CHAPTER 5: Modulation of TRPA1/TRPV1/NR2B signaling by Bergenin in a Rat Model of Chemotherapy-induced Neuropathic Pain

5.1 Introduction

Chemotherapy-induced neuropathic pain is one of the most prevalent somatosensory disorder associated with anti-cancer drug therapy (Baron et al., 2023; Luo et al., 2021). Chemotherapeutics may induce persistent bilateral neuropathic pain in cancer patients receiving treatment with first-line antineoplastic agents for breast, ovarian, and testicular cancer (Brandolini et al., 2022). Despite significant efforts over the last few decades, there are currently no US-FDA-approved drugs for the treatment of CINP and currently available therapeutics in the clinic do not produce effective analgesia also coupled with several severe side effects (Uniyal et al., 2022a). Therefore, there is an unmet need for the discovery of novel analgesics for the treatment of CINP devoid of classical side effects associated with opioids and other non-opioid drugs.

Several members of the TRP family are found to be involved in the development and progression of pain modalities during CINP. It was reported that treatment of cultured DRG neurons with oxaliplatin increases the mRNA expression of TRPV1, TRPA1, and TRPM8 (Wu et al., 2019). In addition, paclitaxel-induced cold and mechanical allodynia are found to be associated with increased TRPA1 and TRPV1 expression in the DRG neurons of mice, rats, and humans. These findings suggest that the activation and/or upregulation of members of the TRP family is critical for development of allodynia during chemotherapy-induced neuropathic pain.

Numerous scientific reports have consistently indicated that Bergenin exhibits a diverse array of pharmacological properties including anti-inflammatory, antioxidant, and neuroprotective effects (Tang et al., 2021). These properties stem from Bergenin's capacity to intricately modulate various molecular targets, encompassing NF-κB, TNF-α, Nrf2, and BDNF. By engaging with these intricate pathways, Bergenin demonstrates the potential to mitigate neuro-inflammation, counteract oxidative stress, and have neuroprotective potential (De Oliveira et al., 2011; Pu et al., 2002; Villarreal et al., 2020). Therefore, the present study was designed with the aim to investigate the effect of Bergenin on chemotherapy-induced neuropathic pain and dissect the mechanism of action involved in its analgesic activity.

Several preclinical findings demonstrated the role of TRP channels including TRPA1, TRPV1 and TRPM8 as major contributors to thermal, mechanical and cold hypersensitivity during chemotherapy-induced neuropathic pain. TRPA1 activation and sensitization is accompanied by increased expression of TRPV1, calcitonin gene-related peptide (CGRP), Substance-P and release of pro-inflammatory cytokines in the DRG and spinal cord tissues Another study demonstrated the upregulation of TRPA1 and TRPV1 co-expression in oxaliplatin-induced neuropathic pain conditions and inhibition of both polymodal nociceptors attenuates pain-like behaviour in CINP animals (Chukyo et al., 2018). Treatment with paclitaxel, bortezomib and cisplatin led to an increase in expression of both TRPA1/TRPV1 mediated mitogen-activated protein kinases (MAPK) pathways followed by the release of pro-inflammatory cytokines and chemokines resulting in neurogenic inflammation (D. Zhang et al., 2020). N-methyl-D-aspartate receptors (NMDARs) are excitatory receptors, and a subset of this receptor unit, NR2B, is essential for NMDAR localization within the synaptic

membrane and plays a vital role in learning, memory, and nociceptive pain. Central sensitization is a key feature for the pathogenesis of peripheral neuropathy induced by NMDAR hyper activation at the spinal and supraspinal levels. NMDAR is known to transmit potent nociceptive signals by interacting with TRPA1, TRRPV1, and TRPM8 nociceptors (Uniyal et al., 2022b).

Bergenin is a C-glucoside of 4-O-methyl gallic acid found naturally in a diverse range of plant species. In the present study, we have investigated the effect of bergenin in animal model of chemotherapy-induced neuropathic pain and dissected the detailed mechanism of action of bergenin involving modulation of TRP channels-mediated NR2B signalling in DRG and spinal cord of the neuropathic rats. Finally, in alignment with our *in vivo* findings, we have further conducted *in-silico* studies to probe the interactions between Bergenin and TRP channels.

5.2 Experimental Design

In this study, adult male Sprague Dawley rats weighing 200-250 g were employed to investigate the impact of chemotherapeutic agents (paclitaxel, cisplatin, and vincristine) on peripheral neuropathy. The chemotherapeutic drugs were administered intraperitoneally (i.p), and a combination of these drugs was prepared by dissolving 4% DMSO in sterile saline. The combined chemotherapy was injected on alternate days (days 0, 2, 4, and 6), while control rats received the vehicle only.

Rats were randomly divided into six groups (n=8-10 rats per group). Group 1 served as the healthy control, group 2 received combined chemotherapy followed by vehicle treatment, and groups 3, 4, and 5 received combined chemotherapy along with Bergenin at doses of 25, 50, and 100 mg/kg, i.p. Additionally, group 6 animals were administered

combined chemotherapy followed by treatment with gabapentin (60 mg/kg, i.p). Baseline pain behavior responses were recorded before administering the chemotherapeutic drugs and on the 14th day post the last injection of combined chemotherapy.

At the end of the study animals were sacrificed, and tissues including dorsal root ganglion (DRG), lumbar region of the spinal cord, and sciatic nerve were harvested and stored at -80°C for subsequent biochemical and molecular biology assessments. in alignment with *in-vivo* experiments, computational techniques were employed to explore Bergenin's binding affinities with transient receptor potential (TRP) channels specifically TRPA1, TRPV1, and TRPM8, known for their roles in sensory and pain perception. Molecular docking and other energy calculation were done subsequently, advanced computational simulations, spanning 100 ns, were conducted to observe the molecular dynamic interactions between Bergenin and these channels by using Schrodinger suit. The simulations provided insights into the stability of the protein-ligand complexes, including information on binding energies, residue contacts, and dynamic behavior.

5.3 Results and discussion

5.3.1 Assessment of general toxicity

During the study, animals subjected to combined chemotherapy and Bergenin were closely monitored for any indications of toxicity and mortality. The results revealed a mortality rate of 10% among animals that received combined chemotherapy throughout the entire experimental period. However, noteworthy clinical observations such as diarrhea, alopecia, and respiratory distress were not apparent in rats treated with the combined chemotherapy regimen.

5.3.2 Bergenin ameliorates chemotherapy-induced mechanical allodynia (static and dynamic) in rats

Mechanical allodynia is one of the most prominent pain behavior phenotypes in patients on chemotherapy. We have assessed the static mechanical allodynia and dynamic mechanical allodynia using von Frey test and paint brush test in our rat model of CINP. Animals treated with combined chemotherapy exhibited a significant drop in paw withdrawal thresholds (PWT) of both left and right hind paws ($p<0.001$) as compared to their pre-chemo baseline and healthy naïve rats. Treatment with Bergenin at doses of 25, 50, and 100 mg/kg i.p. significantly attenuated PWT in both hind paws of chemotherapy-exposed rats as compared to rats treated with vehicle alone. Utilizing Two-Way ANOVA followed by Bonferroni's multiple comparison test, suggest a significant effect across the experimental groups [$F (35, 432) = 7.98$; $p<0.001$] and various time points [$F (7, 432) = 139$; $p<0.001$] in paw withdrawal thresholds during the von-Frey hair test. The maximum possible effect of Bergenin (100 mg/kg, i.p.) was found to be around 85% at 2 hrs post drug treatment **(Figure 5.1 A-C)**.

Paint brush test was used to investigate the effect of Bergenin on dynamic mechanical allodynia of neuropathic rats. Combined chemotherapy-treated rats develop [$F (35, 432) = 27.46$; $P<0.0001$) dynamic mechanical allodynia as evidenced by significant decrease in paw withdrawal latency post- innocuous stimuli application. Notably, Bergenin (25, 50, and 100 mg/kg i.p) treatment significantly reduced combined chemotherapy-induced dynamic allodynia in rats as exhibited by increase PWL **(Figure 5.1 D-E)**. Two-way ANOVA followed by Bonferroni's multiple comparison tests was employed and we observed statistically significant difference across the groups [$F (45, 452) = 27.46$; $P<0.0001$). Moreover, we did not observe any

significant effect of gabapentin on mechanical dynamic allodynia in combined chemotherapy-treated rats as compared with vehicle administered group.

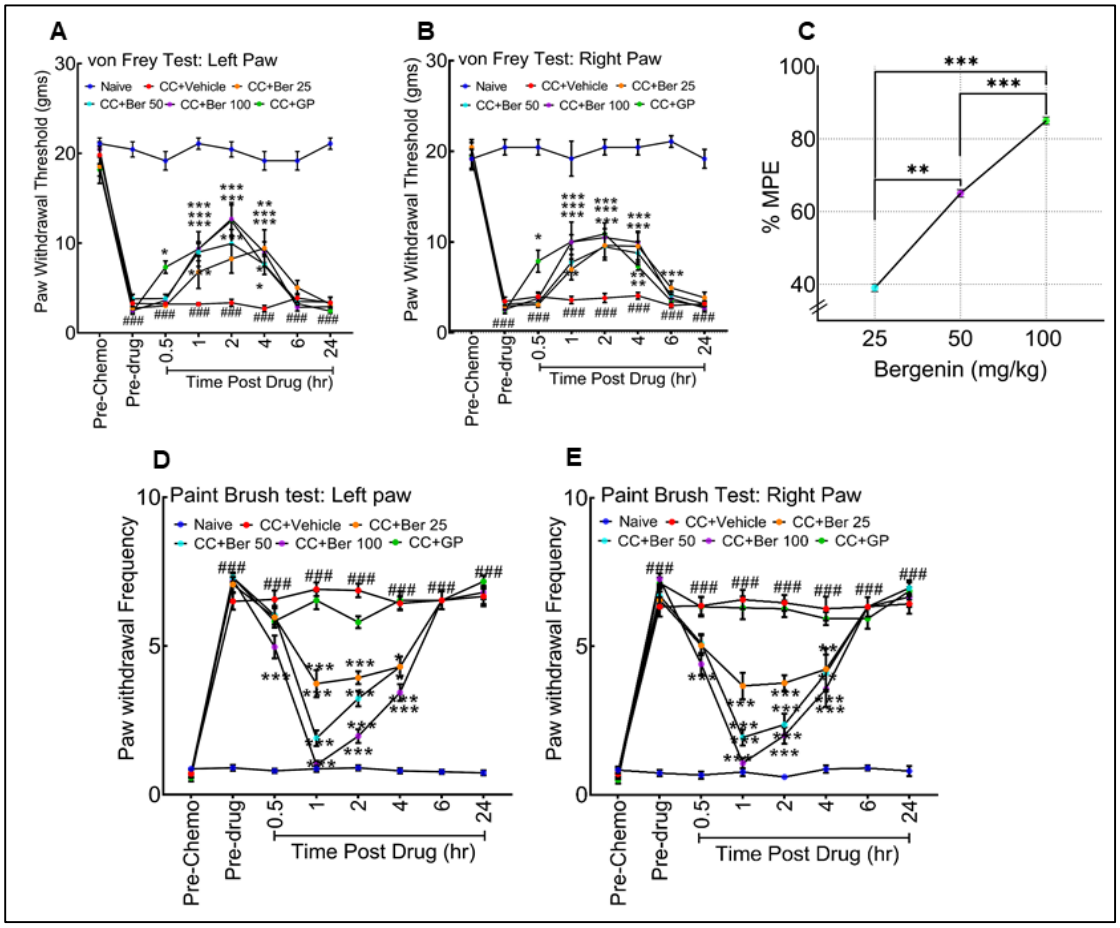

Figure 5.1 Effect of Bergenin on static and dynamic mechanical allodynia in neuropathic rats. von Frey hair test: Chemotherapy induces a significant reduction in paw withdrawal threshold (PWT) of rats compared to the naïve control, which was substantially restored following Bergenin treatment (25, 50, and 100 mg/kg i.p) **A)** left hind paw **B)** right hind paw. **C)** % MPE. **Paint brush test:** Combined chemotherapy-induced significantly decreased paw withdrawal frequency (PWF) of rats as compared to their pre-chemo baseline. Bergenin treatment (25, 50, and 100 mg/kg i.p.) resulted in a significant increase in PWF of chemotherapy induced neuropathic rats as compared to their pre-treatment baseline. **D)** left hind paw **E)** right hind paw. Data were reported as mean ± SEM. $^{###}$P<0.001 indicates statistical significance as compared to the naïve rats. *p<0.05, **p<0.01, and ***p<0.001 indicates statistical significance as compared to the vehicle-treated rats. P<0.05 was considered statistically significant.

5.3.3 Bergenin alleviates chemotherapy-induced thermal hyperalgesia in rats

Thermal hyperalgesia is a notable manifestation of hypersensitivity in chemotherapy patients. Rats treated with chemotherapy exhibited a significant decrease ($p<0.001$) in paw withdrawal latency compared to their pre-chemotherapy baseline and the healthy control group. Treatment with Bergenin at doses of 25, 50, and 100 mg/kg (i.p) significantly mitigated chemotherapy-induced heat hyperalgesia, demonstrated by an increase in paw withdrawal latency (PWL) in neuropathic rats compared to their pre-drug baseline (**Figure 5.2 A-B**). A substantial effect ($p<0.001$) of Bergenin on thermal hyperalgesia was evident at 1 hour post-Bergenin administration at all doses. The peak effect occurred at 2 hours' post-drug administration and persisted for up to 6 hours post-Bergenin administration, particularly with the highest dose (100 mg/kg, i.p.). Furthermore, the maximum possible effect of Bergenin was determined to be approximately 92% at 2 hours with a dose of 100 mg/kg (**Figure 5.2 C**). These findings underscore the therapeutic potential of Bergenin in alleviating chemotherapy-induced thermal hyperalgesia in rats.

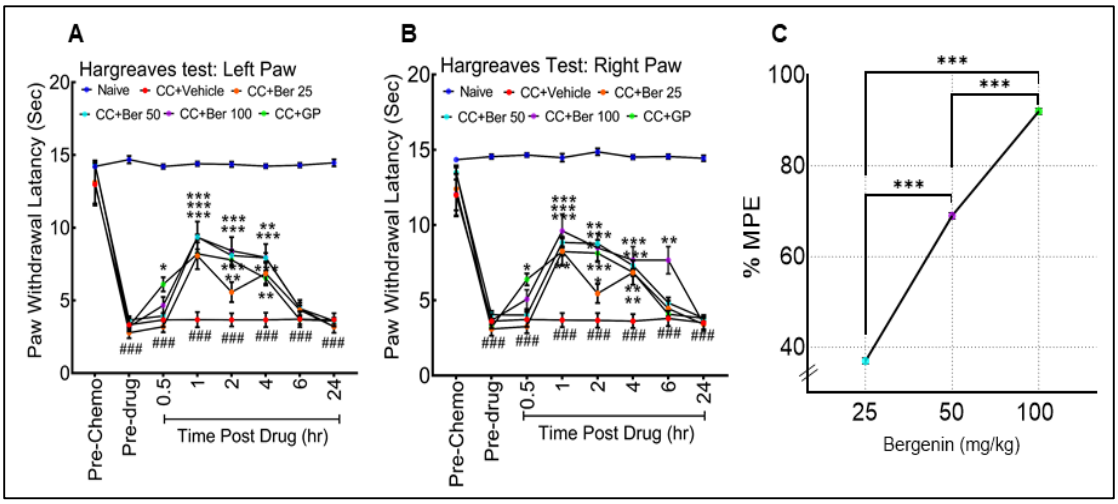

Figure 5.2 Effect of Bergenin on thermal hypersensitivity in rats. **Hargreaves test:** Combination chemotherapy significantly reduced rats paw withdrawal latency (PWL) as compared to their pre-injury baseline. Bergenin (25, 50, and 100 mg/kg i.p.) and

gabapentin (60 mg/kg i.p.) administration resulted in a significant restoration in PWL of chemotherapy-treated rats compared to their pre-drug baseline. **A)** Left hind paw **B)** Right hind paw **C)** % MPE**.** Data were reported as mean ± SEM. $^{###}$P<0.001 indicates statistical significance as compared to the naïve rats. *p<0.05, **p<0.01, and ***p<0.001 indicates statistical significance as compared to the vehicle-treated rats. P<0.05 was considered statistically significant.

