NEUROPROTECTIVE EFFECT OF *PTEROCARPUS MARSUPIUM* MARKETED CAPSULES AGAINST VINCRISTINE INDUCED NEUROPATHY IN RATS

A Dissertation Submitted to

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IN

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Submitted By

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This is to certify that the dissertation work entitled "**Neuroprotective effect of *Pterocarpus marsupium* marketed capsules against vincristine induced neuropathy in rats"** submitted by University **Reg. No. 261525904** is a bonafide work carried out bythe candidate under my guidance and submitted to The Tamil Nadu Dr. M.G.R. Medical University, Chennai, in partial fulfillment for the Degree of Master of Pharmacy in Pharmacology at the Department of Pharmacology, PSG College of Pharmacy, Coimbatore, during the academic year 2016-2017.

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DECLARATION

I do hereby declare that the dissertation work entitled "**Neuroprotective effect of *Pterocarpus marsupium* marketed capsules against vincristine induced neuropathy in rats"** submitted to The Tamil Nadu Dr. M.G.R. Medical University, Chennai, in partial fulfillment for the Degree of Master of Pharmacy in Pharmacology, was done by me under the guidance of **Mr. G.Venkatesh, M.Pharm.,** at the Department of Pharmacology, PSG College of Pharmacy, Coimbatore, during the academic year 2016-2017**.**

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EVALUATION CERTIFICATE

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Dedicated

To The Lord Almighty

My beloved Parents,

Brother

&

Respectful Guide

ABBREVIATIONS

5- HT	**:**	*5*-hydroxytryptamine
TNF-α	**:**	Tumor necrosis factor – alpha
IL	**:**	Interleukin
NGF	**:**	Nerve growth factor
TRP	**:**	Transient receptor potential channel
GABA	**:**	Gamma amino butyric acid
NMDA	**:**	N-methyl-D-aspartate
TCAs	**:**	Tricyclic antidepressants
SSRIs	**:**	Selective serotonin reuptake inhibitors
PHN	**:**	Post-herpetic neuralgia
CCI	**:**	Chronic constriction injury model
PSL	**:**	Partial sciatic nerve ligation model
SNL	**:**	Spinal nerve ligation model
SCN	**:**	Sciatic cryoneurolysis model

CIPN	**:**	Chemotherapy induced peripheral neuropathy
DRG	**:**	Dorsal root ganglia
ROS	**:**	Reactive oxygen species
VIPN	**:**	Vincristine induced peripheral neuropathy
IENF	**:**	Intra epidermal nerve fibers
PM	**:**	*Pterocarpus marsupium*
SNCV	**:**	Sensory nerve conduction velocity
NADP	**:**	Nicotinamide adenine dinucleotide phosphate
NF-κB	**:**	Nuclear factor kappa light chain enhancer of activated B- cells
SNRIs	**:**	Serotonin norepinephrine reuptake inhibitors
NA	**:**	Nor adrenaline
IFNγ	**:**	Interferon gamma
CSF	**:**	Cerebrospinal fluid
ETC	**:**	Electron transport chain
NFAT	**:**	Nuclear factor of activated T -cells

VCR	**:**	Vincristine
SARM-1	**:**	Sterile alpha and TIR motif containing protein -1
ATP	**:**	Adenosine tri phosphate
TBARS	**:**	Thiobarbituric acid reactive substances
IPA	**:**	Isopropyl alcohol
ALA	**:**	Aldose reductase activity
AGE	**:**	Advanced glycation end product
α-DHC	**:**	α-Dihydroxychalcone-glycoside
MAPKs	**:**	Mitogen activated protein kinases
Nrf-2	**:**	Nuclear factor –E2-related factor -2
HO-1	**:**	Hemeoxygenase -1
COX	**:**	Cyclooxygenase
i NOS	**:**	Inducible nitric oxide synthase
NO	**:**	Nitric oxide
MPO	**:**	Myeloperoxidase

mRNA	**:**	Messenger RNA
SOD	**:**	Superoxide dismutase
CAT	**:**	Catalase
GPx	**:**	Glutathione peroxidase
GSH	**:**	Reduced glutathione
GR	**:**	Glutathione reductase
PPAR-γ	**:**	Peroxisome proliferator activated receptor gamma
CPCSEA	**:**	Committee for the purpose of control and supervision on experimental animals
CMC	**:**	Carboxy methyl cellulose

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Introduction

1. INTRODUCTION

The International Association for the Study of Pain (IASP), 2011 states that pain is an unpleasant sensory and emotional experience associated with actual or potential tissue damage or described in terms of such damage. Epidemiological survey reported that chronic neuropathic pain greatly affects physical and mental health as well as the social and daily lives of those with this condition. It significantly reduced the quality of life and imposed economic burdens on individuals and society (Inoue S *et al*., 2017). The estimate of prevalence of pain with neuropathic characteristic probably varies between 6.9 and 10%. Broadly pain is classified into three types nociceptive pain, (caused by the stimulation of peripheral A-delta and C- polymodal pain receptors and by algogenic substances such as histamine, bradykinin, substance P) inflammatory pain (associated with tissue damage and infiltration of immune cells) and neuropathic pain (Manoj *et al.* ,2016).

- **Nociceptive pain**: Represents the normal response to noxious insult or injury of tissue such as skin, muscles, visceral organs, joints, tendons or bones.
- **Neuropathic pain**: Pain initiated or caused by a primary lesion or disease in the somatosensory nervous system.
- **Inflammatory pain**: It occurs as a resultof activation and sensitization of the nociceptive pain pathway by a variety of mediators released at a site of tissue inflammation.

In general pain is classified into acute and chronic pain depending on the duration.

- **Acute pain**: pain of less than 3 to 6 months duration.
- **Chronic pain**: pain lasting for more than 3-6 months, or persisting beyond the course of an acute disease, or after tissue healing is complete.

The general causes of pain include muscle spasms, increased central sensitization to pain, pathological sensitization, vitamin D deficiency ,drug side effects (especially bisphosphonates and statins), analgesic rebound.

1.1.PATHOPHYSIOLOGY OF PAIN

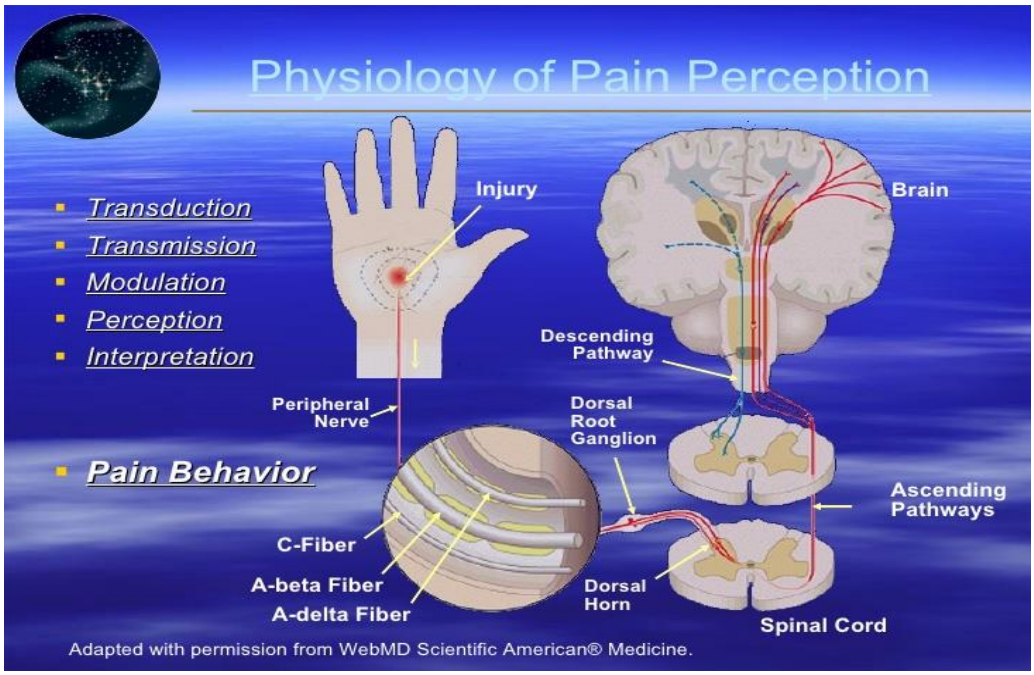


Fig: 1. Pathophysiology of pain

It involves the following processes:

a) Transduction:

Pain stimulus is converted into electrical energy. This electrical energy is known as Transduction. This stimulus sends an impulse across a peripheral nerve fiber (nociceptor).

b) Transmission:

Aδ fibers (myelinated) send sharp, localized and distinct sensations and C fibers (unmyelinated) relay impulses that are poorly localized, burning and persistent pain. Pain stimulus is then travelled to the spinothalamic tract.

c) Modulation:

Inhibitory neurotransmitters like endogenous opioids hinder the pain transmission. This inhibition of the pain impulse is known as modulation.

d) Perception:

In thisperson is aware of pain and unfolds a complex reaction and the somatosensory cortex identifies the location and intensity of pain. (Physiological and behavioral responses is perceived).

e) Interpretation and pain response

Neuropathic pain as defined by The International Association for the Study of Pain is "pain initiated or caused by a primary lesion in the nervous system" (Jaggi *et al*., 2014).Neuropathic pain is a type of chronic pain which is caused by lesion or disease of the somatosensory system, including peripheral fibres (Aβ, Aδ and C fibres) and central neurons and affects 7–10% of the general population. The somato-sensory system allows for the perception of touch, pressure, pain, temperature, position, movement and vibration. The somatosensory nerves arise in the skin, muscles, joints and fascia and they include thermoreceptors, mechanoreceptors, chemoreceptors, pruriceptors and nociceptors that send signals to the spinal cord and eventually to the brain for further processing (Abbott CA *et al*., 2011). Most sensory processes involve a thalamic nucleus receiving a sensory signal that is then directed to the cerebral cortex. Lesions or diseases of the somatosensory nervous system can lead to altered and disordered transmission of sensory signals into the spinal cord and the brain (Colloca *et al.,* 2017). Imbalances between excitatory and inhibitory somatosensory signalling, alterations in ion channels and variability in the way that pain messages are modulated in the central nervous system all have been implicated in neuropathic pain.

Neuropathic pain may result from disorders of the peripheral nervous system or the central nervous system (brain and spinal cord). Thus, neuropathic pain may be divided into peripheral neuropathic pain (nerves of peripheral nervous sytem are damaged), central neuropathic pain (nerves of central nervous system are damaged) or mixed (peripheral and central) neuropathic pain. Peripheral neuropathic pain is caused due to certain conditions such as painful diabetic neuralgia, trigeminal neuralgia, lumbar radiculopathy, nerve damage, pain because of cancer tumor infiltration, alcohol neuropathy. Central neuropathic pain is caused due to several factors such as Post-stroke pain, multiple sclerosis, chemotherapy-induced pain**.**

1.2.ETIOLOGY

Neuropathic pain is caused by several factors such as:

- **Diabetes:** It is one of the conditions which is most commonly associated with neuropathy. The characteristic symptoms of peripheral neuropathy often seen in diabetic people known as diabetic neuropathy.
- **Vitamin deficiencies:** Deficiencies of the vitamins B_{12} and folate as well as other vitamins can cause damage to the nerves.

- **Autoimmune neuropathy:** Autoimmune diseases such as rheumatoid arthritis, systemic lupus and Guillain-Barre syndrome can cause neuropathies.
- **Infection:** Some infections, including HIV/AIDS, Lyme disease, leprosy, and syphilis can damage nerves.
- **Post-herpetic neuralgia:** Post-herpetic neuralgia, a complication of shingles (varicella-zoster virus infection) is a form of neuropathy.
- **Alcoholic neuropathy:** Alcoholism is often associated with peripheral neuropathy. Although the exact reasons for the nerve damage are unclear, it probably arises from a combination of damage to the nerves by alcohol itself along with the poor nutrition and associated vitamin deficiencies that are common in alcoholics.
- **Genetic or inherited disorders:** Genetic or inherited disorders can affect the nerves and are responsible for some cases of neuropathy disease.

  In general neuropathic pain occurs as a result of the increased neuronal expression and/or activation of ion channels and receptors operating at the peripheral, spinal cord and supra-spinal levels, which causes alterations in the pain conduction pathway. This may also develop secondary to some pathological conditions like diabetes mellitus, cancer, herpes infection, autoimmune diseases and HIV infection (Jaggi *et al*., 2014).

  Neuropathic pain could be a blend of many sensory indications such as paresthesias (numbness or tingling), dysesthesias (electric shock phenomenon), hyperesthesia (increased sensitivity to mild painful stimuli), hyperalgesia (increased sensitivity to normally painful stimuli), hyperpathia (pain produced by subthreshold stimuli), spontaneous pain and allodynia (pain produced by normally non painful stimuli (Nischaltyagi *et al.*, 2014. Sensations of burning pain may be occur due to the continuous discharge in C-fibers, whereas dysesthesias (unpleasant abnormal sensations) and paresthesias (abnormal sensations) may arise from intermittent spontaneous discharges in A-δ or A- β fibers (Meena S *et al*., 2011). Peripheral sensitization mediated through C-fiber primary afferent neurons, may be the mechanism responsible for hyperalgesia. Two types of hyperalgesia are frequently described a) Primary hyperalgesia, which is increased pain and sensitivity at the site of injury. It is caused due to the peripheral changes that occur after tissue damage.b) Secondary hyperalgesia, which is increased sensitivity and pain in uninjured tissue that surrounds the site of injury. It results from the events that occur within the dorsal horn of the spinal cord after injury.

Allodynia is mediated by Aβ fibres .It is caused due to the central changes that occur after tissue damage.

