CHAPTER ONE

INTRODUCTION

1.1 BACKGROUND OF THE STUDY

Neuropathic pain (NP) is a chronically painful condition resulting from injury to the somatosensory neurons. It may result from chemotherapeutic drugs, metabolic disorders (e.g., diabetes, herpes zoster and infections), traumatic injury (chronic or acute), stroke, surgical lesion, chronic inflammation (arthritis, neurofimbrine), neurodegenerative diseases (amylolateral sclerosis), and neurotoxicity of peripheral or central nerves. All these causes are associated directly with the damage to the structural and functional integrity of the sensory nervous system (Von Hehn *et al.,* 2012; Hori *et al.,* 2016). Development of both peripheral and central neuronal potentiation is mediated and maintained by the activities of neuroinflammation, neuronal and synaptic plasticity, and oxidative and nitrosative stress which initiate and maintain the painful sensation (Berrocoso *et al.*, 2011; Gilron *et al.*, 2015).

Clinically, NP is diagnosed through sensory tools as well as medical history and clinical investigation. Hyperalgesia (exaggerated response to painful stimuli), allodynia (a painful response to normal stimuli), the spontaneous sensation of pain, and comorbidities (such as sleep deprivation, depression, and anxiety) remains the most cardinal symptoms of NP that are commonly presented by patients (Treede *et al.,* 2008; Zhou *et al.,* 2014; Zhao *et al.,* 2017). These symptoms have been associated with increase neuronal excitation, responsiveness, and decrease synaptic inhibition (Levy and Zochodne, 2004).

The mechanism of development and maintenance in the pathogenesis of NP have been attributed to involving increased sensitisation of peripheral and central neurons that enhanced the responsiveness of neurons to thermal and mechanical physical stimuli. Traumatic assault to the somatosensory neurons causes an imbalance in neuronal ionic homeostasis (Ca^{2+}, K^{+}, Cl^{-}

, and Na^+ ions), and upsurge discharge of excitatory mediators (glutamate, substance P, calcitonin-gene related polypeptide, neurokine-II) that leads to excitotoxic and death of the neurons. Increased glutamatergic excitation and neuronal firing level have been reported, a process that triggers both neuroplastic and excitotoxic cascades following nerve injury (Hudson *et al.,* 2002; Jensen and Baron, 2003; Gegelashvili and Bjerrum, 2014). Injury to somatosensory neurons induced spatiotemporal changes in the glutamate sensing-, transporting- and metabolising machinery and its supporting systems which result in central sensitisation. Differential phosphorylation of glutamate receptors (AMPA, NMDA, and Kainate receptors), have also been documented to mediate central sensitisation (Gegelashvili and Bjerrum, 2014). Furthermore, the episodes of central disinhibition resulting from the depolarising rather than inhibitory effects of gamma-aminobutyric acid (GABA) enhances the maintenance and development of central sensitisation (Payne *et al.,* 2003).

Various ions and their channels have been implicated in the development of neuropathic pains. Derangement in ionic channels expression results in increased neuronal excitabilities, responsiveness, and disinhibition (Price *et al.,* 2009). It has been noted that various forms of nerve damages result in up-regulation in the expression of cation channels (Colloca *et al.,* 2017). Up-regulation of voltage-gated sodium and calcium ion channels in the Dorsal Root Ganglion (DRG) and dorsal horn of the spinal cord has been shown to results in an increased influx of sodium (Na^+) and calcium (Ca^{2+}) ions (André *et al.,* 2003). It is of particular note that abnormal elevation in intracellular Ca^{2+} concentration through increased activities of voltage-gated calcium channel (VGCC) exacerbates the releases of neurotransmitters and neuromodulators in the somatosensory nerves (Jang *et al.*, 2018). It has been well believed that neuronal excitability is increased when voltage-gated K^+ channels are inhibited (this evokes sustained depolarisation of neurons) or when Ca^{2+} flows into the neuron through voltage-gated T-type channels (Linley *et al.,* 2010). Ectopic discharges that characterised NP (Devor, 2009),

aid potentiating increased sensitivity of neurons are probably due to alterations in the expression of ion channels (Gold, 2008).

Disinhibition observed at both central and peripheral nervous systems has been attributed to the loss in maintaining chloride ions (Cl^-) homeostasis of the neurons (Coull *et al.,* 2003; Jin *et al.,* 2005). Neuronal inhibition is mediated by the hyperpolarising effect of the chloride current, which counterbalances the depolarising current caused by excitatory input (Price and Prescott; 2015). The development of neuropathic pain has been associated with central disinhibition, particularly at the neuronal of the spinal dorsal horn, which is presumably susceptible to chloride dysregulation. It has been proposed that chloride dysregulation in projection neurons has devastating consequences due to its direct effects on the signals to the brain (Keller *et al.,* 2007; Lavertu *et al.,* 2014). The development of mechanical allodynia has been attributed to the dysregulation in chloride ion, most especially at the laminar I projecting neurons which lead to the unmasking of low-threshold inputs (Keller *et al.,* 2007).

Further elucidation of the molecular mechanism of NP has shown the important role of inflammatory mediators in mediating hyperalgesia and allodynia. Intrathecal injection of exogenous tumour necrotic factor-α (TNF-α), interleukin-1β (IL-1β), interleukin6 (IL-6), substance P, and prostaglandins E_2 (PGE_2) potentiate allodynia and hyperalgesia in both clinical and experimental studies (Gruber-schoffnegger *et al.,* 2013; Schuh *et al.,* 2014; Tiwari *et al.,* 2014). The neurogenic effects of inflammatory mediators at the site of neuronal injury is characterised by the infiltration of macrophages, T-cells, endothelial, and satellite glial cells (Eliav *et al.,* 2009; Sacerdote *et al.,* 2013). The activation and aggregation of inflammatory cells at the injured neurons play a pivotal role in the initiation, progression, and maintenance of NP (Eliav *et al.,* 2009). Interaction between the afferent neurons and inflammatory mediators increased neuronal excitability and synaptic plasticity.

Inflammatory mediators directly or indirectly activate and sensitise nociceptive ion channels (Linley *et al.,* 2010; Julius 2013). Prostaglandins potentiate hyperalgesia directly via increased phosphorylation of TRPV1 and modulation of ion channels (Na^+, K^+, and Ca^{2+}) (Basbaum *et al.,* 2009; Mizumura *et al.,* 2009). The inhibition of potassium currents by prostaglandins is mediated by an increase in the intracellular Ca^{2+} and voltage-gated Na^+ currents in capsaicin-sensitive primary afferent neurons (Chen *et al.,* 2013).

Studies have shown that the inhibition of inflammatory cytokines (such as TNF-α, IL-1β, and IL-6) effectively relieve neuropathic pain (Gao *et al.,* 2010; Tiwari *et al.,* 2014). These proinflammatory cytokines modulate small-diameter myelinated Aδ and unmyelinated C-fibre by eliciting increased Na^+ and Ca^{2+} currents, lowered excitation threshold, and enhanced membrane excitation of the nociceptors (Schafers and Sorkin, 2008). Centrally, IL-1β potentiate increased glutamate level by inhibiting the activities of glutamate transporter (EAAT1 and EAAT3) (Pitcher *et al.,* 2007). These neuroplasticity changes contribute to central sensitisation that mediates neuropathic pain condition. The involvement of the anterior cingulate cortex (ACC) in chronic pain has been emphasised (Li *et al.,* 2010) and is viewed to be responsible for NP comorbidity. Increased IL-1β in this region, as well as other limbic regions of the brain sample, has been regarded as a common mediator for hyperalgesic and comorbidity symptoms of neuropathic pain (Gui *et al.,* 2016).

The contributions of superoxides (SO) and peroxynitrite (PN) to the development of peripheral and central sensitisation associated with NP are adding evidence that these species are novel targets for pain management. Numerous studies demonstrate that pharmacologic inhibition of SO and PN can prevent and reverse the symptoms of NP (Salvemini *et al.,* 2011). Studies have revealed that increased formation of SO/PN is critically essential in the development of thermal hyperalgesia associated with chronic pain (Ndengele *et al.,* 2008; Tang *et al.,* 2009) in response to spinal activation of the NMDAR (N-methyl-D-aspartate receptor) (Muscoli *et al.,* 2004). SO

are increased in dorsal horn neurons of the spinal cord in the animal model of NP induced by spinal nerve ligation (Park *et al.,* 2006) and neurogenic-induced hyperalgesia (Schwartz *et al.,* 2008). It has also been noted that SO and PN have no role in acute nociception (Ndengele *et al.,* 2008). TRPA1 is a chemosensor that can be activated by ROS, which mediates the development of hyperalgesia and tactile allodynia in animals (Miyake *et al.,* 2016). Systemic injection of ROS scavenger such as Phenyl-N-Tert-Butylnitrone (PBN), 5,5,-dimethyl-1-pyrroline-N-oxide (DMPO), vitamin E, and 4-hydroxy-2,2,6,6,-tetramethylpiperidone-N-oxyl (TEMPOL) have been demonstrated in the spinal nerve ligation model of neuropathic pain to ameliorate mechanical allodynia (Kim *et al.,* 2010; Fidanboylu et al., 2011; Kim *et al.,* 2017).

Bromelain, a major protease enzyme found in pineapple, is recently gaining the attention of researchers for its therapeutic purposes. Bromelain is the collective name for a group of closely related endopeptidase proteolytic enzymes found in the tissue of the plant family of Bromeliaceae, of which pineapple (*Ananas comosus*) is the best known (Benucci *et al.,* 2011). It can be derived from both the stem (stem bromelain: EC 3.4.22.32) and fruit (fruit bromelain: EC 3.4.22.33). It is composed of peroxidase, acid phosphatase, protease inhibitors, and organically bound calcium (Gautam *et al.,* 2010). Bromelain is a native of South America but also popularly grown in West African countries. It is commonly sold in food stores as a nutritional supplement to promote digestive health (Hale, 2004). It possesses a pleiotropic therapeutic effect, i.e. interference with the growth of malignant cells, anti-edematous, anti-inflammatory (Bhui *et al.,* 2009). Furthermore, bromelain has been therapeutically used for the healing of wounds, treatment of arthritis and episiotomy, as well as muscular pain (Majid *et al.,* 2014; Golezar, 2016).

1.2 STATEMENT OF THE PROBLEM

Neuropathic pain (NP) is a challenging worldwide health issue that affect 6.9 % to 10 % of the global population (Ibrahim *et al.,* 2018). The incidence of NP in African countries has been variably put at the ranges between 20 to 50% of the Africa population depending on the method and geographical location of study (Dutta *et al.,* 2005; Assaad-Khalil *et al.,* 2015; Kisozi *et al.,* 2017). In Nigeria, studies show that the prevalence of NP is 35.9% in University of Ilorin Teaching Hospital; 21.6% in University of Lagos Teaching Hospital; and 47.6% in Jos (Bojuwoye, 1995; Ugoya *et al.,* 2006; Asomugha, 2012; Ojo *et al.,* 2016).

NP results in severe distress, dominating and disrupting the quality of life in patients. It is disheartening that less than 2% of drugs used in the treatment of NP yielded about 50% pain reductions in patients (Muthuraman *et al.,* 2014). The treatment of neuropathic pain in the United States of American alone gulp-up millions of dollars yearly (Lippe *et al.,* 2010). Complex mechanisms involved in the development and maintenance of NP contributed to the poor understanding of its processes. This is coupled with the fact that there is no objective diagnostic test which complements the subjective assessment of chronic pain condition. Taken together, it possibly accounted for backsliding in the development of effective drugs that can combat the deteriorating effect of NP. Likewise, NP arises from different etiological causes with varying degrees of manifested symptoms. Patients having the same etiological base may also present various manifestations, all of which complicate a better understanding of neuropathic pain and transition of treatment from the fundamental science study to clinical study.

The current line of drugs that are used in the treatment of neuropathic pain includes non-steroidal anti-inflammatory drugs (NSAIDs), opioids, tricyclic antidepressants, serotonin-norepinephrine reuptake inhibitors, and anticonvulsants. NSAIDs are known to produce several

adverse effects, including gastrointestinal, renal, and hepatic insults (Taylor, 2009). Opioids users have been found to suffer addiction, sedation, respiratory depression, constipation, and itch (Benyamin *et al.,* 2008) to the drug. Furthermore, the tolerance effect of opioids coupled with cardiometabolic disorders and sexual dysfunctions, have limited the use in clinical practice. Tricyclic antidepressants such as amitriptyline, which act by modulation of ion channels and monoamine regulators (serotonin-norepinephrine reuptake inhibitors such as duloxetine) as well as anticonvulsants drugs (ion channel blockers such as gabapentin, pregabalin) are poorly understood in terms of their mechanism of action. Only 40-60% of patients report a 50% reduction in pain sensation (Muthuraman *et al.,* 2014). Gabapentin shows a high level of efficacy but can cause sedation and weight gain (Schmidt *et al.,* 2013). This can be detrimental for obese and diabetic patients where weight gain can exacerbate the disease. Hence, the burden of neuropathic pain is related to the ambiguity of neuropathic symptoms, poor outcomes, and brutal treatment decisions. Impairment in the patient quality of life is due to increased drug prescriptions and visits to health care providers, as well as the morbidity from the pain itself and the inciting disease (Isacchi *et al.,* 2011; Van Hecke *et al.,* 2013).

Therefore, there is a necessity to investigate alternative drugs that will address the efficacy and side effects as well as cost-effective to abate sufferings from the consequence of sensory nerve diseases or lesion. This includes focusing on drugs that target decrease excitation and increases inhibition of the neuronal pain system without/little side effects. Recently, the use of traditional and natural food supplements in the treatment of various ailments, including pain, has increased tremendously.

1.3 JUSTIFICATION

Bromelain, proteolytic enzymes found in the stem and fruit of *Ananas comosus*, have been used traditionally for the treatment of many ailments. Bromelain has been reported to be

therapeutically safe in traditional medicine. It is considered to have very low toxicity, with an LD50 greater than 10 g/kg. In human clinical tests, side effects are generally not observed (AMR, 2010). It is a purely natural supplement from pineapple fruit and stem. It has been used as an anti-inflammatory, anti-tumour, and wound healing agent, among others (Suhrabi and Taghinejad, 2013; Golezar, 2016). Antinociceptive effects of bromelain have been well documented in both clinical and animal studies of acute and inflammatory pain (Sudjarwo, 2005). Clinical trials on analgesic studies of bromelain have been reported to subdue the perineal pain due to episiotomy (Ezeome and Aghaji, 2005; Golezar, 2016), muscular pain, mild acute knee pain, and arthritis pain (Brown *et al.,* 2004). Recently, it has been reported that bromelain improved the symptoms of Parkinson's disease, a neurodegenerative disease (Adu and Mabandla, 2019). Various mechanisms of action of the bromelain are attributable to its effect on nuclear factor-KB, plasmakinin, bradykinin, prekallikrein, and prostaglandins (PGE2 and Thromboxane B_2) (Maurer, 2001; Majid and Al-mashhadani, 2014).

However, its effects and mechanism of actions in painful neuropathic conditions have not been reported. Likewise, nuclear transcription factors have been reported to play a pivotal role against superoxide and peroxynitrite activities. Reports have been non-existent on the contribution of these nuclear transcription factors (NrF-1 and NrF-2) under neuropathic pain conditions. Furthermore, various therapeutic interventions have targeted ion channels and transporters with the aims of modulating both influx and efflux of ions (Gabapentin, ziconotide, and carboxamide subclass, among others, were targeted to block Ca^{2+} and Na^{+} ion channels) (Thomas and Atkinson 2018; Smith, 2018). Increased activities of the Ca^{2+} ATPase and sodium-potassium ATPase (Na-K ATPase) following nerve injury have been reported (Ghosh *et al.,* 2011; Gemes *et al.,* 2012; Paul *et al.,* 2014). Despite interesting reports on the vital role of the electrogenic pumps in neuropathic pain maintenance, therapeutic drugs targeting electrogenic pumps have been poorly explored and grossly underdeveloped.

Hence, the study undertaken was to assess the antinociceptive effects of varied dosage of bromelain in sciatic nerve ligation-induced peripheral neuropathic pain. It also aimed to investigate the protective effect of bromelain on animals pretreated with bromelain before the induction of neuropathic pain. The study further investigates the role of bromelain on comorbidities (anxiety and depression) of neuropathic pain. It is also the interest of the study to unravel its neuroprotective effect against reactive oxygen species and other possible mechanisms of actions that it might be using in ameliorating symptoms and comorbidities of neuropathic pain in the chronic constriction injury model of peripheral neuropathy.

1.4 AIM OF THE STUDY

The current study was designed to investigate the antinociceptive effect of bromelain in a chronically constricted sciatic nerve model of peripheral neuropathy in *Wistar* rats and predict its mechanisms of action to fill the gaps observed in the literature, and to contribute more knowledge in this area of study.

1.5 OBJECTIVES OF THE STUDY

The objects of this study are to:

1. determine the antinociceptive and neuroprotective effects of bromelain on sciatic nerve ligated-induced NP;
2. investigate the effects of bromelain on the comorbidities symptoms (anxiety-like and depressive-like behaviour) in rats induced with NP;
3. assess the role of bromelain on the sciatic functional index of rats;
4. evaluate the anti-stress capabilities of bromelain through nuclear transcription factors and antioxidant system;
5. assessing the effect of bromelain on different families of proinflammatory cytokines in both the peripheral and central nerves;

6. investigate the roles of bromelain on sciatic nerve ions concentration as well as the level of the electrogenic pump;
7. investigate the effect of bromelain on excitatory neurotransmitters; and
8. investigate the effect of bromelain on the structural integrity of the peripheral and central nerves.

1.6 RESEARCH HYPOTHESIS

Null Hypothesis: Bromelain does not protect nor alleviate neuropathic pain in male *Wistar* rats.

Alternative Hypothesis: Bromelain serves as a neuroprotective supplement as well as mitigates NP and its comorbidities in male *Wistar* rats.

1.7 SIGNIFICANCE OF THE STUDY

At the end of this research work, this study should be able to:

1. account for the antinociceptive effect of bromelain on sciatic nerve ligated-induced neuropathic pain symptoms as well as its comorbidities symptoms
2. predict the possible mechanisms of action of bromelain through which it ameliorates neuropathic pain symptoms
3. quantify the neuroprotective effect of bromelain

The researcher believes that the research will contribute to the knowledge of therapeutic usage and mechanisms of action of bromelain as well as promote its further use in clinical settings.

CHAPTER TWO

LITERATURE REVIEW

2.1 INTRODUCTION TO PAIN

Pain is an unpleasant sensation and emotional experience that results from actual or potential tissue damage or described in terms of such damages. It is a subjective experience, with both sensory, affective, and cognitive dimensions that are usually, but not necessarily, associated with tissue damage. Pain is a vital protective mechanism of the body system against further structural damage or dysfunction. However, some normal physiological pain undergoes neurological plasticity with undermine its defence mechanism and result in pain diseases.

2.2 OVERVIEW OF PAIN PATHWAY

The sensation of pain involves the detection of noxious stimuli (thermal, mechanical, and chemical) by the nociceptors which are conducted through the nociceptive fibres to the processing and modulating centres (central nervous system). Peripheral nociceptive fibres are pseudo-unipolar neurons with a cell body that is localised in the dorsal root ganglion (DRG) or the trigeminal ganglion. It has both the peripheral and central axon branches that are terminated at their target organ and spinal cord, respectively. The neurochemical products of the cell body are distributed to both the central and peripheral terminals of the axon, where they are released under the influence of Ca^{2+} ions (Basbaum *et al.,* 2009).

The stimulation of peripheral terminal nociceptor is mediated by both the environmental stimuli (painful heat, cold, and mechanical stimulation) and endogenous molecules (such as Hydrogen ion concentration (H^+), lipids, and neurotransmitters) (Basbaum *et al.,* 2009). Primary afferent nociceptive fibres that convey pain stimuli from the target organ (skin, muscle, joint, etc.) are broadly classified into:

1. medium diameter myelinated Aδ fibre which mediates high localised fast pain.

2. small-diameter unmyelinated C fibre that conveys poorly localised slow pain (Meyer *et al.,* 2008).

The medium diameter myelinated Aδ fibre is a fast pain mediated fibre that can be further classified into type-I and type-II fibre. Type I Aδ fibre (high-threshold mechanical nociceptive fibre) responds to both mechanical and chemical stimuli but has a relatively high heat threshold (> 50 ^{0}C) (Basbaum *et al.,* 2009). Type II Aδ fibre has a much lower heat threshold, but high mechanical threshold activities of this afferent almost certainly mediate the fast acute pain response to noxious heat (Kidd and Urban, 2001). Small diameter unmyelinated C fibre is heterogeneous and polymodal (Perl, 2007). Silent nociceptive fibre is a subtype of C fibre that is heat responsive but mechanical insensitive fibre. Silent nociceptive fibre is more responsive to chemical stimuli and sensitivity to mechanical stimuli only in the advent of injury (Schmidt *et al.,* 1995).

Unmyelinated C-fibre can be differentiated based on the neurochemicals released at the local tissue and synapses into peptidergic and non-peptidergic fibre. Peptidergic C fibre releases neuropeptide, substance P, and calcitonin-gene-related peptide (CGRP) (Basbaum *et al.,* 2009). This fibre expresses tyrosine kinase-A (Trk A) (Kidd and Urban, 2001), neurotrophic receptors that respond to nerve growth factor. Nonpeptidergic C fibre expresses C-Ret neurotrophin receptors that are targeted by a glial-derived neurotrophic factor (GDNF), neuturin, and artemin (Basbaum *et al.,* 2009). They express IB4-isolectin and purinergic receptors (Dong *et al.,* 2001). Myelinated Aδ fibre expresses Trk A and Trk C receptors, high-affinity tyrosine kinase receptors, which bind with brain-derived neurotrophic factor (BDNF) and neurotrophin-3, respectively (Kidd and Urban, 2001).

The central projection of the primary afferent nerve fibre to the dorsal horn of the spinal cord is well organised into anatomical and electrophysiological distinct laminae (Basbaum and

Jessell, 2000) as illustrated in figure 1. Medium diameter Aδ fibre terminated at lamina-I as well as the deeper part of the dorsal horn (lamina V). Fast conducting Aβ afferent fibres, which respond to light touch and project to the deep laminae (III, IV, and V) while C nociceptive fibre project and terminate at the superficial layer of laminae I and II (Braz *et al.,* 2005). Neurochemical staining indicates that the majority of peptidergic C fibres terminate within lamina-I and the most dorsal part of lamina-II while nonpeptidergic fibre terminates in the mid-region of lamina II (Basbaum *et al.,* 2009). Excitatory interneurons that express the gamma isoform of protein kinase C (PKCγ) characterised the ventral part of lamina II (Malmberg *et al.,* 1997).

It has been documented that the PKCγ layer is predominantly targeted by myelinated non-nociceptive fibre (Neumann *et al.,* 2008). In support of the anatomical studies, electrophysiological studies have demonstrated that spinal cord neurons within lamina-I are responsive to noxious stimulation. In contrast, neurons in laminae-III and IV are primarily responsive to innocuous stimulation mediated by via Aβ (Basbaum *et al.,* 2009). Lamina-V neurons receive a convergent of non-noxious and noxious input via Aδ and Aβ (monosynaptic) inputs directly, and C fibre (polysynaptic) inputs indirectly. The latter received a visceral input such that the resultant convergence of somatic and visceral inputs likely contributes to the phenomenon of referred pain (Basbaum *et al.,* 2009).

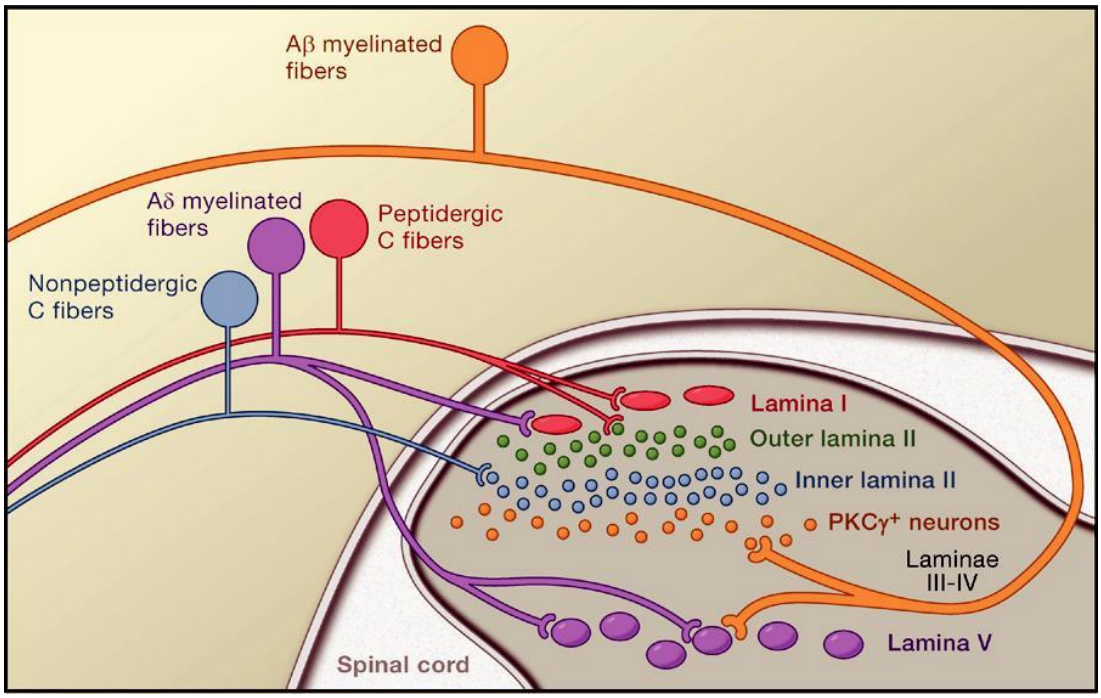

Fig 1: Illustration of the central terminal projection and decussation of the primary afferent fibre within discrete laminae of the dorsal horn of the spinal cord. *There is a precise laminar organisation of the termination of the centre projecting afferent fibre at the dorsal horn of the spinal cord. The unmyelinated, peptidergic C (red) and myelinated Aδ nociceptors (purple) terminate most superficially, synapsing upon large projection neurons (red) located in lamina I and interneurons (green) located in outer lamina II. The unmyelinated, nonpeptidergic nociceptors (blue) target interneurons (blue) in the inner part of lamina II. Contrarily, innocuous input conveyed by myelinated Aβ fibres (orange) terminates on PKCγ expressing interneurons in the ventral half of the inner lamina II. A second set of projection neurons within lamina V (purple) receives convergent input from Aδ and Aβ fibres (Basbaum et al., 2009).*

Projection neurons within laminae-I and V constitute the significant output from the dorsal horn to the brain (Basbaum and Jessell, 2000). These neurons are at the origin of multiple ascending pathways, including the spinothalamic and spinoreticulothalamic tracts, which carry pain messages to the thalamus and brainstem, respectively. The former is particularly relevant to the sensory-discriminative aspects of the pain experience, whereas the latter may be more relevant to poorly localised pains.

Some projecting fibre from the spinal cord to the parabrachial region of the dorsolateral pons provides output for a very rapid connection with the amygdala (figure 2). Information reaches cortical structures via projecting fibre from the brainstem and thalamic loci (Apkarian *et al.,* 2005). Sensory-discriminative properties of pain are mediated by the somatosensory cortex, the cognitive (appraisal) domain of pain is perpetuated by the prefrontal cortex while the limbic system concerns with the emotional aspect of the pain (Basbaum *et al.,* 2009).

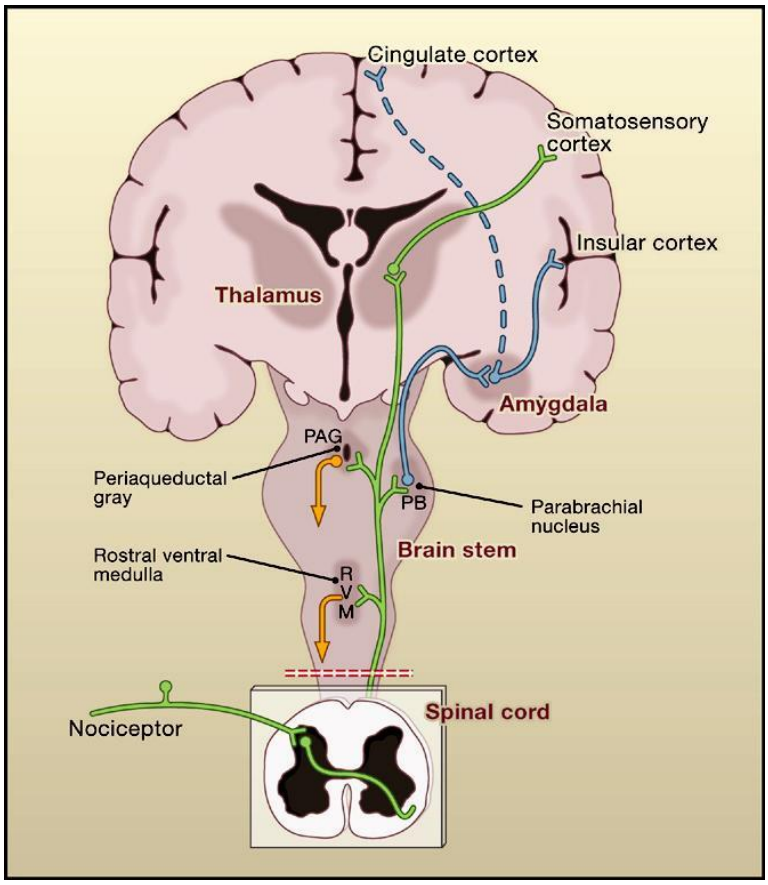

Fig 2: Pain Pathway: *Primary afferent nociceptors convey noxious information to projection neurons within the dorsal horn of the spinal cord. A subset of these projection neurons transmits information to the somatosensory cortex via the thalamus, providing information about the location and intensity of the painful stimulus. Other projection neurons engage the cingulate and insular cortices via connections in the brain-stem (parabrachial nucleus) and amygdala, contributing to the affective component of the pain experience. This ascending information also accesses neurons of the rostral ventral medulla and midbrain periaqueductal grey to engage descending feedback systems that regulate the output from the spinal cord (Basbaum et al., 2009).*

2.3 TYPE OF PAIN

Pain is classified based on the duration of the pain as Chronic or Acute pain. Acute is usually transitory, lasting until the noxious stimulus is removed or the underlying causes have been alleviated. Pains are expected to abate once the causative agent has been ousted. However, some forms of pain outlast their causative agents and persist for a very long duration of time. Such pain is no longer beneficial and protective to the body system but rather inflate discomfort and negatively impacted on patient social life (Schaible *et al.,* 2011). Such pain is described as chronic pain. It does not subside even when the causative agent has been removed.

Chronic pain is a disease of pain that usually lasts for more than six months. Chronic pain includes chronic regional pain syndrome, fibromyalgia, rheumatoid arthritis, idiopathic pain, radiculopathy, neuropathy, complex regional pain syndrome, etc., most of which may persist for years. Chronic pain is clearly distinguished from acute pain in terms of duration, the molecular mechanism of pain development and maintenance. Chronic pain is associated with the development of neuroplasticity through peripheral and central sensitivity which maintained and progressed painful sensation and comorbidity of pain. This literature review focus on neuropathic pain and discus its mechanisms of development and maintenance. It also elucidates the current development and progress made in understanding the mechanism behind peripheral and central sensitisation. In the last section of this chapter, a concise overlook of bromelain and its therapeutic uses were reviewed.

2.4 NEUROPATHIC PAIN

Neuropathic pain (NP) is defined as the "direct consequence of a lesion or disease of the somatosensory nervous system" (Finnerup *et al.,* 2016). It is a chronic type of pain that negatively influences the life quality of patients (Cherif *et al.,* 2020). Epidemiological studies have revealed that about 1% to 8.9% of the general world population is suffering from neuropathic pain (Smith and Torrance, 2012; Colloca *et al.,* 2017). Convincing evidence has

shown that only 40% to 60% of neuropathic patients achieved partial relief following various ranges of treatment (Dworkin *et al.,* 2010; Finnerup *et al.,* 2015).

NP is commonly caused by post-herpetic neuralgia after a herpes zoster infection, painful diabetic polyneuropathy, trigeminal neuralgia, post-amputation pain, persistent post-operative or post-traumatic pain, trauma, autoimmune diseases, pain in cancer patients, and pain in HIV infected patients (Szczudlik *et al.,* 2014). The diagnosis of NP is problematic because it has a subjective nature. NP is described in terms of burning or hot, electric shocks, shooting, pricking or pin sensation, and needless pain evoked by light touching or cold and non-painful sensation such as numbness and tingling (Finnerup *et al.,* 2016). Hence, the hallmarks symptoms of neuropathic pain are characterised by allodynia (where a normally non-painful stimulus results in pain), hyperalgesia (where there is an increased response to a painful stimulus), and spontaneous pain sensation (Austin and Moalem-Taylor, 2010; Sorge *et al.,* 2012; Zhao *et al.,* 2017).

These symptoms of NP are believed to be due to significant alterations of the pain pathway such that pain defied its beneficial purposes and becomes debilitating. The understanding of mechanisms involved in various etiological causes of NP is highly compromised by the involvement of numerous mediators that play key roles in potentiating peripheral and central hypersensitivities. The engagement of highly plastic molecules and circuits, mediate the sustained hyperalgesia and allodynic symptoms of NP (Smith, 2018). These include imbalances between excitatory and inhibitory somatosensory signalling, alterations in ion channels, and variability in the way that pain messages mediate both the peripheral and central sensitisation that account for the development and maintenance of NP.

2.5 DEVELOPMENT AND MAINTENANCE OF NEUROPATHIC PAIN

Lesion or trauma to the somatosensory neurons results in the cascade of events that lead to the development and maintenance of NP. This cascade of events includes neuron plasticity which results in changes in the release of several chemical mediators and expression of receptors and ion channels in both the peripheral and central nervous system (Khan *et al.*, 2015; Terayama *et al.*, 2015; Yadav and Weng, 2017). Summation of these changes in the peripheral axon and central neurons results in peripheral and central sensitisation, respectively (figure 3). An increase in the propensity of neuronal excitation, conduction, and reduced neuronal firing threshold at the peripheral and central nervous system is term peripheral and central sensitisation, respectively. Post-translational and transcriptional changes in the neurons profoundly alter the threshold, excitability, and transmission properties of nociceptors, contributing to pain hypersensitivity and spontaneous pain.

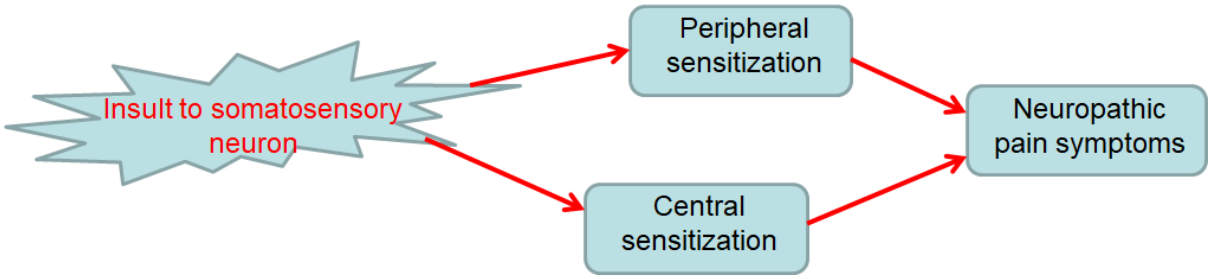

Fig 3: Illustration of the development of neuropathic pain

2.6 PERIPHERAL SENSITISATION

Increased spontaneous firing or alterations in the conduction or neurotransmitter properties of the peripheral nerve fibre is referred to as peripheral sensitisation. Changes in the properties of the peripheral neurons can be localised at the peripheral terminal, the site of axonal injury, or the DRG which can result in general alterations in membrane properties (Ji *et al.,* 2013). Peripheral sensitisation results from changes in the neuronal excitability discharges generated along the nerve fibre. This may be due to the changes in the expression of the genes, receptors, ion channels, and up- or down-regulation of neurotransmitter release (Salvemini *et al.,* 2011; Yan *et al.,* 2014).

Peripheral sensitisation produces a state of heightened sensitivity to peripheral stimuli that are either ordinarily innocuous or are noxious but now produce exaggerated or prolonged effects, both represent stimulus-dependent pains (Woolf and Ma, 2007). Some of the changes are rapid, as with a reduction in heat pain threshold following phosphorylation of the heat transducer receptors, Transient Receptor Potential (TRP: such as vanilloid subtype, e.g. TRPV1, TRPV2; ankyrin subtype, e.g. TRPA1) (Basbaum *et al.,* 2009). Others require retrograde transport of signals to the cell body, activation of signal transduction cascades, changes in transcription, and then orthograde transport of proteins to the peripheral or central terminals (Svensson and Yaksh, 2002).

Heightened sensitivity to peripheral stimuli is characterised by ectopic discharge. Ectopic discharge refers to the spontaneous discharge in the stump of the injured nerve, corresponding sensory nerve in dorsal root ganglia, and neighbouring intact afferent fibres (Schaible *et al.,* 2011). It results in the generation and propagation of action potentials that originate from the axon or cell body which produce sensory inflow in the absence of sensory stimuli or ongoing peripheral inflammation. Ectopic discharge is a self-sustaining activity by pacemaker-like

spontaneous depolarisations resulting from abnormal hyperexcitability of the membrane that occurs after peripheral nerve injury due to alterations in ion channel expression and trafficking (Liu *et al.,* 2002). This can lead to the after-discharge activities of the peripheral fibres and act as signal amplifiers. The discharges are elicited by subthreshold and may persist beyond the end of the stimulation. The subthreshold stimulus can, therefore, elicit a greater-than-normal response.

Ectopic discharge can arise from the stump that is formed at the proximal end of the transected axon. Such a stump gives rise to fine sprouting fibres in the process of regeneration which may elongate and reach the target tissue or the sprouts from the end-bulb neuroma. Ectopic discharges may also arise from partially injured nerves and intact axons close to the injured ones (Basbaum *et al.,* 2009). Increasing evidence has implicated the neighbouring uninjured intact fibres in partial peripheral nerve injury as a more comprehensive source of spontaneous activity (Djouhri *et al.,* 2006). It has been demonstrated that such uninjured axons are exposed to a soup of inflammatory mediators released by degenerating axons, surrounding tissues, immune cells, and peripheral glial cells (Ji *et al.,* 2016).

Although it is not yet known whether such firing elicits hypersensitivity in the area innervated by uninjured axons. However, their increased excitability may contribute to the development of central sensitisation (Wu *et al.,* 2001). Furthermore, sensory neurons in the DRG affected by the nerve injury serve as another source of ectopic activity (Liu *et al.,* 2000). The antidromic propagation of a signal in nerve fibres during ectopic firing causes stimulation of peripheral nerve endings and subsequent release of neurotransmitters such as substance-P and calcitonin gene-related polypeptide. This results in neurogenic inflammation that contributes to the sensitisation of ion channels via activities of inflammatory mediators (Morin *et al.,* 2007).

Peripheral nerves with ectopic sites are hypersensitive to a wide range of mechanical, thermal, and chemical stimuli. Gentle mechanical pressure or brushing may evoke ongoing firing in the injured nerve. When the spot with ectopic activity develops close to tendons or joints, movements and weight-bearing may trigger a persistent pain accompanying various musculoskeletal disorders. Cold allodynia, a common symptom in neuropathy, is related to increased ectopic discharges in unmyelinated C fibres after cold stimulation.

Hypersensitivity and allodynic effect due to ectopic activities are mediated by various events which include alterations in the expression of ion channels and receptors, neurochemical molecules, inflammatory mediators, and receptors. Inflammatory and regeneration processes following nerve injury are mediated by cytokines, peptides, neurotrophins and all of these substances have been shown to contribute to the development of ectopic discharges. Moreover, local metabolic changes at the site of injuries such as tissue ischemia and elevated blood glucose also contribute to the generation of ectopic discharges (Levy *et al.,* 2000; Rivera *et al.,* 2000). These mediators of ectopic activities are discussed subsequently in this chapter.

2.6.1 Changes in the expression and activity of ion channels:

Ectopic discharge is driven by alteration in the expression and activity of ion channels such as cation-nonselective, cyclic nucleotide-modulated channel (Lee *et al.,* 2005), a Ca^{2+}-activated chloride current (Hilaire *et al.,* 2005), as well as potassium channel, sodium channels and ligand-gated transient receptor potential (TRP) channels (Moran and Szallasi, 2018). Changes in the expression and trafficking of voltage-dependent sodium channels play a pivotal role in the generation of ectopic discharge in the injured nerve (figure 4b). Sodium channels seem obvious candidates both because of their intrinsic properties and because sodium channel blockers produce analgesia in some patients with neuropathic pain (Colloca *et al.*, 2017).

However, while antisense knockdown has implicated Nav1.8 in rodent models of neuropathic pain (Gold *et al.*, 2003), surprisingly knockouts of Nav1.8, 1.9, 1.7, and 1.3 have no change in

the neuropathic pain phenotype (Priest *et al.,* 2005; Nassar et al., 2004, 2005, 2006; Amaya *et al.,* 2006). Voltage-gated sodium channels are expressed throughout the nervous system with the most abundant expression in DRG neurons. Some studies have demonstrated that experimental axotomy in adult animal shows a reduction in the level of RNA of NaV1.6, NaV1.7, NaV1.8, and NaV1.9 but upregulates Nav1.3 which usually is not expressed by adult DRGs (Cox *et al.,* 2006; Dib-Hajj *et al.,* 2007; Dib-Hajj *et al.,* 2009).

This suggested that NaV1.3 may be a key player in neuropathic pain and the neurons expressing this channel may exhibit reduced threshold or a high firing frequency (Dib-Hajj *et al.,* 2009; Dib-Hajj *et al.,* 2007). There are also changes in the redistribution of sodium channels, particularly of NaV1.8, increased immunoreactivity of which was observed at the site of nerve injury (Wood *et al.,* 2004; Black *et al.,* 2008). N- and T-type calcium channels are also expressed by C fibres and are upregulated under neuropathic pain conditions. In C-nociceptive fibre, the α2δ subunit is dramatically upregulated after nerve injury and plays a key role in injury-evoked hypersensitivity and allodynia (Luo *et al.,* 2001; Davies *et al.,* 2007).

Transient receptor potential (TRP) channels are a group of cation channels involved in sensory signalling that change after nerve injury and during inflammation (figure 4). The most profound changes have been observed in the expression of capsaicin-activated channel TRPV1 after nerve injury (Baron, 2000; Caterina, 2007, 2008). The involvement of TRPV1 in the development of heat hyperalgesia was demonstrated in experiments where TRPV1 knockout mice did not develop heat hyperalgesia after inflammation (Caterina *et al.,* 2000). The antinociceptive effect of TRPV1 antagonists further supports the idea of the crucial role of TRPV1 in the development of neuropathic pain (Bautista *et al.,* 2006; Kwan *et al.,* 2006; Staaf *et al.,* 2009).

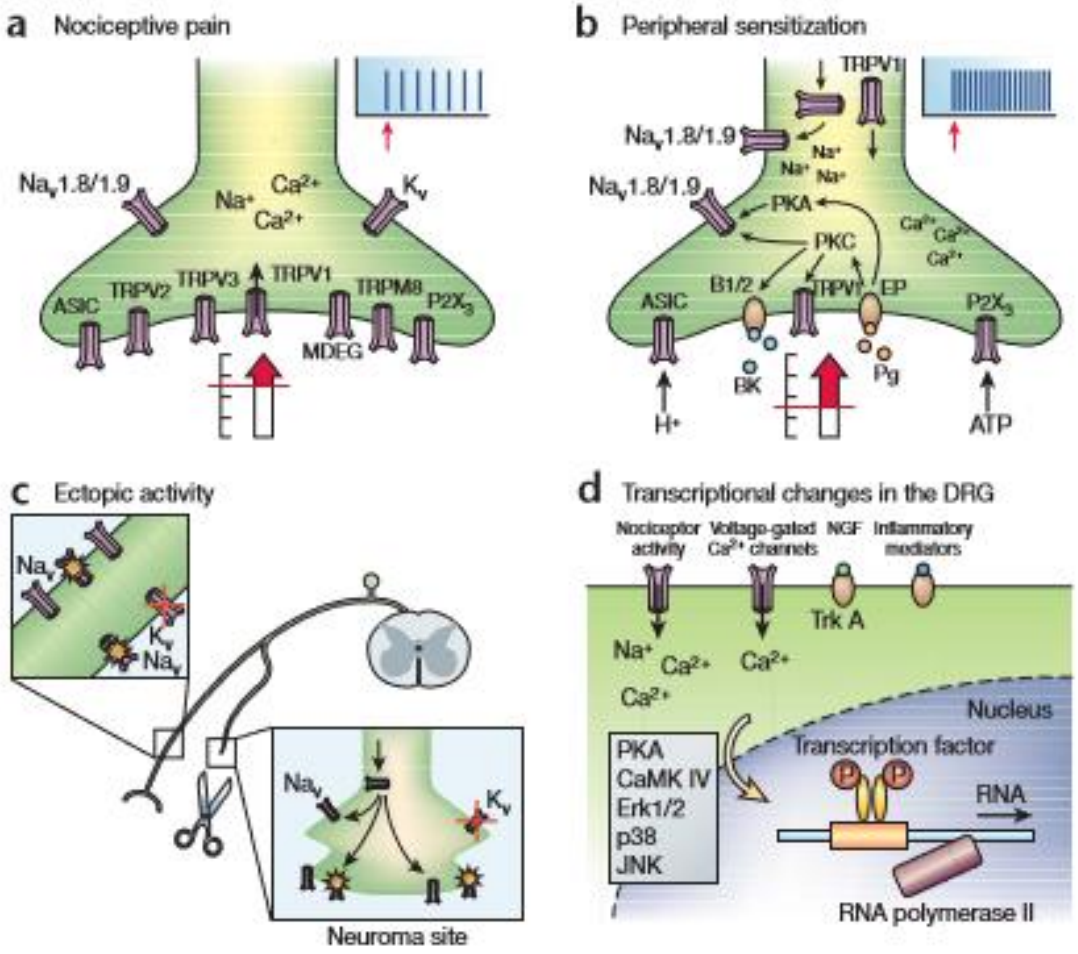

Fig 4: Nociceptor-mediated pain represents those pain conditions driven by activation of peripheral nociceptor sensory fibres. *(a) Nociceptive pain is produced under physiological conditions only by noxious stimuli acting on high-threshold nociceptors. (b) With inflammation, components of the 'inflammatory soup', such as bradykinin or prostaglandins, bind to G-protein-coupled receptors and induce activation of protein kinases A and C in nociceptor ion channels and receptors. As a result, the threshold of activation of transducer receptors such as TRPV1 is reduced, and the excitability of the peripheral terminal membrane increases, producing a state of heightened sensitivity, termed 'peripheral sensitisation'. (c) After injury to nociceptor neurons, increases in transcription as a reduction in potassium channels increases membrane excitability sufficiently so that action potentials are generated spontaneously (ectopic activity). (d) Activity-dependent signal transduction cascades and signaling pathways downstream to receptors bound by cytokines and growth factors act to modify transcription in nociceptor neurons. Altered production of numerous proteins modifies the phenotype of the neurons, changing their transduction, conduction, and transmission properties. (Scholz and Woolf, 2002)*

2.6.2 Inflammatory Mediators:

Peripheral sensitisation commonly results from inflammation-associated changes in the chemical environment of the nerve fibre (McMahon *et al.,* 2008). Traumatic injury to the sensory nerve fibres mediates the release of peptidergic neurochemical mediators at the peripheral axon terminal that result in neurogenic inflammation (Morin *et al.,* 2007). The activation and aggregation of inflammatory cells (satellite cells, Schwann's cells, macrophages, endothelial cells, mast cells, basophils, platelets, neutrophils, keratinocytes, and fibroblasts) at the injured or inflamed neurons play a pivotal role in the initiation, progression, and maintenance of NP via increase concentration of cytokines (Hu and McLachlan 2002; Grace et al. 2014; Ren and Dubner 2016).

Accumulation of endogenous factors released from activated nociceptors or non-neural cells that reside within or infiltrate into the injured area (figure 5) results in a spontaneous discharge of the afferent fibres. These endogenous factors are collectively referred to as the "inflammatory soup," and represent a wide array of signalling molecules, including neurotransmitters, peptides (substance P, CGRP, bradykinin), eicosanoids and related lipids (prostaglandins, thromboxanes, leukotrienes, endocannabinoids), neurotrophins, cytokines, and chemokines, as well as extracellular proteases and protons (Ritner *et al.,* 2008).

