CHAPTER 3: Materials & Methods

3.1 Drugs, Chemicals and Antibodies

Following are 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.	Bergenin (B4349)	TCI Chemicals (India)
2.	Cisplatin (15663-27-1)	Sigma-Aldrich (St. Louis, MO, USA)
3.	Paclitaxel (33069-62-4)	Sigma-Aldrich (St. Louis, MO, USA)
4.	Vincristine (2068-78-2)	Sigma-Aldrich (St. Louis, MO, USA)
5.	Morphine	Sir Sunderlal Hospital Pharmacy (Banaras Hindu University), Varanasi, India
6.	Gabapentin (60142-96-3)	Sigma-Aldrich (St. Louis, MO, USA)
7.	TRPA1 antibody (MBS9612028)	MyBiosource (USA)
8.	TRPV1 antibody (SC398417)	SantaCruz Biotechnology (U.S.A)
9.	NR2B antibody (ab28373)	Abcam (USA)
10.	TNF-α (SC52746)	SantaCruz Biotechnology (U.S.A)
11.	IL-1β (SC52012)	SantaCruz Biotechnology (U.S.A)
12.	β-actin antibody (ACTN05 (C4))	Abcam (USA)
13.	NF-κβ antibody (ab16502)	Abcam (USA)
14.	Iba1 (SC32725)	SantaCruz Biotechnology (U.S.A)
15.	ICAM1 (SC8439)	SantaCruz Biotechnology (U.S.A)
16.	Anti-Mouse IgG H&L (#ab6728)	Abcam (USA)
17.	Goat anti-rabbit IgG H&L (HRP) (ab6721)	Abcam (USA)
18.	TriZol	Thermo scientific (USA)
19.	Maxima SYBR Green/Fluorescein qPCR Master Mix (#K0241)	Thermo scientific (USA)
20.	RevertAid first-strand cDNA synthesis kit (#K1622)	Thermo scientific (USA)
21.	RNA loading buffers (15404849)	Thermo scientific (USA)

22.	Nuclease Free Water (7732-18-5)	Sigma-Aldrich (St. Louis, MO, USA)
23.	Bovine Serum Albumin (BSA)	Sisco Research Laboratories Pvt Ltd (Mumbai)
24.	Bradford reagent (19219)	Sisco Research Laboratories Pvt Ltd (Mumbai)
25.	Enhanced chemiluminescence reagent	Biorad (USA)
26.	(5,5'-dithio bis-(2-nitrobenzoic acid)	Sigma-Aldrich (St. Louis, MO, USA)
27.	Immobilon®-P PVDF Membrane |IPVH00010	Merck, Millipore
28.	Transfer buffer	Biorad (USA)
29.	Malondialdehyde	Sisco Research Laboratories Pvt Ltd (Mumbai)
30.	Griess reagent	Sisco Research Laboratories Pvt Ltd (Mumbai)
31.	Glutathione	Sigma-Aldrich (St. Louis, MO, USA)
32.	Acetone	Sisco Research Laboratories Pvt Ltd (Mumbai)
33.	Chloroform	Sisco Research Laboratories Pvt Ltd (Mumbai)
34.	Ethanol	Sigma-Aldrich (St. Louis, MO, USA)
35.	Tris-hydrochloric acid	Sisco Research Laboratories Pvt Ltd (Mumbai)
36.	EDTA	Sigma-Aldrich (St. Louis, MO, USA)
37.	Disodium hydrogen phosphate	Sigma-Aldrich (St. Louis, MO, USA)
38.	Potassium dihydrogen phosphate	Sigma-Aldrich (St. Louis, MO, USA)
39.	Potassium chloride	Sisco Research Laboratories Pvt Ltd (Mumbai)
40.	PMSF	Sigma-Aldrich (St. Louis, MO, USA)
41.	2- mercaptoethanol	Sigma-Aldrich (St. Louis, MO, USA)
42.	Bromophenol blue dye	Sigma-Aldrich (St. Louis, MO, USA)
43.	Sodium dodecyl sulphate (SDS)	Sisco Research Laboratories Pvt Ltd (Mumbai)

44.	Ammonium persulphate	Sisco Research Laboratories Pvt Ltd (Mumbai)
45.	Tetramethylethylenediamine (TEMED)	Sisco Research Laboratories Pvt Ltd (Mumbai)
46.	TRPA1 siRNA	GenScript Biotech, USA Piscataway NJ
47.	Glycerol	Sisco Research Laboratories Pvt Ltd (Mumbai)
48.	Tris-base	Sisco Research Laboratories Pvt Ltd (Mumbai)
49.	Glycine	Sisco Research Laboratories Pvt Ltd (Mumbai)
50.	Prestained protein ladder	Genetex biotech (India)
51.	DEPC-treated water	Qiagen, Germany
52.	Sodium chloride	Loba Chime (India)
53.	Sodium fluoride	Sigma-Aldrich (St. Louis, MO, USA)
54.	DOTAP (1,2-dioleoyl-3-trimethylammonium-propane	Avanti Polar Lipids, Inc. (Alabaster, AL)
55.	Cholesterol	Avanti Polar Lipids, Inc. (Alabaster, AL)
56.	Carbon-coated TEM grids	EMS (Hatfield, PA, USA)
57.	Triton x100	Loba Chime (India)
58.	Sodium orthovendate	Loba Chime (India)
59.	Sodium deoxycholate	Loba Chime (India)
60.	Acrylamide	Sigma-Aldrich (St. Louis, MO, USA)
61.	Bis-acrylamide	Sigma-Aldrich (St. Louis, MO, USA)
62.	HCL	Sigma-Aldrich (St. Louis, MO, USA)
63.	NaOH	Sigma-Aldrich (St. Louis, MO, USA)
64.	Evans blue	Sisco Research Laboratories Pvt Ltd (Mumbai)

3.2 Equipment and software

Following are the list of equipment and software along with their make, utilized in the present study:

