Chapter-5

Bioactivity Guided Fractionation of *Sida cordifolia* Root Extract: In-vitro and in-vivo Investigations

5.1 Introduction

A European chronic pain report highlighted that prescribed medications were often ineffective (64% reported) or not followed properly (48%), with 14% discontinuing due to side effects. This underscores the need for novel therapies. Our previous study on *Sida cordifolia* extract (SCE) revealed significant anti-allodynic effects, high phenolic and flavonoid content, and potent antioxidant properties. Flavonoids, acting as antioxidants, may have analgesic effects. SCE also suppressed NMDA receptor subunit NR2B and proinflammatory cytokines. Nr2B is implicated in neuropathic pain, and its downregulation has been linked to symptom relief. The study aims to further investigate SCE's therapeutic potential, including fractionation and testing in a chronic constriction injury model in rats, to identify effective fractions and key phytoconstituents, exploring detailed cellular and molecular mechanisms

5.2 Experimental design

Polarity gradient fractionation of the *Sida cordifolia* root crude extract was carried out, utilizing hexane, chloroform, and ethyl acetate in an aqueous medium. This stepwise procedure aimed to isolate distinct fractions with varying polarities and chemical compositions from the original extract. The identification of the most potent fraction was determined through in-vitro egg albumin denaturation assay, which acts as

a valuable indicator of the fraction's efficacy in preventing protein denaturation, a process often associated with inflammation. The fraction that exhibited the highest capability in inhibiting egg albumin denaturation was identified as the most potent, signifying its potential as a bioactive fraction. Subsequently, the anti-nociceptive potential of this identified potent fraction was systematically evaluated in chronic constriction injury (CCI)-induced neuropathic pain model in rats. For this purpose, 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. Rats in the third, fourth and fifth groups belonged to different test groups where nerve-injured rats were administered orally residual aqueous fraction (SAF) 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 and KIF-17 mRNA expression in DRG and Spinal cord. To gain a more profound understanding of the phytoconstituents responsible for the anti-allodynic action, High-Resolution Mass Spectrometry (HRMS) analysis was conducted for the *Sida cordifolia* root (SAF). HR-MS a sophisticated analytical technique with high-resolution allowed for a detailed examination of the various chemical compounds present in the extract. Following the HRAMS analysis, the identified most abundant phytoconstituent was

subjected to quantification using High- Performance Thin-Layer Chromatography (HPTLC). Present study involved successive fractionation, identification, and subsequent evaluation in a neuropathic pain model, allows to characterize the specific fraction of *Sida cordifolia* root extract with the maximum anodynic potential. This study first time reports the potential of aqueous fraction of roots of *Sida cordifolia* in alleviating neuropathic pain like conditions. Besides it also contributes to a deeper understanding in the identification of targeted components for further exploration and development of future medication aimed at reliving pain.

5.3 Results and discussion

5.3.1 *In-vitro* anti-inflammatory activity of successive fractions of crude extract

Whole DCM:Me (1:1) crude extract (SCE) of *Sida cordifolia* roots demonstrated potential anti-nociceptive activity in CCI-induced neuropathic pain model. To identify the most bioactive fraction that could possibly be responsible for the observed effects, whole crude extract SCE was subjected to successive fractionation. SCE on fractionation yielded four different fractions, hexane fraction (SHF), Ethyl acetate fraction (SEF), Chloroform fraction (SCF) and Residual aqueous fraction (SAF). All the fractions were further analysed for in-vitro anti-inflammatory potential employing egg albumin denaturation assay. Anti-inflammatory potential of crude extract of *Sida cordifolia* and its successive fractions was evaluated against denaturation of egg albumin assay. The minimum concentration to achieve the IC-50 was observed in aqueous fraction (SAF) of crude extract at 82.58 mg/ml when compared to the hexane, chloroform, ethyl acetate fractions and crude extract. While

reference drug Diclofenac sodium displayed an IC-50 value of 0.39 mg/ml. Among the all-successive fractions maximum potential of 50% inhibition at lowest concentration was observed in aqueous fraction SAF- 82.58 mg/ml followed by hexane fraction (SHF) - 141.24 mg/ml, ethyl acetate fraction (SEF) - 149.4 mg/ml and chloroform fraction (SCF) - 199.43 mg/ml **(Table 5.1).** Based on these findings, residual aqueous fraction (SAF) was taken forward for further detailed investigations.

Table 5.1 Estimation of *in-vitro* anti-inflammatory activity of crude extract and its successive fractions

EXTRACT/ FRACTION	IC-50 Value
Crude extract (SCE)	**99.16 mg/ml**
Hexane fraction (SHF)	**141.24 mg/ml**
Chloroform fraction (SCF)	**199.43 mg/ml**
Ethyl acetate fraction (SEF)	**149.4 mg/ml**
Residual aqueous fraction (SAF)	**82.58 mg/ml**

5.3.2 *In-vivo* activity of SAF in CCI induced neuropathic pain model in rats

5.3.2.1 SAF attenuates thermal hyperalgesia in nerve injured rats

Efficacy of SAF on thermal hyperalgesia in rats was investigated using Hargreaves apparatus. Residual Aqueous Fraction (SAF) exhibited a pronounced capacity to mitigate thermal hyperalgesia in rats subjected to nerve injuries. Thermal hyperalgesia, characterized by an increased sensitivity to thermal stimuli, is a common manifestation of neuropathic pain. The observed attenuation of this hyperalgesic response in rats treated with SAF suggests a substantial analgesic effect associated with this particular fraction. A significant decline ($p<0.001$) in PWL as compared to

respective pre- injury baseline and nave rats was observed indicating development of thermal hyperalgesia in rats. 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 to vehicle and drug treated nerve injured rats. A significant improvement in PWL was observed on day 2 in SAF treated rats (200 mg/kg, p=0.002; 400 and 800 mg/kg, p<0.001) as compared to vehicle treated nerve injured group (Fig 5A). Standard drug gabapentin (30mg/kg i.p) also decreased the thermal hyperalgesia evident by a 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 (Fig. 5.1)