5.3.4 Bergenin attenuates mechanical hyperalgesia in rats administered with combination chemotherapy

Combination chemotherapy caused a significant increase in paw withdrawal frequency of rats as compared to naïve rats in pinprick assay. Treatment with Bergenin at doses of 25, 50, and 100 mg/kg (i.p.) resulted in a significant reduction in mechanical hyperalgesia in both the left and right hind paws of rats at 1, 2, and 4 hours post-Bergenin administration compared to vehicle-treated neuropathic rats. Similarly, gabapentin treatment also led to a significant decrease in paw withdrawal frequency in both the left and right hind paws at 0.5 hr, 1 hr, 2 hr, and 4 hr post-drug administration **(Figure 5.3 A-B)**. The application of Two-way ANOVA followed by Bonferroni's multiple comparison test indicated a significant effect across the group interaction for both the left and right hind paws [$F(5,58) = 332$; $p<0.001$], [$F(5,44) = 312$; $p<0.001$] respectively.

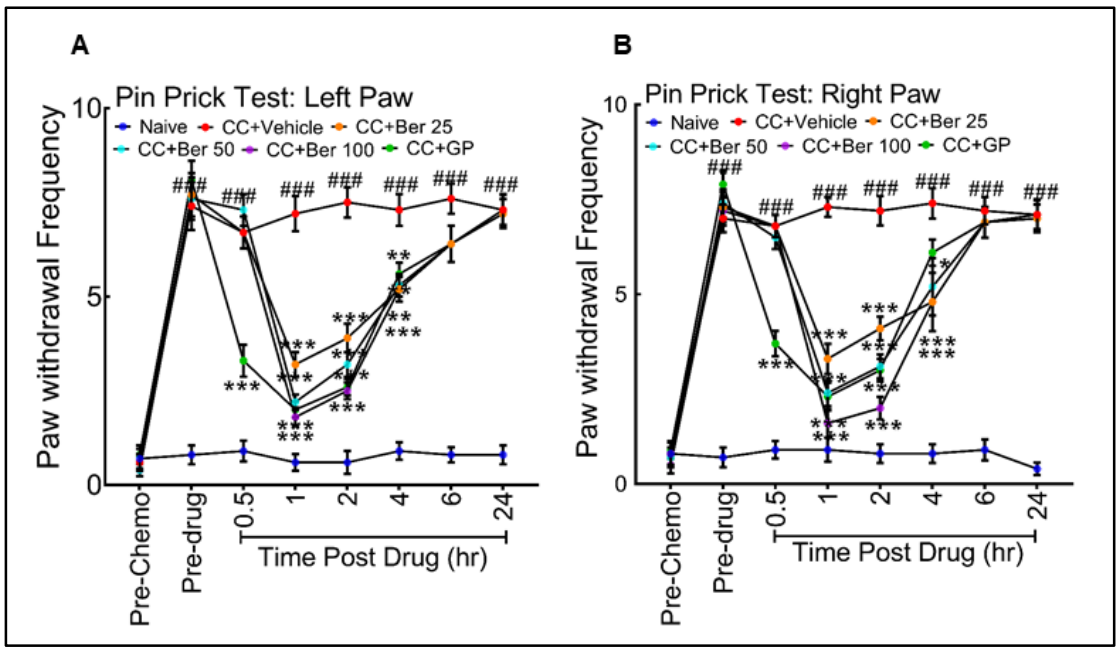

Figure 5.3 Effect of Bergenin on mechanical hyperalgesia in neuropathic rats. Pinprick test: chemotherapy-induced neuropathic pain resulted in increased paw withdrawal frequency (PWF) to noxious mechanical stimuli in rats which was significantly attenuated on treatment with Bergenin (25.50 and 100 mg/kg i.p) and gabapentin (60 mg/kg i.p) as compared to their pre-drug baseline. **A)** left hind paw **B)** right hind paw. Data were reported as mean ± SEM. $^{###}$P<0.001 indicates statistical significance as compared to the naïve rats. *p<0.05, **p<0.01, and ***p<0.001 indicates statistical significance as compared to the vehicle-treated rats. P<0.05 was considered statistically significant.

5.3.5 Bergenin treatment decreased chemotherapy-induced cold pain behavior in rats

Cold allodynia and hyperalgesia were assessed using the acetone test and cold plate test, respectively. The paw withdrawal score of rats significantly increased after chemotherapy administration compared to their pre-chemotherapy baseline and naïve rats **(Figures 5.4 A-B)**. Treatment with Bergenin (25, 50, and 100 mg/kg i.p.) and gabapentin (60 mg/kg i.p.) resulted in a significant reduction in cold hypersensitivity in both the left and right hind paws of rats compared to vehicle-treated animals. Two-way ANOVA followed by Bonferroni's multiple comparison tests indicated a significant effect across the groups' interaction [F (5, 42) = 173; P<0.001] and time

points [F (4.75, 199) = 155; P<0.001]. Our observations revealed that Bergenin treatment led to a significant and dose-dependent alleviation of cold pain-like behavior in rats subjected to chemotherapy. The behavioral findings from our studies align with previous reports indicating a substantial reduction in mechanical and thermal pain hypersensitivity following Bergenin treatment in animal models of diabetes-induced peripheral neuropathy and inflammatory pain. In the cold plate test, chemotherapy administration led to a significant ($p<0.001$) increase in the number of paw lifts compared to the pre-chemotherapy baseline and healthy control rats **(Figures 5.4 C-D).** Interestingly, Bergenin treatment did not result in a substantial decrease in the number of paw lifts and duration of paw licking at any time point. However, gabapentin significantly attenuated the number of paw lifts and duration of paw licking in combined chemotherapy-treated rats compared to vehicle-treated rats. Findings from our behavioral studies are supported by previous reports suggesting notable reduction in mechanical and thermal pain hypersensitivity post-Bergenin treatment in animal models of diabetes-induced peripheral neuropathy and inflammatory pain. Cold allodynia and cold hyperalgesia were assessed using the acetone test and cold plate test respectively. The paw withdrawal score of rats significantly increased after chemotherapy administration compared to their pre-chemotherapy baseline and naïve rats. Treatment with Bergenin (25, 50, and 100 mg/kg i.p.) and gabapentin (60 mg/kg i.p.) resulted in a significant reduction in cold hypersensitivity in both the left and right hind paws of rats compared to vehicle-treated animals. Two-way ANOVA followed by Bonferroni's multiple comparison tests indicated a significant effect across the groups' interaction [F (5, 42) = 173; P<0.001] and time points [F (4.75, 199) = 155; P<0.001].

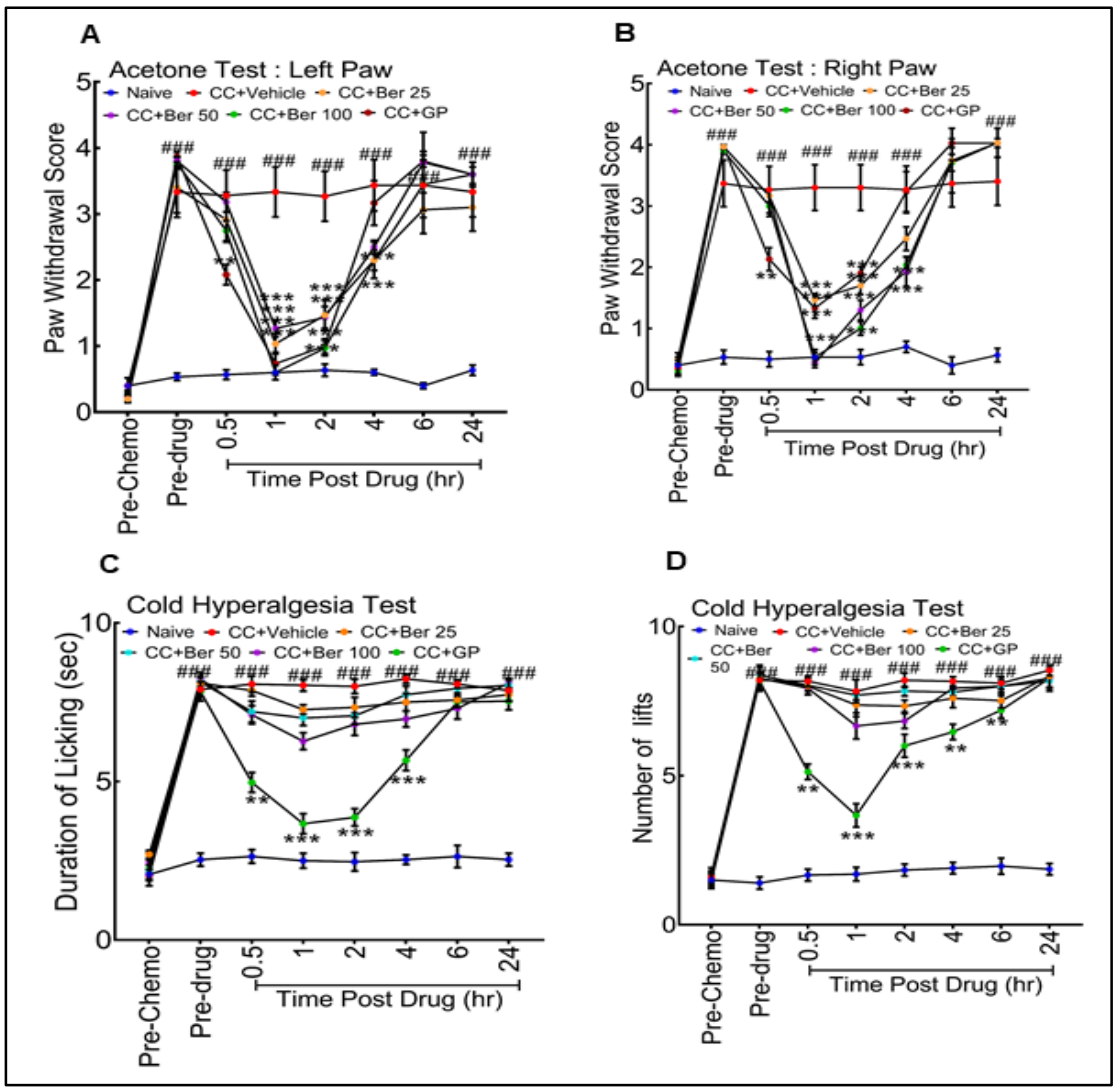

Figure 5.4 Effect of Bergenin on CINP-induced cold hypersensitivity in rats. Acetone test: Chemotherapy-induced significant increase in paw withdrawal score of rats in response to non-noxious cold stimuli. Bergenin (25, 50, and 100 mg/kg i.p.) and gabapentin (60 mg/kg i.p.) treatment leads to significantly decreased paw withdrawal scores **(A)** paw withdrawal score in left hind paws. **(B)** paw withdrawal score in right hind paws. **Cold hyperalgesia test:** Chemotherapy-induced cold hyperalgesia in rats in response to unpleasant cold stimuli. Bergenin (25, 50, and 100 mg/kg i.p.) treatment does not inhibit cold hyperalgesia in chemotherapy-treated rats. However, gabapentin (60 mg/kg i.p.) treatment significantly reduced the frequency of paw lifts and duration of licking in chemotherapy-treated rat to their pre-drug baseline and healthy rats. **(C)** Duration of licking. **(D)** Number of lifts. Data were presented as mean ± SEM. $^{###}P<0.001$ indicates statistical significance as compared to the Naïve rats. *p<0.05, **p<0.01, and ***p<0.001 indicates statistical significance as compared to the vehicle-treated rats. P<0.05 was considered statistically significant.

5.3.6 Bergenin did not altered the pain threshold of healthy naïve rats

To evaluate the impact of bergenin on pain sensitivity in naïve rats, we conducted tail-flick and tail clip tests, using morphine as a positive control. These tests assess the response to noxious thermal and mechanical stimuli, respectively. Analysis via two-way ANOVA, followed by Bonferroni's multiple comparisons, revealed significant effects across groups [$F(25, 252) = 2.675$; $p<0.001$] and time points [$F(5, 252) = 4.559$; $p<0.001$] during the tail-flick test in both naïve and drug-administered rats **(Figure 5.5 A and B)**. Morphine notably increased tail-flick latency compared to naïve healthy rats ($p<0.001$), with the maximal effect observed at 30 minutes ($p<0.001$) and sustained for up to 4 hours.

In the tail clip test, significant effects were observed across groups [$F(25, 252) = 8.11$; $p<0.001$] and time points [$F(5, 252) = 18.88$; $p<0.001$]. Morphine significantly increased latency to mechanical stimuli compared to naïve rats ($p<0.001$), with this effect persisting throughout the 4-hour test duration **(Figure 5.5 C and D)**. However, bergenin administration (25, 50, and 100mg/kg i.p) did not alter nociceptive latency in either test. Similarly, Gabapentin (60 mg/kg, i.p) treatment did not affect pain thresholds compared to naïve rats **(Figure 5.5 D)**. These results collectively suggest that bergenin does not disrupt normal pain thresholds in healthy naïve rats.

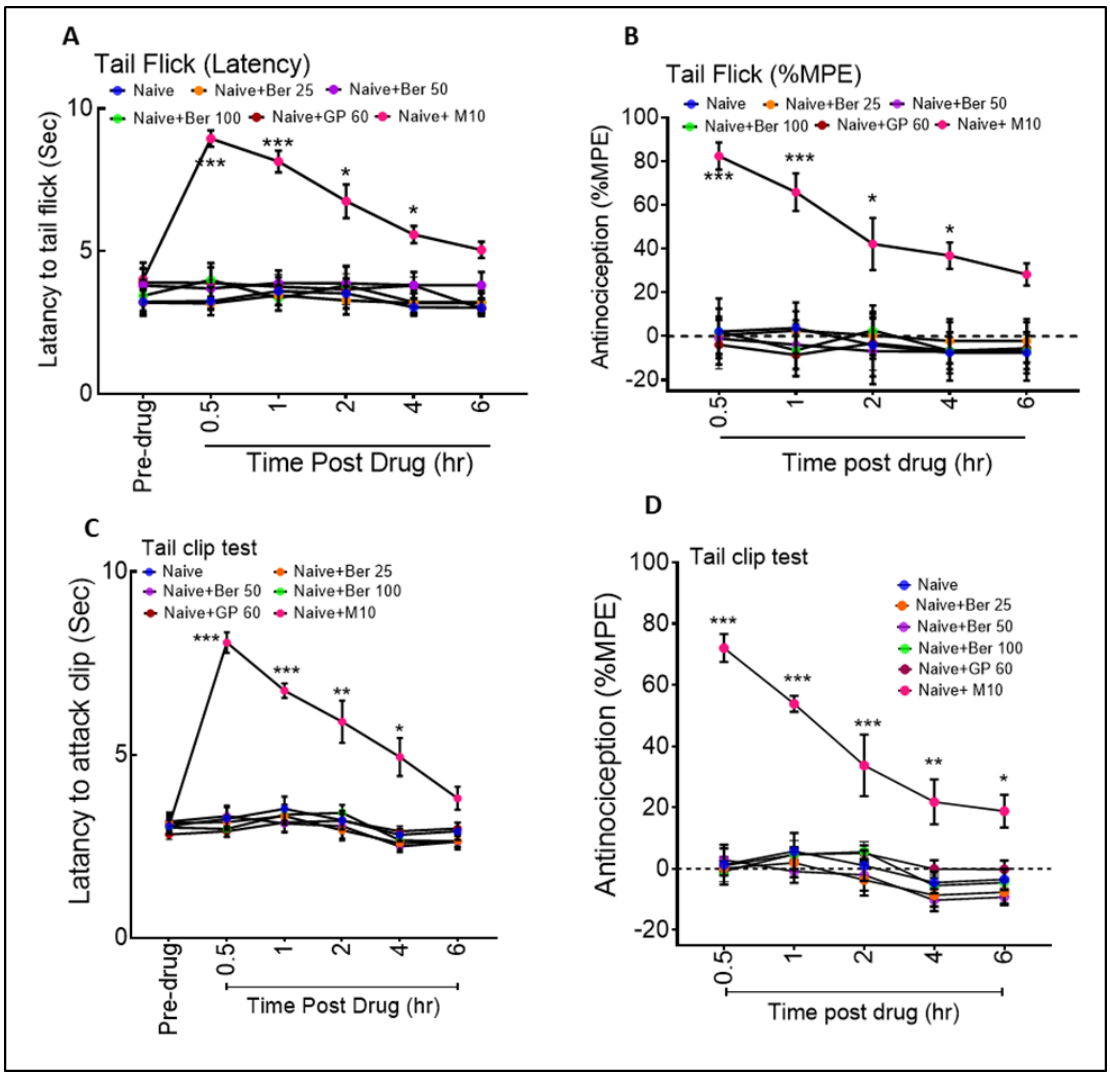

Figure 5.5: Effect of bergenin on basal pain response in healthy naïve rats. (A & B) Tail flick latency and tail flick % maximum possible effect (C & D) Latency to attack clip and percent anitnociception. Data were expressed as Mean ± SEM and analysed by two-way ANOVA (Bonferroni's multiple comparison) (n=8). ### represents significance compared to the naïve group ($p<0.001$), * ($p<0.05$) ** ($p<0.01$) and *** ($p<0.001$) represents significance. $P<0.05$ was considered statistically significant.