Table 3.2 List of equipment & 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.	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.	High Resolution Transmission Electron Microscope (HR- TEM)	FEI Company of USA (S.E.A.) PTE, LTD
18.	High Resolution Scanning Electron Microscope (HR- SEM)	FEI Company of USA (S.E.A.) PTE, LTD
19.	Scanning Electron Microscope (SEM)	Carl Zeiss microscopy ltd. Eds:51n1000 – eds system
20.	Fourier Transform Infrared Spectroscopy (FTIR)	THERMO Electron Scientific Instruments LLC
21.	Thermogravimetric Analysis (TGA)	M/s Shimadzu (Asia Pacific) Pte Ltd.
22.	Differential Scanning Calorimetry (DSC)	M/s Shimadzu (Asia Pacific) Pte Ltd.
23.	X-ray photoelectron spectroscopy (XPS)	Thermo Fisher Scientific
24.	Hargreaves apparatus	Ugo Basile (Italy)
25.	von-Frey filaments	Anesthesio (USA)

26.	Conditioned Place Preference Apparatus	Rolex (India)
27.	Rangour	Fine Science Tools (USA)
28.	Temperature sensor	Aptech Deals (Indian)
29.	von-Frey mesh	Workshop IIT (BHU), Varanasi
30.	Dry Bath	Precious Instrument techno (Delhi, India)
31.	Rocker	Precious Instrument techno (Delhi, India)
32.	Vortex	Remi (India)
33.	Spinwin	Abdos (India)
34.	Rota rod	Orchid Scientific
35.	Weighing balance	Sartorius (Germany)
36.	Surgical tools	Bharat Surgicals (Varanasi, India)/Fine Science Tools (USA)
37.	Matlab software	MathWorks (USA)
38.	Microsoft word	Microsoft (USA)
39.	Microsoft power point	Microsoft (USA)
40.	Microsoft excel	Microsoft (USA)
41.	Gpower software	Heinrich Heine University (Germany)
42.	ChemDraw Software	PerkinElmer (USA)
43.	Image Lab software	Biorad (USA)
44.	Rotor-Gene Q Series software	Qiagen (Germany)
45.	GraphPad Prism 8.0	GraphPad (USA)
46.	Maestro	Schrödinger
47.	SIM alignment tool	Swiss Institute of Bioinformatics
48.	Mutalin Software	Florence Corpet
49.	Origin	Origin Software, Ayanagar, Bangalore
50.	Raptor X	Toyota Technological Institute of Chicago
51.	UCSF chimera 1.13.1	Resources for Biocomputing, Visualization, and Informatics (RBVI)
52.	Glide	Maestro, Schrödinger (USA)

3.3 *In-vivo* studies

3.3.1 Experimental animals

Adult male Sprague Dawley rats, 5-6 weeks old (200-240 g) were used in this study. Rats (four rats/cage) were housed in controlled environment (temperature 21 ± 2°C; 12-h light-dark cycle). Animals were provided with standard laboratory food ad-libitum and sterile water. All rats were handled and acclimatized to the laboratory room before the initiation of the experiment. All the Animals were randomly assigned to different experimental groups (n=8-9/group) for behavioral testing.

3.3.2 Ethical committee approval

All experiments were performed in accordance with the guidelines by International Association for The Study of Pain and the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India, New Delhi. All the experimental protocols were approved by the Institute Animal Ethics Committee of Banaras Hindu University, Varanasi, UP, India (IAEC approval number: Dean/2020/IAEC/2207 and IIT(BHU)/IAEC/2022/013).

3.3.3 Animal models of chemotherapy induced neuropathic pain

3.3.3.1 Paclitaxel Model of Chemotherapy-Induced Neuropathic Pain

The PTX model was used to compare the behavioral and molecular parameters with those of the novel combined chemotherapy model of CINP. Paclitaxel (TCI chemicals) was dissolved in the 4% DMSO in sterile saline at a concentration of 2000 µg/ml. The rats were dosed with 2 mg/kg PTX or an equivalent volume of vehicle solution intraperitoneally on four alternate days (0, 2, 4, and 6) with a final cumulative dose of 8 mg/kg as previously described (Griffiths et al., 2018; Ullah et al., 2021).

3.3.3.2 Combination chemotherapy model of chemotherapy-induced neuropathic pain

Rats were intraperitoneally (i.p.) administered with a combination of chemotherapeutic agents which includes paclitaxel 2mg/kg, cisplatin 2mg/kg and vincristine 0.5mg/kg. The doses chemotherapy were chosen on the basis of contemporary literature (Chiba et al., 2017b; Kuai et al., 2020b; Mangaiarkkarasi et al., 2015; Younan and Rashed, 2013). Drugs were dissolved with 4% DMSO in sterile saline and previous reports suggest that 4% DMSO administration does not interfere with pain-associated outcomes (Zhou et al., 2020). A combination of the drugs was injected on 4 alternate days (days 0, 2, 4 and 6) and control rats were injected with the same volume of the vehicle without combination chemotherapy.

3.3.4 Experimental design

In the present study, we used a standard animal model development paradigm using behavioural, pharmacological and molecular tools to develop a rationalized combination-based rat model of CINP. In our studies, we employed two models of chemotherapy-induced neuropathic pain (CINP): one involving paclitaxel alone and another incorporating a combination of paclitaxel (2 mg/kg), cisplatin (2 mg/kg), and vincristine (0.5 mg/kg) in animal subjects. To induce neuropathic pain, we administered chemotherapy for four alternate days. Subsequently, we conducted pharmacological validation using gabapentin (60 mg/kg, i.p.) as a standard treatment. Gabapentin was administered once daily from day 1 to day 8 during chemotherapy. Following the pharmacological validation, we conducted molecular biological studies to assess the effect of gabapentin on molecular changes in the dorsal root ganglion (DRG) and spinal cord of rats subjected to paclitaxel alone and combined chemotherapy. In a subsequent

set of experiments, we investigated the potential of Bergenin (25, 50, 100 mg/kg, i.p.) to alleviate pain-like behaviour induced by combination chemotherapy in rats. At the conclusion of this study, animals were euthanized, and tissues from the DRG, lumbar region of the spinal cord, and sciatic nerve were harvested and stored at -80°C for further biochemical and molecular analyses. Finally, *in-silico* studies were 39 performed to validate Bergenin's binding affinity with different TRP channels.