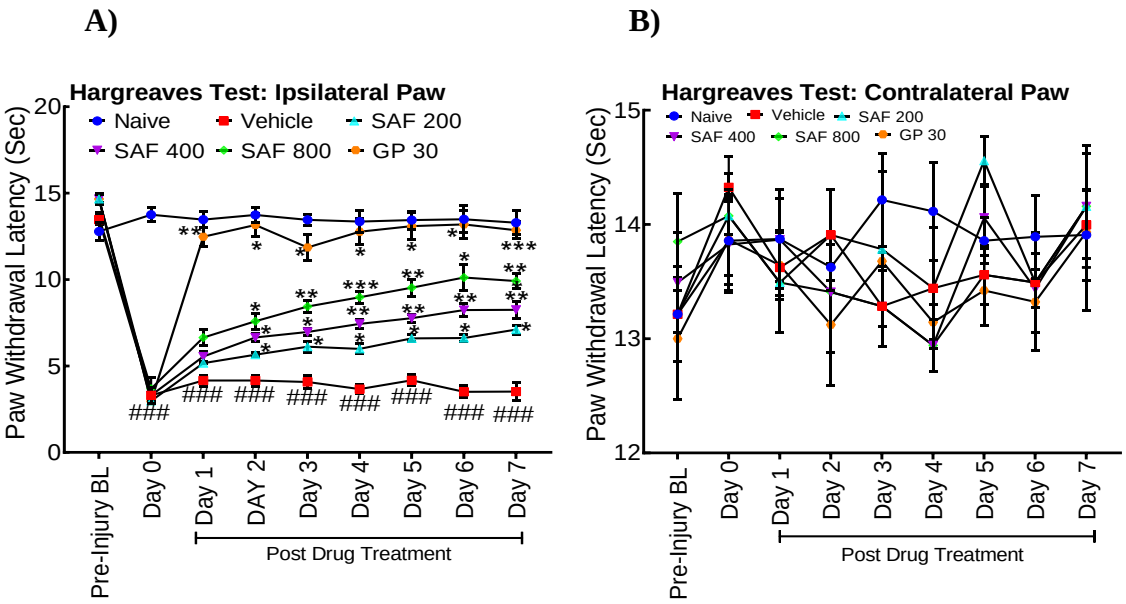


Figure 5.1 Effect of SAF on CCI-induced heat hyperalgesia in nerve injured rats. **A)** Ipsilateral paw withdrawal latency. **B)** Contralateral paw withdrawal threshold. Data were expressed as Mean ± SEM and analyzed by two-way ANOVA (Bonferroni's multiple comparison). ### represents significance compared to the control group (p<0.001), * (p<0.05) ** (p<0.01) and *** (p<0.001) represents significance compared to the CCI group. *Sida cordifolia* aqueous fraction dose SAF200: 200mg/kg, SAF400: 40mg/kg and SAF800 : 800mg/kg, & GP30: Gabapentin 30 mg/kg

5.3.2.2 SAF inhibits mechanical allodynia in nerve-injured rats

Effect of mechanical allodynia on nerve injured rats was determined by using on-Frey hair test. Mechanical allodynia was evident by significant drop in ipsilateral paw-withdrawal threshold PWT ($p < 0.0001$) in nerve injured rats as compared to the naive group, pre-CCI baseline, and contralateral paws **(Fig 5.2 A).** A significant Two-way ANOVA followed by Bonferroni's multiple comparisons demonstrated a significant ($p < 0.0001$; $F (5, 42) = 149$) effect across the groups on ipsilateral paw withdrawal threshold in von-Frey hair test. SAF at doses of (400 and 800 mg/kg p.o.) significantly enhance the PWT in CCI rats on day 2 ($p = 0.0195$, $p = 0.0025$ and $p < 0.0001$, respectively) as compared to the vehicle treated rats. Anodynic effect was observed at 120 minutes while no significant effect was observed at 240 minutes. On day 3 significant improvement in PWT was found at all the three doses of SAF (200, 400 and 800 mg/kg p.o.) starting at 2 hrs and lasting up to 4 hours. These findings demonstrate the anti-allodynic effect of SAF during nerve-injury induced chronic pain in rats. SAF is able to provide an early onset of anodynic effect and at lower dose as compared to the crude extract. Gabapentin (30 mg/kg i.p.) treatment also attenuates the mechanical allodynia in CCI rats at 0.5 h ($p < 0.0001$) and 1 h ($p = 0.0005$) post-drug administration as compared to the vehicle-treated rats. No significant effect was observed in the contralateral paw-withdrawal threshold across the groups **(Fig 5.2 B).**

