present in a lysate will bind with the TBA and form a complex which produces pink color. In the presence of thiobarbituric acid the supernatant was mixed with acetic acid and sodium dodecyl sulfate (SDS) and heated to 100°C for 1hr. Later the samples were allowed to cool and plating was done in a 96-well plate. Using a multimode microplate reader, the absorbance was measured at 532 nm. The concentration of MDA is expressed in µm/mg protein of the tissue.

3.9.8.2 Determination of reduced glutathione (GSH)

Determining reduced glutathione levels is an indirect indicator of the cell's physiological or pathological condition, where GSH is an antioxidant enzyme that prevents the damage caused by reactive oxygen species generation within a cell. The activity of GSH is measured by mixing the tissue lysate with 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB) and incubated until color changes to yellow at 48°C. Later using a multimode microplate reader, the absorbance is recorded at 412 nm. The concentration of GSH is expressed in µm/mg protein of the tissue (Tiwari and Chopra 2011; Tiwari et al. 2018).

3.9.8.3 Quantification of superoxide dismutase (SOD)

The assessment of the antioxidant enzyme such as superoxide dismutase activity was done via adopting the experimental protocol as described (Tiwari and Chopra 2011).The activity of SOD is a measure of the degree of inhibition of the reaction unit of enzyme providing 50% inhibition of nitro blue tetrazolium reduction. Briefly, the reagent was prepared by dissolving 50mM of anhydrous sodium carbonate (Na_2CO3), 0.1 mM of ethylenediaminetetraacetic acid (EDTA) and 25 µM of nitro blue tetrazolium (NBT) in PBS (0.1 M, pH 7.4). For the experiment, 100 µL of prepared reagent, 25 µL of hydroxylamine hydrochloride (NH2OH·HCl) and 50 µL of supernatant were mixed thoroughly. Absorbance was taken at regular intervals at 570 for 3 min. Results was further expressed in U/mgpr.

3.9.8.4 Quantification of Nitrite

To measure the extent of nitrosative stress inside a cell, Nitrite levels were quantified using the Griess reagent. Equal quantities of sample are mixed with the Griess reagent and the mixture is incubated for 30 mins at room temperature. Finally, using the microplate reader, absorbance was recorded at 540nm. The concentration of nitrite is expressed in µM/mgpr of the tissue (Raygude et al. 2012).

To measure the extent of nitrosative stress inside a cell, Nitrite levels were quantified using the Griess reagent. Equal quantities of sample are mixed with the Griess reagent and the mixture is incubated for 30 mins at room temperature. Finally, using the microplate reader, absorbance was recorded at 540nm. The concentration of nitrite is expressed in µM/mgpr of the tissue.

3.9.9 Molecular biology studies

3.9.9.1 Reverse transcription polymerase chain reaction (RT-PCR)

RT-PCR analysis was used for the measurement of mRNA expressions in the DRG and spinal cord of neuropathic rats with and without treatment. Using trizol reagent (Ambion) total RNA was isolated from the tissue sample followed by cDNA synthesis using cDNA kit (K1622 Thermo scientific). Reaction master mix of 10 ml was prepared by combining maxima SYBR Green qPCR Master Mix (K0241 Thermo scientific), template DNA, reverse and forward primer and nuclease-free water (Sigma W4502). Rotor-Gene Q. (Qiagen, Germany) was used to run the reaction master mix and comparative threshold cycle (CT) was calculated to determine the relative quantification of target RNA. Normalization of target threshold cycle number was done using GAPDH (housekeeping gene). All the primers used in RT-PCR and their details are listed in table.

Table 3.3 Primers used in RT-PCR analysis

SN	Gene	Sequence	Primer 5'<-----Sequence----->3'
1	GAPDH	Forward	CAGTGCCAGCCTCGTCTCAT
		Reverse	CAAGAGAAGGCAGCCCTGGT
2	NR2B	Forward	GGCAGGGGCGTCAAAAACAA
		Reverse	CACACAGGGGTTGGACTGGT
3	KIF17	Forward	CAGCCATCCTCCACTGACCT
		Reverse	ACCACCTCCTCAGCCACTTT
4	TNF-α	Forward	GTAGCCCACGTCGTAGCAAAC
		Reverse	ACCACCAGTTGGTTGTCTTTGA
5	NF-κβ	Forward	ACGACGATCCTTTCGGAACT
		Reverse	TCCTCTCTGTTTCGGTTGCT
6	IL1β	Forward	CCTATGTCTTGCCCGTGGAG
		Reverse	CACACACTAGCAGGTCGTCA
5	IL6	Forward	TCTGGTCTTCTGGAGTTCCGTT
		Reverse	GAGAGCATTGGAAGTTGGGGT