5.3.7 Bergenin attenuates spontaneous ongoing pain in neuropathic rats without causing drug addiction

Spontaneous ongoing pain is one of the important components of neuropathic pain and is increasingly being accepted as a parameter of overall well-being in pain patients (Z. Li et al., 2017; Zilliox, 2017). Preclinical studies have almost entirely

focused on evoked hypersensitivity, whereas the existence of spontaneous ongoing pain is a significant concern of patients with CINP. This disparity could be a reason for the poor translation of preclinical findings to clinically useful therapies. Therefore, we have performed the spontaneous ongoing pain assay using the conditioned place preference (CPP) apparatus after Bergenin, gabapentin and morphine (positive control) administration in rats. we have identified bergenin as a compound that demonstrates efficacy in alleviating spontaneous ongoing pain in rats with neuropathic conditions **(Figure 5.6 A-B)**. Bergenin (100 mg/kg *i.p.)* treatment significantly ($p<0.001$) inhibits the spontaneous ongoing pain in chemotherapy-treated rats as demonstrated by their increased preference for Bergenin-paired chamber and decreased preference in saline paired chamber during a post-conditioning. Additionally, there was a significant ($p<0.001$) increase in difference score in the Bergenin-paired chamber compared to the vehicle-paired chamber in neuropathic rats **(Figure 5.6 C).** Most importantly, Bergenin does not produce place preference behavior in healthy rats suggesting place preference behavior of Bergenin is purely dependent on pain relief and does not have any abuse liability potential.

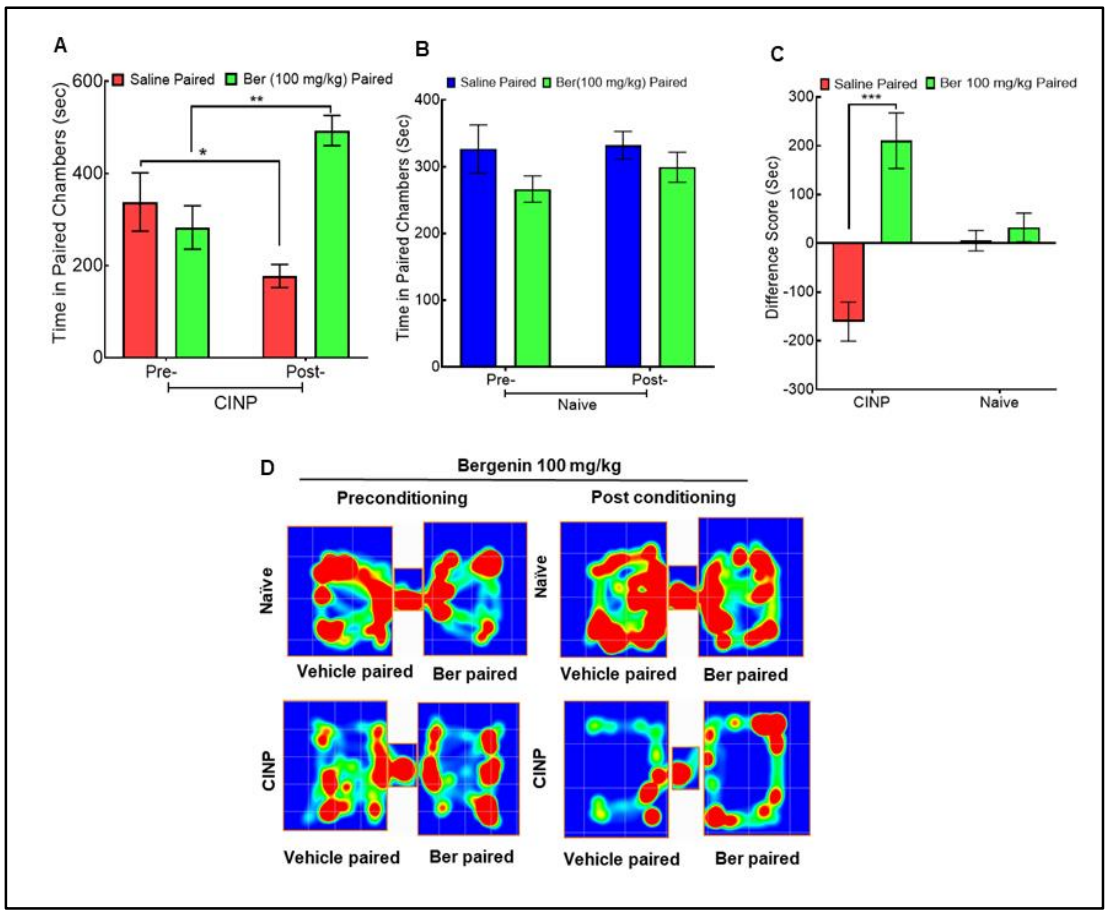

Figure 5.6 Effect of Bergenin on spontaneous ongoing pain in chemotherapy-treated rats. (A, B and C) Conditioned place preference (CPP) for Bergenin: Combined chemotherapy-treated rats showed significant place preference behavior in the Bergenin (100mg/kg *i.p.*) paired chamber as compared to the pre-conditioning baseline. Interestingly, Bergenin (100mg/kg *i.p.*) treatment did not produce CPP in healthy naïve rats. (**D**) Heat maps recorded during preconditioning and post conditioning with vehicle v/s Bergenin (100mg/kg *i.p*) paired chambers. Data were presented as mean ± SEM. $^{###}$P<0.001 indicates statistical significance as compared to the Naïve rats. *p<0.05, **p<0.01, and ***p<0.001 indicates statistical significance as compared to the vehicle-treated rats. P<0.05 was considered statistically significant.

In our subsequent investigation, we assessed the impact of gabapentin on the spontaneous ongoing pain behavior in rats. Our findings revealed a preference for the gabapentin-paired chamber during the post-conditioning trial in neuropathic rats, indicating a significant alleviation of ongoing pain in rats treated with chemotherapy upon gabapentin administration at a dose of 60 mg/kg intraperitoneally. Notably, in

naïve rats, we did not observe any place preference behavior following gabapentin administration, suggesting a no- addictive potential (**Figure 5.7 A-D**).

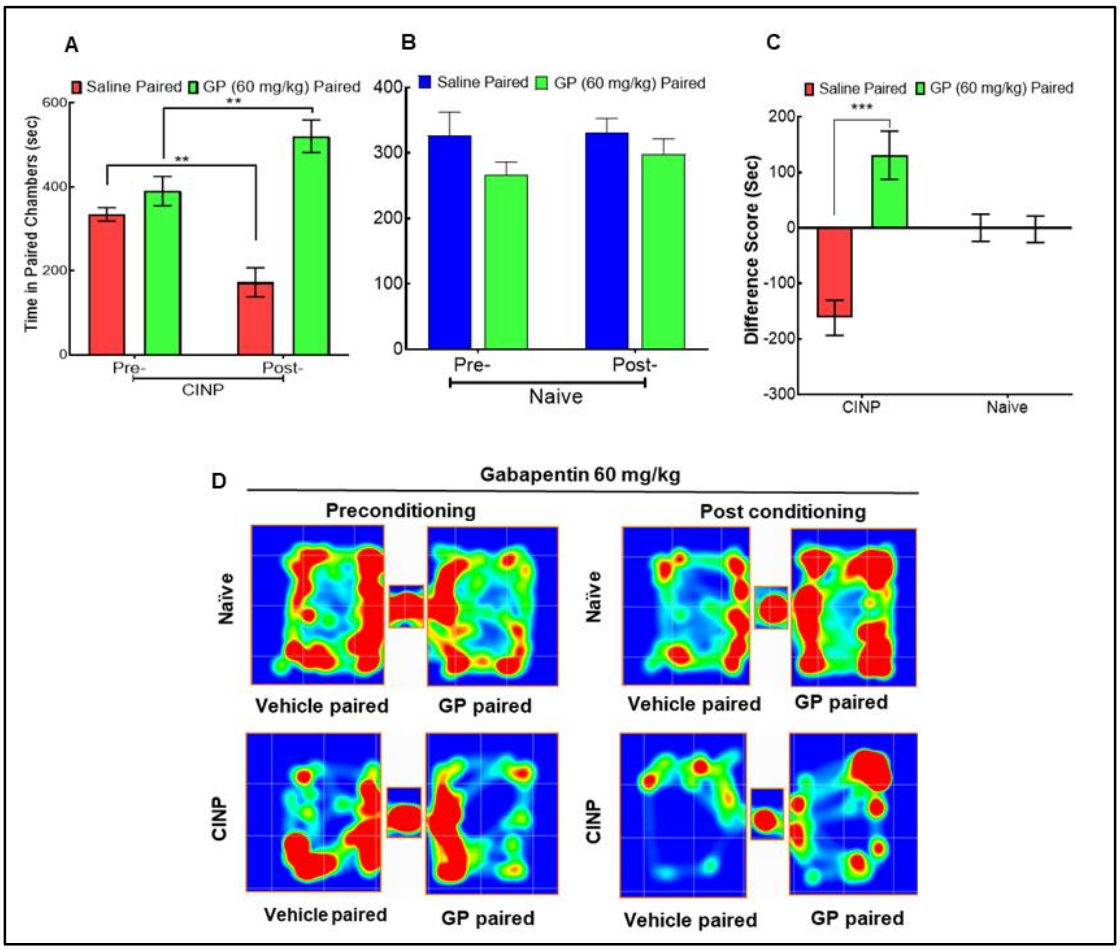

Figure 5.7 Effect of gabapentin on spontaneous ongoing pain in chemotherapy-treated rats. (A, B and C) Conditioned place preference (CPP) for Gabapentin: Combined chemotherapy-treated rats showed significant place preference behavior in the gabapentin (60mg/kg *i.p.*) paired chamber as compared to the pre-conditioning baseline. Interestingly, gabapentin (60 mg/kg *i.p.*) treatment did not produce CPP in healthy naïve rats. **(D)** Heat maps recorded during preconditioning and post conditioning with vehicle v/s gabapentin (60mg/kg *i.p)* paired chambers. Data were presented as mean ± SEM. $^{###}$P<0.001 indicates statistical significance as compared to the Naïve rats. *p<0.05, **p<0.01, and ***p<0.001 indicates statistical significance as compared to the vehicle-treated rats. P<0.05 was considered statistically significant.

Next, we conducted the CPP test for morphine, uncovering noteworthy findings in both neuropathic and naïve rats. Intriguingly, the results indicated a substantial

preference for chambers paired with both morphine and saline in both groups, underscoring not only the analgesic effects but also highlighting the addictive potential of morphine **(Figure 5.8 A-D)**.

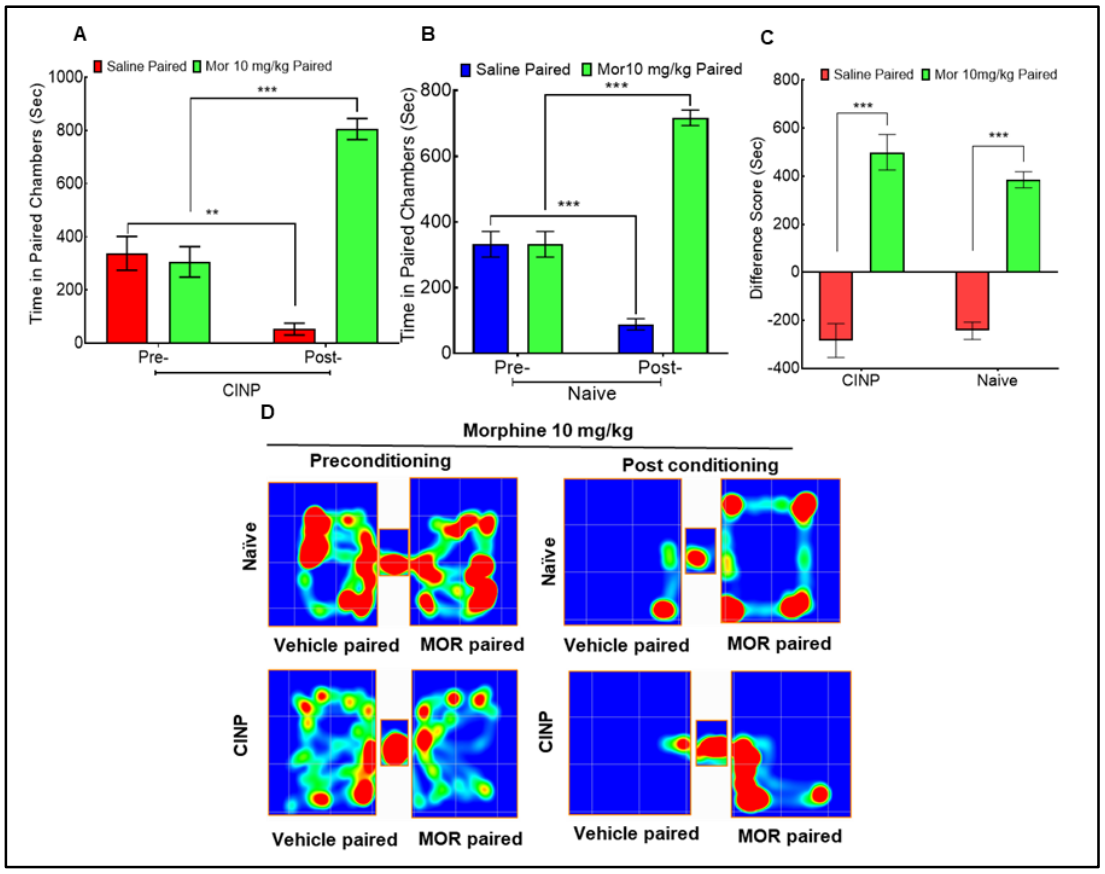

Figure 5.8 Effect of Morphine on spontaneous ongoing pain in chemotherapy-treated rats. (A, B and C) Conditioned place preference (CPP) for Morphine: Combined chemotherapy-treated rats showed significant place preference behavior in the morphine (10mg/kg *i.p.*) paired chamber as compared to the pre-conditioning baseline. Surprisingly, morphine (10mg/kg *i.p.*) treatment produce CPP in healthy naïve rats. **(D)** Heat maps recorded during preconditioning and post conditioning with vehicle v/s morphine (10 mg/kg *i.p.*) paired chambers Data were presented as mean ± SEM. $^{###}P<0.001$ indicates statistical significance as compared to the Naïve rats. *p<0.05, **p<0.01, and ***p<0.001 indicates statistical significance as compared to the vehicle-treated rats. P<0.05 was considered statistically significant.

5.3.8 Bergenin treatment does not cause locomotor impairment and motor in-coordination in rats

Locomotor impairment and motor in-coordination are very common side effects associated with centrally acting analgesics which creates a substantial barrier to the therapeutic management of chronic pain. We have examined the effect of Bergenin, gabapentin and morphine on locomotor activity and motor in-coordination of rats using open field and Rota-rod test. Results from the open field test suggests that treatment with Bergenin and gabapentin did not affect the total distance travelled and average speed of rats as compared to the vehicle treated rats. However, morphine treatment significantly decreased the average speed and total distance travelled by rats in the open-field assay indicating its sedative property **(Figure 5.9 A, B and C)**. In Rota-rod test, both Bergenin and gabapentin treatments demonstrated no induction of motor incoordination in rats. In contrast, morphine-treated rats exhibited a significant decrease in latency on the rota-rod compared to their pre-drug state **(Figure 5. 9-D)**. Given that central nervous system (CNS) side effects pose a significant concern in the clinical use of current analgesic drugs for the treatment of CINP [27], we conducted *in-vivo* neurotoxicity assays, including open-field and rota-rod tests post-drug treatment. Morphine-treated rats displayed a significant reduction in total distance traveled and average speed in the open-field test, coupled with a decrease in performance on the rota-rod test. However, Bergenin, even at its highest tested dose of 100 mg/kg intraperitoneally, did not elicit any adverse effects on the locomotor and spontaneous exploratory activity of rats in the open-field test. Furthermore, Bergenin did not affect the latency to fall in the rota-rod test at this dose. These findings suggest that Bergenin treatment inhibits both evoked and spontaneous ongoing pain in chemotherapy-treated rats without inducing CNS toxicities or drug addiction in rats. Overall, finding suggests

that Bergenin does not induce CNS side effects and toxicity which are usually associated with classical opioid like and other centrally acting analgesic drugs.