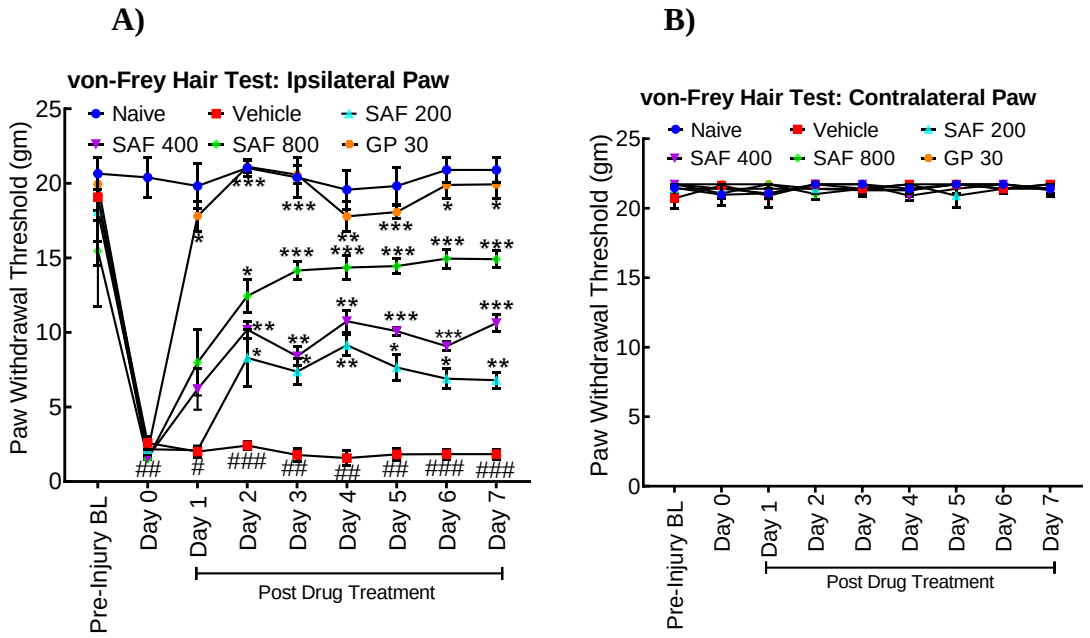


Figure 5.2 Effect of SAF on mechanical allodynia induced by CCI injury. A) Ipsilateral paw withdrawal threshold, **B)** Contralateral paw withdrawal threshold. Data were expressed as Mean ± SEM and analyzed by two-way ANOVA (Bonferroni's multiple comparison) . ### represents significance compared to the control group ($p<0.001$), * ($p<0.05$) ** ($p<0.01$) and *** ($p<0.001$) represents significance compared to the CCI group. *Sida cordifolia* aqueous fraction dose SAF 200: 200mg/kg, SAF 400: 400mg/kg and SAF 800: 800mg/kg, & GP30: Gabapentin 30 mg/kg

5.3.2.3 *Sida cordifolia* aqueous fraction (SAF) restored biochemical alterations in sciatic nerve of injured rats

Biochemical estimations of oxidative markers MDA, nitrite and antioxidant enzyme, GSH were performed using sciatic nerve tissues from nerve injured rats with and without treatment. The *Sida cordifolia* aqueous Fraction (SAF) restored biochemical imbalances in the sciatic nerve of injured rats, specifically affecting key markers such as reduced glutathione (GSH), malondialdehyde (MDA), and nitrite. MDA is a marker of lipid peroxidation and oxidative stress. The normalization of MDA levels in the sciatic nerve indicates that SAF may play a role in mitigating lipid peroxidation and preventing oxidative damage to cellular membranes. This suggests a

protective effect against oxidative stress-induced damage to nerve tissues. SAF at the doses of 400 and 800 mg/kg were able to abate the elevated levels of MDA ($p<0.001$) and restore the GSH levels ($p<0.001$) in sciatic nerve tissue of nerve injured rats after 7-day treatment **(Fig 5.3 A,C).** GSH is a vital antioxidant involved in cellular defense against oxidative stress. The restoration of GSH levels in the sciatic nerve of injured rats by SAF suggests its potential to counteract oxidative damage and is an indicator of protective effect on the cellular antioxidant defense system. Whereas, nitrite levels were significantly restored at 400 mg/kg and 800 mg/kg ($p<0.001$) as well as gabapentin (30m mg/kg) as compared to saline treated group **(Fig 5.3 B)**. Nitrite levels are indicative of nitric oxide (NO) production, which can be associated with inflammation and oxidative stress. The restoration of nitrite levels in the sciatic nerve by SAF suggests a potential modulation of the nitric oxide pathway. This modulation may further be related to reduced inflammation and oxidative stress, contributing to the overall neuroprotective effects of SAF. These biochemical alterations are critical indicators of oxidative stress and inflammation, and the ability of SAF to restore balance in these markers suggests its potential in mitigating the deleterious effects associated with nerve injuries. Findings from this study suggests a robust antioxidant profile of SAF at 400 and 800 mg/kg with comparable potency as of standard drug gabapentin.