In the next phase of our research, we explored the therapeutic efficacy of a liposomal formulation containing TRPA1 siRNA in animal model of chemotherapy-induced neuropathic pain. Using the combination chemotherapy-induced model, we administered the TRPA1 siRNA liposomal formulation intravenously and intrathecally on the 14th day, when pain symptoms were at their peak. This treatment was repeated for three consecutive days at a dose of 5 µg/15 µl. We then assessed pain behavioral responses and subsequently euthanized the animals and tissues were harvested and stored at -80°C for further biochemical and molecular studies. The detailed timeline of the study was depicted in Figure 3.1.

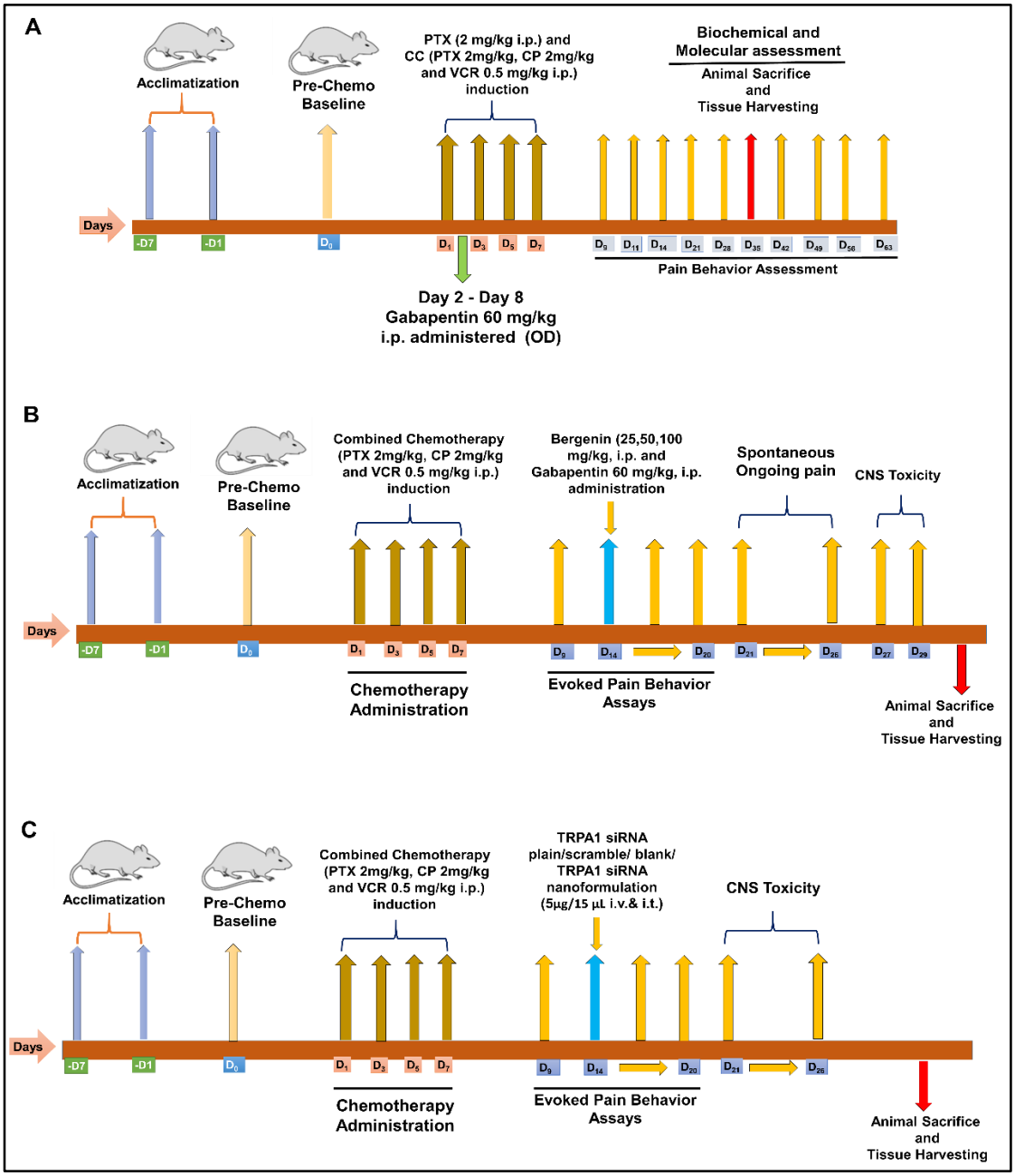

Figure 3.1 Experimental Timeline

3.3.5 Intrathecal siRNA knockdown of TRPA1 expression

The siRNA against TRPA1 was customized and purchased from GenScript Biotech. The following siRNA sequences were used for TRPA1 gene, sense 5-CUGGCAGACUACCUAAUUUCA-3' and antisense 5 AAAUUAGGUAGUCUGCCAGGU-3'. Firstly, we have checked the in-vivo efficacy

of TRPA1 siRNA in healthy rats through mRNA and protein expression employing RT-PCR and western blotting analysis. Intrathecal injections once a day, 10 µg administered as previously described (Chan et al., 2021; Haghiralsadat et al., 2018; Hattori et al., 2019). Briefly, rats were anesthetized with ketamine 80mg/kg. i.p. and xylazine 20 mg/kg i.p. and intrathecal administration were performed by lumbar puncture into the L4-L5 intervertebral space using a 21-gauge scalp vein set (Nipro Co.) fitted to a Hamilton 25-µL syringe. A correct injection was confirmed when a 10–15 minutes' bilateral hind limb motor deficit developed within 1 min after the injection (Chan et al., 2021; Hattori et al., 2019; Sahu et al., 2022). The optimal administration dosages of siRNA were selected based on previous studies.

3.3.6 Preparation of liposomal formulation

3.3.6.1 Preparation of blank and TRPA1 siRNA based liposomal formulation

1,2-Dioleoyl-3-trimethylammonium propane (DOTAP)/cholesterol lipid-nanoparticle were prepared by the thin-film evaporation method. Briefly, liposomes were prepared using a thin-film hydration method. Then IQ, DOTAP, and cholesterol were dissolved in methanol at concentrations of 0.075, 1.5, and 0.83 mg, respectively. Using a rotary evaporator, the lipid mixture was kept under a vacuum condition for 3 h at 30◦C to completely remove the organic solvent. The thin film was hydrated with RNAase-free water containing the desired amount of indocyanine at 50◦C for 1 h. The suspension was subsequently probe sonicated at 40% power for 1 min to obtain the naked liposomes. The unilamellar liposomal solution was extruded through a 200-nm and then a 100-nm polycarbonate membrane 10 times using a manual extruder from Avanti Polar Lipid (Alabaster, AL). The cationic liposomes were then complexed to

anionic siRNA (1µg), by spontaneous electrostatic interaction for 30 min at room temperature (Berger et al., 2021).

