Chapter-4

Phytochemical and Pharmacological Investigations of *Sida cordifolia* Root Extract on Nerve Injury-induced Chronic Pain

4.1 Introduction

Neuropathic pain is defined as pain caused by lesion or disease of somatosensory system, that involves abrasion in peripheral fibers and central neurons resulting in wide variety of complex chronic pain syndromes (Ameenudeen et al. 2022). According to a report, worldwide epidemiological pervasiveness of neuropathic pain ranges between 6.9-10 % and is likely to increase significantly in years to come. Recent COVID pandemic has further added to the neuropathic pain burden as few studies suggests that up to 2.3% of hospitalized patients being treated for corona virus reports neuropathic pain (Zouhri and El Baroudi 2021). The concern is raised even more due to inadequate efficacy of currently available analgesics and their off-target side effects.

Sida cordifolia, a blossoming herb widely distributed across tropical and subtropical areas in India and Sri Lanka, is commonly known as "Bala" or "country mallow" within the Malvaceae family. Traditionally, the roots of the plant are utilized as a tonic to fortify the central nervous system and treat neurological disorders like hemiplegia, facial paralysis, cervical spondylosis, and neurosis. Furthermore, historical records highlight the use of the roots in managing neuropathic pain conditions such as neuralgia and sciatica (Justino et al. 2020). Up to the present moment, there exists a notable absence of scientific documentation that corroborates the traditional assertion regarding the efficacy of *Sida cordifolia* in the management of neuropathic pain.

Rigorous scientific investigations and empirical evidence supporting the purported benefits of *Sida cordifolia* for alleviating neuropathic pain are currently lacking in the available literature. the potential effect of *Sida cordifolia* roots on neuropathic pain has not been explored. Hence, the primary goal of this study is to explore the potential anti-allodynic properties inherent in the root extract derived from *Sida cordifolia*. Present study specifically targets the assessment of the extract's effectiveness within a carefully designed rat model simulating chronic constriction-induced neuropathy. The aim is to comprehensively examine and discern the impact of *Sida cordifolia's* root extract on alleviating allodynia, a heightened sensitivity to pain typically associated with neuropathic conditions. This investigation contribute valuable insights into the potential therapeutic applications of *Sida cordifolia* roots in the context of neuropathic pain management.

Chronic pain resulting from nerve injury remains a significant clinical challenge, impairing the lives of millions worldwide. Traditional analgesic approaches often fall short, prompting the exploration of alternative therapies. *Sida cordifolia,* a medicinal plant with a rich history in traditional medicine, has emerged as a potential candidate for addressing nerve injury-induced chronic pain. The roots of *Sida cordifolia*, known as Bala, contain a diverse array of phytochemicals, including alkaloids, flavonoids, tannins, saponins, and polyphenols. These bioactive compounds have demonstrated antioxidant, anti-inflammatory, and analgesic properties in preclinical studies. Animal models simulating neuropathic pain have shown promising results, suggesting that *Sida cordifolia* root extract may alleviate pain behavior, inflammation, and oxidative stress. This research aims to comprehensively explore the phytochemical composition of *Sida*

cordifolia root extract and investigate its pharmacological effects on nerve injury-induced chronic pain. The study's outcomes could contribute valuable insights into the development of innovative and effective therapies for managing chronic neuropathic pain, potentially offering a natural and safer alternative to existing treatments.

4.2 Experimental procedure

For the purpose to investigate the neuropathic pain combating potential of the root extract of *Sida cordifolia* DCM: Me extract of 2 kg of roots were cold maceration for 7 days with intermittent stirring. Resulting extract was evaporated, completely dried and stored at 4 °C, for further use. The quantification of total phenolic and flavonoid content in the obtained crude extract (SCE) was carried out utilizing gallic acid and quercetin as reference standards. This provided a quantitative measure of the phenolic and flavonoid constituents present in the crude extract (SCE) enhancing our understanding of its chemical composition and potential bioactive properties. The antioxidant capacity of the extract through the employment of the DPPH (2,2-diphenyl-1-picrylhydrazyl) assay was evaluated. This involves measuring the ability of the extract to neutralize DPPH radicals, thereby providing valuable insights into its potential as an antioxidant, hence, serves as an indicative measure of the extract's capacity to scavenge free radicals, reflecting its anti- oxidative capability.