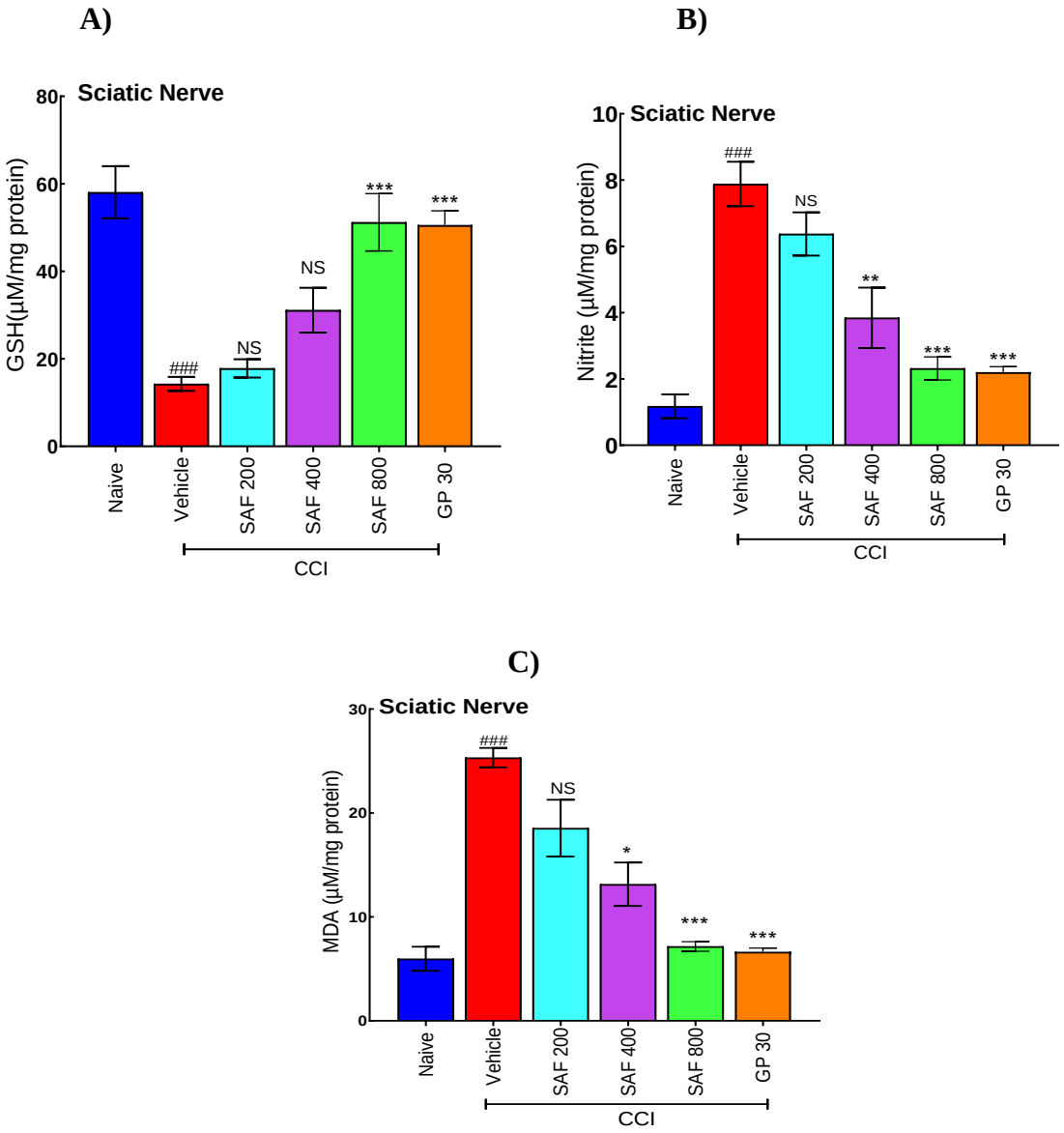


Fig. 5.3 Effect of SAF on biochemical parameters in sciatic nerve of injured rats. A) MDA **B)** Nitrite and **C)** GSH. Data were expressed as Mean ± SEM and analyzed by one-way ANOVA (Post hoc analysis: Tukey's post hoc analysis test). *($p<0.05$), **($p<0.01$) and ***($p<0.001$) represents significance compared to the vehicle group. ### represents significance compared to the naïve. Sida cordifolia aqueous fraction dose SAF200: 200mg/kg, SAF400: 400mg/kg, SAF800: 800mg/kg & GP30: Gabapentin:30 mg/kg.

5.3.2.4 SAF supressed mRNA protein expressions of pro inflammatory cytokines and neuropeptides in DRG of neuropathic rats

Calcitonin gene–related peptide (CGRP), a 37-amino acid peptide majorly found in the C and Aδ sensory fibers arising from the dorsal root ganglia also plays an important role in chronic pain signaling. Release of neuropeptides from C terminal of primary sensory neurons leads to activation of CGRP receptor signaling cascades followed by sensitization of NMDA receptor causing central sensitization (Smriti Iyengar 2017). Pain modulating peptides Substance P and CGRP are long reported to be involved in development and maintenance of neuropathic pain after peripheral injury. Chronic constriction injury leads to significant increase in release of Substance P and CGRP in both DRG and spinal cord of rats. SAF treatment significantly attenuates substance P and CGRP release in DRG and spinal cord of nerve injured rats. mRNA and protein expressions of pro-inflammatory cytokines were examined in ipsilateral L4-L5 DRG tissues of nerve injured rats. Nerve injury leads to significant upregulation in pro-inflammatory cytokines (IL-1β, TNF-α) and neuropeptides (CGRP and Substance P) in DRG and spinal cord of rats. SAF treated group displayed significant ($p<0.001$) suppression in mRNA expressions of IL-1β in DRG tissues of rats at 200 mg/kg, 400 mg/kg and 800 mg/kg **(Fig. 5.4 D).** While, TNF-α expression were significantly ($p<0.01$) downregulated at only 800 mg/kg in DRG tissues of SAF treated rats as compared to vehicle treated group **(Fig. 5.4 C).** Further, peripheral nerve-injury increased mRNA expressions of CGRP and substance P in DRG tissue of rats which was significantly attenuated by SAF at different doses of 200 mg/kg, 400 mg/kg and 800 mg/kg **(Fig 5.4 A-D).**