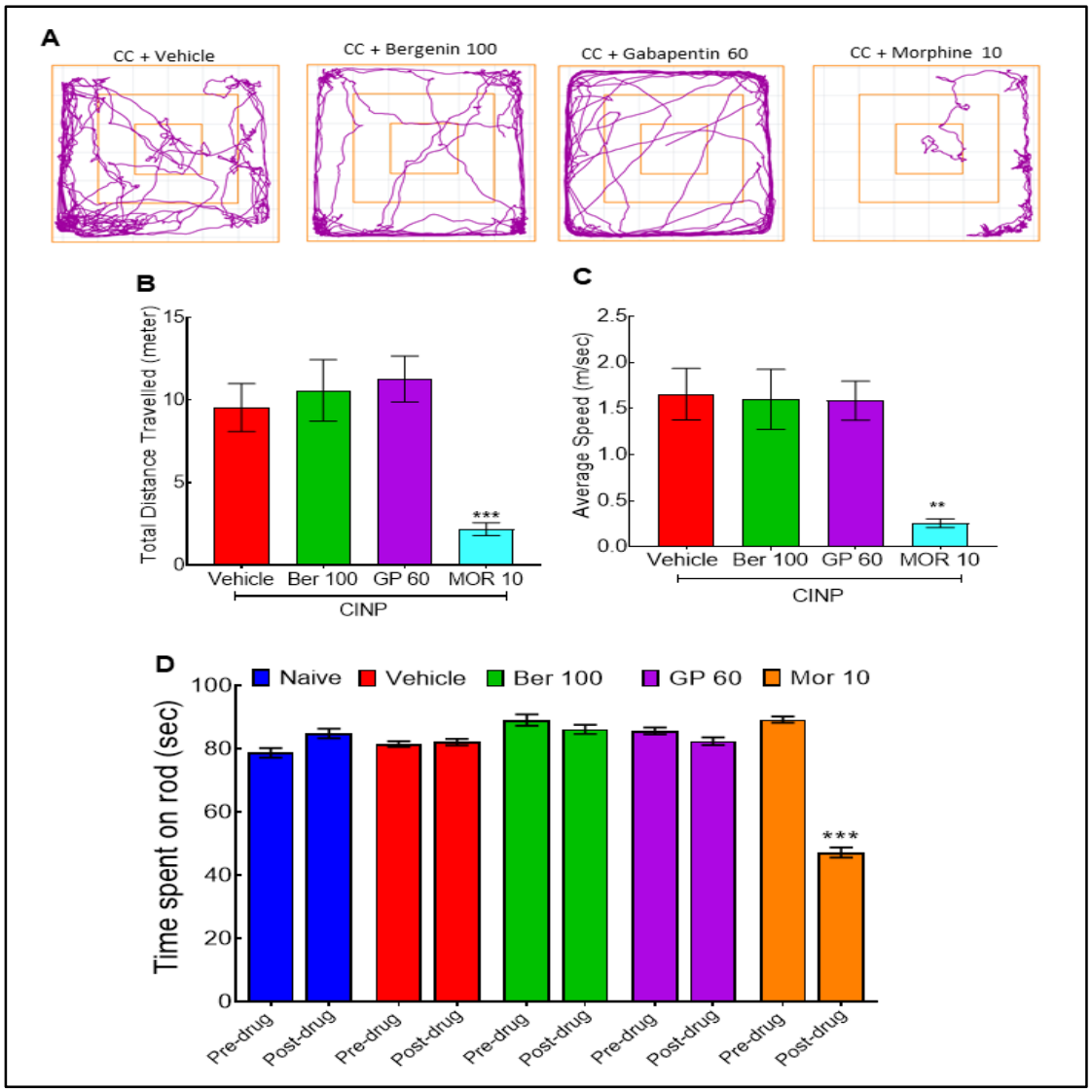

Figure 5.9 Effect of Bergenin on locomotor and motor coordination activity of rats (A, B and C) Open field test: Bergenin (40mg/kg i.p.) and gabapentin (60 mg/kg i.p.) treatment did not affect the locomotor activity of chemotherapy-administered rats in the open field arena as compared to the vehicle-treated rats. However, morphine (10mg/kg *i.p.*) treated rats showed a significant decline in total distance travelled and average speed. **(A) Open field track plots** of vehicle-treated rats, Bergenin, gabapentin and morphine. **(B) Total distance travelled** by rats after treatment with vehicle, Bergenin, gabapentin and morphine. **(C) Average speed** of chemotherapy-treated rats in open field arena after treatment with vehicle, Bergenin, gabapentin and morphine. **(D) Rotarod test:** Bergenin (100 mg/kg i.p.) and gabapentin (60 mg/kg i.p) treated rats did not show a significant decrease in fall-time in rota-rod test as compared to their pre-

treatment baseline. However, morphine (10mg/kg i.p.) treatment significantly decreased fall time of rats as compared to the pre-morphine baseline. Data were presented as mean ± SEM. $^{###}$P<0.001 indicates statistical significance as compared to the Naïve rats. *p<0.05, **p<0.01, and ***p<0.001 indicates statistical significance as compared to the vehicle-treated rats. P<0.05 was considered statistically significant.

5.3.9 Bergenin modulates mRNA and protein expressions of TRP channels in DRG and spinal cord of neuropathic rats

TRP channels are sensory receptors activated by chemicals (capsaicin, allicin) and noxious stimuli. TRPA1, TRPV1 and TRPM8 are major contributors to the progression and development of neuropathic pain due to chemotherapy (Naziroğlu and Braidy, 2017c). Chemotherapy administration leads to significant upregulation in mRNA expression of TRAP1, TRPV1 and TRPM8 in the DRG and spinal cord of rats as compared to the healthy rats **(Figure 5.10 A1, A2, A3, B1, B2, B3).** We also found that chemotherapy treatment significantly increased the protein expression of TRPA1 in DRG (p<0.001) and spinal cord (p<0.001) **(Figure 5.11 A2, B2)** and TRPV1 in DRG (p<0.001) and spinal cord (p<0.001) of rats **(Figure 5.12 A1, B1).** Bergenin treatment (25, 50 and 100 mg/kg *i.p.*) significantly (p<0.001) decreased the mRNA and protein expression of TRPA1 and TRPV1 in both DRG and spinal tissues neuropathic rats as compared to the vehicle-treated rats **(Figure 5.10A1, A2, B1, B2, Figure 5.11 A2, B2 and Figure 5.12 A1, B1).** However, Bergenin at the dose of 25 mg/kg *i.p.* did not show any significant effect on TRPV1 mRNA in the spinal tissues of CINP rats **(Figure 5.10 B2)**. Moreover, we did not observe any significant effect on TRPM8 mRNA expressions of neuropathic rats after Bergenin administration **(Figure 5.10 A3, B3)**. Gabapentin treatment also reduced the mRNA and protein expression of all three nociceptors in DRG and spinal cord of neuropathic rats (**Figure 5.10 A & B).**

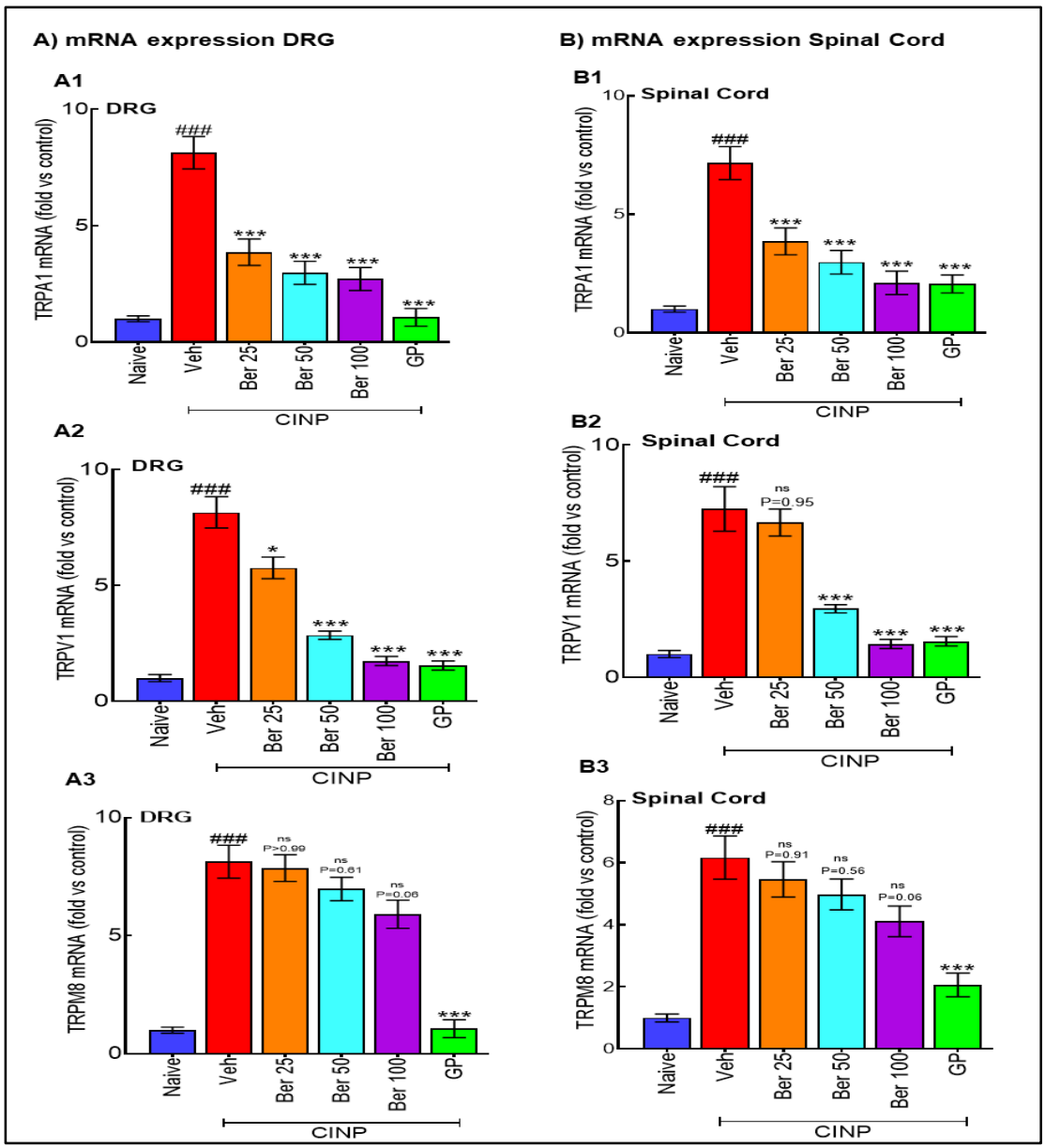

Figure 5.10 Effect of Bergenin on CINP-induced increase in TRP channel mRNA expressions in L4-L5 dorsal root ganglion (DRG) and spinal cord of rats. Chemotherapy-induced increased mRNA expressions of TRPA1, TRPV1, and TRPM8 in DRG and spinal cord of rats which were significantly reversed on treatment with Bergenin (25, 50 and 100 mg/kg *i.p.*). However, Bergenin does not have any effect on TRPM8 expression in the chemotherapy-treated rats. (A1-A3) TRPA1, TRPV1, TRPM8 mRNA expression in DRG. (B1-B3) TRPA1, TRPV1, TRPM8 mRNA expression in the spinal cord. Data were presented as mean ± SEM.

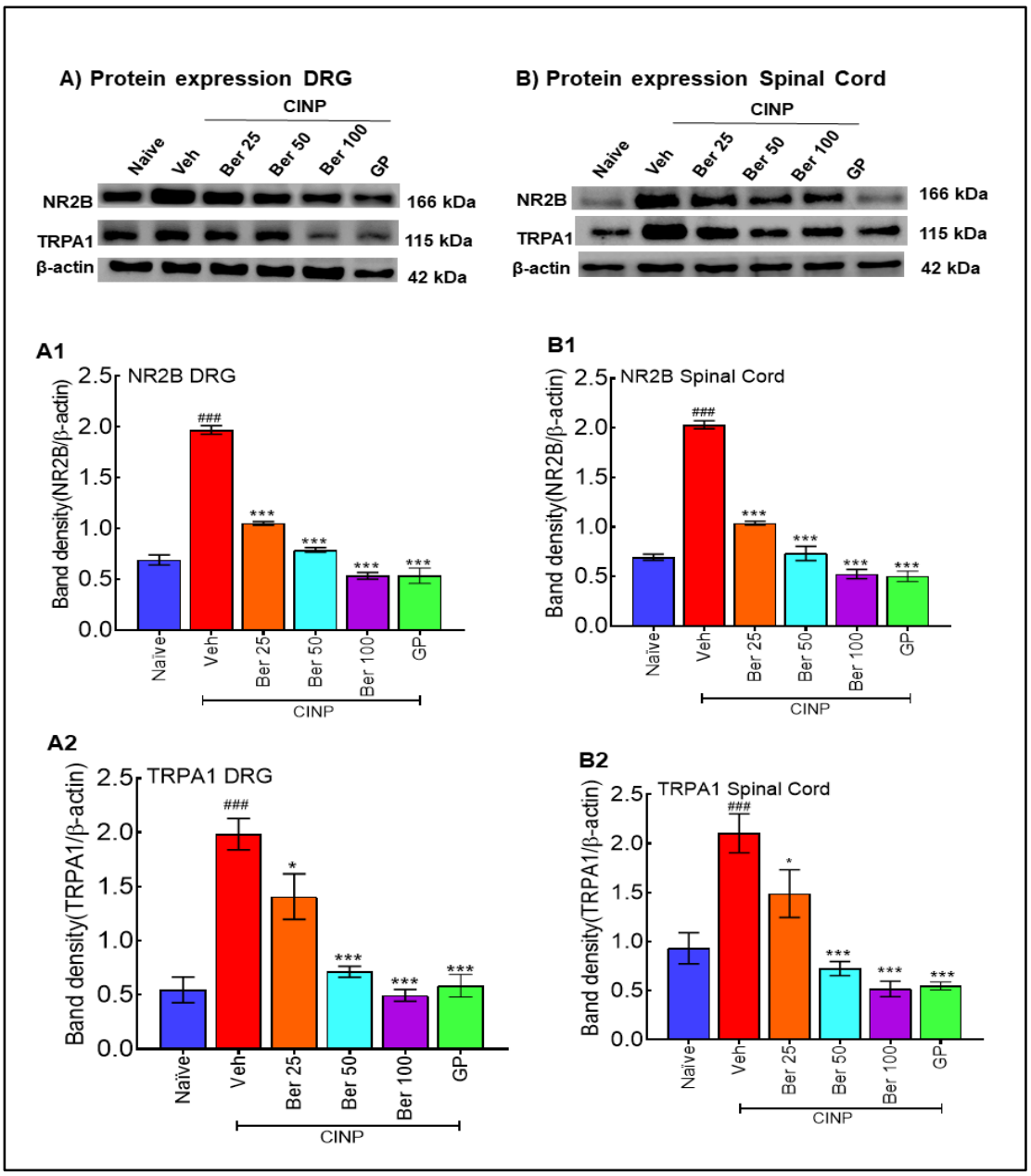

Figure 5.11 Effect of Bergenin on CINP-induced increase in NR2B and TRPA1 protein expressions in dorsal root ganglion and spinal cord of rats. Bergenin treatment (25,50, and 100 mg/kg i.p.) significantly attenuates chemotherapy-induced protein expressions of NR2B and TRPA1 in DRG and spinal cord of rats **(A) DRG protein expressions of NR2B & TRPA1 (A1-A2)** and **(B) Spinal protein expressions of NR2B and TRPA1 (B1-B2)** The data was presented as mean ± SEM and statistical significance was indicated as ###P<0.001 for comparison with Naïve rats, while *p<0.05, **p<0.01, and ***p<0.001 were used for comparison with vehicle-treated rats.