1.3.PATHOGENESIS OF NEUROPATHIC PAIN

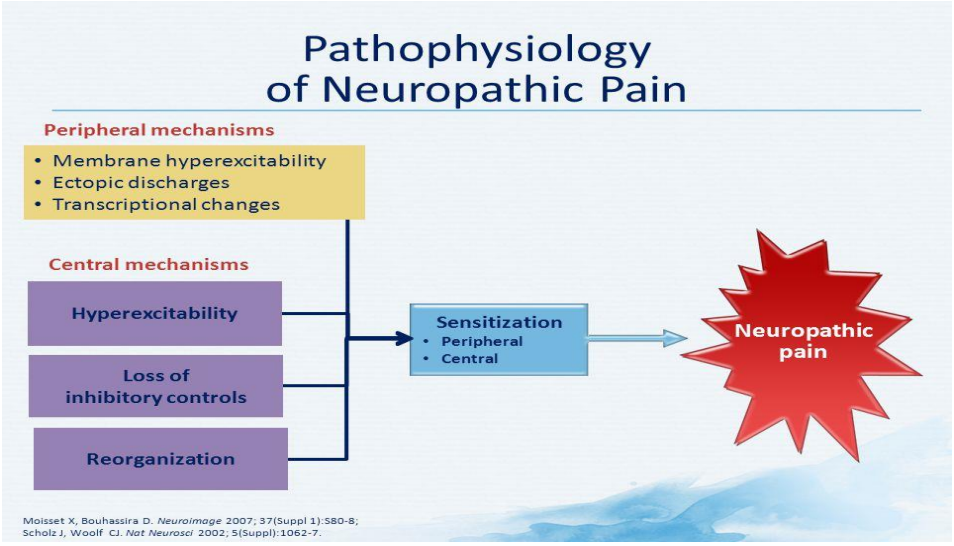


Fig: 2. Pathogenesis of neuropathic pain

1.3.1. PERIPHERAL MECHANISMS OF NEUROPATHIC PAIN

Neuropathic pain in the peripheral nervous system may occur due to the following reasons:

1. Ectopic discharges in lesioned fibers and their corresponding ganglia
2. Abnormal activity in axons undamaged by the lesion
3. Alterations in the expression and regulation of intracellular Ca^{2+} ion and modulatory receptors on primary afferent terminals
4. Neuroimmune interactions resulting in enhanced and/or altered production of inflammatory signaling molecules
5. Sensory-sympathetic coupling and other alterations in receptor signaling

1. Ectopic discharges in injured fibres

After an injury in the peripheral nervous system, chemicals such as noradrenaline, bradykinin, histamine, prostaglandins, potassium, cytokines, 5HT, and neuropeptides are released from damaged cells and inflammatory cells (e.g., mast cells, lymphocytes).These cellular mediators act to sensitize nociceptors to further neural input.This produces changes in the number and location of ion channels especially sodium ion channels in the injured nociceptor nerve fibers

and their dorsal root ganglia (Campero M *et al.,* 1998). As a result, the threshold for depolarization is lowered and spontaneous discharges known as ectopic discharges can occur in abnormal locations. Consequently, the response of nociceptors to thermal and mechanical stimuli is increased, this phenomenon is known as peripheral sensitization.In some disease processes, nerve demyelination from diminished blood supply may also contribute to the production of ectopic discharges along the nerve fiber.

2. Abnormal activity in axons not damaged by lesions

Persistent neural activity and changes that occur because of nerve injury can cause chemically mediated electrical connections between injured nerve fibers and nearby uninjured fibers (Devor M *et al.,* 2006). It is assumed that this transmission, known as ephaptic conduction ("cross excitation" or "cross talk"), causes nonpainful stimuli to evoke activity in these normally "silent" nociceptors and thereby produce pain.

3. Alterations in the expression and regulation of intracellular Ca^{2+} and modulatory receptors on primary afferent terminals

Neurotransmitter release by nociceptive terminals is triggered by Ca^{2+} entry and therefore depends on voltage-dependent channels (Cao YQ *et al.,* 2006). Spinal nerve ligation in rats induces over expression of the α_2/δ Ca^{2+} channel subunit in the corresponding dorsal ganglia, which might lead to an enhanced neurotransmitter release (in particular glutamate) by these terminals thereby increasing the pain sensation as well transmission.

4. Neuroimmune interactions resulting in enhanced and/or altered production of inflammatory signaling molecules

Nerve injury causes activation of peripheral immune cells. Cytokines and chemokines released by these immune cells, such as tumor necrosis factor-a (TNF-α), interleukins (ILs), nerve growth factor (NGF), and CC chemokine ligands, can cause sensitization of channels, such as TRPV1, and result in firing of nociceptors at normal body temperature (Chris Pasero *et al.,* 2004).

5. Sensory-Sympathetic coupling and other alterations in receptor signaling

Neuropathic pain is sometimes dependent on sympathetic nervous system activity. This is often referred to as 'sympathetically maintained pain' (Campbel *et al.,* 2006). It has recently been demonstrated that an abnormal contact develops between the sympathetic nervous system and the sensory nervous system following peripheral nerve injury, which may underlie the enhanced sensitivity to catecholamines which in turn leads to neuropathic pain (Dworkin R. H *et al.,*

2002). The basic observation must be that activity in the sympathetic nervous system initiates abnormal impulse in sensory neurons that leads to pain perception. Several sites of coupling between sensory and sympathetic nervous systems have been proposed and tested in animal models. The following have received experimental support:-

- Direct chemical coupling within peripheral effector sites between the noradrenergic and sensory neuron terminals.
- Ephaptic nerve coupling: Observed between sensory fibres in a damaged nerve but not between sympathetic and sensory fibres.
- Indirect coupling via peripheral sensitizing mechanisms involving the release of inflammatory mediators from sympathetic terminals and the sensitization of primary sensory neuron axons.
- Direct coupling between the sympathetic nervous system and the sensory nervous system in the dorsal root ganglion.

1.3.2. CENTRAL MECHANISMS

1. Spinal cord—anatomical re-organization

Spinal cord reorganization occurs in response to peripheral nerve injury. Consequence of this rearrangement is that second-order neurons within the spinal cord, that normally receive predominantly high-threshold sensory input, begin to receive inputs from low-threshold mechanoreceptors (Ducreux D *et al.*, 2006). This misinterpretation of information within the spinal cord may result in low-threshold sensory information being interpreted as nociceptive and causes an allodynia condition (Cameron AA *et al.*,1997).

2. Spinalcord –hyperexcitability

Due to peripheral injury, hyperexcitability of central nociceptor neurons (central sensitization) occurs in the spinal cord and causes the release of tachykinins (neuropeptides substance P and neurokinin) and neurotransmitters [glutamate, calcitonin gene-related peptide, and γ-aminobutyric acid (GABA)]. Prolonged release and binding of these substances to neural receptors activate the N-methyl-D-aspartate (NMDA) receptor, which causes an increase in intracellular calcium levels (Attal N *et al.*, 2000). The increase in calcium levels occur through N-type calcium channels. These changes lead to a series of biochemical reactions in dorsal horn neurons. The threshold for activation is lowered, the response to stimuli is increased (in both magnitude and duration), and the size of the receptive field is enlarged (a greater area on the

neuron surface is available to receive stimuli). Collectively, these changes results in an increased excitability and sensitivity of spinal cord neurons to pain.

3. Central dis-inhibition

Central dis-inhibition occurs when control mechanisms along inhibitory (modulatory) pathways are lost or suppressed. This, in turn, causes abnormal excitability of central neurons thereby increasing the pain sensitization (Bridges D *et al.*, 2001).

Neuropathic pain is very challenging to manage because of the heterogeneity of its etiologies, symptoms and underlying mechanisms (Beniczky *et al.*, 2005).Commonly used pharmacological treatments include antidepressants (tricyclic antidepressants [TCAs]), (selective serotonin reuptake inhibitors [SSRIs]), antiepileptic (anticonvulsant) drugs, topical treatments and opioid analgesics. Tricyclic antidepressants like (amitriptyline, clomipramine, desipramine) inhibit the re-uptake of biogenic amines and are also strong sodium-channel modulators (Beydounand Backonja, 2003; Sanchez and Hyttel, 1999). Thus TCAs may act both by enhancing dorsal horn inhibition and by diminishing peripheral sensitization. The serotonin noradrenaline reuptake inhibitors (SNRIs) such as duloxetine and venlafaxine enhance endogenous inhibitory mechanism thereby decreasing the pain sensitization. Anti- epileptics such as gabapentinoids, gabapentin and pregabalin bind to presynaptic voltage-gated calcium channels in the dorsal horn, reducing the release of excitatory neurotransmitters such as glutamate and substance P (Taylor CP, 2004).Topical lidocaine reduces pain by blocking the sodium channels at the periphery.Opioids like Oxycodone, Morphine sulphate act through the descending inhibitory pathways modulating nociceptive impulses in the dorsal horn**.** Tramadol is a centrally-acting analgesic, which has both direct opioid action and indirect monoaminergic action (S. Beniczky *et al.*, 2005; Moulin D *et al.*, 2014).

Animal models are important for understanding the mechanism of neuropathic pain and development of effective therapy for its optimal management. Neuropathic pain models are developed for manifestation of clinical pain conditions. Animal models have been used widely in pain research as these models are capable to serve as substitute assay that can dependably evaluate the potency and efficacy of the pharmacological intervention (Nischaltyagi *et al.*, 2014). Different types of animal models for neuropathic pain includes peripheral nerve injury,spinal

cord injury induced peripheral and central pain, chemotherapeutic drugs induced pain model, cancer and HIV-induced pain model, post-herpetic neuralgia (PHN), diabetes and chronic ethanol-induced pain model, trigeminal neuralgia and orofacial pain model have been developed (Jaggi *et al* .,2009)

Central Pain Model

The commonly used models for central pain include spinal cord compression. Nocifensive signs crushing of spinal cord with forceps or aneurysm, photochemically induced injury, excitatory neurotoxin methods, and spinal hemi-section are generally employed (Claire E *et al.,* 2009).

Peripheral nerve injury models

Numerous pain models have been developed that employ injury to a peripheral nerve for instance sciatic nerve which produces short-term or everlasting behavioral hypersensitivity and animal becomes susceptible to various behavioral tests such as tactile allodynia or thermal hyperalgesia (Zimmermann M *et al.,* 2001).It includes Chronic constriction injury model (CCI or Bennett model), Partial sciatic nerve ligation model (PSNL or Seltzer model), L5/L6 spinal nerve ligation model (SNL), Sciatic cryoneurolysis model (SCN), L5 spinal nerve ligation (Bennett GJ *et al.,*1988).

Neuropathic pain due to medical conditions

In human beings certain ailments such as diabetes, post herpeticneuralgia and cancer are responsible for causing neuropathic affliction.

Chemotherapy-induced peripheral neuropathy (CIPN) is defined as toxic neuropathy that results from the direct injury of the peripheral nervous system by chemotherapeutic agents such as taxanes, platinum compounds, vinca alkaloids, proteasome inhibitors, antiangiogenic or immunomodulatory agents. It is a frequent, dose-dependent complication of anticancer drugs**.** CIPN is considered to be one of the most common non-hematological adverse effects of a number of effective chemotherapeutic agents (Park SB *et al.,* 2013). CIPN can usually affect the dorsal root ganglia (DRG) of the primary sensory neurons, but other sites, i.e., the nerve terminals (distal terminations of the branches of an axon), may also be involved. CIPN is characterized by paresthesia, dysesthesia and often pain, primarily in the hands and feet (Mols F *et al.,* 2014). Patients most often report sensory symptoms of numbness and tingling, followed by symptoms described as burning, shooting, throbbing and stabbing. All of these symptoms are

also associated with diminished ability to detect touch and pinprick sensations as well as sensorimotor impairment (M. Ewertz *et al.*,2015).

1.4.PATHOPHYSIOLOGY

Although the mechanism of development of neuropathic pain due to chemotherapeutic drugs is unknown, various hypotheses have been suggested. They are as follows:

a) **Mitochondrial dysfunction**

Abnormal mitochondrial structure and function are considered as a possible etiology of CIPN. Most chemotherapeutic agents cause damage to neuronal and non-neuronal mitochondria, leading to increased production of ROS and thus causes increased oxidative stress. The pathological increase in ROS production in turn can cause damage to intracellular biomolecules such as enzymes, proteins and lipid molecules, which in turn leads to demyelination and disruption of the cytoskeleton of peripheral nerves as well as sensitization of signal transduction processes (Carozzi *et al*., 2015). Furthermore, ROS can cause the activation of apoptotic pathways and increase production of pro-inflammatory mediators. These processes can cause further damage to mitochondria, amplifying the production of ROS and pathological processes of oxidative stress.

b) **Pain mediators**

After anticancer drug treatment changes in many pain mediators like cytokines, growth factors, ion channels etc. in the peripheral nerve, dorsal root ganglion, and spinal cord have been postulated. Mitogen-activated protein kinases and extracellular signal-related kinases may play important roles in CIPN; activation of these kinases is involved in chemotherapy induced apoptosis (Lobert *et al.,* 1996). In CIPN animal model it was found that there was an increase in the TNF-α level, IL-1, and IL-6 in the sciatic nerve and spinal cord. Fos expression was activated significantly in the superficial, intermediate, and deep layers of the spinal cord in a CIPN animal model

c) **Abnormal spontaneous discharge in A and C fibers**

Peripheral and central sensitization may be involved in the development of CIPN. Abnormal spontaneous activity in Aβ, Aδ, and C fibers has been observed in vincristine, paclitaxel, oxaliplatin, and bortezomib induced peripheral neuropathy (Park, 2014). Moreover, C-fiber

nociceptors showed significant hyper responsiveness to supra-threshold tactile and thermal stimuli in a CIPN animal model.

d) **Others**

Peripheral neuropathy induced by paclitaxel, oxaliplatin, and vincristine induced is caused by decreased numbers of intra-epidermal sensory fibers and increased numbers of epidermal Langerhans cells and resembles a traumatic-nerve-injury-induced neuropathic pain model (CCI, sciatic nerve transection) (Jessica *et al.*, 2015). The CNS has also been shown to be involved in CIPN both directly and indirectly.