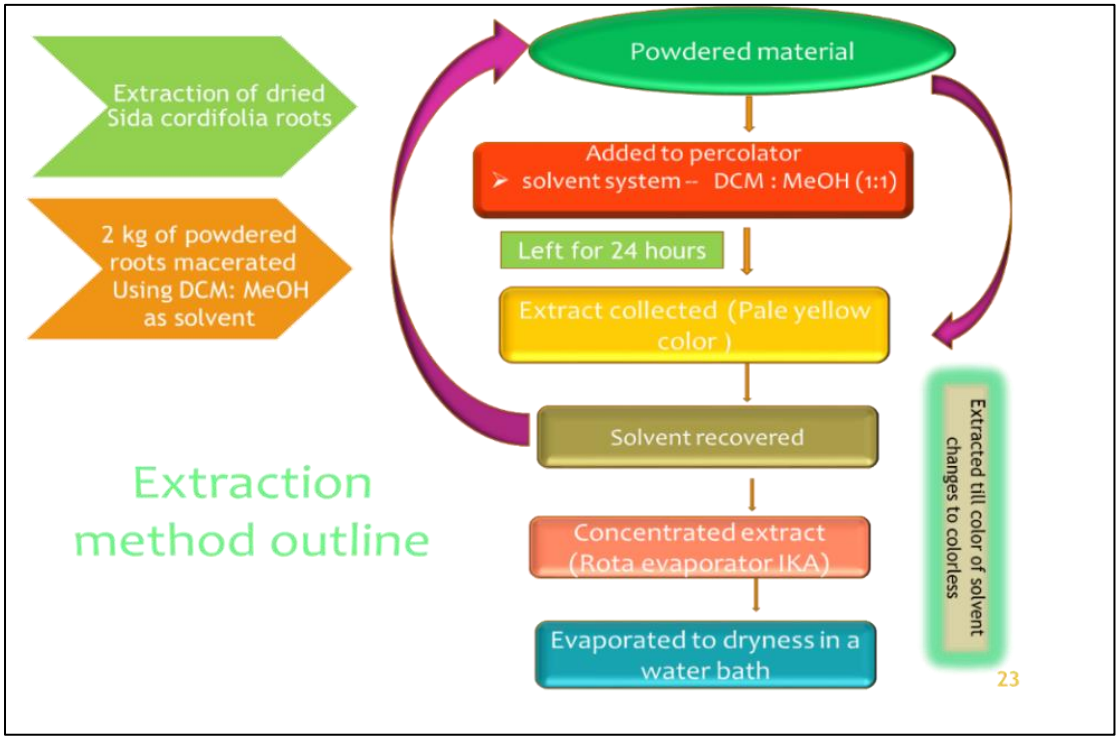

Fig. 4.1 Extraction method outline for *Sida cordifolia* roots

Liquid Chromatography-Mass Spectrometry/Time-of-Flight (LC-MS/TOF) analysis on the crude extract (SCE) was also conducted. This advanced analytical technique allowed for a detailed and comprehensive examination of the chemical composition of the extract. anti-nociceptive activity of *Sida cordifolia* crude extract (SCE) in CCI-induced neuropathic pain model in rats was evaluated. Rats were divided into six experimental groups with a minimum of eight rats/group. The first group consists of naïve healthy rats, while the second group was disease control group where nerve-injured rats were administered with the vehicle for SCE. Rats in the third, fourth and fifth groups belonged to different test groups where nerve-injured rats were administered orally crude extract (SCE) at 200 mg/kg, 400 mg/kg, and 800 mg/kg respectively. The sixth group consists of the standard control group in which nerve-injured rats were treated with gabapentin (30 mg/kg *i.p*). On 14th-day post nerve injury

pain behaviors including heat hyperalgesia (Hargreaves test) and static mechanical allodynia (von-Frey test) were assessed at 0, 0.5, 1, 2, 4 hr post administration of drugs each day for next seven days. Next the animals were sacrifices and DRG, spinal cord and sciatic nerve were harvested for molecular analysis. RT-PCR and western blotting was performed to assess inflammatory cytokines and NR2B mRNA expression in DRG and Spinal cord.

4.3 Results and discussion

4.3.1 Phytochemical analysis

4.3.1.1 Total Phenolic and flavonoid content of SCE

Total phenolic content was measured using a standard calibration curve (R2 = 0.991) and was quantified and expressed as milligrams of gallic acid equivalents per gram of extract. SCE was found to have 2.10 ± 0.04 mg gallic acid equivalents/g of total phenolic content. Flavonoid concentration was determined using the spectrophotometric method and was expressed in terms of quercetin equivalents. The concentration of flavonoids in SCE was found to be 1.12 ± 0.05 mg equivalent of quercetin. The related observation and calculated data is presented in (Table 4.1.)

4.3.1.2 Antioxidant assay of SCE

Total free radical scavenging activity of the DCM: Me (1:1) extract of root (SCE) was evaluated by using DPPH assay. The IC-50 results of the SCE showed at 0.45 ± 0.03 mg/ml concentration, whereas the IC-50 of Gallic acid was 0.002 mg/ml which was used as a positive control. The related observation and calculated data is presented in (**Table 4.1)**

Table 4.1 Estimation of Antioxidant, total phenolic and flavonoid content

***Sida cordifolia* Root Extract**	
Total phenolic content (mg gallic acid equivalents/g)	**2.10 ± 0.04**
Total flavonoid content (mg rutin equivalents / g)	**1.12 ± 0.05**
Anti-oxidant activity (%)	**0.45 ± 0.03**

4.3.1.3 LC-MS/TOF analysis of SCE

LC-MS/TOF (time of flight) analysis of the *Sida cordifolia* DCM:Me crude extract (SCE) of roots indicate the presence of profuse phytoconstituents with different molecular mass showing 30 min retention time (RT). LC-MS scan displayed distinct RTs for various compounds present in the crude extract sample **(Fig. 4.1).**