The spontaneous role of inflammatory mediators in the development and maintenance of neuropathic pain remains one of the vital mechanism through which therapeutic intervention can be developed. Precipitous increase in the concentration of proinflammatory cytokines (interleukin-1-beta (IL-1β), interleukine-6 (IL-6) and tumour necrotic factor-alpha (TNF-α)) released by these inflammatory cells induces hyperalgesia and allodynia symptoms in both clinical and experimental studies (Andratsch et al. 2009; Vallejo et al. 2010; Tiwari, *et al.,* 2014).

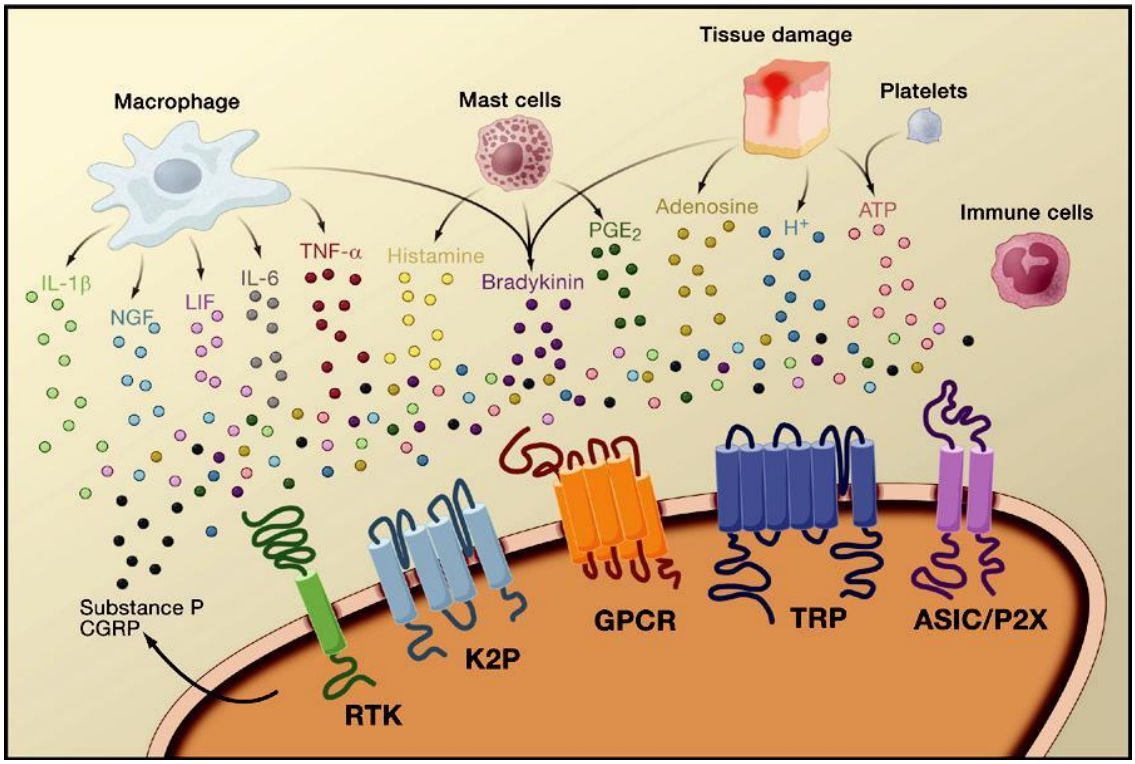

Fig 5: Peripheral Mediators of Inflammation. *Tissue damage leads to the release of inflammatory mediators by activated nociceptors or non-neural cells that reside within or infiltrate into the injured area, including mast cells, basophils, platelets, macrophages, neutrophils, endothelial cells, keratinocytes, and fibroblasts. This "inflammatory soup" of signaling molecules includes serotonin, histamine, glutamate, ATP, adenosine, substance P, calcitonin gene-related peptide (CGRP), bradykinin, eicosanoids prostaglandins, thromboxanes, leukotrienes, endocannabinoids, nerve growth factor (NGF), tumor necrosis factor α (TNF-α), interleukin-1β (IL-1β), extracellular proteases, and protons. These factors act directly on the nociceptor by binding to one or more cell surface receptors, including G protein-coupled receptors (GPCR), TRP channels, acid-sensitive ion channels (ASIC), two-pore potassium channels (K2P), and receptor tyrosine kinases (RTK), as depicted on the peripheral nociceptor terminal. Adapted from Meyer et al. (2008)*

The increased expression of IL-1 and TNFα in the DRGs is closely correlated with the reduced mechanical and thermal withdrawal threshold in animals (Sorkin and Doom, 2000; Schafers *et al.,* 2003). Clinical studies showed that patients with mechanical allodynia have higher levels of serum soluble TNFα receptors. Evidence has shown that NP signs are reduced in the IL-1β receptor knocked out mice (Kleibeuker *et al.,* 2008) or treatment with anti-inflammatory cytokines (Vale *et al.,* 2003; Milligan *et al.,* 2012). Reflexively, the inhibitory effect of anti-inflammatory agents on neuropathic pain has been postulated as an effective therapeutic intervention by many researchers (Khan et al. 2015; Yadav and Weng 2017).

Furthermore, literature has extensively implicated prostaglandin E_2 (PGE_2) in the development and maintenance of peripheral sensitisation (figure 6). The hyperalgesic properties of PGE_2 in the periphery are traditionally considered to be due to its sensitising effect on primary afferent nerves by both direct and indirect mechanisms. Prostaglandin E_2 (PGE_2) can directly modulate ion channel function by acting on EP receptors, in particular EP_1 and EP_4. Potentiation or sensitisation of TRPV1 activity, mainly through protein kinase C (PKC)- and PKA-dependent mechanisms, is perhaps the most critical mechanism underlying the peripheral nociceptive actions of PGE_2 (Chen *et al.,* 2013). TRPV1 channels, which are constitutively expressed in primary peripheral terminals of nociceptors, are activated by temperatures higher than 43 ^{o}C in the absence of inflammation. Increases in neuronal exposure to PGE_2 reduces the firing threshold to temperature stimulus as low as 35 ^{o}C, a response that was not observed in TRPV1-deficient mice (Moreyama *et al.,* 2005).

Activation of tetrodotoxin-resistant sodium channels (TTX-R Na^+) and inhibition of voltage-gated potassium currents are also components of PGE_2-mediated pain signalling transduction. For example, genetic deletion of Nav1.8, a voltage-gated TTX-R Na^+ channel, dramatically inhibited PGE_2-induced inflammatory mechanical hyperalgesia (Villareal *et al.,* 2005; Abrahamsen *et al.,* 2008), while reduced inflammatory thermal hyperalgesia was observed in

Nav1.9 null mice. These changes in TTX-R Na+ and voltage-gated potassium currents involve the activation of the PKA and PKC pathways (Chen *et al.,* 2013).

Similarly, PKA-dependent sensitisation of T-type calcium channels (Kawabata, 2011) and activation of purinergic P_2X_3 receptors (Wang *et al.,* 2007) was also reported to mediate PGE_2-evoked mechanical and thermal pain hypersensitivity. Also, PGE_2 inhibits potassium currents by increasing intracellular calcium in capsaicin-sensitive primary afferent neurons. There is evidence that PKA phosphorylation mainly contributes to the early phase of hyperalgesia evoked by peripheral PGE_2 administration, while PKC predominantly affects the late stage of PGE_2-evoked hyperalgesia (Sachs *et al.,* 2009).

In addition to modulating ion channels directly, PGE_2 also possesses indirect effects, including enhancing the sensitivity of peripheral neurons to other excitatory chemical agents such as bradykinin acting on B_2-receptors or capsaicin acting on TRPV1-receptors (Chen *et al.,* 2013). Although PGE_2 alone did not produce spontaneous pain in human volunteers, a small dose of PGE_2 injected subcutaneously evoked long-lasting hyperalgesia to mechanical and chemical stimuli. In animal studies, monoclonal antibodies directed against PGE_2 inhibited carrageenan-induced hyperalgesia in rats and reduced the phenyl benzoquinone-induced nociceptive response in mice. The sensitisation of sensory receptors to heat and bradykinin stimulation was mediated by EP_2 and EP_3 receptors, respectively (Chen *et al.,* 2013).

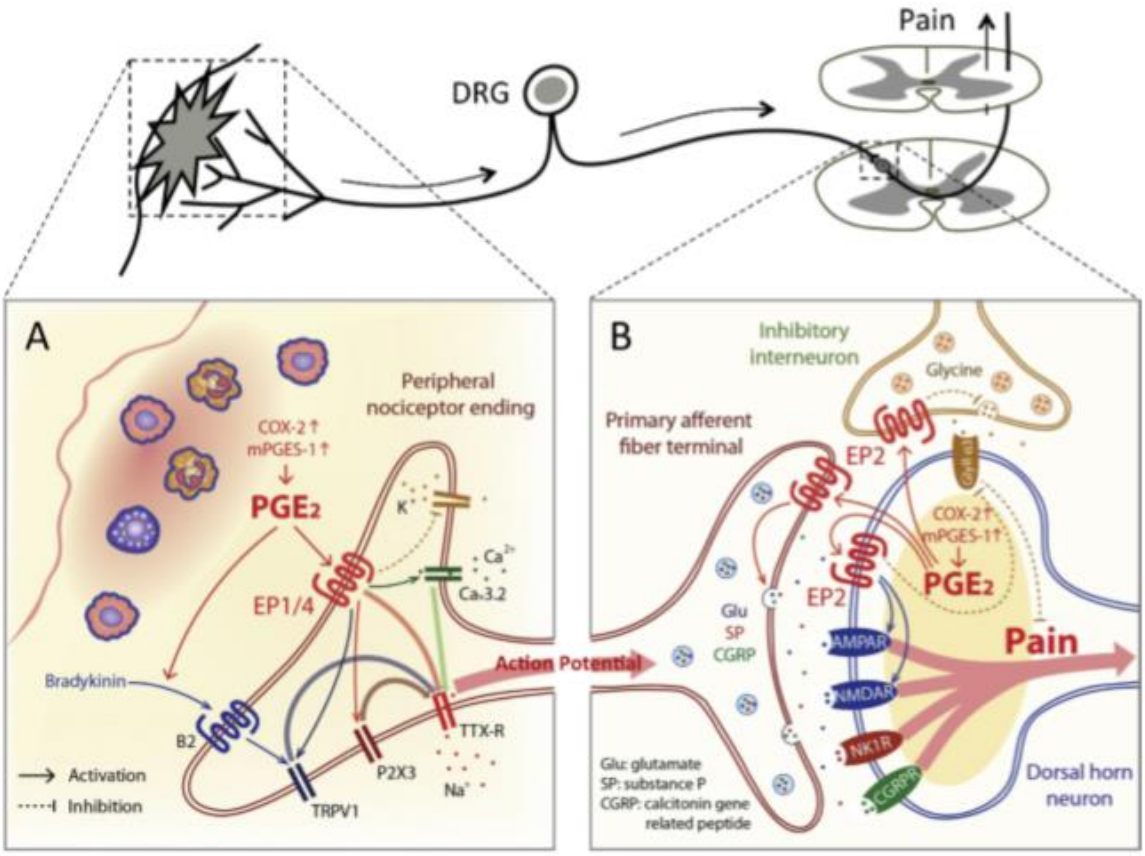

Fig 6: Peripheral and Central sensitisation of prostaglandin E_2 (PGE2). *A. Peripherally, PGE_2 acted via EP1 and EP4 to potentiate TTX-R, Ca^{2+} channel directly, and TRPV1 and bradykinin indirectly. It also inhibits the influx of K^+ current via voltage-gated K^+ channel. B. Centrally, through the activation of EP2, it promotes the release of peptidergic transmitters and glutamate. It also enables the phosphorylation of AMPAR and NMDAR in the dorsal horn of the spinal cord. Chen et al., 2013*

Nerve growth factor (NGF) is a neurotrophic factor required for the survival and development of sensory neurons during embryogenesis (Rittner *et al.,* 2008). Among its many cellular targets, NGF acts directly on peptidergic C fibre which expresses the high-affinity NGF receptor tyrosine kinase, TrkA, as well as the low-affinity neurotrophin receptor, p75 (Snider and McMahon, 1998; Chao, 2003). NGF produces profound hypersensitivity to heat and mechanical stimuli through two temporally distinct mechanisms.

At first, an NGF-TrkA interaction activates downstream signalling pathways, including phospholipase C (PLC), mitogen-activated protein kinase (MAPK), and phosphoinositide 3-kinase (PI3K). This results in functional potentiation of target proteins at the peripheral nociceptor terminal, most notably TRPV1, leading to a rapid change in cellular and behavioural heat sensitivity (Chuang *et al.,* 2001). NGF is also retrogradely transported to the nucleus of the nociceptor, where it promotes increased expression of pronociceptive proteins, including substance P, TRPV1, and the Nav1.8 voltage-gated sodium channel subunit (Chao, 2003; Ji *et al.,* 2002). Together, these changes in gene expression enhance the excitability of the nociceptor and amplify the neurogenic inflammatory response.

2.7 CENTRAL SENSITISATION

Central sensitisation refers to the process through which a state of hyperexcitability is established in the central nervous system, leading to enhanced processing of nociceptive (pain) messages (Woolf and Ma, 2007). It is the state of the enhanced response of pain circuits in the spinal cord and brain. Central sensitisation is generally mediated by increased excitation of the central neurons via alteration in glutamatergic neurotransmission; loss of tonic inhibitory controls (disinhibition); neuronal plasticity via reorganisation; and neuroinflammation via dysrangement in glial-neuronal interactions as indicated in figure 7 and 8.

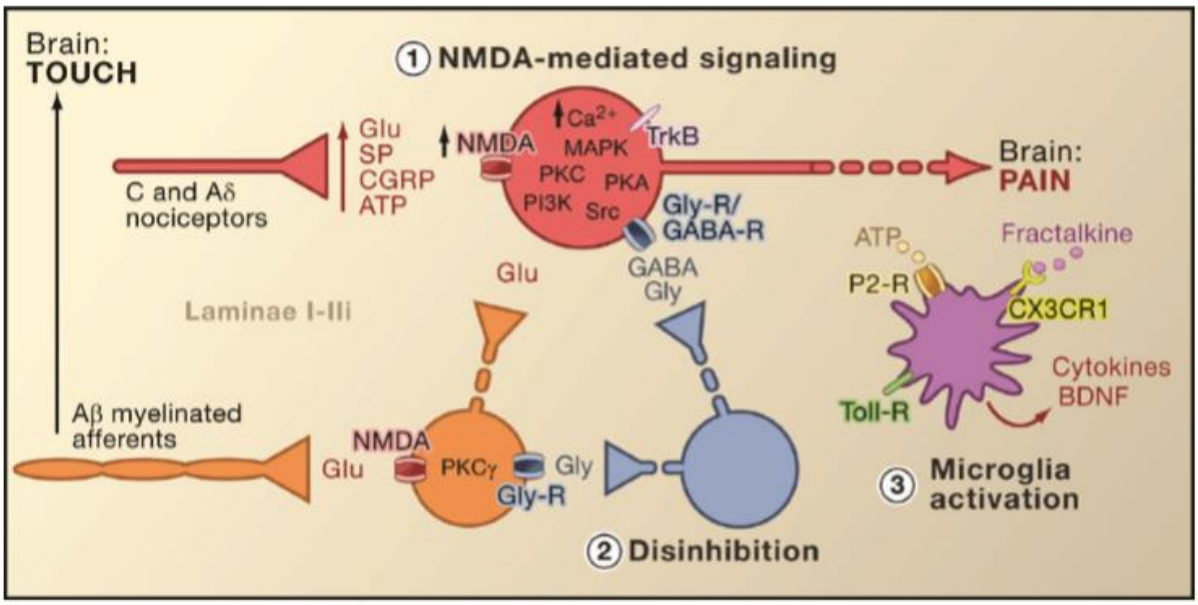

Fig 7: Central Sensitization. *(1) Glutamate/NMDA receptor-mediated sensitisation. After intense stimulation or persistent injury activated C and Aδnociceptors release a variety of neurotransmitters, including glutamate, substance P, calcitonin gene-related peptide (CGRP), and ATP, onto output neurons in lamina I of the superficial dorsal horn (red). As a consequence, ordinarily silent NMDA glutamate receptors located in the postsynaptic neuron can now signal, increase intracellular calcium, and activate a host of calcium-dependent signalling pathways and second messengers including mitogen-activated protein kinase (MAPK), protein kinase C (PKC), protein kinase A (PKA), phosphatidylinositol 3-kinase (PI3K), and Src. This cascade of events will increase the excitability of the output neuron and facilitate the transmission of pain messages to the brain. (2) Disinhibition. Under normal circumstances, inhibitory interneurons (blue) continuously release GABA and/or glycine (Gly) to decrease the excitability of lamina I output neurons and modulate pain transmission (inhibitory tone). However, in the setting of injury, this inhibition can be lost, resulting in hyperalgesia. Additionally, disinhibition can enable non-nociceptive myelinated Aβ primary afferents to engage the pain transmission circuitry such that ordinarily innocuous stimuli are now perceived as painful. This occurs, in part, through the disinhibition of excitatory PKCγ expressing interneurons in inner lamina II. (3) Microglial activation. Peripheral nerve injury promotes the release of ATP and the chemokine fractalkine that will stimulate microglial cells. In particular, activation of purinergic P2-R receptors, CX_3CR1, and Toll-like receptors on microglia (purple) result in the release of brain-derived neurotrophic factor (BDNF), which through activation of TrkB receptors expressed by lamina I output neurons, promotes increased excitability and enhanced pain in response to both noxious and innocuous stimulation (that is, hyperalgesia and allodynia). Activated microglia also release a host of cytokines, such as tumour necrosis factor α (TNFα), interleukin-1β and 6 (IL-1β, IL-6), and other factors that contribute to central sensitisation (*Basbaum et al., 2009)

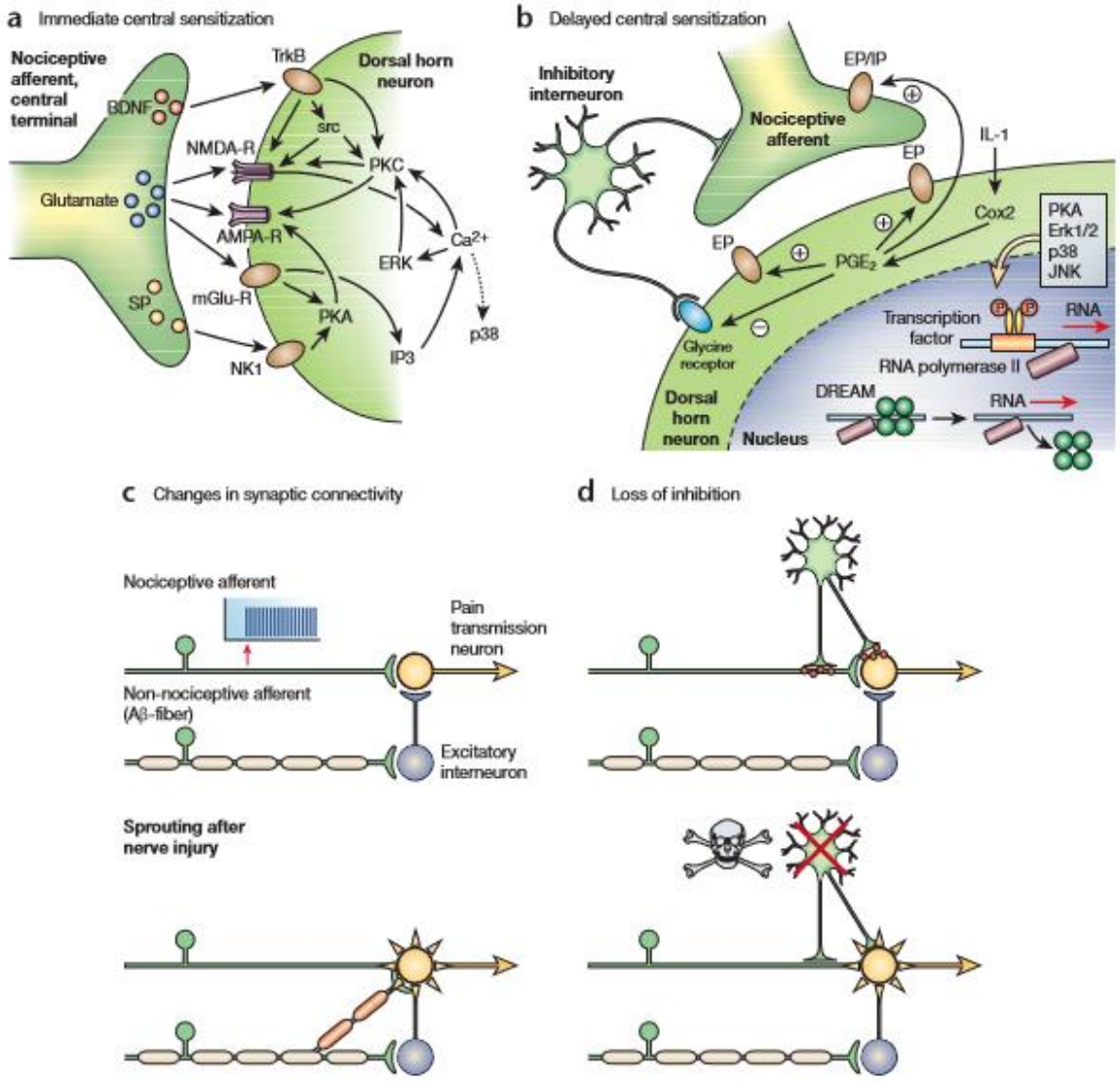

Fig 8: Non-nociceptor-mediated pain is generated by sensory inputs that would naturally produce an innocuous sensation, and reflects a change in the functioning of central neurons. *(a) Activity-dependent central sensitisation. An immediate and relatively short-lasting increase in the excitability and responsiveness of pain transmission dorsal horn neurons, which is due to phosphorylation of ion channels and receptors and follows nociceptor-driven transmitter release and activation of intracellular kinases. Eventually, the response to normally subthreshold inputs is increased. (b) Transcription-dependent central sensitisation. Cox2 induction leads to PGE2 production, which acts pre- and postsynaptically to facilitate excitatory and reduce inhibitory transmission; hence, results in long-lasting changes in the function of dorsal horn neurons. (c) After peripheral nerve injury, the central terminals of myelinated non-nociceptive Aβ-afferents sprout in the dorsal horn and form new connections with nociceptive neurons in laminae I and II. This re-wiring of the circuitry of the spinal cord may contribute to persistent pain hypersensitivity. (d) Disinhibition. Reduced synthesis of the inhibitory neurotransmitters GABA and glycine or loss of these inhibitory interneurons after the excessive release of the excitotoxic amino acid glutamate following peripheral nerve injury increases the excitability of pain transmission neurons such that they begin to respond to ordinarily innocuous inputs (Scholz and Woolf, 2002).*

2.7.1 Enhanced excitatory neurotransmission-induced hypersensitivity:

Glutamate is a significant neurotransmitter mediating the fast excitatory transmission at central synapses and plays critical roles in synaptic plasticity and development. An imbalance in the homeostasis of the glutamatergic system mediates enhanced central hypersensitivity. Increases in the synaptic release of glutamate, upregulation of glutamate receptors (N-methyl-D-aspartate receptors (NMDAR), α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptors (AMPAR) and kainite receptors) as well as down-regulation of glutamate transporters are responsible for the development of central potentiations in the central neurons (Yan and Weng 2013).

Incessive afferent presynaptic release of glutamate into the dorsal horn of the spinal cord with concurrent with endocytosis of glutamate transporters plays a pivotal role on increases activation of glutamate receptors (Sung *et al.,* 2003; Kanai *et al.,* 2013; Yan *et al.,* 2014). Down-regulation of glutamate transporters has been linked with an increase in the expression and activities of glutamate receptors (Gegelashvili and Bjerrum 2019). Up-regulation of glutamate receptors in the dorsal horn of the spinal cord has been observed in various models of NP (Ramos *et al.,* 2010; Napier *et al.,* 2012; Yan and Weng, 2013; Inquimbert *et al.,* 2012, 2018). Reduced clearance of synaptic glutamate majorly results in neurotoxicity with repetitive activation of glutamate receptors that pronounce central potentiation (Weng *et al.,* 2014; Gegelashvili and Bjerrum 2019) that mediate hyperalgesia (Ramos *et al.,* 2010) and allodynia symptoms (Mao and Yang, 2010) of NP.

The involvement of central glial cells (microglia and astrocyte) has been overemphasised as a mediator of central sensitisation (Milligan and Watkins, 2009; Ji *et al.,* 2013). It is now well established that nerve damage via activation of glial cells leads to neuroanatomical and neurochemical transformations in the central nervous system (CNS) that result in neuropathic pain (Ji *et al.,* 2013). Both astrocytes and microglia play essential roles in mediating

neuropathic pain by releasing proinflammatory cytokines, chemokines, and other factors known to facilitate pain signallings, such as calcitonin gene-related peptide (CGRP), substance P, and glutamate (Wieseler-Frank *et al.,* 2005; Milligan and Watkins, 2009). Studies have also reported that nerve damage induces up-regulation of glutamate receptors and intracellular invagination of ionotropic glutamate transporters (Yan and Weng, 2013; Yan *et al.,* 2014). This results in synaptic neurotoxicity with prolonging excitation of glutamate receptors (Gegelashvili and Bjerrum, 2014).

The upregulation of dynorphin in the spinal cord after nerve injury has been reported in rats and mice (Gardell *et al.,* 2004). A strain of mouse that does not develop the signs of neuropathic pain does not show an increased level of dynorphin (Gardell *et al.,* 2004). Dynorphin may increase the release of excitatory neurotransmitters from primary afferent fibres as suggested from studies showing that release of calcitonin gene-related peptide, substance P, and excitatory amino acids into the dorsal horn is potentiated by dynorphin (Gardell *et al.,* 2003; Koetzner *et al.,* 2004). Also, the pharmacological inhibition of dynorphin abolished the development of mechanical and thermal hypersensitivity in rats and mice after nerve injury; genetic knockout of dynorphin led to only transient hypersensitivity after nerve injury (Gardell *et al.,* 2004). Those studies point to the vital role of spinal dynorphin in persistent pain.

2.7.2 Disinhibition:

The sensory input into the spinal cord is under the regulation of inhibitory circuitry maintained by sensory afferents, spinal interneurons, and descending inhibition. Disruption of these circuitry accounts for sustained hyper-excitation of the central neurons and contributes to the genesis of neuropathic pain (Bonin and De Koninck, 2013). ϒ-aminobutyric acid (GABA), glycine, serotonin, and nor-epinephrine remain the primary inhibitory neurotransmitters in the dorsal horn of the spinal cord. GABAergic inhibitory synaptic activities in the spinal dorsal horn are mediated on pre- and post-synaptic neurons. GABA activates ionotropic $GABA_A$ and

metabotropic $GABA_B$ receptors at the presynaptic terminal to reduce the presynaptic release of glutamate (Bardoni *et al.,* 2013). GABA acts post-synaptically on $GABA_A$ receptor to induced an influx of Cl^- ion that mediate hyperpolarisation (Yadav *et al.,* 2015). This increases the threshold of sensory neurons, therefore, abolished the propagation of pain sensation.

The hyperexcitability of dorsal horn neurons in neuropathic pain has been explained by a disruption of inhibitory tone in the spinal cord (Gwak *et al.,* 2006). Peripheral nerve injury, spinal cord injury, and other traumatic injuries to the afferent neurons are associated with central disinhibition. Dysfunction in the activities of primary inhibitory neurotransmitters system decreases tonic inhibition, enhances depolarisation, and hyper-excitation of projection neurons in response to painful (hyperalgesia) and non-painful (allodynia) stimulation (Keller *et al.,* 2007; Ossipov *et al.,* 2010; Lee and Prescott, 2015; Lopez-Alvarez *et al.,* 2018).

The blockage of GABA signalling in the spinal GABAergic neurotransmission by intrathecal antagonists has been demonstrated to produce hypersensitivity to innocuous tactile stimuli (Gwak *et al.,* 2006). Transgenic mice lacking specific subunits of GABA receptors have been reported to developed hyperalgesia and allodynia (Schuler *et al.,* 2001). Spinal administration of GABA or glycine receptor antagonists (bicuculline or strychnine, respectively) in rodents (Malan *et al.,* 2002) produces behavioural hypersensitivity resembling that observed after peripheral injury. A reduction of GABA and GABA synthesising enzyme (GAD) has been reported after spinal cord injury and peripheral nerve injury (Keller *et al.,* 2007). It has been suggested that GABA transporter (GAT-1) increases following nerve injury, which reduces synaptic GABA concentrations (Yadav *et al.,* 2015). Hence, result in loss of tonic inhibition by the spinal interneurons, which is associated with dysregulation of sodium-potassium-chloride cotransporter-1 (NKCC-1) and potassium chloride cotransporter-2 (KCC-2) (Lopez-Alvarez *et al.,* 2018). These induce an inversion of GABAergic depolarising current (Kahle *et*

al., 2014; Modol *et al.,* 2014) in which the inhibitory effect of the GABAergic system reverses to an excitatory effect mostly in the lamina I projection neurons (Coull *et al.,* 2003).

It has also been documented in a mouse study that the deletion of the gene encoding PKCγ leads to a marked decrease in nerve injury-evoked mechanical hypersensitivity (Malmberg *et al.,* 1997). The blockade of glycinergic inhibition with strychnine induces activation of PKCγ-positive interneurons in lamina II (Miraucourt *et al.,* 2007), as well as projection neurons in lamina I following stimulation with innocuous brushing of the hind paw activates. This suggested that PKCγ-positive interneurons are essential for the expression of nerve injury-evoked persistent pain and that disinhibitory mechanisms lead to their hyperactivation. Other studies indicate that changes in the projection neuron itself contribute to the disinhibitory process.

Another disinhibition mechanism that involves the action of prostaglandins in the spinal cord has been suggested to mediate the activity via glycinergic signalling (Harvey *et al.,* 2004). Specifically, prostaglandin E_2 (PGE_2) which acts on EP2 receptors expressed by excitatory interneurons and projection neurons in the superficial dorsal horn. The resultant stimulation of the cAMP-PKA pathway phosphorylates GlyRa3 glycine receptor subunits and renders the neurons unresponsive to the inhibitory effects of glycine (figure 9). Indeed, mice lacking the GlyRa3 gene show reduced heat and mechanical hypersensitivity in models of nerve injury (Zeilhofer, 2007).

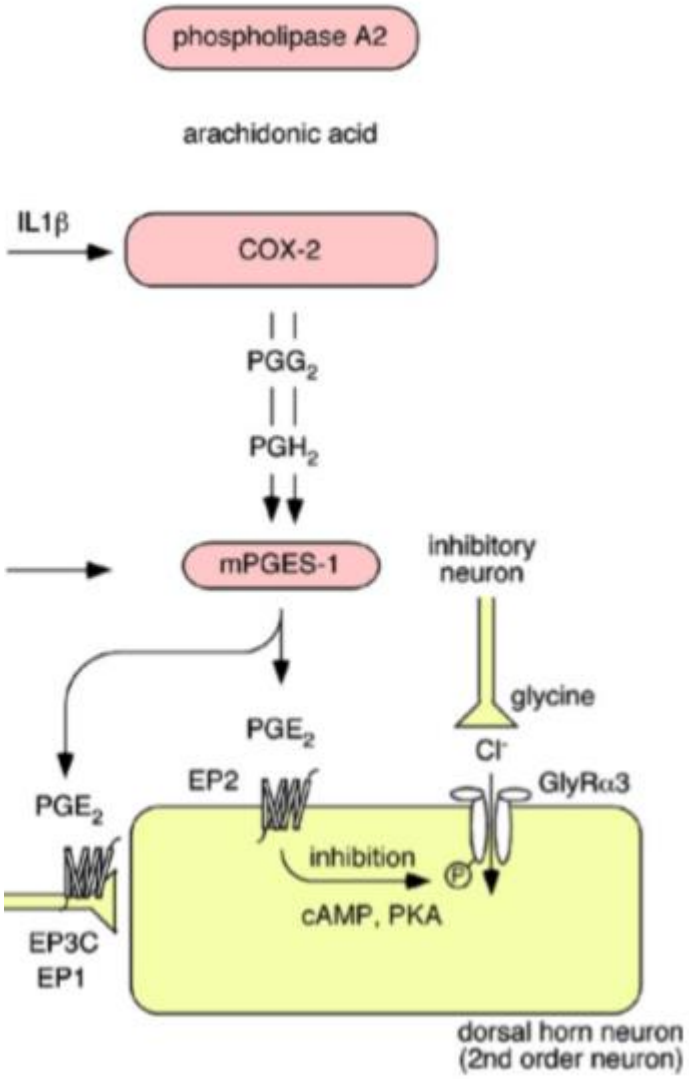

Fig 9: prostaglandin E_2 mediates central disinhibition in the spinal cord. *(Zeilhofer, 2007).*

Regulation of the spinal and ascending nociceptive fibre is also achieved by the inhibitory input of descending supra-spinal fibres (prefrontal cortex, cingulate cortex, amygdala, ventrolateral periaqueductal grey, locus cerullus, rostral ventromedial medulla). This is collectively known as descending inhibition (Millan, 2002; Andreoli *et al.,* 2017). This polysynaptic pathway releases serotonin and norepinephrine (NE) from their terminal into the dorsal horn modulating the sensation of neurons. Serotonin and NE modulate spinal signals via pre- and post-synaptic mechanisms by activating spinal enkephalinergic and GABA/glycinergic interneurons. Enkephalin act on the opioid receptors located on the presynaptic terminal of primary afferent fibre. Hence, reducing calcium ion influx that consequently inhibits the release of excitatory neurotransmitter (Ossipov *et al.,* 2010; Lopez-Alvarez *et al.,* 2018).

Loss of this descending inhibitory input contributes to the maintenance of central sensitisation. This is evidence by the use of serotonin-norepinephrine reuptake inhibitors as a basis of a treatment to ameliorate the symptoms of neuropathic pain (Ossipov *et al.,* 2010). The lesion of the significant pathways connecting RVM to the spinal cord, dorsolateral funiculus, also abolished the development of neuropathic pain (Burgess *et al.,* 2002; Ren and Dubner, 2008). Electrophysiological experiments demonstrated that a specific population of RVM cells expressing the opioid receptor is directly related to the descending facilitatory input (Heinricher and Neubert, 2004). The selective ablation of these cells prevented the development of neuropathic pain (Burgess *et al.,* 2002).

2.7.3 Reorganisation:

Studies have shown that the nerve injury culminates into structural reorganisation in the spinal dorsal horn allowing the signal from non-nociceptive Aβ fibres to reach and activate nociceptive neurons (Scholz and Woolf, 2002). This idea was based on the observation of positive labelling of superficial dorsal horn laminae with retrograde tracer cholera toxin B (CTB) after nerve injury (Shehab *et al.,* 2003). In familiar animals, labelling is found in the

deeper dorsal horn laminae. However, under neuropathic conditions, CTB labelled neurons were expressed in the superficial lamina (of nociceptive neurons termination), which suggested the sprouting of Aβ fibres from deeper laminae.

This partly explains the development of allodynic symptom experience via an input of non-nociceptive signalled by Aβ fibres that excite nociceptive neurons. However, subsequent studies showed that there is a phenotypic switch in DRG neurons after axotomy as the expression of GM1 receptors was also found on the unmyelinated nociceptive DRG neurons. Likewise, mechanical allodynia may also result from increase expression and activation of silent neurons and type II Aδ fibres (Basbaum *et al.,*2009). Therefore, CTB is transported by both myelinated and unmyelinated afferents which explain its appearance in upper dorsal horn laminae, termination of unmyelinated fibres (Bao *et al.,* 2002; Shehab *et al.,* 2003). Although the structural reorganisation does not seem to be a case for abnormal Aβ signal processing, electrophysiological experiments showed the increased Aβ input into the dorsal horn (Shehab *et al.,* 2003).

The role of Aβ fibres in the development of tactile allodynia is further supported by evidence showing that injection of sodium channel blocker lidocaine into the supraspinal termination of Aβ fibres- nucleus gracilis - block tactile allodynia (Sun *et al.,* 2001). An interesting finding in this study was that thermal allodynia was not changed by this treatment (Ossipov *et al.,* 2002). The expression of neuropeptide Y in DRG neurons after peripheral or spinal nerve injury seems to be related to the activity of Aβ fibres. This protein is not present in DRGs during physiological conditions. After nerve injury, its expression is found in DRGs and spinal cord in the area of termination of Aβ fibres.

Neuropeptide Y is also upregulated in the ipsilateral nucleus gracilis after nerve injury (Ossipov *et al.,* 2002). It has been shown that its presence originates from the DRG since neither dorsal rhizotomy nor lesion of the dorsal column blocked its appearance in nucleus gracilis. The

significance of this supraspinal centre for the development of pain was demonstrated in experiments where injection of neuropeptide Y into nucleus gracilis evoked tactile allodynia (Ossipov *et al.,* 2002). Interestingly, thermal sensitivity was not changed. The findings of these studies indicated that the tactile hypersensitivity is mediated by Aβ input through the upregulation of neuropeptide Y in the DRGs and that the development of tactile hypersensitivity can be modulated from supraspinal areas. Also, the modulation of tactile hypersensitivity without affecting thermal sensitivity points out to different processing of mechanical and thermal nociceptive input.

2.7.4 Prostaglandin:

The central role of prostaglandin E_2 (PGE_2) in mediating central hyperalgesia has been described in various experimental models. PGE_2 mediated central sensitisation via both presynaptic and postsynaptic mechanisms (figure 10). At the presynaptic level, PGE_2 causes enhancement of nociception by facilitating the spinal release of the excitatory neurotransmitter glutamate or neuropeptides including glutamate, substance-P, and calcitonin gene-related peptide (CGRP) (figure 10) (Chen *et al.,* 2013). This can be mediated by an increase in the influx of calcium currents that potentiate the release of neurochemicals.

PGE_2 act via the EP_2 receptor to activate directly deep dorsal horn neurons, then potentiating AMPA (2-amino-3-(5-methyl-3-oxo-1, 2-oxazole-4-yl) propanoic acid) and NMDA currents (Kohno *et al.,* 2008). This leads to the activation of the nonselective cation channels (Baba *et al.,* 2001), which reduce neuronal threshold and hyperalgesia. Furthermore, PGE_2 can also facilitate central inflammatory pain sensitisation by blocking the glycinergic receptor α_3 subunit and reducing inhibitory glycinergic neurotransmission (Harvey *et al.,* 2004) as discussed above. Intrathecal administration of substance P, N-methyl-D-aspartic acid (NMDA) and kainite, and systemic administration of cytokines significantly increased spinal PGE_2 formation (Samad *et al.,* 2001; Ricciotti and FitzGerald, 2011).

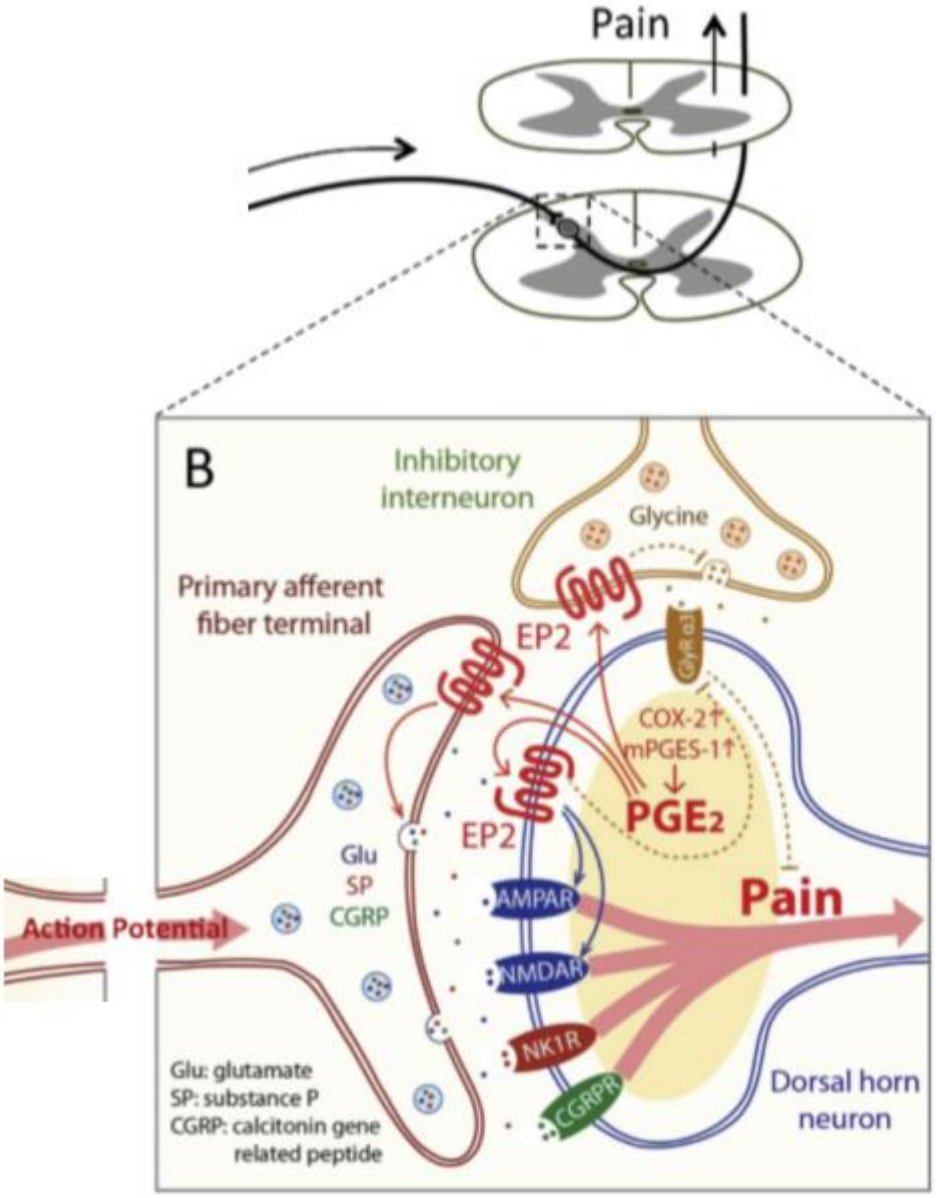

Fig 10: Central sensitisation of prostaglandin E_2 (PGE2). *Chen et al., 2013*

2.7.5 Macrophage, immune cell, Glial-Neuronal Interactions:

The vital role of non-neuronal cells in the development of central sensitisation is well documented in the literature. Infiltration of leukocytes and activation of glial cells plays an active role in pain, exhibiting diverse mechanisms that mediate hyperalgesia. In most cases, these non-neuronal cells produce pain through the release of proinflammatory mediators such as tumour necrosis factor-alpha (TNF-α), prostaglandin E_2 (PGE_2), interleukin-6 (IL-6), and interleukin-1β (IL-1β) (Grace *et al.*, 2014; Ji *et al.*, 2016). These mediators result in enhanced pain transduction and conduction via modulation of ion channels, neurotransmitters, and phosphorylation of excitatory receptors (Basbaum *et al.*, 2009; Yan *et al.*, 2014; Grace *et al.*, 2014; Ji *et al.*, 2014). They can then fine-tune both excitatory and inhibitory synaptic transmission, which ultimately enhances pain signal transmission to the brain (Kawasaki *et al.*, 2008).

Indecent releases of proinflammatory mediators have been linked with allodynia and hyperalgesia symptoms of neuropathic pain. Studies have shown that TNF-α, IL-6, IL-1β, and PGE_2 induces endocytosis of glutamate transporter (Yan *et al.*, 2014), increase presynaptic primary afferent Ca^{2+}-dependent glutamate release in the spinal dorsal horn (Yan and Weng, 2013), phosphorylation of NR1 NMDA glutamate receptor (Grace *et al.*, 2014), and up-regulation of glutamate receptors (Yadav and Weng, 2017) as indicated in figure 11. Proinflammatory mediators also result in disinhibition by inhibiting the spontaneous release of GABA and glycine from the interneurons (Kawasaki *et al.*, 2008). It inhibits the activation of GABA receptors as well as descending inhibitory projection (Grace *et al.*, 2014) resulting in reduced hyperpolarisation (Zhang, 2010) and a shift in depolarisation of GABAergic transmission (Kahle *et al.*, 2014; Modol *et al.*, 2014; Ren and Dubner, 2016).

It has been demonstrated that the ATP-evoked activation of P_2X_4 receptors induces the release of the brain-derived neurotrophic factor (BDNF) from microglia (Coull *et al.*, 2005). The

BDNF, in turn, acts upon TrkB receptors on lamina I projection neurons to generate a change in the Cl^- gradient, which, as described above, would shift the action of GABA from hyper-polarisation to depolarisation neurons (Coull *et al.*, 2005, Ferrini *et al.*, 2013). Activation of microglia will sensitise lamina I neurons such that their response to monosynaptic inputs from nociceptors, or indirect inputs from Aβ afferents, is enhanced (Kim *et al.*, 2007) resulting in allodynia. Selective activation of spinal microglia by fractalkine (CX3CL1) is sufficient to rapidly facilitate synaptic strength between primary afferent C-fibres and lamina I projection neurons (Clark *et al.*, 2015).

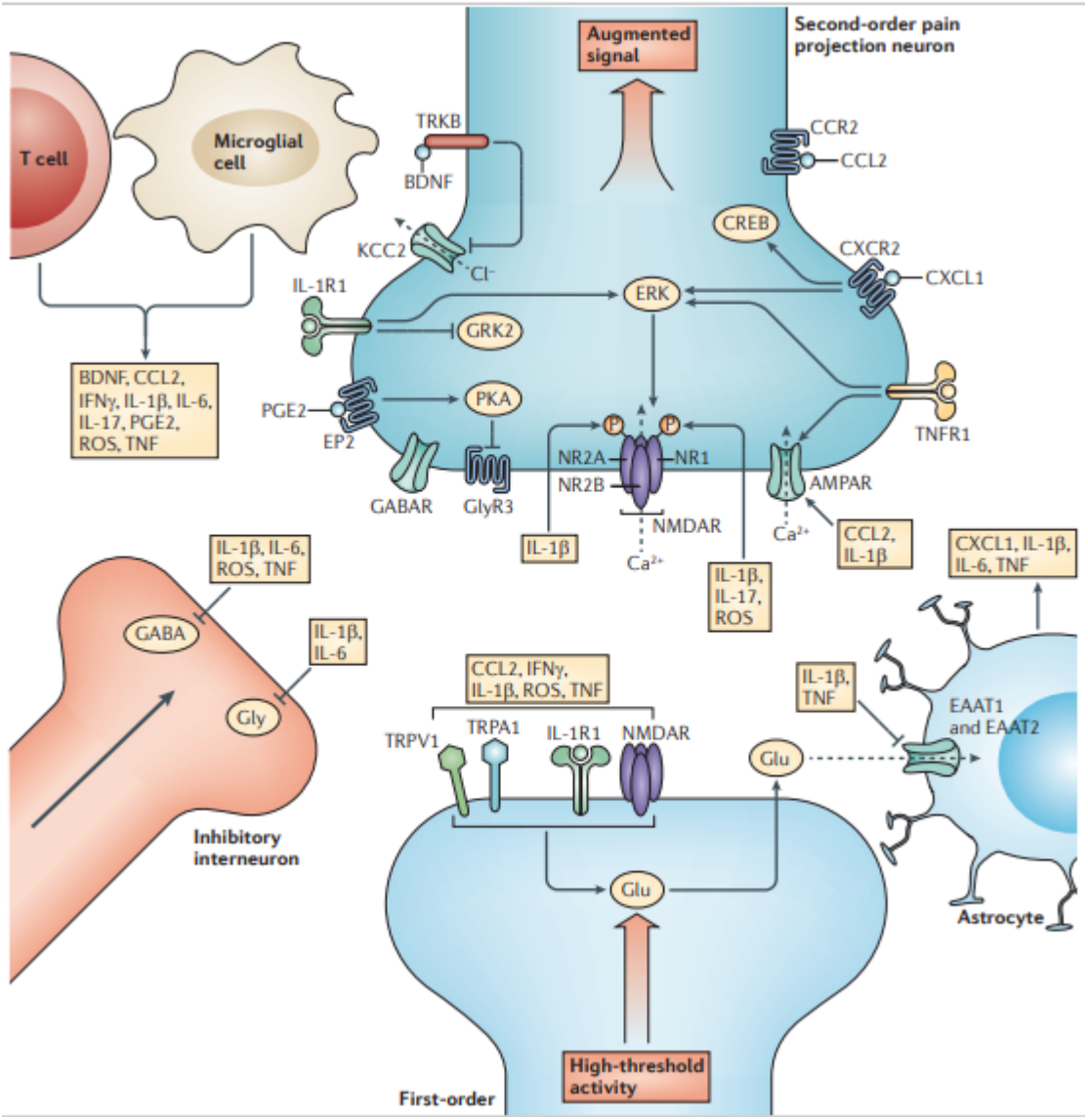

Fig. 11: ***Proinflammatory Mediators' enhancement of nociception:*** *proinflammatory mediators bind to receptors on presynaptic and postsynaptic terminals in the spinal dorsal horn to modulate excitatory and inhibitory synaptic transmission, resulting in nociceptive hypersensitivity. Tumour necrosis factor (TNF), and interleukin-1β (IL-1β), increase glutamate (Glu) release from central terminals, partly due to the activation of transient receptor potential channel subtypes (TRPV1 and TRPA1), and functional coupling between interleukin-1 receptor 1 (IL-1R1) and presynaptic ionotropic glutamate receptors (NMDAR). TNF, IL-1β, and IL-6 decrease GABA (γ-aminobutyric acid) and glycine (Gly) release by inhibitory interneurons. IL-1β also increases AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid) signalling, and TNFR1 signalling increases the expression of Ca2+-permeable AMPA receptors (AMPARs). TNF increases NMDAR activity through the phosphorylation (P) of extracellular signal-regulated kinases (ERKs). IL-1β increases the calcium permeability of NMDAR through phosphorylation of the NR1, NR2A, and NR2B subunits. Brain-derived neurotrophic factor (BDNF) signalling via TRKB downregulates the potassium-chloride cotransporter KCC2, increasing the intracellular Cl– concentration and weakening the inhibitory GABAA and glycine channel hyperpolarisation of second-order nociceptive projection neurons. Prostaglandin E2 (PGE2) signalling at the EP2 receptor activates protein kinase A (PKA), thereby inhibiting glycinergic neurotransmission via GlyR3. TNF and IL-1β downregulate astrocyte expression of the Glu transporters excitatory amino acid transporter 1 (EAAT1) and EAAT2, leading to enhanced glutamatergic transmission (*Grace *et al., 2014).*

Immune mediating cells (innate and adaptive immune systems) are critical in the development of central sensitization. These cells infiltrate the peripheral and central nerve fibres following nerve injury releasing proinflammatory cytokines (figure 12) that intensify pain sensation (Uceyler and Sommer, 2008; Andratsch et al., 2009; Martini and Willison, 2015). At the dorsal root ganglia (DRG) after nerve injury, T cells gain access and releases proalgesic mediator leukocyte elastase (LE), resulting in mechanical allodynia. Consistently, an inhibitor of LE reduces nerve injury-induced allodynia (Vicuña *et al.*, 2015). The role of the T-cell in the spinal cord towards the development of mechanical hypersensitivity as also be indicated by a reduction of pain behaviours in T-cell-deficient Rag1-null mice (Costigan *et al.*, 2009).