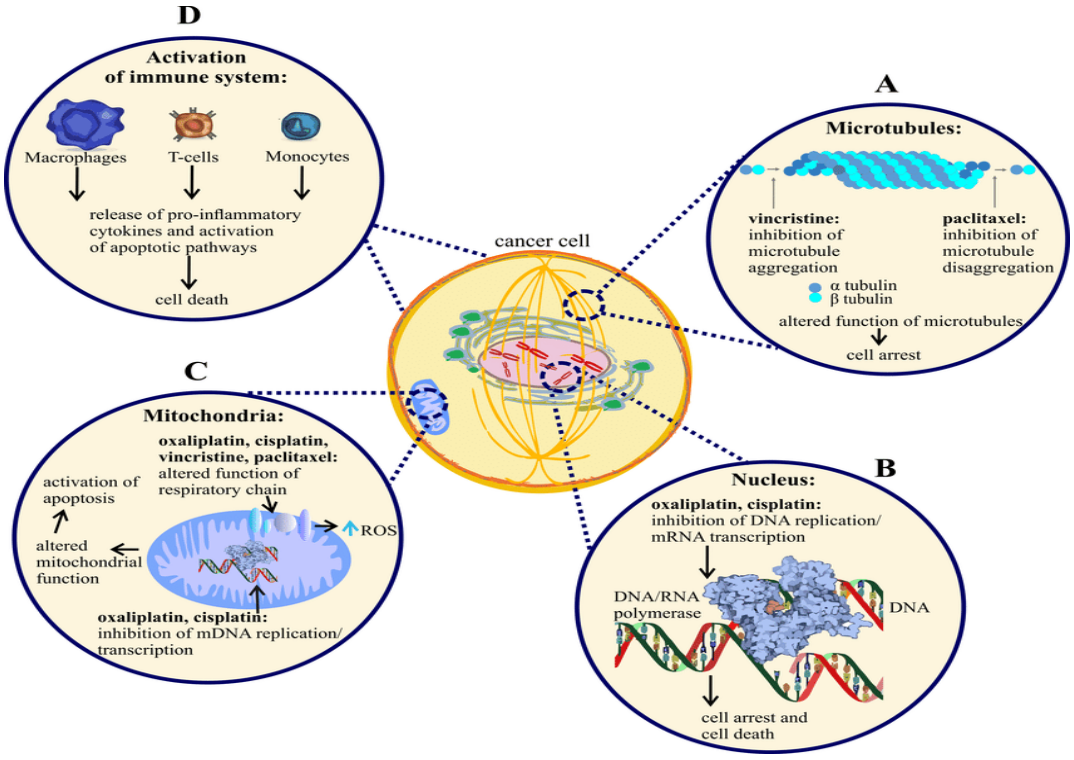


Fig: 3. Pathophysiology of chemotherapy induced neuropathic pain

Vincristine, also known as leurocristine is one of the most common chemotherapeutic agent used to treat several types of malignanacies such as leukaemia, lymphomas and sarcomas (Balaji *et al.*, 2015).These agents exert their anti-tumor efficacy by inhibiting mitosis through binding to tubulin in mitotically active cells, disrupting microtubule formation in the mitotic spindle. Thus, clinical application of these agents is often associated with dose-dependent painful neuropathy owing to damages to the peripheral axons (Emiliano S. Higuera *et al.*, 2004).

Vincristine belongs to the family of vinca alkaloids that can be obtained from the Madagascar periwinkle *Catharanthus roseus.* Vincristine is created through the semi-synthesis coupling of indole alkaloids vindoline and catharanthine in the vinca plant. It can also be synthesized through a stereocontrolled total synthesis technique which retains the correct stereochemistry at C_{18} and C_2. The absolute stereochemistry at these carbons is responsible for vincristine's anticancer activity.

Vincristine treatment is limited by a progressive sensorimotor peripheral neuropathy (Smith *et al.*,2016). This occurs in two major stages. In the early stage, the peripheral axons are damaged by vincristine and the principal symptoms are paresthesias and dyesthesias. In the later stage,which occurs more frequent at higher doses, axons are lost and the principal finding is loss of motor function**.** Vincristine affects the structure and function of peripheral nerve fibres.It has a great binding affinity to protein subunits of microtubules, tubulin, dissolution of axonal microtubules and causes the proliferation of neurofilaments.

Symptoms of neuropathy can be present soon after initiation of vincristine therapy but usually develops within several weeks and generally worsen with increasing cumulative doses of the drug.VIPN can be divided into sensory, motoric and autonomic components, with tendon reflexes, vibration sensitivity and strength mostly affected in the first year of treatment. Sensory neuropathy is characterized by numbness, tingling and neuropathic pain in the upper and lower extremities (Grisold W *et al.,* 2012).Vincristine-induced motor neuropathy is characterized by weakness in the upper and lower extremities and the development of wrist- or foot-drop due to impaired dorsiflexion that arises from damage to peripheral nerves. This is accompanied by gait abnormalities, cramps, and loss of or reduction in deep tendon reflexes which can be severe. Typical symptoms of autonomic neuropathy are constipation, urinary retention, and orthostatic hypotension.

1.5. PATHOLOGICAL MECHANISMS CONTRIBUTING TO VIPN

1.Oxidative stress and apoptotic pathways

Vincristine causes dysregulation and structural modification of neuronal mitochondria, leading to activation of apoptotic pathways, alteration in neuronal excitability and dysfunction of glial cells (Higuera E. S *et al.,* 2004). The effect of vincristine on mitochondria involves altered mitochondrial Ca^{2+} signaling.

2. Calcium homeostasis

Changes in the intracellular Ca^{2+} concentration influence membrane excitability, neurotransmitter release and gene expression of neuronal and glial cells

3. **Axon degeneration**

Long-term administration of chemotherapeutic agents results in axonal degeneration.There will be loss of large myelinated, small unmyelinated (more rarely), and intra-epidermal nerve fibers (IENF) which may be connected to the development of sensory-motoric peripheral neuropathy. Intraepidermal nerve fibers are unmyelinated or thinly myelinated nociceptors located in the dermis and are necessary for the sensation of pain arising from the periphery (Legha S.S *et al., 1*986). Additionally, loss of myelin and changes to the axonal cytoskeleton may alter the structure and function of peripheral nerves, which in turn may contribute to the development of altered pain perception. Vincristine causes the impairment of β-tubulin assembly and leads to severe alterations in axonal microtubules, axonal swelling and myelinated and unmyelinated fiber damage.

4. Changes in neuronal excitability

Chemotherapeutic agents cause changes to peripheral nerve excitability that contribute to the development of sensory peripheral neuropathy. These are likely caused by altered expression and function of a range of ion channels—including voltage-gated sodium (NaV), voltage-gated potassium (KV) and transient receptor potential (TRP) channels (Johnson I. S *et al.,* 1963). Vincristine-induced neuropathy has also been attributed to activation of $TRPA_1$ and $TRPV_4$ via the generation of reactive oxygen species and also may be due to the disruption of TRP channel function due to altered association with microtubules.

5. **Activation of the immune system and inflammation**

Vincristine induces the expression of integrins (immune markers) on the surface of endothelial cells which allows macrophages expressing the CX3CR receptor to adhere to the endothelium and to migrate into nervous tissue. Activation of monocyte-macrophages by the chemokine CX3CL1 also lead to production of ROS and subsequent activation of $TRPA_1$ (Hana Starobova and Irina Vetter 2017). Vincristine enhances the binding of the $STAT_3$ (Signal Transducer and Activator of Transcription 3) to the CXCL12 gene promotor (Xu *et al.,* 2017), causing up-regulation of C-X-C Motif Chemokine Ligand 12 in dorsal horn ganglia. The activation of

CXCR4 receptors in turn leads to an increase in intracellular Ca^{2+} and chemotaxis of immune cells to the site of inflammation.

Pterocarpus marsupium Roxb. (Leguminosae), (PM) Malabar kino, Indian kinotree or vijayasar is a plant drug belonging to the group called rasayana in ayurvedic system of medicine .These rasayana drugs are immunomodulators and relieve stress in the body. It is widely distributed in central, western and southern regions of India. Parts of the Indian kino have long been believed to have many medicinal properties. Heartwood juice of this plant is known to contain polyphenolic compounds (like flavonoids, diphenylpropane derivatives, and sesquiterpenes), which show strong antioxidant, anti-inflammatory, antidiabetic, antimicrobial, and anticancer activities and is used for the treatment of diabetes, jaundice, ulcer, gastritis, etc. In the ayurvedic Pharmacopoeia of India, it has been described to be used in the treatment of krmiroga (worm infection), kustha (leprosy), prameha (diabetes), pandu (anemia), and medodosa (obesity).

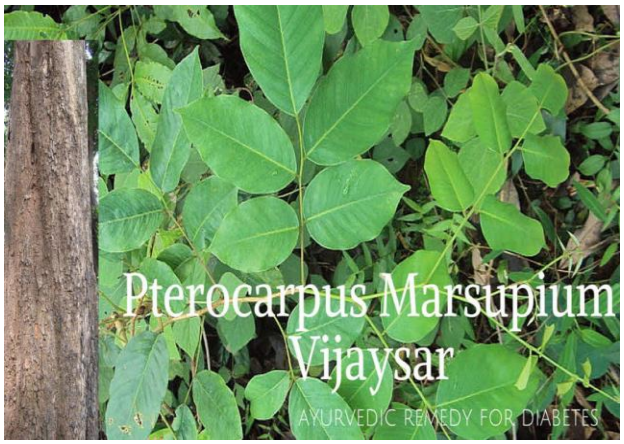


Fig: 4. *Pterocarpus marsupium* plant

Previous studies have showed the antioxidant (Gupta P *et al*.,2016) (Chakraborty A *et al.*,2010), antimicrobial(Gayathri M *et al.*,2009), antidiabetic, anti-inflammatory, and analgesic activities of different extracts of *P. marsupium* (Dipak Raj Pant *et al.*, 2017). Its main chemical constituent is a glucosidal tannin namely kinotannic acid. Several other chemical constituents like pterostilbene, (-)-epicatechin, pterosupin, marsupsin, etc. have been identified and isolated.

Neuropathic pain is widely recognized as one of the most difficult pain syndromes to manage, and outcomes often are unsatisfactory. Epidemiological research in this area can be problematic and the reasons for this are multifactorial. Patients experiencing pain may try numerous therapies, including traditional medicinal approaches for relief. Pain relief is the most frequently

cited reason that people seek complementary and alternative medicine. Since *Pterocarpus marsupium* capsules has shown its activity in diabetic neuropathy as well as analgesic activity in the previous studies the present study was designed to evaluate its protective effect in vincristine induced peripheral neuropathy.

Chapter 2
Literature review

2. LITERATURE REVIEW

Ahmet Hoke, 2012 discussed about the animal models which were used to induce peripheral neuropathies such as diabetic peripheral neuropathy, chemotherapy-induced peripheral neuropathy and HIV-associated sensory neuropathy. It was found that the similarity between these models is the comprehensive use of pathological, electrophysiological, and behavioral outcome measures (evaluation of distal sensory axons in the skin using a simple immunohistochemical stain) that mimic the human disease.

Xia Zhang et al., 2017 suggested that peripheral neuropathy is one of the major side effects associated with chemotherapeutic agents. This study mainly focused on the various mechanisms of neuropathic pain such as peripheral, central, inflammatory as well as chemotherapy induced neuropathic pain.

Park, 2014 examined that anti- cancer chemotherapeutics can cause painful peripheral neuropathy. It affects the sensory, motor and autonomic functions. Symptoms may occur at any time during the course of chemotherapy or even after discontinuation of treatment. In this study possible mechanism of neuropathy such as mitochondrial dysfunction, various pain mediators, abnormal spontaneous discharge in A and C fibers were discussed.

Ahmet Hoke et al., 2014 evaluated CIPN in rodents by behavioral, electro-physiologic and pathologic parameters. Behavioral testing was assessed by various methods such as mechanical allodynia, mechanical hyperalgesia,thermal hypo- or hyperalgesia,sensory–motor coordination. They are used to evaluate evoked sensory responses. Electrophysiological testing was assessed by motor nerve conduction studies and sensory nerve conduction studies. It evaluates the integrity of large myelinated axons. Histologic analyses were done by evaluating theneuronal morphology and numbers, nerve morphometry and by intraepidermal nerve fiber density. It evaluates the degree of axonal degeneration or changes in neuronal cell body.

Yaqin Han et al., 2013 evaluated the structural changes in peripheral nerves such as neuronopathy, axonopathy and/or myelinopathy, especially intra-epidermal nerve fiber (IENF) degeneration which are mainly caused by mitochondrial dysfunction and oxidative stress caused by the cancer chemotherapeutic agents. . It was found that CIPN was characterized by multiple

sensory changes including the development of (i) mechanical allodynia, in which light pressure or touch that would normally be perceived as innocuous, evokes pain (ii) cold allodynia whereby cold temperature evokes a painful sensation (iii) slowing of SNCV and (iv)loss of heat sensitivity.

Jessica A Boyette-Davis et al., 2015 provided information regarding the mechanism of chemotherapy induced neuropathic pain which include both cellular and molecular mechanisms. It has been found that processes found to be influenced during CIPN include an increased expression of inflammatory mediators, primarily cytokines, which can create cascading effects in neurons and glia. It was noted that in CIPN there was a change in ion channels, neurotransmission,intracellular signaling and structures.

Preet G. S. Makker et al ., 2017evaluated the distinct pathological changes caused by Paclitaxel (PTX) and Oxaliplatin (OXA) in the peripheral immunity and neuroinflammation, It was found that PTX and OXA were associated with an increase in CD_{4+} and CD_{8+} T-cells in the blood. OXA caused a significant reduction in immunosuppressive cells PTX selectively caused marked phenotypic changes in both the peripheral and central nervous systems.

Chuanyin Hu et al., 2016 evaluated the efficacy of Fucoidan on vincristine-induced neuropathic pain. It was observed that only with repeated Fucoidan (50, 100 or 200 mg/kg) administration there was attenuation in vincristine-induced mechanical and cold allodynia in a dose-dependent manner. The analgesic effects of Fucoidan contributed to an upregulation in the expression of GABA-B receptor in the spinal cord.

Gong SS et al., 2016 designed a study to investigate the effect of Matrine (MT) on VCR-induced neuropathic pain. MT ameliorated the nociceptive pain sensation (mechanical and cold allodynia, thermal and mechanical hyperalgesia), and alleviated electrophysiological and histopathological injury. It was found that Matrine caused a suppression of inflammatory response through regulating the expression of MPO, TNF-α, IL-6 and IL-10, as well as inhibiting oxidative stress and also decreased the calcium accumulation.

Jing Xu, et al., 2016 demonstrated the effect of Methyl-cobalamin on vincristine induced neuropathy. In this study it was found that MeCbl inhibited the loss of IENF and decreased

atypical mitochondrial prevalence. Moreover MeCbl not only inhibited the overproduction of TNF-α but also increased the expression of IL-10 in the spinal dorsal horn. It also inhibited the activation of NADPH oxidase and the downstream NF-κB pathway which resulted in the rebalancing of pro-inflammatory and anti-inflammatory cytokines in the spinal dorsal horn.