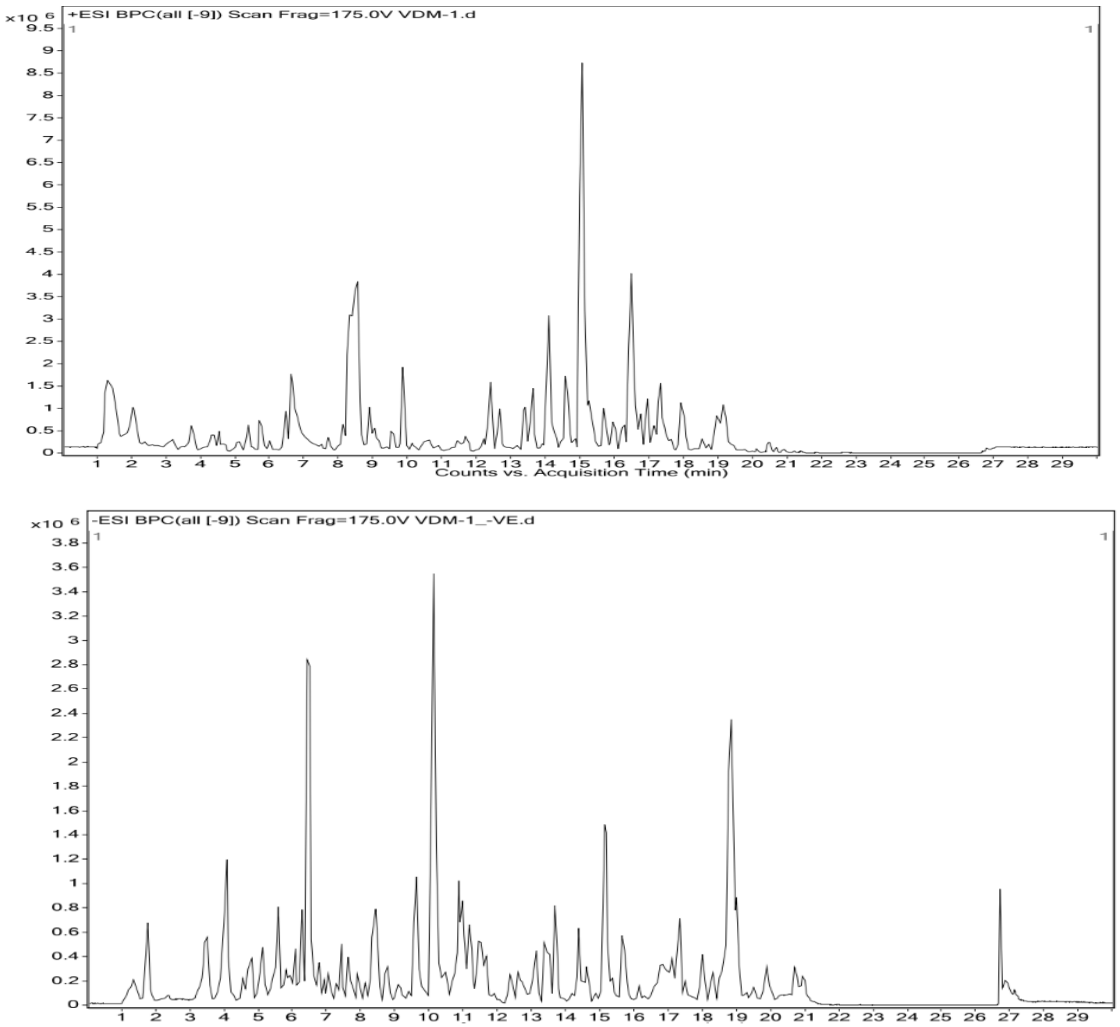

Figure 4.2 Sida cordifolia DCM: ME (SCE) extract LC-MS/TOF chromatogram showing different phytoconstituents

4.3.2 In-vivo evaluation of *Sida cordifolia* root crude extract (SCE)

4.3.2.1 Anti-nociceptive activity of *Sida cordifolia* crude extract (SCE) in CCI-induced neuropathic pain model

Chronic pain is accompanied by the lower pain threshold to the innocuous stimuli. Hypersensitivity induced by non-noxious thermal as well as mechanical stimuli is an illustrating feature among neuropathic pain patients. Hence, potential of SCE in mitigating mechanical allodynia and thermal hyperalgesia was evaluated using von