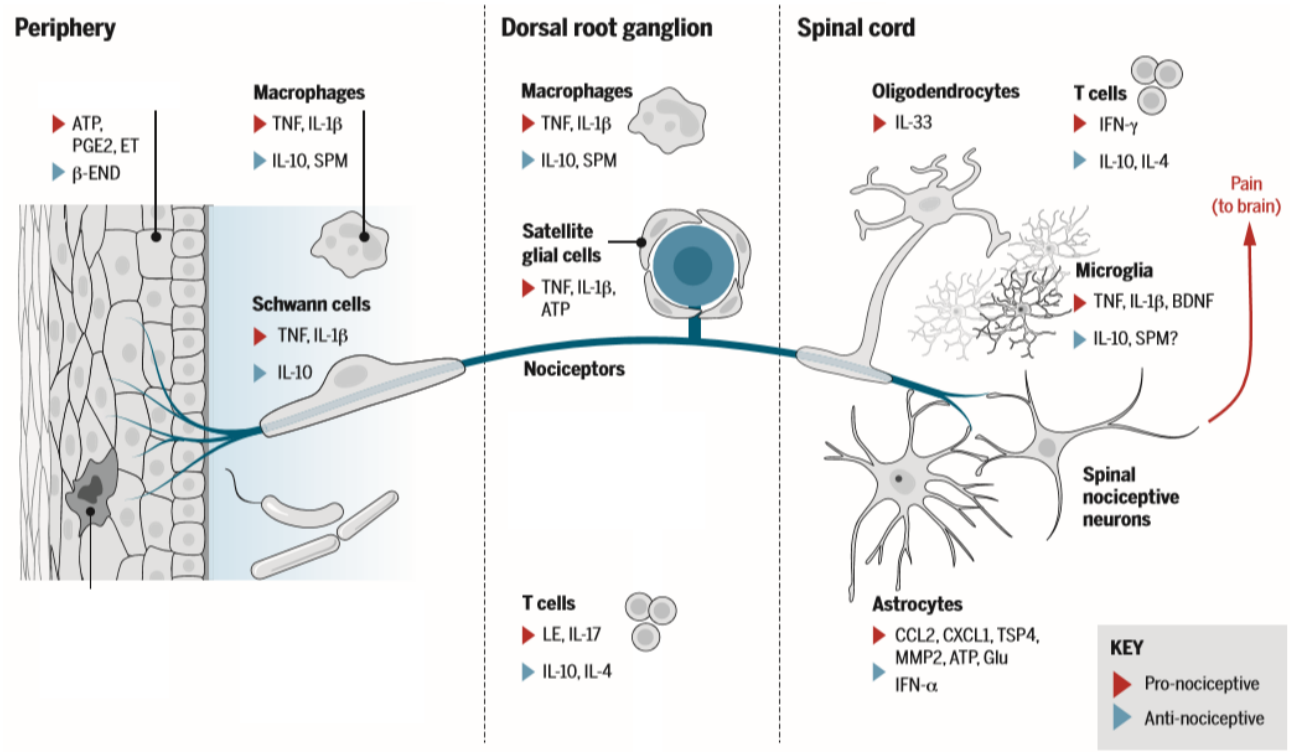

Fig 12: Role of non-neuronal cells in mediating central sensitization. *Included are Schwann cells, satellite glial cells, oligodendrocytes, and astrocytes, as well as immune cells (for example, macrophages and T-cells), microglia, cancer cells, and stem cells. These non-neuronal cells produce both pro-nociceptive (highlighted in red) and anti-nociceptive (highlighted in blue) mediators, which can bind their respective receptors on the nociceptor to modulate its sensitivity and excitability. The central terminal of the nociceptor forms a nociceptive synapse with a postsynaptic neuron in the spinal cord dorsal horn to mediate pain transmission in the CNS. END, endorphin; ET, endothelin; Glu, glutamate; IFN-a, interferon-a; LE, leukocyte elastase (Ji et al., 2016).*

Furthermore, glial cells (microglia, astrocytes, Schwann's and satellite cells) role in the development of the central sensitization process has been illustrated in the various models of nerve injury-induced neuropathic pain (Ji *et al.,* 2013; Grace *et al.,* 2014; Tsuda, 2016). In the shortest hours following peripheral nerve injury, microglia accumulate in the superficial dorsal horn within the termination zone of injured peripheral nerve fibres. Microglia also surround the cell bodies of ventral horn motor neurons, whose peripheral axons are concurrently damaged. The activated microglia release a panoply of signalling molecules, including cytokines (such as TNF-α and interleukin-1β and 6), which enhance central neuronal sensitization and nerve injury-induced persistent pain (Basbaum *et al.,* 2009; Zhang, 2010; Yadav and Weng, 2017).

Activation of microglia after physical damage of the peripheral afferent has been suggested to be due to the release of specific signals by the damaged nerve that is detectable by microglia (Clark *et al.,* 2007; Denk *et al.,* 2016; Guan *et al.,* 2016). These specific signals include ATP, colony-stimulating factor–1 (CSF1), chemokines (CCL2 and CX3CL1), and proteases, which can originate from injured or activated sensory neurons (Denk *et al.,* 2016). Chief among these is ATP, which targets microglial P2-type purinergic receptors. Of particular interest are P_2X_4 (Tsuda *et al.,* 2003), P_2X_7 (Chessell *et al.,* 2005), and P_2Y_{12} (Haynes *et al.,* 2006; Kobayashi *et al.,* 2008) receptor subtypes. In parallel, expression of the receptors for ATP and CX_3CL_1 (P_2X_4, P_2X_7, P_2Y_{12}, and CX_3CR_1) is increased selectively on spinal microglia in response to nerve injury (figure 13) (Ji *et al.,* 2013; Grace *et al.,* 2014).

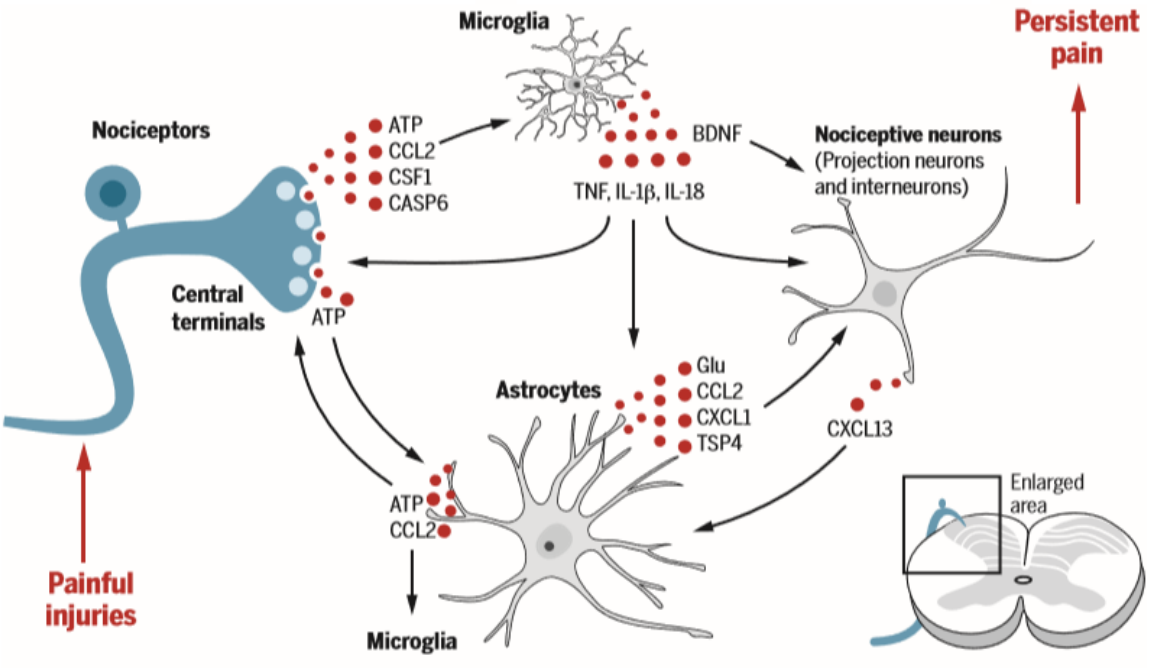

Fig 13. Neuron-glial interactions in the spinal cord for the amplification of chronic pain. *Painful injuries such as nerve injury, arthritis, cancer, and treatment (chemotherapy) cause hyperactivity of nociceptors and secretion of glial modulators from their central terminals, leading to the activation of microglia and astrocytes in the spinal cord dorsal horn. Upon activation, microglia and astrocytes secrete neuromodulators to drive chronic pain by inducing synaptic and neuronal plasticity. Pre- and postsynaptic neurons can both "listen" and "talk" to microglia and astrocytes. CASP6, caspase-6 (Ji et al., 2016).*

Astrocytes are unquestionably induced in the spinal cord after an injury to either tissue or nerve (Ren and Dubner, 2008). But, in contrast to microglia, astrocyte activation is generally delayed and persists much longer, up to several months. One exciting possibility is that astrocytes are more critical to the maintenance, rather than to the induction of central sensitization. One suggestion is that nerve injury induces various changes in astrocytes that lead to enhanced pain. After nerve injury, astrocytes lose their ability to maintain the homeostatic concentrations of extracellular potassium (K^+) and glutamate, leading to neuronal hyperexcitability (Ji *et al.,* 2013). Astrocytes can also signal directly to neurons through physically coupled networks mediated by gap junctions to facilitate intercellular transmission. Gap junction communication is mediated by connexin-43 (Cx43), the predominant connexion expressed in astrocytes. Nerve injury induces persistent up-regulation of Cx43 in astrocytes and switches the function of Cx43 from gap junction communication to paracrine modulation (Chen *et al.*, 2014). This paracrine regulation leads to the increased release of glutamate, ATP, and chemokines through a paracrine mechanism (Jiang *et al.,* 2016).

Oligodendrocytes create the myelin sheath that provides support and insulation to axons in the CNS. Despite their ubiquity and importance, only recently has a role for oligodendrocytes been revealed in pain (Zou *et al.,* 2016). In the chronic constriction injury model of nerve injury-induced neuropathic pain, oligodendrocyte-derived IL-33 contributes to pain hypersensitivity via MAP kinases and nuclear factor-kB (NFkB) (Zarpelon *et al.,* 2016). Conversely, toxin-mediated ablation of oligodendrocytes induces neuropathic pain symptoms, suggesting a potential protective role of these cells (Gritsch *et al.,* 2014). These divergent findings indicate that as with other non-neuronal cells, oligodendrocytes play active and context-specific roles in pain.

Schwann cells and satellite glial cells (SGCs) are principal glial cells of the peripheral nervous system, both of which contribute to pain hypersensitivity (Ji *et al.,* 2016). Peripheral glial cells

are activated before central glia in response to painful stimuli and release various inflammatory mediators, sensitizing nociceptors at axons (Schwann cells) and cell bodies (SGCs). After nerve injury, activated Schwann cells mediate the breakdown of the blood-nerve barrier via the secretion of matrix metalloproteinase 9 (MMP-9), which promotes the recruitment of immune cells from the vasculature and their subsequent release of more pro-nociceptive mediators (Calvo *et al.,* 2012).

Satellite glial cells (SGCs) surround the somata of DRG neurons and are directly coupled to each other via gap junctions. After nerve injury, SGCs become activated and proliferate (Calvo *et al.,* 2012). SGCs contribute to chronic pain sensitization by producing cytokines and MMPs that regulate the cleavage and activation of cytokines (Ji *et al.,* 2013). The nociceptive activity also causes ATP release from neuronal soma to activate P_2X_7 in SGCs, leading to TNF release from SGCs and subsequent increase in neuronal excitability (Zhang *et al.,* 2007). Activation of P_2X_7 receptors in SGCs also reduces pain through the down-regulation of P2X3 receptors in nociceptive neurons (Chen *et al.,* 2008).

2.8 REACTIVE OXYGEN SPECIES (ROS) AND REACTIVE NITROGEN SPECIES (RNS)

The contribution of Reactive Oxygen Species (ROS) and reactive nitrogen species (RNS) are very crucial to the development of NP via peripheral and central sensitization. Various published reports documented the critical role of oxidative stress in the pathogenesis of peripheral nerve injury (Areti *et al.,* 2016). The direct effects of superoxide (SO) and peroxynitrite (PN) was demonstrated by intraplantar injection of SO or PN, which results in the development of hyperalgesia (Ndengele *et al.,* 2008). Indecent increases in SO and PN in the spinal dorsal horn neurons, dorsal root ganglion (DRG), as well as the inflamed or traumatized neurons, have been noted in many studies (Ahlawat *et al.,* 2014; Castany *et al.,* 2016; Garg *et al.,* 2017).

Peripheral and central neuropathies result in an imbalance in the redox activities of the nervous system mediating the hypersensitivity of the neurons. Increased endoneurial Lipid Peroxidation (LPO) level, SO, and nitric oxide resulting in PN, as well as concurrent reduced antioxidant concentration, are shreds of evidence in many animal studies (Ndengele *et al.,* 2008; Kanyadhara *et al.,* 2014; Garg *et al.,* 2017; Liang *et al.,* 2017). Accumulation of ROS and RNS usually results in hyper-excitotoxicity of central and peripheral neurons that mediate peripheral and central sensitization. It was reported that the production of mitochondrial SO by intrathecal injection of inhibitors of the electron transport complex (i.e., antimycin A or rotenone) in mice leads to mechanical hyperalgesia (Kim *et al.,* 2008).

ROS activate a cascade of intracellular reaction such as the mitogen-activated protein kinase (MAPK) system and nuclear factor kappa-B-cell- light-chain-enhancer (NF-κB) to mediate painful neuropathies (Garg *et al.,* 2017). It also enhances excitatory neuro-transmission by modifying and activating transient receptor potential (TRP) channels (Sawada *et al.,* 2008; Nishio *et al.,* 2013; Jang *et al.,* 2018). It has been noted that NADPH-oxidase-derived SO production in dorsal root ganglion neurons, was essential for the development of thermal and mechanical hyperalgesia (Ibi *et al.,* 2008).

It has also been proposed that GABAergic transmission is vulnerable to increased production of ROS. Superoxide contributes to the presynaptic inhibition of GABA release in the hypothalamic paraventricular nucleus by angiotensin II (Chen and Pan, 2007), indicating the susceptibility of GABA neurons to oxidative stress. Yowtak and his colleague (Yowtak *et al.,* 2011) suggested that the increase of spinal ROS may cause a reduction of inhibitory transmission in the spinal dorsal horn by selective attenuation of GABAergic transmission. Resveratrol, an antioxidant found in red wine, imparted neuroprotective effects against kainate-induced excitotoxicity by selective attenuation of depleting synthesis of glutaminic acid decarboxylase enzyme (Virgili, 2000). This enzyme catalyzes the synthesis of GABA from

glutamine.

Nitric oxide (NO) is produced through nitric oxide synthase (NOS) enzymatic conversion of *L*-arginine to citrulline using NADPH, FMN, FAD, calmodulin, heme, and tetrahydrobiopterin as co-factors. Increase production of NO results in raise in the concentration of PN which is critically involved in the pathological process of neuropathic pain (Ahlawat *et al.,* 2014; Mukherjee *et al.,* 2014; Liang *et al.,* 2017). PN induced nitroxidative stress to the peripheral and central neurons that mediate hyperalgesia and allodynia. PN mediate central sensitization through the modulation of protein kinases, alterations in glutamatergic neurotransmission, neuroinflammation, and modulation of ion channels. These modifications occur at the peripheral, spinal, and supraspinal neurons (Lee *et al.,* 2007). In the spinal cord, nitroxidative species sensitized wide dynamic range neurons of the spinal dorsal horn neurons (Salvemini *et al.,* 2011). The roles for RNS in mediating neuropathy was supported using a variety of nonselective RNS scavengers such as phenyl-N-tert-butyl nitrone (PBN) and 4-hydroxy-2, 2, 6, 6-tetramethylpiperidine-1-oxyl (TEMPOL) to mitigate hyperalgesia and allodynia in a painful neuropathy model (Batinić-Haberle *et al.,* 2010).

A major mechanism involved in PN potentiation of central sensitization is via modulation of protein kinase (PK) activation. PKC is a known regulator of NADPH oxidase activity (Brennan *et al.,* 2009). PN increased autophosphorylation and activation of PKC (Hongpaisan *et al.,* 2004; Robles-Flores *et al.,*2008) as well as enhanced PKC-modulated persistent sodium currents (Lai *et al.,* 2006) which mediate central hypersensitivity. Activation of PKC can induce enhanced NR1-subunit of NMDA receptor phosphorylation that results in neurotoxicity. RNS activate PKA and calmodulin kinase (CaMK)-II to induce hyperalgesia. This was suggested in a study in which the antioxidant PBN reduced the levels of PKA-specific NMDAR phosphorylation and attenuated capsaicin-induced hyperalgesia (Gao *et al.,* 2007). The study of the decomposition of the PN showed that reduced PN prevent PGE_2-mediated

thermal hyperalgesia and potential downstream PKA activity after intraplantar SO administration (Ndengele *et al.*, 2008).

Indirectly, PN can potentiate increase production of ROS to mediate central sensitization further. PN is involved in the inactivation of mitochondrial manganese superoxide dismutase (MnSOD) to prevent the clearance of ROS (figure 14). This enzymatic deactivation involves nitration of Tyr-34 by PN in a manganese-catalyzed process (Salvemini *et al.*, 2011). Series of studies revealed that spinal nitration and inactivation of MnSOD provide a critical "feedforward" mechanism that allows for the accumulation of SO and PN during the development and maintenance of central sensitization (Schwartz *et al.*, 2008, 2009; Ndengele *et al.*, 2009).

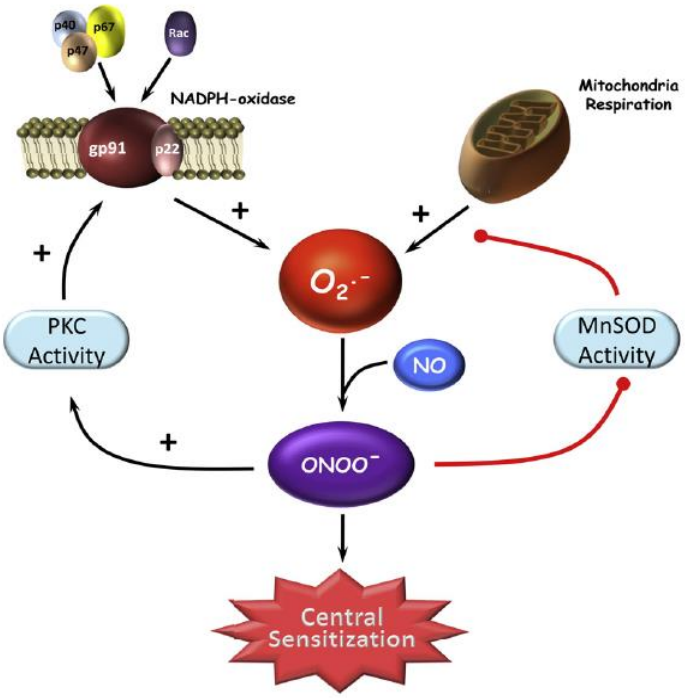

Fig 14: Peroxynitrite-reinforced superoxide production in central sensitization: two feedforward mechanisms. *Two significant sites of superoxide ($O^{\bullet}{}_2^-$) production, NADPH oxidase, and mitochondrial respiration are active in the development of central sensitization. Peroxynitrite ($ONOO^-$) formed from NADPH oxidase- and mitochondrial-derived superoxide nitrates and inactivates the manganese SOD (MnSOD) enzyme, preventing the removal of mitochondrial-derived superoxide. Peroxynitrite enhances protein kinase C (PKC) activity and, in turn, enhances the translocation of NADPH oxidase regulatory subunits to the membrane to increase the NADPH oxidase-derived superoxide production. These two mechanisms amplify superoxide-derived peroxynitrite formation leading to the development of central sensitization* (*Salvemini et al., 2011*).

Nitration of the glutamatergic system is a vital component of hypersensitivity (Watkins *et al.,* 2005). PN modify protein function via posttranslational nitration of tyrosine (Radi, 2004; Szabó *et al.,* 2007). Glutamate receptors (Zanelli *et al.,* 2002), ionotropic glutamate transporters (Salvemini *et al.,* 2011), and glutamine synthetase (GS) (Bidmon *et al.,* 2008; Görg *et al.,* 2010) are categories of protein that are modified in the presence of RNS (figure 15). Disruption in the biological functions of these proteins due to PN nitration result in critical consequences in events underlying central sensitization (Salvemini and Neumann, 2009; Little *et al.,* 2012). Irreversible nitration of the tyrosine residues of NR1 subunits of NMDAR by peroxynitrite (Zanelli *et al.,* 2002), sustain the potentiation of the synaptic currents and calcium influx. This results in an increased presynaptic release of excitatory neurotransmitters, overt activation of NMDARs, and ultimately excitotoxicity (Schmidtko *et al.,* 2009; Salvemini *et al.,* 2011; Ahlawat *et al.,* 2014).

Extracellular glutamate concentration needs to be kept low for quick termination of glutamate receptor activation to prevent glutamate excitotoxicity of the nociceptive neurons. Synaptic glutamates are uptake by the sodium-dependent high-affinity glutamate transporters (GTs) proteins in the plasma membranes of neurons and glia (Grewer *et al.,* 2014; Gegelashvili and Bjerrum, 2019). Nitration of the glutamate transporters (GTs) results in dysregulation in the homeostasis of extracellular glutamate that mediate central hypersensitivity (Salvemini *et al.,* 2011). Inadequate clearance of the synaptic glutamate induces neurotoxic-mediated hyperalgesia and allodynia. Studies have shown that PN-mediated nitration of GTs particularly excitatory amino acid channel 1 (EAAC1), that mediate 90% uptake of cysteine (involve in the production of glutathione reductase), in neurons reduces the uptake capacity of cysteine, leading to a depletion of intracellular glutathione and neuronal cell death (Aoyama *et al.,* 2008). This implicates that central sensitization may develop from a decrease in neuronal thiol redox state due to reduced intracellular glutathione reductase (GSH).

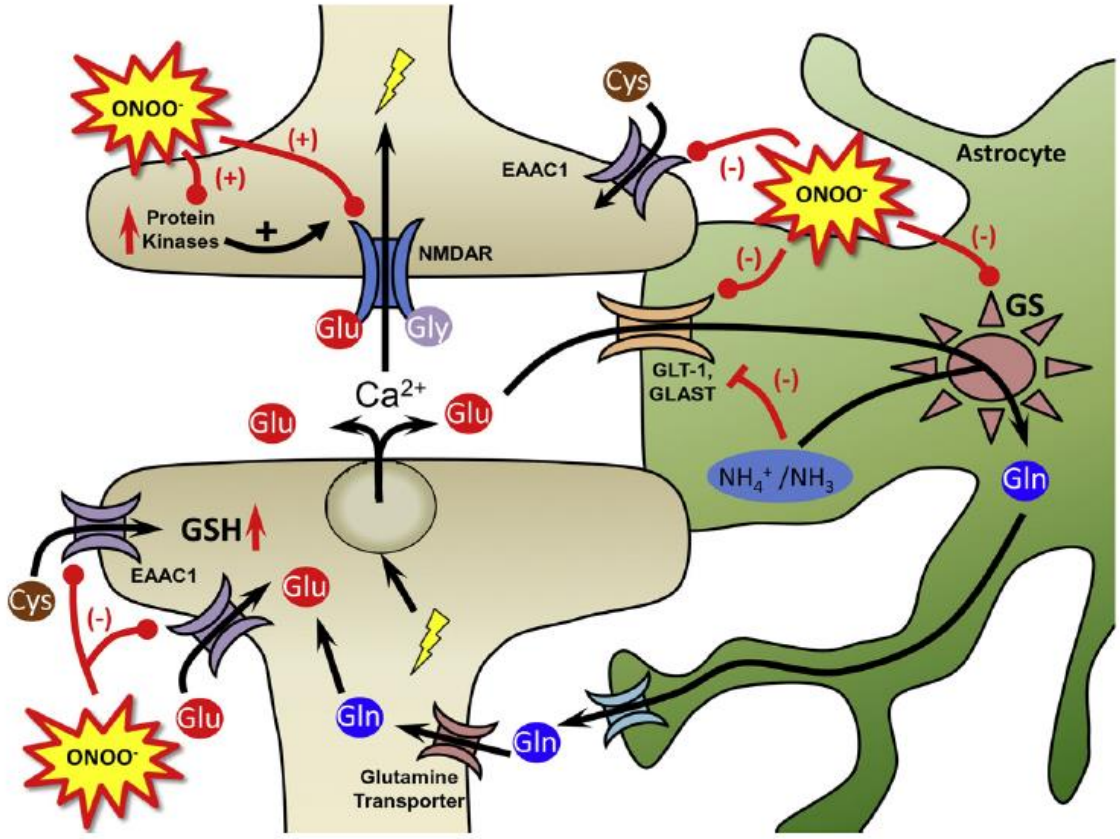

Fig 15: The role of peroxynitrite in glutamatergic homeostasis and signalling modulating central sensitisation. *EAAC1: Excitatory amino acid channel 1; Glu: Glutamate; Gly: Glycin; Gln: Glutamine; Cys: Cysteine; GLT-1: Glutamate transporter 1; GLAST: Glutamate Aspartate transporter; GS: Glutamine synthase (Salvemini et al., 2011)*

Nitroxidative species may regulate TRPV1 expression through growth factors, transcription factors, and MAPK kinases. Nerve growth factor, a well-described regulator of TRPV1 expression, acts through the tyrosine kinase receptor A and depends upon the Rac1/NADPH oxidase pathway-mediated activation of p38 MAPK to increase TRPV1 expression in spinal dorsal horn neurons and dorsal root ganglion cells (Suzukawa *et al.,* 2000). Numerous studies suggest that TRPV1 sensitization results in the production of nitroxidative species (Pall and Andason, 2004). Expression of TRPV1 has been documented to maintain peripheral neuron integrity and pain perception (Puntambekar *et al.,* 2005). TRPV1 receptors also contribute to the development of hyperalgesia and allodynia through activation by endogenous lipids, the oxidized linoleic acid metabolites (9- and 13-hydroxyoctadecadienoic acid), which act as TRPV1 agonists in the periphery and spinal cord (Patwardhan *et al.,* 2010).

Nuclear factor erythroid-derived-2-related factor (Nrf) protein, and Nuclear factor kappa-light-chain enhancer of activated B-cells (NF-kB) inhibitory regulation coordinates the redox status of a healthy cell (Catanzaro *et al.,* 2017; Sant *et al.,* 2017; Arruri *et al.,* 2017; Silva-Islas and Maldonado, 2018). NrF plays a significant role in the biosynthesis of innate antioxidant by binding with a specific region of DNA sequence, antioxidant response element (ARE), found in the promoter region of chemoprotective genes (Sant *et al.,* 2017). Under physiological conditions, NrF remains inactive in the cytoplasm as part of kelch-like ECH-associated protein -1 (keap -1)-NrF complex (Zhou *et al.,* 2020). The keap-1-NrF complex is fragmented into Keap-1 and NrF in the presence of RNS and ROS (figure 16). NrF translocates, bind with ARE, and regulate the transcription of target genes such as heme oxygenase-1 (HO-1), superoxide dismutase (SOD), GSH, and NAD(P)H: quinone oxidoreductase-1 (Raghunath *et al.,* 2018; Zhao *et al.,* 2018).

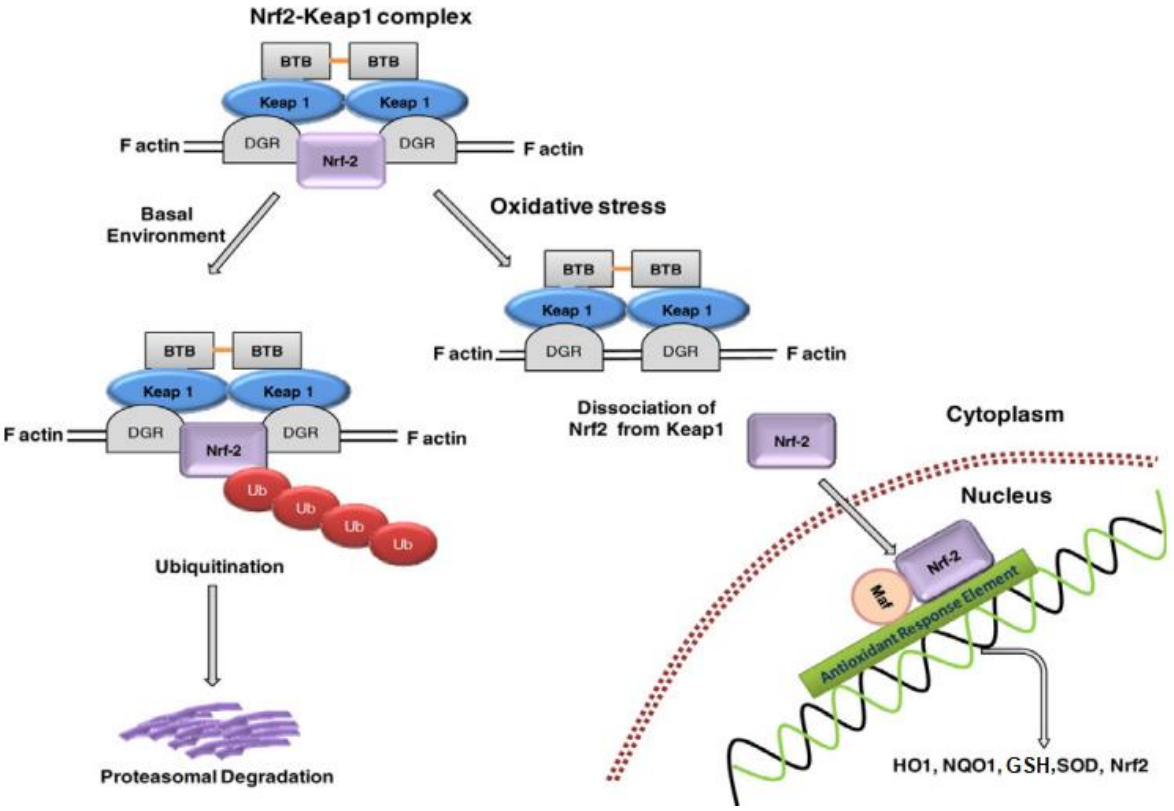

Fig 16: Interaction between Nrf2-Keap1 and Nrf2 activation by oxidative stress. *Keap1 acts as a negative regulator of the Nrf2 activation pathway and Nrf2 mediated antioxidant response. Under basal or unstimulated environment, two molecules of Keap1 keep Nrf2 sequestered in the cytoplasm. Keap1 molecules are dimerized through the BTB domain and anchored to the F-actin cytoskeleton through a Kelch (DGR) domain. Keap1 complex also recruits the ubiquitin machinery to Nrf2, leading to proteasomal degradation of Nrf2. However, an encounter with reactive oxygen species brings about a change in the conformation of Keap1, which results in the dissociation of Nrf2 from this inhibitory subunit. The free form of Nrf2, less prone to proteasomal degradation, translocates to the nucleus where it interacts with the antioxidant response element (ARE) to increase the expression of many antioxidant and detoxifying enzymes and proteins. BTB: broad complex, tram track, and bric-a-brac; DGR: double glycine repeat; Ub: ubiquitin; HO1: haemeoxygenase 1; NQO1: NADPH-quinone oxidoreductase 1; GSH: glutathione reductase; SOD: superoxide dismutase (Negi et al., 2011).*

Injury to the sensory neurons induces increases in the concentration of NF-kB and NrF leading to exaggerated oxidative stress and neuroinflammation (Negi *et al.,* 2011; Areti *et al.,* 2016; Komirishetty *et al.,* 2017; Arruri *et al.,* 2017). Pharmacological agents that stimulate production and activation of Nrf2 have been reported to induce the production of antioxidant which attenuate hyperalgesia and allodynia symptoms of NP (de Vries *et al.,* 2008; Arruri *et al.,* 2017; Zhou *et al.,* 2020).

2.9 COMORBIDITY OF PAIN

Anxiodepressive symptoms remain one of the most frequent emotional disturbances that are closely related to NP. Patients suffering from chronic neuropathic pain are at high risk of comorbid emotional deficits such as depression, which burdens healthcare (Kim *et al.,* 2012). Clinical and various models of experimental studies have demonstrated the development of mood disorder in painful neuropathic conditions (Zhuo *et al.,* 2016; Bravo *et al.,* 2020; Cherif *et al.,* 2020; Kremer *et al.,* 2020).

Anxiety and depressive symptoms increase the duration and intensity of pain experienced by NP patient, which influences their quality of life (Goyal *et al.,* 2014; Zhuo *et al.,* 2016). Studies have put the prevalence of anxiety disorder associated with NP at the ranges of 20 – 30% (Bravo *et al.,* 2020) while the prevalence of depressive symptoms was 65.6% (Cherif *et al.,* 2020). Alleviation of anxiodepressive symptoms and molecular mechanisms underlying the supraspinal structures has suggested being a standard optimal procedure in NP management (Alvarado *et al.,* 2013; Mutso *et al.,* 2014).

Many supraspinal structures such as the thalamus, amygdala, medial prefrontal cortex, insular cortex, nucleus accumbens, and hippocampus are involved in the central regulation of chronic pain (Bushnell *et al.,* 2013; Tajerian *et al.,* 2014; Zhuo *et al.,* 2016). Interconnection and

activities of these cortico-limbic structures play vital roles in emotional behaviours. Overlapping neurobiological processing, as well as morphological changes, results in deterioration of emotional behaviours associated with NP. Morphological changes in the medial prefrontal cortex (Baliki *et al.*,2008; Ong, 2019), anterior cingulate cortex (Obermann *et al.,* 2013), hippocampus (Mutso *et al.,* 2014); amygdala (Liu *et al.,* 2014) and thalamus mediate emotional disturbance in NP.

The amygdaloid complex is well-known to be relevant to fear learning, anxiety, and pain (Tye *et al.,* 2011; Li *et al.,* 2013). The central nucleus of the amygdala (CeA), referred to as the 'nociceptive amygdala', serves as the central output nucleus for amygdala functions (Neugebauer *et al.,* 2004; Zhou *et al.,* 2019). Highly processed, polymodal information reaches the CeA from the thalamus, cortical areas, and brainstem (Han *et al.,* 2015, Zhou *et al.,* 2019). Furthermore, the CeA forms widespread connections with forebrain areas and the brainstem, which have been implicated in mediating fear and mood disorders (Penzo *et al.,* 2015; Zhou *et al.,* 2019). These studies suggest that the CeA is a possible convergent point of chronic pain and depression.

Biochemical imbalances in the cortico-limbic system play pivotal roles in comorbid anxiodepressive systems. Functional reorganization of the processing in the hippocampal neurons and between the hippocampus and cortex was deeply involved in the emotional disturbances (Mutso *et al*., 2014). Likewise, pre- and post-synaptic long term plasticity in the anterior cingulate cortex were linked positively between pain and anxiodepressive disorder. Long term potentiation in the presynaptic neuron in the anterior cingulate cortex mediated mood disorder trigger by NP (Zhuo, 2016). Increases in the calcium ion influx via NMDA receptors into the post-synaptic neurons explain one of the mechanisms through which long term potentiation mediated negative emotional behaviour (Tsuda *et al.,* 2017).

Dysfunction in the serotoninergic (Martin *et al.,* 2017; Zhou *et al.,* 2019) and glutamatergic (Wang *et al.,* 2015) systems have also been implicated in the development of anxiodepressive disorder. Depletion in the hippocampal serotonin and dysregulation in the expression of glutamate receptors have suggested their vital roles in the emotional disturbances associated with NP. These are thought to results in pronounced anxiety, reduced reward, and enhanced-learned helplessness (Zhou *et al.,* 2008; Wang *et al.,* 2015).

Studies on experimental animals have revealed that emotional disorders in NP can result from the development of neuroinflammation. The elevated release of pro-inflammatory cytokines, chemokines, and prostaglandins by the glia and macrophage cells mediated emotional imbalance (Walker *et al.,* 2013; Stuart and Baune, 2014; Torta *et al.,* 2017). A study by Gui and his colleagues noted that IL-1β plays a crucial role in the development of comorbid emotional symptoms (Gui *et al.,* 2016). Treatment of NP-induced *Wistar* rats with IL-1β neutralizing antibody or deletion of IL-1 receptor type-1 in mice alleviates emotional disorder (Gui *et al.,* 2016).

2.10 NEUROPATHIC PAIN MODELLING

For many centuries, researchers have developed several models for neuropathic pain to evaluate different neuropathic pain etiologies and manifestations. Continuous strives towards an understanding of the mechanism that mediated neuropathic pain (NP) result in models that elucidate peripheral mechanisms and central mechanisms (Jaggi *et al.,* 2011). Human neuropathy can be mimicked by various established rodent models of peripheral and central neuropathic pain. This comprises spinal nerve ligation (SNL), partial nerve injury, chronic constriction injury (CCI), and spared nerve injury (SNI). Peripheral animal models are more frequently used than central neuropathy models since, in patients, peripheral neuropathies are also more frequent than central neuropathic pain states (Niederberger *et al.,* 2008). A good

model of neuropathic pain is described as one that produces sensory deficits only (Sousa *et al.,* 2016). The hallmarked behavioural characteristics in NP include allodynia, hyperalgesia, and spontaneous pain.

2.10.1 Axotomy:

This is an extensive injury in which the sciatic nerve is wholly transected in the medial region of the thigh. This model of neuropathic pain involves anaesthetizing the animal, exposing the common sciatic nerve before trifurcation, and removal of about 5mm of the nerve. During the procedure, the saphenous nerve has been damaged as well. The animal develops painful anaesthesia and autonomy (self-mutilation) symptoms. Due to the denervation of a limb that encompasses this model, it is nearly impossible to evaluate pain-related behaviour mainly because of motor dysfunction. Hence other models were developed based on the various degree of sciatic nerve injury to address these challenges (Jigga *et al.,* 2011).

2.10.2 Chronic sciatic nerve constriction:

Chronic constriction injury is a model that was developed by Bennett and his colleague (Bennett and Xie, 1988). This model involves placing three to four loose ligatures on the common sciatic nerve causing swelling and constriction of the nerve at the site of ligation (figure 17) (Sousa *et al.,* 2016). Constriction resulting majorly from oedema reduces the diameter of the sciatic nerve up to 75% (Siddall *et al.,* 2003). Oedema is a result of an immune reaction triggered by suture thread and compression, in addition to axotomy and Wallerian degeneration. Evidence from the histological examination shows massive loss of myelinated Aα and Aβ fibres as well as Aδ to some lesser degree (Munger *et al.,* 1992). Likewise, studies from electrophysiology revealed that almost 90% loss of myelinated and 30% of unmyelinated fibres were lost within 14 days (Coggeshall *et al.,* 1993). Injury of the sensory fibres results in neuropathic pain symptoms (Catheline *et al.,* 1999). Ventroflexed toes display of the limb is probably due to injury to motor fibres. This model of neuropathic pain is similar to that of

complex regional pain syndrome in humans. The disadvantage of this model is the variable degree of nerve injury as it is nearly impossible to inflate the same degree of constriction in each animal.

2.10.3 Partial sciatic nerve ligation (Seltzer's model): This is a model of neuropathic pain that involved ligation of the dorsal third (or half) of the sciatic nerve. It is a model that mimics causalgia symptoms after nerve trauma (Seltzer *et al.,* 1990; Shir and Seltzer, 1990). Animal display protection behaviour, licking of the affected limb, mechanical and thermal hyperesthesia. Variability of the degree of nerve injury between animals, producing different extent of injured is the principal disadvantage of this model.

2.10.4 Spinal roots ligation (SNL): This model negates the variability of the degree of nerve injury that is associated with previously discussed models. Kim & Chung developed the model in 1992 (Kim and Chung, 1992). In this model, only spinal L5 and L6 branches of the sciatic nerve are ligated (figure 17) so that the corresponding DRGs and spinal cord segments reflect changes related to nerve injury. Uninjured L4 branch and its DRG help to identify how the nerve injury influences surrounding intact nerves. The development of pain-related behaviour is observed 1-2 days post-injury as tactile and thermal allodynia (Ossipov *et al.,* 1999; Chaplan *et al.,* 1994) which persist up to 10 weeks. It may be considered an animal model of pain mediated by sympathetic since sympathetic block relieves hyperalgesia.

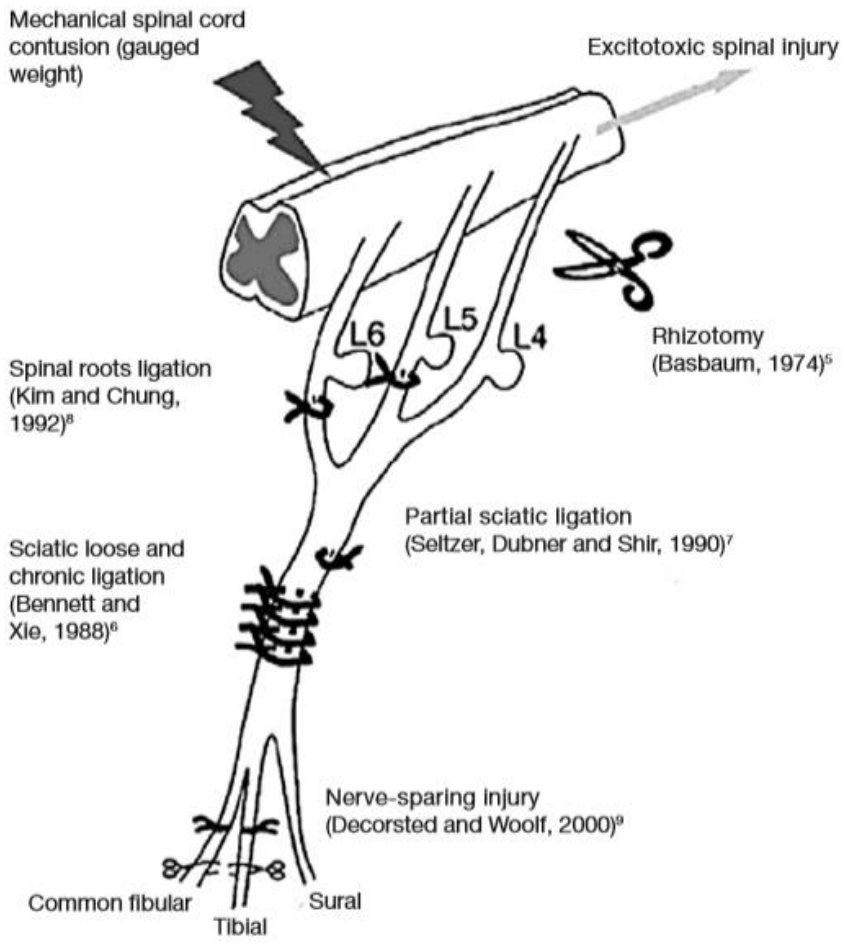

Fig 17: Illustration of various peripheral ligating loci for inducement of neuropathic pain (Sousa et al., 2016)

2.10.5 Spared nerve injury (SNL): Another way of modelling neuropathic pain is by ligating the tibial and common peroneal branches of the sciatic nerve (Decosterd and Woolf, 2000). In this model, the sural nerve is spared out. This technique produces increased responses to nociceptive and non-nociceptive stimuli in the territory of the ipsilateral sural nerve and a lesser extent, in the territory of the saphenous nerve. Pain induced by this model is independent of the sympathetic nervous system. It allows the comparison of thermal and mechanical sensitivity of non-injured territories close to injured areas. This makes it possible to assess the contribution of injured primary sensory afferent neurons to hypersensitivity. Mechanical and thermal hyperalgesia are said to be present on the fourth post-surgical days and persist for several weeks, up to six months (Boyette-Davis *et al.,* 2015).

2.10.6 Central Injury Modelling of Neuropathic Pain

Central neuropathy can be induced in animals to model some of the features that are associated with spinal cord injuries in humans. This involves producing spinal cord injury that results in the development of neuropathic pain. The prevalence of centre neuropathy is about 70-80% (Ravenscroft *et al.,* 1999). Neuropathic pain that results from spinal cord injuries is classified as at-level, or below-level pain with an incidence of about 30-40% (Siddall *et al.,* 2003). Below-level pain is a severe pain that develops in months or years after the initial injury, while at-level pain is a pain that probably arises from the spinal cord above the injury site. Several models of central neuropathy include contusion (Hulsebosch *et al.,* 2000; Lindsey *et al.,* 2000), irradiation (Xu *et al.,* 1992), and excitotoxicity (Yezierski and Park, 1993). Although the mechanism is different, the presence of spinal cord injuries pain in these models manifest within a couple of days and persists for several weeks to months. An electrophysiological study showed an increased background activity of spinal cord neurons, increased responsiveness to peripheral stimuli, and prolonged after discharges (Hains *et al.,* 2003).

2.10.7 Contusions Injury Model:

This is a technique that model spinal cord injuries in humans as a result of fracture/luxations induced by compressive contusions which is more relevant to evaluate clinical phenomena. It is also called the weight-drop model, which is the oldest and most widely used spinal cord injury model. As described by Allen (Allen, 1911), spinal cord injury is produced by dropping a weight on the surgically exposed spinal dorsal surface at the lower thoracic-lumbar level (Sousa *et al.,* 2016). This results in paraplegia and complete segmental necrosis. Some modifications have been made to this technique to give better control of the degree of spinal cord injury (Hulsebosch *et al.,* 2000).