Soh Katsuyama et al., ***2014*** investigated the attenuation of vincristine-induced mechanical allodynia in mice by Milnacipran and Duloxetine. It was observed that SNRIs such as Milnacipran and Duloxetine were used for the treatment of acute, persistent, neuropathic pain as supplementary analgesics and also they showed significant antinociceptive effects against nociceptive and inflammatory pain. 5-HT and NA are implicated in modulating descending inhibitory pain pathways. The antiallodynic effects of SNRI in neuropathic pain models were predominantly attenuated by the noradrenergic system rather than by the serotonergic system at the spinal level. It has been reported that activation of the descending noradrenergic system and a subsequent increase of NA in the spinal dorsal horn in a rat neuropathic pain model contributed to the antihyperalgesic effects of antidepressants such as TCAs and SNRIs. The neuronal NA transporter is functionally expressed in cultured rat astrocytes and SNRI potently inhibits NA uptake in these cells.

Byoung Yoon Park et al., ***2010*** examined the antinociceptive effect of Memantine (N-methyl-D-aspartate receptors antagonist) and Morphine (opioid agonist) on vincristine-induced peripheral neuropathy.In this study it was examined that Memantine produced anti nociceptive effect due to its antagonistic activity on NMDA receptor. Memantine has been used to treat Parkinson's disease, spasticity, convulsions, vascular dementia, Alzheimer's disease, complex regional pain syndrome and phantom limb pain. Morphine exhibited its analgesic activity by acting through μ-opioid receptors.

Bilin Nie et al., 2017 demonstrated that intrathecal injection of Dexmedetomidine (DEX) or intraperitoneally administered Ulinastatin (UTI) significantly reduces mechanical allodynia induced by vincristine. It was observed that the antinociceptive effect was due to the upregulation of IL-10 expression and activation of α_2-adrenergic receptor in dorsal root ganglion. It was also noted that when DEX and UTI were given in combination there was a synergistic interaction. It was demonstrated that inflammation was closely related to neuropathic pain induced by chemotherapeutic drugs. IL-10 may be a powerful anti-inflammatory factor that can

suppress pro-inflammatory cytokines such as TNFα, IL-1, IL-6 and IFNγ implicated in neuropathic pain. IL-10 was able to reduce nociceptor sensitization and produce analgesia. Intrathecal IL-10 also suppresses CSF levels of at least IL-1and IL-1-mediated pain induced by intrathecal dynorphin.

Terumasa Chiba et al., 2017 investigated that there was an up-regulation of transient receptor potential vanilloid 1 ($TRPV_1$) in peripheral neuropathy caused by anti-cancer drug treatment. In this study it was found that vincristine-treated rats developed mechanical allodynia/hyperalgesia and there was an increased level of $TRPV_1$ protein expression in the small diameter DRG neurons. It was noted that C-fiber neurons began to express $TRPV_1$ after vincristine treatment. In contrast, $TRPV_2$ protein expression did not change in DRGs after treatment with vincristine.

Ting Xu et al., 2017 demonstrated that oral application of Magnesium-L-Threonate attenuated vincristine-induced allodynia and hyperalgesia. From this study it was evident that vincristine treatment activated TNF-α/NF-κB signaling by reduction of intracellular free Mg^{2+} of DRG neurons. It was found that oral application of L-TAMS completely blocked up-regulation of TNF-α but partially blocked activation of NF-κB induced by vincristine. Thus it clearly demonstrated that L-TAMS could not completely prevent the vincristine-induced allodynia and hyperalgesia, as activation of NF-κB also leads to transcription of many other pro-inflammatory genes, such as interleukin-1β, which are considered to be important for nociceptive sensitization.

Kiguchi N et al., 2009 examined the involvement of inflammatory mediators in neuropathic pain caused by vincristine. It was observed that systemic treatment with vincristine damages Schwann cells and DRG neurons. It was observed that the inflammatory mediators released by these cells may recruit macrophages in the PNS. Macrophages released the inflammatory cytokine IL-6, which elicited neuro-inflammation and led to the development of neuropathic pain. Secondary signal transduction of pain to the CNS activated microglia and astrocytes, and these activated glial cells contributed to the development and maintenance of neuropathic pain in the CNS through production of inflammatory cytokines (e.g., TNF- α).

Elizabeth K. Joseph et al., 2006 demonstrated that mitochondrial electron transport chain and ATP play an important role in peripheral pain mechanisms. It was found that inhibitors of

mitochondrial electron transport chain complexes I, II, III, IV, V exhibited antinociceptive effect in models of toxic and metabolic painful peripheral neuropathy and also for the mechanical hyperalgesia induced by TNF α. Since the mitochondrion is the major source of cellular ATP, this study provided further support for the role of mETC in peripheral pain mechanisms including a role of its end product, ATP. From the study it can be concluded that mETC and its end product, ATP, play an important role in peripheral pain mechanisms. mETC chain was important in inflammatory as well as neuropathic pain models whereas ATP contributed only to peripheral neuropathy.

Anita Barzegar-Fallah et al., 2014 designed a study to investigate the suppressive effect of Tropisetron (5-HT_3 receptor antagonist) against VCR induced neuropathy and also to ensure whether this effect was exerted through the 5-HT_3 receptor. Tropisetron is used as an antiemetic or in chemotherapy-induced emesis. In -vitro and in vivo studies have shown that tropisetron exerts immune modulatory and anti-inflammatory properties. It also inhibited IL-2 gene transcription and proliferation in antigen-stimulated human T cells via blockade of calcineurin/NFAT-dependent signaling pathway. It was found that due to nerve injury there was an increase in the intracellular calcium level which led to calcineurin activation and dephosphorylation of NFAT and its translocation to the nucleus. Finally these events resulted in the release of several cytokines, including IL-2, IL-4 and TNF and neuronal cell apoptosis. Tropisetron act as a neuroprotective agent for prevention of VCR-induced neuropathy via a receptor-independent pathway.

Stefanie Geisler et al., 2016 designed a study to evaluate whether genetic deletion of SARM-1 decreases axonal degeneration in a mouse model of neuropathy induced by VCR. It was observed that SARM-1 is necessary for the development of VIPN. Activation of the SARM-1 pathway rapidly depleted NAD^+ followed by loss of ATP which resulted in morphological degeneration of the axon. To block SARM-1-induced axonal degeneration in –vitro, NAD^+ has to be maintained.

Seunguk bang et al., 2016 demonstrated the anti-allodynic (mechanical and cold allodynia) effect of TheoesberivenF (*Melilotus* extract and proxyphylline) in VCR induced neuropathy. It was reported that ethyl acetate fraction of *Melilotussuaveolens* reduced the production of pro-inflammatory cytokines, including IL-6 and TNF-α via the suppression of nuclear factor-

κBactivation. Coumaric acid, a component of *Melilotus* extract, has been reported to exert an anti-inflammatory effect by reducing the expression of TNF-α. A coumarin derivative has showed its activity against neurotoxicity by improving the mitochondrial function. *Melilotus* extract exhibited its antioxidant effect by inhibiting the formation of TBARS, pro-oxidant H_2O_2 and superoxide anion.

Dipak Raj Pant et al., 2017 designed a study to evaluatethe phytochemical constituents and to study the antioxidant, antimicrobial, anti-diabetic, anti-inflammatory and analgesic activities of extracts from stem wood of PM. The study revealed that PM is a source of various phytoconstituents such as alkaloids, glycosides, saponins, tannins, proteins, carbohydrates, cardiac glycosides, flavonoids, and terpenoids. The acetone and IPA extract and ethanol extract showed antioxidant activity and this may be due to the presence of phenolic compounds.Acetone and IPA extract showed antibacterial activity only against Gram-positive bacteria and ethanol extract was inactive against all the bacteria. The acetone and IPA extract also showed potential anti-inflammatory activity. Ethanol extract exhibited anti -diabetic and analgesic activities.

Pankaj Gupta et al., 2016 evaluated the effect of alcoholic extract of heartwood of PM on in-vitro antioxidant, anti-glycation, sorbitol accumulation and inhibition of aldose reductase activity (ALA). It was found that the alcoholic extract exhibited antioxidant activity due to the presence of phenolic compounds. Due to the presence of high phenolic compounds and flavonoid contents in the alcoholic extract it inhibited the AGEs formation (antiglycation) either by decreasing the ROS formation or by scavenging .The alcoholic extract inhibited the ALR and prevented the accumulation of intracellular sorbitol.

Prarthana Chakraborty et al ., 2014 demonstrated that α-Dihydroxychalcone-glycoside (α-DHC) isolated from the heartwood of Pterocarpus marsupium inhibited LPS induced MAPK activation and up regulated HO-1 expression in murine RAW 264.7 macrophage.Theantiinflammatory activity of α-DHC was exerted by down-regulating MAPKs and restricting nuclear stabilization of NF-κB that inhibited the over expression of pro-inflammatory cytokines as well as inflammation mediating enzymes. It disrupted the Nrf-2–Keap-1 complex leading to induction of cytoprotective enzymes that indirectly repress the expression of inflammatory mediators. It has been observed that α-DHC reduces production of

NO as well as release of pro-inflammatory cytokines (IL-6, IL-1β, TNF-α) and also suppressed COX-2 and iNOS expression at both protein and mRNA levels.

Ajanta Chakraborty et al., 2010 evaluated the cytotoxic, anti-proliferative and anti-oxidant properties of pterostilbene isolated from PM. The study demonstrated that pterostilbene plays an important role in the apoptosis of two different types of cancer cell lines MCF-7 and PC3.It inhibited cellular proliferation and induces apoptosis by the production of nitric oxide

Mohammadi M et al ., 2009 designed a study to evaluate the antioxidant properties of extract of stem bark extract of PM .The results demonstrated that during acute exposure to alcohol, PM extract helps to maintain cholesterol as well as triglyceride levels. PM extracts reduced the free radical generation and this was due to the presence of flavonoids. PM extract showed strong antioxidant activity in all in vitro assays and protects liver cells from ethanol induced oxidative stress

Maruthupandian A et al., 2011 investigated the antidiabetic, antihyperlipidaemic and antioxidant activity of PM in alloxan induced diabetic rats. The ethanolic extract of PM showed hypoglycemic effect by the induction of insulin release from pancreatic cells of diabetic rats .The hypolipidemic effect was due to the inhibition of fatty acid synthesis. It was also observed that there was a reduction in the activity of SOD, CAT, GPx, GSH and GR in alloxan induced rats and this was due to the decrease lipid peroxidation and by normalizing the antioxidant system.

Kirana Halagappa et al., 2010 designed a study to investigate the effect of aqueous extract of PMon elevated inflammatory cytokine, tumor necrosis factor (TNF)-α in Type II diabetic rats. It was observed that there was a reduction in blood glucose level which was due to the enhanced insulin secretion by regeneration of β-cells.Decrease in elevated TNF-α by the aqueous extract of PM along with its blood glucose lowering effect suggested that the immunomodulatory property of the drug could be related with its potential anti-diabetic activity. It was noted that modulation of cytokine TNF-α by the aqueous extract of PM had indirect effect on PPAR-γ expression.

Dhanabal SP et al., 2006 evaluated the antidiabetic activity of various subfractions of the alcohol extract of the bark of PM in alloxan-induced diabetic rats. From the results it can be concluded that the PM decreased the glucose level (antihyperglycaemic) by increasing the

insulin secretion from the remnant or regenerated beta cells. It also resulted inhyperlipidaemic conditions.

Suresh K et al., 2012 investigated the insulinotrophic and insulin-like effects of a high molecular weight aqueous extract of PM (10KR). It was observed that the aqueous extract of PM (PME) stimulated the insulin secretion in response to hyperglycaemic culture conditions .The results demonstrated that both PME and 10KR had beneficial effects on lowering blood glucose and sensitizing the insulin responsiveness after a glucose challenge. It was noted that 10KR had potent antidiabetic properties both in -vitro and in- vivo

M. Gayathri et al., 2009 examined the antimicrobial activity of *Hemidesmus indicus, Ficus bengalensis* and *Pterocarpus marsupium.* The aqueous extracts of the roots of *H. indicus* and the bark *F. bengalensis* and *P. marsupium Roxb* exhibited significant antibacterial activity against tested bacterial strains. Presence of tannins and saponins in higher concentration than the other phytochemicals suggested that these phytochemicals are responsible for the antibacterial activity.

Chapter 3

Aim & Objective

3. AIM AND OBJECTIVE

AIM

- To evaluate the neuroprotective effect of ***Pterocarpus marsupium*** marketed capsules against **vincristine** induced neuropathy in rat model.

OBJECTIVE

- To study the effect of ***P.marsupium*** on behavioural models for neuropathic pain.
- To study the effect of ***P.marsupium*** on excitatory neurotransmitters (glutamate and aspartate) in vincristine induced rats.
- To study the effect of ***P.marsupium*** on histopathology of sciatic nerve.