Frey hair test and Hargreave's test respectively. Paw withdrawal latency (PWL) and paw withdrawal threshold (PWT) of nerve injured rat's day 14 post CCI surgery was recorded as outcome measures of thermal hyperalgesia and mechanical allodynia respectively. Nerve injured rats showed significantly ($p<0.001$) lowered PWL and PWT as compared to their preinjury baseline indicating development of hyperalgesia and allodynia post CCI surgery. Administration of SCE (200, 400, and 800 mg/kg p.o) for seven consecutive days leads to gradual and time dependent increase in both PWL and PWT of nerve injured rats as compared to vehicle treated group. Two-way ANOVA followed by Bonferroni's multiple comparison suggested a significant effect across the groups [$F (5, 42) = 173$; $P<0.001$] and time points [$F (4.75, 199) = 155$; $P<0.001$] in comparison of vehicle and drug treated nerve injured rats. An observable reversal in decreased PWL was first appeared at 400 and 800 mg/kg on day 4 post SCE treatment with peak effect at 2 hours which lasted for 4 hours. From day 5 to day 7 a dose dependent significant effect on PWL was observed at 200, 400 and 800 mg/kg as compared to the vehicle administered group. While, maximum effect was achieved at a dose of 800 mg /kg on day 6 and day 7 ($p<0.001$) and was found to be statistically significant when compared to the reference drug pregabalin (30 mg/kg) (Fig 4.2 A). Gabapentin (30mg/kg i.p) also decreased the thermal hyperalgesia as evident by significant increase in ipsilateral PWL at 0.5 hr ($p=0.007$), 1 hr ($p<0.001$), 2 hr ($p<0.001$) and 4 hr ($p<0.001$) post administration as compared to the vehicle treated nerve injured rats. 800 mg/kg p.o SCE administration significantly ($p<0.05$), increased the innocuous stimuli-induced PWT in CCI rats on day 2. SCE from day 4 onwards exhibits significant anti-allodynic effect at 200mg/kg ($p<0.05$), 400 mg/kg ($p<0.01$), and 800 mg/kg ($p<0.001$) at 120 mins post SCE administration which lasts up to 240

mins. Significant effect was observed at 400 and 800 mg/kg on day 6 and day 7 ($p<0.001$) displaying maximum peak effect at 2hrs which lasts for up to 4 hrs. Gabapentin (30mg/kg i.p) also decreased the paw withdrawal threshold evident by a significant increase in ipsilateral PWT at 0.5 hr ($p=0.007$), 1 hr ($p<0.001$), 2 hr ($p<0.001$) and 4 hr ($p<0.001$) post administration as compared to the vehicle treated nerve injured rats (Fig 4.2 B).

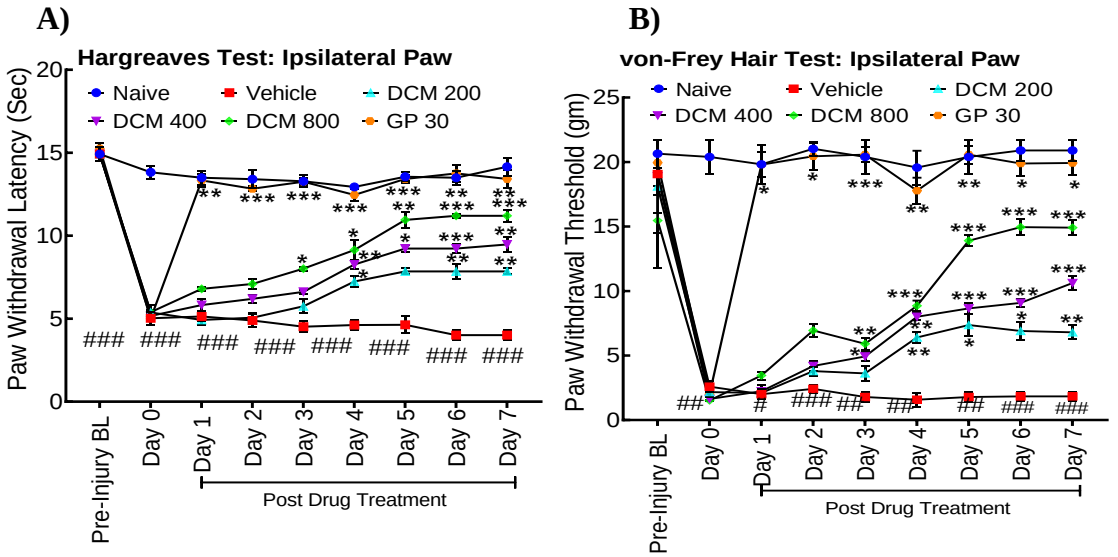

Figure 4.3 Effect of SCE (DCM:Me) extract on thermal and mechanical pain in nerve-injured rats. Administration of (200 mg/kg, 400 mg/kg, 800 mg/kg, p.o.) showed significant improvement in the paw withdrawal threshold in the nerve-injured rats from day 3, and peak effect is observed from day 5 and maintained up to day 7. **A)** Thermal Pain **B)** Mechanical allodynia**.** Data were expressed as mean ± SEM and analyzed by two-way ANOVA (Bonferroni's multiple comparisons) (n=4/group). *($p<0.05$), **($p<0.01$) and ***($p<0.001$) represents significance compared to the vehicle group. ### represents significance compared