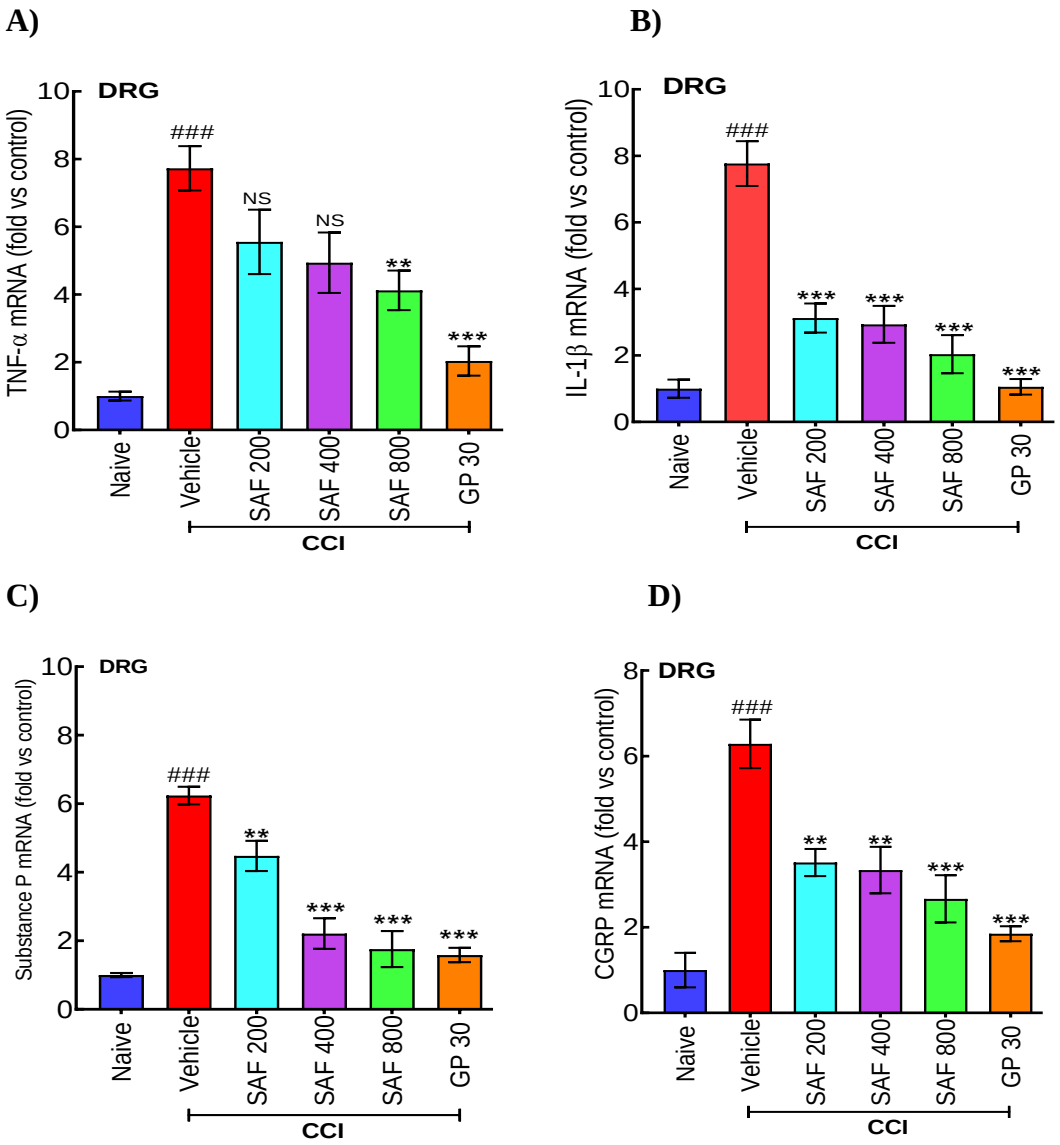


Fig. 5.4. Effect of SAF on neuropeptides & pro-inflammatory cytokines in DRG of nerve-injured rats. A) TNF-α **B)** IL-1β **C)** Substance P **D)** CGRP. Data were expressed as mean ± SEM and analyzed by two-way ANOVA (Bonferroni's multiple comparisons). *($p<0.05$), **($p<0.01$) and ***($p<0.001$) represents significance compared to the vehicle group. ### represents significance compared to the naïve. Sida cordifolia aqueous fraction dose SAF200: 200mg/kg,SAF400: 400mg/kg, SAF800: 800mg/kg & GP30: Gabapentin:30 mg/kg