2.10.8 Excitotoxic Spinal Cord Injury Model:

This is a model of neuropathic pain that is based on making significant neurochemical changes in the spinal cord injury. Intraspinal injection of some neurochemicals (quisqualic acid, AMPA-metabotropic receptor agonist) produces abnormal pain mimicking spinal cord injury pain in humans (Yezierski *et al.,* 1998; Yezierski and Park, 1993). The excitotoxic spinal cord injury model also includes the following:

Diabetic neuropathy (Streptozocin-induced neuropathy (Akbarzadeh *et al.,* 2007), chemotherapy-induced neuropathy (Vincristine), Platinum Platinum-derived products (oxaliplatin, cisplatin, and carboplatin are agents are inhibiting DNA synthesis and replication (Cavaletti *et al.,* 2002), cancer-induced neuropathies (Wacnik *et al.,* 2003), alcohol-induced neuropathy, pyridoxine-induced, trigeminal neuralgia, and Orofacial pain.

2.11 BROMELAIN

Bromelain is the collective name for a group of closely related cysteine endopeptidase proteolytic enzymes found in the tissue of the plant family of *Bromeliaceae*, of which pineapple, *Ananas comosus*, is the best known (Benucci *et al.,* 2011). Pineapple is a native of

South America, but it is now widely grown in various parts of the world including Africa. In Africa, Nigeria was ranked as the eighth largest producer of pineapple (Babagana, 2015).

Bromelain has been known chemically since the year 1876 (Tochi *et al.,* 2008). In the year 1891, bromelain was first identified by Marcano (Upadhyay *et al.,* 2010) and subsequently isolated in the year 1894 (Neta *et al.,* 2012). The biochemical constituents of bromelain include endopeptidases (ananian, comosain), phosphatases, glucosidases, peroxidases, escharase, cellulases, glycoproteins, proteinase inhibitors (cystatin), organically-bound calcium, and carbohydrates (Nadzirah *et al.,* 2013; de Lencastre Novaes *et al.,* 2016).

In the pineapple plant, bromelain is accumulated in the whole part with different extent and properties depending on its source. The two sources of bromelain are the fruit: fruit bromelain; and the stem: stem bromelain. The primary enzyme fraction found in the pineapple fruit is named 'fruit bromelain' (FBM) tag: EC3.4.22.33. Aspartate is the most common amino acid that is found in FBM. It has a molecular weight of 24.5-32 KDa with an optimum pH of 3-8 (Manzoor *et* al., 2016).

Stem bromelain is tag EC3.4.22.32 which is a family of sulfhydryl (cysteine) proteinases, is the major endopeptidase present in extracts of the plant stem. It is a group of enzymes that depends on the thiol group (SH) of a cysteine residua for its activities. It contains a glycoprotein with one oligosaccharide moiety per molecule that is covalently bound to the peptide chain (Mahmood and Sleemuddin, 2007). It contains 285 amino acids with alanine, and glycine has been the highest and histidine and methionine the lowest (Arshad *et al.*, 2014). Stem bromelain has a molecular weight of 23.4 – 35.73 KDa and optimum pH of 6.5 – 8 (Arshad *et al.,* 2014; Benucci, 2011). Its activities are no longer susceptible to pH once it is combined with the substrate (Gautam *et al.*, 2010). Bromelain is represented structurally in figure 18.

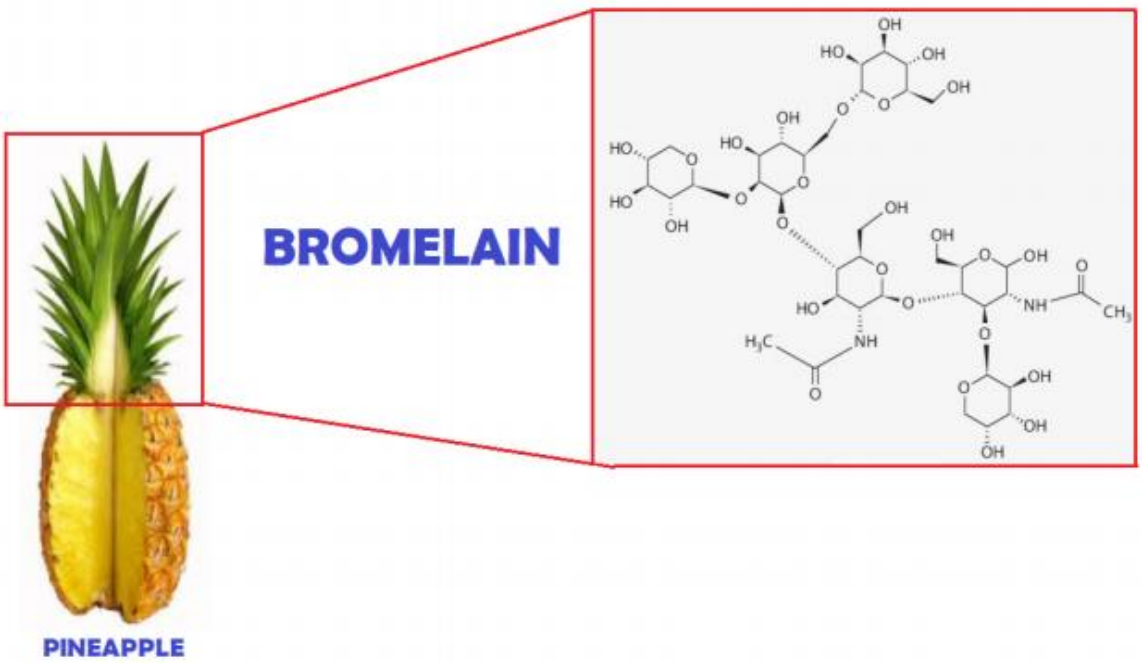

Figure 18: Molecular structure of bromelain from pineapple stem

Commercially, bromelain is extracted in large quantity from the stem. It is well absorbed in the intestine after oral administration (Pavan *et al.,* 2012). In humans, it has been reported to be absorbed from the intestine without degradation and without losing its biological activities (Agarwal *et al.,* 2016). *Cysteine* proteases, in general, are involved in a myriad of homeostatic as well as pathological processes in physiological conditions (Manoury *et al.,* 2011). Bromelain can be activated by calcium chloride, cysteine, bisulphate salt, NaCN, H_2S, Na_2S, and benzoate. However, bromelain is usually sufficiently active without the addition of activators. Bromelain is inhibited by Hg^{++}, Ag^{+}, Cu^{++}, antitrypsin, estatin A, and B, iodoacetate (Gautam *et al.,* 2010).

2.12 APPLICATIONS OF BROMELAIN

Bromelain is well absorbed after oral application, and it has no negative impact on health after prolonged use (Bhattacharyya, 2008). As a result of this, bromelain has been widely applied both industrially and clinically for various purposes (figure 19). Bromelain has been exploited in various industrial and food industries as well as in medicine (Grzonka *et al.,* 2007). Industrially, it has been used as tenderization, food drinks, detergents, baking cookies, and as textile materials (Arshad *et al.,* 2014). Folk medicines have used bromelain for the treatment of digestive disorder and skin diseases (Golezar *et al.,* 2016). Furthermore, it has been used for solubilization of grain proteins, stabilization of beer, production of protein hydrolyzates, softening skins in leather (Ketnawa *et al.,* 2009).

Therapeutically, bromelain is utilized to modulate tumour growth, blood coagulation, antibiotic activities, antiviral effects, and anti-inflammatory (Nadzirah *et al.,* 2013; Ali *et al.,* 2015; Manzoor *et al.,* 2016; Abdulmuhammed *et al.,* 2017). It has also been reported to speed up the healing of wounds, burns, and ease out pain (Golezar *et al.,* 2016; Benucci *et al.,* 2011). Clinical studies have shown that bromelain may help in the treatment of several disorders such as: relieve the symptoms of sinusitis, relieve joint injuries, stimulate immune function, increase immune system hormones, reduce some side effects of cancer therapy and possibly some signs

of cancer itself, treat diarrhoea related to E. coli infections, speed recovery time, remove damaged tissue from burns without surgery, and aid digestion (Manzoor *et al.,* 2016; Golezar *et al.,* 2016; Abdulmuhammed *et al.,* 2017). It reduces pityriasis *lichenoides chronica* skin diseases (Massimiliano *et al.,* 2007).

Bromelain is as good as diclofenac on the standard pain assessment scale and possibly better than many drugs used in treating acute pain. It has good efficacy, effective and safe alternative to NSAIDs (indomethacin, paracetamol, and ibuprofen) in the treatment of painful osteoarthritis (Akhtar *et al.*, 2004). Walker and his colleagues (Walker *et al.,* 2002) reported bromelain to be dose-dependent in its effectiveness for subsiding mild knee pain. These findings submitted that bromelain might provide safe, effective relief from acute and chronic joint pain. Furthermore, bromelain is effective in attenuating muscular pain, episiotomy pain, and perineal pain (Ezeome and Aghaji, 2005; Golezar, 2016). Majid and Al-mashhadani (2014), also reported that bromelain improves life quality and subsides pain measures after mandibular third molar surgery.

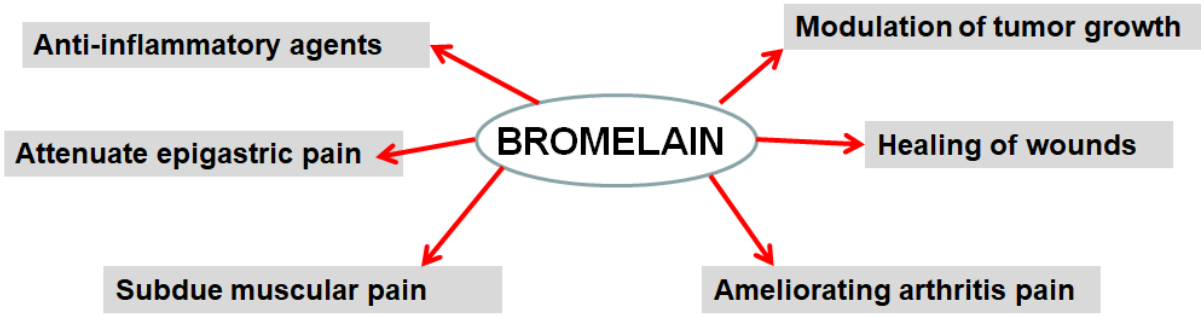

Fig 19: Therapeutic effects of Bromelain

The therapeutic effect of bromelain has been attributed to its direct effect on inflammatory mediators such as cytokines, bradykinin, and prostaglandin E2 (Majid and Al-mashhadani, 2014). It has been documented that bromelain inhibits the release of interleukin (IL)-8, IL-1β, IL-6, interferon (INF)- γ, and TNF-α from the neutrophil and already activated immune cells under the condition of inflammatory-induced overproduction of cytokines (Hale *et al*., 2005; Onken *et al.,* 2008; Rathnavelu *et al.,* 2016). It was suggested that bromelain decreases transient rolling and adhesion of the leukocytes to the blood vessel at the inflammation site (Fitzhugh *et al.,* 2008). CD-62L/L-selectin, which mediate the rolling and CD-128 that regulate firm adhesion of the leukocytes is possible down-regulated by the effects of bromelain (Fitzhugh *et al.,* 2008).

Bromelain decreases free cytokine NF-κB concentration that promotes the synthesis of proinflammatory cytokines (Rathnavelu *et al.,* 2016; Habashi *et al.,* 2017). It also inhibits p38mitogen-activated protein kinase (MAPK), extracellular signal-regulated protein kinase (EPK-1/2), and COX-2 (Bhui *et al.,* 2009; 2012), hence it reduces prostaglandin-E_2 concentration (Manzoor *et al*., 2016; Rathnavelu *et al.,* 2016; Abdulmuhammed *et al.,* 2017). Its anti-inflammatory effects are further mediated by inhibiting CD-44 and iNOS level (Manzoor *et al.,* 2016; Habashi *et al.,* 2017)

However, it is important to note that bromelain has a bidirectional modulatory role on proinflammatory cytokines depending on the context. In response to infection (microbial or viral), bromelain increases in the release of IL-1β, IL-6, interferon (INF)- γ, and TNF-α from the macrophages and peripheral blood mononuclear cells (Barth *et al.,* 2005; Rathnavelu *et al.,* 2016). Bromelain enhances the releases of IFN-γ-mediated nitric oxide (NO) and TNF-α by the macrophages and natural killer cells (Habashi *et al.,* 2017).

Stimulatory effects of bromelain on CD4+ mediated its antiviral effect. It has been reported that HIV patients treated with bromelain shows increased CD4+ counts after four months

(Pandjaitan *et al*., 2014). Bromelain activates type-1 helper T (TH_1)-cells that increase the production of IFN-γ, which plays pivotal roles in defending the body from viral aggressive devastating effects (Suthihono et al., 2011). Bromelain's mode of action as an analgesic agent was said to be multifaceted and thought to be both as a secondary effect of reducing pain-inducing factors, such as oedema, debris, and immune complexes (Klein and Kullich, 2000) and through a direct influence on pain mediators, such as bradykinin (Walker *et al.,* 2002). It also increases the release of an endogenous encephalin-related peptide to mediates its analgesic effects (Orlandi-Mattos *et al.,* 2019).

2.13 DRUG INTERACTIONS

Bromelain has been documented to increase blood and urine levels of some antibiotics in humans. Because of its anti-platelet aggregation (Gläser and Hilberg, 2006) and fibrinolytic effects, bromelain may theoretically potentiate the development of blood thinners. However, it has historically been used pre-surgery to speed healing (AMR, 2010).

2.14 SIDE EFFECTS AND TOXICITY OF BROMELAIN

Bromelain is considered to be generally safe with rare reports of nausea, vomiting, allergic reaction, and menstrual bleeding (Worm *et al.,* 2015). It has very low toxicity, with an LD50 greater than 10 g/kg. Toxicity tests on dogs, with increasing levels of bromelain up to 750 mg/kg administered daily, showed no toxic effects after six months (AMR, 2010). Dosages of 1.5 g/kg/day administered to rats showed no carcinogenic or teratogenic effects. In human clinical tests, side effects are generally not observed; however, caution is advised if administering bromelain to individuals with hypertension, since one report indicated individuals with pre-existing hypertension might experience tachycardia following high doses of bromelain (AMR, 2010).

Anti-bromelain antibody titers (IgG) have been detected in both serum and stool after long-term oral therapy in mice. Repeated exposure is required for the development of anti-bromelain

antibodies. The proteolytic activity appears to be a prerequisite for this response. These results indicate that bromelain can trigger systemic and mucosal immunoglobulin responses; however, the clinical relevance of this has yet to be determined (Hale, 2004; Hale *et al.*, 2006). Bromelain, as well as other proteolytic enzymes, can cause IgE-mediated respiratory allergies of both the immediate type and the late-phase of immediate type. Information regarding safety in pregnancy and lactation is lacking (AMR, 2010).

CHAPTER THREE

RESEARCH METHODOLOGY

3.1 Materials

3.1.1 Drugs:

Bromelain powder 3,000 gelatin digesting units per gram was obtained from Maple Lifesciences (Subhash Nagar, India). Gabapentin was a product of Teva UK limited (Eastbourne, UK). Ketamine Hydrochloride injection was a product of Laborate pharmaceutical (India).

3.1.2 Reagents:

Prostaglandin E_2 ELISA kits (Cloud-Clone Corp, Texas, USA), Glutamate assay kits (Abcam, Cambridge, USA), IL-1 Beta ELISA kits (Abcam, Cambridge, USA), IL-6 ELISA kits (Abcam, Cambridge, USA), TNF Alpha (Abcam, Cambridge, USA), Protein assay kits (Randox Laboratories Ltd., UK), NF-kB ELISA kits (Cayman chemical, USA), NrF-1 ELISA kits (Cayman chemical, USA), NrF-2 ELISA kits (Cayman chemical, USA), Chloride assay kits (Teco Diagnostic, USA), Potassium assay kits (Teco Diagnostic, USA), Calcium assay kits (Teco Diagnostic, USA), Sodium assay kits (Teco Diagnostic, USA), Na^+-K^+ ATPase assay kits (Teco Diagnostic, USA), Ca^{2+}ATPase assay kits (Teco Diagnostic, USA), primary Rabbit anti-iNOS (Bio-Science, USA), secondary goat anti-rabbit (Bio-Science, USA), Phospate buffer tablet (Sigms Aldrich, USA), Griess reagent kits (Molecular Probe Inc., USA), Ellman reagent kits (Molecular Probe Inc., USA), Thiobarbituric acid assay kits (Biocombiotect Nigeria LTD), Superoxide dismutase assay kits (Biocombiotect Nigeria LTD), Sodium phosphate dibasic (Loba Chemie Pvt., India), Sodium phosphate monobasic (Loba Chemie Pvt., India), Sucrose (Loba Chemie Pvt., India), Formaldehyde solution (ACS reagent, Germany), Tris (hydroxymethyl) aminomethane (RGT reagent, London, UK).

3.2 Ethical Approval

This research work complies with the University of Ilorin ethical guidelines for the Care and Use of Laboratory Animals which is in line with the international ethical guidelines. Ethical clearance was obtained from the University of Ilorin Ethical Review Committee (UERC/ASN/2017/936), University of Ilorin, Nigeria.

3.3 Study Setting

This research was conducted at the Physiology Laboratory, Physiology Department, Faculty of Basic Medical Sciences, University of Ilorin.

3.4 Experimental Animal

3.4.1 Animal Choice:

Male *Wistar* rats were used for these studies. The use of rats for pain study has been known for many decades ago, mainly because of the subjective nature of the measurement of the behavioural parameters. Animal models of neuropathic pain are mostly based on peripheral nerve injury for reproducibility and simplicity. Male rats were preferred since they do not undergo a menstrual cycle that may cause hormonal fluctuations which may influence the behaviours of the rats during the experiment.

3.4.2 Study Population:

The research work comprises of two study groups. Sixty-four (64) randomly selected male *Wistar* rats were used for each study group based on power analysis. The average weight of the *Wistar* rats that were used for the study was 160 g ± 10 g.

3.4.3 Study Design:

The first phase of the study focused on the antinociceptive effect of bromelain and its mechanism of actions. Various pain-related behavioural paradigms were used, and antioxidant, neuronal ionic regulation, nuclear transcription factors, and some anti-inflammatory effects of bromelain were investigated. The second phase of the study focused on the anti-comorbid

effect of bromelain using anxiety-like and depression-like paradigms. Histological examination, excitatory neurotransmitters, as well as some anti-inflammatory effect of bromelain was investigated.

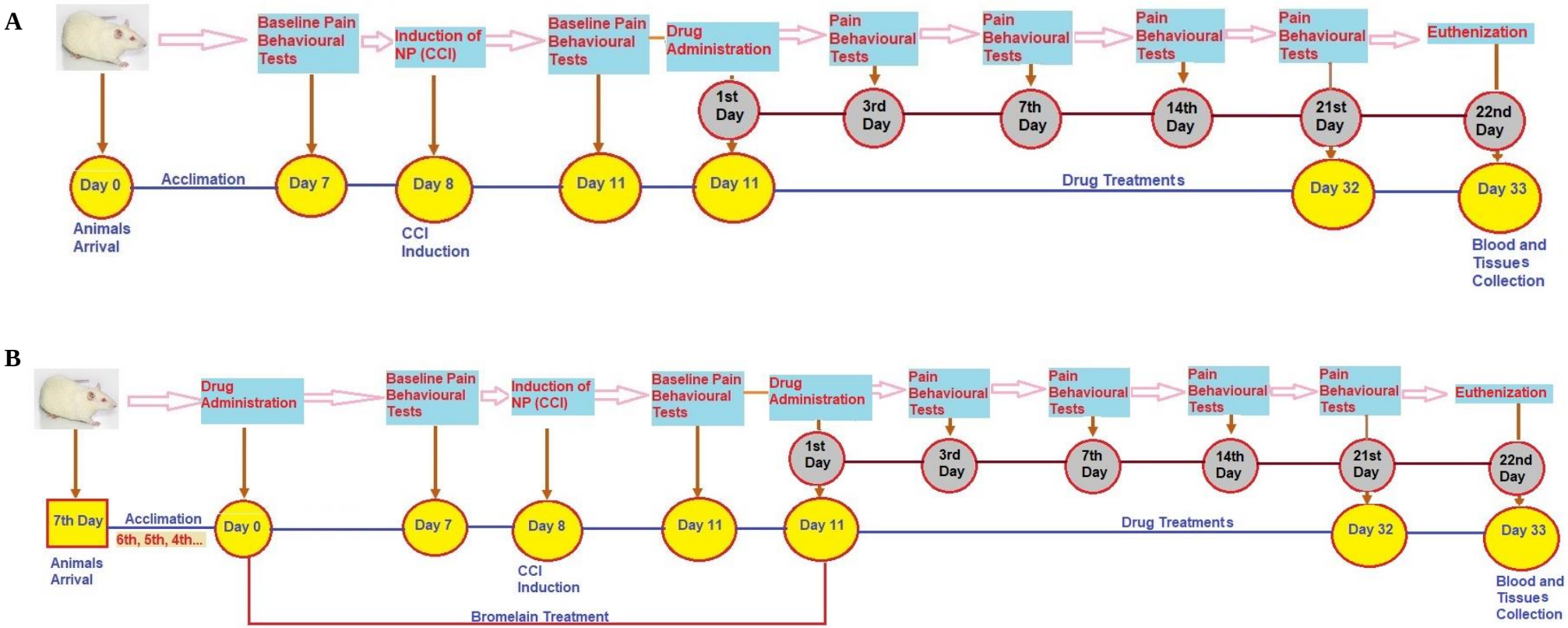

Figure 20: Schematic representation of drug treatments and behavioural tests schedule in Phase one Study: Anti-noociceptive effect of bromelain. (A). Post-treatment schedule. (B). Pre-treatment schedule

3.4.4 Animal Source and Acclimatization:

Male *Wistar* rats bred at Central Animal House, Osun State University, Nigeria, were used for the study. The rats were housed in the animal facilities of the Faculty of Basic Medical Sciences, University of Ilorin, Nigeria, under standard conditions of natural day and light cycle with free access to food and water. They were acclimatized to the animal facility for ten days before the commencement of the experiments.

3.4.5 Animal Grouping and Treatment:

The rats used for the study were divided into eight (8) groups for each study group. Each group consists of eight randomly selected male *Wistar* rats. Bromelain, normal saline or Gabapentin were administered orally to the *Wistar* rats for twenty-one (21) consecutive days. Animals were grouped as follows:

Group A (Unligated Control): 10ml/Kg of normal saline

Group B (Sham Control): 10ml/Kg of normal saline

Group C (Ligated control): 10ml/Kg of normal saline

Group D (Ligated reference control): 30mg/kg of Gabapentin drug

Group E (Ligated Test 1): Low dose of bromelain (30 mg/kg/d)

Group F (Ligated Test 2): High dose of bromelain (50 mg/kg/d)

Group G (Ligated pretreated Test 3): Low dose of bromelain (30 mg/kg/d). Animals in this group received bromelain for seven consecutive days before ligation

Group H (Ligated pretreated Test 4): High dose of bromelain (50 mg/kg/d). Animals in this group received bromelain for seven consecutive days before ligation.

Animals were treated for twenty-one (21) consecutive days after the development of neuropathic pain symptoms. Bromelain and gabapentin were dissolved in normal saline (0.9% NaCl) and administered orally (figure 20), while Ketamine hydrochloride was given via

intraperitoneal route. The dosages were carefully selected based on literature reports (Sudjarwo, 2005; Kilic *et al.,* 2012).

3.5 Neuropathic Pain Induction: Sciatic Nerve Ligation

Chronic constriction injury (CCI) model of peripheral neuropathic pain was adopted in this study by slightly modifying the method described by Jiang and his Colleague (Jiang *et al.,* 2018). Chronic constriction of the sciatic nerve just before the trifurcation at the mid-thigh level without disrupting the epineural blood supply leads to the required nerve injury. In brief, male *Wistar* rats were deeply anaesthetized with ketamine hydrochloride (100 mg/kg, intraperitoneal injection [i.p.]). The hairs on the lower back and thigh of the rats were shaved. The skin of the lateral surface of the right thigh was incised, and a cut was made bluntly through the biceps. The sciatic nerve was then exposed and free from adhered tissue (figure 21). Four ligatures (silk sutures size 4–0) were placed loosely around the sciatic nerve before the trifurcation without disrupting the epineural blood supply. The intensity of the constriction was carefully guage by ensuring that ligature was not too loose nor tight. This was done by terminating the constriction when a small degree of constriction is visually observed in gastrocnemius muscle. After performing the ligation, the muscular and skin layers were sutured in layers with catgut and suturing thread, respectively. Sham operation was performed similarly to the procedure described above except for placing suture silk around the sciatic nerve. Penicillin powder was then applied on the surface of sutured skin to prevent infection by the microbes. Animals were then placed in a dry cage, rested on the opposite side of the body, to recover before returning it to its cage with an adequate supply of food and water. Animals that showed neurological symptoms (seizure), or loss 30% of weight after surgical ligation were excluded from the experiment, and the humane endpoint was executed to them.

Fig 21: Exposition, ligature, and suturing of the skin during the induction of neuropathic pain procedure.

3.6 BEHAVIORAL TEST

3.6.1 Pain Behavioural Paradigms: Baseline Test:

Presurgical Test: All the *Wistar* rats were assessed on behavioural pain paradigms before the induction of neuropathic pain. Animals were familiarized with the experimental room as well as all the equipment used for the study to minimize the effect of anxiety on the study before the conduction of the behavioural tests. Animals that showed signs of hyperalgesia and allodynia were excluded from the study and replaced.

Validatory Test: Following the surgical induction of peripheral neuropathy, animals are expected to developed spontaneous pain within three to seven days. Animal behavioural characters such as guarding behaviour of the ipsilateral hind paw and licking of the suture area were observed. Post induction pain behavioural test before the commencement of treatment was conducted three days after CCI procedure. Ligated animals that do not show symptoms of neuropathic pain were not treated until these behavioural signs were observed or were excluded from the study and replaced.

3.6.2 Measurement of the Bodyweight

Wistar rats were weighed and recorded with digital weighting balance in every three days. Briefly, after zero the weight of rat restrictor on the weighing scale, each rat was placed on the surface of the weighing scale and digitally displayed rat weigh taken when the rat has calmed down. The weight of the rats was taken before the induction of neuropathic pain as well as during the treatment period. The percentage change in weight was calculated as

$$\% \text{ change} = \frac{Change\ in\ weight}{initial\ weight} x\ 100$$

3.6.3 Mechanical Allodynia Test:

von Frey filaments (Cat No: 37450-275; Ugo Basile, Gemonio, Italy, figure 22) were used to assess mechanical hypersensitivity by measuring the paw withdrawal threshold to the applied stimuli as described by Zhu *et al.,* (Zhu *et al.*, 2012). A series of von Frey filaments were used in a sequential order that has approximately equal logarithmic incremental bending forces (equivalent to 1, 1.4, 2, 4, 6, 8, 10, 15, 26, 60, and 100 g forces, respectively). The rats were placed in a transparent perspex box (figure 23) with a wire mesh floor for at least 15 minutes of habituation time before the behavioural test. The filaments were applied to the plantar surface of the hind paw for approximately 5 seconds of considerable force (figure 24) in ascending order. The behaviour of each rat was observed for each applied filament force. The mechanical threshold was defined as the minimal force that caused at least three consecutive withdrawals, sudden flinching, or paw licking. Positive responses were recorded for the animals in which the von Frey filament force produce entirely upliftment of the rat paw. A duration of 60 seconds was given between each filament test. Mechanical allodynia was assessed on the baseline and validatory test day as well as on 3rd, 7th, 14th, and 21st days of treatment.

Fig22: von Frey filaments, Cat No: 37450-275; Ugo Basile, Gemonio, Italy.

Fig 23: von Frey filaments cage

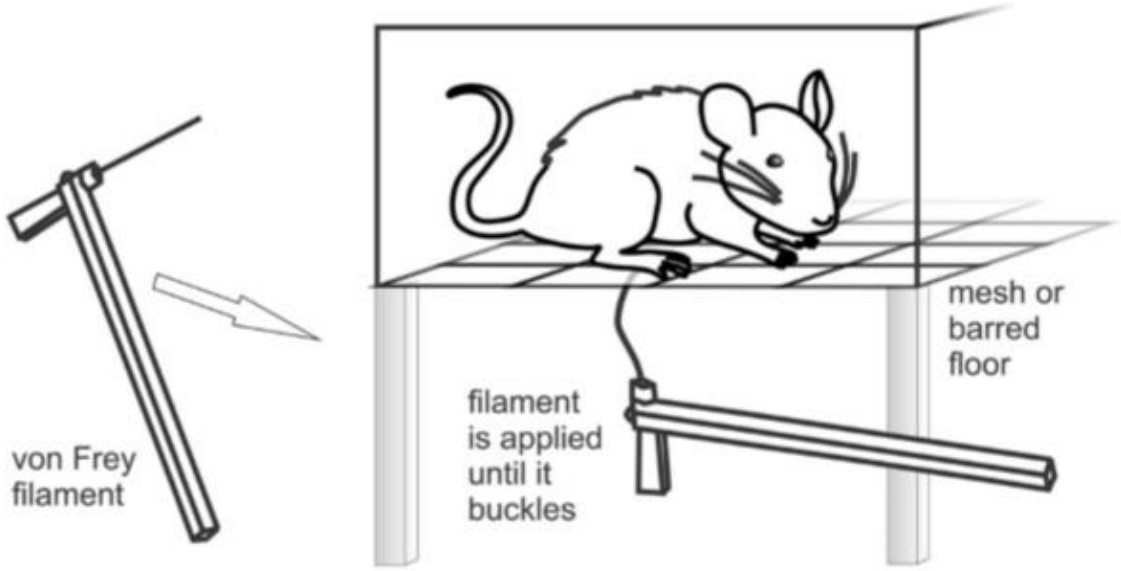

Fig 24: Mechanical allodynia test setup

3.6.4 Cold Plate Hyperalgesia Test:

Cold plate test was performed by placing the hind paw on the ice block. The animal was restricted by firmly holding the rat, however not too tight to minimize discomfort, between the second finger and middle finger. The paw withdrawal latency (PWL) to cold stimuli was recorded in seconds (with the help of a stopwatch) as the time taken by the rat to withdraw or flick its paw from the surface of the ice block. The latency of paw withdrawal from ice block was established three times, 5 minutes apart, and averaged (Coderre *et al.,* 2005; Naik *et al.,* 2006). This was taken as the threshold to Cold stimulus. Cold plate hyperalgesia test was assessed on the baseline and validatory test day as well as on 3rd, 7th, 14th, and 21st days of treatment.

3.6.5 Cold immersion Tail Flick Test:

Cold immersion tail-flick test was another paradigm that was used to assess cold hyperalgesia in *Wistar* rats by slightly modified the procedure described by Dawane and his colleagues (Dawane *et al.,* 2016). The cold tail-flick test was carried out by using an improvised closed rat restrainer. The lower half of the rat tail was immersed in a beaker of ice-cold water maintained at a temperature range of 0–4 °C. Time (in seconds) taken the rat to flick or withdraw its tail from cold water was taken as the tail withdrawal time latency. A cut-off time of 60 seconds was used. The cold tail-flick test was assessed on the baseline and validatory test day as well as on 3rd, 7th, 14th, and 21st days of treatment.

3.6.6 Acetone Evaporation Test:

Cold chemical allodynia was assessed by using 1 ml syringe to spray 100 µl of acetone onto the plantar surfaces (figure 25) of the ligated right paw of rats (placed over a wire mesh), without touching their skin (Yamamoto *et al.,* 2016). The time (in seconds) used by the animal in licking their hind paw in response to the cold effect of acetone was noted for the first 120 s

after application. Acetone spray test was assessed on the baseline and validatory test day as well as on 3rd, 7th, 14th, and 21st days of treatment.

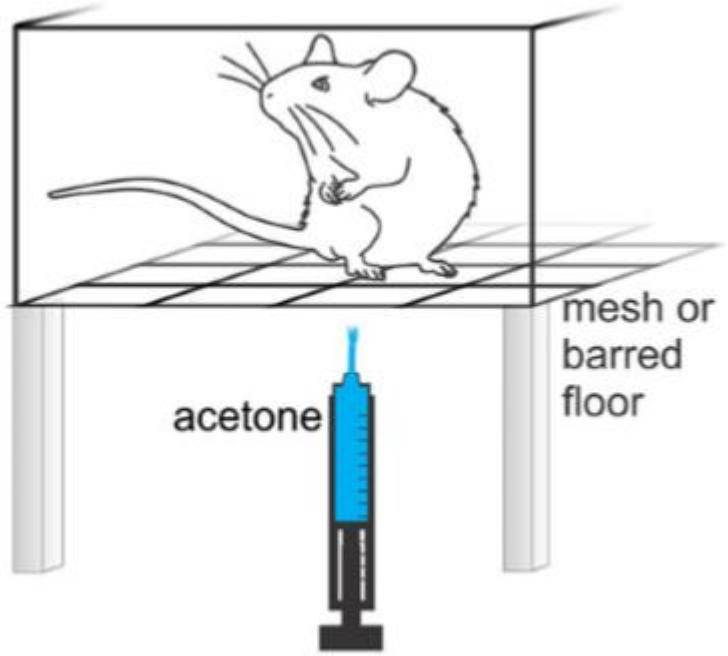

Fig 25: Acetone spray test paradigm

3.6.7 Hotplate Hyperalgesia Test:

Thermal hyperalgesia was assessed as described in the work of AbdulAbdulmajeed and Owoyele (2015), using the hotplate (DB-1A; Wincom Company Ltd., Hunan, China) model. The threshold to the thermal stimulus was measured using mean paw withdrawal latency (PWL) of the rat paw when placed on the hotplate surface maintained at 55 ± 0.5°C (figure 26). Paw withdrawal latency (PWL) of the rat was measured chronologically and as the time taken by the rat to lick the ligated hind limb or jump from the surface of the hotplate. A cutoff time of 20 seconds was imposed on each animal to avoid paw tissue damage. Hotplate test was assessed on the baseline and validatory test day as well as on 3rd, 7th, 14th, and 21st days of treatment.

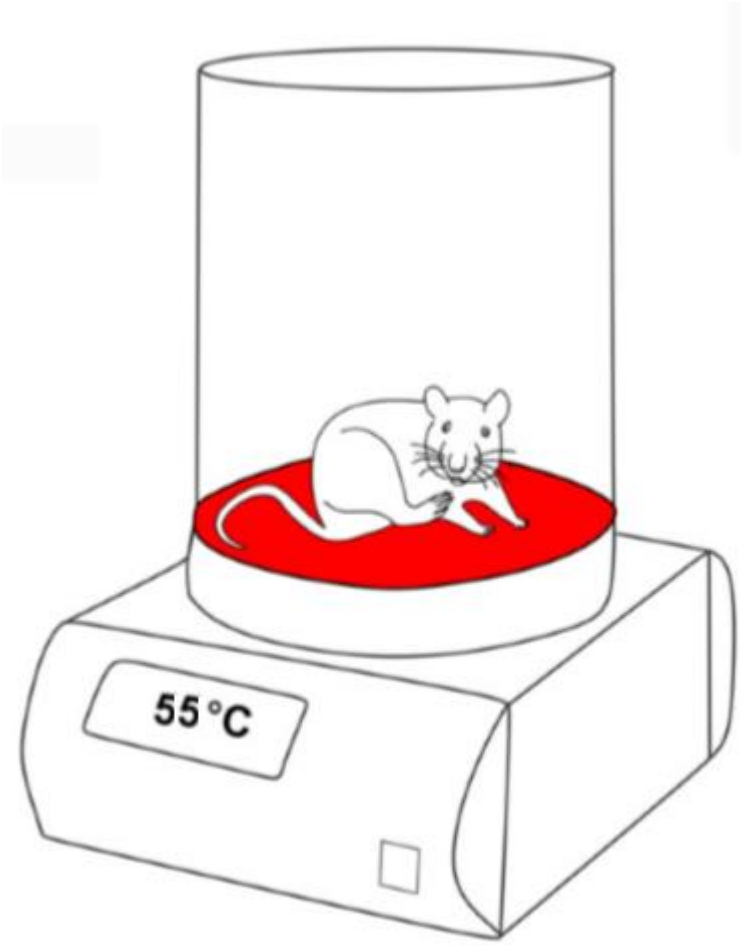

Fig 26: Illustration of hotplate test

3.6.8 Thermal tail immersion Test:

A thermal tail immersion test was carried out to check for thermal hyperalgesia by slightly modified the procedure described by Dawane and his colleagues (Dawane *et al.,* 2016). Briefly, the lower tip of the rat tail was immersed in a water bath at a maintained temperature of 55 ± 0.5°C (figure 27). Tail withdrawal latency was defined as the time taken (seconds) by the rat to flick or withdraw its tail from the water bath. A cut-off time of 20 seconds was imposed to avoid tail injury. Tail immersion test was assessed on the baseline and validatory test day as well as on 3rd, 7th, 14th, and 21st days of treatment.

Fig 27: Illustration of tail immersion test

3.6.9 Sciatic Functional Index (SFI) Test:

The level of demyelination of the nerve and degree of locomotive dysfunction was assessed using the SFI paradigm as described by Zhang *et al.* (Zhang *et al.*, 2017). The SFI was measured by estimating the footprinted image of *Wistar* rat hind paw using the formula derived by Bain *et al.*, (1989). Briefly, the animal was subject to walk freely in a wooden walking corridor (9×10×60 cm) that is bilaterally walled by plywood plates, containing a darkened carton box at the end (figure 28). The floor of the corridor was covered with a white sheet of paper (8.6×59.4 cm). The rat hind paws will be a dip in black ink and then placed at the entrance to walk freely in the corridor mentioned above. Three footprints were selected from the several impressions of each rat paw, and the following parameters were measured:

(1) distance from the heel to the third toe (print length; PL);

(2) distance from the first toe to the fifth toe (toe spread; TS); and

(3) distance from the second toe to the fourth toe (intermediate toe spread; ITS).

The above-assessed parameters were used to estimate SFI using:

(I) Print length factor (PLF) = (EPL- NPL)/NPL;

(II) Toe spread factor (TSF) = (ETS - NTS)/NTS;

(III) Intermediary toe spread factor (ITF) = (EIT - NIT)/NIT.

SFI = -38.3 x PLF + 109.5 x TSF + 13.3 x ITF - 8.8.

Where E is the experimental (ligated hind limb) footprint, and N is the control (unligated hind limb) footprint. A value of “0” indicates normal function while “-100” indicates total impairment. The mean value for the SFI was calculated from these three footprints. SFI was assessed on the baseline and validatory test day as well as on 3rd, 7th, 14th, and 21st days of treatment.

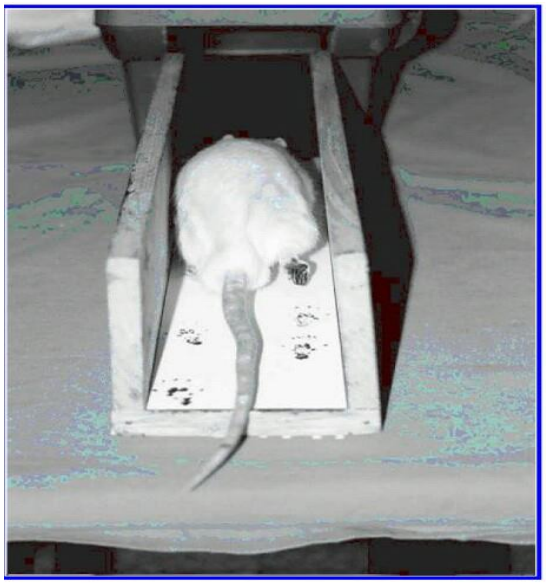

Fig 28: Sciatic functional index wooden walking corridor

3.6.10 Open Field Test:

Anxiety, exploratory, and locomotive activities of the rats were investigated using an open field maze (OFM). The OFM was built with plywood measured 72 cm x 72 cm x 36 cm (length x breadth x height). The floor of the OFM was divided into sixteen equal square boxes (18 x 18 cm) with a central square (18 cm x 18 cm) drawn in the middle (figure 29). The maze was placed in an isolated test room (1.8 x 4.6 m) and lighten by a 60-watt red lamp for background lighting. OFM test was carried out by slightly modification of the procedure described by Bailey and Crawley, (Bailey and Crawley, 2009). Briefly, rats were carried to the test room in their home cages and were handled by the base of their tails at all times. Each rat was placed at the centre of the OFM and allowed to explore the apparatus for 5 minutes. After the 5 minute test, the rat was removed from OFM and returned to its cage. The OFM was subsequently cleaned with 70 % ethyl alcohol and allowed to dry between tests. The OFM test was recorded for later analysis, using a detachable webcam camera positioned above the apparatus. Total line crossing, centre square entries, centre square duration, rearing, stretch, grooming, Freezing, and defecation were analyzed from the recorded section. An open field test was conducted during the night time on 16th days of treatment after induction of neuropathic pain.

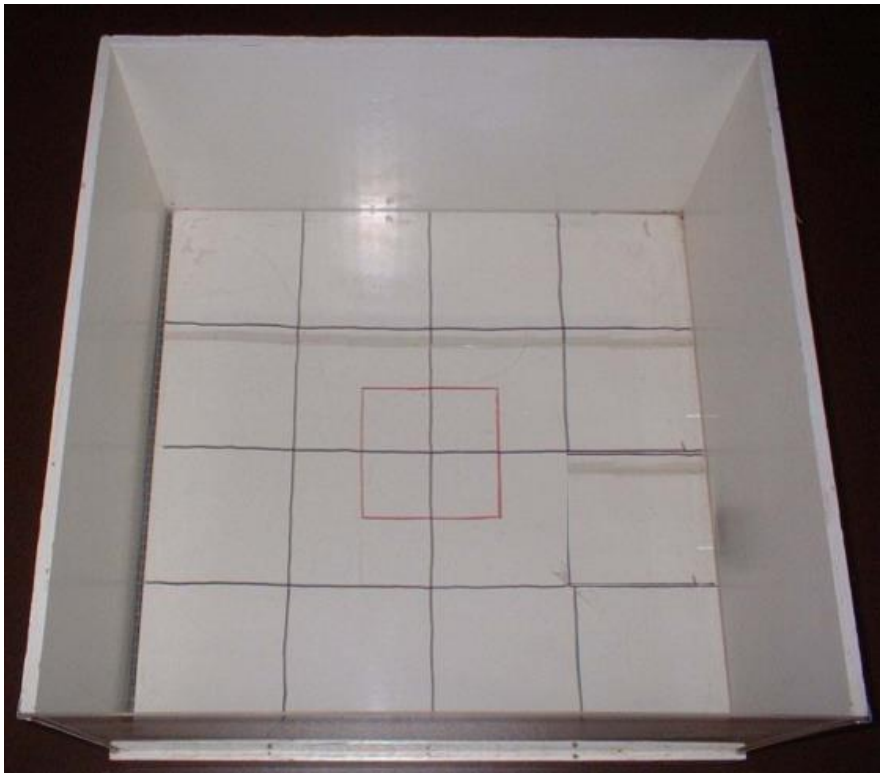

Fig 29: Open field maze box

3.6.11 Elevated Plus Maze (EPM) Test:

EPM paradigm was used to assess the ambulatory and anxiety-like behaviour in *Wistar* rats. The apparatus was made up of two (2) open arms and two (2) closed arms, crossed in the middle perpendicularly to each other with a central platform (10cm x 10cm). The closed arms are bordered with plywood walls of 30 cm height, with each arm measures 50 cm x 10 cm and 75cm elevated above the ground (figure 30). Rats were placed at the junction of the four arms of the maze, facing an open arm, and are allowed to move freely between them for a total duration of 5 minutes (Walf and Frye, 2007). The EPM was cleaned with 70 % ethyl alcohol and permitted to dry between tests. The entire section was recorded by a video-tracking system and observed later for further analysis. The number of entries into the open arms, percentage of open arms entries, close arms entries, percentage of close arm entries, time spent in the open arms, time spent in closed arms, the number of entries into the centre, centre time duration and rearing were estimated from the recorded video and used to estimate anxiety-like and ambulation in the animals (Komada *et al.*, 2008). EMP was conducted on 11th days of treatment after induction of neuropathic pain.

Fig 30: Elevated maze plus

3.7 BIOCHEMICAL ASSAY

After 21 post-treatment days, animals were euthanized using an i.p. of ketamine. Blood samples were collected into heparinized sample bottle via cardiac puncture technique. The rat was subsequently transcardiac perfused with phosphate buffer (pH 7.4). The sciatic nerve proximal to the point of ligature was excised and washed with cold Phosphate buffer solution. 80 mg mass of Sciatic nerve tissue homogenate was prepared with 1ml each of either ice-cold 0.1 M Tris buffer (pH 7.4) or phosphate buffer (pH 7.4,). The homogenate was then centrifuged at 15,000 RPM for ten minutes. The supernatant derived from the phosphate buffer homogenate was employed in the biochemical estimation of tissue protein, malondialdehyde (MD), superoxide dismutase, reduced glutathione, and nitric oxide. The supernatant of the TIS buffer homogenate was used in the estimation of sodium ions, potassium ions, calcium ions, chloride ions, sodium-potassium ATPase, calcium ATPase and glutamate level using laboratory kits and a spectrophotometer. Also, the supernatant was utilized for the determination of interleukin-1β, interleukin-6, tumour necrotic factor-alpha (TNF-α), prostaglandin E_2 (PGE_2), nuclear factor kappa-B (NF-κB), and nuclear transcription factors (NrF-1 and NrF-2) using their respective ELISA kits.

Collected cardiac blood was centrifuge at 3000 RPM for 15 minutes, and the blood plasma was collected into a plain bottle with the aid of micropipette (1000 µl). The blood plasma was utilized in the biochemical estimation of plasma protein, malondialdehyde (MD), superoxide dismutase, reduced glutathione, and nitric oxide level using laboratory kits and a spectrophotometer.

Furthermore, the brain of the rats was quickly excised on ice. The cerebra cortex (Ipsilateral cerebral cortex excluding the olfactory bulb, but including frontal and parietal lobes, and their deep brain structures such as the hippocampus, amygdala) was used to prepare brain homogenate using 0.32 Molar of sucrose solution. The homogenate was centrifuged at 15,000

RPM for 10 minutes, and the supernatant was collected. The supernatant was used for the estimation of interleukin-1β, interleukin-6, tumour necrotic factor-alpha (TNF-α), prostaglandin E_2 (PGE_2) concentration using their respective ELISA kits. Glutamate level was estimated from the brain tissue homogenate using spectrophotometry technique.

3.8 Estimation of Total Protein

Randox total protein assay kit was used to estimate the total protein concentration in both the blood plasma and tissue supernatant samples using the manufacturer procedure, as indicated in table 1 below.

Table 3.1: Procedure for determination of total protein

Pipette into cuvette			
	Reagent Blank	**Standard**	**Sample**
Distilled water	0.02 ml	-----	-----
Standard (Cal)	------	0.02 ml	-----
Sample	-----	-----	0.02 ml
RI	1 ml	1 ml	1 ml

The mixture in the table above was incubated for 30 minutes at room temperature (25^0C). The absorbance of the sample (A_{sample}) and standard ($A_{Standard}$) against the reagent blank was read at 546 nm using an automated spectrophotometer. Total protein concentration was estimated using

Total protein concentration = $\frac{\text{A sample}}{\text{A Standard}}$ $X\ standard\ concentration$

Standard concentration = 59 g/l

3.9 Estimation of Lipid Peroxidation

The biochemical method described by Hunter *et al.* (1963), modified by Gutteridge and Wilkins (1982) was used in the estimation of malondialdehyde (an end product of lipid

peroxidation). In brief, the Stock solution of TCA-TBA-HCl composed of 15g of TCA, 0.375g of TBA, and 0.25N of HCl was prepared. 1 ml of sample homogenates supernatant was combined with 2ml of TCA-TBA-HCl reagent and mixed thoroughly. The mixed solution was heated for 15 minutes in a boiling (100^0C) water bath. After cooling, the flocculent precipitate was removed by centrifugation at 1000 rpm for 10 minutes. The absorbance of the supernatant was measured at 532 nm against a blank that contains all the reagents minus the sciatic homogenate.

TABLE 3.2: Procedure for determination of malondialdehyde concentration

Reagent	**Sample cuvette (ml)**	**Blank cuvette (ml)**
Plasma sample	1.0	-
TCA-TBA-HCl	2.0	3.0

The absorbance value was used in the analysis of MDA concentration.