3.3.7 Characterization of blank and TRPA1 siRNA based liposomal formulation

3.3.7.1 Determination of particle size, polydispersity index and zeta potential

The hydrodynamic diameter, polydispersity index (PDI), and zeta potential of Blank liposome and TRPA1 siRNA loaded formulation were determined by dynamic light scattering (DLS) measurements using Zetasizer (Nano ZS, Malvern Instruments, Malvern, United Kingdom). PBS (pH 7.4) was used as a dispersion medium to prepare samples. DLS analysis was carried out at a scattering angle of 45° to 150° with 8° intervals and a refractive index of 1.10. Sample sonication was carried out using a probe sonicator. The hydrodynamic diameter of the particles was recorded based on the cumulated analysis of intensity distribution. Zeta potential was recorded following laser Doppler velocimetry with an average of 5 measurements per sample in duplicate. The size distribution study was carried out using a Zetasizer equipped with a diode laser operated at 659 nm. All the measurements were carried out in triplicate (Gonçalves et al., 2014).

3.3.7.2 Morphological characterization

We conducted a thorough morphological characterization of both blank and liposome TRPA1 siRNA formulations using various advanced imaging techniques. The bench-top scanning electron microscope (BT-SEM, EVO-SEM, MA15/18), high-resolution transmission electron microscope (HR-TEM) (Technai G2 T20, FEI, USA), and NTEGRA prima atomic force microscope (AFM, NT-MDT Service & Logistics Ltd., Ireland) were employed for this purpose. For HR-TEM imaging, particles were

dispersed in PBS (1 mg/mL), sonicated for 30 minutes, and then transferred to TEM grids. After drying for 12 hours at 40°C, analysis was performed at 200 kV, and images were captured using a 135 mm CCD camera, processed using Digital Micrograph 3.1 software. SEM analysis utilized gold-coated air-dried samples placed on a silica wafer, with images captured at 8-10 kV, a working distance of 4.4 mm, and a spot size of 200 µ. AFM analysis involved spreading a drop of sample dispersion on a thin glass slide using a spin coater, with images captured at 256 x 256 pixels' resolution, 300 kHz cantilever frequency, and a nominal force constant of 40 N/m at room temperature. Additionally, a bench-top scanning electron microscope (Carl Zeiss Microscopy Ltd.) equipped with a TEAM Pegasus integrated energy dispersive spectroscopy system (EDS-EBSD, EDAX Inc., Tokyo, Japan) was employed for dimensional and elemental analysis of blank and liposome. Gold-coated samples were dried at 40°C for 20 minutes using a micro-processed controlled oven, and image processing was carried out at 200 kV with a Mega View III CCD camera. These comprehensive analyses allowed for a detailed understanding of the morphology and composition of the formulated particles.

3.3.7.3 Infra-red (IR) spectroscopy

IR spectrums of dried samples were recorded by the Attenuated Total Reflectance (ATR) method using an IR spectrophotometer (L160000A, Perkin Elmer, USA) at a resolution of 4 cm-1 from 4100 cm-1 to 400 cm-1.

3.3.7.4 X-ray photoelectron spectroscopy (XPS) analysis

X-ray photoelectron spectroscopy was carried out on a PHI 5000 Versa Probe III supplied by ULVAC-PHI Inc., Japan equipped with a monochromatic Al Kα radiation source. XPS spectra were recorded. Calibration was done by using the C 1s

peak at 284.5 eV as an internal standard to identify the characteristic binding energies of lipids. The XPS scanning was conducted for important elements such as Si, O, C, S, P, and N (Hattori et al., 2019).

3.3.7.5 Thermogravimetric analysis

The weight loss of the 5 mg sample was assessed utilizing a thermogravimetric analyzer (TGA-50, Shimadzu, Japan). The measurements were conducted over a temperature range from 25 to 1200°C, with a temperature increment of 10°C per minute.

3.3.7.6 Differential scanning calorimeter analysis

Differential Scanning Calorimetry (DSC) is a thermal analysis technique used to investigate the heat flow associated with physical and chemical transitions in a material as a function of temperature. The DSC is based on the comparison of the heat flow to a sample and a reference material as they both undergo controlled temperature changes. DSC measurements, a sample size of 5.0±0.5 mg was heated up to 550°C in a covered aluminum sample pan at 10°C/min and a flow of 40 ml/min of nitrogen gas

3.3.8 Animal pain behavior tests

3.3.8.1 Tail flick test: Analgesic assay

The test was conducted to assess the analgesic activity of Bergenin in naïve rats (Manning and Mayer, 1995). Rats were gently restrained and a heat source was applied on 4-7 cm from the tail distal end (Ugo Basile, Italy). The cut-off time for each measurement was set at 10 secs to avoid any tissue damage of rats. The heat intensity was standardized by maintaining the average response time between 3-4 seconds. The response time was measured using an automatic detector (Ugo Basile, Italy).

3.3.8.2 Tail clip test: Mechanical threshold

This test is used to assess the mechanical threshold in rodents by subjecting their tail to a noxious stimulus (Xie et al., 2021). Alligator clips are affixed to the tail of the rats. The point at which the rat reaches the clip with its mouth is used as the endpoint measurement. In general, rodents reach and begin licking their tails within 3-4 seconds. Furthermore, the study assesses the potency of Bergenin on whether it can elevate the pain threshold in rats.

3.3.8.3 Pinprick test: Mechanical hyperalgesia

This test was used to evaluate the mechanical hyperalgesia in rats. To perform the test, a 22-gauge needle was securely glued to a von-Frey hair (Xie et al., 2021). This modified device was then gently applied to the hind paw of the rats, with meticulous care taken to prevent puncture of the skin. Each paw underwent ten trials, and a blinded observer recorded the number of paw withdrawals occurring during these ten trials.