5.4 Bergenin decreased ICAM-1 and iba1 expression in DRG and spinal cord of neuropathic rats

Microglial cells are vital players in the immune response to injury and are largely implicated in CINP and these glial cells are versatile, responding to numerous cues in their microenvironment, and spinal cord microglia have been found to be particularly responsive to peripheral nerve injury caused by chemotherapy (Di Cesare Mannelli et al., 2014; Y. Li et al., 2017) . Within a day, these cells up-regulate specific marker proteins such as intercellular adhesion molecule-1 (ICAM-1) or the ionized calcium-binding adapter protein (Iba-1), a process that has traditionally been considered as 'microglial activation'. There is ample evidence that microglial activation in the spinal cord contributes to pain hypersensitivity seen in CINP. Indeed, pharmacological modulation of spinal microglial responses effectively mitigates neuropathic pain hypersensitivity, and the introduction of 'activated microglia' to the spinal cord can elicit neuropathic pain. Numerous studies suggest that NR2B subunit of NMDA receptors is involved in inflammatory signalling and central sensitization which is responsible for the development and maintenance of CINP (Liang et al., 2021). Other hand activation of TRPA1/TRPV1 also contributes to the over-expression of NR2B in the DRG and spinal cord through similar downstream signalling (Chia et al., 2020b). We have found that NR2B protein expression was significantly alleviated in DRG and spinal cord of chemotherapy-treated rats and was significantly downregulated on treatment with Bergenin **(Figure 5.11 A1 & B1).** Gabapentin treatment also decreased the NR2B expression in the spinal cord and DRG tissues of chemotherapy-treated rats and the findings are in line with the previously published studies (Ruyang et al., 2015; YIN and YU, 2015). Previous studies suggested that TRPV1 and TRPA1 directly

interact with NMDA receptors and causes transduction of central sensitization during chronic pain condition. Chemotherapy treatment significantly upregulated the protein expression of NR2B in DRG [F (5, 12) = 151; P<0.001] and spinal cord [F (4, 12) = 165; P<0.001 respectively] of rats as compared to the healthy control rats. Bergenin (25, 50,100 mg/kg *i.p.)* and gabapentin (60 mg/kg. *i.p.)* significantly inhibited the upregulation of NR2B protein expression in DRG and spinal cord of neuropathic rats as compared to the vehicle-treated group **(Figure 6 A1 & B1)**. The increased expression of ICAM-1 and Iba1 may be attributed to several factors. Notably, one major contributor is the NR2B subunit of NMDA receptors, which is known to induce microglial activation, playing a significant role in chronic pain observed in neuropathic rats. Therefore, we have further measured the protein expression of ICAM-1 and iba1 to investigate the involvement of microglia activation during CINP and its modulation by Bergenin. Our results show that chemotherapy administration significantly upregulates the protein expression of ICAM-1 and Iba1 in the spinal cord of rats which was attenuated on treatment with Bergenin and gabapentin **(Figure 5.12 C1 & C2).**

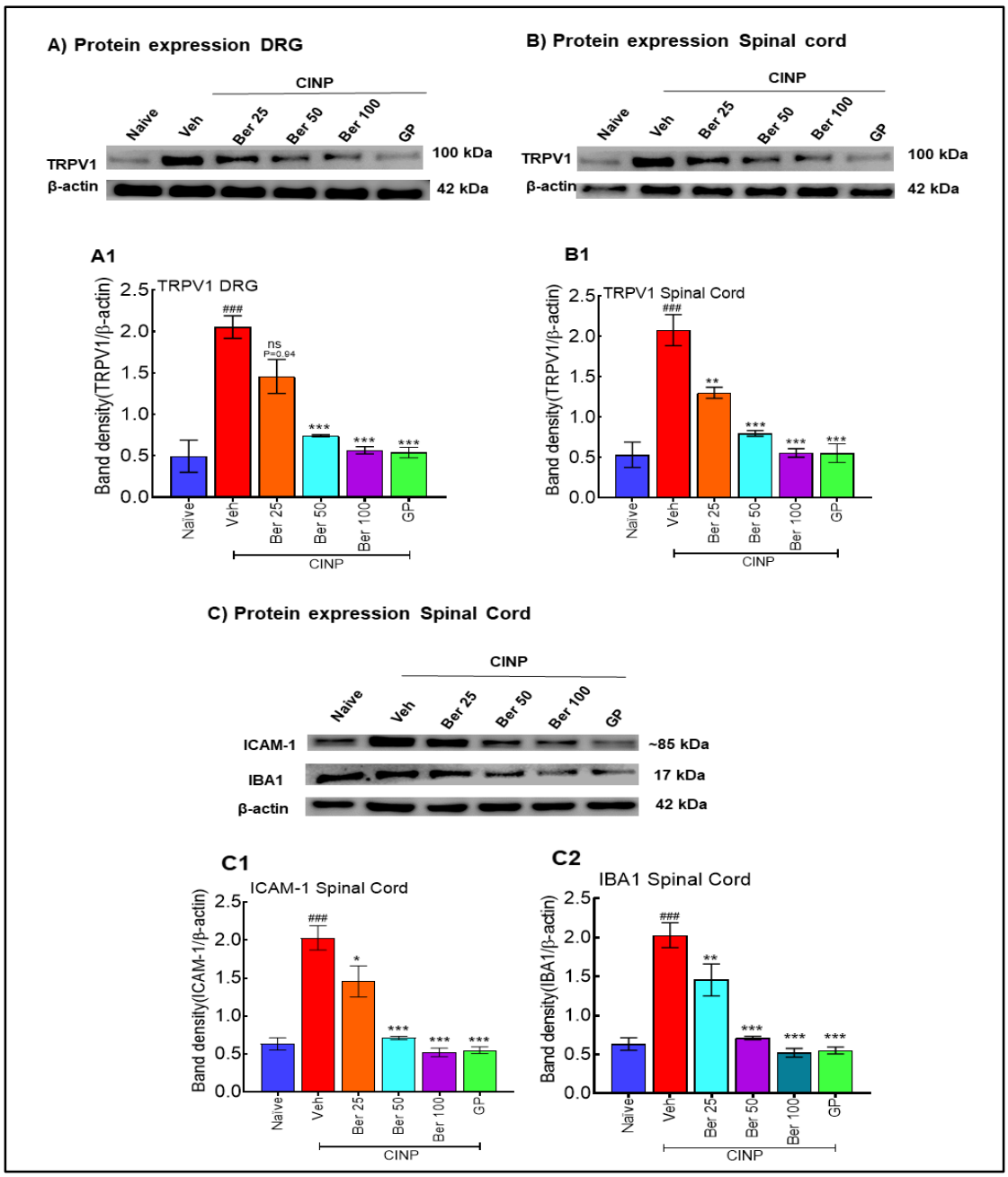

Figure 5.12 Effect of Bergenin on chemotherapy-induced protein expressions of TRPV1, ICAM-1 and IBA1 in dorsal root ganglion and spinal cord of rats. The protein expressions of TRPV1 were upregulated in the dorsal root ganglion and spinal cord of rats following chemotherapy administration **(A) DRG protein expressions (A1)** Bergenin treatment inhibits chemotherapy-induced increase in protein expressions of TRPV1 in the DRG of rats. **(B) Spinal protein expressions (B1)** chemotherapy increases TRPV1 protein expression in spinal cord of rats which was significantly attenuated on treatment with Bergenin **(C) Spinal protein expressions (C1)** Bergenin treatment significantly reduced the chemotherapy-induced upregulation of ICAM-1 **(C2)** and IBA1. Data were presented as mean ± SEM. $^{###}P<0.001$ indicates statistical significance as compared to healthy rats. *p<0.05, **p<0.01, and ***p<0.001 indicates

statistical significance as compared to the vehicle-treated rats. $P<0.05$ was considered statistically significant.

5.5 Bergenin restored blood-spinal cord barrier integrity in chemotherapy-administered rats

The blood-spinal cord barrier (BSCB) plays a crucial role in maintaining the homeostasis of the spinal cord microenvironment by selectively regulating the passage of substances between the blood circulation and the spinal cord parenchyma (Fumagalli et al., 2021). Disruptions in BSCB integrity have been implicated in various neurological conditions, including chemotherapy-induced neuropathic pain (CINP). Chemotherapeutic agents, while crucial for combating cancer, often exhibit neurotoxic side effects, potentially compromising the structural and functional integrity of the BSCB. The precise mechanisms underlying chemotherapy-induced alterations in BSCB integrity remain elusive, and understanding these changes is essential for developing targeted therapeutic interventions. Previous research has primarily focused on the blood-brain barrier (BBB), and extrapolating findings to the BSCB may oversimplify the unique challenges posed by the spinal cord microenvironment. Moreover, the specific impact of chemotherapy on BSCB integrity in the context of neuropathic pain remains an underexplored area. Given the intricate interplay between the central nervous system and systemic chemotherapy, it is imperative to investigate the effects of chemotherapeutic agents on BSCB integrity. Notably, assessing BSCB integrity in chemotherapy-administered rats provides a valuable opportunity to elucidate the pathophysiological changes occurring within the spinal cord microvasculature. Understanding these alterations is critical not only for comprehending the etiology of CINP but also for identifying potential targets for therapeutic intervention.

Findings from the Evans blue assay suggests that chemotherapy treatment breached the blood-spinal cord integrity causing infiltration of pro-inflammatory cytokines in the spinal cord. Spectrophotometric analysis revealed that Bergenin treatment maintains the permeability of blood-spinal cord barrier and prevents the invasion of immune cells and inflammatory cytokines-mediated damage thereby preventing peripheral sensitization (Kuai et al., 2020c; Montague-Cardoso et al., 2020). Taken together findings from the present study suggests that TRPA1/TRPV1 mediated NR2B activation leads to glial cell activation and neuro-inflammation in both DRG and spinal cord of chemotherapy-treated rats which was significantly attenuated with Bergenin treatment. Evans blue assay to determine the integrity of the blood-spinal cord barrier as it is a crucial component responsible for CNS infiltration and central sensitization. The tight junction proteins including claudin-5 and occludin are required or maintaining blood-spinal cord barrier integrity and preventing the invasion of pro-inflammatory cytokines such as TNF-α, and IL-6 into the CNS, thereby protecting the spinal cord from any potential damage. We found a significant increase in Evan's blue dye concentration in the spinal cord and brain of vehicle-treated rats as compared to the healthy control rats and which was restored on treatment with Bergenin and gabapentin in a dose-dependent manner **(Figure 5.13 A, B & C)**. Moreover, we also found significantly decreased mRNA levels of both occludin and claudin-5 in chemotherapy-treated rats as compared to the healthy control rats which were significantly restored post Bergenin (25, 50, and 100 mg/kg *i.p*) treatment **(Figure 5.14 A1 & A2)**. Gabapentin treatment also restored the decreased expressions of claudin-5 post-chemotherapy administration. However, we did not find any significant effect of

gabapentin on mRNA expression of occludin in the spinal cord of neuropathic rats **(Figure 5.14 A2)**.

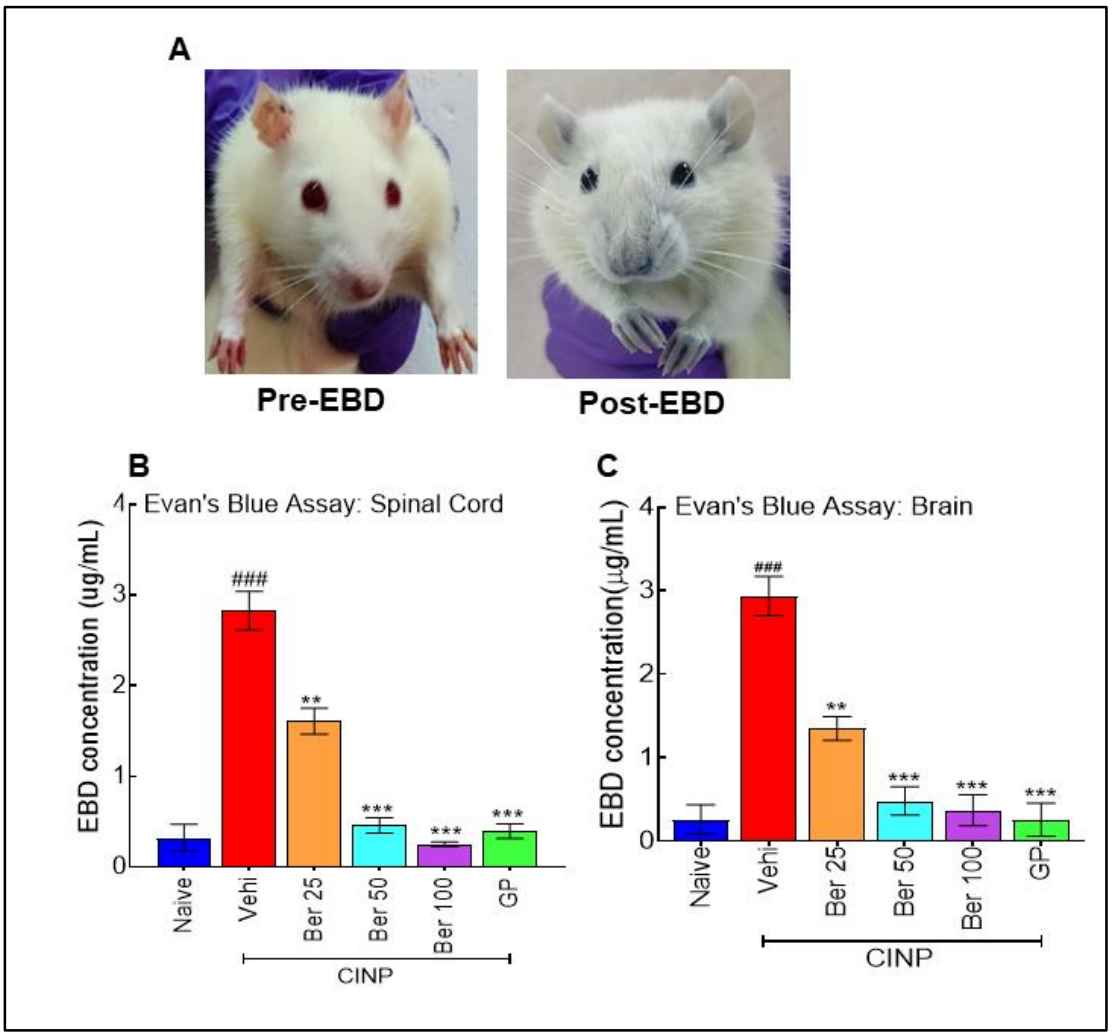

Figure 5.13 Effect of Bergenin on CINP-induced disruption in blood spinal cord integrity of rats A) Representative images of rats before and after receiving 1mL of 2% EBD i.v. into the tail vein. Post-injection images were taken at 2 hrs. post-injection. **B)** EBD concentration was found to be significantly increased in the chemotherapy-treated rats' spinal cord tissues as compared to the healthy controls which were considerably reduced post Bergenin and gabapentin administration as compared to vehicle-treated rats. **C)** EBD concentration was found to be significantly increased in the chemotherapy-treated rats' brain as compared to the healthy controls which were considerably reduced post Bergenin and gabapentin administration as compared to vehicle-treated rats. Data were presented as mean ± SEM. $^{###}P<0.001$ indicates statistical significance as compared to the Naïve rats. *p<0.05, **p<0.01, and ***p<0.001 indicates statistical significance as compared to the vehicle-treated rats. P<0.05 was considered statistically significant.