5.3.2.5 SAF supressed nerve injury induced glial cell activation and neuro-inflammation in spinal cord of CCI rats

Central sensitization along with NMDA receptor activation induce the release of cytokines leading to functional and morphological changes in microglia signalling molecules. Glial cells make up almost 70% of the total cell population in the central nervous system (CNS), and are classified into microglia, astrocytes and oligodendrocytes. Microglial are also known as resident macrophages and are ubiquitously distributed throughout the CNS. They have small cell bodies having branched and motile hair like structure, which might play a role in monitoring the local environment within the CNS (Nimmerjahn et al. 2005). Growing body of evidences over the past couple of years suggest that peripheral nerve injury leads to activation of microglia in the spinal dorsal horn as evident by cell body hypertrophy with thickened processes, an increase in cell number and an increase in microglial markers, such as Iba-1 and ICAM1. Nerve injury increased Iba-1 and ICAM-1 expressions in lumbar spinal cord of neuropathic rats. In this study, two common microglia activation markers, Iba-1 and ICAM-1 in spinal cord tissues of neuropathic rats were analyzed. Further, SAF efficacy on protein expressions of Iba1 and ICAM-1 in spinal cord tissues of nerve injured rats was also analysed with Western blot studies. Significant effect across the groups in one-way ANOVA followed by Tukey's multiple comparison test on Iba1, ICAM-1, TNFα and IL-1β protein expression in L4–L5 spinal cord ($p < 0.0001$) tissues were observed. Similarly, oral administration of SAF (200, 400 and 800 mg/kg p.o.) for 7 days significantly supressed protein expressions of Iba1, ICAM-1, TNFα and IL-1β in L4–L5 spinal cord as compared to the vehicle-treated rats **(Fig. 5.5 A-D).** Gabapentin treatment also decreased the Iba1, ICAM-1, TNFα and IL-1β protein

expressions in spinal cord of neuropathic rats ($p<0.001$) as compared to the vehicle treated group.

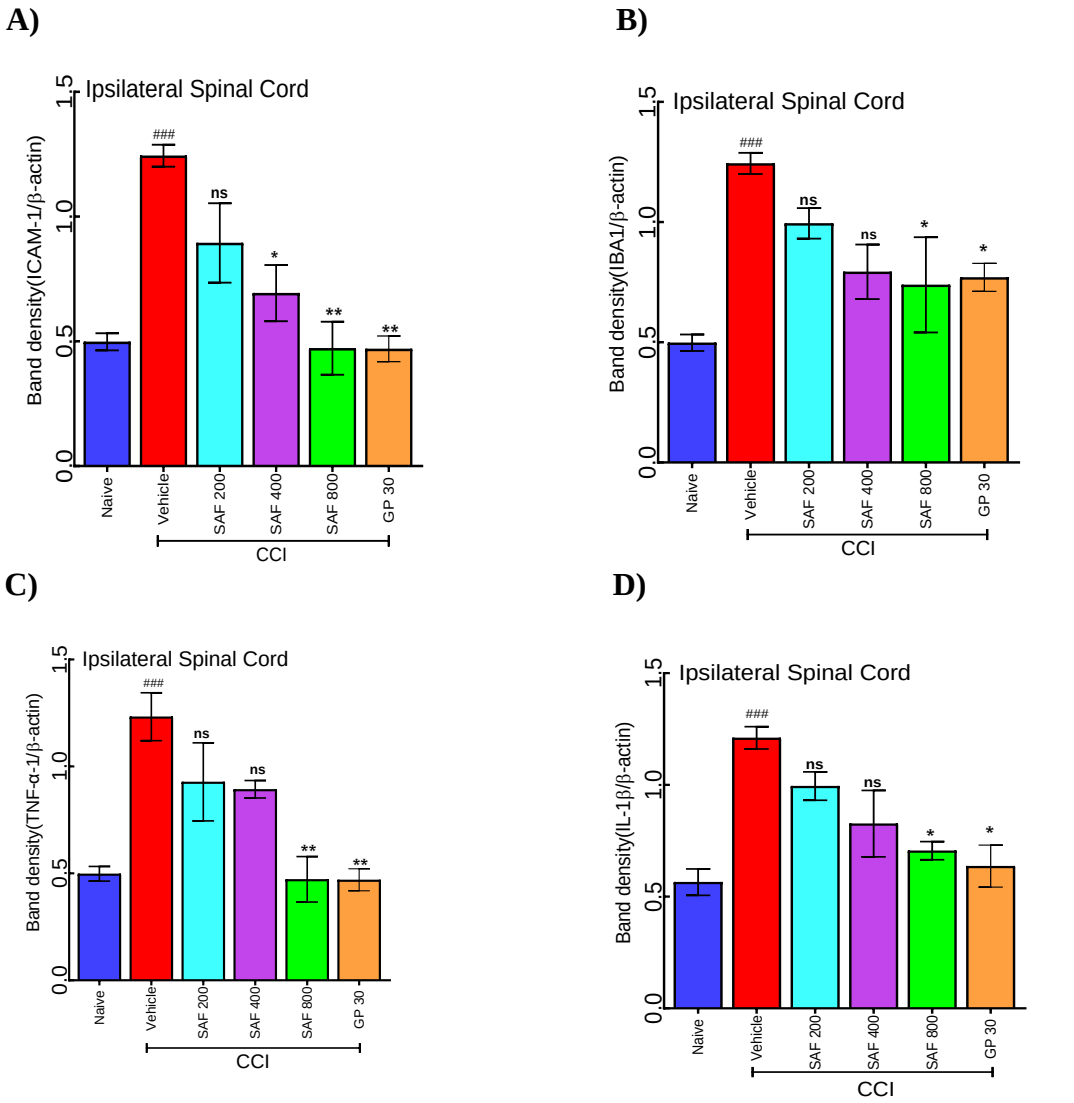


Figure 5.5. Effect of SAF on ICAM-1, IBA1, and TNF-α and IL-1β protein expression in spinal cord of nerve-injured rats. Aqueous fraction SAF (200 mg/kg, 400 mg/kg, 800 mg/kg, p.o.) significantly restored the ICAM-1 **A)** and IBA1 **B)** Protein expression in the spinal cord of nerve-injured animals **B)**. **C)** TNF-α and **D)** IL-1β Protein expression in the spinal cord 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.