3.9.9.2 Western blotting

Tissues samples were homogenized using the procedure described in the literature (Uniyal et al. 2021a). Ice cold lysis buffer, RIPA buffer (50 mM Tris, 150 mM NaCl, 1 mM EDTA, 0.5% Triton X-100, and 1% SDS) (Table 3.3) along with

protease inhibitor cocktails was used to homogenized the tissue samples followed by cold centrifugation at 16000 g, 4°C. The supernatant was pipetted out and total protein concentration was determined in the same using Bradford assay. All the samples were loaded on equivalent protein basis on 10% SDS-PAGE (Sodium dodecyl sulfate - polyacrylamide gel electrophoresis) (Table 3.4) followed by separation of proteins on gel using constant current and voltage. Proteins were transferred to nitrocellulose membrane using transblot assembly followed by blocking of non-specific binding sites using bovine serum albumin (3% BSA) for 2 hours. Afterwards, membranes were incubated overnight with primary antibodies of beta-actin (1:1000), NR2B (1:1000), Iba-1 (1:1000), ICAM-1 (1:1000), TNF-α (1:1000) and IL-1 β (1:1000) at 4°C. Membrane washing was done with Tris- buffered saline Tween-20 for 10 minutes, thrice and incubated with respective secondary antibodies for 2 hours at room temperature. Bands were detected using an enhanced chemiluminescence substrate (Biorad, USA) on Chemi-DocTM, BioRad, Hercules, California, USA. Image J software was used for quantification of blots and the data was normalized to the respective beta-actin bands.

Table 3.4 Composition of RIPA buffer

S.N	Chemical	Concentration
1	Tris-HCL (pH 8.0)	50mM
2	NaCl	150mM
3	Triton X	0.1%
4	Sodium deoxycholate	0.3%
5	SDS	0.1%
6	Sodium fluoride	1mM
7	Sodium orthovanadate	1mM
8	EDTA	2mM
9	EGTA	2mM
10	PMSF	1mM
11	Protease inhibitor	5µl/100 mg of tissue

Table 3.5 Composition of Loading buffer

SN	Composition	Concentration
1	SDS	4%
2	2-mercaptoethanol	10%
3	Glycerol	20%
4	Bromophenol blue	0.004%
5	Tris HCl	0.125 M

Table 3.6: Running buffer recipe

SN	Composition	Concentration
1	Tris	25mM
2	Glycine	190mM
3	SDS	0.1%

3.10 High performance thin layer chromatography HPTLC Profiling

CAMAG HPTLC equipment with automatic TLC sampler 4 (ATS4), TLC plate heater III, visualizer and TLC scanner coupled to vision CATS version 2.1 software was used for quantitative estimation of betaine in SCE and SAF. The reference standard solutions of Betaine were prepared at concentration of 100 mg/ml in methanol. Dragendroff's reagent was prepared according to Bregoff–Delwiche for quaternary nitrogen compounds (Mehta et al. 2011). 200 mg of solid samples of both SCE (Crude Extract) and SAF (Aqueous fraction) were weighed individually followed by addition of 5 ml of methanol. The resultant mixture was shaken for 10 min at 300 rpm and centrifuged at 5000 rpm for 5 min. Supernatant was transferred into individual vials, and used for further HPTLC analysis. Chromatography was performed on 10 cm × 10 cm aluminium foil HPTLC plates coated with 0.25 mm layers of silica gel 60 F254 (E. Merck, Darmstadt, Germany). Serial dilutions of Betaine as standard and extracts SCE and SAF were applied on precoated plates using spray-on technique with a CAMAG

(Switzerland) Linomat 5 sample applicator fitted with a 100-µL Hamilton syringe.6 mm bands were applied under nitrogen at 4 bar. Number of tracks in plate were 8. After application, the plate was allowed to dry in air for 15 min then developed with methanol–ammonia 3:1 (v/v) in a CAMAG vertical twin-trough chamber (10 cm × 10 cm with stainless-steel lid). Development chamber was allowed to saturate with 10 ml of mobile phase for 30 minutes. After development, plate was dried and inspected under short and long-wavelength UV light in a UV cabinet. Derivatisation of plates was performed by dipping in modified Dragendroff's reagent and dried in air for 15 minutes and heated at 120°C for 20 mins on the TLC plate heater. The plate was photographed using CAMAG documentation system DigiStore2. Densitograms were obtained using CAMAG TLC scanner equipped with winCATS planar chromatography manager and were recorded at 540 nm.

3.11 Statistical analysis

One-way ANOVA and two-way ANOVA followed by Tukey's multiple or Bonferroni multiple comparison respectively was performed to analyse the behavioral and molecular data. Microsoft Excel was used to interpret the raw data and calculate the mean, standard error mean (SEM), % maximum possible effect (MPE), fold change for RT-PCR and western blot data. The statistical analysis was carried out using GraphPad Prism 8.0. The data was provided as mean and standard error of the mean ± SEM $P<0.05$ was considered as statistically significant data.

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