Chapter 4

Plan of study

4. PLAN OF STUDY

Phase I:

Optimization of vincristine and induction of neuropathic pain in Female Sprague Dawley rats

Phase II:

BEHAVIOURAL TESTS

- Hot plate test
- Randall-Sellito test
- Electronic Von Frey test
- Formalin test
- Sciatic functional index

Phase III:

NEUROCHEMICAL ESTIMATION

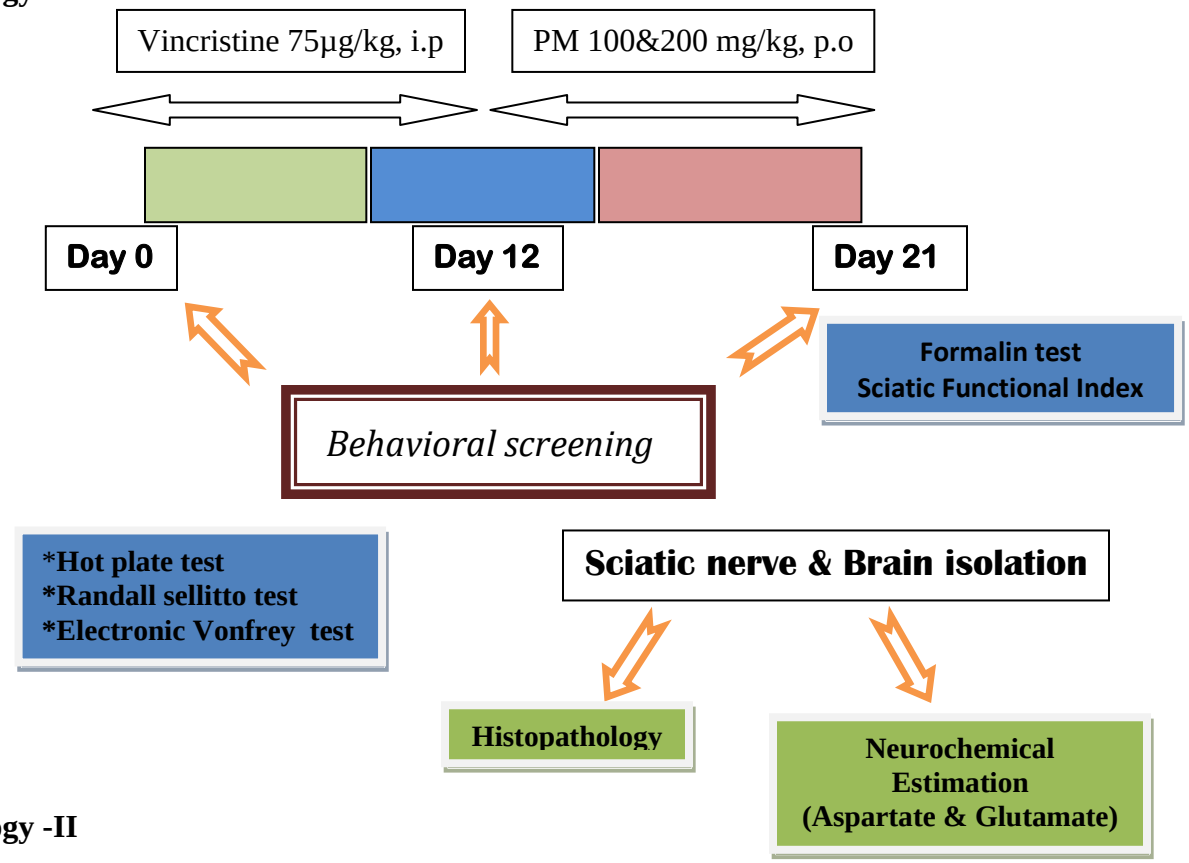
- Glutamate
- Aspartate

Phase IV:

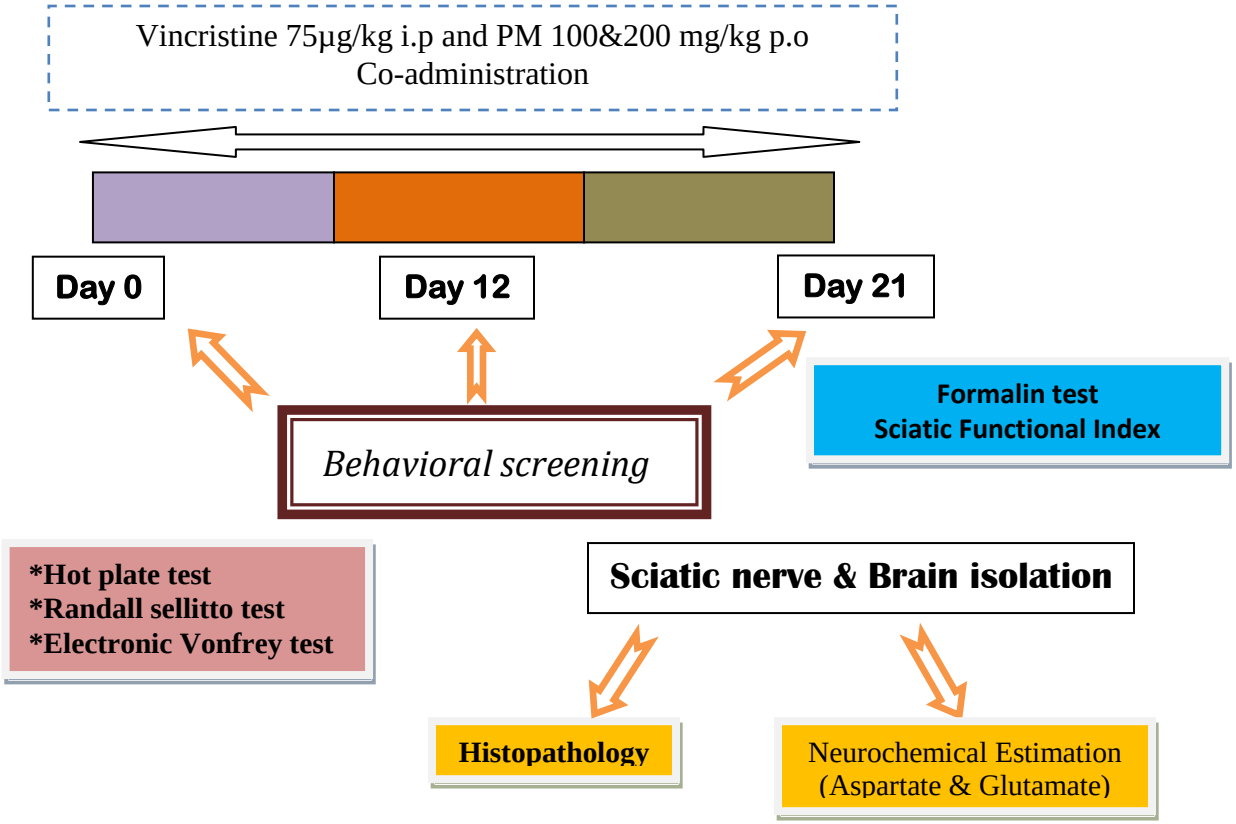
HISTOPATHOLOGICAL EVALUATION

- Histopathology of sciatic nerve

Methodology -I



Methodology -II



Chapter 5
Materials & Methods

5. MATERIALS AND METHODS

5.1. Chemicals and instruments used:

The chemicals used for the present study were tabulated in table 1.

Table 1:List of chemicals used

Chemicals	***Manufacturer***
Vincristine	Cipla, India.
Pterocarpus marsupium	VasuPharma, India.
Pregabalin	Sun pharma, India.
Chloroform	Reachem, India.
Sodium citrate	HI-MEDIA, India.
Formaldehyde	Reachem, India.
Carboxy Methyl Cellulose	OTTO, India.
Millipore water	Direct-Q, India.

The instruments used for the present study were tabulated in table 2.

Table 2:List of instruments used

Instrument	***Manufacturer***
Hot plate Analgesiometer	Dolphin
Vonfrey Filament	BIOSEB
Randall- Sellitto	Harvard
Refrigerated centrifuge	Eppendrof
Deep freezer	Cryo scientific systems
Vortex	TARSONS
HPTLC	CAMAG

5.2. Animals:

Female wistar rats of average weight (150-250g) were used for this study.The animals used for this study were obtained from PSG IMS&R (PSG Institute of Medical Science and Research) animal house and all the experimental protocol was approved by the institutional animal ethics committee(IAEC) proposal number 357/2017/IAEC. The rats were grouped and housed (6animals/cage) with husk as a bedding material in a room with controlled temperature of 25±1°C and a reversed light –dark cycle (12h\12h). Normal feed pellets and pure water ad libitum were provided for the animals throughout the experiment. Animals were acclimatized to laboratory condition for 1 week prior to experiments. The experiments were performed according to the guidelines of the committee for the purpose of supervision and control of experiment on animals (CPCSEA), Government of India

5.3. Pterocarpus marsupium capsules:

Pterocarpus marsupium capsules were obtained from VASU Healthcare Pvt. Ltd,Gujarat. It is a safe and effective herbal preparation that has been found to reduce glucose absorption from the gastrointestinal tract and improves insulin and pro-insulin levels. Each capsule contains 300mg bark extract of *P.marsupium*.

Fig: 5. *Pterocarpus marsupium* capsules

5.4. Methodology:

5.4.1. Induction and assessment of peripheral neuropathy by vincristine

Table: 3. Groupings of animals

GROUPS	TREATMENT	NUMBER OF ANIMALS
GROUP –I	Control (CMC + Saline)*	6
GROUP –II	Vincristine +Saline (75µg/kg,i.p)*	6
GROUP –III	Vincristine +Pregabalin (75µg/kg, i.p+10mg/kg ,p.o)*	6
GROUP –IV	Vincristine+*P.Marsupium* (75µg/kg, i.p+100mg/kg ,p.o)	6
GROUP –V	Vincristine+*P.Marsupium* (75µg/kg ,i.p+200mg/kg ,p.o)	6
GROUP –VI	Vincristine+*P.marsupium*(100mg/kg) (co-administration)	6
GROUP-VII	Vincristine+*P.marsupium*(200mg/kg) (co-administration)	6

* Indicates that the animals were obtained from Department Animal House.

Vincristine sulphate injection (1mg/ml) was diluted in normal saline just before the administration to get a concentration of 75µg/ml. The injection was given in a dose of 75µg/ kg (IP) once per day for 10 days in 5days on 2 days off and 5 days on cycle for the rats depending on their body weight. Peripheral neuropathy was confirmed after 10 consecutive days of vincristine administration. Nociceptive thresholds in these animals were assessed by subjecting

them to behavioral studies such as hot plate test for thermal hyperalgesia , Randall Sellito test for mechanical hyperalgesia, electronic Von Frey test for mechanical allodynia (0^{th} day,12^{th} day, 21^{st}day), formalin test for chemical induced nociception and sciatic functional index test for screening the neurophysiology of the nerve (21^{st} day) (Muthuraman *et al.* ,2011). Neurochemical estimation of excitatory neurotransmitters such as glutamate and aspartate in rat brain were done. Histopathological study of sciatic nerve was carried out.

5.4.2. Treatment schedule

The animals were divided into seven groups of six animals each. The study period was for 21 days. The first group is normal control in which the animal receives 0.3%CMC, second group consists of negative control (only vincristine treated), third group consists of standard in which the animal is treated with Pregabalin10mg/kg after the induction of neuropathic pain by vincristine, fourth and fifth group consists of animals treated with test drug (*Pterocarpus marsupium* 100 and 200 mg/kg post administration) after the induction of neuropathic pain,sixth and seventh group consists of animals receiving vincristine as well as test drug *Pterocarpusmarsupium* 100 and 200 mg/kg(co-administration) with a time interval of 1 hour. The standard (Pregabalin 10mg/kg) and the test drug (*P.Marsupium* 100 & 200mg/kg) dissolved in 0.3%w/v carboxymethylcellulose and were given orally.

5.5. Behavioral examination :

The animals were subjected to various behavioural studies such as hot plate, Randall-Sellito, Von Frey, formalin and sciatic functional index test.

5.5.1. Hot plate test (Thermal hyperalgesia)

The animals were individually placed on a hot plate (Eddy's hot plate) maintained at 55±1°C within the restrainer. The timer was started .The reaction time (in seconds) or latency period or nociceptive threshold was determined as the time taken for the rats to react to the thermal pain by either licking their paws or jumping . The cut off time was fixed to 10 sec in order to avoid damage to the paw (Sook-Ha Fan *et al.*, 2014).

5.5.2. Randall Sellito test (Mechanical hyperalgesia)

Mechanical hyperalgesia was evaluated by the Randall Sellito test using a paw pressure analgesia instrument. Before the test each animal received 5mins of handling and then it was placed into a cloth and immobilized with the same hand used to hold the tested paw. The test consists of application of mechanical force in which the tip of the device was applied on to the dorsum of the rat's hindpaw. The nociceptive threshold (expressed in grams)was determined when the rat exhibited a flinch response and attempted to withdraw its hind paw. The cut off pressure of 450g was maintained to avoid damage to the paws (Muthuraman *et al.*, 2011).

5.5.3. Electronic Von Frey test (Mechanical allodynia)

Mechanical allodynia was assessed by measuring the withdrawal threshold of the hind paw in response to a mechanical stimulus using an electronic Von Frey esthesiometer. Each animal was placed on a metallic grid floor in a plastic observation chamber that provided access to the plantar surface of the hind paw. The animals were allowed to acclimate in a quiet, subdued light environment to the environment for 10 min. A rigid tip attached to the meter was applied to the plantar surface of the hind paw from under the floor. At threshold the rats respond by flicking its hind paw away from the stimulus. The cut off force between 150-200grams was maintained to avoid damage to the paws. (Yoshihiro Takahashi *et al*., 2015)

5.5.4. Formalin test (Chemical induced pain model)

The experiment was conducted in a quiet, temperature controlled (20-30°C) room. Rats were acclimated to the individual observation chambers 1hr prior to the testing. 50 µL of 2.5%w/v solution of formalin was injected into the intraplantar surface of the rat hind paw. After injection the animals were placed immediately into the observation chamber. The amount of time the animal spent in licking and showing the elevation of the injected paw were taken as the indicator of pain response .The response was noted for the initial 10 mins (early phase) which starts immediately after the formalin injection. This phase is probably due to the direct chemical stimulation of nociceptors. The response was then noted for the last 20mins (delayed phase) which starts approximately 15 to 20 mins following formalin injection and it probably suggests that peripheral and inflammatory processes are involved. Time interval between the two phases

was 10mins .Control and test drugs were administered 30mins prior to the formalin injection (Meunier *et al.*, 1998).

5.5.5. Determination of Sciatic functional index (SFI) (Functional loss)

SFI has been used as one of the evaluation methods of walk track analysis. It is used in assessing the nerve function and motor function. The rat hind paws were dipped in ink and they were allowed to walk freely and SFI was measured from the images obtained on a white paper. A single walk by each animal was enough to obtain adequate footprints on the white paper. The footprints were analysed and the following foot lengths are obtained.

- Print length (PL): The distance from heel to toe
- Toe spread (TS): The distance from the first toe to the fifth to
- Intermediary toe spread(IT): The distance from the second to the fourth toe

All these measurements were taken from the experimental (E) and normal sides (N) .

The factors were calculated as follows:

Print Length Factor (PLF) = (EPL-NPL)/NPL

Toe Spread Factor (TSF) = (ETS-NTS)/NTS

Intermediary Toe Spread Factor (ITF) =(EIT-NIT)/NIT

Where, EPL = experimental print length

NPL = normal print length

ETS = experimental toe spread

NTS = normal toe spread

EIT = experimental intermediary toe spread

NIT = normal intermediary toe spread

These factors were incorporated into the SFI formula derived by Bain *et al.*,1989;

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

An SFI of 0 is normal and -100 indicates total impairment due to a complete transaction of the sciatic nerve. (Bain JR *et al*., 1989)

5.6. Neurochemical Estimation:

Simultaneous estimation of glutamate and aspartate in rat brain using HPTLC:

Excitatory neurotransmitters like L-glutamic acid and L-aspartic acid were measured in rat brain using high performance thin layer chromatography (HPTLC) coupled with densitometer.