5.6 Bergenin attenuates oxido-inflammatory cascade in DRG and spinal cord of neuropathic rats

Anti-neoplastic drugs may cause peripheral neuropathy by disrupting the integrity of the blood-spinal cord barrier and the resulting infiltration of inflammatory cytokines along with microglial responses and exacerbating neuronal damage (Montague-Cardoso et al., 2020). Tight junctions span the apical region of the intercellular cleft of barrier tissues and allow for the restriction of ion flux and paracellular diffusion (Branca et al., 2018). We also confirmed the distortion of the blood spinal cord integrity by the rt-PCR analysis for the tight junction proteins and found the expression of claudin-5 and occludin to be significantly decreased after chemotherapy which was restored after Bergenin treatment. NMDA receptor activation is linked with upregulation of microglia activity (IBA1, ICAM-1), and TNF-α pathways (Guerrini et al., 1995; Palazzo et al., 2018). This pathway, in turn, activates parallel cytokine signalling including the release of IL1B, IL-6, and TNF-α. Increased activity of these mediators is known to be associated with enhanced pain signalling and central sensitization, thereby, promoting the pathophysiology of chronic pain (Kawasaki et al., 2008). We have measured the protein expression of ICAM-1 and IBA1 in the spinal cord and found that chemotherapy-treated rats showed significant upregulation of both ICAM-1 and IBA1 which was attenuated by Bergenin. Findings from the biochemical assay revealed that Bergenin treatment effectively lowered MDA and nitrite levels the in sciatic nerves of CINP rats. Additionally, Bergenin restored reduced GSH levels in chemotherapy-treated rats, aligning with prior findings.

Compromised blood-spinal cord integrity due to chemotherapy leads to significant upregulation of IL6 and TNF-α in spinal cord of rats. Bergenin (25, 50,100 mg/kg *i.p.)* and gabapentin (60 mg/kg *i.p.)* treatment significantly suppressed the overexpression of IL6 and TNF-α in the spinal cord of chemotherapy-treated rats **(Figure 5.14 A3 & A4)**. According to recent studies, antineoplastic agents such as

cisplatin, paclitaxel, and others may activate TRP channels, resulting in the generation of reactive oxygen species and reactive nitrogen species in the peripheral sites followed by the release of pro-inflammatory cytokines and central sensitization. An increase in the levels of nitrite and MDA was observed in the sciatic nerve of the vehicle-treated rats which were significantly attenuated on treatment with Bergenin and gabapentin **(Figure 5.15 A-B)**. Furthermore, the levels of anti-oxidant enzymes GSH were found to be significantly reduced in vehicle-treated rats which were significantly restored post-Bergenin and gabapentin treatment **(Figure 5.14 C)**.

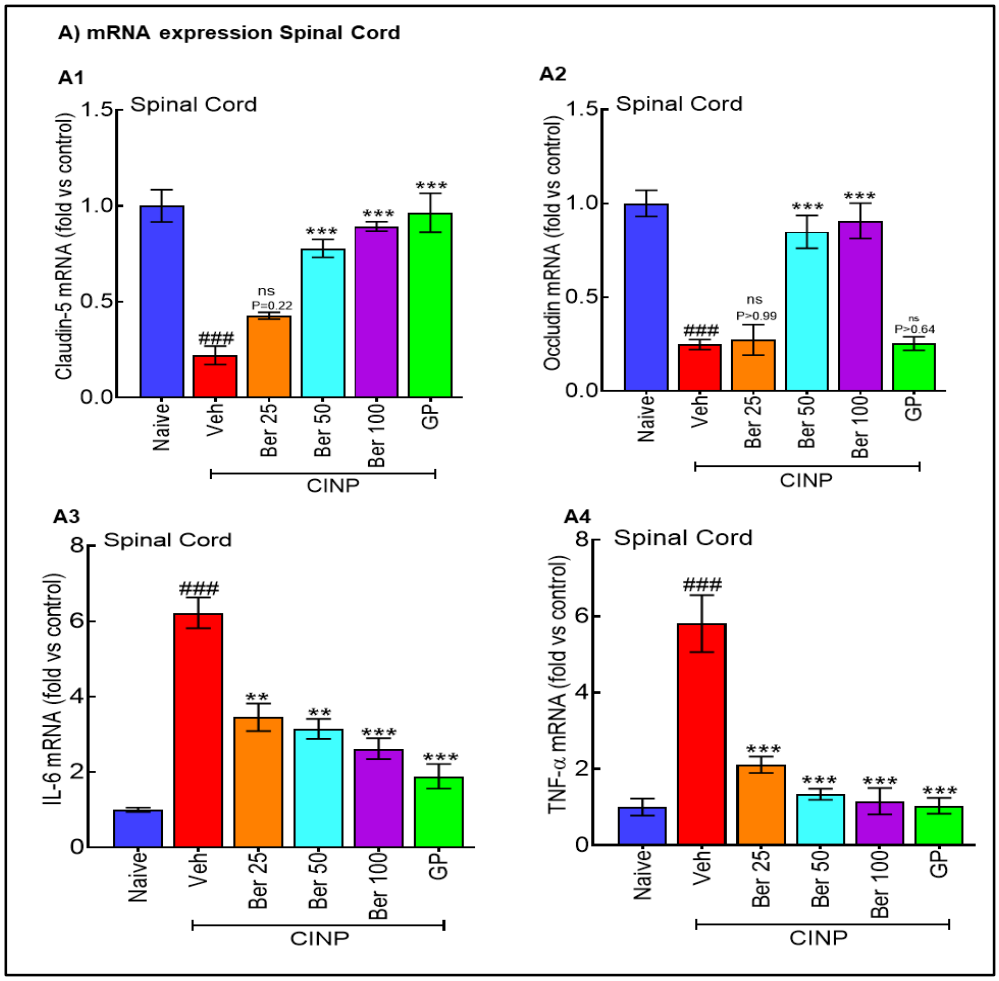

Figure 5.14 Effect of Bergenin on blood-spinal tight junction proteins and neuro-inflammation in L4-L5 spinal cord of neuropathic rats. Chemotherapy treatment significantly decreased the mRNA expression of claudin-5 and occludin and increased pro-inflammatory cytokines in L4-L5spinal cord of rats. Bergenin treatment significantly restored decreased mRNA expressions of claudin-5 and occludin in spinal

cord of neuropathic rats. However, gabapentin treatment only restored claudin-5 without modulating occludin mRNA expressions in chemotherapy-treated rats. Bergenin and gabapentin treatment significantly decreased IL-6 and TNF-α mRNA expressions in spinal cord of rats. **A1)** mRNA expression of claudin-5 in spinal cord **A2)** mRNA expression of occludin in spinal cord **A3**) mRNA expression of IL-6 in the spinal cord **A4)** mRNA expression of TNF-α in the spinal cord. Data were presented as mean ± SEM. ###P<0.001 indicates statistical significance as compared to the Naïve rats. *p<0.05, **p<0.01, and ***p<0.001 indicates statistical significance as compared to the vehicle-treated rats. P<0.05 was considered statistically significant.

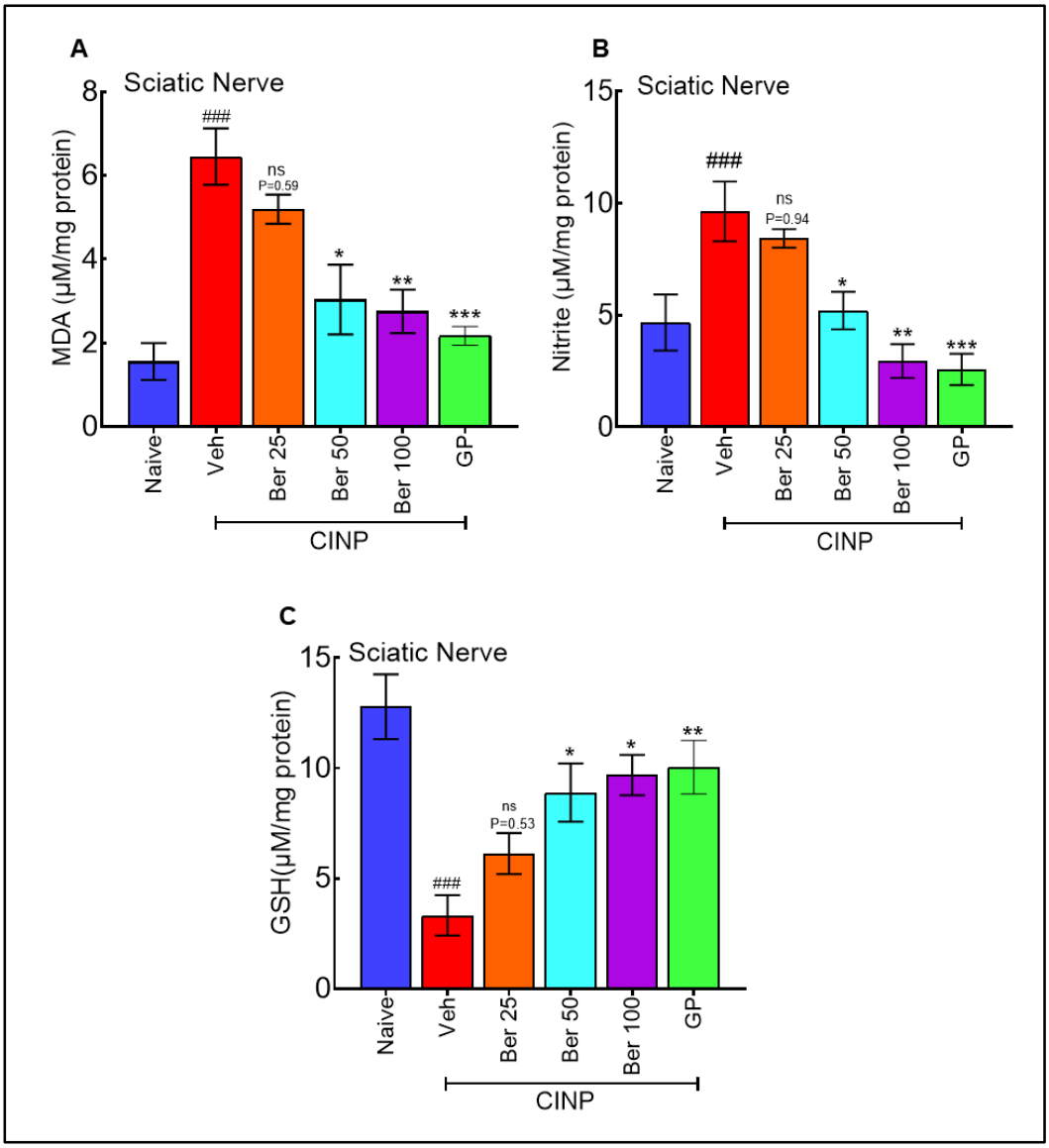

Figure 5.15 Effect of Bergenin on oxidative stress induced by combination chemotherapy in rats. Chemotherapy-treated rats show significantly increased MDA and nitrite levels in the sciatic nerves, which were significantly restored after Bergenin at the dose of 50 and 100 mg/kg *i.p.* and gabapentin 40mg/kg *i.p.* However, Bergenin does not affect oxidative stress marker at the dose of 25mg/kg *i.p. (***A)** MDA levels **(B)**

Nitrite level. Bergenin and gabapentin administration also increased the level of antioxidant enzyme GSH in the sciatic nerves of combination chemotherapy treated rats as compared to the vehicle administered rats **(C)** GSH levels. Data were reported as mean ± SEM. ***p<0.001and **p<0.01 indicates statistical significance as compared to the vehicle rats. p<0.05 was considered statistically significant. n=5 biological and n=3 technical replicates. Ber 50, Bergenin 50 mg/kg; Ber 100, Bergenin 100 mg/kg; GP, Gabapentin 60 mg/kg. CC, Combined chemotherapy-induced neuropathic pain.

5.7 *In-silico* studies

5.7.1 Homology modelling

Based on our behavioral observations, it becomes evident that bergenin does not exhibit the capacity to alleviate cold hyperalgesia nor does it induce modulation of the TRPM8 expression. However, it reduces the expression of TRPA1 and TRPV1, hence these discernible outcomes stimulated our contemplation of an *in-silico* approach. Consequently, we have performed *in-silico* studies that were precisely tailored to delve into the intricate dynamics underpinning the interactions between Bergenin and TRPA1, TRPV1, and TRPM8. The three-dimensional structure of TRPA1 and TRPM8 proteins were constructed using Swiss Model tools and quantified using Ramachandran plot (RC) analysis. We have chosen the two best-predicted models of both proteins based on the residue lying in the most favoured region in the RC plot. The illustration was depicted using a degree of confidence, the total number of residues in the most favoured region and energy minimized model of both TRPA1 and TRPM8 were selected for further analysis. The RC plot displays the x-axis Phi and y-axis Psi angles, respectively, which have four quadrants including the area of a disallowed region, a generously allowed region, an allowed region and a low-energy region. We observed that the most recommended regions for TRPA1 and TRPM8 comprised 93.95% and 93.83% of the residues, however, 5.2% and 4.9% within the additional allowed region, 0.85 % and 1.2 % in the generously allowed region, whereas in the disallowed region

0% and 0.2% were found respectively **(Figure 5.16 A-B)**. Using Verify3D and ERRAT software supported the generated model having 84.21% and 81.25%. Further, the PROSA Z-score and QMEANS values for TRPA1 and TRPM8 were observed as −2.6 and −4.26 and−3.23 and −4.56 respectively **(Figure 5.16)**. Thus, the finding from the RC plot, ERRAT, Verify-3D, and QMEANS confirm that the generated model of TRPA1 and TRPM8 are reliable and has good quality.

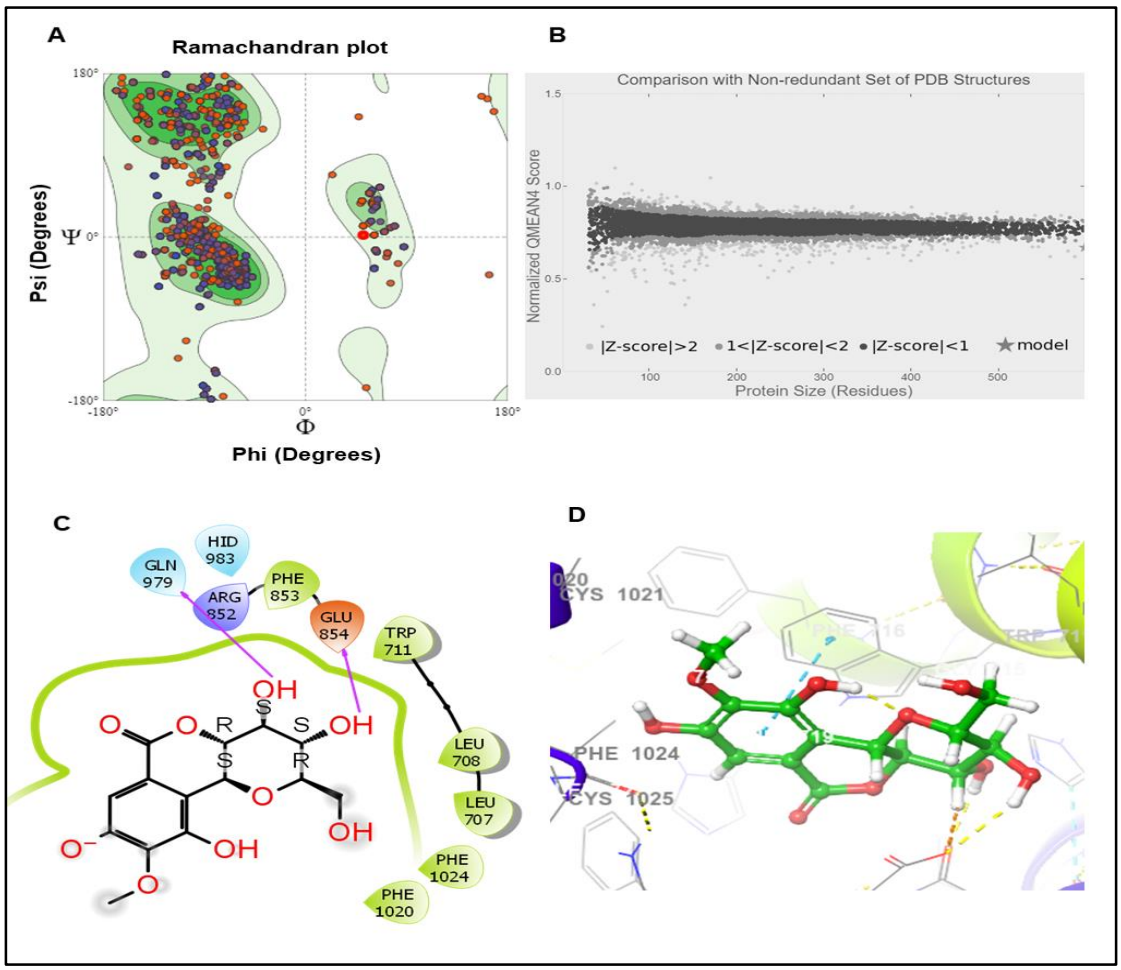

Figure 5.16 ***In-silico*** **studies of Bergenin with TRP channels (A)** Homology modelling of TRPA1 protein using SWISS-MODEL workspace: TRPA1 protein analysis was performed using Ramachandran plots and quantification was conducted using PROCHECK software, indicating that 98.6% of TRPA1 residues were detected in the most preferred areas. **B)** the QMEAN-dependent structure validation. **C)** Illustration of 2D ligand interlinkage and **D)** 3D binding posture of Bergenin and TRPA1 protein.