3.3.8.4 Hargreaves test: Thermal hyperalgesia

Thermal hyperalgesia was assessed in chemotherapy-treated rats using the Hargreaves apparatus from Ugo Basile in Italy (Hargreaves et al., 1988). To ensure accurate measurements of paw withdrawal latency, the rats were acclimatized to the testing environment and acclimatized on Hargreaves apparatus enclosed in transparent plexiglass chambers. A mobile radiant infrared (IR) beam source was then directed onto the plantar surface of both the right and left hind paws, with a cutoff time of 20 seconds. To ensure precision and reliability, the experiment was conducted in triplicate for each of the experimental groups. Thermal hyperalgesia was evaluated in all experimental

groups both before chemotherapy, after chemotherapy, and before and after the administration of Bergenin at different time intervals.

3.3.8.5 von-Frey hair Test: Static mechanical allodynia

von-Frey hair test was used to evaluate the mechanical allodynia in chemotherapy-treated rats. Mechanical allodynia in rats treated with chemotherapy was evaluated using the von-Frey hair test (Steen et al., 1992). The test involved using a set of von-Frey filaments with different strengths, ranging from 0.40 g to 15 g, to determine the paw withdrawal threshold in rats. To perform the test, the animals were first placed in plexiglass chambers on a stainless-steel mesh floor and allowed to acclimatize to the environment for 15 minutes prior to the experiments. Blunted von-Frey filaments were then applied perpendicular to the sub-plantar region of each rat's hind paw. The up-down method was employed to assess mechanical sensitivity both before and after chemotherapy treatment. A positive response to the von-Frey filament was defined as any of the following behaviours: paw withdrawal, licking, or shaking of the paw. The test began with a lower force of 1.8 g, and if there was no response, a filament with a higher force was used. Conversely, if a positive response occurred, the filament with a lower force was tested. This process was repeated until the maximum and minimum five withdrawal thresholds were determined, corresponding to higher and lower filament intensities, respectively. For all experimental groups, the pre-drug baseline (day 14) and 0.5 hr, 1 hr, 2 hr, 4hr, and 24 hr post-drug/vehicle administration thresholds were assessed for both the right and left hind paws.

3.3.8.6 Paint brush test: Dynamic mechanical allodynia

To evaluate dynamic mechanical allodynia in rats, we performed a paint brush test in accordance with previously reported study (Lim et al., 2022). To ensure minimal stress, rats were handled with care and placed in transparent plexiglass chambers positioned on elevated wire mesh for habituation. A stimulus was applied ten times, with a 5-second interval between each application, and the number of paw withdrawal were recorded. A single cumulative score for mechanical dynamic allodynia was obtained by taking average of total withdrawals observed across three separate trials. This cumulative score ranged from a minimum of 0 to a maximum of 10, providing an effective measure of dynamic mechanical allodynia.

3.3.8.7 Cold plate test: Cold hyperalgesia

Rats were subjected to an experimental setup involving a transparent plexiglass chamber equipped with an ice-cooled floor (D. Zhang et al., 2020). A precise temperature sensor was placed on the surface of the floor to continuously monitor and regulate temperature, which was maintained within a range of 4°C ± 2°C throughout the experiment. Moderate ambient illumination was provided throughout the entire duration of the assessment. The experimental procedure was captured via video recording for a duration of 1 minute, with subsequent analysis carried out by a blind experimenter. Paw latency and licking responses were assessed as indicators of nociceptive behaviour. These measurements were obtained at various time points: pre-drug baseline (day 14), and at 0.5 hours, 1 hour, 2 hours, 4 hours, and 24 hours following the administration of the test drug or vehicle in neuropathic rats.

3.3.8.8 Acetone evaporation test: Cold allodynia

Cold allodynia was assessed using the acetone evaporation test in rats (Chukyo et al., 2018). Prior to testing, rats were habituated to plexiglass chambers on von-Frey mesh. Subsequently, a 100 µl droplet of acetone was sprayed to the dorsal surface of the hind paw. This procedure was conducted in triplicate to assess the cold allodynia responses. Each trial was recorded through video recording for a duration of 1 minute and assessed by a blind observer who was unaware of the treatment conditions. The scoring system employed encompassed four distinctive response levels: 0, signifying no response; 1, single instance of paw flicking; 2, representing repeated paw flicking; and 3, signifying recurrent paw flicking coupled with licking behavior.

3.3.8.9 Conditioned place preference test: Spontaneous ongoing pain

Spontaneous ongoing pain was assessed using a Conditioned Place Preference (CPP) apparatus, following a previously established protocol (Little et al., 2015; Van Acker et al., 2014). The CPP apparatus consisted of three chambers, denoted as A, B, and C. Chamber 'C' served as a corridor connecting chambers A and B, with each of these chambers presenting distinct visual and tactile cues to rats during the preconditioning, conditioning, and post-conditioning phases. Video tracking of rat behavior was conducted using Any Maze software (Version 7.0, Stoelting, USA). The experimental procedure commenced with a two-day habituation phase. Rats were acclimated to the testing room for 30 minutes on each of these days, followed by an additional 30-minute period of acclimatization within the CPP apparatus. On the subsequent day, a preconditioning was performed for each rat by video recording their chamber preference prior to drug intervention. During this preconditioning phase, rats

were permitted to freely explore all chambers, and their behavior was recorded for 15 minutes to quantify the time spent in each chamber. Rats exhibiting a preference of less than 20% or more than 80% for any particular chamber were excluded from further testing. The conditioning phase spanned two days and comprised two sessions each day. In the morning session, animals received a vehicle administration and were confined to one of the chambers for 30 minutes. Four hours later, a second session was conducted, during which animals were administered drugs and placed in a different chamber within the CPP apparatus for 30 minutes. During the post-conditioning trial, rats were gently placed in the corridor and given unrestricted access to all chambers. This post-conditioning trial was video-recorded for 15 minutes, and the time spent by each rat in each chamber was recorded. The results were presented as a difference score, reflecting the changes in chamber preference following drug conditioning.