Chromatographic conditions:-

- Stationary phase: Silica gel 60 F 254
- Mobile phase: n-butanol : glacial acetic acid : water (65:15:25v/v)
- Chamber saturation time: 3hrs
- Instrument : HPTLC(Camag-version1.3.4)
- Applicator (injector): Linomat V
- Scanner: Camag TLC scanner III
- Developing chamber: Twin trough glass chamber (20×10)
- Developing mode: Ascending mode(multiple development)
- Detection reagent: 0.2% ninhydrin in acetone
- Scanning wavelength: 486nm
- Experimental condition : 25±2°C
- Temp/RH: 55–65%

Preparation of Standard Solutions:-

0.1 N HCl was prepared in 80% ethanol which was used to dissolve L-glutamic acid and L-aspartic acid.

Preparation of Stock Solution of L-Glutamic Acid and L-Aspartic Acid:-

Stock solutions of L-glutamic acid and L-aspartic acid were prepared by dissolving 1mg,2mgand 4mg of the respective amino acid in 1mL of 0.1 N HCl (1 mg/mL).

Preparation of 0.2% Ninhydrin Solution:-

In 100 mL standard flask, 200 mg of ninhydrin was taken and dissolved in acetone. Then add

1mL of pyridine and make up the volume with 100 mL acetone

Preparation of Brain Tissue Samples:-

Rats were sacrificed under ether anesthesia and their brains was excised out quickly in ice cold conditions and stored at -80°C. The hippocampus region was homogenized in 0.1 N HCl (for every 10 mg tissue/200 mL) in a manual homogenizer. The homogenates were transferred to polypropylene tubes and centrifuged at 4500 rpm for 20 min at 25°C. The supernatant was then transferred into micro centrifuge tubes and used at the earliest for spot application.

Procedure:

A 5µL volume of the standard solutions of (L-glutamic acid, L-aspartic acid) and brain samples was applied on a prewashed (ethanol) HPTLC plate. Spots were dried in a hot air oven at 60–65°C for 1–2 min and the plate was developed in the mobile phase n-butanol: glacial acetic acid: water (65:15:25). When the solvent front reached the top of the plate, the plates were removed and dried at 60–65°C for 3–4 min in a hot air oven.The plates were then dipped (1 sec) in 0.2% ninhydrin reagent and dried in a hot air oven at 60–65°C for 3–4 min. The spots were scanned at 486 nm and the peak areas were recorded. Calibration curves of L-glutamic acid and L-aspartic acid were prepared by plotting areas vs. concentration (Babu *et al*., 2007).

5.7. Histopathological Evaluation:

The histopathological examination of the myelin sheath level in the sciatic nerve will be analyzed qualitatively for the extent of axonal degeneration. Samples of sciatic nerve will be stored in a fixative solution (10% formalin) and staining will be done by using hematoxylin and eosin as described by method of (Oznur*et al.*,2016).

5.8. Statistical Analysis:

Data were given in the form of arithmetical mean values and standard error mean of variance. Data's of hot plate test, Randall Sellito test, electronic Von Frey test were subjected to Two way

ANOVA followed by Bonferroni's post hoc test for comparison. The data's of formalin test and sciatic functional index were subjected to One way ANOVA (Analysis of variance) followed by post hoc Tukey's multiple comparison test. The level of significance was accepted at $p<0.05$.The analysis was carried out by using Graph pad prism software of version 5.03.

Chapter 6

Results

6. RESULTS

6.1. Effect of PM on hot plate test in vincristine induced neuropathic pain rat model

Vincristine (75µg/kg) administration for ten days resulted in significant development of thermal hyperalgesia as reflected by increase in paw licking and jumping from the hot plate surface. The increased pain perception is the symptoms of neurotoxicity or neuropathic pain. Administration of PM attenuated vincristine induced decrease in the nociceptive threshold for thermal hyperalgesia .Control group showed significant difference in pain threshold on 12^{th} and 21^{st} day when compared to the other groups. $F(6, 35) = 11.81$, $p<0.05$[PM 200mg; post-admin, 12^{th} day], $p<0.001$[PM 200mg; co-admin, 12^{th} day] and $p<0.001$[vincristine 12^{th} & 21^{st} day, PM 100mg; post & co-admin 12^{th} & 21^{st} day, PM 200mg; co-admin 21^{st} day, pregabalin 21^{st} day]. It showed significant increase in pain threshold on 12^{th} and 21^{st} day when compared to the vincristine treated group ($p<0.001$). Vincristine treated group showed significant reduction in the pain threshold on 12^{th} and 21^{st} day when compared to the other groups ($F(6, 35) =11.81$, $p<0.001$). Treatment with standard drug Pregabalin (10mg/kg) showed significant increase in pain threshold on 12^{th} and 21^{st} day when compared to treatment with test drug PM post & co-administration (100mg/kg) $F(6, 35) = 11.81$, $p<0.01$[PM 100mg; co-admin 21^{st} day], $p<0.001$[PM 100mg; post-admin 12^{th} & 21^{st} day]. Treatment with test drug PM post administration (100mg/kg) showed significant difference in pain threshold on 12^{th} and 21^{st} when compared to treatment with test drug PM post administration (200mg/kg) and co – administration (200mg/kg) $F(6, 35) =11.81$, $p<0.05$[PM 200mg; post-admin 21^{st} day], $p<0.01$[PM 200mg; co-admin 12^{th} & 21^{st} day] and $p<0.001$[PM 200mg; post-admin 12^{th} day]. Treatment with test drug PM post administration (200mg/kg) showed significant difference in pain threshold on 12^{th} and 21^{st} when compared to treatment with the test drug PM co-administration (100mg/kg) $F(6, 35) = 11.81$, $p< 0.05$).

6.2. Effect of PM on Randall Sellito test in vincristine induced neuropathic pain rat model

Vincristine administered rats exhibited a significant development of mechanical hyperalgesia as reflected by either a flinch response or withdrawal of hind paw. Administration of PM attenuated vincristine induced decrease in the nociceptive threshold for mechanical hyperalgesia. Control group showed significant difference in pain threshold on 12^{th} and 21^{st} day when compared to

the vincristine treated group, treatment with the test drug PM post administration (100mg/kg & 200mg/kg) F (6, 35) =13.16, p<0.05[PM 200mg; post-admin 12th day] and p<0.001[vincristine, PM 100mg; post-admin 12th & 21st day]. Vincristine treated group showed significant reduction in the pain threshold on 12th and 21st day when compared to the other groups (F (6, 35) =13.16, p<0.001). Treatment with standard drug Pregabalin (10mg/kg) showed significant difference in pain threshold on 12th and 21st day when compared to treatment with test drug PM post & co-administration (100mg/kg and 200mg/kg) F (6, 35) =13.16, p<0.05[PM 200mg; co-admin 12th & 21st day], p<0.01[PM 100mg; co-admin 12th day], p<0.001[PM 100mg; co-admin 21st, PM 100& 200mg; post-admin 12th & 21st day].Treatment with test drug PM post administration (100mg/kg) showed significant difference in pain threshold on 12th and 21st when compared to treatment with test drug PM co – administration (100mg/kg and 200mg/kg) F(6, 35) =13.16, p< 0.05[PM 100mg; co-admin 12th day, PM 200mg; co-admin 21st day], p< 0.01[PM 200mg; co-admin 12th day].

6.3 Effect of PM on electronic Von Frey test in vincristine induced neuropathic pain rat model

Administration of Vincristine (75µg/kg) for ten days resulted in a significant development of mechanical allodynia as reflected by the significant increase in hind paw withdrawal reflux. Administration of PM attenuated vincristine induced decrease in the nociceptive threshold for mechanical allodynia. Control group showed significant difference in pain threshold on12th and 21stday in comparison to vincristine treated group and treatment with test drug PM post administration of 100mg/kg and 200mg/kg F (6, 35) =1.219 p<0.05[PM 200mg; post-admin 12th day], p<0.01[PM 100mg; post-admin 12th day], p<0.001[vincristine 12th & 21st day]. Vincristine treated group showed significant reduction in the pain threshold on 12th and 21st day when compared to the other groups F (6, 35) =1.219, p<0.05[PM 100mg; post-admin 21st day, PM 200mg; co-admin 12th day], p<0.01[PM 100mg; co-admin 21st day, PM 200mg; post-admin 21st day] and p<0.001[PM 200mg; co-admin 21st day].

6.4. Effect of PM on formalin test in vincristine induced neuropathic pain rat model

The effect of PM in formalin test was measured in two different phases, acute and delayed phase based on paw licking and paw elevation parameters.

6.4.1. Effect of PM on acute phase (0-10mins)

Paw licking

Formalin injection in control group showed significant difference in paw licking when compared to vincristine treated group, treatment with test drug PM post and co – administration (100mg/kg and 200mg/kg) F (6, 35) =82.93, p<0.01[PM 200mg; co-admin], p<0.001[vincristine; PM 100& 200mg; post-admin, PM 100mg; co-admin]. Vincristine treated group showed significant increase in paw licking when compared to treatment with standard drug Pregabalin (10mg/kg) as well as the test drug PM (100mg/kg and 200mg/kg) post and co-administration (F (6, 35) =82.93, p<0.001). Treatment with standard drug Pregabalin (10mg/kg) showed significant difference in paw licking when compared to treatment with test drug PM post and co-administration (100mg/kg and 200mg/kg) (F (6, 35) =82.93, p<0.001).Treatment with test drug PM post administration (100mg/kg) showed significant difference in paw licking when compared to treatment with test drug PM co-administration (100mg/kg and 200mg/kg) F (6, 35) =82.93, p<0.05[PM 100mg; co-admin], p<0.001[PM 200mg; co-admin].Treatment with test drug PM post administration (200mg/kg) showed significant difference in paw licking when compared to treatment with test drug PM co-administration (200mg/kg) (F (6, 35) =82.93, p<0.01).

Paw elevation

Formalin injection in control group showed significant difference in paw elevation when compared to vincristine treated group, treatment with test drug PM post and co – administration (100mg/kg and 200mg/kg) F (6, 35) =82.93, p<0.01[PM 200mg; co-admin], p<0.001[vincristine, PM 100 & 200mg; post-admin, PM 100mg; co-admin]. Vincristine treated group showed significant increase in paw elevation when compared to treatment with standard drug Pregabalin (10mg/kg) as well as the test drug PM (100mg/kg and 200mg/kg) post and co-administration (F (6, 35) =82.93, p<0.001). Treatment with standard drug Pregabalin (10mg/kg) showed significant difference in paw elevation when compared to treatment with test drug PM post and co-administration (100mg/kg and 200mg/kg) (F (6, 35) =82.93, p<0.001). Treatment with test drug PM post administration (100mg/kg) showed significant difference in paw elevation when compared to treatment with test drug PM co-administration (100mg/kg and 200mg/kg) F (6, 35)

=82.93, p<0.05[PM 100mg; co-admin], p<0.001[PM 200mg; co-admin]. Treatment with test drug PM post administration (200mg/kg) showed significant difference in paw elevation when compared to treatment with test drug PM co-administration (200mg/kg) (F (6, 35) =82.93, p<0.01).

6.4.2. Effect of PM on delayed phase (20-40mins)

Paw licking

Formalin injection in control group showed significant difference in paw licking when compared to vincristine treated group, treatment with test drug PM post (100mg/kg and 200mg/kg) and co–administration (100mg/kg) F (6, 35) = 22.58, p<0.01[PM 100mg; co-admin], p<0.001[vincristine, PM 100 &200mg; post-admin]. Vincristine treated group showed significant increase in paw licking when compared to treatment with standard drug Pregabalin (10mg/kg) as well as the test drug PM (100mg/kg and 200mg/kg) post and co-administration F (6, 35) =22.58, p<0.01[PM 100mg; post-admin], p<0.001[PM 200mg post-admin]. Treatment with standard drug Pregabalin (10mg/kg) showed significant difference in paw licking when compared to treatment with test drug PM post administration (100mg/kg and 200mg/kg) F (6, 35) =22.58, p<0.05[PM 200mg; post-admin], p<0.01[PM 100mg; post-admin].Treatment with test drug PM post administration (100mg/kg) showed significant difference in paw licking when compared to treatment with test drug PM co-administration (200mg/kg) (F (6, 35) =22.58, p<0.05).

Paw elevation

Formalin injection in control group showed significant difference in paw elevation when compared to vincristine treated group, treatment with test drug PM post (100mg/kg and 200mg/kg) and co –administration (100mg/kg) F (6, 35) = 22.58, p<0.01[PM 100mg; co-admin], p<0.001[vincristine PM 100& 200mg; post-admin]. Vincristine treated group showed significant increase in paw elevation when compared to treatment with standard drug Pregabalin (10mg/kg) as well as the test drug PM (100mg/kg and 200mg/kg) post and co-administration F (6, 35) =22.58, p<0.01[PM 100mg; post-admin], p<0.001[pregabalin, PM 100mg; co-admin, PM 200mg; post & co-admin]. Treatment with standard drug Pregabalin (10mg/kg) showed significant difference in paw elevation when compared to treatment with test drug PM post

administration (100mg/kg and 200mg/kg) F (6, 35) =22.58, p<0.05[PM 200mg; post-admin], p<0.01[PM 100mg; post-admin]. Treatment with test drug PM post administration (100mg/kg) showed significant difference in paw elevation when compared to treatment with test drug PM co-administration (200mg/kg) (F (6, 35) =22.58, p<0.05).

6.5. Effect of PM on sciatic functional index in vincristine induced neuropathic pain rat model

Vincristine (75µg/kg) administration for ten days resulted in significant sciatic functional loss and administration of PM significantly attenuated the vincristine induced rise in sciatic functional index. Control group showed significant difference in sciatic functional index level when compared to vincristine treated group. Treatment with standard drug Pregabalin (10mg/kg)as well as the test drug PM (100mg/kg & 200mg/kg) post and treatment with co – administration (F (6, 35) =1304, p<0.001). Vincristine administered rats resulted in sciatic functional loss as reflected by a significant rise in sciatic functional index level when compared to treatment with standard drug Pregabalin (10mg/kg) as well as the test drug PM (100mg/kg & 200mg/kg) post and with co–administration (F (6, 35) =1304, p<0.001).Treatment with Standard drug Pregabalin (10mg/kg) showed significant difference in sciatic functional index level when compared to treatment with the test drug PM (100mg/kg & 200mg/kg) post and co-administration (F (6, 35) =1304, p<0.001). Treatment with the test drug PM post administration (100mg/kg) showed significant difference in sciatic functional index level when compared to treatment with the test drug PM post (200mg/kg) and co-administration (100mg/kg & 200mg/kg) (F (6, 35) =1304, p<0.001). Treatment with the test drug PM post administration (200mg/kg) showed significant difference in sciatic functional index level when compared to treatment with the test drug PM co-administration (100mg/kg & 200mg/kg) (F (6, 35) =1304, p< 0.001).