4.3.3 SCE restored oxido-nitrosative stress markers in sciatic nerve of nerve injured rats

Any physical insult may cause an imbalance between production, accumulation and detoxification of reactive oxygen species in cell and tissues leading to onset of severe oxidative stress in biological system. Biochemical assays were performed to observe the levels of oxidative markers MDA and nitrite along with antioxidant enzymes GSH in sciatic nerve of rats. SCE (400 and 800 mg/kg) was able to abate the elevated levels of GSH ($p<0.001$) and restored the MDA levels ($p<0.001$) in sciatic nerve tissue of nerve injured rats **(Fig. 4.3 A-B).** Whereas, nitrite levels were restored at only 800 mg/kg SCE ($p<0.001$) as well as gabapentin (30 mg/kg) as compared to vehicle treated rats **(Fig 4.3 C).**

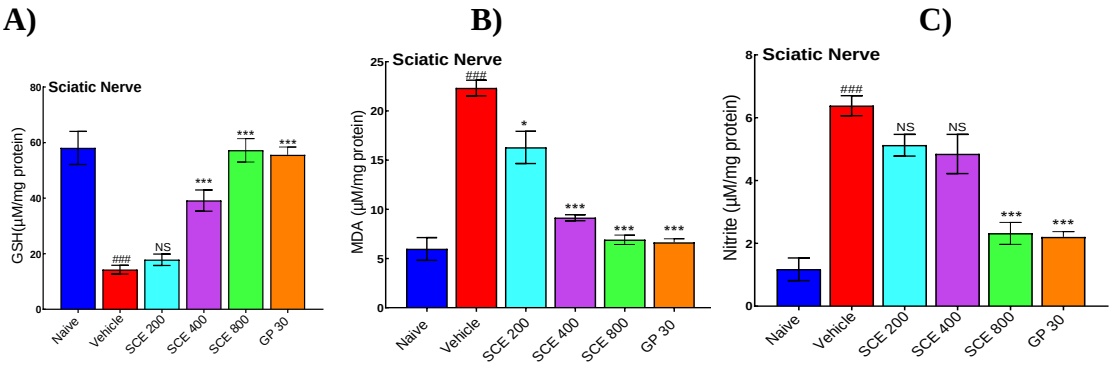

Figure 4.4 Effect of *Sida cordifolia* root extract (SCE) on biochemical markers in sciatic nerve of injured rats. A) GSH **B)** MDA and **C)** Nitrite. Data were expressed as Mean ± SEM and analyzed by one-way ANOVA (Post hoc analysis: Tukey's post hoc analysis test).*($p<0.05$), **($p<0.01$) and ***($p<0.001$) represents significance compared to the vehicle group. ### represents significance compared to the naïve. Sida cordifolia rioot extract dose SCE200 : 200mg/kg, SCE400: 400mg/kg and SCE800: 800mg/kg & GP30: Gabapentin:30 mg/kg

4.3.4 Effect of SCE on mRNA expression of pro inflammatory cytokines and neuropeptides in DRG and spinal cord of nerve injured rats

Pro inflammatory cytokines, key regulators of inflammation play a critical role in development and maintenance of chronic pain conditions. TNF-α, IL1β, CGRP and Substance P mRNA expressions were found to be significantly upregulated in ipsilateral L4-L5 DRG and spinal cord of nerve injured rats. Treatment with SCE (200, 400 and 800 mg/kg) leads to significant attenuation in DRG and spinal pro-inflammatory cytokines and neuropeptides of nerve injured rats. One-way ANOVA followed by Tukey's multiple comparison test suggest, SCE at a dose of 200, 400 and 800 mg/kg along with standard drug gabapentin (30 mg/kg) was able to significantly suppress ($p<0.001$) the overexpression of IL1β and TNF-αin the DRG as well as spinal cord of treated rats as compared to saline treated group **(Fig 4.4 A1-B1 & A2- B2).**

Calcitonin gene–related peptide (CGRP), a 37-amino acid peptide primarily present in C and Aδ sensory fibers originating from the dorsal root ganglia (DRG), plays a crucial role in chronic pain signaling. The activation of CGRP receptor signaling cascades, triggered by neuropeptides released from the C-terminal of primary sensory neurons, leads to NMDA receptor sensitization, contributing to central sensitization. Substance P and CGRP, remains well-established pain-modulating peptides, and have long been implicated in the initiation and perpetuation of neuropathic pain followed by peripheral nerve injury. In cases of chronic constriction injury, there is a significant elevation in the release of Substance P and CGRP in both the dorsal root ganglia (DRG) and spinal cord of rats. Elevated levels of CGRP and substance P were significantly subsided by SCE administration in DRG and spinal cord of nerve injured rats as

compared to the vehicle treated group **(Fig 4.5 A1-B1 & A2- B2).**One-way ANOVA followed by Tukey's multiple comparison test demonstrated a significant effect across the groups on DRG tissues and spinal cord CGRP [F (6, 18) = 8.66; p<0.001 and F (8, 18) = 9.64; p<0.001 respectively] and Substance P [F (5, 18) = 11.1: p<0.001 and F (5, 18) = 18.4; p<0.001 respectively] levels in vehicle and drug treated nerve injured rats.