5.3.2.6 *Sida cordifolia* Aqueous Fraction (SAF) Downregulates NR2B mRNA & Protein Expressions in DRG and Spinal Cord of Neuropathic Rats

Plenty of scientific communications reveals downregulation NR2B results in attenuation of chronic and as well as neuropathic pain symptoms. Therefore, efficacy of SAF on mRNA and protein expressions NR2B, in DRG and spinal cord tissues of nerve injured rats was analyzed with Western blot studies. A significant effect across the groups in one-way ANOVA followed by Tukey's multiple comparison test on NR2B, protein expression in L4–L5 spinal cord ($p < 0.0001$) tissues was observed. Upregulated protein expression of NR2B due to nerve injury were significantly downregulated in spinal cord tissues ($p<0.01$ and $p<0.001$) at 400 and 800 mg/kg respectively post SAF treatment, as compared to the naïve group **(Fig. 5.6 B).** Gabapentin treatment also decreased NR2B protein expressions in spinal cord of neuropathic rats ($p<0.001$) as compared to the vehicle treated group.

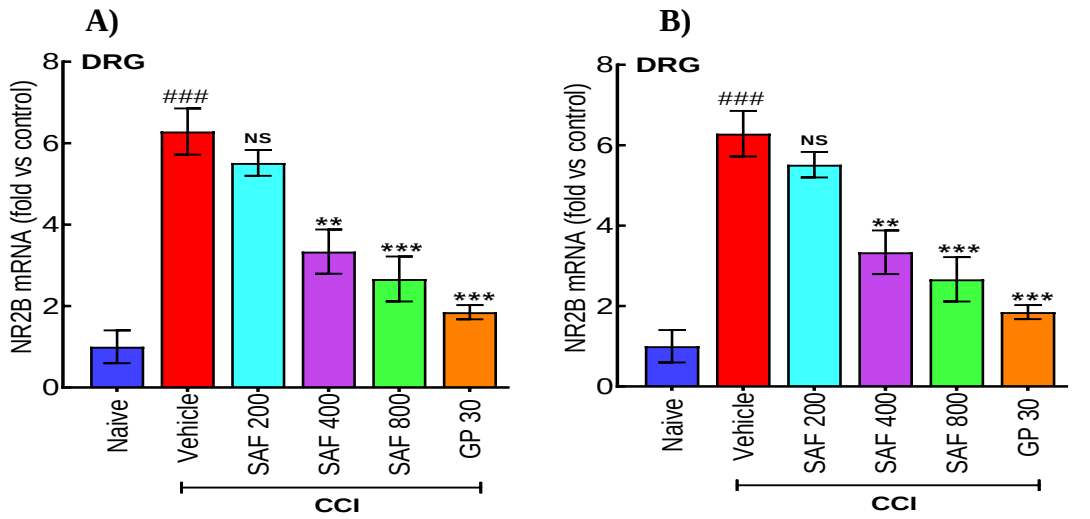
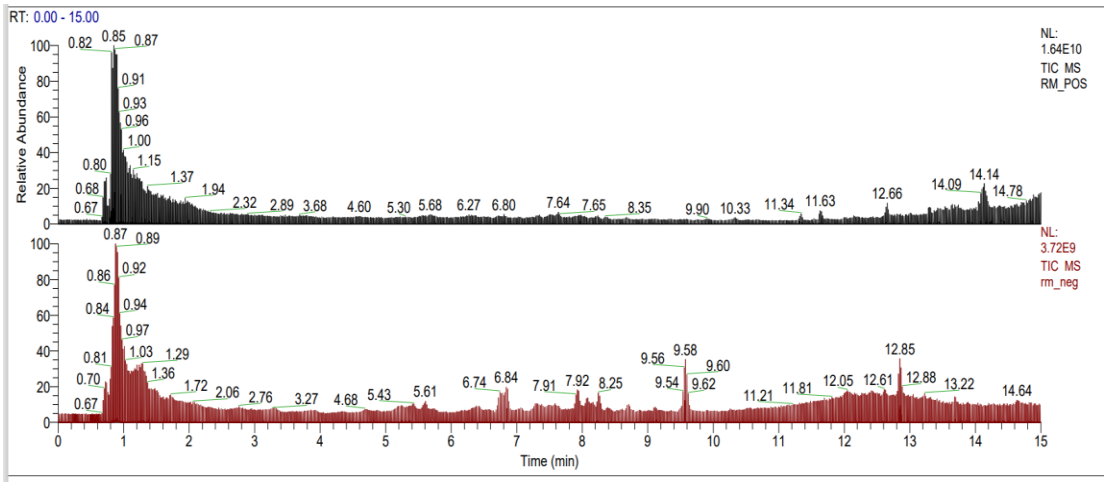


Figure 5.6 Effect of SAF on DRG & spinal NR2B expressions in nerve-injured rats. SCE (200 mg/kg, 400 mg/kg, 800 mg/kg, p.o.) significantly restored the NR2B mRNA expression level in the **A)** DRG of nerve-injured animals and **B)** Spinal Cord. Data were expressed as mean ± SD (n=4/group) ###($p<0.001$) 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 Your text

5.3.3 HR-MS analysis of SAF

The results from the in-vivo studies revealed that both the crude extract (SCE) and the Aqueous Fraction (SAF) derived from *Sida cordifolia* roots exhibited significant anti-allodynic effects in a rat model of chronic constriction injury (CCI)-induced neuropathic pain. Notably, SAF demonstrated anodynic behavior starting from day 2, and at a considerably lower dose than required for SCE. To have a deeper understanding of the composition of SAF and identify the specific phytoconstituents responsible for its observed activity, the fraction was subjected to High-Resolution Mass Spectrometry (HR-MS) analysis. The HR-MS analysis of the enriched aqueous fraction revealed the presence of various phytoconstituents, each exhibiting distinct retention times (RTs), with a 15-minute retention time being particularly noteworthy **(Fig. 5.7).**