5.7.2 Molecular Docking

Molecular docking is a computational technique widely employed in structural bioinformatics and drug discovery to predict the binding orientation and affinity of a small molecule to a target macromolecule. (Baidya et al., 2023). In the molecular docking study, we have prepared and constructed 3D structures of TRPV1, TRPA1 and TRPM8 and binding mode with Bergenin using Protein Wizard, Maestro, Schrodinger suits. Interestingly, docking studies revealed that Bergenin induced a favorable binding upon interacting with TRPA1 proteins through different catalytic residues such as for GLU-854, GLN-797, TRP-711, ARG-852 LEU-708 through carbon-hydrogen bonding. Binding residues for TRPV1 protein were ASP-427, GLN-423, TRP-426, ARG-701 and PHE-559 through hydrogen and hydrophobic interaction, etc. Moreover, TRPM8 interacted with different catalytic residues such as PHE-738 and SER-738. The Bergenin-achieved docking score with TRPA1, TRPV1 and TRPM8 were found to be −11.8, −11.4 and −4.4 kcal/mol respectively. It indicates that Bergenin has a strong binding affinity with TRPA1 and TRPV1 but a low binding affinity with TRPM8. 2D and 3D interaction position of targeted proteins with Bergenin was displayed in figure **(Figure 5.16 C and D; Figure 5.17; Figure 5.18 C and D).**

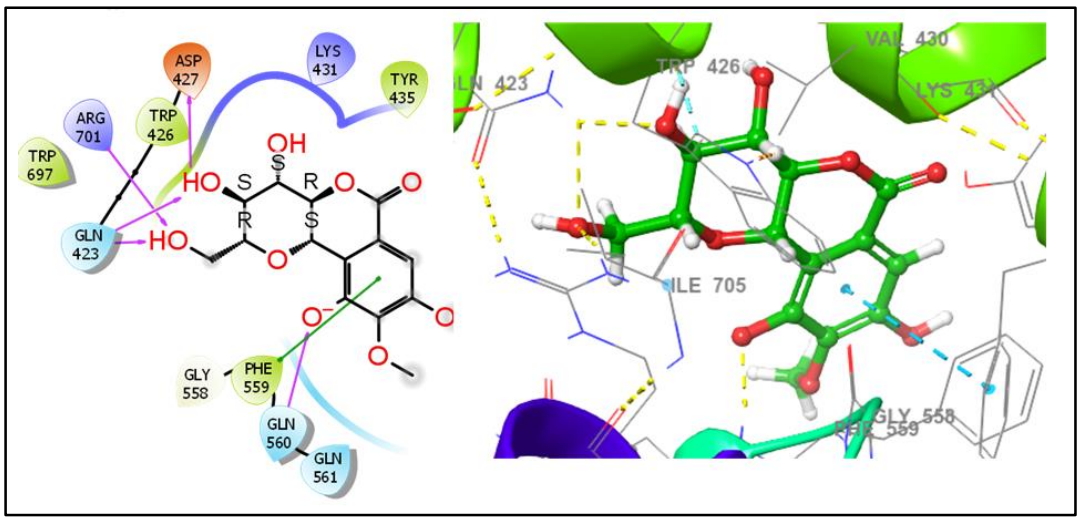

Figure 5.17 Illustration of 2D ligand interlinkage and 3D binding posture of Bergenin and TRPV1 protein.

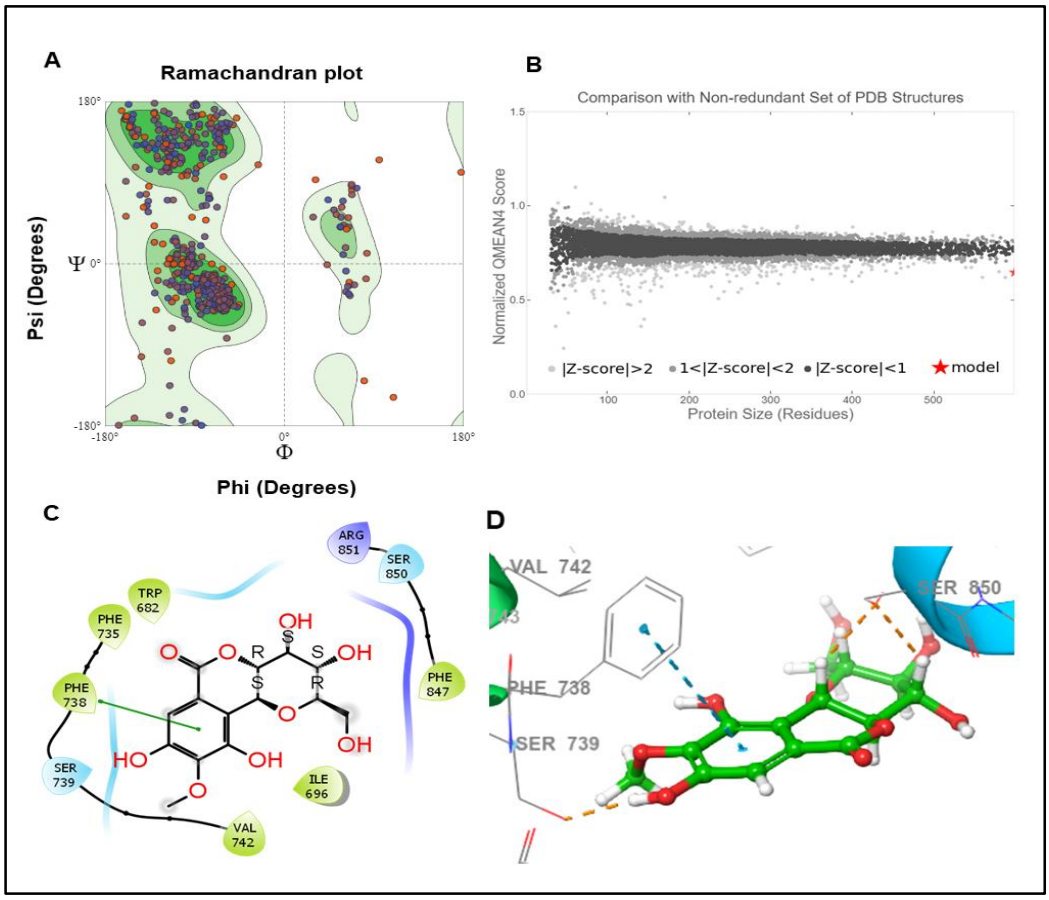

Figure 5.18 Ramachandran plots for TRPM8 protein structure. A) The PROCHECK software was employed to quantify the graph, demonstrating 96% of all TRPM8 residues were found in the most favored regions as per the RC graph quantification. B) the QMEAN validation of targeted protein. C) Illustration of 2D ligand interlinkage D) 3D binding pose of Bergenin and TRPM8 protein.

5.7.3 Molecular Dynamics Simulation

Several factors like docking score and binding energy of the protein-ligand complexes were considered to select Bergenin having a better affinity with TRPA1 and TRPV1 however TRPM8 showed less binding efficiency with it. Further *in-silico* investigations were carried out using molecular dynamic simulation in the Schrödinger Desmond modules for each complex. MD simulation provides much valuable information about the precise estimation of the binding strength of a docked complex and the dynamicity of the biological systems. The root mean square deviation (RMSD) and root mean square fluctuation (RMSF) were analyzed during MD trajectory under a physiological environment with a simulation time of 100 ns. The figure (Figure 10A (e-j) illustrates the MD trajectory analysis of Bergenin with TRPA1. The RMSD value was in the accepted range of 1.2 to 4.1 Å for protein and 0.8 to 2.8 Å for the ligand during simulation time 100ns **(Figure 5.19 A-E)**. The RMSF plots value between 1 to 8 Å indicated that there was no fluctuation observed in the ligand-protein complex during the simulation time interval of 100ns **(Figure 5.19 A-F)**. The protein-ligand interaction of each amino acid residue within the TRPA1 binding pockets was analyzed and displayed in **(Figure 5.19A-G)**. It can be clearly observed from the plot of the protein-ligand interaction that the active site with ARG_499 binds mainly through the H bond. Residues SER_693, PHE_358, LEY_571, and VAL_567 have been connected through ligands by hydrophobic interaction. GLN_700 additionally assisted a supportive performance for the binding interaction of the active site of the ligand with protein through the water bridge. However, VAL_567 residues are responsible for interacting with the ligand through both hydrogen and water bridges 10A-h. **(Figure 5.19 A-)** illustrates the ligand interaction through the binding action and active site

amino acid residues of the target protein. Based on the result we perceive the nucleus core alongside the replacement of the side chain engaged with the residues of amino acid through several hydrogen bonds and hydrophobic interactions. Finally, the interaction of ligand-protein was found in the form of different interactions such as H bond, ionic interaction, hydrophobic bond and water bridge which plays an important role in the established interconnection between ligand-protein complex the interaction is shown in **(Figure 5.19 A-F)** throughout the simulation period of 100 ns.

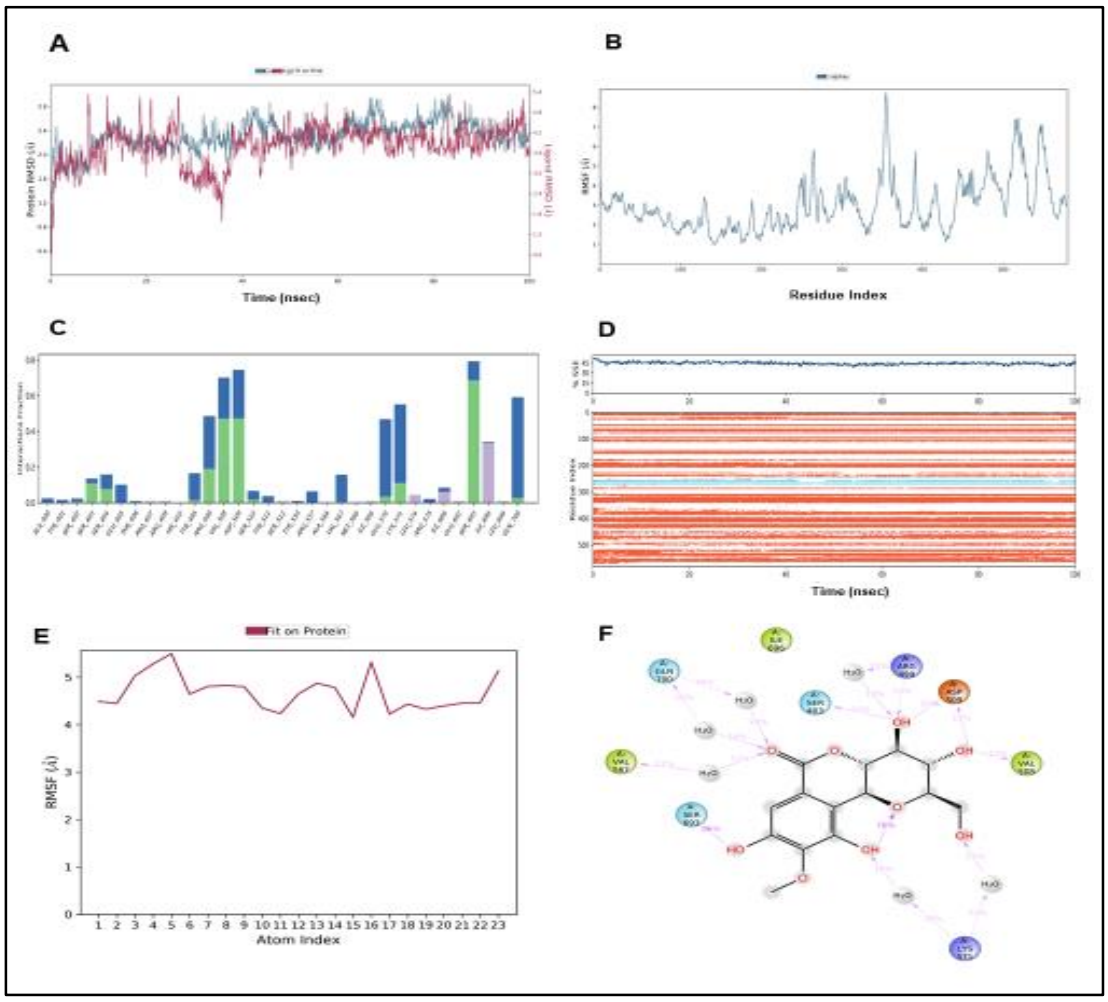

Figure 5.19 MD trajectory data of Bergenin with TRPA1 interaction A) Protein-ligand RMSD; **B)** RMSF for protein; **C)** Protein-ligand contact distribution histogram**; D)** protein-ligand contacts**; E)** RMSF for Bergenin. **F)** Interaction of TRPA1 and Bergenin

Next, we conducted data analysis on the molecular dynamics (MD) trajectory of the complex between Bergenin and the TRPV1 protein. The analysis aimed to investigate the dynamic behavior of the complex and gain insights into the binding mechanism of Bergenin to TRPV1. The RMSD plot shows slight instability at the beginning of simulation up to 40ns however, the protein-ligand interaction was stable after 40ns to throughout simulation time 100ns (RMSD was observed between 0.6 to 2.4 Å and 5 to 7Å for protein and ligand respectively) which suggests little induction of conformational change in the TRPV1 during simulation **(Figure 5.20 B**-E). Additionally, in order to predict the dynamic behaviour of residues and flexibility of protein structure during the simulation recording was performed. The root mean square fluctuation (RMSF) indicated that there are no internal fluctuations were observed during the simulation time interval of 100 ns. The RMSF value was in an acceptable range between 0.4 to 5.6 Å. The interaction of ligand and amino acid residue of TRPV1 binding pockets was amylase and displayed in **(Figure 5.20 B-F)**. The plot of Bergenin and TRPV1 diagram showed that the active site of the target protein binds with different residues such as THR_359 and LEU_549 interconnected through hydrogen bonding, with ILE_696 and GLN_700 via hydrophobic interaction, however, SER_693 was bound through both hydrogen and hydrophobic bonding. Meanwhile, SER_693 and LYS_571 are interconnected with the water bridge. The graphical plot revealed that hydrophobic interactions played a crucial role in the binding of ligands to the active site of TRPV1. The analysis suggested that the hydrophobic nature of the ligands enabled them to form stable interactions with the protein, thereby facilitating their binding to the TRPV1 active site. Moreover, hydrogen bonding is also one of the essential forces involved in the protein-ligand interaction and it has been considered an essential factor used in stabilizing the protein-ligand complexes (Du et al. 2016). RMSF graph of ligand **(Figure 5.19 B-)** suggested that the general way the ligand is strictly bound to the active site of the amino acids. (**Figure 5.20 B-)** from the plot we observed that the ligand was

bound to the TRPV1 through the specific amino acid interaction. The same graph also represents the substitution action on the side chain that was highly involved in the active site of the amino acid residues. Finally, there are different ligand and protein interactions were present in the form of H Bond, hydrophobic bond, ionic interaction, and water bridges were plotted in (**Figure 5.20 B-)** throughout the 100 ns simulation time. We have observed that the top panel indicates a total number of specific protein-ligand contact.