Concentration of MDA in the tissue (moles MDA/ml) = $\frac{O.D\ X\ V\ X\ 1000}{A\ X\ v\ X\ L}$

O.D = absorbance of the test at 532nm

V = Total volume of the reaction mixture = 3ml

A = molar extinction coefficient of the product = 56 x 10^5 $m^{-1}cm^{-1}$

L = length of light path = 1cm

v = volume of the plasma = 1ml

3.10 Estimation of Superoxide Dismutase (SOD)

The concentration of SOD was estimated by a slight modification of the method described by Misra and Fridovieh (1972). Adrenaline auto-oxidizes rapidly in aqueous solution to adrenochrome, whose concentration can be determined at 480nm using a spectrophotometer. The auto-oxidation of adrenaline depends on the presence of superoxide anions. The enzyme SOD inhibits the auto-oxidation of adrenaline by catalyzing the breakdown of superoxide

anions. The degree of inhibition is thus a reflection of the activity S.O.D. and was determined at one unit of the enzyme activity. In brief, having set the spectrophotometer at 480nm, it was zero with blank that contain 3.0 ml of distilled water. 0.2 ml of distilled water was added to the reference cuvette, while 0.2ml of the appropriate enzyme sample was added to the appropriately labelled test cuvette. To each of these was added 2.5ml of carbonate buffer, followed by equilibration at room temperature. Subsequently, 0.3 ml of 0.3Mm adrenaline solution was added to the reference and each of the test solutions and mixed by inversion. The absorbance of each mixture in the cuvette was read at 480 nm using an automated spectrophotometer at a duration of 30 seconds for 120 seconds

Calculation:

% inhibition = $\frac{\Delta ABS_{REF} - \Delta ABS_{TEST}}{\Delta ABS_{REF}}$ X 100

Δ ABS = change in absorbance at a duration of 60 seconds

However, 1 unit of SOD activity was taken as the amount of SOD required to cause 50% inhibition of the auto-oxidation of adrenaline to adrenochrome per minute. Therefore enzyme activity is calculated as

Unit/ml = $\frac{\%\ inhibition}{50}$

3.11 Estimation of Lipid Catalase

The continuous activities of the catalase in the blood plasma and sciatic nerve homogenates were determined with a spectrophotometer (modification of the method used by Claiborne, 1985). The continuous spectrophotometric rate reduction of catalase activity (A_{480nm}, light path = 1cm) was based on the following reaction

$H_2O_2 \xrightarrow{catase} 2H_2O + O_2$

TABLE 3.3: Procedure for determination of catalase activities

Reagent	**Sample cuvette (ml)**	**Blank cuvette (ml)**	**Standard cuvette (ml)**
Plasma sample	0.5	-	-
30mM H_2O_2	5.0	5.0	-
Distilled water	-	0.5	-
Mix by inversion, stand for 3 minutes			
6M H_2SO_4	1.0	1.0	1.0
0.05M Phosphate buffer (pH 7.4)	-	-	5.5
Mix by inversion			
0.01M $KMnO_4$	7.0	7.0	7.0
Mix by inversion			

Catalase activity was estimated by preparing the mixture in a labelled curvet as indicated in the table above. The absorbance of each mixture was read at 480nm at an interval of 30 seconds for 3 minutes.

Calculation:

Abs of blank = Abs_B

Abs of standard = Abs_{std}

Abs of test = Abs_T

Unit/m = $\frac{2.303}{\Delta T}$ x log $\frac{O.D_1}{O.D_2}$

3.12 Estimation of Reduced Glutathione

Reduced glutathione (GSH) was measured by slightly modification of the method described by Ellman (1959). Briefly, the sample was diluted by mixing 0.1 ml of the sample with 0.9 ml distilled water. The diluted solution was then deproteinized by adding 3 ml of 4% sulfosalicylic

acid solution. The resulting mixture was centrifuged at 3000 RPM for 10 minutes, and the supernatant was collected. Thereafter, 0.5 ml of the supernatant was added to 4 ml of 0.1 M phosphate buffer (pH 7.4) and 4.5 ml Ellman's reagent. A blank was prepared with a reaction mixture of 4 ml of 0.1 M phosphate buffer (pH 7.4), 0.5 ml diluted precipitating mixture (addition of 3 ml of precipitating solution and 2 ml distilled water), and 4.5 ml Ellman's reagent. The mixture was mixed by vortex, and the absorbance was read spectrophotometrically at 412 nm within 5 minutes. A standard curve was plotted using the absorbance of the concentration of the standard solution. The concentration of GSH from each sample was interpolated from the standard curve (Fig. 54, appendix II) and it was expressed as "g/mg of protein".

3.13 Estimation of Nitric Oxide (NO)

Nitric oxide was estimated indirectly by determining the concentration of both Nitrite and Nitrate in the supernatant using the Griess diazotization reaction method. The supernatant was first to be deproteinized via the addition of $ZnSO_4$ (15g/l) and then centrifuged at 10000g for 5 minutes under room temperature. For estimation of nitrite (NO_2^-), 100µm of Griess reagent (50µm of 1% sulfanilamide in 5% HCl and 50µm of 0.1% naphthyl-ethylenediamine dihydrochloride) was added to 300µm of the sample followed by 2.6ml of deionized water. The mixtures were incubated for 30 minutes at room temperature, and absorbance was measured at 548nm using a spectrophotometer. For the estimation of nitrate (NO_3^-), activated cadmium granules (2.5g-3g) was added to the deproteinized supernatant. The mixtures were stirred and incubated for 90 minutes so that nitrate in the supernatant is reduced to nitrite, and estimation of total nitrite was carried out as described above. A calibration curve (Fig. 55, Appendix II) used to determine the concentration of nitrite was generated by preparing different concentrations of sodium nitrite in distilled water ranges 0 - 500µM/l. The absorbance of each

sample was read spectrophotometrically at 548 nm. The standard curve of nitrite concentration was plotted against reading absorbance.

3.14 Estimation of Glutamate Concentration

Microplate reading technique was used in measuring the glutamate concentration in prepared homogenate samples. The glutamate assay kit was used in the estimation of glutamate concentration using the procedure described by the manufacturer. In brief, the reaction mix sample and background reaction mix were prepared according to the manufactural instruction. The reaction mix sample (100 µL) was added into each standard (six standard samples were prepared according to manufacturer direction) and sample wells. Likewise, 100 µL of the background reaction mix was added into Background sample wells. The mixture above was incubated at 37°C for 30 minutes protected from light. Absorbance was read at 450 nm on a microplate reader. The corrected absorbance of the standard was used to generate the standard curve (Fig. 56, Appendix II) from which the samples glutamate concentration was extrapolated from.

Calculation:

Sa = $\frac{(corrected\ absorbance - (y - intercept))}{slope}$

Corrected absorbance = Sample absorbance – blank absorbance

The concentration of glutamate = $\frac{Sa}{Sv}\ X\ Df$

Sa = Amount of sample (nmol) from the standard curve.

Sv = Volume of the sample (µL) added into the well.

Df = Sample dilution factor.

3.15 Estimation of Chloride Concentration

Chloride ion concentration was estimated spectrophotometrically according to the kit manufacturer procedure. In brief, 0.01 ml of sample was mixed with 1.5 ml of chloride reagent by Voltex. The resulting mixture was incubated at room temperature (25 ^{0}C) for five minutes,

and absorbance was read at 480 nm from automated spectrophotometer zero with reagent blank.

Calculation:

Chloride concentration (mEq/L) = $\frac{Abs\ of\ sample}{Abs\ of\ standard}\ X\ concentration\ of\ standard$

Concentration of standard = 100 mEq/L

3.16 Estimation of Sodium, Potassium and Calcium Ions Concentration

The method described by the Association of Official Analytical Chemists [AOAC 2005] was used for the estimation of sodium, potassium, and calcium ions concentration. In brief, the samples were ashed at 550°C. The ash was boiled with 10 ml of 20% hydrochloric acid in a beaker and then filtered into a 100 ml standard flask. Deionized water was used to make up the filtrate up to the marked level. The sodium and potassium ions concentration were determined from the resulting solution using Atomic Absorption Spectrophotometer (AAS Model Bulk Scientific Accuzy 211). All values were expressed as ppm (mg/100 g). Calcium ion concentration was determined spectrophotometrically using UV/Vis Spectrophotometer model 752N.

3.17 Estimation of Sodium/Potassium ATPase Activities

The Na^+/K^+-ATPase activity was measured as described earlier by (Maurya *et al.,* 2013). The final assay mixture contained 0.4 to 0.9 mg membrane protein per ml, 140 mM NaCl, 20 mM KCl, 3 mM $MgCl_2$, 30 mM imidazole (pH 7.25), ± 5 x 10-4 M ouabain and 6 mM ATP. The mixture above was incubated for 30 min at 37°C and subsequently stopped by adding 3.5 ml of a solution containing 0.5 M H_2SO_4, 0.5% ammonium molybdate, and 2% SDS. The amount of liberated inorganic phosphate was estimated. The Na+/K+-ATPase activity was expressed in terms of a micromole of Pi released / hr/mg membrane protein at 37°C.

3.18 Estimation of Calcium ATPase Activities

The Ca^{2+}-ATPase activity was assayed as described by (Maurya *et al.,* 2013). Briefly, 2.25 ml of the assay mixture contained 80 mM NaCl, 15 mM KCl, 3 mM MgCl2, 18 mM Tris-HCl (pH 7.4), 0.1 mM ouabain, 0.1 mM EGTA, 0.2 ml of the membrane containing 0.4 to 1.5 mg protein per ml and ± 0.2mM $CaCl_2$. The reaction was initiated by the addition of 0.1 ml of 30 mM ATP. After 30 min at 37°C incubation, the reaction was stopped by adding 3.5 ml of a solution containing 0.5 M H2SO4, 0.5% ammonium molybdate, and 2% SDS. The amount of liberated inorganic phosphate was estimated. The Ca^{2+}-ATPase activity was expressed in terms of micromole of Pi (inorganic phosphate) released / hr/mg membrane protein at 37°C.

3.19 Biochemical Estimation of Tumor Necrotic Factor-Alpha (TNF- α) Concentration

Tumor Necrotic Factor-Alpha (TNF-α) Concentration was determined using the ELISA technique as described by the kit manufacturer. In brief, all the reagents, working standards, and samples were prepared as instructed. Briefly, 50 µL of TNF alpha Standard and samples were added per well. The wells were cover with a sealing tape and incubated for two hours. The wells were then washed five times with 200 µL of 1X Wash Buffer and then invert the plate, decant the contents. Subsequently, 50 µL of 1X Biotinylated TNF alpha Antibody was added to each well and incubated for two hours, followed by washing of the microplate as described above. Conjugate (50 µL of 1X SP) was added to each well and incubated for 30 minutes and then washed. Chromogen Substrate (50 µL) was added per well and incubated for about 20 minutes or till the optimal blue colour density develops. Finally, 50 µL of Stop solution was added to each well, and the absorbance was read on a microplate reader at a wavelength of 450 nm immediately. Standard Curve (Fig. 57, Appendix II) was generated by plotting the standard concentrations on the x-axis and the corresponding 450 nm absorbance on the y-axis. The concentration of TNF-α in the samples were determined from the Standard Curve and multiply by the dilution factor.

3.20 Biochemical Estimation of Interleukin-1β (IL-1β) Concentration

IL-1β Concentration was determined using the ELISA technique as described by the kit manufacturer. In brief, all the standards, controls, and samples were prepared as instructed. The standard and sample (100 µL each) were added into appropriate wells, covered and incubated for two and half hours (2 ½ hrs) at room temperature. The solution was then discarded and washed four (4) times with 1X Wash solution. Biotinylated IL-1β Detection Antibody (100 µL of 1X) was added to each well and incubated for one (1) hour at room temperature with gentle shaking. The solution was after that discarded and washed. HRP-Streptavidin solution (100 µL of 1X) was added to each well and incubated for 45 minutes at room temperature with gentle shaking and subsequently discarded and washed. TMB One-Step Substrate Reagent (100 µL) was added to each well and incubated for 30 minutes at room temperature in the dark with gentle shaking. Finally, 50 µL of Stop Solution was added to each well and read with a microplate reader at 450 nm immediately. The absorbance reading for standards and samples was subtracted from the zero standard optical density. The standard curve (Fig. 58, Appendix II) was plotted with standard concentration on the x-axis and absorbance on the y-axis. It was used to determine the IL-1β concentration from the sample absorbance.

3.21 Biochemical Estimation of Interleukin-6 (IL-6) Concentration

IL-6 concentration was determined using the ELISA technique as described by the kit manufacturer. In brief, all reagents, working standards, and samples were prepared as directed. The microplate was washed twice with approximately 400 µL 1X Wash Buffer per well. The prepared standards (100 µL) were added to appropriate wells. Assay Buffer (50 µL of 1X) was added to the sample wells followed by the addition of 50 µL of each sample to appropriate wells. Biotin Conjugated Antibody (50 µL of 1X) was then added to all the wells, covered, incubated at room temperature (25°C) for 2 hours and then washed. Streptavidin-HRP (100 µL of 1X) was added to all wells, including the blank wells, and incubated at room temperature

(25°C) for 1 hour and then washed. TMB Substrate solution (100 µL) was Pipetted into all wells and Incubated at room temperature (25°C) for 10 minutes. The substrate reaction was stopped by the addition of 100 µL stop solution as colour development was observed. The absorbance of each microplate was read at 450 nm. The absorbance of the control blank was subtracted from that of standard and samples. The standard curve (Fig. 59, Appendix II) was generated by plotting corrected standard absorbance readings against their concentrations.

3.22 Biochemical Estimation of Prostaglandin E_2 (PGE_2) Concentration

PGE_2 concentration was determined using the ELISA technique as described by the kit manufacturer. In brief, 50µL each of dilutions of standard, blank, and samples were added into appropriate wells, followed by 50µL of Detection Reagent A. The mixture was incubated for 1 hour at 37 ^oC, aspirate and washed with 350µL of 1X Wash Solution. Detection Reagent B working solution (100µL) was added into each well, incubated for 30 minutes at 37^oC, and then washed. Substrate solution (90µL) was added to each well, incubated for 10 - 20 minutes at 37^oC, and finally, 50µL of Stop solution was added. The absorbance of the resulting microplate was then read at 450 nm immediately. A standard curve (Fig. 60, Appendix II) was generated on log-log graph paper, with the log of PGE2 concentration on the y-axis and absorbance on the x-axis. The samples PGE2 concentrations were read from the standard curve and multiplied by the dilution factor.

3.23 Biochemical Estimation of Nuclear Factor kappa-B (NF-κB) Concentration

NF-κB Concentration was determined using the ELISA technique as described by the kit manufacturer. In brief, 100 µL OF CTFB was added each into NSB and blank wells, while 90 µL CTFB followed by 10 µL samples and 90 µL, CTFB followed by 10 µL standard serial dilution were added into their appropriate wells. The microplate was covered and incubated for 1 hour at room temperature (25 ^oC) and washed with 200 µL of 1X Wash Buffer. NF-κB primary antibody (100µL) was added into each well except blank well, incubated for 60

minutes at room temperature (25 ^{o}C), and then washed. Transcription factor Goat anti-Rabbit HRP conjugate (100µL) was added to each well except the blank well, incubated for 60 minutes at room temperature (25 ^{o}C), and then washed five times. Transcription factor developing solution (100 µL) was added to each well and incubated for 15 - 45 minutes at room temperature (25 ^{o}C). Finally, 100 µL of Stop Solution was added. The absorbance of the resulting microplate was then read with a microplate reader at 450 nm immediately. A standard curve (Fig. 61, Appendix II) was generated with the absorbance of the standard on the y-axis and concentration on the x-axis. The samples NF-κB concentrations were read from the standard curve and multiplied by the dilution factor.

3.24 Biochemical Estimation of Nuclear Factor Erythroid-Derived 2-Like 1 and 2 (NrF-1 and NrF-2) Concentration

ELISA technique was used to assess for the NrF concentration in samples homogenate as described in the manufacturer instruction manual. Briefly, 40 µL CBB containing wt was added to the Competitive Binding Control wells. CBB (40 µL) was added to each of the other wells, and 10 µL of sample diluted was added into CLB Sample wells. H_2O_2 treated MCF-7 nuclear extract (2 µL), and CLB (8 µL) was added into Positive control wells. CLB (10 µL) was added to Blank wells. The mixture was incubated for 1 hour at room temperature 25 ^{o}C with mild agitation and subsequently washed three times with 200 µL 1X Wash Buffer. Diluted primary antibody (100 µL) was added to all wells being used, incubated for 1 hour at room temperature without agitation, and then washed three times with 200 µL 1X Wash Buffer. Diluted anti-rabbit HRP-antibody (100 µL) was added to all wells, incubated for 1 hour at room temperature without agitation, and washed. RT Developing solution (100 µL) was added to all wells being used and incubated for 2 – 10 minutes at room temperature. Stop solution (100 µL) was then added, and the absorbance was read at 450 nm. A standard curve (Fig. 62-63, Appendix II) was generated with the absorbance of the standard on the y-axis and concentration on the x-axis.

3.25 Histology Study

Structural integrity of the sciatic nerve was investigated using haematoxylin and eosin (H & E) stain as described by Muthuraman *et al.,* 2008. Sciatic was fixed in a 10% formalin solution and blocked. It was further sectioned longitudinally into 5 μm before staining with H & E and observed under a light microscope.

3.26 Immunohistochemistry

Expression of inducible nitric oxide synthase (iNOS) was assessed using immunohistochemistry technique as described by Emokpae *et al.,* 2020. Briefly, rats were anaesthetized with ketamine hydrochloric (100 mg/kg, i.p.) and perfused transcardially with phosphate buffer (pH 7.4) followed by 10% formalin. The sciatic nerve was isolated and post-fixed in the same 10% formalin for 18 hours. It was then blocked with paraffin wax and further sectioned into 5 μm transversely and laid on a gelatin-coated glass slide. The sections were deparaffinized, hydrated, and antigen retrieval procedure was carried out. Tissue sections were incubated with primary antibody rabbit-iNOS (1:500, Bioscience) for 20–30min at room temperature (25°C) according to the manufacturer's instructions. The sections were then incubated for 20–30min secondary antibodies at room temperature (1:500, Bioscience). Few drops of ready to use 3, 3′- diaminobenzidine (DAB) reagent were added on each tissue sections and allowed to incubate for 6–10min at room temperature (25°C) before washing with PBS 5–7 times and then with distilled water. The slides were incubated with hematoxylin for 30–60s, rinsed with distilled water, and allowed to drain before mounting with appropriate mountant. Images acquired using Amscope Digital Camera connected to a computer and light microscope, and the expression of immunopositive cells was analyzed using Image J software (NIH, Bethesda, MD, USA).

3.27 Statistical Evaluation

Graph pad prism version 5 software was used to analyze all data. Data were expressed as mean ± SEM. Two way ANOVA parametric test was used to analyze behavioural pain tests while one-way ANOVA was used to analyze behavioural emotion tests and biochemical parameters. Bonferroni's and Tukeys' *post hoc* multiple comparison tests were used for comparison among groups with a significant level set at $p < 0.05$. Data that were not evenly distributed were transformed before the use of parametric ANOVA test.

CHAPTER FOUR

RESULT

4.1 Pre-surgical Baseline Behavioral Test

There were no signs of hyperalgesic nor allodynic observed in all the *Wistar* rats. The sciatic functional index (SFI) was normal in all the *Wistar* rats. Statistical comparison among groups shows no significant differences on SFI, mechanical allodynia and thermal hyperalgesia tests except for those groups that were pretreated with bromelain whose thermal and mechanical threshold was significant ($p < 0.05$) higher compared with other groups as represented in Fig. 32 – 38.

4.2 Validatory Post-surgical Baseline Behavioral Test

Following CCI, ligated *Wistar* rats developed spontaneous pain characterized by guarding behaviour of the ipsilateral hind paw. There was occasional licking of the ligated hind limb starting within a few hours after recovering from sedation. Also, all the ligated *Wistar* rats displayed symptoms of allodynia, thermal hyperalgesia, and impaired SFI, which were significantly different ($p < 0.05$) in comparison with unligated control. These latter observations were noticed on the third day after the induction of neuropathic pain.

4.3 Body Weight

Sciatic nerve ligation does not result in significant ($p < 0.05$) changes in *Wistar* rats' body weight. Pretreatment and post-treatment of animals with bromelain yielded no significant difference ($p < 0.05$) in Percentage changes in body weight of *Wistar* rats compared with other groups. It was observed that the sham ligated group shows minimal changes in their body weight, as illustrated in fig 31. Likewise, bromelain treated groups did not show any form of sweeping changes in their percentage of body weight changes compared with other groups.

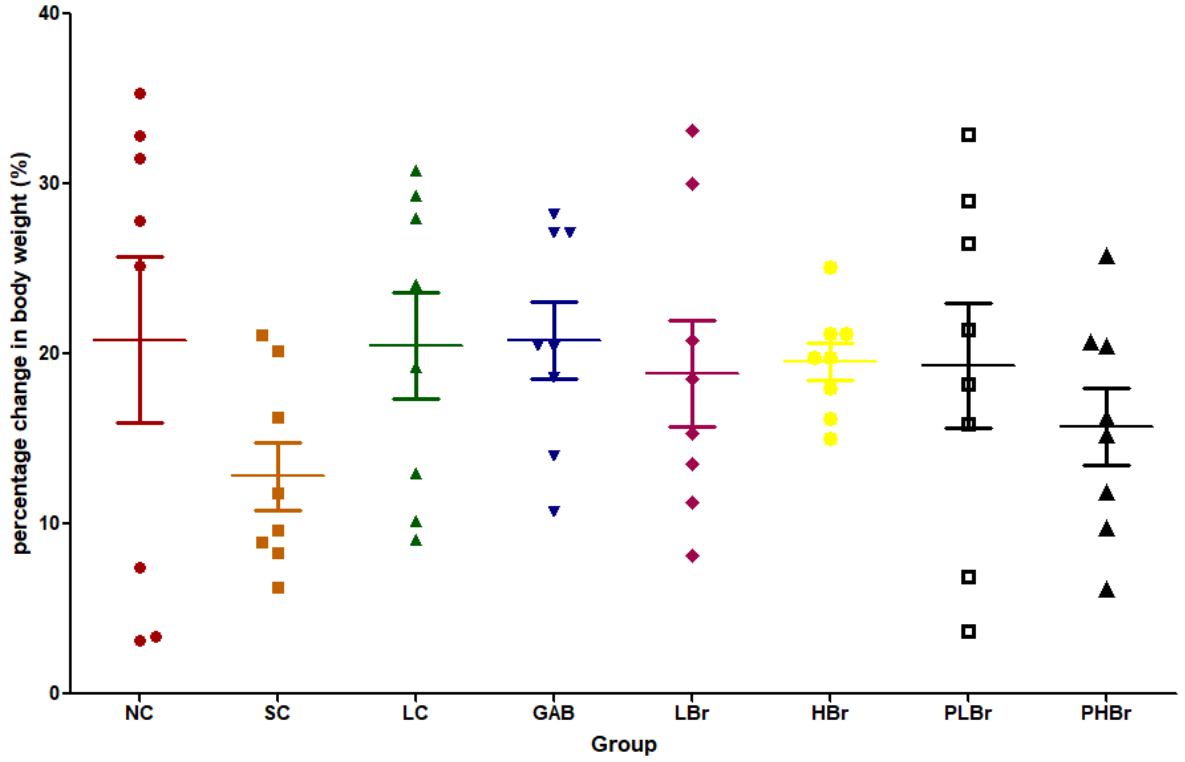

Fig 31: Bromelain negligible effect on Wistar rats body weight. *Each value represents the mean ± SEM of each group. NC: Unligate Normal Control; SC: Sham Control; LC: Ligated Control; RC: Reference Control (30mg/kg gabapentin); LD: Low Dose Bromelain (30mg/kg); HD: High Dose Bromelain (50mg/kg); PLD: Pretreated Low Dose Bromelain (30mg/kg); PHD: Pretreated High Dose Bromelain (50mg/kg)*

4.4 Mechanical Allodynia

Mechanical allodynia signs were observed in all the sciatic nerve ligated *Wistar* rats, as indicated in figure 32. The observed mechanical allodynia was highly pronounced in the ligated control group ([3.67 ± 0.33 g] – [5.67 ± 0.62 g] average bending force). All the ligated *Wistar* rats treated with bromelain show significant ($p < 0.05$) improved mechanical allodynic signs. However, both low and high dose bromelain treated groups have a low mechanical von Frey filament force threshold compared with unligated control groups. Hence, bromelain treated groups have a low significant ($p < 0.05$) response to mechanical force compared with the unligated control group. Pretreating *Wistar* rats with bromelain before the induction of neuropathic pain did not yield significant ($p > 0.05$) improvement to the mechanical von Frey filaments perception compared with post-treated bromelain groups. The effect of bromelain on mechanical allodynia sign is comparable with that of gabapentin as there was no significant difference ($p < 0.05$) between the two groups. There was no significant difference ($p > 0.05$) between the standard control and sham group.

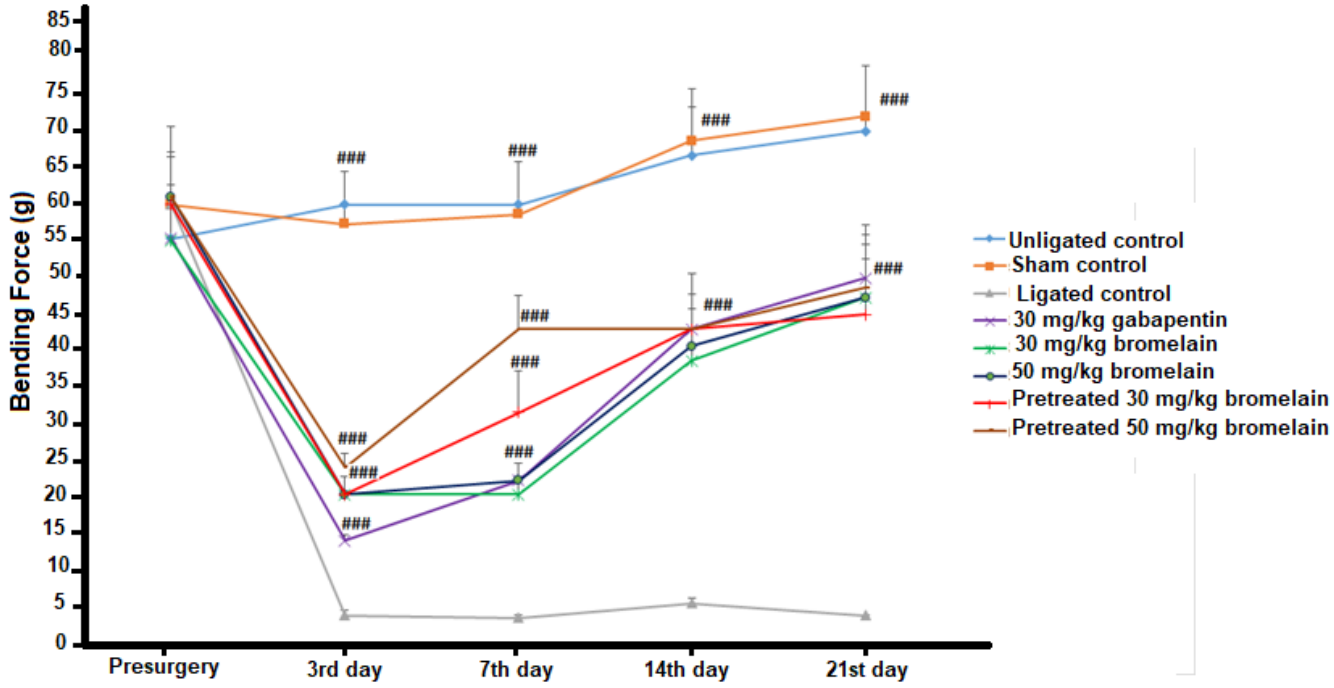

Fig 32: Bromelain improved mechanical threshold to von Frey filaments in CCI in Wistar rats. *Each value represents the mean ± SEM of each group.* $^{###}p < 0.001$ *significant compared with the ligated control.*

4.5 Acetone Evaporation Test

Ligated *Wistar* rats showed an increased response to the chemical cold-induced allodynic test. There were increases in the frequency and response time (seconds) to acetone in all the ligated *Wistar* rats. Increased responses (paw reaction duration) to acetone in the ligated control group were significantly ($p < 0.05$) higher compared with all other groups throughout the test days. Bromelain administered groups showed significant ($p < 0.05$) reduced paw-licking/flicking time compared with the ligated naïve group as represented in figure 33. Bromelain treated groups showed a dose-dependent response to acetone on 3rd, 7^{th,} and 21st days of post-surgical operation. There were no significant differences between the unligated control group, reference control group, and high dose bromelain administered group on the 21st-day post-surgical test.

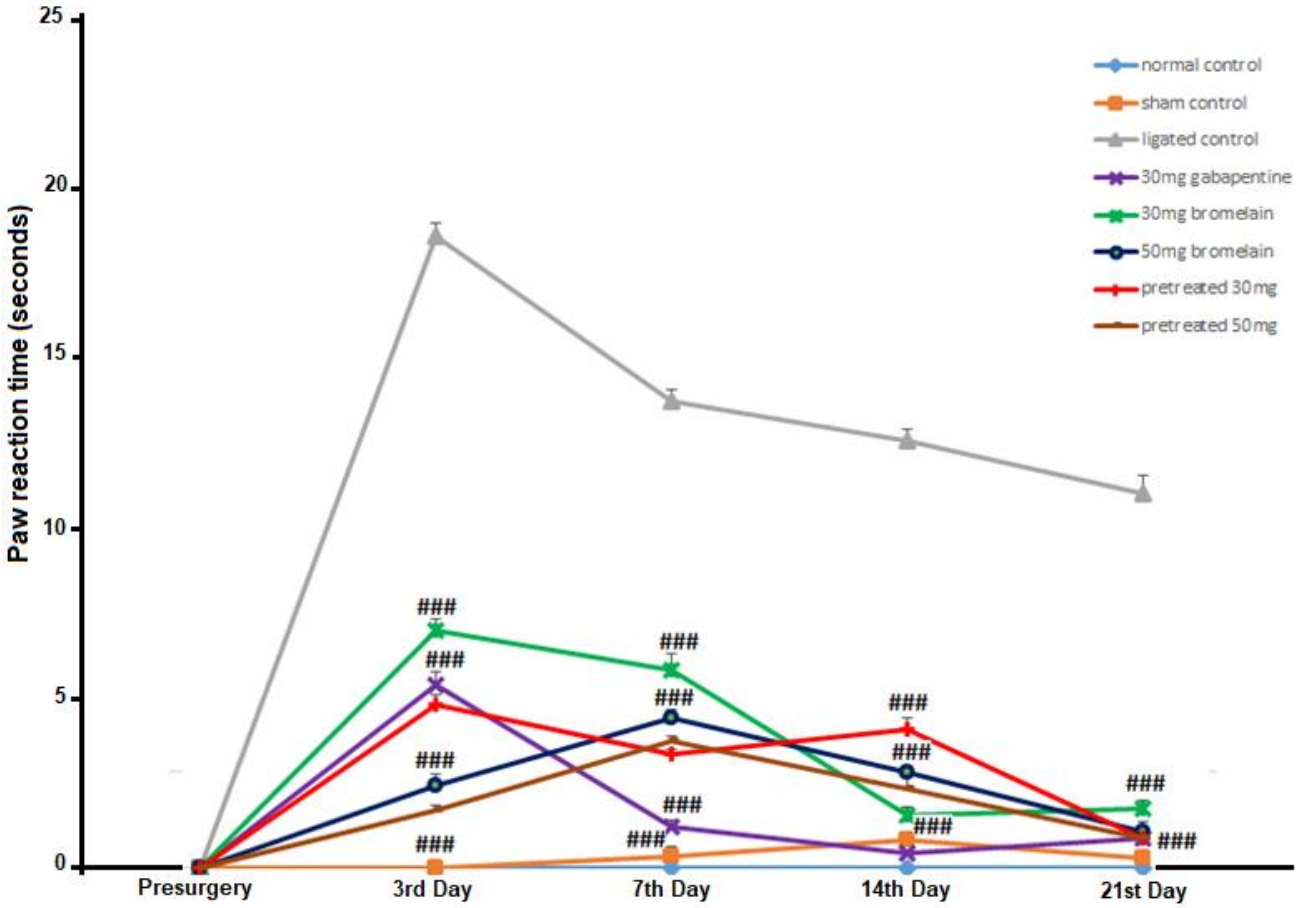

Fig 33: Bromelain reversed acetone-induced cold allodynia in the CCI model of neuropathic pain. *Each values represent mean ± SEM of each group.* $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ *compared with ligated control.*

4.6 Cold-plate Hyperalgesia Test

Chronic constriction injury (CCI) induces cold hyperalgesia in *Wistar* rats. There were significant differences ($p < 0.05$) in the cold hyperalgesia sign between the ligated and unligated groups. Treatment with bromelain completely reversed the observed cold hyperalgesia signs in ligated *Wistar* rats. Bromelain significantly increased the threshold to cold stimulus compared with the unligated control group. Likewise, high dose bromelain treated groups have increased withdrawal threshold to a cold stimulus, that was highly significant different ($p<0.05$) compared with gabapentin reference control group on the third-day post-treatment hyperalgesia test. However, both drugs can be said to have a similar therapeutic effect after twenty-one days of treatment as there was no significant difference ($P < 0.05$) between them, as indicated in figure 34. There was no significant difference ($P < 0.05$) between bromelain pretreated and post-treated group except on seventh-day post-treatment hyperalgesia test.

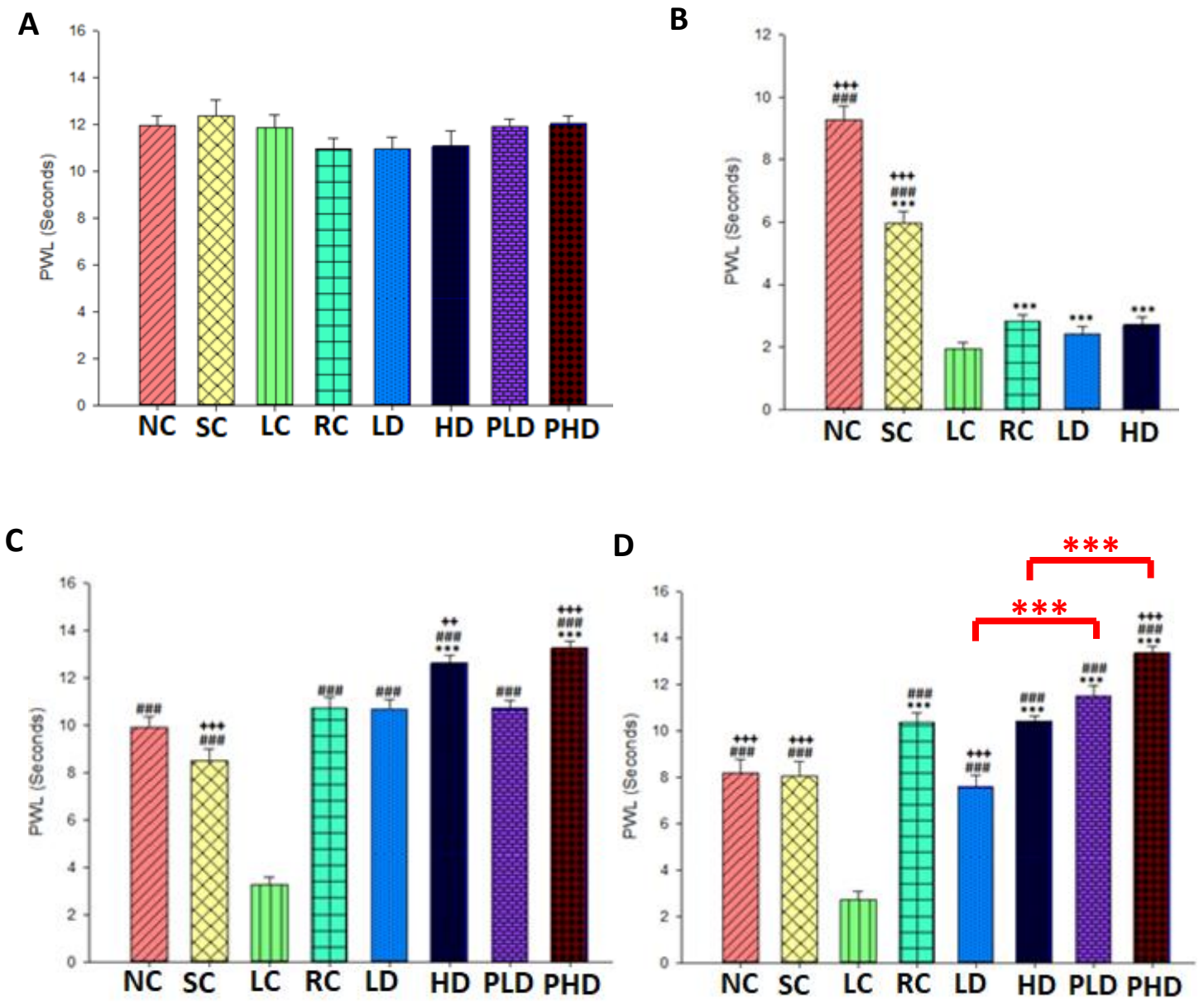

Fig 34: Bromelain subdue cold plate-induced hyperalgesic symptoms in the CCI model of neuropathic pain in Wistar rats. *Each values represent mean ± SEM of each group. (A) Presurgical baseline cold plate test. (B) Baseline cold plate test. (C) 3rd-day post-surgery cold plate test (D) 7th-day post-surgery cold plate test.* $^{\#}p < 0.05$ *compared with ligated control; *p < 0.05 compared with normal control;* $^{+}p < 0.05$ *compared with reference control. NC: Unligate Normal Control; SC: Sham Control; LC: Ligated Control; RC: Reference Control (30mg/kg gabapentin); LD: Low Dose Bromelain (30mg/kg); HD: High Dose Bromelain (50mg/kg); PLD: Pretreated Low Dose Bromelain (30mg/kg); PHD: Pretreated High Dose Bromelain (50mg/kg)*

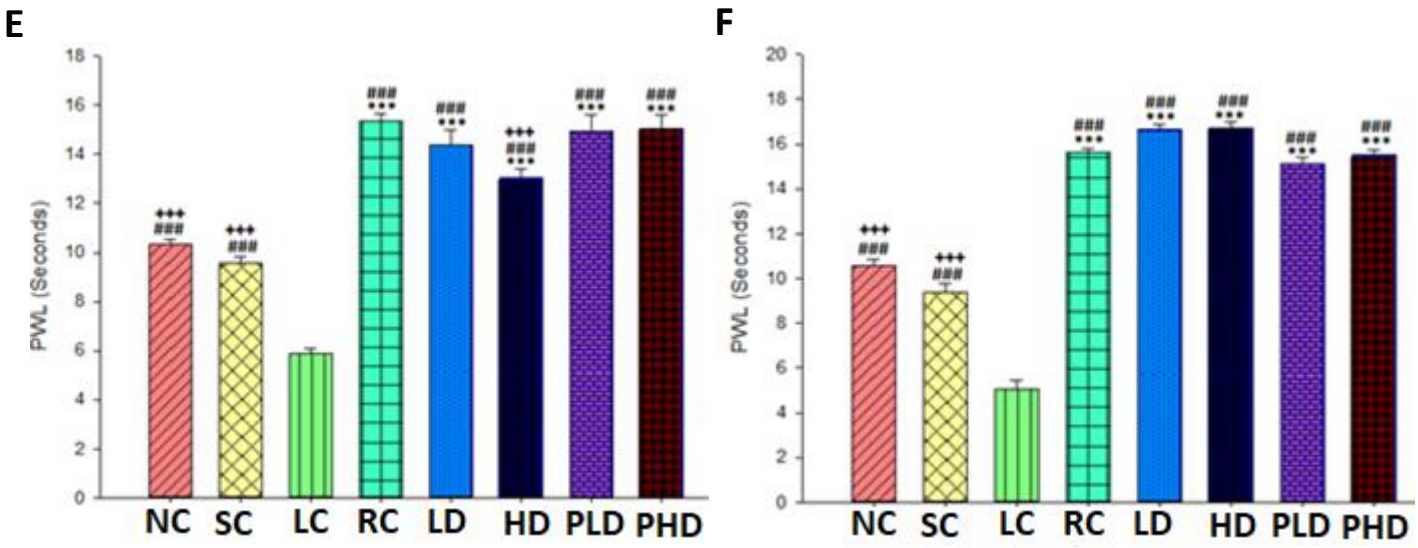

Fig 34: Bromelain subdue cold plate-induced hyperalgesic symptoms in the CCI model of neuropathic pain in Wistar rats. *Each values represent mean ± SEM of each group. (E) 14th-day post-surgery cold plate test (F) 21st-day post-surgery cold plate test.* $^{\#}p < 0.05$ *compared with ligated control;* $^{*}p < 0.05$ *compared with normal control;* $^{+}p < 0.05$ *compared with reference control. NC: Unligate Normal Control; SC: Sham Control; LC: Ligated Control; RC: Reference Control (30mg/kg gabapentin); LD: Low Dose Bromelain (30mg/kg); HD: High Dose Bromelain (50mg/kg); PLD: Pretreated Low Dose Bromelain (30mg/kg); PHD: Pretreated High Dose Bromelain (50mg/kg)*

4.7 Cold Tail Flick Immersion Test

The cold tail-flick immersion test result was similar to what was observed in the cold plate test. There was a marked degree of cold hyperalgesia in all the ligated *Wistar* rats. Bromelain completely reversed cold hyperalgesia in the ligated *Wistar* rats to the basal level. Bromelain significantly ($p < 0.05$) increased tail withdrawal latency (TWL) of ligated *Wistar* rats compared with the unligated control group, as indicated in figure 35. The effect of bromelain on cold-induced hyperalgesia is similar to gabapentin. Likewise, there was a significant ($p < 0.05$) effect of pretreatment with bromelain at the 7^{th}-day test compared with post-treatment with bromelain.

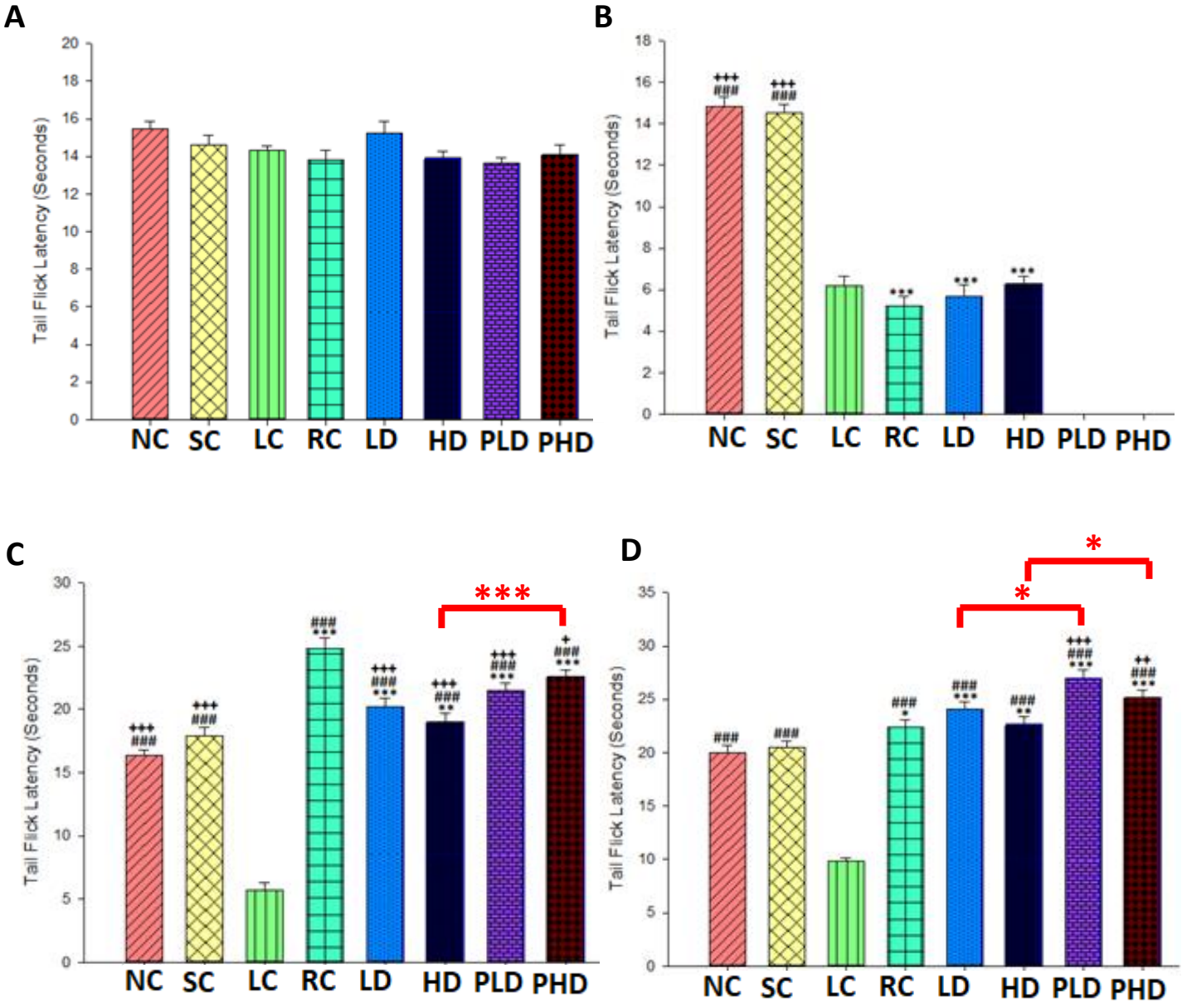

Fig 35: Bromelain reversed cold hyperalgesia in tail-flick immersion test in sciatic nerve-induced neuropathic pain. *Each values represent mean ± SEM of each group. (A) Presurgical baseline cold flick test. (B) Post-surgical Baseline cold flick test. (C) 3rd day post-surgery cold flick test (D) 7th day post-surgery cold flick test.* $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ *compared with ligated control;* $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ *compared with normal control;* $^{+}p < 0.05$, $^{++}p < 0.01$, $^{+++}p < 0.001$ *compared with reference control. NC: Unligate Normal Control; SC: Sham Control; LC: Ligated Control; RC: Reference Control (30mg/kg gabapentin); LD: Low Dose Bromelain (30mg/kg); HD: High Dose Bromelain (50mg/kg); PLD: Pretreated Low Dose Bromelain (30mg/kg); PHD: Pretreated High Dose Bromelain (50mg/kg)*

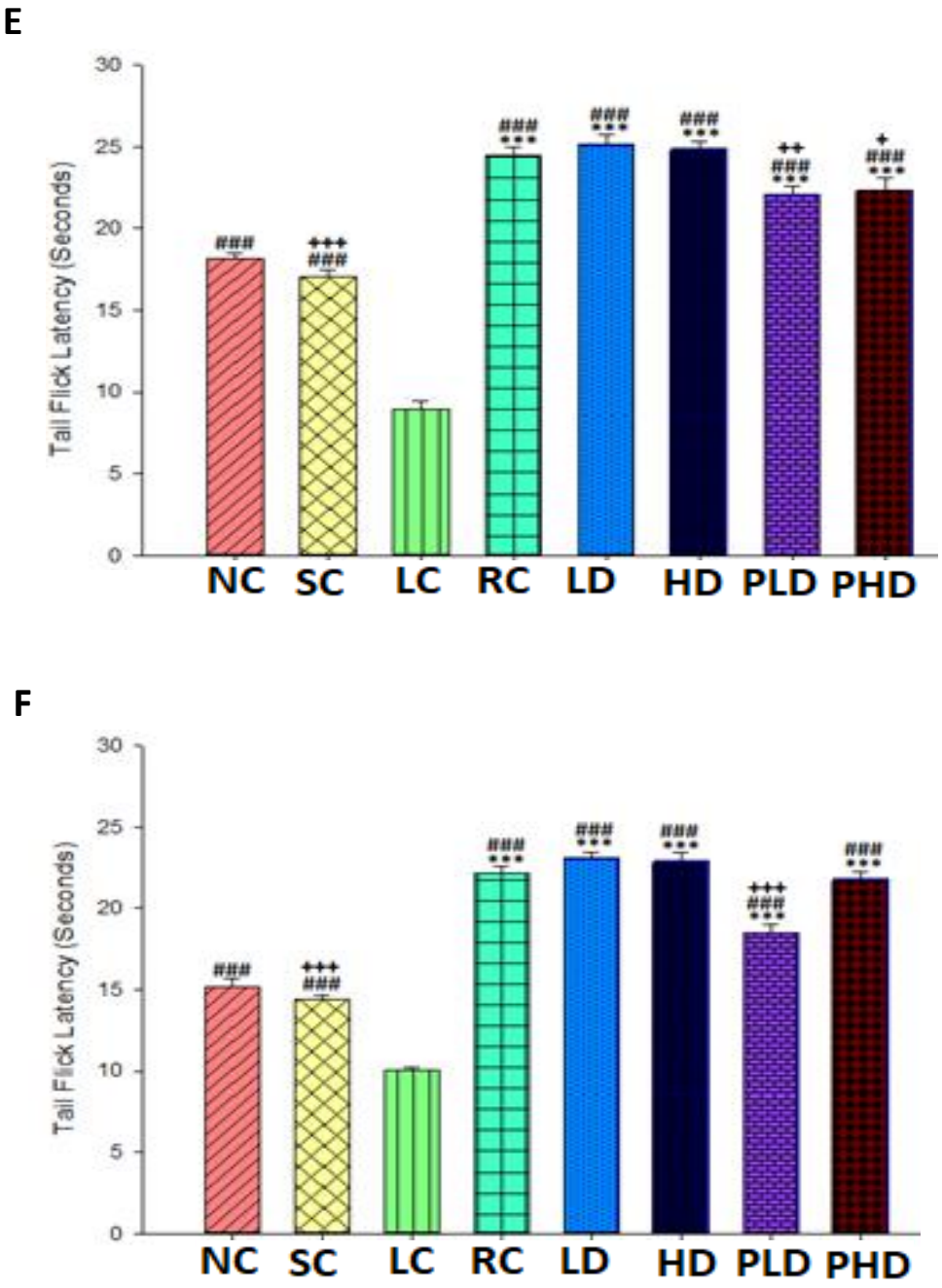

Fig 35: Bromelain reversed cold hyperalgesia in tail-flick immersion test in sciatic nerve-induced neuropathic pain. *Each values represent mean ± SEM of each group. (E) 14th day post-surgery cold flick test (F) 21st day post-surgery cold flick test.* $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ *compared with ligated control;* $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ *compared with normal control;* $^{+}p < 0.05$, $^{++}p < 0.01$, $^{+++}p < 0.001$ *compared with reference control. NC: Unligate Normal Control; SC: Sham Control; LC: Ligated Control; RC: Reference Control (30mg/kg gabapentin); LD: Low Dose Bromelain (30mg/kg); HD: High Dose Bromelain (50mg/kg); PLD: Pretreated Low Dose Bromelain (30mg/kg); PHD: Pretreated High Dose Bromelain (50mg/kg)*

4.8 Hotplate Thermal Hypersensitive Test:

There was increased hypersensitivity to the thermal stimulus (hotplate: 55 ± 0.5°C) in *Wistar* rats whose sciatic nerve was ligated. This was characterized in ligated *Wistar* rats which shows reduced paw withdrawal latency (PWL) that was significantly different ($p < 0.05$) compared with unligated control groups. Bromelain effectively attenuated hyperalgesia caused by noxious temperature by increased dramatically in the PWL. The third-day post-surgical test indicated that high dose bromelain administered group PWL was significantly ($p < 0.05$) higher compared with the unligated control group (14.43 ± 0.35 vs 9.19 ± 0.36 seconds) and all other groups. Treatment with bromelain completely obtunded the developed hyperalgesia to thermal noxious stimulus as there were significant differences between the bromelain treated groups and the ligated control group throughout the test days. It was observed that pretreatment with bromelain prevents the development of thermal hyperalgesia. Indeed, pretreatment with a high dose of bromelain significantly increases the threshold to thermal noxious stimulus compared with the unligated control group, as indicated in figure 36.