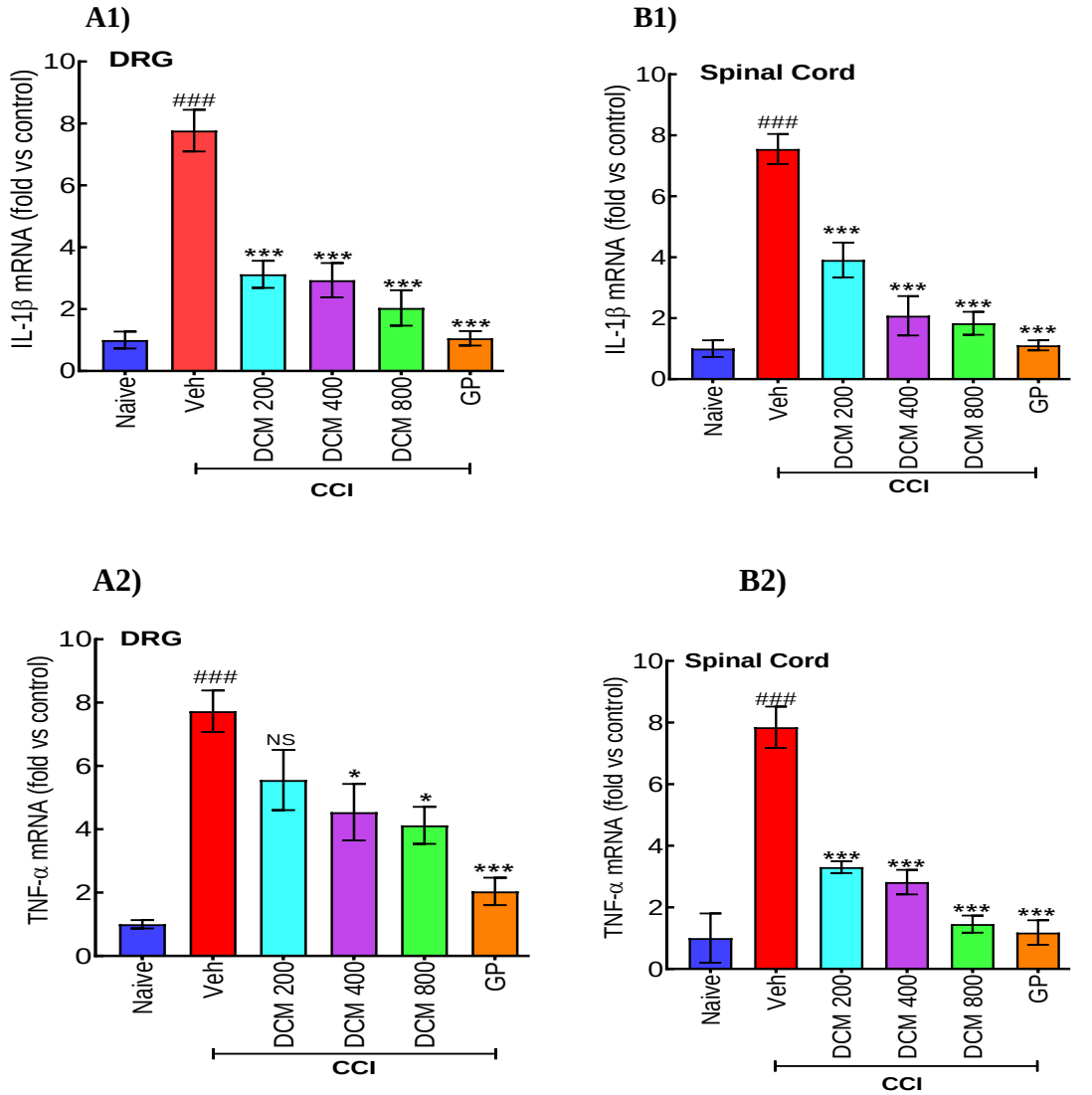

Fig 4.5 Effect of SCE on Pro-inflammatory cytokines in nerve-injured rats. DCM extract (200 mg/kg, 400 mg/kg, 800 mg/kg, p.o significantly restored the level of IL-1β and TNF-α mRNA in DRG **(A1 & A2)** and IL-1β and TNF-α spinal cord **(B1&B2)** of nerve-injured animals. Data were expressed as mean ± SD (n=4/group) # # # (p<0.001) represents significance compared to the naïve group. *(p<0.05), **(p<0.01) and ***(p<0.001) represents significance compared to the nerve-injured group.