3.3.9 Behavioral neurotoxicity assays

3.3.9.1 Rota-rod test

Motor coordination in rats was evaluated through the rota-rod test, both prior to and following drug administration (Lopes et al., 2013). On the training session, the rats underwent training on a rota-rod apparatus (Orchid Scientific, India) characterized by a gradual acceleration from 5 to 30 revolutions per minute (rpm) over a duration of 180 seconds. Subsequently, their performance on the rota-rod testing was assessed in two phases: first, pre-drug, and then 30 min post-drug administration. During the testing, the rats were subjected to a consistent speed of 25 rpm. The primary measure of interest was the total duration (in seconds) each rat was able to remain on the accelerating rod without experiencing a fall.

3.3.9.2 Open field test

Open field test was performed to measure the effect of Bergenin on the locomotor activity of rats (Fernandes and Gupta, 2019). Briefly, habituation was carried out to acclimatize the rats to the new environment by placing them in open field apparatus for 15 minutes. On the day of testing, rats were placed in the open field apparatus (45x45x75cm) and the session was video recorded for 10 minutes. Total distance travelled and average speed of rats was calculated and analysed by using Any Maze software (Version 7.0 Stoelting, USA).

3.3.10 Tissue harvesting and storage

After completion of all behavioral studies, animals were subjected to humane euthanasia. A longitudinal incision was made above the vertebral column, allowing for the exposure and subsequent removal of the spinal cord using a rongeur and Dumont forceps, thereby achieving complete spinal cord exposure. The lumbar region of the spinal cord was meticulously identified and isolated. Additionally, the dorsal root ganglia and sciatic nerve tissues of rats were harvested. All harvested tissues were preserved at -80°C for subsequent biochemical and molecular analyses.

3.3.11 Biochemical assays

3.3.11.1 Lipid peroxidation

Malondialdehyde (MDA) is a byproduct resulting from the peroxidation of lipids, and its quantification was conducted utilizing the Thiobarbituric Acid Reactive Substances (TBARS) assay (Krishnaraju et al., 2009). This analytical method relies on the formation of a pink-colored complex between MDA and TBA. Specifically, a mixture containing acetic acid, sodium dodecyl sulfate (SDS), and thiobarbituric acid

was combined with the sample supernatant, and the resulting solution was subjected to a heating process at 100°C for one hour. Following this heat treatment, the samples were allowed to cool before being transferred to a 96-well plate, and the absorbance was measured at 532 nm using a multimode microplate reader. The concentration of MDA was expressed in micromoles per milligram of tissue protein. A predetermined standard curve equation was used to calculate the amount of MDA present in per mg of tissue protein.

3.3.11.2 Nitrite estimation

Nitrite levels were quantified utilizing the Griess reagent as an indicator of nitrosative stress within cellular systems (Chopra and Tiwari, 2013). The Griess reagent was blended in equimolar proportions with the test samples and subsequently incubated for a 30-minute duration at ambient temperature. The resulting mixture was then subjected to absorbance measurement at 540 nanometers using a microplate reader, enabling the determination of nitrite concentration in units of micromoles per milligram of tissue (µM/mgpr). Levels of nitrite per mg of protein were calculated using an equation that has been derived from the standard curve.

3.3.11.3 Glutathione estimation

Glutathione (GSH) serves as a vital antioxidant enzyme that shields the cell from such oxidative harm (Ertilav et al., 2021). Assessing the levels of reduced glutathione indirectly provides insight into the pathological state of the cell. To determine reduced glutathione levels, a process involves mixing tissue lysate with 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB) and subsequently incubating this mixture until a color change to yellow occurs, typically at a temperature of 48°C. Subsequently, the

absorbance of the resulting solution is quantified at a wavelength of 412 nm using a multimode microplate reader. The concentration of GSH is then expressed in units of micromoles per milligram of protein within the tissue sample.

3.3.11.4 Quantification of superoxide dismutase (SOD)

Superoxide dismutase (SOD) enzymes constitute a vital class of catalysts that facilitate the dismutation of superoxide radicals, playing a critical role in the modulation of CINP. The assessment of SOD activity was conducted following a specific experimental protocol (Borzan and Meyer, 2009). This protocol involved the utilization of the xanthine-xanthine oxidase system to generate a flux of superoxide radicals, with nitro blue tetrazolium (NBT) employed as a marker to indicate the production of superoxide radicals. The activity of SOD was quantified based on the degree of inhibition exhibited by the enzyme in reducing NBT by 50%. In brief, a reagent solution was prepared by dissolving 50 mM of anhydrous sodium carbonate (Na_2CO3), 0.1 mM of ethylenediaminetetraacetic acid (EDTA), and 25 µM of nitro blue tetrazolium (NBT) in phosphate-buffered saline (PBS) at a pH of 7.4 (0.1 M). For the experimental procedure, 100 µL of the prepared reagent, 25 µL of hydroxylamine hydrochloride ($NH_2OH{\cdot}HCl$), and 50 µL of the supernatant were thoroughly mixed. Absorbance readings were recorded at 570 nm at regular intervals over a 3-minute period. The results were subsequently expressed in units per milligram of protein (U/mgpr).

3.3.12 Evans blue assay

Blood-spinal cord barrier (BSCB) integrity was evaluated by performing the Evans Blue assay (West et al., 2018). Rats from each experimental group (n=6) received a tail vein injection of 2% Evans Blue solution dissolved in sterile saline, administered

at a dosage of 1 mL per kilogram on the 14th day following the final chemotherapy injection. Subsequently, a two-hour circulation period was allowed for the dye to distribute systemically. To ensure the animals were adequately anesthetized, a combination of ketamine and xylazine was administered. Following anaesthesia, a continuous infusion of 180 mL of normal saline, delivered at a rate of 60 mL per minute, was transcardially introduced. This procedure aimed to prevent any potential vascular injury. The lumbar segment of the spinal cord was meticulously dissected and promptly preserved at -80°C for subsequent analysis. Tissue samples were homogenized in 400 µL of dimethylformamide (DMF) and incubated for a duration of 3 days at 50°C. The resulting lysates were subjected to centrifugation for 30 minutes at 14,000 rpm to collect the supernatant. For the quantification of extravasated dye, 200 µL of the supernatant was employed in duplicate within a 96-well plate. Optical density (OD) readings at 620 nm were recorded using a multimode plate reader. The determination of the Evans Blue concentration in the samples was achieved by reference to a standard linear curve, allowing for a precise assessment of the amount of extravasated dye.