Likewise, bromelain showed a high anti-hyperalgesic effect on the third-day post-treatment test that was significantly ($p < 0.05$) higher compared with the gabapentin reference group. However, there was no significant difference ($p > 0.05$) between the gabapentin and bromelain group on the twenty-first-day hotplate test. The bromelain administered groups show dose-dependent responses to a thermal stimulus. It was noted that the PWL of the sham group increased significantly ($P < 0.05$) compared with the ligated control group on the 7th, 14th, and 21st days of post-surgical tests only.

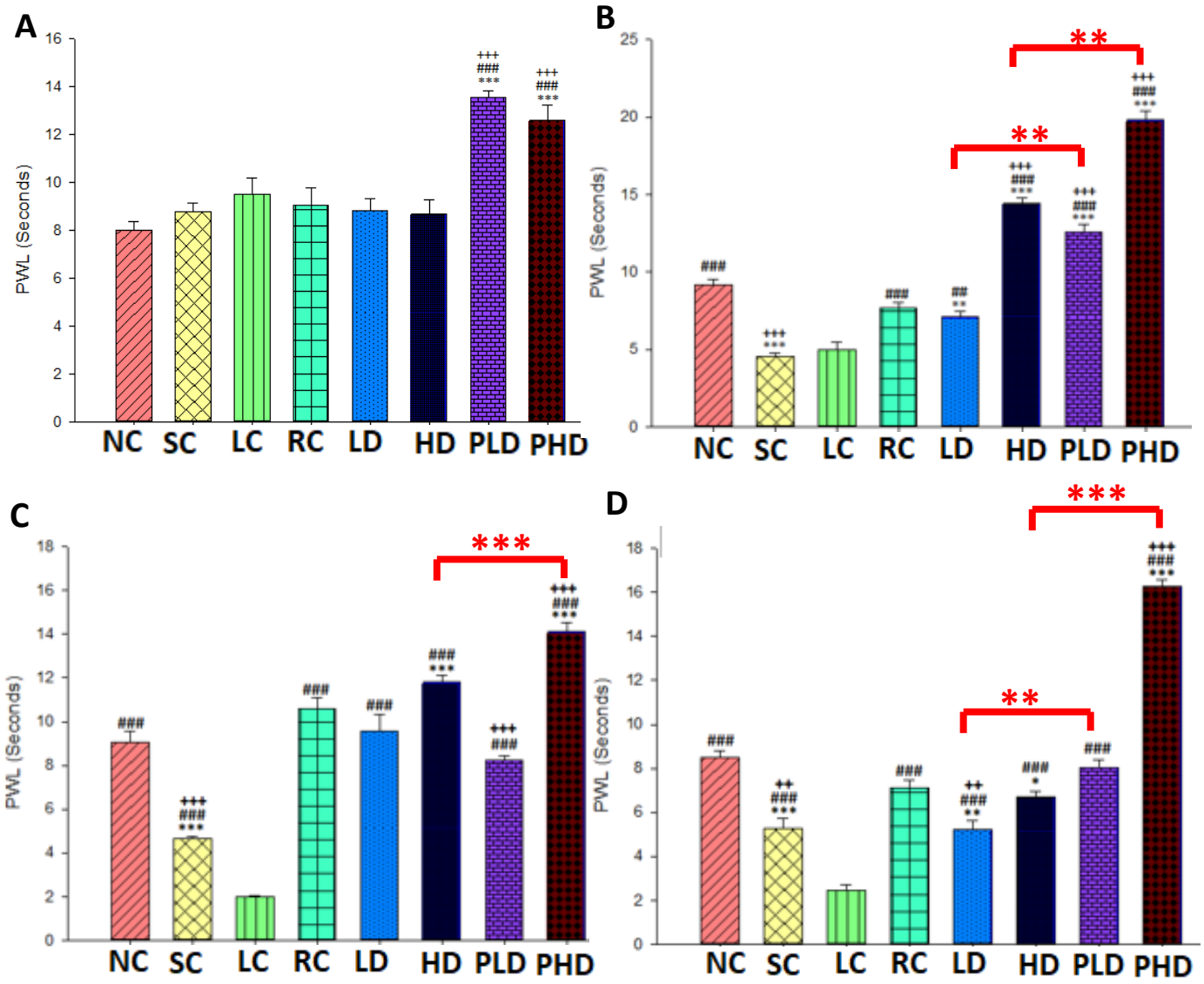

Fig 36: Bromelain reversed thermal hyperalgesia to hotplate in sciatic nerve induced neuropathic pain. *Each values represent mean ± SEM of each group. (A) Presurgical hotplate test. (B) 3rd day post-surgery hotplate test (C) 7th day post-surgery hotplate test (D) 14th day post-surgery hotplate test.* $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ *compared with ligated control;* $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ *compared with normal control;* $^{+}p < 0.05$, $^{++}p < 0.01$, $^{+++}p < 0.001$ *compared with reference control. NC: Unligate Normal Control; SC: Sham Control; LC: Ligated Control; RC: Reference Control (30mg/kg gabapentin); LD: Low Dose Bromelain (30mg/kg); HD: High Dose Bromelain (50mg/kg); PLD: Pretreated Low Dose Bromelain (30mg/kg); PHD: Pretreated High Dose Bromelain (50mg/kg)*

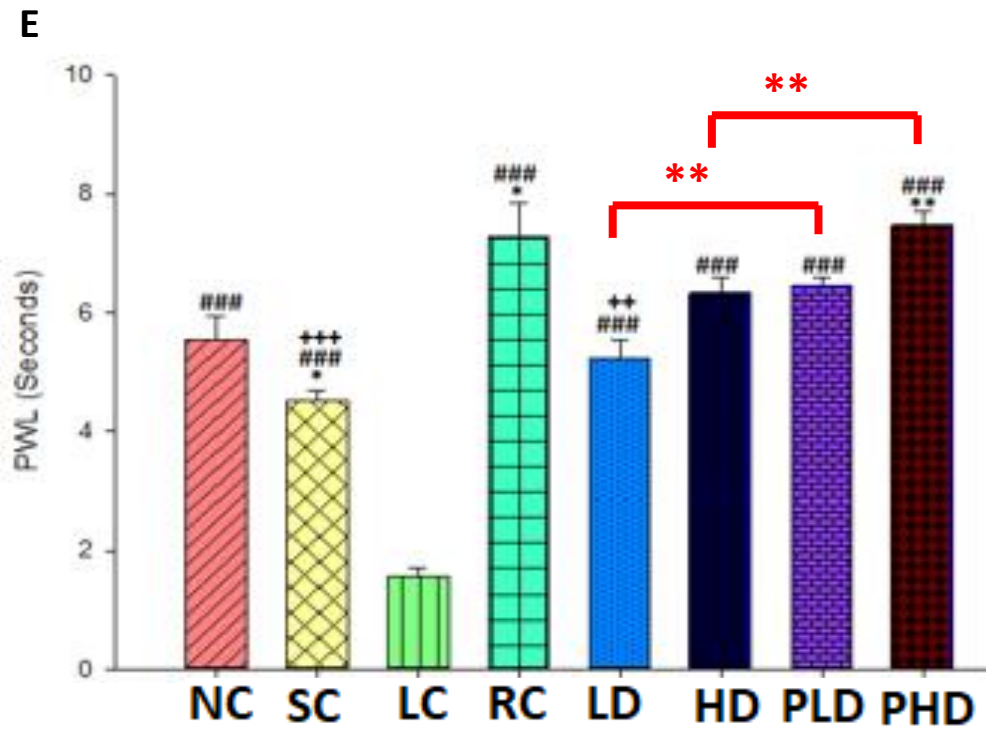

Fig 36: Bromelain reversed thermal hyperalgesia to hotplate in sciatic nerve induced neuropathic pain. *Each values represent mean ± SEM of each group. (E) 21st day post-surgery hotplate test. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ compared with ligated control; *p < 0.05, **p < 0.01, ***p < 0.001 compared with normal control; $^{+}p < 0.05$, $^{++}p < 0.01$, $^{+++}p < 0.001$ compared with reference control. NC: Unligate Normal Control; SC: Sham Control; LC: Ligated Control; RC: Reference Control (30mg/kg gabapentin); LD: Low Dose Bromelain (30mg/kg); HD: High Dose Bromelain (50mg/kg); PLD: Pretreated Low Dose Bromelain (30mg/kg); PHD: Pretreated High Dose Bromelain (50mg/kg)*

4.9 Tail Flick Immersion Thermal Hypersensitive Test

As observed with the hotplate test, CCI shows reduced tail withdrawal latency (TWL) to hot water (55 ± 0.5°C) stimuli which were significantly different ($p < 0.05$) compared with the unligated control group. Treatment with bromelain reversed the observed hyperalgesia, which was characterized by significant increases in TWL compared with the ligated vehicle-treated group, as indicated in figure 37. Both low and high doses of bromelain completely mitigated thermal hyperalgesia as there were no significant differences ($p>0.05$) in the TWL between the bromelain administered groups and the unligated naive group. Bromelain increases the TWL compared with the unligated control group ($p < 0.05$) on the 7th-day test through the 21st-day test. Likewise, there were significant differences between bromelain pretreated and post-treated groups on the 7th-day test through the 21st-day test. Bromelain pretreated groups show better antinociceptive effects to thermal stimulus compared ($p < 0.05$) with gabapentin as it significantly increased the threshold to thermal noxious stimulation.

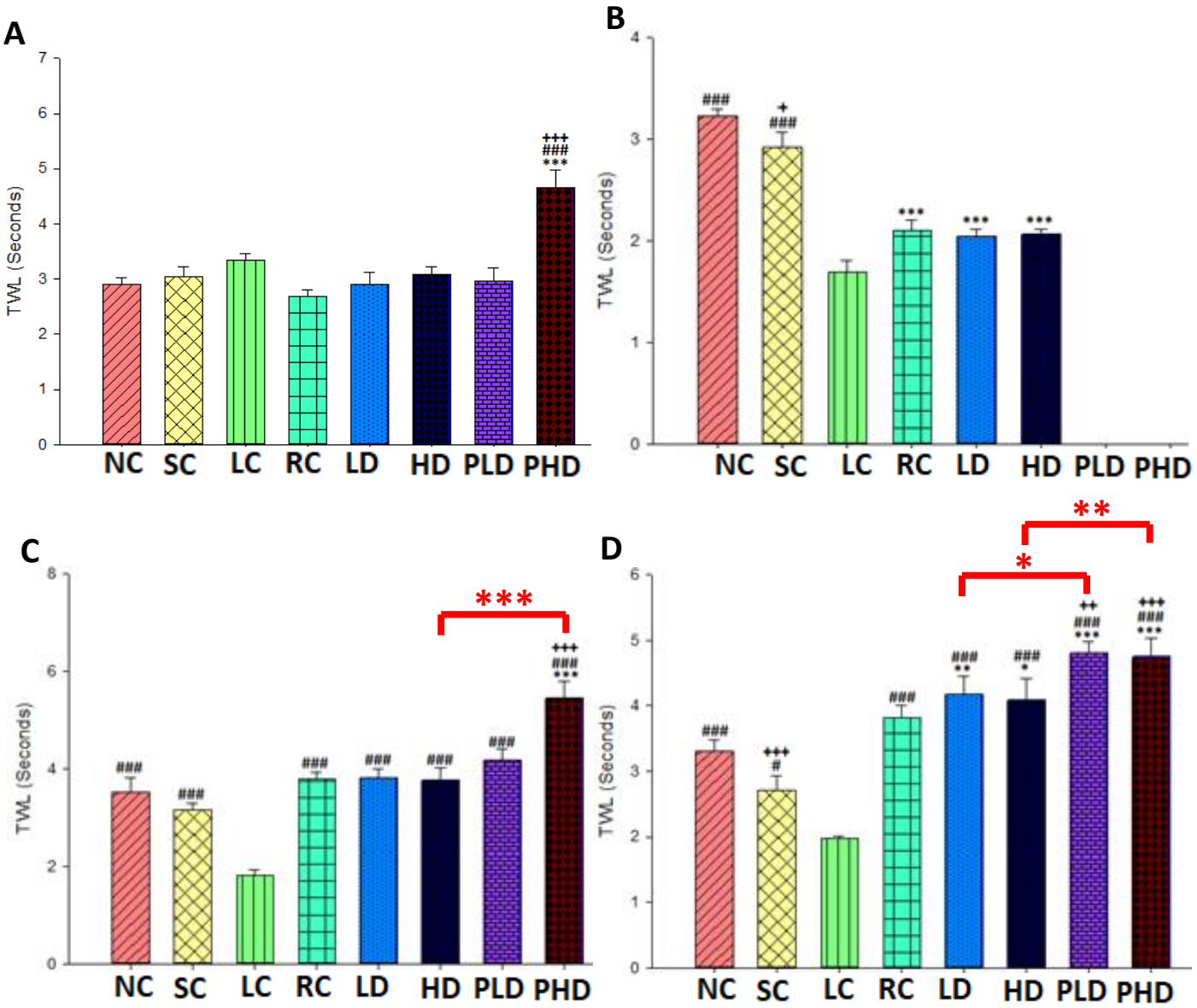

Fig 37: Bromelain reversed thermal hyperalgesia to tail immersion hypersensitive test in sciatic nerve induced neuropathic pain. *Each values represent mean ± SEM of each group. (A) Presurgical baseline tail flick immersion test. (B) Post-surgical Baseline tail flick immersion test (C) 3rd day post-surgery tail flick immersion test (D) 7th day post-surgery tail flick immersion test.* $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ *compared with ligated control;* $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ *compared with normal control;* $^{+}p < 0.05$, $^{++}p < 0.01$, $^{+++}p < 0.001$ *compared with reference control. NC: Unligate Normal Control; SC: Sham Control; LC: Ligated Control; RC: Reference Control (30mg/kg gabapentin); LD: Low Dose Bromelain (30mg/kg); HD: High Dose Bromelain (50mg/kg); PLD: Pretreated Low Dose Bromelain (30mg/kg); PHD: Pretreated High Dose Bromelain (50mg/kg)*

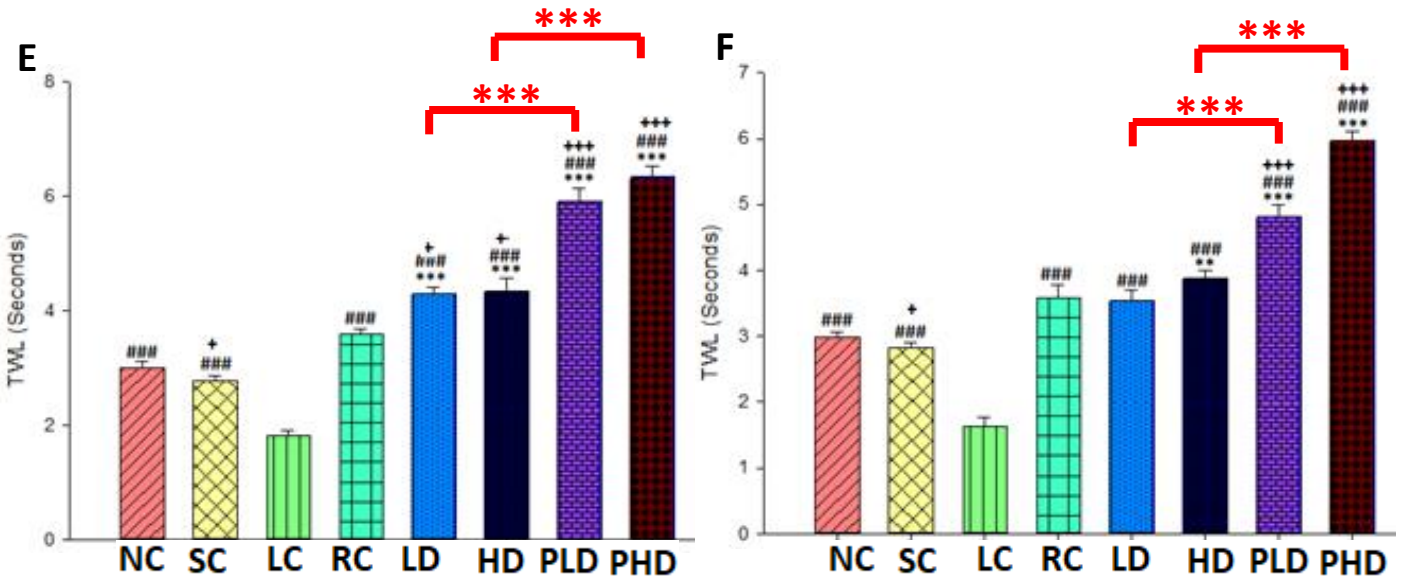

Fig 37: Bromelain reversed thermal hyperalgesia to tail immersion hypersensitive test in sciatic nerve induced neuropathic pain. *Each values represent mean ± SEM of each group. (E) 14th day post-surgery tail flick immersion test (F) 21st day post-surgery tail flick immersion test.* $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ *compared with ligated control;* $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ *compared with normal control;* $^{+}p < 0.05$, $^{++}p < 0.01$, $^{+++}p < 0.001$ *compared with reference control. NC: Unligate Normal Control; SC: Sham Control; LC: Ligated Control; RC: Reference Control (30mg/kg gabapentin); LD: Low Dose Bromelain (30mg/kg); HD: High Dose Bromelain (50mg/kg); PLD: Pretreated Low Dose Bromelain (30mg/kg); PHD: Pretreated High Dose Bromelain (50mg/kg)*

4.10 Sciatic Nerve Functional Index (SFI) Test

The defects in the sciatic nerve functional index observed in ligated animals were significantly higher ($P < 0.05$) compared with the unligated control groups. Bromelain significantly ($P < 0.05$) improved the SFI, as indicated in Fig. 38. It was observed that a high dose post-treated bromelain group, as well as a pretreated bromelain group, showed improved SFI progressively. The improvement was significantly greater ($P < 0.05$) compared with the reference control (30 mg/kg gabapentin) on the 21st day post-surgical SFI test. However, neither treatment with gabapentin nor bromelain restored SFI of ligated *Wistar* rats to the basal level (-8.0) after 21 days of treatment

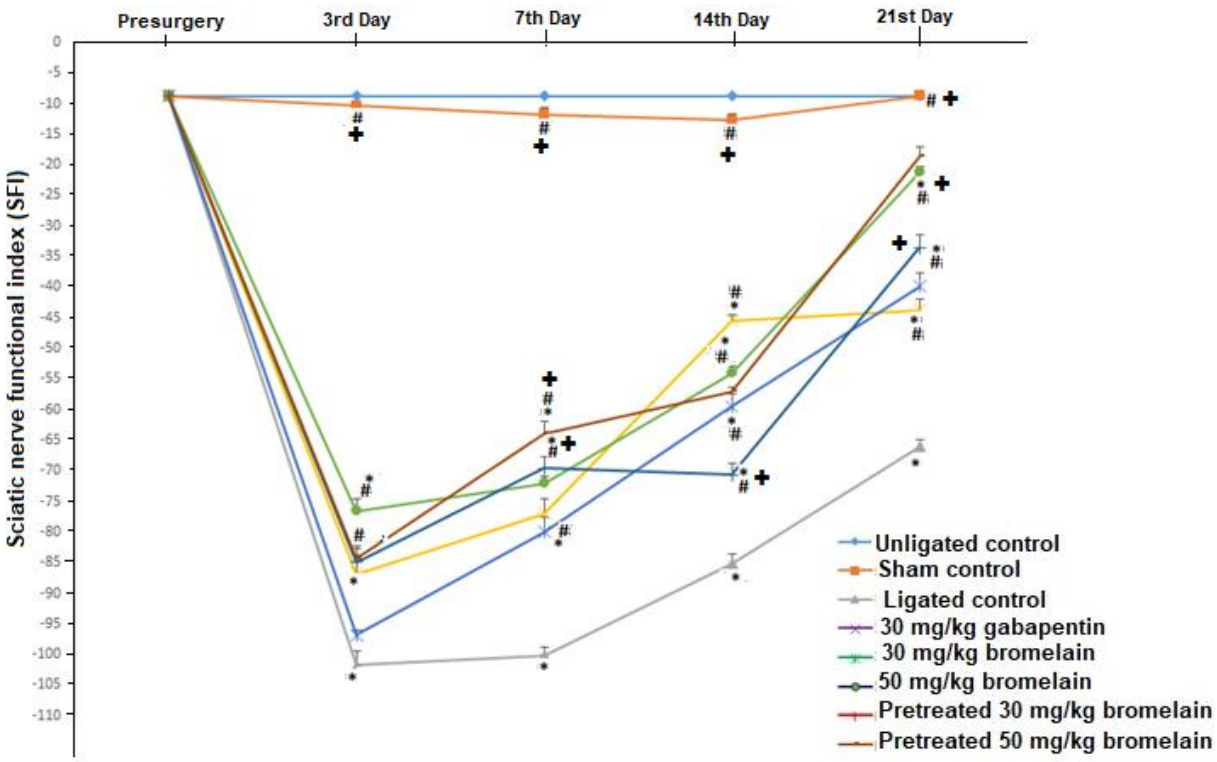

Fig 38: Bromelain improved the physiological function of the sciatic nerve in SFI test. *Each values represent mean ± SEM of each group.* $^{\#}p < 0.05$ *compared with ligated control;* $^{*}p < 0.05$ *compared with unligated control;* $^{+}p < 0.05$ *compared with reference (gabapentin) control. NC: Unligate Normal Control; SC: Sham Control; LC: Ligated Control; RC: Reference Control (30mg/kg gabapentin); LD: Low Dose Bromelain (30mg/kg); HD: High Dose Bromelain (50mg/kg); PLD: Pretreated Low Dose Bromelain (30mg/kg); PHD: Pretreated High Dose Bromelain (50mg/kg)*

4.11 Open Field Tests (OFT)

OFT was used to assess the anxio-depressive effects of bromelain on CCI-induced neuropathic pain model in *Wistar* rats. The locomotive activities, exploratory and anxiety-like behaviours of rats in various groups were indicated in fig. 39 A–J. Chronic constriction injury (CCI) resulted in reduced ambulatory, exploratory, and increased anxiety-like behaviour in ligated *Wistar* rats. There were reduced locomotive activities in both the vertical and horizontal exploration in ligated rats ($p < 0.05$) compared with unligated control. Taken together, pretreated bromelain groups increased the locomotive activities in ligated *Wistar* rats. Pretreatment with bromelain significantly increases ($p < 0.05$) the number of lines crossed but not the rearing frequency in a novel open-field arena. Post-treatment with bromelain does not improve ($p > 0.05$) both vertical and horizontal locomotion activities. Gabapentin, pretreated bromelain, and unligated control groups were indifferent ($p > 0.05$) in the number of lines crossed in the open field maze.

Anxiety-like behaviours characterized by decreases in both centre time duration and frequency were observed in rats induced with CCI, as shown in fig. 39 C-D. Vehicle administered ligated *Wistar* rats do not visit the centre box of the open-field arena. However, pretreatment with bromelain increases the centre time duration and frequency in ligated rats. High dose bromelain post-treated *Wistar* rats significantly showed an increased in the centre frequency but not the time spent in the centre arena. Low dose bromelain post-treated *Wistar* rats were insignificantly different ($p > 0.05$) in both the centre time duration and frequency compared with naive ligated rats. However, both doses of bromelain significantly reduced the time taken for rats to make their first move after being introduced at the centre square of the open field maze compared with ligated control *Wistar* rats. The ligated control group generally spent significant time agitating before making the first successful line cross, as indicated in figure 39 E.

Likewise, chronic constriction injury (CCI) increases the number of stretches and the rate of defecation observed in rats. Stretching and the number of faecal boli produced by CCI ligated rats were significantly reduced ($p<0.05$) following treatment with bromelain or gabapentin as indicated in figure 39 F - G. Bromelain substantially reduces the frequency of stretches and number of faecal boli in ligated *Wistar* rats compared with the ligated control group. There were insignificant differences ($p > 0.05$) in the frequency of stretching between bromelain pretreated, post-treated, and unligated control group. Furthermore, there were decreased in the grooming duration and frequency in the ligated *Wistar* rats. Both pretreated and post-treated bromelain groups significantly increase the grooming duration in rats (figure 39 H). Indeed, CCI showed reduced grooming frequency which was significantly increased following treatment with bromelain. Treatment with gabapentin failed to improve the grooming frequency in ligated rats. Nevertheless, the effect of pretreatment with bromelain was similar to gabapentin as there were no significant differences ($p > 0.05$) between the two in all the parameters measured in the OFT.

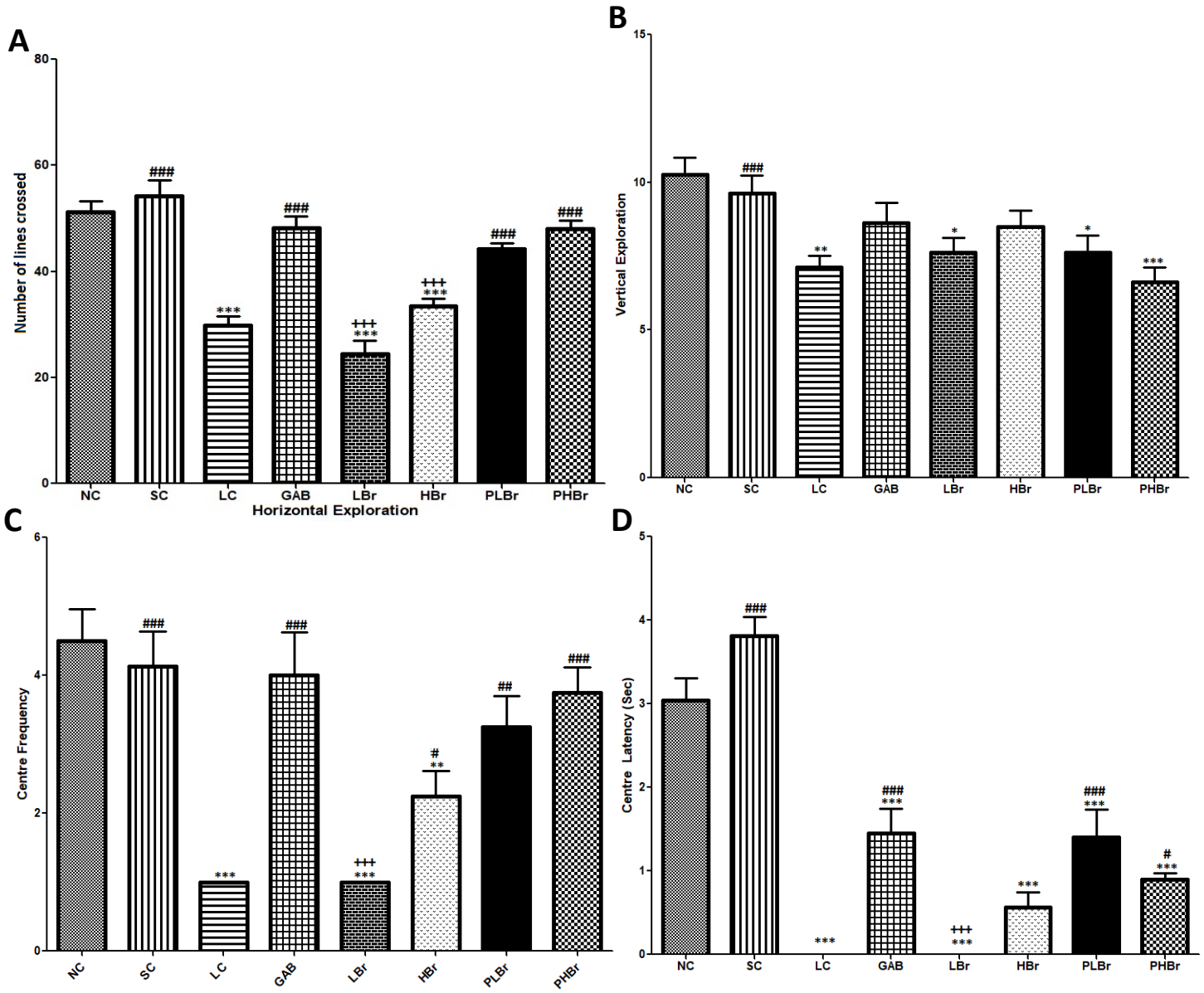

Fig 39: Bromelain reduces anxiety-like behaviour in sciatic nerve induced neuropathic pain. *(A) Horizontal line cross (B) rearing (C) Centre frequency (D) Centre square duration.* $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ *compared with ligated control;* $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ *compared with normal control;* $^{+}p < 0.05$, $^{++}p < 0.01$, $^{+++}p < 0.001$ *compared with reference control. NC: Unligate Normal Control; SC: Sham Control; LC: Ligated Control; RC: Reference Control (30mg/kg gabapentin); LD: Low Dose Bromelain (30mg/kg); HD: High Dose Bromelain (50mg/kg); PLD: Pretreated Low Dose Bromelain (30mg/kg); PHD: Pretreated High Dose Bromelain (50mg/kg)*

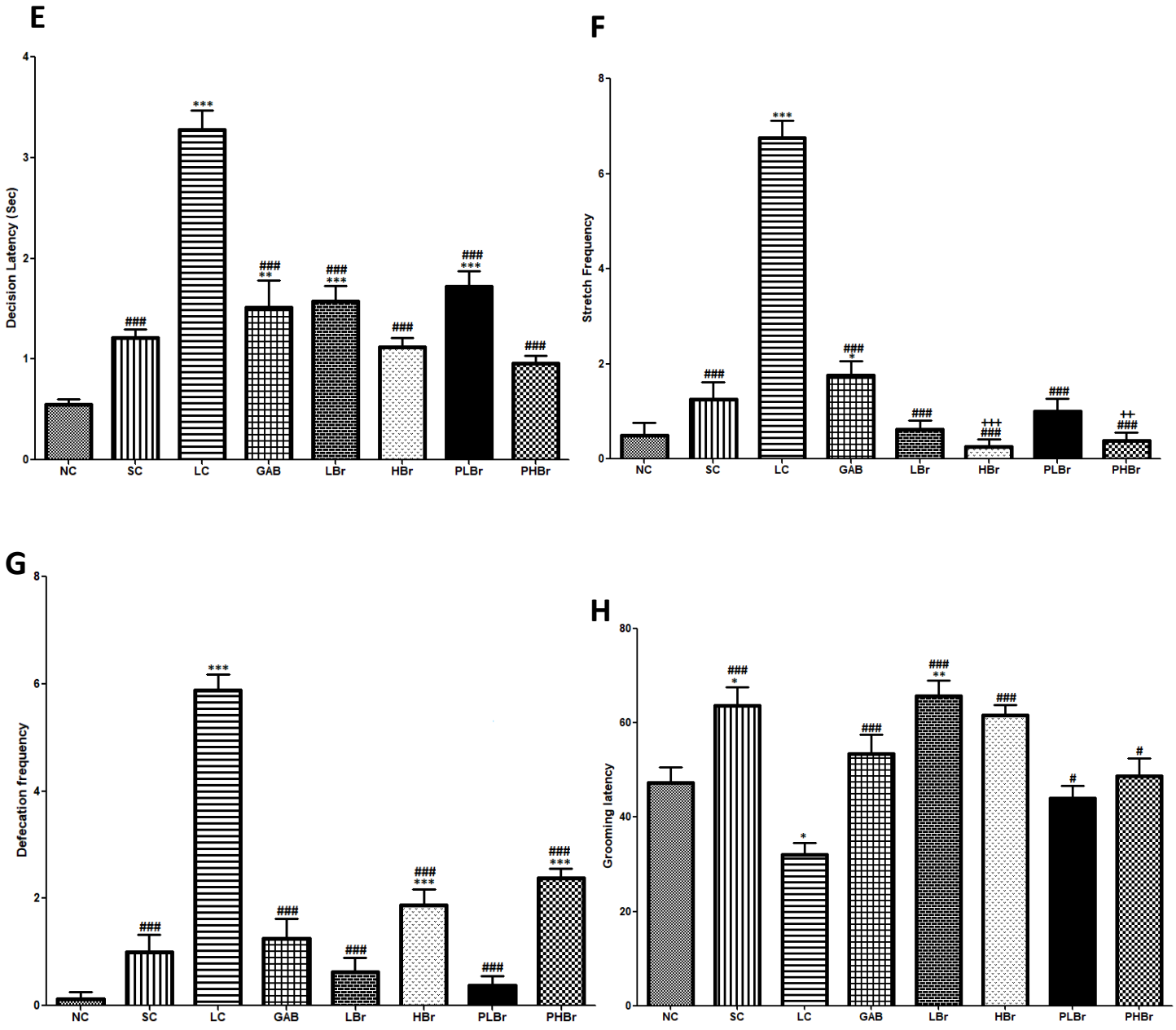

Fig 39: Bromelain reduces anxiety-like behaviour in sciatic nerve induced neuropathic pain. *(E) Decision latency (F) Stretch frequency (G) Defecation frequency (H) Grooming duration. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ compared with ligated control; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ compared with normal control; $^{+}p < 0.05$, $^{++}p < 0.01$, $^{+++}p < 0.001$ compared with reference control. NC: Unligate Normal Control; SC: Sham Control; LC: Ligated Control; RC: Reference Control (30mg/kg gabapentin); LD: Low Dose Bromelain (30mg/kg); HD: High Dose Bromelain (50mg/kg); PLD: Pretreated Low Dose Bromelain (30mg/kg); PHD: Pretreated High Dose Bromelain (50mg/kg)*

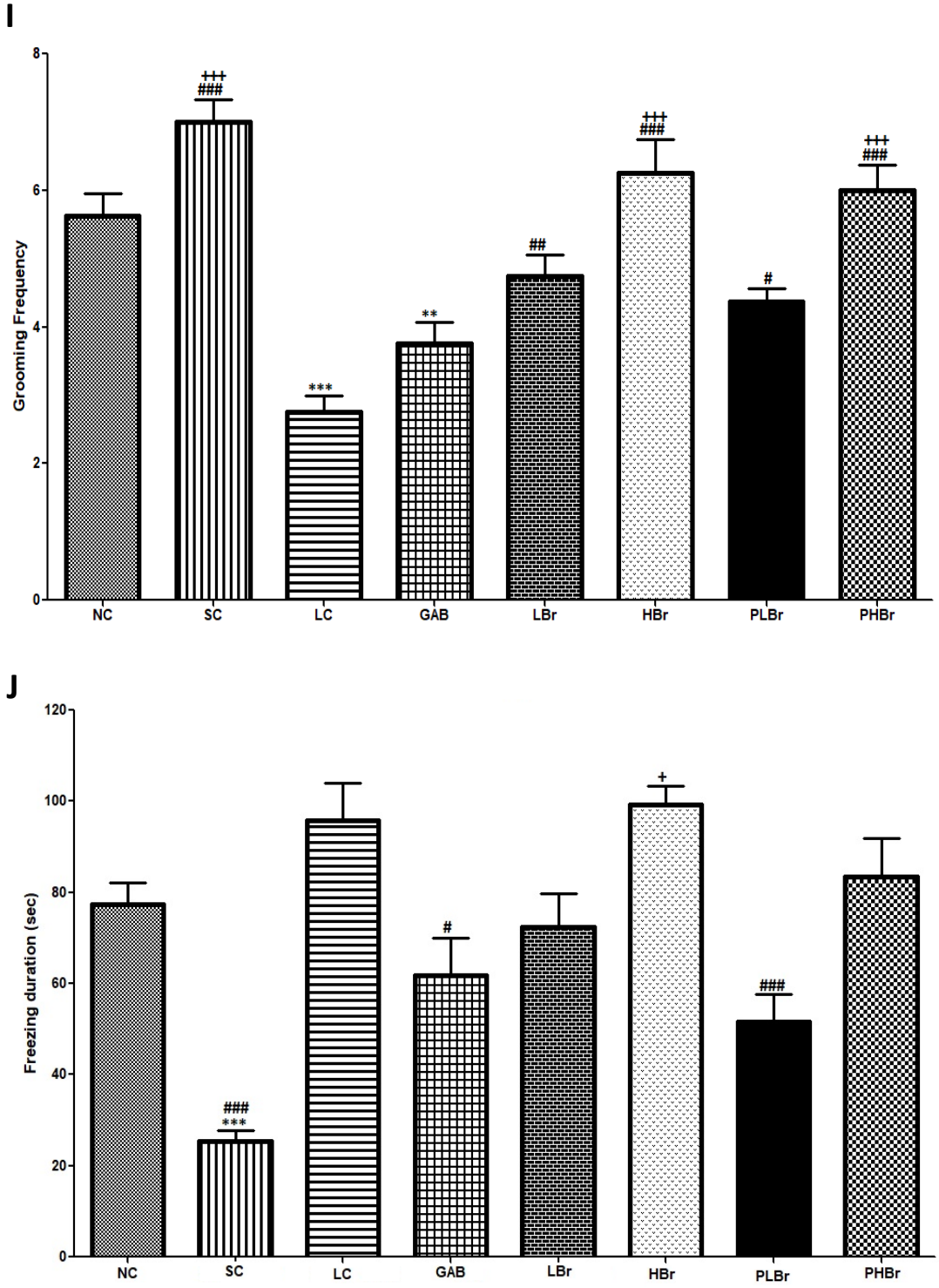

Fig 39: Bromelain reduces anxiety-like behaviour in sciatic nerve induced neuropathic pain. *Each values represent mean ± SEM of each group. (I) Grooming frequency (J) Freezing duration. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ compared with ligated control; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ compared with normal control; $^{+}p < 0.05$, $^{++}p < 0.01$, $^{+++}p < 0.001$ compared with reference control. NC: Unligate Normal Control; SC: Sham Control; LC: Ligated Control; RC: Reference Control (30mg/kg gabapentin); LD: Low Dose Bromelain (30mg/kg); HD: High Dose Bromelain (50mg/kg); PLD: Pretreated Low Dose Bromelain (30mg/kg); PHD: Pretreated High Dose Bromelain (50mg/kg)*

3.12 Elevated Plus Maze Test

Figure 40 A-F illustrated the percentage of time spent at the open-arm, number of entry into the open-arm, percentage of time spent at the close-arm, number of entry into the close-arm, time spent on decision making at the centre (centre duration) and rearing frequency in elevated plus maze (EPM) test. CCI decreased ($p < 0.05$) the time and frequency at which *Wistar* rats enter into the open-arm, rearing frequency, centre time duration. There was a significant increase in the time duration at which CCI rats spent in the closed-arm compartment. It was observed that pretreatment with bromelain and high dose post-treated bromelain groups significantly increased the percentage of time spent in the open-arm and centre time duration in the EPM test. Both pretreated and high dose bromelain post-treated group shows significant decreases in the percentage of time spent in the close-arm. Bromelain generally increased significantly the frequency at which ligated rats visit the open arms. However, neither the pretreated nor the post-treated bromelain group affect the rearing frequency. There were no significant differences ($p > 0.05$) between the pretreated bromelain group and unligated group in the percentage of time spent at the open-arm, open-arm entry frequency, percentage of time spent at the close-arm, and close-arm entry frequency. Likewise, there were insignificant differences ($p > 0.05$) in the time spent at the centre of EPM between high dose pretreated bromelain, post-treated bromelain, and unligated control group.

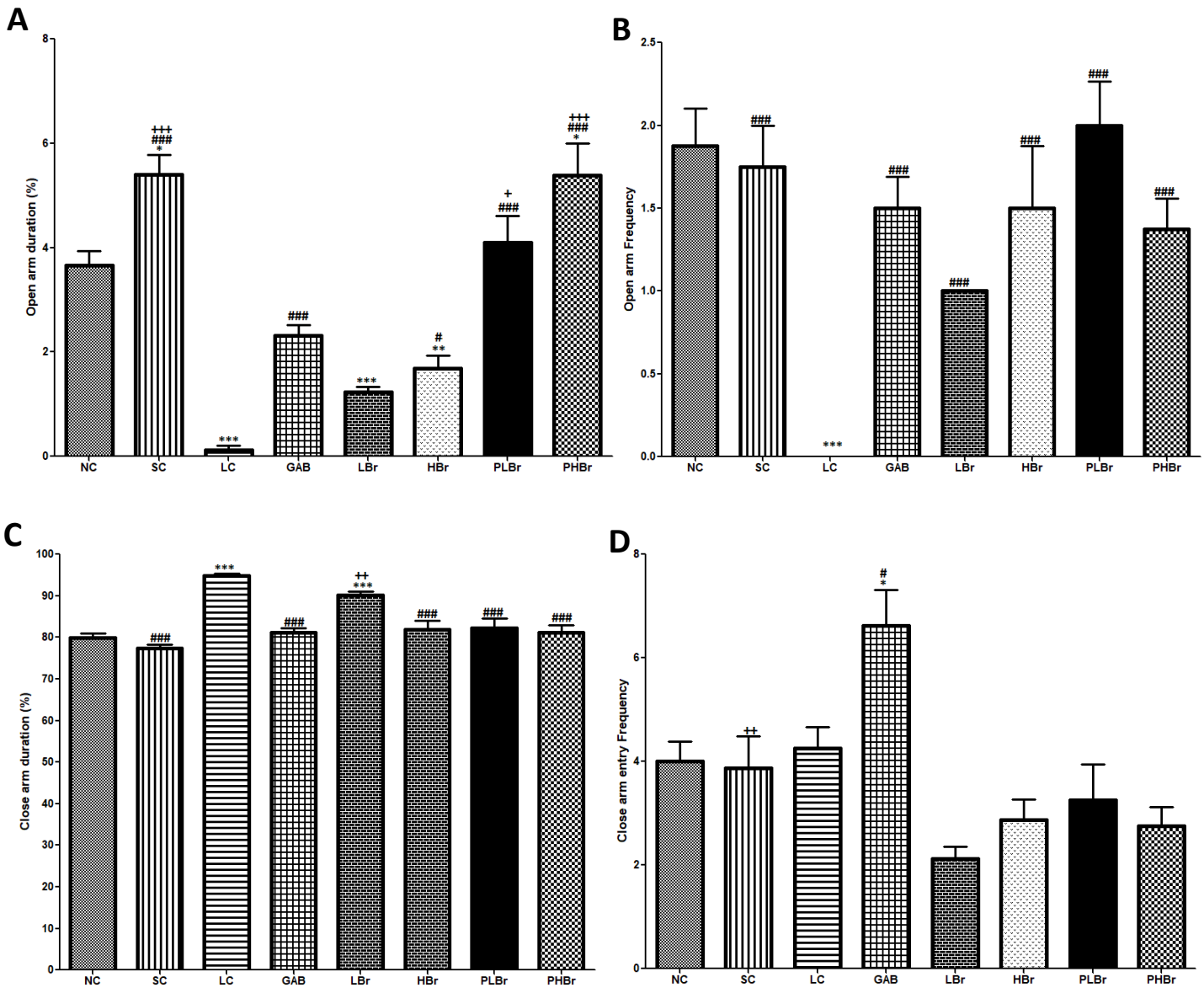

Fig 40: Bromelain reduces anxiety-like behaviour in sciatic nerve induced neuropathic pain. *Each values represent mean ± SEM of each group. (A) Percentage of time spent at the open arm of the elevated maze (B) Number of entry into the open arm of the maze (C) Percentage of time spent at the close arm of the elevated maze (D) Number of entry into the close arm of the maze.* $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ *compared with ligated control;* $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ *compared with normal control;* $^{+}p < 0.05$, $^{++}p < 0.01$, $^{+++}p < 0.001$ *compared with reference control. NC: Unligate Normal Control; SC: Sham Control; LC: Ligated Control; RC: Reference Control (30mg/kg gabapentin); LD: Low Dose Bromelain (30mg/kg); HD: High Dose Bromelain (50mg/kg); PLD: Pretreated Low Dose Bromelain (30mg/kg); PHD: Pretreated High Dose Bromelain (50mg/kg)*

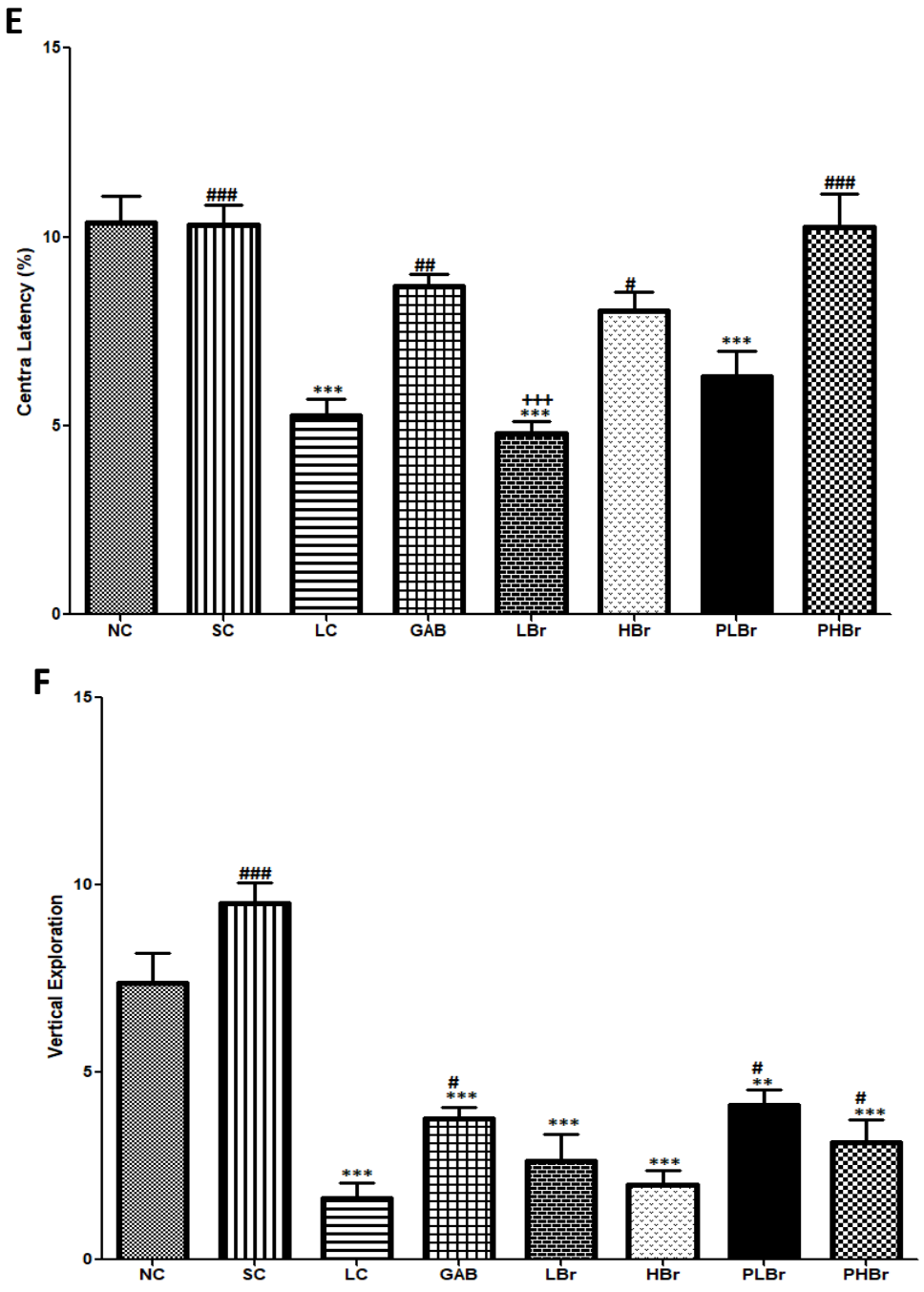

Fig 40: Bromelain reduces anxiety-like behaviour in sciatic nerve induced neuropathic pain. *Each values represent the mean ± SEM of each group. (E) Time spent on decision making at the centre (centre duration) (F) Rearing frequency. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ compared with ligated control; $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ compared with normal control; $^{+}p < 0.05$, $^{++}p < 0.01$, $^{+++}p < 0.001$ compared with reference control. NC: Unligate Normal Control; SC: Sham Control; LC: Ligated Control; RC: Reference Control (30mg/kg gabapentin); LD: Low Dose Bromelain (30mg/kg); HD: High Dose Bromelain (50mg/kg); PLD: Pretreated Low Dose Bromelain (30mg/kg); PHD: Pretreated High Dose Bromelain (50mg/kg)*

4.13 Effect of Bromelain on Brain Weight

There was insignificant ($p > 0.05$) decreased in the effect of CCI on the brain of *Wistar* rats, as illustrated in figure 41. Likewise, bromelain insignificantly increases the mass of the *Wistar* rats brain after twenty-one days of treatment.