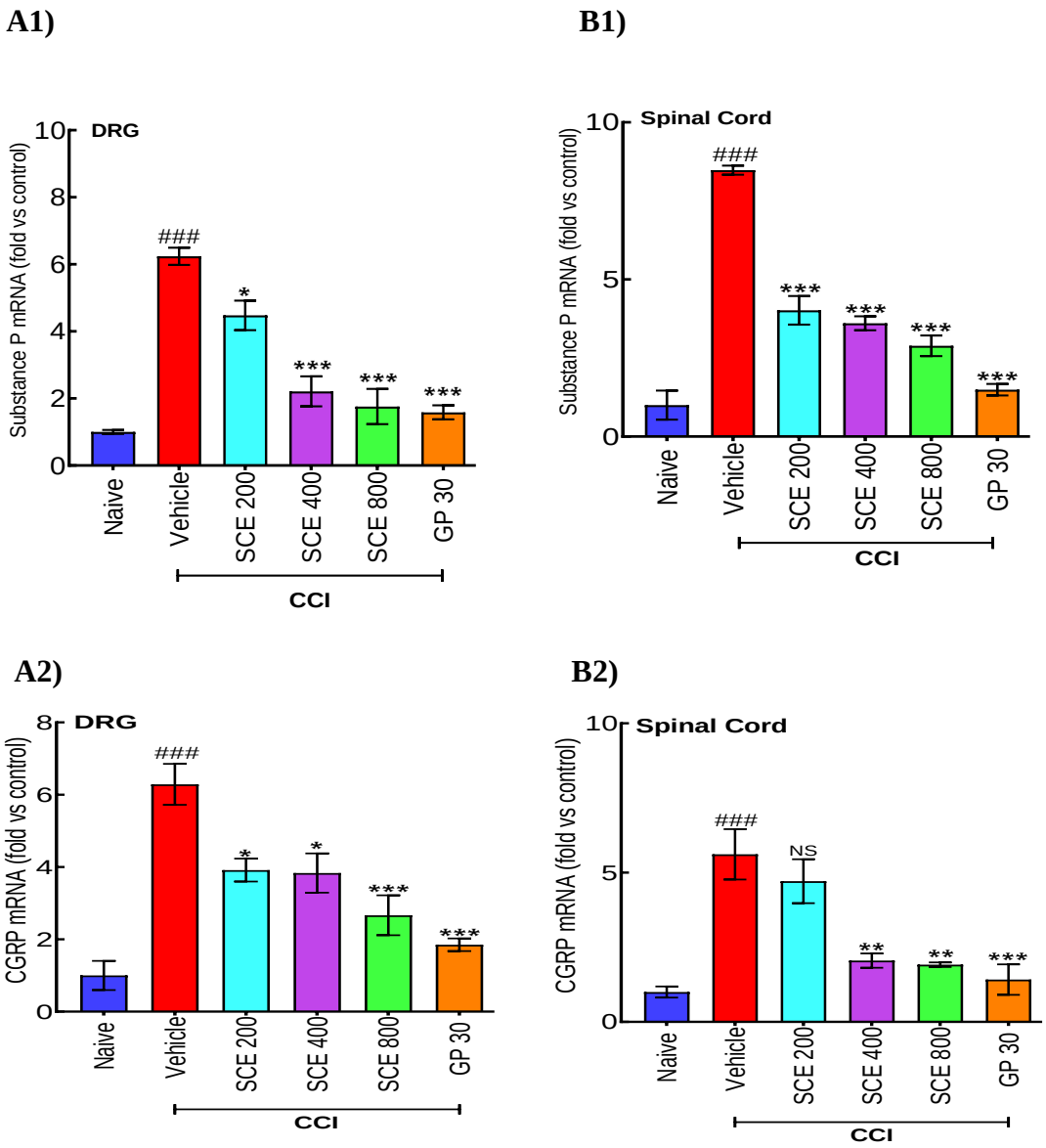

Figure 4.6 Effect of SCE on Substance P and CGRP in nerve-injured rats. SCE (200 mg/kg, 400 mg/kg, 800 mg/kg, p.o.) significantly restored the level of Substance P and CGRP mRNA expression in DRG **(A1&A2)** and spinal cord **(B1&B2)** of nerve-injured animals Data were expressed as mean ± SEM and analyzed by two-way ANOVA (Bonferroni's multiple comparisons). *($p<0.05$), **($p<0.01$) and ***($p<0.001$) represents significance compared to the vehicle group. ### represents significance compared to the naïve. Sida cordifolia rioot extract dose SCE200: 200mg/kg, SCE400: 400mg/kg and SCE800: 800mg/kg & GP30: Gabapentin:30 mg/kg

4.3.5 *Sida cordifolia* root extract (SCE) Downregulates NR2B mRNA Expressions in DRG & Spinal Tissues of Neuropathic Rats

NR2B plays a crucial role in development and maintenance of central sensitization during chronic pain condition. Several scientific evidences suggest downregulation of NR2B is linked to reduced chronic and neuropathic pain symptoms. Enhanced pain perception after peripheral nerve injury involves increased nociceptor sensitivity and NMDA receptor-mediated synaptic changes). While competitive NMDA receptor blocking can cause CNS side effects, partially antagonizing the receptor allows therapeutic value by inhibiting pathological activation without impairing physiological activity. NR2B subunit, predominantly expressed in layer II spinal dorsal horn and DRG, plays a key role in initiating and sustaining neuropathic pain, contributing to lasting spinal excitability. NR2B mRNA expressions were found to be significantly upregulated in both lumbar DRG and spinal cord of neuropathic rats. Treatment with SCE, medium (400 mg/kg) and high (800 mg/kg) dose, leads to significant ($p<0.01$) downregulation of NR2B mRNA expressions of nerve injured rats. A significant effect was observed across the groups in one-way ANOVA followed by Tukey's multiple comparison test on NR2B, mRNA expression in L4–L5 spinal cord ($p < 0.0001$) and DRG tissue samples **(Fig.4.6 A, B).**