6.6. Effect of PM on excitatory neurotransmitters in rat brain

6.6.1. HPTLC fingerprinting

Finger printing of reference standards such as glutamate and aspartate along with brain samples was done using HPTLC and the spots were visualized at 486 nm. The solvent system used for the development of chromatogram was n-butanol, glacial acetic acid, water (65:15:25)v/v/v).The pink colour spots obtained on the chromatogram after spraying of ninhydrin indicated the

presence of glutamate and aspartate in the brain samples. A defined peak at Rf between 0.40 and 0.60 were obtained for standard glutamate and aspartate. The 3D display of the densitogram at 486nm and chromatogram indicated the presence of glutamate and aspartate in the brain samples.

6.6.2. Effect of PM on glutamate levels in vincristine induced neuropathic pain rat model

The concentration obtained from HPTLC was analysed by One way ANOVA followed by Tukey's multiple comparison test. Data's were expressed in ng/mg of wet tissue. It was found that PM significantly attenuated vincristine induced rise in glutamate levels. Control group showed significant difference in glutamate levels when compared to vincristine treated group. Treatment with standard drug Pregabalin (10mg/kg) as well as the test drug PM (100mg/kg & 200mg/kg) post and treatment with co – administration ($F(6, 35) = 664.4$, $p<0.001$). Vincristine administered rats resulted in significant rise in glutamate level when compared to treatment with standard drug Pregabalin (10mg/kg) as well as the test drug PM(100mg/kg & 200mg/kg) post and co – administration ($F(6, 35) = 664.4$, $p< 0.05$, $p<0.001$).Treatment with Standard drug Pregabalin (10mg/kg) showed significant difference in glutamate level when compared to treatment with the test drug PM (100mg/kg & 200mg/kg) post and co-administration ($F(6, 35) = 664.4$, $p<0.05$, $p<0.001$). Treatment with the test drug PM post administration (100mg/kg) showed significant difference in glutamate level when compared to treatment with the test drug PMpost (200mg/kg) and co-administration (100mg/kg & 200mg/kg) ($F(6, 35) =664.4$, $p<0.001$). Treatment with the test drug PM post administration (200mg/kg) showed significant difference in glutamate level when compared to treatment with the test drug PM co-administration (100mg/kg & 200mg/kg) ($F(6, 35) =664.4$, $p<0.001$).

6.6.3. Effect of PM on aspartate levels in vincristine induced neuropathic pain rat model

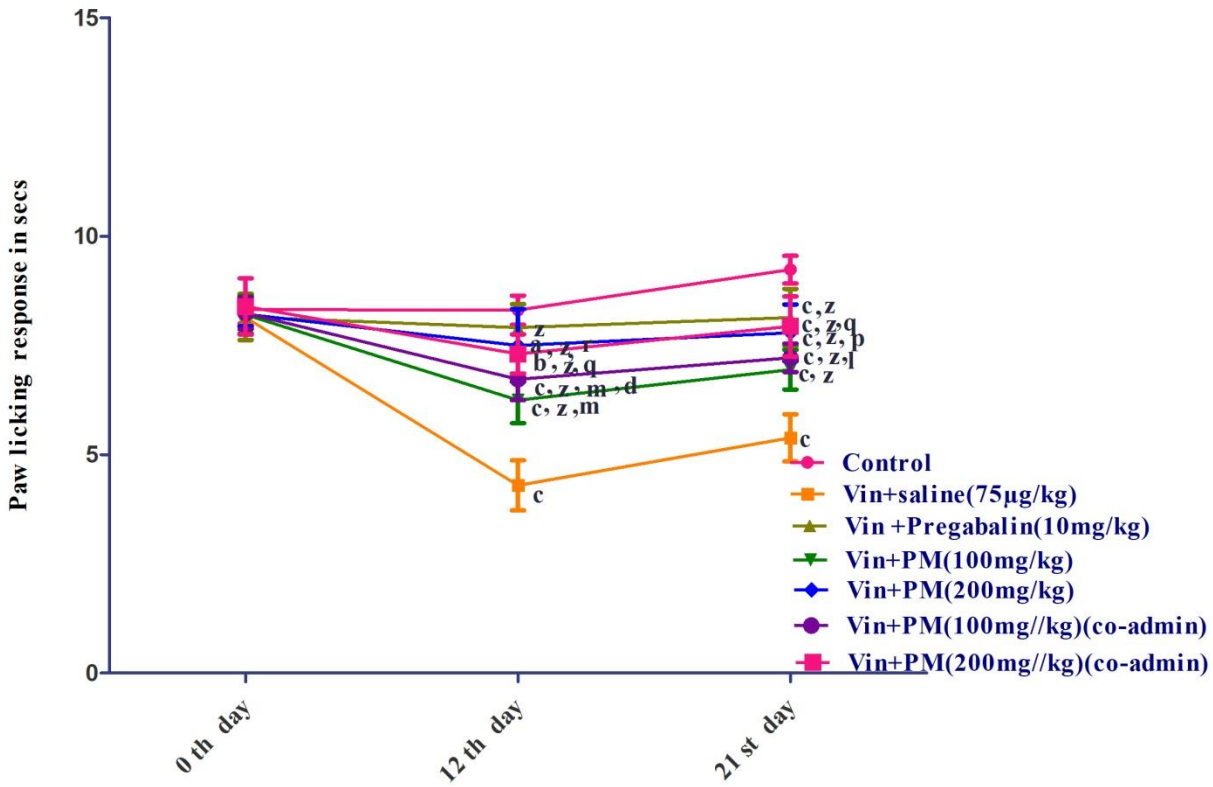
The concentration obtained from HPTLC was analysed by One way ANOVA followed by Tukey's multiple comparison test. Data's were expressed in ng/mg of wet tissue. It was found that PM significantly attenuated vincristine induced rise in aspartate levels. Control group showed significant difference in aspartate levels when compared to vincristine treated group. Treatment with the test drug PM (100mg/kg & 200mg/kg) post and treatment with co–administration ($F(6, 35) =141.8$, $p<0.001$). Vincristine administered rats resulted in significant rise in aspartate level when compared to treatment with standard drug Pregabalin (10mg/kg) as

well as the test drug PM post (200mg/kg) and with co – administration (100mg/kg & 200mg/kg) ($F (6, 35) = 141.8$, $p<0.001$).Treatment with Standard drug Pregabalin (10mg/kg) showed significant difference in aspartate level when compared to treatment with the test drug PM(100mg/kg & 200mg/kg) post and co-administration ($F (6, 35) = 141.8$, $p< 0.05$, $p<0.001$). Treatment with the test drug PM post administration (100mg/kg) showed significant difference in aspartate level when compared to treatment with the test drug PM post (200mg/kg) and co-administration(100mg/kg & 200mg/kg) ($F (6,35)=141.8$, $p<0.001$). Treatment with the test drug PM post administration (200mg/kg) showed significant difference in aspartate level when compared to treatment with the test drug PM co-administration (200mg/kg) ($F (6, 35) = 141.8$, $p<0.001$). Treatment with the test drug PM co-administration (200mg/kg) showed significant difference in aspartate level when compared to treatment with the test drug PM co-administration (200mg/kg) ($F (6, 35) = 141.8$, $p<0.05$).

6.7. Histopathology

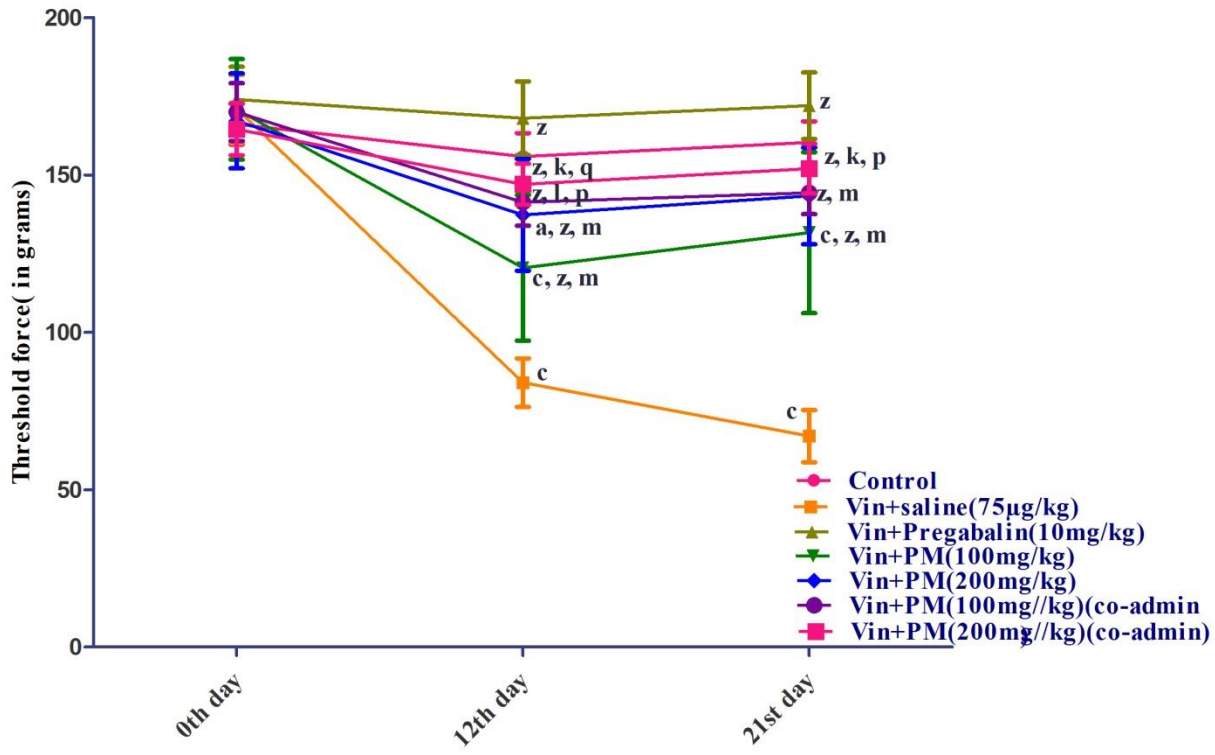
Vincristine treated group resulted in significant histopathological changes when compared to the other groups such as axonal degeneration, schwann cell hyperplasia, damage of myelin sheath, fibrosis which were assessed in the transverse sections of sciatic nerve. Treatment with standard drug Pregabalin (10mg/kg) as well the test drug PM (100mg/kg & 200mg/kg) post and co-administration significantly attenuated vincristine induced axonal degeneration (caused axonal regeneration) and other histopathological changes.

Fig: 6. Effect of PM on hot plate test in vincristine model



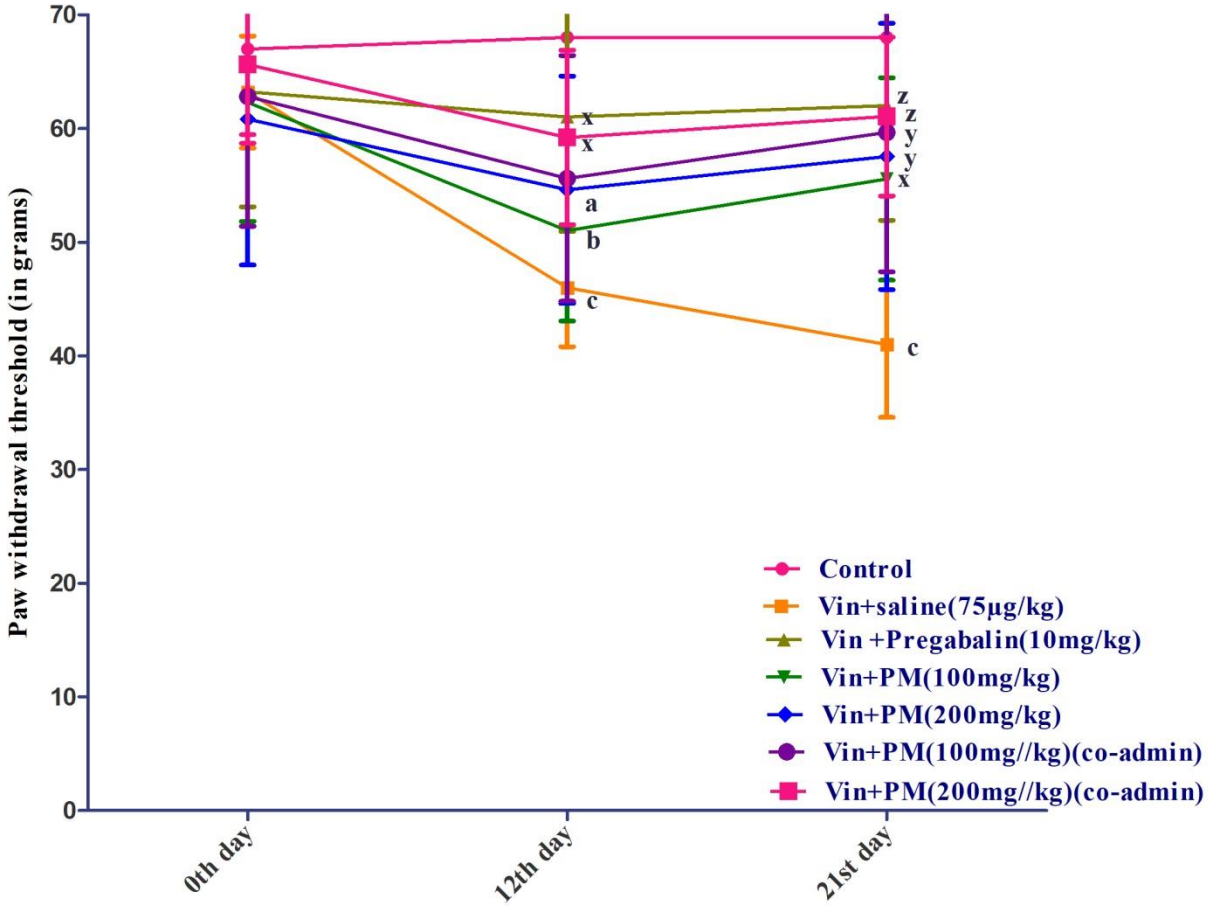
All the data's are expressed as mean ± SD n=6 rats per group. Two way ANOVA followed by Bonferonni's post hoc- test. **a, b** and **c** denotes statistical significance at $p<0.05$, $p<0.01$ and $p<0.001$ respectively in comparison to the control group. **z**denotes statistical significance at $p<0.001$ in comparison to the vincristine treated group. **l , m** denotes statistical significance at $p<0.01$ and $p<0.001$ respectively in comparison to the treatment with standard Pregabalin group. **p ,q ,r** denotes statistical significance at $p<0.05$, $p<0.01$ and $p<0.001$ respectively in comparison to the treatment with test drug PM post administration group(100mg/kg).**d** denotes statistical significance at $p<0.05$ in comparison treatment with test drug PM to the post administration group(200mg/kg).