3.3.13 Molecular biology studies

3.3.13.1 Western blot analysis

DRG and spinal cord tissue lysates from rats were prepared using radio-immunoprecipitation assay (RIPA) buffer (Table 3.3). The estimation of protein in each tissue sample was done by the Bradford method (Wang et al., 2019). Briefly, the 198µl of Bradford reagent was added to the 2µl of sample in 96 well plates followed by 10min incubation at room temperature and absorbance was taken at 595 nm using a multimode plate reader. The protein concentration in each sample was determined using the standard curve plotted with bovine serum albumin (BSA).

Table 3.3 Composition of RIPA buffer

S.No	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

The samples were prepared by mixing the 20µg of protein with loading buffer (Recipe shown in Table 3.4) and volume was made up to 20µl with deionized water. Next, the samples were heated on a dry bath at 100°C for 5 mins and briefly centrifuged at room temperature. The gel was set for electrophoresis and an equal amount of sample (20µl) and protein ladder (3µl) were loaded into the wells of polyacrylamide gel and protein were allowed to separate using Sodium Dodecyl Sulphate-PolyAcrylamide Gel Electrophoresis (SDS-PAGE) with running buffer (Recipe is given in Table 3.5). The initial voltage was maintained at 70 V till the lanes reached the resolving buffer and then made run at 90 V.

Table 3.4 Loading buffer recipe

S.No	Composition	Concentration
1	SDS	4%
2	2-mercaptoethanol	10%
3	Glycerol	20%
4	Bromophenol blue	0.004%
5	Tris HCl	0.125 M

Table 3.5 Running buffer recipe

S.No	Composition	Concentration
1	Tris	25mM
2	Glycine	190mM
3	SDS	0.1%

After the gel electrophoresis, the proteins were transferred to PVDF membranes using transfer buffer (Biorad, USA). Briefly, the transfer stacks and nitrocellulose/PVDF membranes were soaked into the transfer buffer and a sandwich of PVDF and gel was prepared. The cassettes were loaded into the transblot system and protocol was made run at 0.7amp, 15V for 45 mins. After the completion of the protein transfer, the membranes were blocked to reduce the nonspecific binding using 3% BSA solution prepared in TBST (tris buffer saline-tween-20) over the rocker system for 2hr. Membranes were washed with TBST for 3 times using an orbital shaker for 5 min each. Further, the membranes were incubated with primary antibodies at 4°C for overnight. Following incubation, membranes were washed with TBST and incubated with their respective secondary antibodies which are conjugated with HRP for 2hr. Immune complexes formed were detected using ECL (chemiluminescent agent) (Invitrogen, Carlsbad, California, United States). Blots were visualized in the ChemiDocTM system (ChemiDoc^{TM}, BioRad, Hercules, California, United States) and quantified band density using Image J software.

3.3.13.2 Reverse transcription polymerase chain reaction (RT PCR) analysis

Total RNA was isolated from the spinal cord and DRG tissues using the Trizol reagent method. Briefly, desired trizol was added to the samples, and tissue was homogenized using an automated homogenizer at an ice-chilled platform. The resulting

mixture was kept at room temperature for 10 mins and then chloroform was added followed by centrifugation at 12000g at 4°C. Next, the supernatant was collected and an equal amount of isopropyl alcohol was added to the same followed by incubation for 10 min at RT and centrifugation at 12000g at 4°C for 15 min. The observed pellet was RNA content and washed with 75% ethanol twice to remove organic contents. Finally, the resulted pellet was dissolved in nuclease-free water and total RNA was quantified using NanoDrop (Thermo Scientific).

Further, cDNA was prepared using commercially available cDNA Synthesis kit (K1622 Thermo scientific) by mixing the template RNA, random primers, reaction buffer, RNAse inhibitor, dNTP mix, revert aid reverse transcriptase and final volume was made up using nuclease-free water. The cDNA synthesis reaction was thermocycled for 60 min at 45°C and terminated at 70°C for 5 min. A reaction master mix of 15µl was prepared by mixing forward primer, reverse primer, template cDNA, Maxima SYBR Green/Fluorescein qPCR Master Mix, and nuclease-free water. The run was performed on Rotor-Gene Q (Qiagen, Germany). Table 3.6 shows the list of used primers for RTPCR.

Table 3.6 Primers used in RT PCR analysis

S.No	Gene	Sequence	Primer 5'<-----Sequence----->3'
1	GAPDH	Forward	CAGTGCCAGCCTCGTCTCAT
		Reverse	CAAGAGAAGGCAGCCCTGGT
2	SUBSTANCE P	Forward	CTGGTCCGACAGTGACCAAA
		Reverse	CTTTCATAAGCCATTTTGTGA
3	NR2B	Forward	GGCAGGGGCGTCAAAAACAA
		Reverse	CACACAGGGGTTGGACTGGT
4	CGRP	Forward	CAGCCATCCTCCACTGACCT
		Reverse	ACCACCTCCTCAGCCACTTT
5	TRPA1	Forward	TGGCAGTTGGGGACATTGCT

		Reverse	CCGTGCCTGGGTCTATTCGG
5	TRPV1	Forward	ACGACGATCCTTTCGGAACT
		Reverse	TCCTCTCTGTTTCGGTTGCT
6	TRPM8	Forward	AGAAGCTCTTGGCTGTTTGAGC
		Reverse	TTTCTGCGGCTCCTCATGCT
7	TNF-α	Forward	GTAGCCCACGTCGTAGCAAAC
		Reverse	ACCACCAGTTGGTTGTCTTTGA
8	IL-1β	Forward	CCTATGTCTTGCCCGTGGAG
		Reverse	CACACACTAGCAGGTCGTCA
9	IL-6	Forward	TCTGGTCTTCTGGAGTTCCGTT
		Reverse	GAGAGCATTGGAAGTTGGGGT
10	Occludin	Forward	TCTGGTCTTCTGGAGTTCCGTT
		Reverse	GAGAGCATTGGAAGTTGGGGT
11	Claudin-5	Forward	TCTGGTCTTCTGGAGTTCCGTT
		Reverse	GAGAGCATTGGAAGTTGGGGT