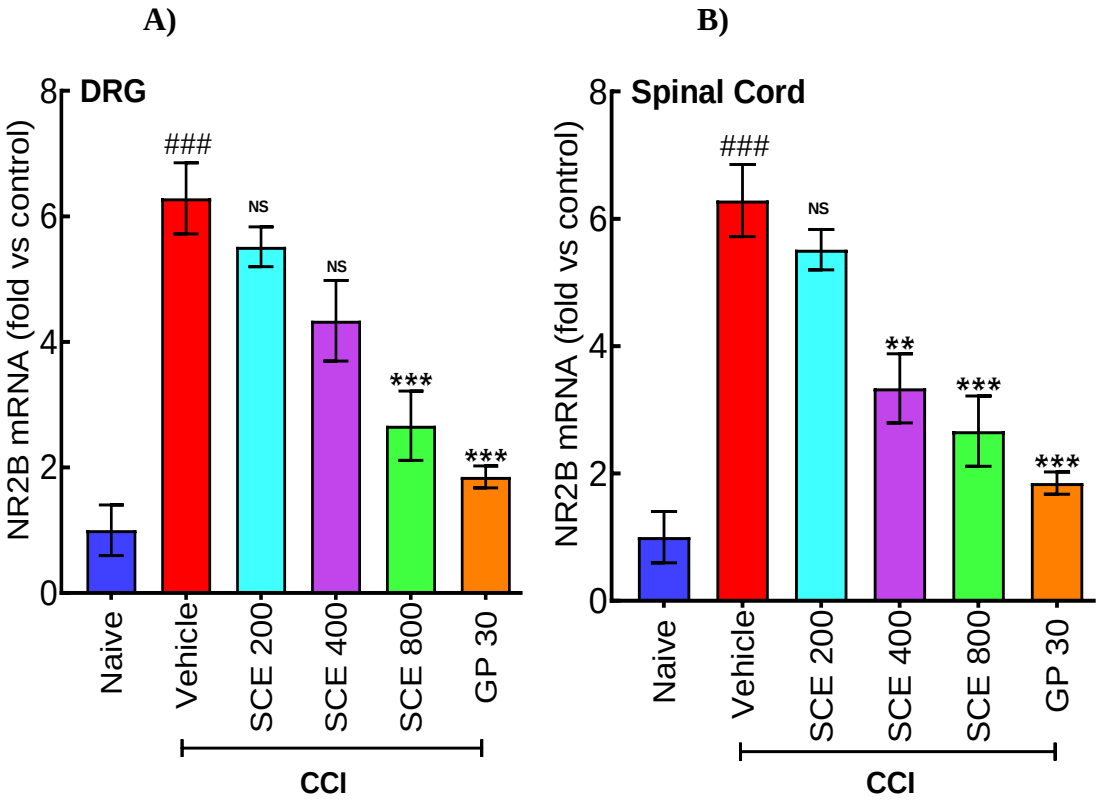

Figure 4.7 Effect of SCE on NR2B mRNA levels in DRG & Spinal Cord of Nerve Injured Rats. SCE (200 mg/kg, 400 mg/kg, 800 mg/kg, p.o.) significantly restored the NR2B mRNA expression level in the **A)** DRG of nerve-injured animals and **B)** Spinal Cord. Data were expressed as mean ± SD (n=4/group) ###($p<0.001$) represents significance compared to the naïve group. *($p<0.05$), **($p<0.01$) and ***($p<0.001$) represents significance compared to the nerve-injured group.

4.6 Outcome

- The administration of *Sida cordifolia* root extract (SCE) at varying doses (200, 400, and 800 mg/kg p.o) resulted in a dose-dependent attenuation in both thermal hyperalgesia and mechanical allodynia in CCI rats. This indicates a potential analgesic effect of SCE, with the degree of pain alleviation correlating with the administered dose.

- SCE demonstrated a mitigating effect on oxido-nitrosative & inflammatory cascade in the sciatic nerve, dorsal root ganglion (DRG), and spinal cord of rats with nerve injuries. Furthermore, the treatment with SCE supress mRNA expressions of Substance P, Calcitonin Gene-Related Peptide (CGRP), in both the DRG and spinal cord of rats with neuropathic conditions.

- NR2B is a subunit of the N-methyl-D-aspartate (NMDA) receptor implicated in the amplification of pain signals. Treatment with SCE also decreased Substance P, CGRP & NR2B mRNA expressions in DRG & spinal cord of neuropathic rats

- The downregulation of these molecular markers indicates that SCE exerts its effects not only at the behavioral level but also at the molecular level, potentially disrupting key signaling pathways involved in neuropathic pain transmission.

- Overall, the findings suggest that *Sida cordifolia* root extract exhibits promising therapeutic potential against neuropathic pain by addressing both behavioral manifestations and underlying molecular mechanisms, favouring further exploration of its analgesic properties and potential clinical applications.

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