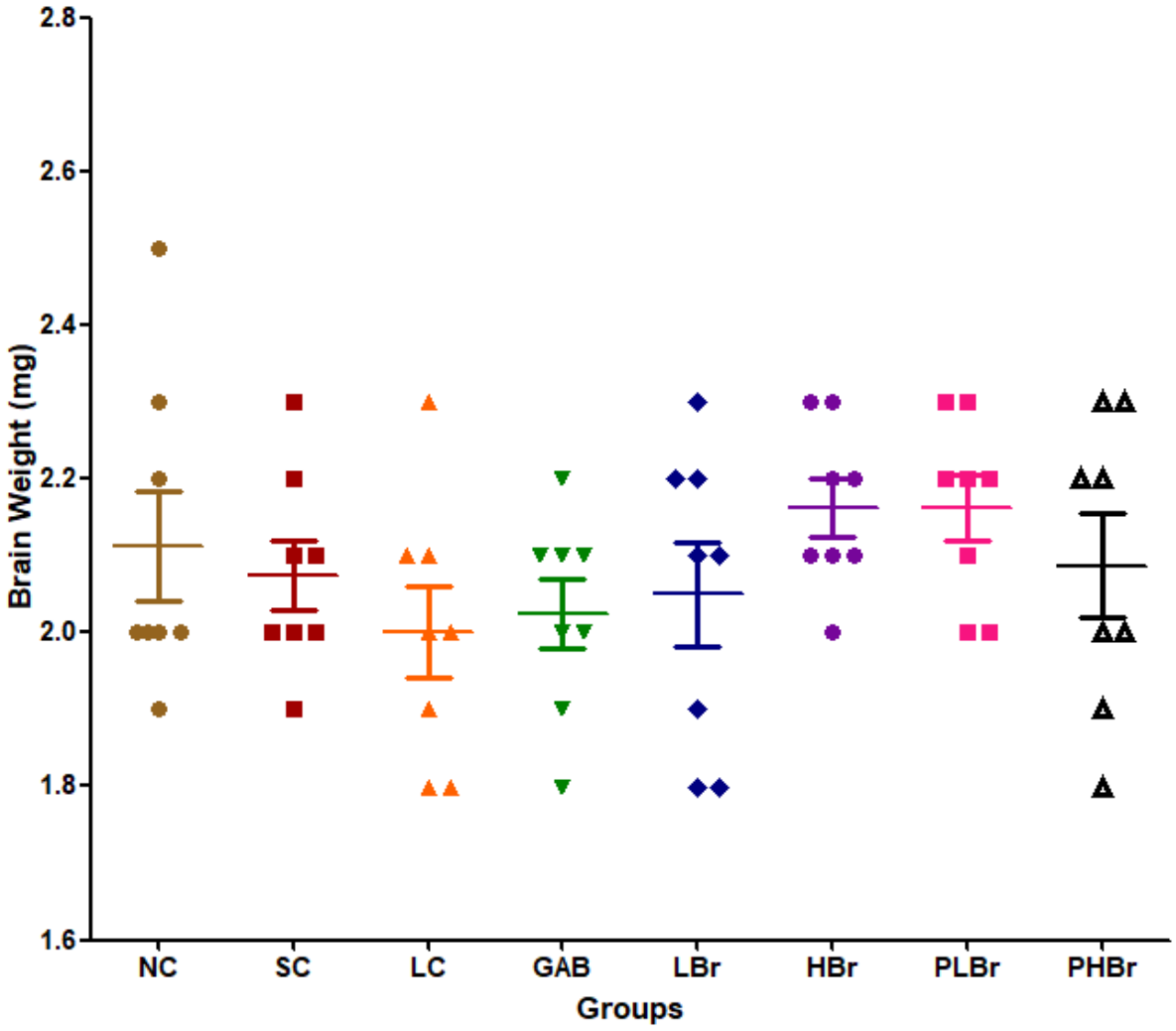

Fig 41: Bromelain insignificantly (p > 0.05) increases the brain weight of Wistar rats in sciatic nerve induced neuropathic pain. *Each value represents the mean ± SEM of each group. NC: Unligate Normal Control; SC: Sham Control; LC: Ligated Control; RC: Reference Control (30mg/kg gabapentin); LD: Low Dose Bromelain (30mg/kg); HD: High Dose Bromelain (50mg/kg); PLD: Pretreated Low Dose Bromelain (30mg/kg); PHD: Pretreated High Dose Bromelain (50mg/kg)*

4.14 Effect of Bromelain on neuronal protein level

Chronic constriction injury-induced neuropathic pain rats showed significantly ($p < 0.05$) reduced total protein level in the brain of *Wistar* rats. However, there was insignificantly ($p > 0.05$) reduction in the sciatic nerve, as indicated in figure 42. Pretreatment with a high dose of bromelain significantly ($p < 0.05$) increases the brain protein level. Both pretreated and post-treated bromelain groups show significant increases in the sciatic nerve total protein level, as indicated in figure 42 B.

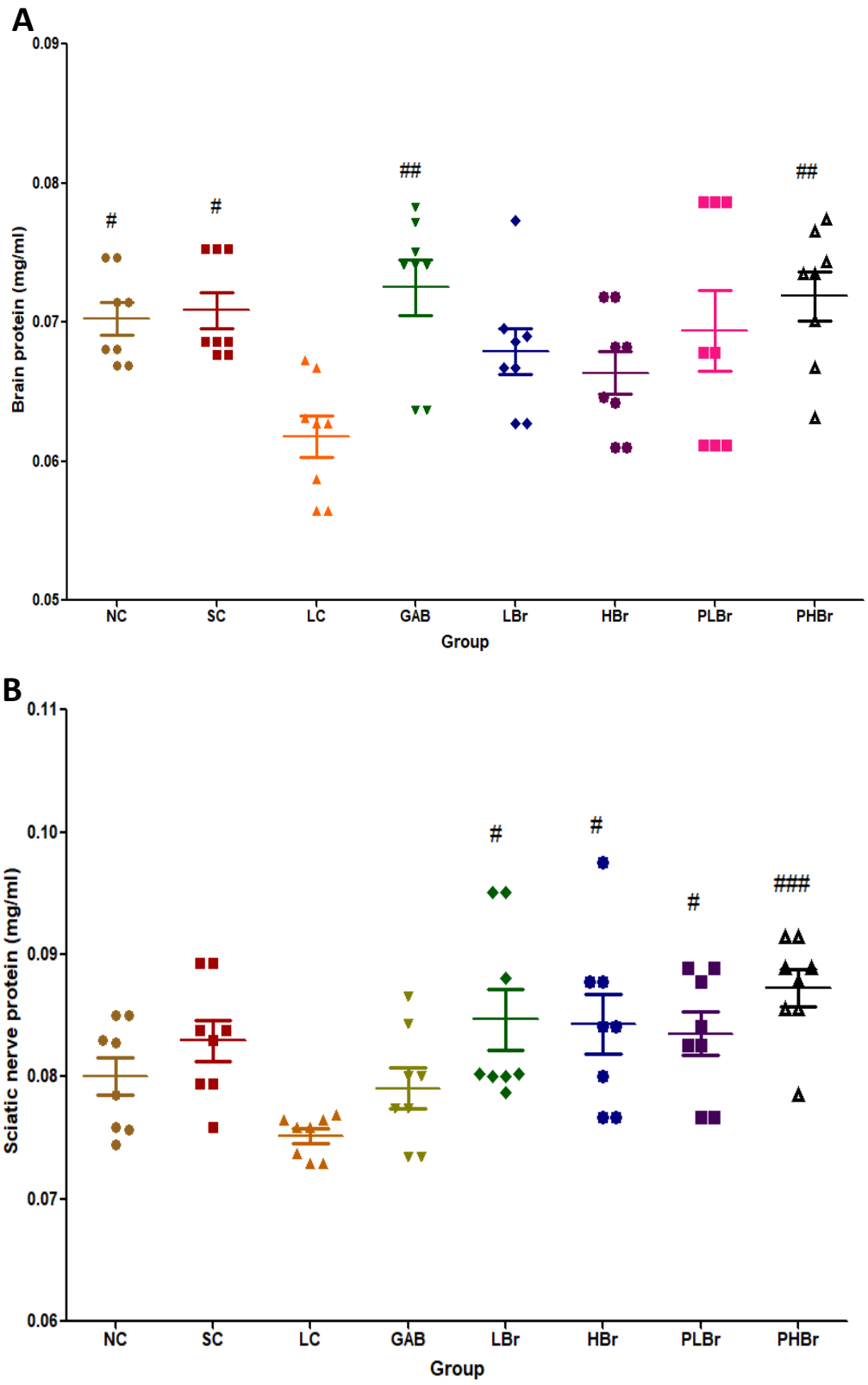

Fig 42: Effect of bromelain on tissue protein in sciatic nerve induced neuropathic pain. *Each values represent the mean ± SEM of each group. (A) Total protein in the rats' brain (mg/ml) (B) Total protein in the rats' sciatic nerve (mg/ml).* $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ *compared with ligated control. NC: Unligate Normal Control; SC: Sham Control; LC: Ligated Control; RC: Reference Control (30mg/kg gabapentin); LD: Low Dose Bromelain (30mg/kg); HD: High Dose Bromelain (50mg/kg); PLD: Pretreated Low Dose Bromelain (30mg/kg); PHD: Pretreated High Dose Bromelain (50mg/kg)*

4.15 Effect of Bromelain on Malondialdehyde (MDA) Level in CCI *Wistar* Rats

There were significant increases in the lipid peroxidation level in the sciatic nerve and blood plasma in the ligated *Wistar* rats compared with the unligated control group. CCI induces an increase in the MDA concentrations in the sciatic nerve and blood plasma. Treatment with bromelain significantly ($p < 0.05$ mitigated increases in lipid peroxidation level as indicated by the reduced MDA concentration observed. There was an insignificant difference ($p>0.05$) in the MDA level between bromelain treated group and unligated control group. Conversely, pretreated high dose bromelain groups showed significant ($p<0.05$) reduced MDA concentration in the sciatic nerve compared with the unligated control group (figure 43 A).

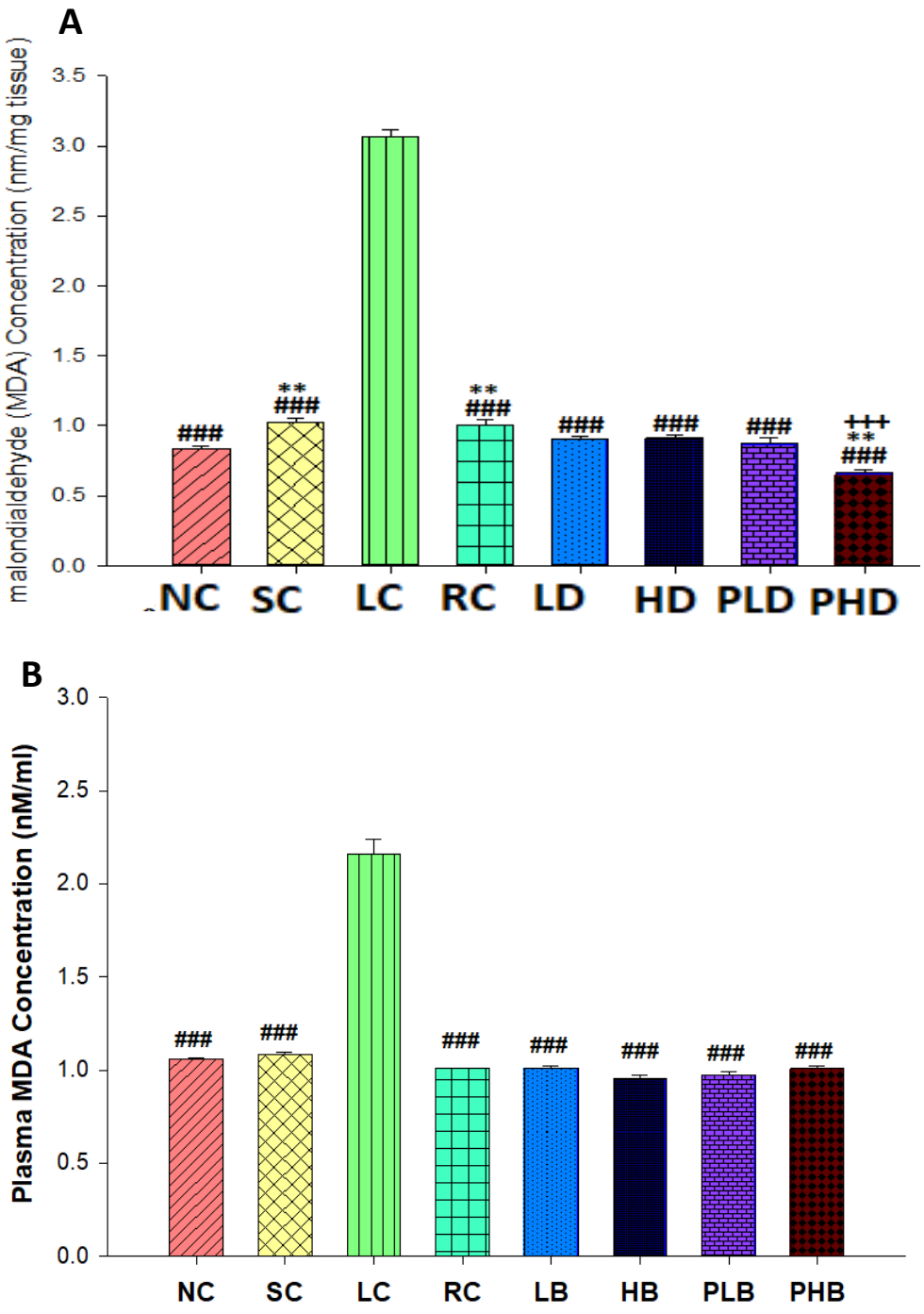

Fig 43: Bromelain reduces MDA level in sciatic nerve-induced neuropathic pain. *Each values represent mean ± SEM of each group. (A) MDA level in the sciatic nerve (B) MDA level in the blood plasma. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ compared with ligated control; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ compared with normal control; $^{+}p < 0.05$, $^{++}p < 0.01$, $^{+++}p < 0.001$ compared with reference control. NC: Unligate Normal Control; SC: Sham Control; LC: Ligated Control; RC: Reference Control (30mg/kg gabapentin); LD: Low Dose Bromelain (30mg/kg); HD: High Dose Bromelain (50mg/kg); PLD: Pretreated Low Dose Bromelain (30mg/kg); PHD: Pretreated High Dose Bromelain (50mg/kg)*

4.16 Effect of Bromelain on Antioxidant Enzymes (Superoxide Dismutase [SOD], Catalase and Reduced Glutathione [GSH]) in CCI rats

Chronic constriction injury significantly reduced the activities of antioxidant enzymes (SOD and GSH) in the blood plasma and sciatic nerve (figure 44 A-F). CCI significantly ($p > 0.05$) reduced blood plasma catalase activities. However, there was an insignificant different ($p > 0.05$) between the sciatic nerve catalase activities across all the groups, as indicated in figure 44 E-F. It was observed both SOD activities, and GSH concentration increased significantly ($p< 0.05$) in the blood plasma as well as in the sciatic nerve after twenty-one days treatment with bromelain. Likewise, bromelain substantially increases the blood plasma catalase activities compared with the ligated control group but has no significant effect on sciatic nerves catalase activities. The effect of bromelain on the concentration level of GSH was more pronounced compared with the gabapentin treated group, as indicated in Fig 44 C-D. The study showed that there was a significant ($P < 0.05$) increased in the sciatic nerve SOD activities of the groups administered with bromelain compared with the unligated control group (18.68 ± 0.45 U/mg protein vs 6.58 ± 0.18 U/ mg proteins).

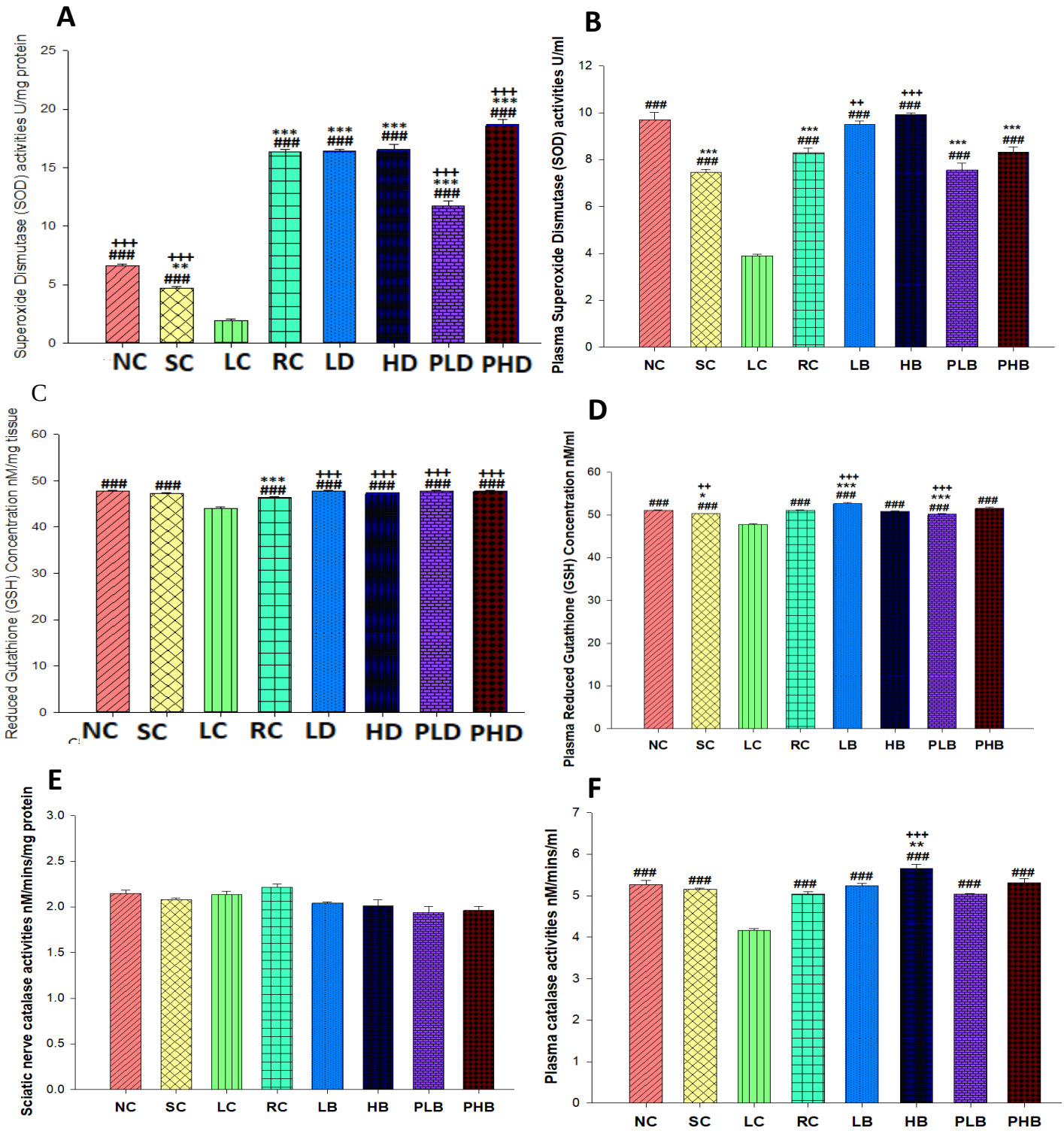

Fig 44: Bromelain increases antioxidant enzymes activities in sciatic nerve-induced neuropathic pain. *Each values represent mean ± SEM of each group. (A) SOD level in sciatic nerve (B) SOD level in the blood plasma (C) GSH level in sciatic nerve (D) GSH level in the blood plasma (E) Catalase level in sciatic nerve (F) Catalase level in the blood plasma.* $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ *compared with ligated control;* $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ *compared with normal control;* $^{+}p < 0.05$, $^{++}p < 0.01$, $^{+++}p < 0.001$ *compared with reference control. NC: Unligate Normal Control; SC: Sham Control; LC: Ligated Control; RC: Reference Control (30mg/kg gabapentin); LD: Low Dose Bromelain (30mg/kg); HD: High Dose Bromelain (50mg/kg); PLD: Pretreated Low Dose Bromelain (30mg/kg); PHD: Pretreated High Dose Bromelain (50mg/kg)*

4.17 Effect of Bromelain on Nitric Oxide (NO_2) Level in CCI rats

Chronic constriction injury induces increased in the sciatic nerve and blood plasma nitrite concentration which was significantly different from the unligated control group. Raised in the nitrite concentration was ameliorated following twenty-one days treatment with bromelain, as represented in figure 45 A-B. Bromelain completely reversed increased nitrite level in the plasma as there was no significant difference between the bromelain treated group and unligated control group. Furthermore, the was no significant difference ($p<0.05$) in the sciatic nerve nitrite concentration between the pretreated high dose bromelain treated group and unligated control group. The post-treated high dose and pretreated bromelain groups were significantly different ($p<0.05$) in the sciatic nerve nitrite concentration compared with the gabapentin treated group.

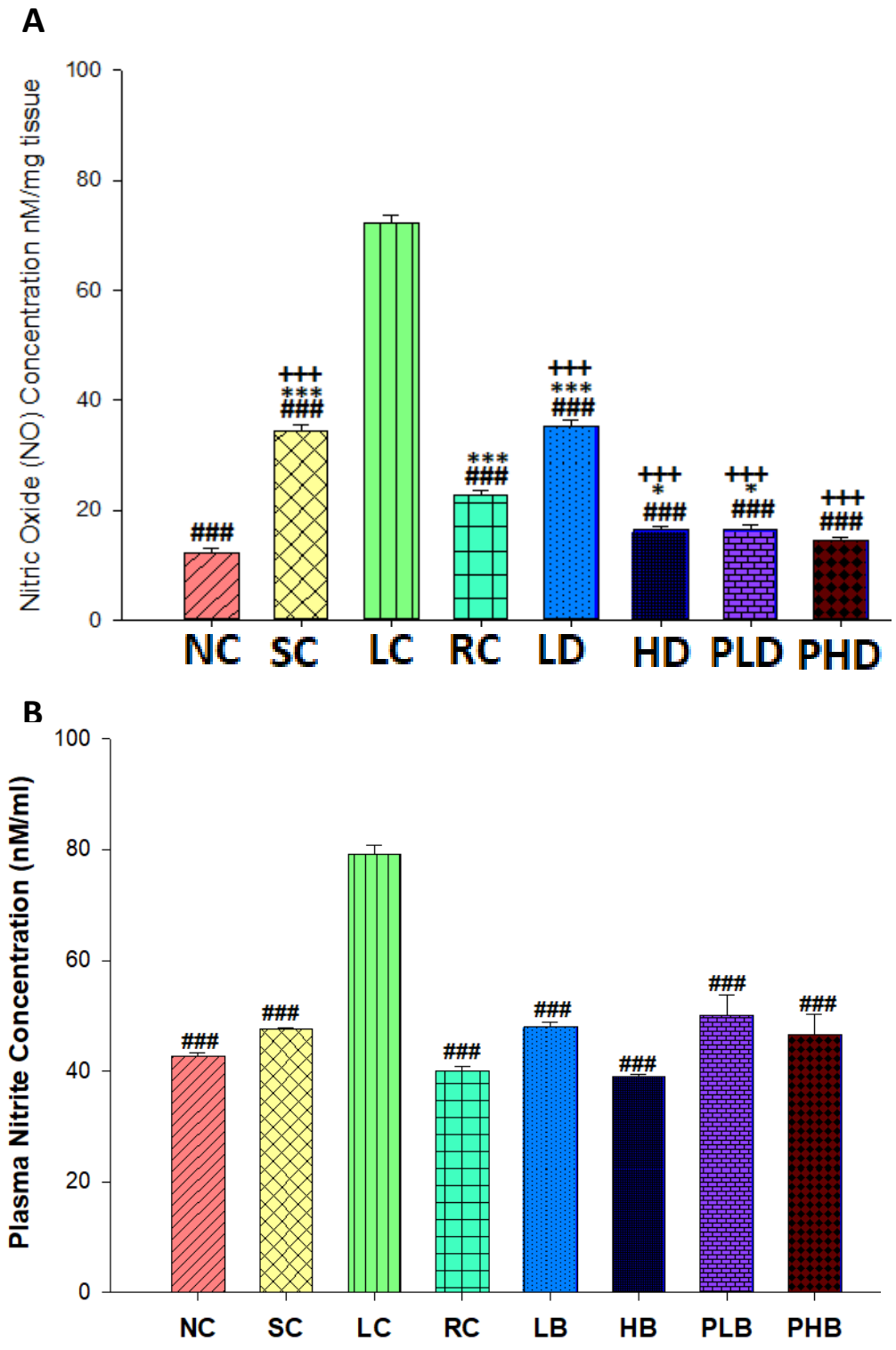

Fig 45: Bromelain reduces plasma and sciatic nerve nitrite level in sciatic nerve-induced neuropathic pain. *Each values represent mean ± SEM of each group. (A) Nitrite level in sciatic nerve (B) nitrite level in the blood plasma. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ compared with ligated control; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ compared with normal control; $^{+}p < 0.05$, $^{++}p < 0.01$, $^{+++}p < 0.001$ compared with reference control. NC: Unligate Normal Control; SC: Sham Control; LC: Ligated Control; RC: Reference Control (30mg/kg gabapentin); LD: Low Dose Bromelain (30mg/kg); HD: High Dose Bromelain (50mg/kg); PLD: Pretreated Low Dose Bromelain (30mg/kg); PHD: Pretreated High Dose Bromelain (50mg/kg)*

4.18 Effect of Bromelain on Sciatic Nerve Nuclear Transcription Factors in CCI rats

Chronic constriction injury (CCI) resulted in reduced activities of nuclear factor erythroid-derived-2-related factors- 1 and 2 (NrF-1 and NrF-2). There was significant reduction in the activities of NrF-1 and NrF-2 in the sciatic nerve of ligated *Wistar rats* compared with unligated control (Fig 46 A-B). At the end of 21 days of bromelain administration, there were significant ($P < 0.05$) increased in nuclear transcription factors NrF-1 (14.42 ± 0.32 μg/mL/mg protein vs 11.05 ± 0.24 μg/mL/mg protein) and NrF-2 (34.50 ± 1.61 μg/mL/mg protein vs 18.26 ± 1.06 μg/ mL/mg protein) compared with the ligated control group. Bromelain completely reversed the detrimental effect of CCI on NrF-1and NrF-2 activities back to the basal level. These effects were more pronounced in groups administered with a high dose of bromelain. There was no significant difference between the pretreated and post-treated bromelain groups.

The activities of nuclear factor Kappa light chain enhancer B-cell inhibitor (NFκB) in the sciatic nerve also increases significantly in rats induced with CCI compared with the unligated control group. Treatment with various doses of bromelain significantly reversed this effect. The effect of bromelain was insignificantly different from unligated control. Furthermore, the pretreated bromelain group shows significant reduced NFκB activities compared with the gabapentin administered group, as indicated in figure 48 C.

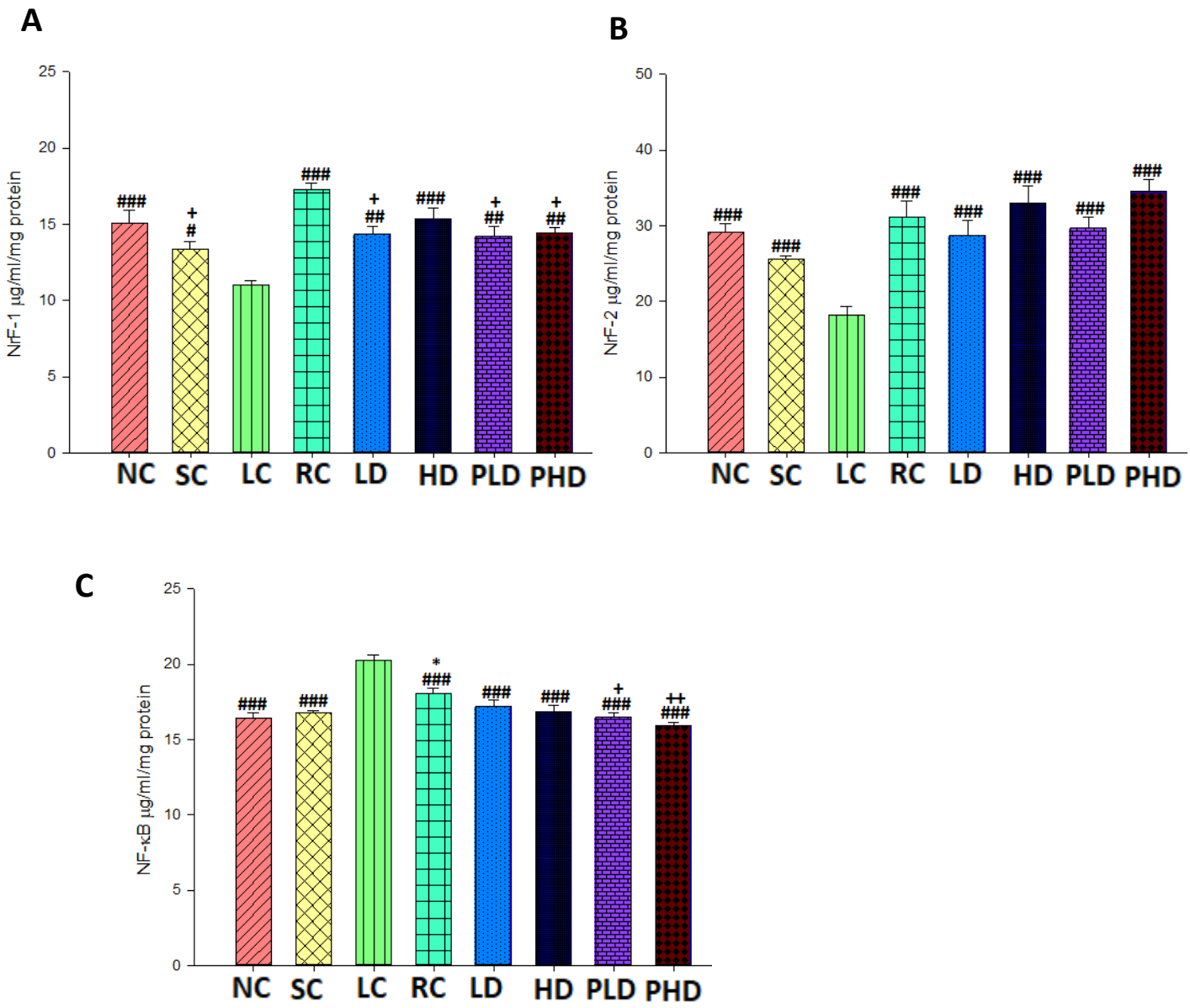

Fig 46: Effect of bromelain on sciatic nerve nuclear transcription factors. *Each values represent mean ± SEM of each group. (A) Nuclear factor erythroid-derived 2 related factor-1 (B) Nuclear factor erythroid-derived 2 related factor-2 (C) Nuclear factor kappa-light-chain enhancer of activated B-cells (NF-kB).* $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ *compared with ligated control;* $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ *compared with normal control;* $^{+}p < 0.05$, $^{++}p < 0.01$, $^{+++}p < 0.001$ *compared with reference control. NC: Unligate Normal Control; SC: Sham Control; LC: Ligated Control; RC: Reference Control (30mg/kg gabapentin); LD: Low Dose Bromelain (30mg/kg); HD: High Dose Bromelain (50mg/kg); PLD: Pretreated Low Dose Bromelain (30mg/kg); PHD: Pretreated High Dose Bromelain (50mg/kg)*

4.19 Effect of Bromelain on Sciatic Nerve Ionic concentration in CCI rats

Chronic constriction of the sciatic nerve resulted in the derangement of the electrolyte levels in the sciatic nerve. There were significant increases in sodium (34.49 ± 1.17 vs 40.96 ± 1.24 mg/ml/ mg protein) and calcium (1.70 ± 0.21 vs 6.65 ± 0.30 mg/ml/mg protein) ions concentration and decreases in potassium (2.30 ± 0.03 vs 0.36 ± 0.05 mg/ml/mg protein) and chloride (18.26 ± 0.27 vs 15.82 ± 0.21 mg/ml/mg protein) ions concentration in ligated *Wistar* rats compared with unligated naive groups, as shown in Fig. 47 A-D. Treatment with bromelain (pretreatment and post-treatment) significantly decreased sodium and calcium levels in the sciatic nerve, while the levels of potassium and chloride concentration were increased.

CCI increases the activities of calcium-ATPase and sodium-potassium pumps which were significantly different ($p < 0.05$) compared with the unligated control group. Treatment with bromelain significantly mitigated increases in Ca^{2+} ATPase activities (13.68 ± 0.27 vs 16.90 ± 0.50 ngiP/min/mg protein). However, neither bromelain nor gabapentin has a significant effect ($p > 0.05$) on the activities of Na^{+}-K^{+} ATPase.

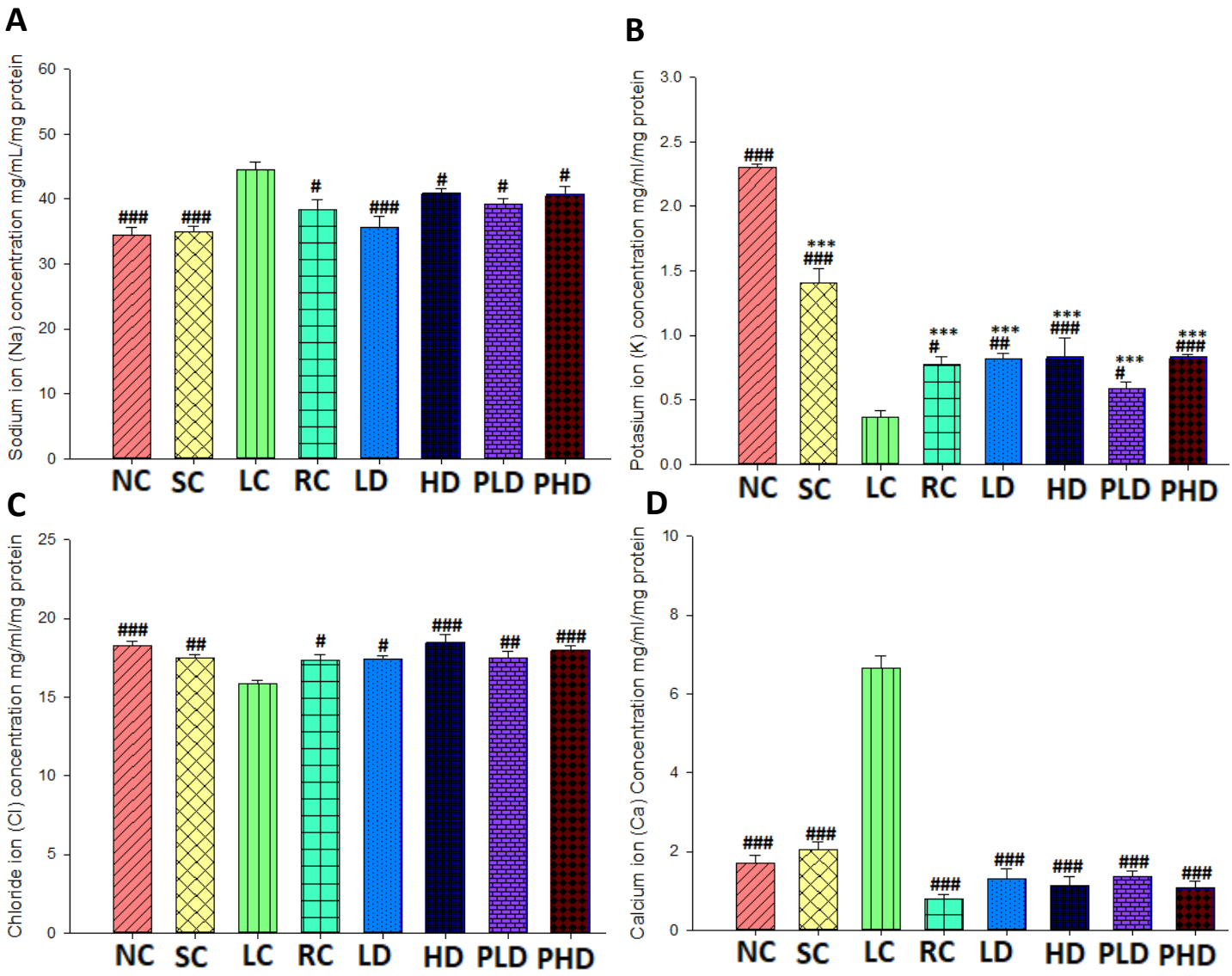

Fig 47: Effect of bromelain on sciatic nerve ions concentration and electrogenic pumps activities. *Each values represent mean ± SEM of each group. (A) Sodium ion concentration (B) Potassium ion concentration (C) Chloride ion concentration (D) Calcium ion Concentration.* $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ *compared with ligated control;* $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ *compared with normal control;* $^{+}p < 0.05$, $^{++}p < 0.01$, $^{+++}p < 0.001$ *compared with reference control. NC: Unligate Normal Control; SC: Sham Control; LC: Ligated Control; RC: Reference Control (30mg/kg gabapentin); LD: Low Dose Bromelain (30mg/kg); HD: High Dose Bromelain (50mg/kg); PLD: Pretreated Low Dose Bromelain (30mg/kg); PHD: Pretreated High Dose Bromelain (50mg/kg)*

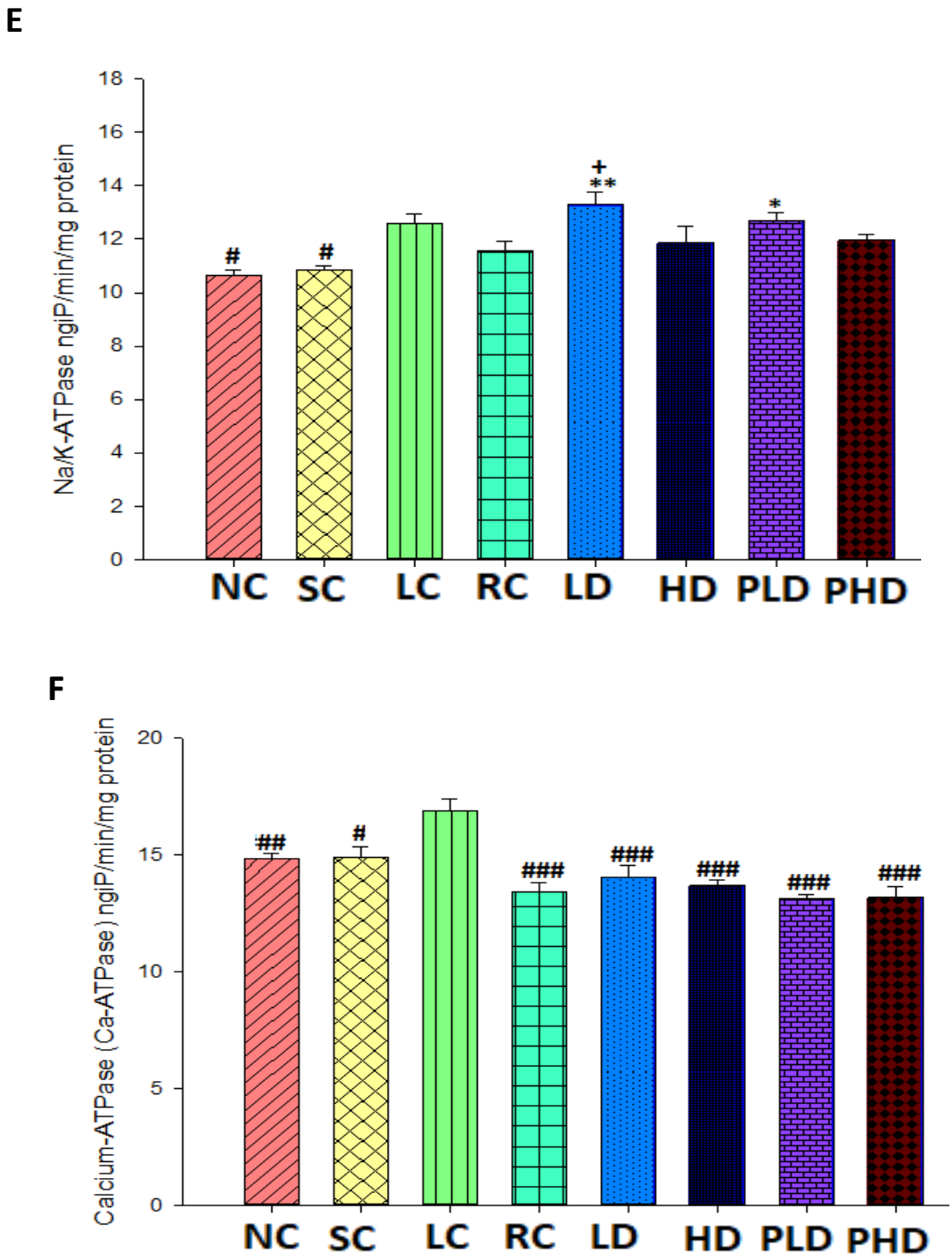

Fig 47: Effect of bromelain on sciatic nerve ions concentration and electrogenic pumps activities. *Each values represent mean ± SEM of each group. (E) Sodium-Potassium AtPase (F) Calcium ATPase.* $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ *compared with ligated control;* $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ *compared with normal control;* $^{+}p < 0.05$, $^{++}p < 0.01$, $^{+++}p < 0.001$ *compared with reference control. NC: Unligate Normal Control; SC: Sham Control; LC: Ligated Control; RC: Reference Control (30mg/kg gabapentin); LD: Low Dose Bromelain (30mg/kg); HD: High Dose Bromelain (50mg/kg); PLD: Pretreated Low Dose Bromelain (30mg/kg); PHD: Pretreated High Dose Bromelain (50mg/kg)*

4.20 Effect of Bromelain on Sciatic Nerve Glutamate concentration in CCI rats

Chronic constriction injury increased the sciatic nerve glutamate concentration, which was significantly different from the unligated control group, as represented in figure 48. Increased glutamate concentration in the sciatic nerve was alleviated by the administration of different doses of bromelain. A high dose of bromelain exclusively reversed sciatic nerve glutamate concentration back to an average level as there was no significant difference between it and the unligated control group.

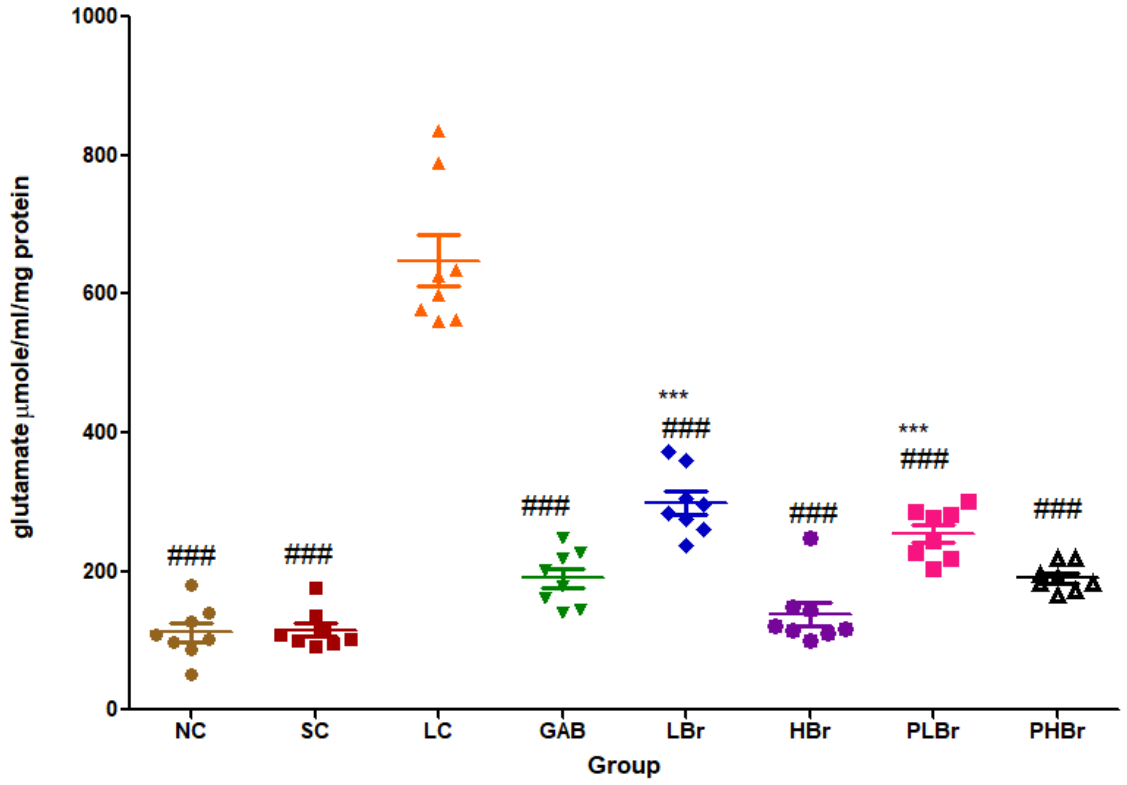

Fig 48: Effect of bromelain on sciatic nerve glutamate. *Each values represent mean ± SEM of each group. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ compared with ligated control; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ compared with normal control; $^{+}p < 0.05$, $^{++}p < 0.01$, $^{+++}p < 0.001$ compared with reference control. NC: Unligate Normal Control; SC: Sham Control; LC: Ligated Control; RC: Reference Control (30mg/kg gabapentin); LD: Low Dose Bromelain (30mg/kg); HD: High Dose Bromelain (50mg/kg); PLD: Pretreated Low Dose Bromelain (30mg/kg); PHD: Pretreated High Dose Bromelain (50mg/kg)*

4.21 Effect of Bromelain on Proinflammatory Mediators in CCI rats

Chronic constriction injury significantly increased the activities of inflammatory mediators (IL-1β, IL-6, TNF-α, and PGE_2) in the sciatic nerve and brain cortex (figure 49 A-F and figure 50 A-B) in *Wistar* rats. Administration of bromelain significantly mitigated the effect of CCI on inflammatory mediators. Pretreated high dose bromelain group significantly reduced both the sciatic nerve and brain IL-1β compared to the unligated control group. However, low dose bromelain showed insignificant ($p > 0.05$) differences in the brain IL-1β concentration compared with naïve ligated control group. Also, only pretreated high dose bromelain group was able to reverse the IL-6 concentration back to the basal level as there was an insignificant difference between pretreated high dose bromelain group and unligated control group. It was observed that gabapentin insignificantly reduced IL-6 concentration in the sciatic nerve and brain cortex. The ligated rats treated with gabapentin was indifferent from those of ligated control group in its sciatic TNF-α concentration. CCI have no significant effect on the brain cortex TNF-α level as there were insignificant differences between the ligated and unligated *Wistar* rats. Bromelain significantly reduces the concentration of PGE_2 in the sciatic nerve compared to the unligated control group. Both the pretreated and post-treated high dose of bromelain effectively reduces the sciatic nerve and brain cortex PGE_2 below the basal level, as indicated in figure 50 A-B.