3.4 *In-silico* study

3.4.1 Homology modeling

3.4.1.1 NCBI Database and Sequence Retrieval

We used NCBI database (https://www.ncbi.nlm.nih.gov) to examine the available information of target proteins i.e. TRPV1, TRPA1, and TRPM8. The three-dimensional structure of rat's TRPA1 and TRPM8 were not available in the protein data bank. Therefore, we constructed 3D structures for both proteins using homology modelling employing multiple software including Swiss-Model, Raptor X, and Robetta (Kim et al., 2004). The exact amino acid sequences for target proteins were obtained from the UniProt database (www.uniprot.org). TRPA1 and TRPM8 have six and four predicted protein sequences, respectively. Q6RI86-TRPA1 and Q8R455-TRPM8 were chosen for further investigation. The amino acid sequence information was downloaded in FASTA format. The crystal structure of rat TRPV1 was available in the protein data

bank with PDB ID-3J5R and the same was used for binding interaction studies with Bergenin.

3.4.1.2 Sequence Alignment

TRPA1 and TRPM8 amino acid sequences were analyzed using the NCBI database BLASTp tool www.blast.ncbi.nlm.nih.gov and the UniProt database. To evaluate the similarity index, we conducted a correlation analysis with the OSM-3 protein data https://www.rcsb.org/structure/6PQP and https://www.rcsb.org/structure/7WRE respectively. The preliminary BLAST optimization was performed on the non-reducing database to isolate the information from the available sequence. Thereafter, a second BLAST was run to assess the degree of similarity between the sequences in the protein data bank www.rcsb.org. The percentage similarity index was calculated according to previous methods for further analysis.

3.4.1.3 Structure prediction and homology modelling of TRPA1 and TRPM8

The SWISS-MODEL repository contains models generated by a fully automated homology modelling approach. Homology modelling typically includes the following steps: template identification, synchronization of target sequence and template structure, model construction, energy minimization and/or refining, and model quality assessment. The 3D structure of a query protein through the sequence alignment of template proteins. The amino acid sequence of TRPA1 and TRPM8 was searched to identify suitable templates. Based on the sequence alignment between a target protein and the template structure, a 3D structure model was constructed. The similarity between the designed 3D structures was validated using BLASTp, Robetta, RaptorX, and SWISS models (Hagens et al., 2020), hence confirming the suitability of designed

templates for further analysis. SWISS-MODEL (https://swissmodel.expasy.org) a structural bioinformatics tool was used to evaluate the selected model of TRPA1 and TRPM8 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 (Sahay et al., 2020). In the current study, 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 the differentiation of unfavourable and favorable regions was also predicted. The residues falling outside the favoured zone were considered to be unfavourable outliers. Later on, the outliers in the generated model were used to analyze the confirmations of the confidence scores. We have used the visualizer tool UCSF chimera 1.13.1 to predict and modify the 3D structures of the rats' proteins TRPA1 and TRPM8. Finally, we have assessed the accuracy and performance of the designed model by using ERRAT (Ghosh et al., 2021) and ProCheck (Farid et al., 2022).

3.4.1.4 Structure-based virtual screening

The generated 3D structures of TRPA1 and TRPM8 as well as the PDB of TRPV1 proteins were subjected to molecular docking to assess the binding interaction between Bergenin with targeted proteins. The Maestro interface of the Schrodinger tool was used to carry the docking studies further, docking score and binding affinity of Bergenin to TRPV1, TRPA1 and TRPM8 were recorded. To obtained findings were quantified or analysed using Glide-XP to interpret the detailed interaction of Bergenin and targeted proteins.

3.4.1.5 Molecular dynamics simulation NCBI Database and Sequence Retrieval

To validate *in-silco* docking prediction, we have performed molecular dynamic interaction between Bergenin and TRPV1, TRPA1 and TRPM8. We have used the Desmond molecular dynamic program. The complex of TRPV1, TRPA1 and TRPM8 protein and Bergenin were deposited in the dynamic simulation suit for 100 ns simulation time using Desmond suits Schrodinger as described previously. Briefly, the ligand and protein complex of Bergenin and targeted proteins were kept inside the orthorhombic box with a dimension of 10 x 10 and the solvated system was a buffer of 10 Å TIP3P. Furthermore, 0.15 molar Na+ and Cl- ions were taken to neutralize the net charge of the simulation system. OPLS3e was used to produce a force field for the system (Schrödinger, 2022). The system was relaxed for the MTK-NPT simulation and in the beginning stage of the techniques, the system required two minimizations which have 2200 steps. The ligand and protein complexes were restrained with a force constant at 50 kcal A°, after complete release of the system without any restraint, the first Brownian dynamics NVT simulation was applied with 10K temperature and 100 PS in the meantime. NVT simulation was successfully applied for their limited time procedure with 300K temperature and 1B pressure by using a Nose-Hoover chain thermostat and Berendsen- thermostat- barostat respectively. A final simulation was applied to the system at 25NP/NPT ensemble at 300K and 1013 bar and a production simulation of a 100 ns run was carried out. Resultant the simulation interaction diagrams and total simulation analysis were subjected to quantify the MD trajectory such as contact with residues and binding energy of protein-ligand complexes of TRPV1, TRPA1 and TRPM8 with Bergenin. Moreover, REPSA integrator and PME methods were used to calculate long-range electrostatic interaction and within a 2s time

interval. The coordinates and energy were kept in the trajectory at every 10ps (Morris and Corte, 2021).

3.5 Statistical analysis

Behavioral data was analyzed using one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test and two-way ANOVA followed by Bonferroni's multiple comparisons. For two-way ANOVA we used a repeated measure model of statistical analysis. Microsoft Excel was used to interpret the raw data and calculate the mean, standard error mean (SEM), % maximum possible effect (MPE) for behavioral data, fold changes for RTPCR and band density for western blot data respectively. Molecular studies data was analyzed by using one-way ANOVA followed by Tukey's multiple comparison test. GraphPad Prism 8.0 was used to perform the statistical analysis. Data was presented as mean ± SEM. $P<0.05$ was considered statistically significant. The sample size was calculated using Gpower statistical analysis tool based on our previous studies (Sharma et al., 2018; Uniyal et al., 2021a).