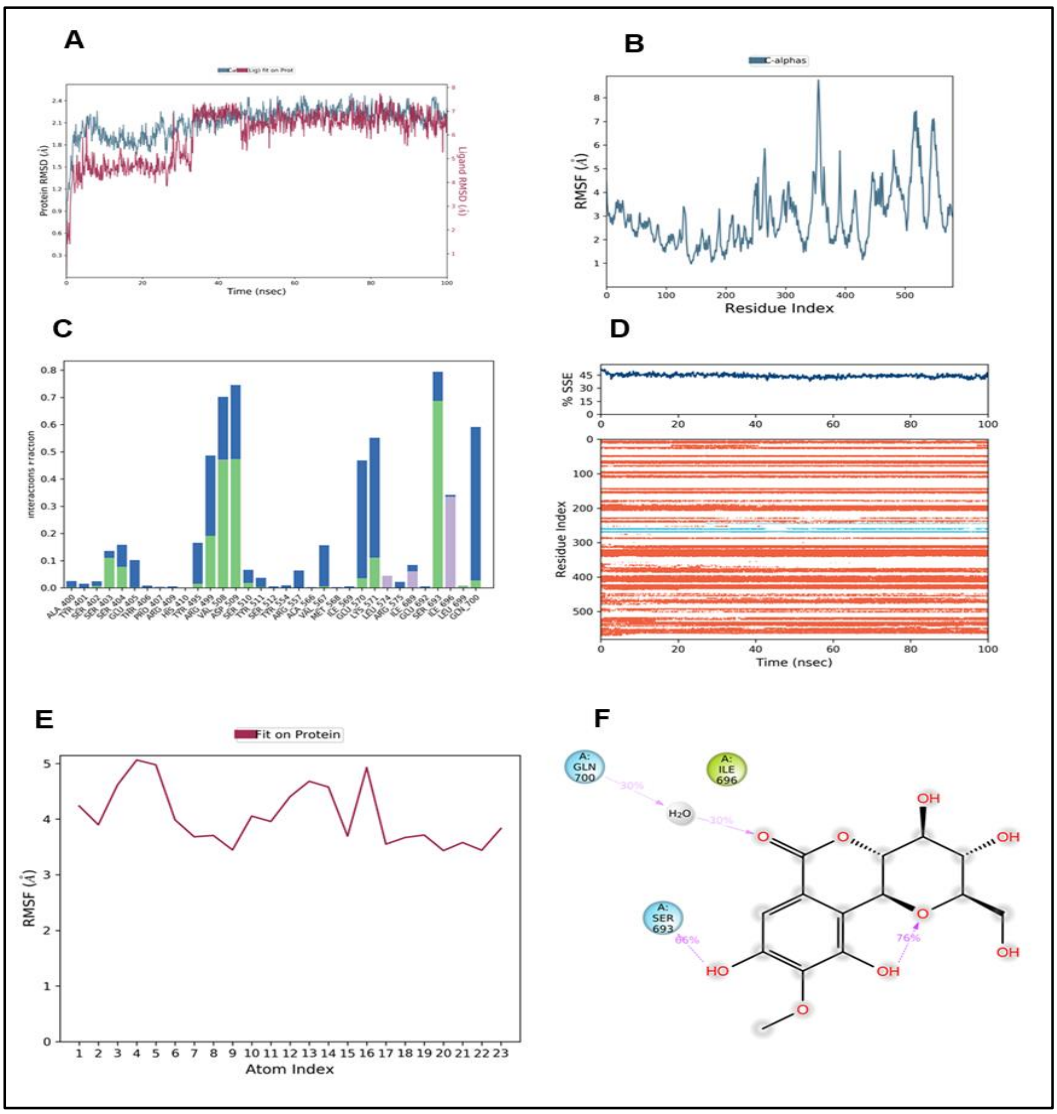

Figure 5.20 MD trajectory data of Bergenin with TRPV1 interaction A) Protein-ligand RMSD; **B)** RMSF for protein; **C)** Protein-ligand contact distribution histogram**; D)** protein-ligand contacts**; E)** RMSF for Bergenin. **F)** Interaction of TRPV1 and Bergenin.

Further, we have performed the MD simulation of TRPM8 with Bergenin complex and figure describes the MD trajectory analysis for Bergenin and TRPM8 protein. The graphical representation of the RMSD plot demonstrates that Bergenin with TRPM8 complex is unstable for the 100 ns simulation time. We observed that RMSD values were not detected under the range of 2 to 14 Å for the protein and 6 to 18 Å ligand system **(Figure 5.21 C-E)**. Moreover, it was observed that the intramolecular interactions within the amino acid residues exhibited moderate fluctuations throughout the entire simulation time interval. Additionally, the ligand was found to have poor interactions with the active site amino acid residues located within the internal binding pocket associated with TRPM8. These results were represented and illustrated in **Figure 5.21 C-F**. Based on the Bergenin-TRPM8 interaction we have observed that the active site of TRPM8 interconnects with amino acid residues such as ARG_432 through H bonding. However, TRP_426, PHE_429, and GLN_560 interacted with water bridges. The RMSF graphical representation of ligand and protein complex recommended the binding affinity of the Bergenin with the active site of amino acid residues of the TRPM8. **Figure 5.21 C** illustrates that the binding affinity of the ligand with TRPM8 was specific for the active site of amino acids. In the end, a timeline for the protein-ligand interactions was drawn up for the duration of simulation time of 100 ns and the results indicated that TRPM8 has minimum contact with the ligand. To better understand conformational, orientational, and dynamic characteristics of the Bergenin with TRPM8. The results showed that there was a fluctuation in the conformation, orientation, and dynamics of Bergenin with TRPM8 throughout 100 ns simulation time.

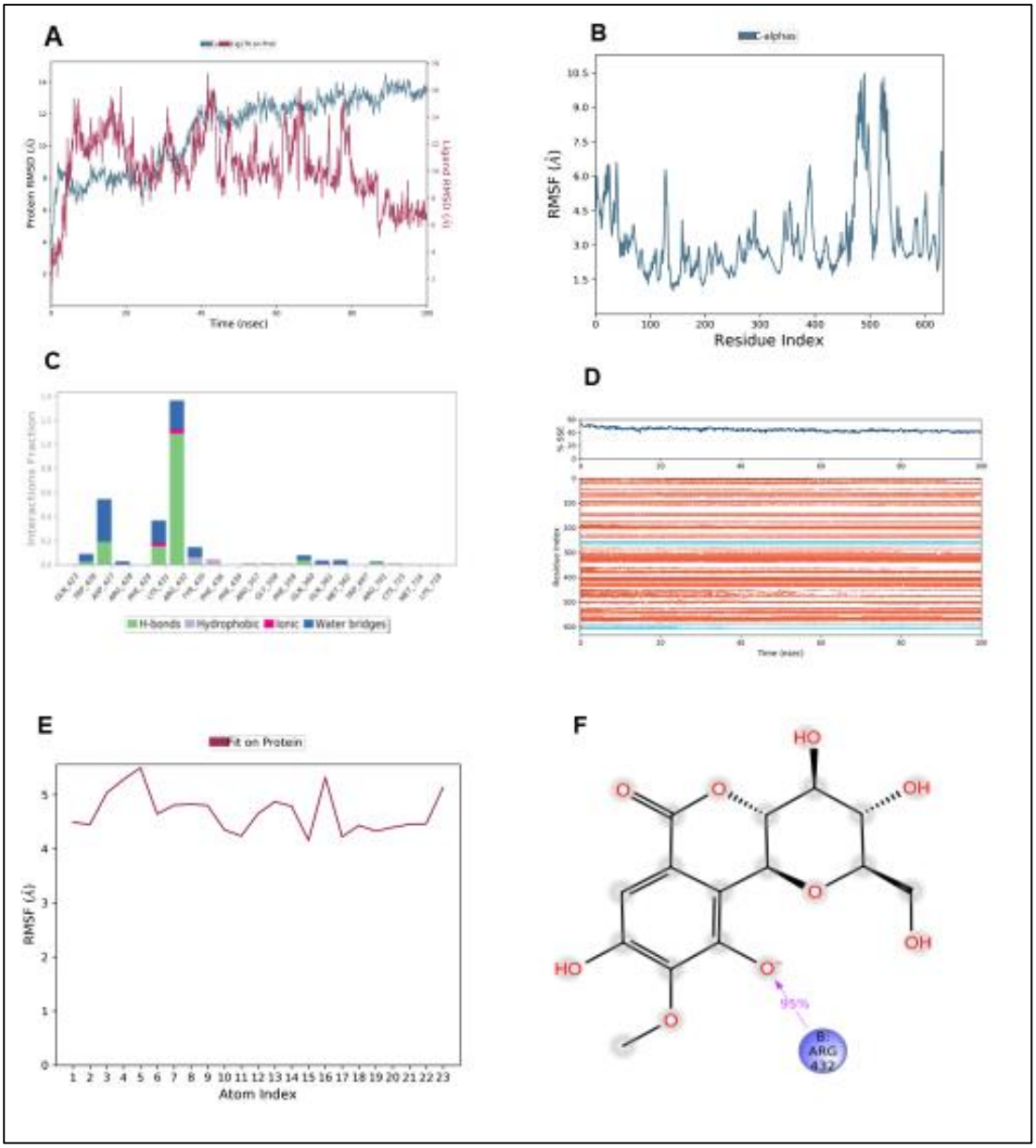

Figure 5.21 MD trajectory data of Bergenin and TRPM8 interaction c) RMSD of Protein-ligand; **d)** RMSF for protein; **e)** Protein-ligand contact distribution histogram; **f)** protein-ligand contacts; **g)** Interaction of TRPM8 and Bergenin **h)** RMSF for Bergenin.

Overall, the MD simulations confirmed the stable nature of the interactions between Bergenin, TRPA1, and TRPV1 with their respective active sites. Furthermore, the simulation analysis indicated that the binding poses of the compounds were maintained during the simulation period. However, Bergenin was quite unstable with

TRPM8 during a simulation time of 100 ns. Based on these findings we conclude that Bergenin has a better binding affinity and interaction with TRPA1 and TRPV1 but poor binding efficiency and interaction with TRPM8

Findings from the biochemical assay revealed that Bergenin treatment effectively lowered MDA and nitrite levels the in sciatic nerves of CINP rats. Additionally, Bergenin restored reduced GSH levels in chemotherapy-treated rats, aligning with prior findings. Notably, Bergenin does not modulate TRPM8 expression, while it significantly restored increased TRPA1 and TRPV1 expressions during neuropathic pain. These observations prompted *in-silico* investigations into Bergenin's dynamic interactions with TRP channel including TRPA1, TRPV1, and TRPM8 aiming to decipher their implications in CINP. Computationally, we examined Bergenin's interactions with these TRP channels, unravelling potential insights for CINP treatment. Homology modelling is currently the most precise computational tool for generating accurate structural models and is commonly utilized in many biological applications (Waterhouse et al., 2018). Molecular docking findings suggested that Bergenin has strong binding affinity for TRP channels in the order of decreasing affinity TRPA1>TRPV1>TRPM8. Further, molecular dynamics studies, RMSD and RMSF remarks values were obtained which suggests that Bergenin forms stable complex with TRPA1 and TRPV1. However, Bergenin and TRPM8 complex showed fluctuation in the beginning and throughout time intervals of 100 ns. Findings from the in-silico studies corroborate with the results obtained from *in-vivo* behaviour and molecular biology studies pointing towards the potential of Bergenin in ameliorating chemotherapy-induced neuropathic pain.

5.7.4 Overall Summary

The study aimed to assess Bergenin's impact on chemotherapy-induced neuropathic pain (CINP) in rat models, investigating its cellular and molecular mechanisms. Behavioral assays were conducted before and after inducing CINP, followed by Bergenin treatment. The study evaluated changes in tight junction proteins, pro-inflammatory cytokines, TRP channels, and NR2B in the lumbar dorsal root ganglion (DRG) and spinal cord. *In-silico* studies validated Bergenin's binding affinity with various TRP channels. Key findings showed Bergenin effectively reduced pain-like behavior in neuropathic rats without causing central nervous system toxicity. Bergenin restored chemotherapy-induced alterations, including TRP channel activation, disruption of spinal cord tight junction proteins, pro-inflammatory cytokine infiltration, and NR2B activation, except for TRPM8. *In-silico* investigations revealed Bergenin's strong binding affinity with TRPA1 and TRPV1, with a weaker interaction with TRPM8. In conclusion, Bergenin holds therapeutic potential for alleviating CINP by modulating the TRPA1/TRPV1/NR2B signaling pathways, providing valuable insights into CINP mechanisms and suggesting Bergenin as a candidate for effective treatments. Overall summary of the findings was depicted in **Figure 5.22.**

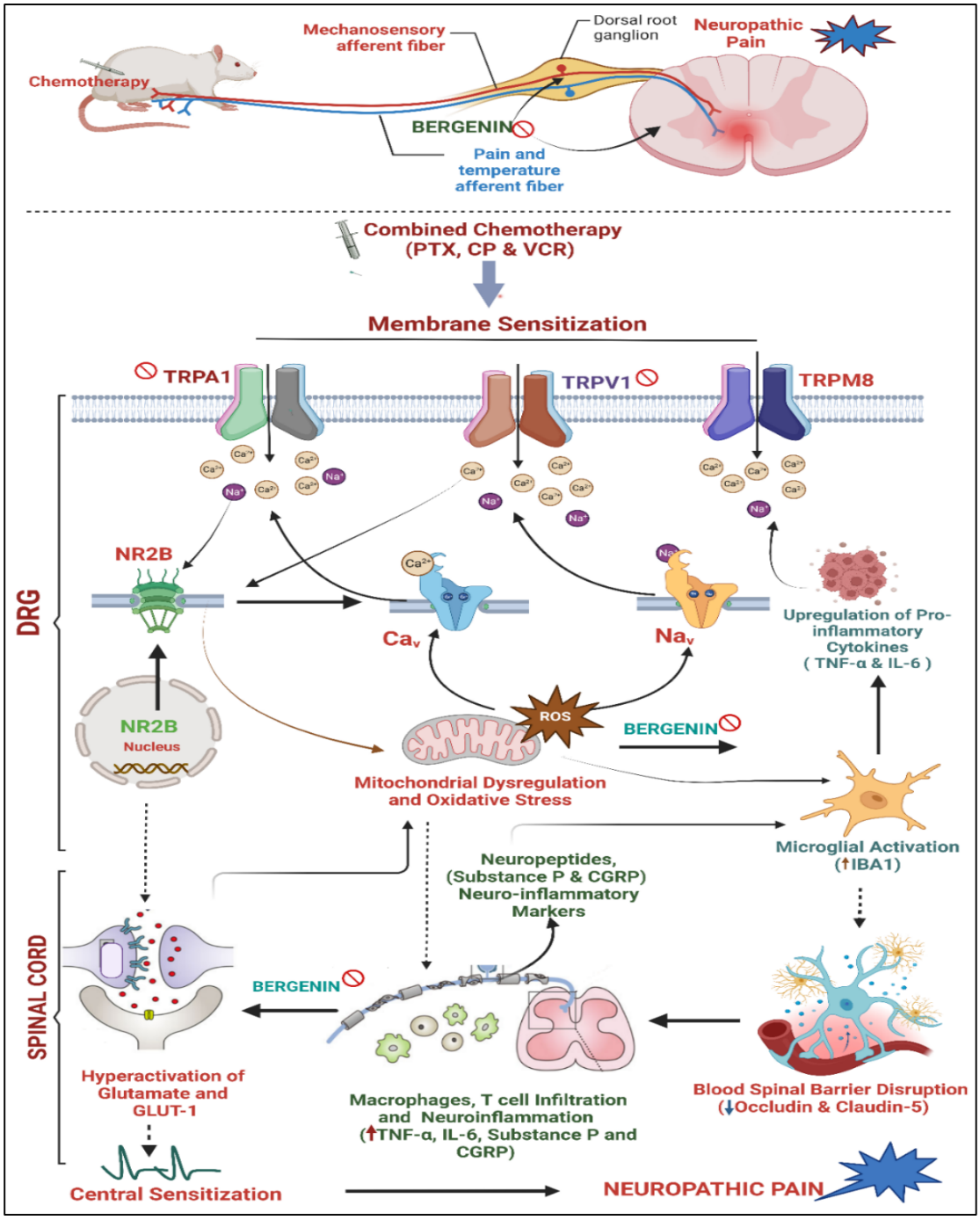

Figure 5.22 Working hypothesis and overall summary of the results. Bergenin attenuates chemotherapy-induced neuropathic pain (CINP) by regulating TRPA1/TRPV1 mediated NR2B activation and neuroinflammation.

5.8 Outcomes

In summary, the findings presented in this study reveal, for the first time, that Bergenin effectively reduces both evoked and spontaneous pain in animal model of chemotherapy-induced peripheral neuropathy. Furthermore, it's important to highlight that the administration of Bergenin did not result in motor incoordination, sedation, or the development of drug dependence in the treated rats. Therefore, Bergenin may serve as a potential and safer alternative to alleviate chronic pain in cancer patients undergoing chemotherapy.