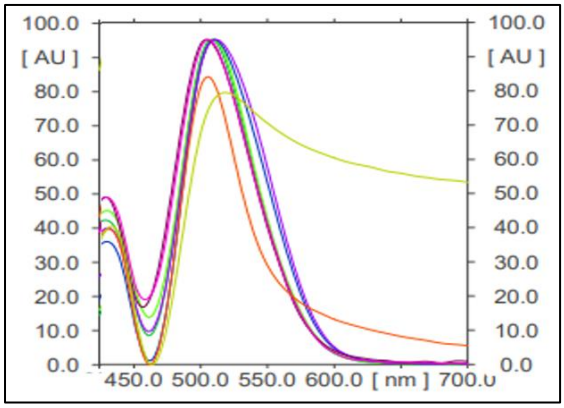
Fgure 5.7 *Sida cordifolia* aqueous fraction (SAF) HRAMS chromatogram displaying different phytoconstituents.

The findings of this study suggest the existence of several phytoconstituents in SAF, with betaine identified as the major component **(Table-5.2)**. The abundance of betaine,

as indicated by the substantial area under the curve (Max. 1.24E+11), implies its prominence within the phytochemical composition of SAF.

5.3.4 HPTLC Quantification of Betaine in *Sida cordifolia* aqueous fraction (SAF)

Several reports suggest the presence of betaine in *Sida cordifolia*. Since, HRMS study of SAF displayed betaine as major phytoconstituents, further, quantification of betaine in crude extract (SCE) as well as enriched aqueous fraction (SAF) using HPTLC was performed. HPTLC analysis of SCE and SAF demonstrated 13.059 % of betaine in enriched aqueous fraction (SAF) while concentration of betaine in crude extract was found to be below detection limits **(Fig.5.9).** Suggesting, betaine along with other phytoconstituents may be responsible for superior anodynic efficacy of enriched aqueous fraction as compared to the crude extract.



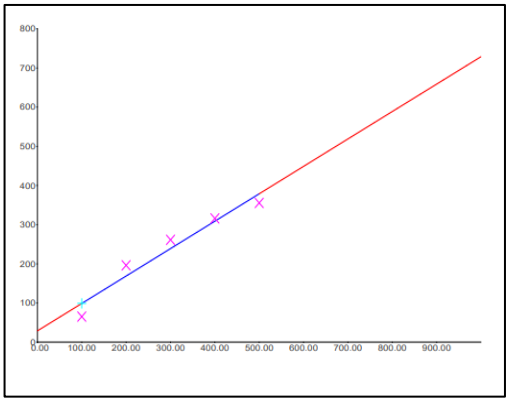


Figure 5.8 Overlain spectra of betaine standards, SCE and SAF employing winCATS Planar Chromatography Manager @ 540 nm Manager

Substance: Betaine @ 540 nm

Regression via height: Linear Y = 28.84 + 0.6999 * X
Regression via area: Linear Y = -2355 + 61.89 * X

Track	Vial	Rf	Amount	Height	X(Calc)	Area	X(Calc)
1	1						
2	1	0.57	100.00 µg	65.32		2501.57	
3	1	0.57	200.00 µg	196.05		10456.60	
4	1	0.56	300.00 µg	261.37		17301.46	
5	1	0.56	400.00 µg	316.02		24253.21	
6	1	0.56	500.00 µg	355.29		26549.10	
7	2						
8	2	0.54		98.86	100.04 µg	5727.46	130.59 µg

5.4 Outcome

The administration of the Aqueous Fraction (SAF) from *Sida cordifolia* roots demonstrated a significant inhibitory effect on evoked pain behavior in rats subjected to chronic constriction injury (CCI)-induced nerve injury. This indicates a potent anti-nociceptive activity associated with SAF treatment in the experimental model. Moreover, the CCI-induced nerve injury led to a substantial increase in neuroinflammatory markers, specifically tumor necrosis factor-alpha (TNFα), interleukin-1 beta (IL-1β), and interleukin-6 (IL-6). Besides, treatment with SAF resulted in a significant reduction in the elevated levels of these neuroinflammatory markers, suggesting its potential in modulating the inflammatory response associated with nerve injury. Additionally, SAF treatment exhibited a notable reduction in neuropeptides, including Substance P and Calcitonin Gene-Related Peptide (CGRP), both of which are implicated in pain signaling. Furthermore, the activation of microglia, as indicated by Iba1 expression, was significantly decreased following SAF treatment in rats with nerve injuries. This suggests that SAF not only impacts neuroinflammation but also influences neuropeptide release and microglial activation, contributing to its comprehensive anti-nociceptive effects. The identification of betaine as the major phytoconstituent in the aqueous fraction of *Sida cordifolia* extract suggests a potential link between betaine and the observed anti-nociceptive activity exerted by SAF. Betaine's role in modulating pain perception and neuroinflammation merits further exploration and provides a molecular basis for understanding the therapeutic mechanisms of SAF in neuropathic pain conditions. In summary, these findings collectively suggest anti-nociceptive properties of SAF, and its potential in modulating

neuroinflammation, neuropeptide release, and microglial activation in the context of nerve injury-induced pain.

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