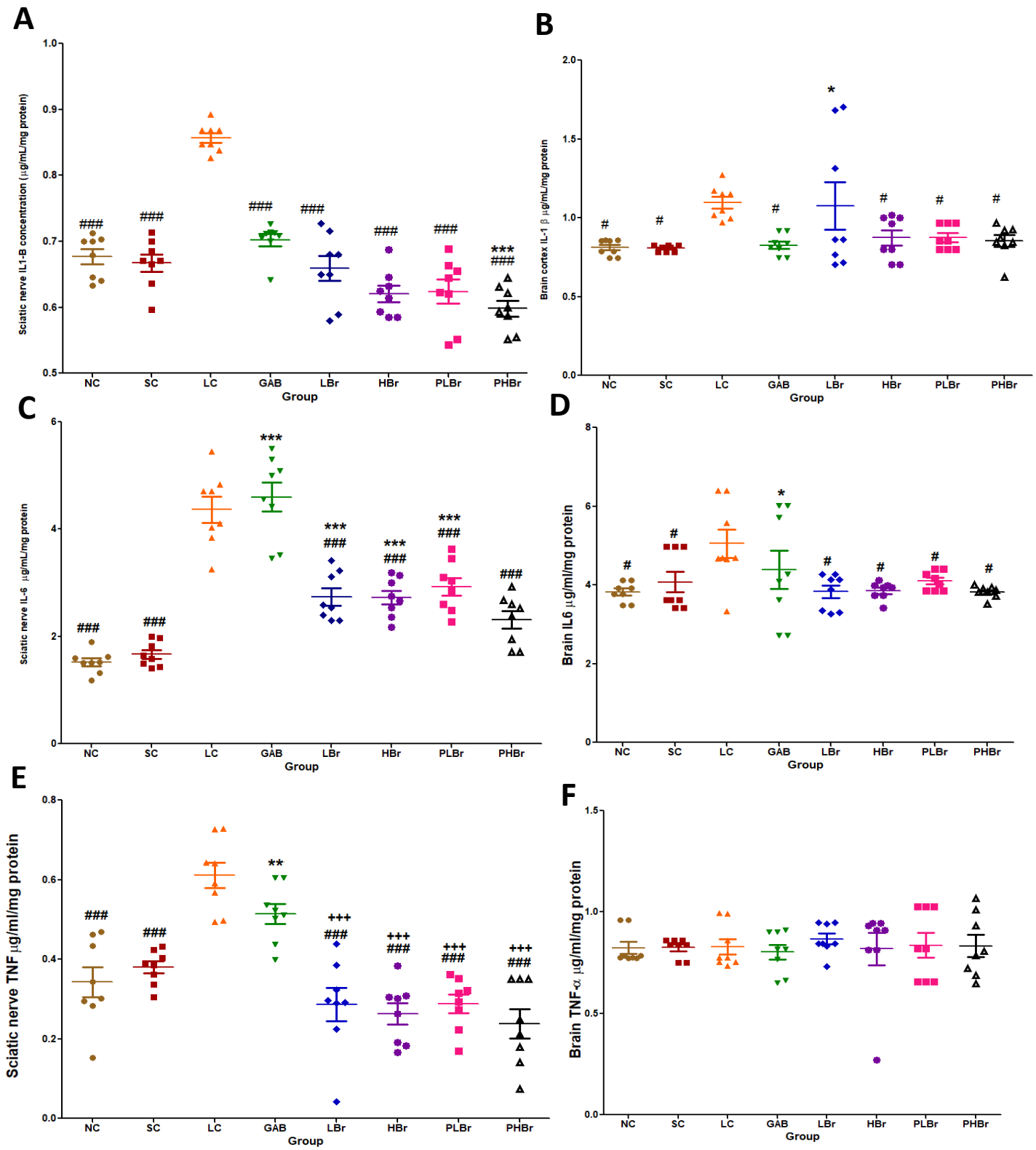

Fig 49: Effect of bromelain on sciatic nerve and brain proinflammatory cytokines. *Each values represent mean ± SEM of each group. (A) Sciatic nerve IL-1β concentration (B) Brain cortex IL-1β concentration (C) Sciatic nerve IL-6 concentration (D) Brain cortex IL-6 concentration (E) Sciatic nerve TNF-α concentration (F) Brain cortex TNF-α concentration.* $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ *compared with ligated control;* $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ *compared with normal control;* $^{+}p < 0.05$, $^{++}p < 0.01$, $^{+++}p < 0.001$ *compared with reference control. NC: Unligate Normal Control; SC: Sham Control; LC: Ligated Control; RC: Reference Control (30mg/kg gabapentin); LD: Low Dose Bromelain (30mg/kg); HD: High Dose Bromelain (50mg/kg); PLD: Pretreated Low Dose Bromelain (30mg/kg); PHD: Pretreated High Dose Bromelain (50mg/kg).*

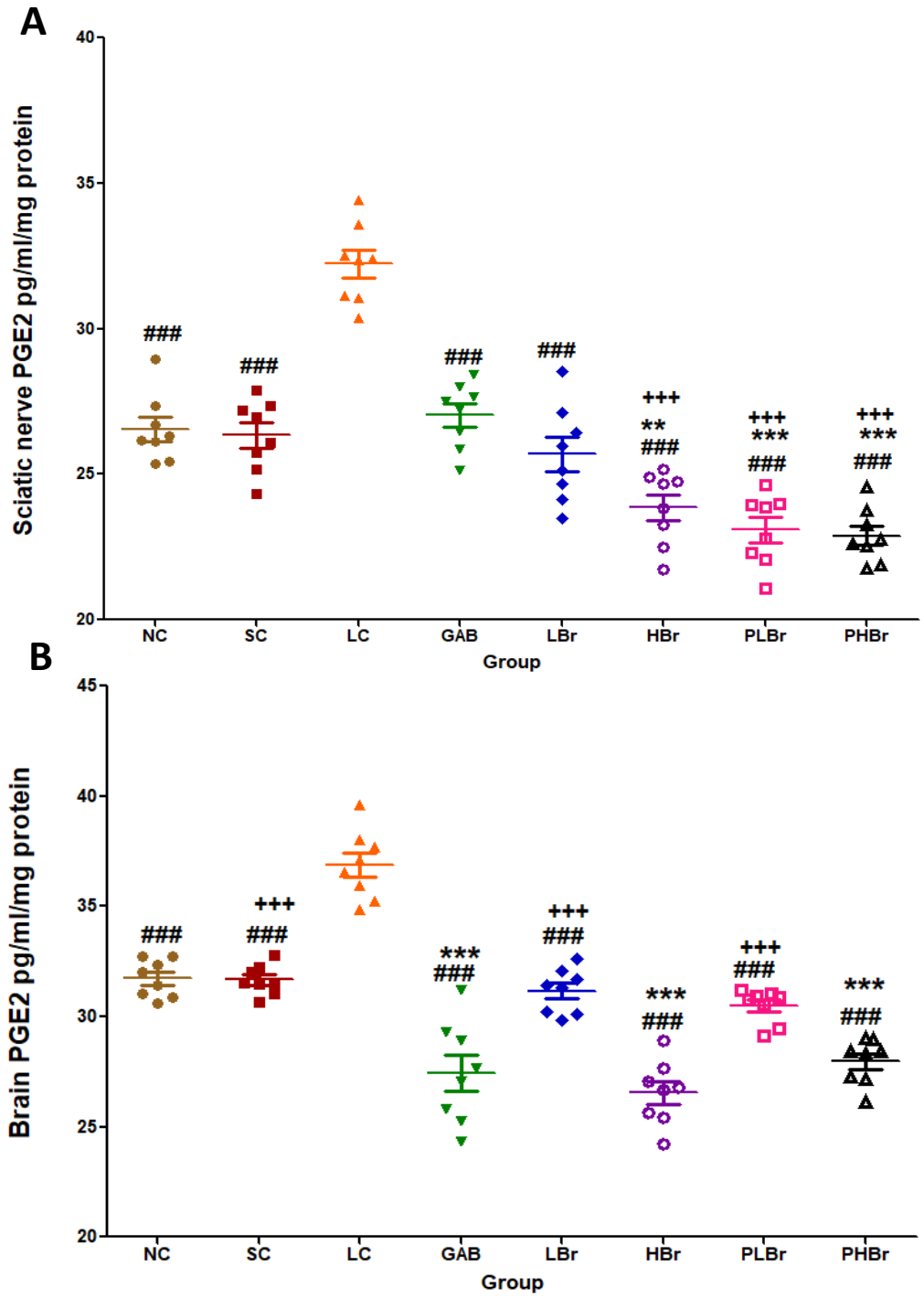

Fig 50: Effect of bromelain on the sciatic nerve and brain PGE_2. *Each values represent mean ± SEM of each group. (A) Sciatic nerve PGE_2 concentration (B) Brain cortex PGE_2 concentration. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ compared with ligated control; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ compared with normal control; $^{+}p < 0.05$, $^{++}p < 0.01$, $^{+++}p < 0.001$ compared with reference control. NC: Unligate Normal Control; SC: Sham Control; LC: Ligated Control; RC: Reference Control (30mg/kg gabapentin); LD: Low Dose Bromelain (30mg/kg); HD: High Dose Bromelain (50mg/kg); PLD: Pretreated Low Dose Bromelain (30mg/kg); PHD: Pretreated High Dose Bromelain (50mg/kg)*

4.22 Histological Study

There was axonal degeneration in the sciatic nerve of the ligated *Wistar* rat (Fig. 51 C). Increased number of proliferated myelinated and non-myelinated neurons were observed in the sciatic nerve of ligated rats. The presence of Schwann's cells significantly reducted in ligated control compared with the unligated control group. Animal treated with bromelain showed increased in myelinated neurons, reduced number of swollen myelinated and non-myelinated neurons, and reduced vacuolation in the sciatic nerve (Fig. 51 E, F). The sciatic nerve of the pretreated bromelain group (Fig. 51 G, H) showed nearly fully recovered structural integrity similar to the unligated control group.

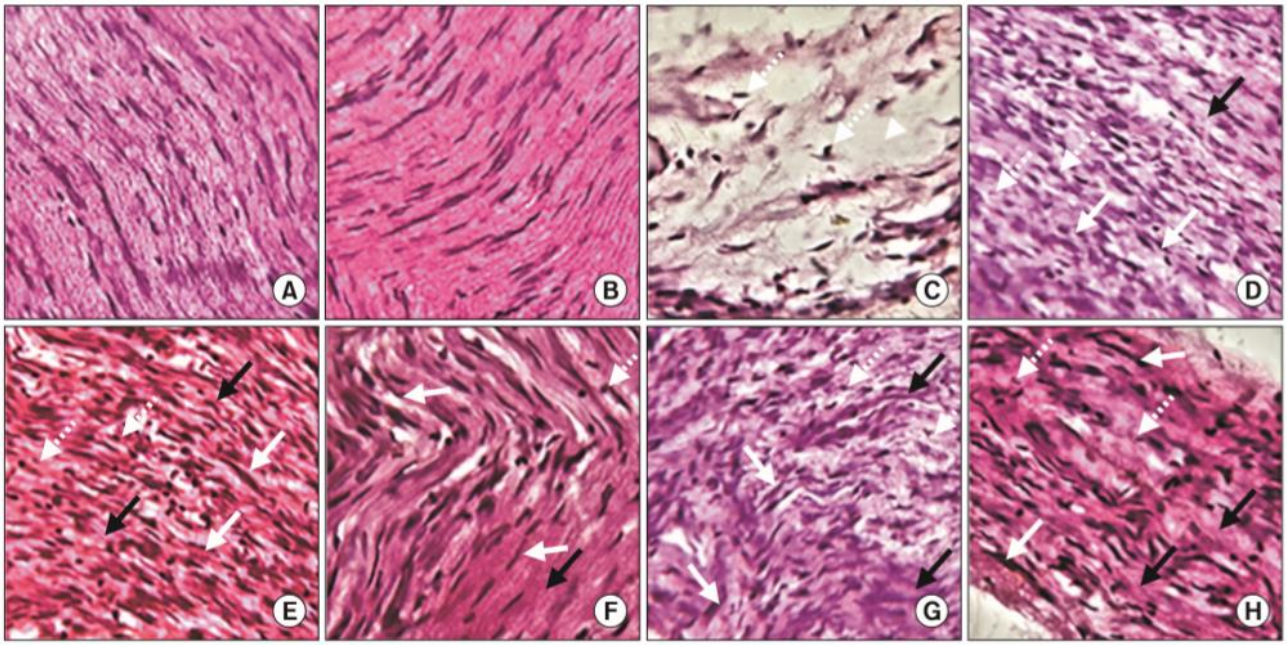

Fig 51: Bromelain improved myelinated (white arrows) and unmyelinated nerve (black arrows) integrity. *It attenuated swollen and degeneration of myelinated neurons (white arrows) and increases the occurrence of Schwan's cells. Arrowhead indicates swollen and deranged non-myelinated neurons, while dashed arrows indicate reduced and swollen myelinated neurons (H&E, ×400). (A) Normal control, (B) sham control, (C) ligated control, (D) reference control (30 mg/kg gabapentin), (E) low dose bromelain (30 mg/kg), (F) high dose bromelain (50 mg/kg), (G) pre-treated low dose bromelain (30 mg/kg), (H) pre-treated high dose bromelain.*

4.23 Immunohistological Study

Chronic constriction injury increased the expression of iNOS in the sciatic nerve of rats (figure 52 C, red arrow). There were also a reduced number of myelinated and non-myelinated neurons and increased neuronal swollen (dark orange arrow) as indicated in figure C. These showed highly disorganized structural integrity of the axons in the sciatic nerve. Treatment with bromelain mitigated the expression of iNOS, increased axonal myelination, and reduced axonal swelling (figure 52 E-H). The increased structural integrity of the sciatic nerve was comparable with the unligated control group (figure 52 A-B).

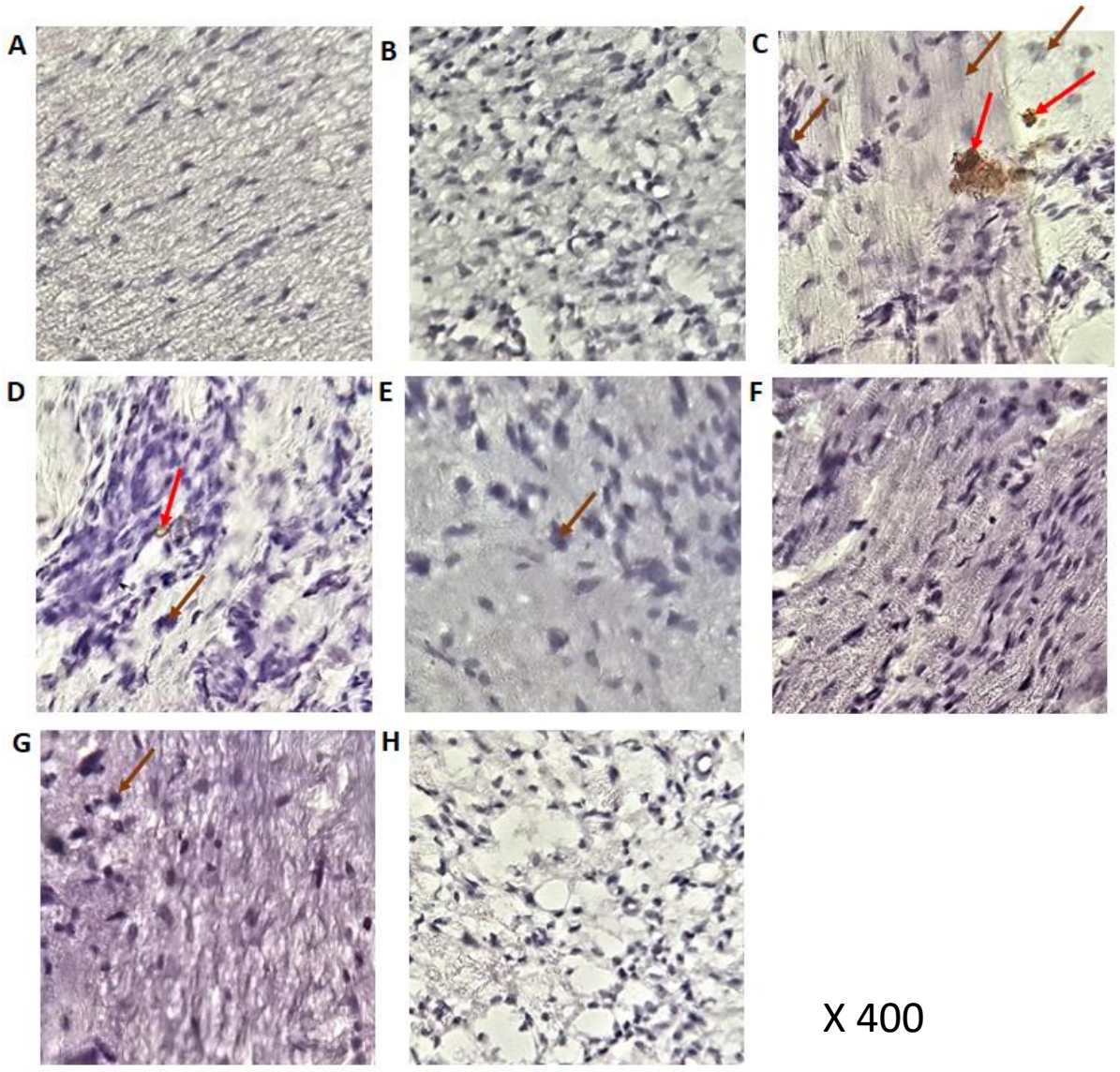

Figure 52: Bromelain improved neuronal myelination and attenuated induceable nitric oxide synthase (iNOS) expression. *It attenuated swollen and degeneration of nerve. The dark orange arrow indicates swollen and deranged non-myelinated neurons while Red arrow indicates the expression of iNOS. (A) Normal control (B) Sham control (C) Ligated control (D) Reference control (30 mg/kg gabapentin) (E) Low dose bromelain (30 mg/kg) (F) High dose bromelain (50 mg/kg) (G) Pre-treated low dose bromelain (30 mg/kg) (H) Pre-treated high dose bromelain.*

CHAPTER FIVE

DISCUSSION

5.1 DISCUSSION

This research investigated the antinociceptive effect of orally administered bromelain and its possible mechanism of action in the chronic constriction injury (CCI) model of peripheral neuropathic pain (NP) in *Wistar* rats. The manifestation of hyperalgesic and allodynic signs are the main hallmarks of NP in clinical and experimental settings (Berrocoso *et al.*, 2011; Gilron *et al.,* 2015; Wahab and Owoyele, 2015; Liang *et al.,* 2017). Chronic constriction injury is a known standard minor surgical procedure that has been widely used in the induction of NP and silk ligature has been shown to induce stable NP (Van der Wal *et al.,* 2015). It was evident from this study that CCI induced autotomy, thermal hyperalgesia, mechanical and cold allodynia, and emotional disturbances (anxiodepressive-like behaviour). It has been documented that ligated animals developed spontaneous pain behaviours, allodynia, hyperalgesia, autotomy, anxiety, and depression (Gilron *et al.,* 2015; Sousa *et al.,* 2016). Emotional dysfunction and sensory disorders in the CCI model have been said to correlate with symptoms of NP in humans' after peripheral nerve injury (Sorge *et al.,* 2012).

The anti-nociceptive effects of bromelain were investigated by the use of tactile, chemical, and thermal stimuli as well as gaits analysis in CCI-induced NP in *Wistar* rats. The use of von Frey filaments to assess mechanical allodynia has been reported extensively in the literature (Wahab and Owoyele, 2015; Verma *et al.,* 2020). Bromelain improved the mechanical allodynia in the ligated *Wistar* rats. However, it could not completely reverse the mechanical allodynia sign. Meanwhile, the results of the study revealed that pretreatment of ligated rats with bromelain did not prevent them from developing mechanical allodynia sign. The anti-allodynic effect of bromelain was further supported by the result from the chemical-induced (acetone evaporation

test) cold allodynia test. The use of acetone evaporation tests to induce cold allodynia has been validated in multiple models of neuropathic pain in rats (Colburn *et al.,* 2007; Yamamoto *et al.,* 2016). Observation from the study showed that bromelain completely reversed chemical-induced allodynia. Hence, this suggested that bromelain effectively mitigated chemical-induced allodynia than mechanical allodynic sign. Cold allodynia is related to increased ectopic discharge in unmyelinated C fibre after cold stimulation (Yin *et al.,* 2015). Whereas, tactile allodynia is believed to be conveyed by Aβ fibre (Ossipov *et al.,* 2002; Basbaum *et al.,* 2009). Both results showed that bromelain possesses strong anti-allodynic properties. Bromelain is as effective as gabapentin in the reduction of allodynia as observed in this study. Although, bromelain offered to have a quicker anti-allodynia response compared to gabapentin. Gabapentin is a generally accepted anti-convulsant drug for the treatment of neuropathic pain.

The anti-hyperalgesic effect of bromelain was assessed by conducting hotplate, cold-plate, and tail immersion tests on the ligated rats. The use of hotplate and cold-plate for the screening of antinociceptive drugs were well documented (Allen and Yaksh 2004) for both spinal and supra-spinal acting drugs. This study showed that bromelain completely reversed thermal hypersensitivity. Surprisingly, pretreatment with bromelain prevented the development of thermal hyperalgesia. Pretreatment with bromelain may have possibly resisted neuronal plasticity which underlies the development of hyperalgesia. Both low and high doses of bromelain effectively abated the hyperalgesic consequence of NP. Bromelain serves as prophylaxis for hyperalgesia.

Evaluation of sciatic functional index correlates with the extent of myelin degradation and nerve injury (Arruri *et al.,* 2017). The study showed that bromelain improved the sciatic functional index arise from CCI. It is suggested that bromelain possesses neuroprotective properties that improved sciatic nerve structural integrity. Impairment of sciatic functional index as observed may account for abnormal gait observed in CCI animals. The defect in

animal gait has been associated with the loss of the myelinated fibre (Arruri *et al.,* 2017). An improved sciatic function has been linked with improvement in peripheral nerve regeneration and correlated with morphology of the peripheral nerves (Shen and Zhu, 1995; Monte-Raso *et al.,* 2008).

Anxiety and depression are common comorbid emotional deficits that are associated with NP (Cherif *et al.,* 2020; Khan *et al.,* 2020). The open-field and elevated-plus mazes have been widely used in the study of anxiodepressive-like behaviours in experimental animals (Gambeta *et al.,* 2017; Kremer *et al.,* 2020). Bromelain does not only abated the hyperalgesia and allodynia but as well as mitigated the comorbid neuropathic pain. It was revealed in the study that only a high dose of bromelain was effective in reversing the anxiety-like and depressive-like characteristic of neuropathic pain. Treatment with a low dose of bromelain has weak anti-anxiodepressive effects. Pretreatment with bromelain yielded a better anti-comorbidity result which suggests that bromelain could be used primarily as both therapeutic and prophylaxis agents. Likewise, Observations from the EPM and OFM suggest that bromelain does not have sedative effects. There were improved locomotive activities in the rats treated with bromelain. Locomotive activities in rats correlated with sedative and depressive-like behaviours.

Long-term administration of bromelain did not evoke a sporadic increase in body mass of animals which is a common side effect associated with some known analgesic drugs such as opioids, gabapentinoid (Ricardo Buenaventura *et al.*, 2008; Schmidt *et al.*, 2013). It has been reported that weight gain may be problematic in a diabetic population where weight gain can exacerbate the NP disease (Moran and Szallasi, 2018). The study showed that usage of bromelain might have lesser cardiovascular adverse risk.

The involvement of reactive oxygen species (ROS) and reactive nitrogen species (RNS) in the development and maintenance of neuropathic pain has been well documented (Salvemin *et al.,*

2011; Duggett *et al.,* 2016; Shim *et al.,* 2019). Concurrent activities of accumulated ROS and RNS in the central and peripheral nervous system result in the development of hyperalgesia and allodynia (Park *et al.,* 2006; Duggett *et al.,* 2016). Administration of bromelain produced neuroprotective effects by improving the level of antioxidant enzymes in the neurons. Bromelain significantly increased the production of superoxide dismutase (SOD) and reduced glutathione (GSH) production and catalase activities. It is hereby suggested that bromelain is a potent neuroprotective agent that contributes to its antinociceptive effects. Antioxidant enzymes neutralize ROS and RNS, and there harmful deleterious effects on pain perception. It has been reported that ROS increases phosphorylation of NMDA receptor, inactivate GABA, and increased the expression of TRPA1, TRPV1, and TRPV4 via increase IB4+ neuron expression, all of which are critical in the maintenance and development of NP (Gao *et al.,* 2007; Yowtak *et al.,* 2011; Kilic, 2012; Duggett *et al.,* 2016). Reactive nitrogen species modulates neuropathic pain via modification of some protein kinase, receptors, enzymes, and glutamate transporters (Gorg *et al.,* 2005, 2010; Kilic, 2012; Little *et al.,* 2012). This results in inactivation of SOD, GS, GLUTs, and increased phosphorylation of both peripheral and central receptors as well as secondary messengers that mediate hypersensitivities (Zhang *et al.,* 2014; Miyake *et al.,* 2016). Decreased firing threshold of nociceptive fibre and activation of silence neurons have also been linked with increased activities of accumulated RNS and ROS (Park *et al.,* 2006).

The imbalance in nuclear transcription molecules such as nuclear factor erythroid-derived-2-related factors- 1 and 2 (NrF-1 and NrF-2) and nuclear factor Kappa light chain enhancer B-cell inhibitor (NF-κB), as the aftermath of nerve injury remain critical factors that are contributed to neuroplasticity which results in neuropathy (Kumar *et al.,* 2013; Arruri *et al.,* 2017). Transcription of the cytoprotective protein such as catalase, SOD, glutamine is under the control of NrF-1 and NrF-2. These nuclear transcriptor molecules play a pivotal role in the

regulation of cellular redox status via promoting antioxidant enzyme synthesis (Biswas and Chan, 2010; Negi *et al.,* 2011; Zhou *et al.,* 2020). Under normal homeostatic conditions, NrF transcription is repressed by negative regulator kelch-like ECH-associated protein 1 (keap-1) (Nguyen *et al.,* 2003). However, upon exposure to ROS, NrF2 dissociates from cytosolic keap-1 and translocates to the nucleus, where it binds to the antioxidant response element (ARE) in the promoter region of genes encoding antioxidant enzymes, thereby inducing the production of endogenous antioxidant enzymes (Nguyan *et al.,* 2003). Findings from the study showed that CCI repressed the activities of both NrF-1 and NrF-2. Reduced activities of the nuclear transcription factors compromised the synthesis of antioxidant enzymes and their downstream signal molecules. This explained the reduced antioxidant enzymatic activities and increased lipid peroxidation noted in CCI rats which is inconsonant with other previously reported studies (Komirishetty *et al.,* 2017). Dysregulation of NrF-1 and NrF-2 have been implicated in various neurodegenerating diseases (Alzheimer's, Parkinson's, and Huntington's diseases) (de Vries *et al.,* 2008) as well as chemotherapy-induced neuropathy (Zhou *et al.,* 2020).

This study observed that bromelain increased the activities of NrF-1 and NrF-2 and subsequently stimulates their translocation. It is therefore presumed that increases in the neuronal antioxidant enzymes (SOD and GSH) activities were due to the stimulating effect of bromelain on transcription factors. Activation of endogenous nuclear factor (erythroid derived-2) like-2 (Nrf2) helps in scavenging the reactive oxygen and nitrogen species by hemoxygenase-1 (HO-1) and glutathione (GSH) (de Vries *et al.,* 2008). The reduction in the level of MDA observed in this study further confirm the neuroprotective effect of bromelain. This is indicative of reduced neuronal lipid peroxidation result from oxidative stress. Studies have suggested that endoneurial lipid peroxidation (LPO) increases as a consequence of chronic constriction injury (CCI) to the sciatic nerve (Kanyadhara *et al.,* 2014). It has been suggested that oxidative stress triggers the activation of sensory transient receptors' potential

channels as well as the release of chemical mediators that result in hyperalgesia (Hassler *et al.,* 2014; Goel and Tyagi, 2016). It is hereby proposed that bromelain acted by increasing the activities of nuclear transcription factors that increase antioxidant enzyme production and activities which mopped up RNS and ROS to ameliorate hyperalgesia and allodynia.

Also, structural disruption of the sciatic nerve has been linked with the accumulation of ROS in the nervous system (Duggett *et al.,* 2016). The loss of myelinated fibres and related postural defects after the CCI of the sciatic nerve can be due to neuropathic pain associated with oxidative stress and neuroinflammation (Arruri *et al.,* 2017). Bromelain improved the structural integrity of the neurons by reducing vacuolation of the neuron as well as increased density of myelinated neurons. This accounts for the improved SFI and locomotive activities observed in ligated rats. Hence, it is suggested that the antioxidant effects of bromelain aid improved structural neuronal integrity.

Furthermore, the study showed that CCI induced increases in NF-kβ and up-regulation in the expression of nitric oxide synthase (iNOS), both of which mediate neuroinflammation. Marked increases in NF-κB levels in sciatic nerve and DRG following nerve injury have been reported (Arruri *et al.,* 2017). Nuclear factor-kappa B (NF-κB) transmigrate into the nucleus, and it binds to DNA response elements in gene promoter regions to control transcription of genes, such as inducible NO synthase (iNOS), cyclo-oxygenase-2 (COX2) and induces production of proinflammatory cytokines (Dou *et al.,* 2013; Han *et al.,* 2017; Komirishetty *et al.,* 2017). Accumulated evidence showed that iNOS mediates production of peroxynitrite nitrogen species (PNS) and COX_2 both of which through a cascade of enzymatic processes and increased phosphorylation of excitatory receptors, potentiate hyperalgesic and allodynic signs of neuropathic pain (Liang *et al.,* 2017). Inhibition of iNOS expression by bromelain is hereby suggested to be a mechanism that further enhanced the neuroprotective effect of bromelain that mediates its anti-nociceptive effects. This is consistent with the previously reported

downregulatory effect of bromelain on nitric oxide synthase (Wen *et al.,* 2006). In addition to oxidative and nitrosative stress, CCI induced an increase in the Ca^{2+} concentrations in the neuron. Calcium ion contributes towards the buildup of ROS (Muthuraman *et al.,* 2008) and nitric oxide by stimulating the production of nitric oxide synthase (iNOS) (Salvemini *et al.,* 2011). The results from this study showed that bromelain reversed the increase in Ca^{2+} level in the neuron. On this account, reduced iNOS expression by bromelain may be due to its mitigating effects on the elevation of Ca^{2+} concentrations in the neuron. This further contributes to its neuroprotective role that aids its anti-nociceptive and anti-anxiodepressive properties.

Likewise, increases in COX_2 following nerve injury (Zeilhofer, 2007) potentiate the production of prostaglandin E_2 (PGE_2) in central and peripheral neurons that mediate neuropathic pain (Kohno *et al.,* 2008; Kawabata, 2011; Komirishetty *et al.,* 2016; Liang *et al.,* 2017). Prostaglandin E_2 causes pronounced hyperalgesia via peripheral and central sensitization. Finding from the study showed that bromelain mitigated increased PGE_2 concentration resulting from the CCI. The level of PGE_2 was found to be elevated in the brain cortex and sciatic nerves of ligated rats. This showed that PGE_2 is critical for the development of hyperalgesia, allodynia, and anxiodepressive disorder. It is suggested that PGE_2 serve as one of the common mediators of NP and emotional disorder. Hence, bromelain versatility in mitigating anxiodepressive disorder and NP signs in rats was due to its inhibitory effect on PGE_2. It is suggested that bromelain may have blocked cascades of enzymes that stimulate PGE_2 synthesis. Nuclear factor kappa-B (NF-κB) is a perfect candidate that fitted in this cascade of enzymes. In the study, it was observed that CCI increased NF-κB concentration. It has been reported that NF-κB potentiates the release of COX2 mRNA and iNOS, both of which induce the production of PGE_2 (Secor *et al.,* 2005; Komirishetty *et al.,* 2016; Liang *et al.,* 2017). Hence, the downregulation effect of bromelain on NF-κB mediates the inhibition of PGE_2 concentration in the central and peripheral neurons. This is in support of other findings

that have reported the inhibitory effect of bromelain on PGE_2 (Gaspani *et al.,* 2002; Bhui *et al.,* 2009), COX2 mRNA (Secor *et al.,* 2005; Rathnavelu *et al.,* 2016), and NF-κB (Wen *et al.,* 2006; Habashi *et al.,* 2017).

Precipitous increase in the activities of interleukin-1-beta (IL-1β), inter-leukine-6 (IL-6) and tumour necrotic factor-alpha (TNF-α) released by the glial, macrophages, and endothelial cells mediate painful experience via increased sensitization of peripheral and central receptors (Tiwari *et al.,* 2014; Yan *et al.,* 2014; Komirishetty *et al.,* 2016). This study showed that CCI induces remarkable increases in proinflammatory cytokines which indicated the development of neuroinflammation. Such cytokines have been reported to induce long-lasting excitability and synaptic plasticity via an excessive spontaneous discharge of afferent nociceptive fibre and upregulation of receptor expressions (Üceyler *et al.,* 2009; Schaible *et al.,* 2010; Julius, 2013). Classical inflammatory mediators such as proinflammatory cytokines (Kiguchi *et al.,* 2010; Von Hehn *et al.,* 2012; Hori *et al.,* 2016; Komirishetty *et al.,* 2017) and prostaglandins (Basbaum *et al.,* 2009; Mizumura *et al.,* 2009) are critical mediators of thermal hyperalgesia, mechanical allodynia as well as emotional dysfunction of NP (Sawada *et al.,* 2014; Yalcin *et al.,* 2014; Yadav and Weng, 2017).

Findings from the study showed that Interleukin-1β and interleukin-6 mediated both NP signs and emotional comorbidity in ligated rats. Whereas, TNF-α was observed to mainly mediated hyperalgesia and allodynia of NP. Hence, the study suggests that IL-1β and IL-6 are the common mediators of hyperalgesia, allodynia, and anxiodepressive disorder in NP. The previous study has also suggested that IL-1β plays a common role in mediating emotional disorder and NP signs in rodents (Gui *et al.,* 2016). Bromelain further mediated its anti-nociceptive effect through its anti-inflammatory properties. Central and peripheral effects of bromelain are evidenced by its inhibitory actions on proinflammatory cytokines in the cerebral cortex and sciatic nerve. Inhibition of inflammatory cytokines has been shown to effectively

relieve neuropathic pain (Gao *et al.,* 2011). The blockade of the TNF-α signalling pathway, as well as IL-1β and IL-6, have been documented to reduce hyperalgesia and allodynia (Schaible *et al.,* 2011; Gruber-schoffnegger *et al.,* 2013; Ishikawa *et al.,* 2013; Schuh *et al.,* 2014; Tiwari *et al.,* 2014). This recent study shows that bromelain inhibits TNF-α. The report has shown that bromelain inhibits the activation of both TNF-α receptors (TNFR1 and TNFR2) (Zhou *et al.,* 2017). Bromelain ameliorated CCI-induced IL-1β and IL-6. It can be further inferred that bromelain reduced exacerbatory potentiation of cortical neurons via decreases in IL-1β and IL-6 synthesis. Hence, the anti-anxiety-like effect of bromelain is hereby suggested to be a result of its inhibitory effect on PGE_2, IL-1β, and IL-6 rather than TNF-α.

The inhibitory effect of bromelain on proinflammatory cytokines may be due to its inhibitory effect on nuclear factor kappa light chain enhancer B cell inhibitor (NF-κB). Bromelain has been reported to block the activation and transmigration of NF-κB (Bhui *et al.,* 2009; Juhasz *et al.,* 2008). Proinflammatory cytokines such as TNF, IL-1, IL-6, PGE_2, and iNOS can be activated by NF-κB (He *et al.,* 2014; Komirishetty *et al.,* 2016; Liang *et al.,* 2017). Hence, inhibition of NF-κB plays a pivotal role in mitigating the elevation of proinflammatory cytokines.

Bromelain further mediated anti-nociceptive effects by reducing the neuronal glutamate through its inhibitory effects on pro-inflammatory cytokines. Glutamate is an excitatory neurotransmitter that mediates pain perception. Elevated glutamatergic neurotransmission in the central nervous system is widely observed in neuropathic pain conditions which correlates with what was found from this study. Proinflammatory cytokines such as IL-1β, act centrally by inhibiting the activities of glutamate transporters (Pitcher *et al.,* 2007). Convincing evidence has shown that IL-1β stimulate the release of glutamate in the afferent nerves that contributes to central sensitisation (Yan and Weng, 2013). This results in an increased in the synaptic glutamate level which potentiates central sensitization. Therefore, bromelain inhibited pro-

inflammatory cytokines which result in the reduction in the bioavailability of synaptic excitatory glutamate. The use of anti-inflammatory agents to mitigate glutamate-induced hyperalgesia in NP has been gaining recognition as an effective therapeutic intervention (Eliav *et al.,* 2009; Khan *et al.,* 2015).

Findings on the changes in ionic concentration and pump activities showed that bromelain may be inducing its anti-nociceptive properties by hyperpolarising the sensory neuron. Neuropathic pain has been well known to alter the rate of influx and efflux of ion currents. Impairment in the expression and distribution of ion channels, pumps, and transporters were deeply involved in the derangement of intracellular ionic balance (Paul *et al.,* 2014; Thiagarajan and Shanmugam 2014). This results in hyperexcitation and disinhibition of the sensory neurons that cause hyperalgesia and allodynia. Increased Na^+ concentration results in hyperexcitability of the nociceptive fibres. It has been shown that an excessive influx of Na^+ is an indication of the upregulation of Na^+ channel expression with simultaneous increases in the activities of Na-K^+ ATPase (Paul *et al.,* 2014). It is hereby hypothesized that bromelain modulates the electrical activities of neurons by reducing the concentration of Na^+ and Ca^{2+} ions which leads to reduced hyperalgesia as a result of hyperexcitation. Convincing evidence has shown that imbalance in spinal Na^+ and Ca^{2+} ions produce hyperalgesia and allodynia (Malan *et al.,* 2002; Schoffnegger *et al.,* 2008). This study showed that CCI results in decreased Cl^- and K^+ ions. Disorder in the regulation of Cl^- regulation may account for the oxymoronic excitation of the neuron that causes hyperalgesia. Increases in Cl^- and K^+ in ligated rats treated with bromelain could be an indication of the elimination of possible disinhibition in neurons. Regulation of neuronal Cl^- gradient modulates the synaptic inhibition which may be independent of neither neurotransmitter (GABA and Glycine) nor receptor activation (Price *et al.,* 2009). The hyperchloremia brought about by bromelain was possibly lead to a reduction in the depolarization and increases in the hyperpolarization of the neurons. The previous report

showed that the increased expression of sodium and calcium ion channels leads to increased activities of Na^{+}-K^{+} ATPase and Ca^{2+} ATPase (Gemes *et al.*, 2012; Paul *et al.*, 2014). A distinct role of calcium-ATPase as a homeostasis control mechanism for the clearance of intracellular calcium has been established. The study was in line with a previously reported observation on increased activities of Ca^{2+}-ATPase following traumatized sensory nerves (Gemes *et al.*, 2012). Reduction in the activities of Ca^{2+}-ATPase is an indication that bromelain modulates Ca^{2+} trafficking. Increases in the activities of Ca^{2+}-ATPase are associated with a reduction in the magnitude of afterhyperpolarization and increases in neuronal excitation (Ghosh *et al.*, 2011). Therefore, it is suggested that the antinociceptive effects of bromelain are linked with its effect on Ca^{2+}-ATPase.

CHAPTER SIX

SUMMARY, CONCLUSION, AND RECOMMENDATION

6.1 SUMMARY

Neuropathic pain is a neurological disease of pain that posed greater challenges to society which consumed millions of dollars in every nation. The study investigated the anti-nociceptive effects and mechanisms of action of bromelain as a potential supplement for relieving NP in CCI *Wistar* rats model of peripheral neuropathy. Findings from the study are summarized as follow:

1. Bromelain mitigated hyperalgesia and allodynia signs of NP in ligated rats. Bromelain increases the mechanical threshold to von Frey filaments and thermal threshold to cold and heat stimuli. Both doses of bromelain administered were effective in ameliorating CCI-induced NP.
2. Bromelain acted as a prophylactic agent to hyperalgesia signs of NP, as pretreatment with bromelain prevented the development of thermal hyperalgesia.
3. Bromelain ameliorated emotional comorbidity signs of NP. Pretreated bromelain group and high dose bromelain post-treated rats showed improved emotional behaviour to anxiodepressive disorder.
4. Bromelain served as a potent neuroprotective agent. It improved the activities of antioxidant enzymes by mitigating decreases in the NrF-1 and NrF-2 transmigration that regulate antioxidant enzyme production. Reduced oxidative stress was confirmed by inhibition of lipid peroxidation by bromelain.
5. Bromelain inhibited the expression of iNOS that modulate NO production. This further demonstrated the neuroprotective capacity of bromelain. Bromelain reduced NO concentration in the sciatic nerve and blood plasma.

6. Bromelain reduced inflammatory mediators by mitigating increases in the concentration of proinflammatory cytokines and PGE_2 in the cerebral cortex and sciatic nerve.
7. Bromelain inhibited the concentration of NF-κB in the neurons. This explained the inhibiting effects of bromelain on the inflammatory mediators.
8. Bromelain reduced the concentration of glutamate in the sciatic nerve.
9. Bromelain modulate the sciatic nerve electrolytes by reducing the concentration of Ca^{2+}-ATPase, Na^{+}, and Ca^{2+} but increased the concentration of K^{+} and Cl^{-}.
10. Bromelain improved the structural integrity of the nerve by increasing the density of myelinated neurons, and reduced vacuolation of the nerve.

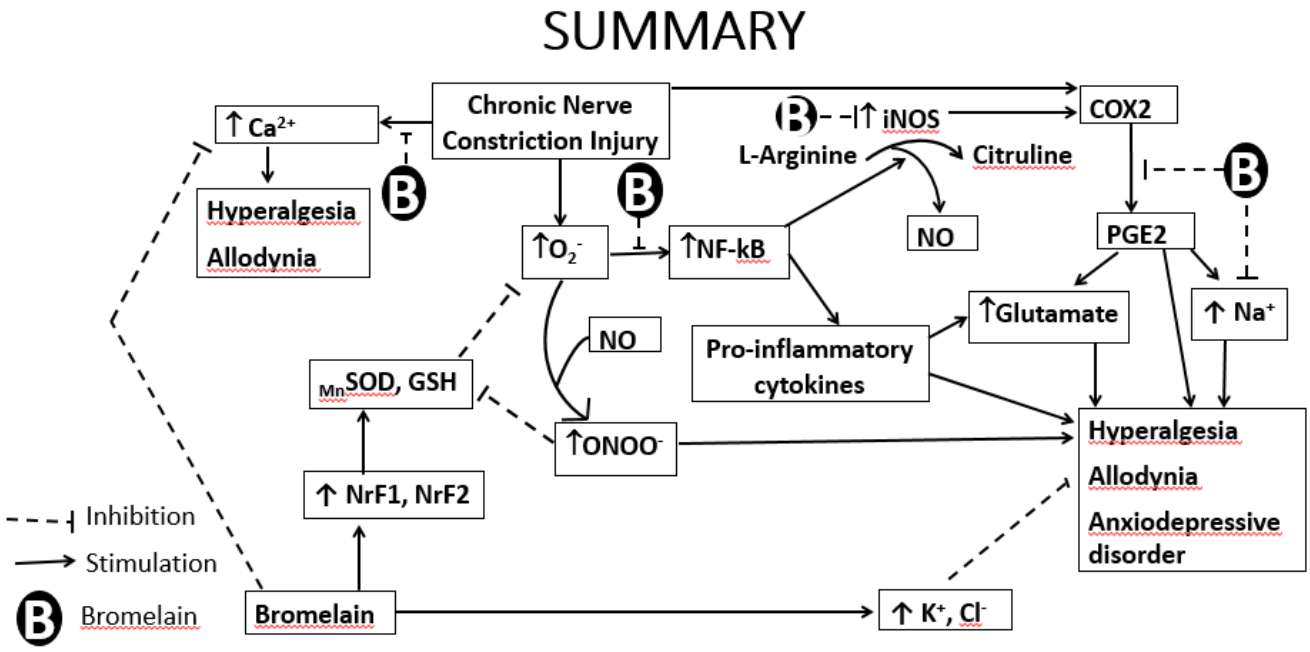

Fig 53: Graphical illustration of mechanism mediating anti-nociceptive effects bromelain

6.2 CONCLUSION

The study concludes that bromelain is an effective anti-nociceptive supplement against CCI-induced neuropathic pain. Bromelain anti-nociceptive effects were mediated by its neuroprotective mechanisms through anti-inflammatory and antioxidant properties, as well as its modulatory effects on the neuronal electrolytes homeostasis.

6.3 Recommendation

The study demonstrated the anti-nociceptive properties of bromelain in CCI-induced NP in Wistar rats. Hence, the following are the recommendations:

1. Since pretreatment of rats with bromelain do not effectively prevent the development of neuropathic pain, bromelain should be applied as a therapeutic agent rather than prophylactic
2. Bromelain could be used as a substitute of gabapentinoids and other tradition drugs that are in use for the treatment of neuropathic pain since its effects are comparable to that of gabapentin and have minimal side effects.

6.4 Suggestions for Further Studies

1. Further investigation should be carried out to determine the specific receptor through which bromelain acted upon.
2. More accurate techniques should be used to verify the electrical functions of the sciatic nerve, DRG and dorsal horn of the spinal cord to confirm the regulatory effects of bromelain on the neuronal electrolytes.
3. The specific corticolimbic area should be identified to ascertain the exact region of the brain that characterized by an increased level of proinflammatory mediators.

6.5 Contribution to Knowledge

The study showed that bromelain possesses anti-nociceptive properties to neuropathic pain (NP). It ameliorated the hyperalgesia, allodynia and anxiodepressive disorder associated with NP. The study further revealed that anti-nociceptive properties of bromelain are mediated by its neuroprotective effects by promoting transmigration of NrF to stimulate the production of antioxidant enzymes. Bromelain also possessed anti-inflammatory properties and regulate neuronal electrolytes balance. Findings from the study showed that IL-1β, IL-6 and PGE_2 might be the overlapping neurobiology molecules that mediated NP and anxiodepressive emotional disorder.