Fig: 7. Effect of PM on Randall Sellito test in vincristine model



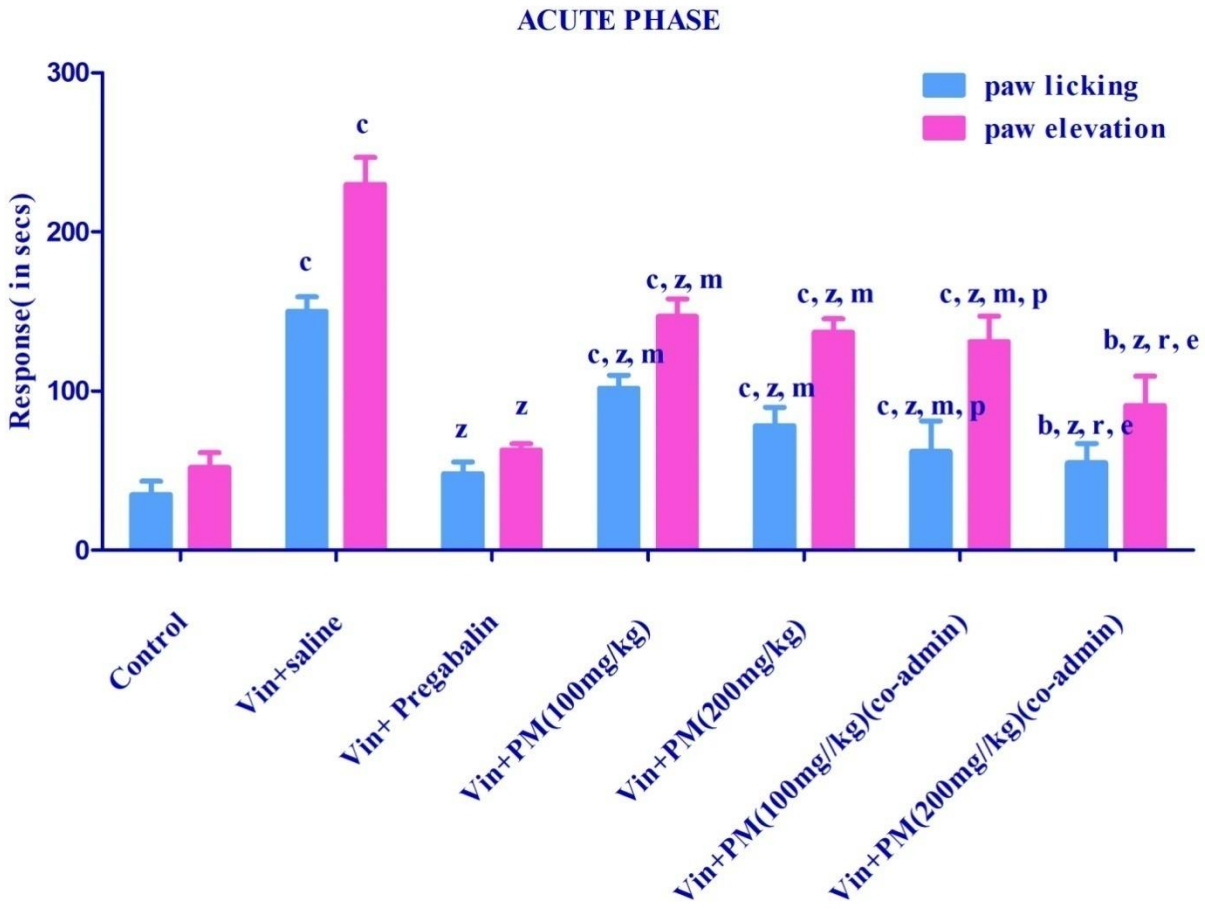
All the data's are expressed as mean ± SD n=6 rats per group. Two way ANOVA followed by Bonferonni's post hoc- test. **a**, **c** denotes statistical significance at $p<0.05$, $p<0.001$ in comparison to the control group. **z**denotes statistical significance at $p < 0.001$ in comparison to the vincristine treated group. **k**, **l , m** denotes statistical significance at $p <0.05$ $p < 0.01$ and $p < 0.001$ respectively in comparison to the treatment with standard drug Pregabalin group. **p ,q** denotes statistical significance at $p < 0.05$ and $p < 0.01$ respectively in comparison to the treatment with test drug PM post administration group(100mg/kg).

Fig: 8. Effect of PM on electronic Von Frey test in vincristine model



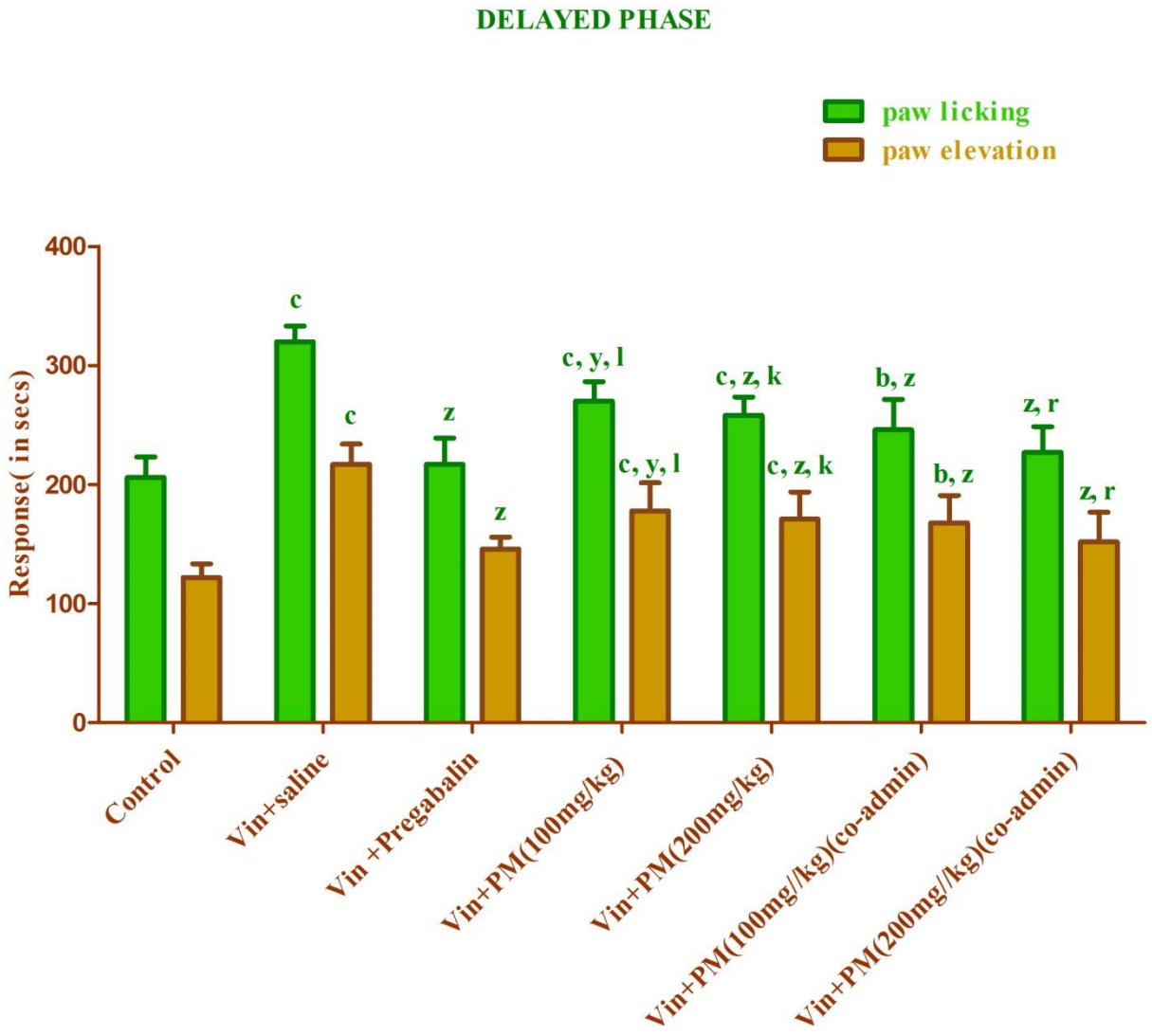
All the data's are expressed as mean ± SD n=6 rats per group. Two way ANOVA followed by Bonferonni's post hoc- test. **a, b, c** denotes statistical significance at $p<0.05$, $p<0.01$, $p<0.001$ in comparison to the control group. **x, y, z** denotes statistical significance at $p<0.05$, $p< 0.01$ and $p<0.001$ in comparison to the vincristine treated group.

Fig: 9. Effect of PM on formalin test in vincristine model (acute phase)



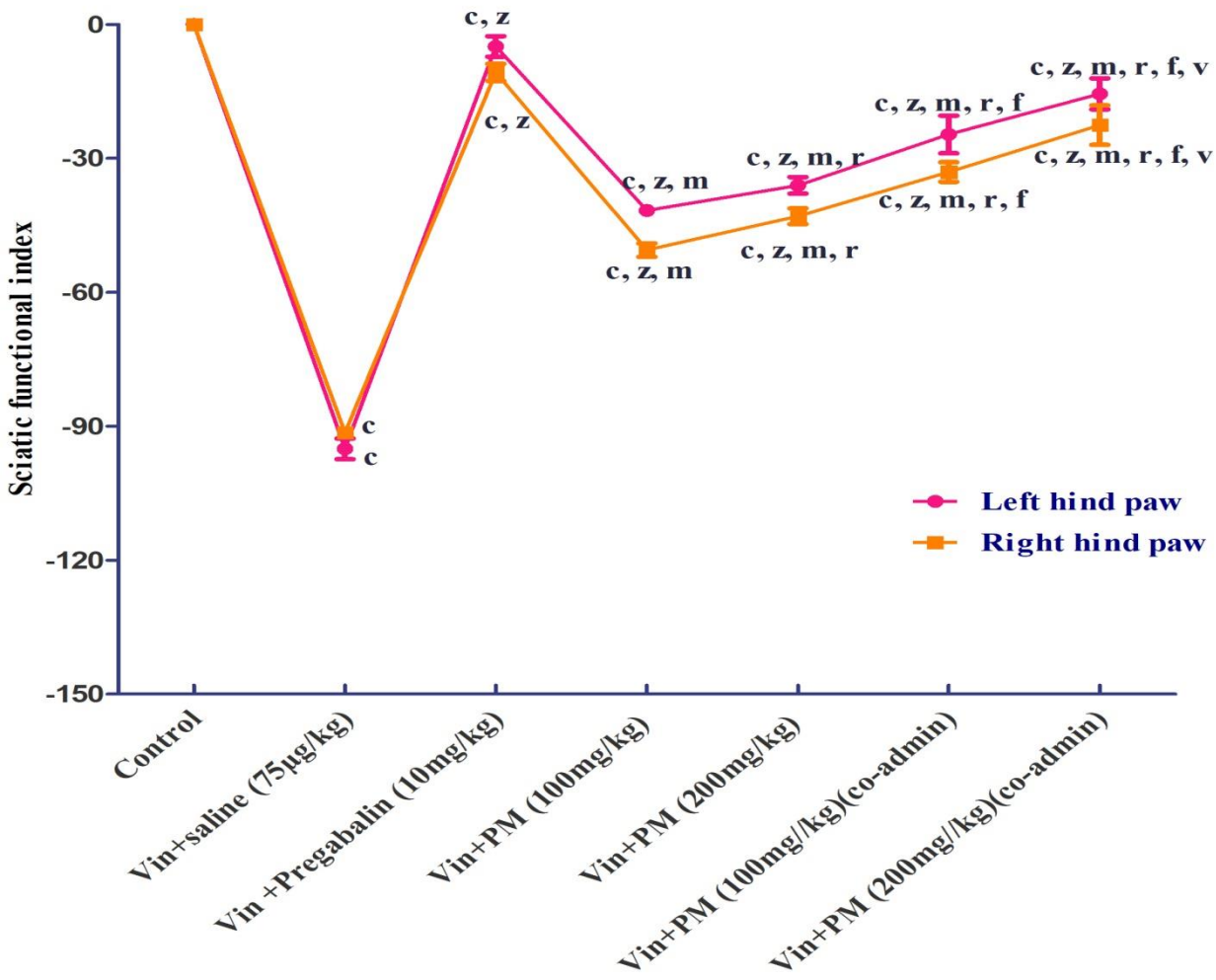
All the data's are expressed as mean ± SD n=6 rats per group.One way ANOVA followed by post hoc Tukey's multiple comparison test. **b, c** denotes statistical significance at $p<0.01$, $p<0.001$ in comparison to the control group**.** **z** denotes statistical significance at $p<0.001$ in comparison to the vincristine treated group. **m** denotes statistical significance at $p< 0.001$ respectively in comparison to the treatment with standard drug Pregabalin group. **p, r** denotes statistical significance at $p<0.05$ and $p<0.001$ respectively in comparison to the treatment with test drug PM post administration group (100mg/kg). **e** denotes statistical significance at $p<0.01$ comparison to the treatment with test drug PM post administration group (200mg/kg).

Fig: 10. Effect of PM on formalin test in vincristine model (delayed phase)



All the data's are expressed as mean ± SD n=6 rats per group. One way ANOVA followed by post hoc Tukey's multiple comparison test. **b, c** denotes statistical significance at $p<0.01$, $p<0.001$ in comparison to the control group. **y, z** denotes statistical significance at $p<0.01$, $p<0.001$ in comparison to the vincristine treated group. **k, l** denotes statistical significance at $p<0.05$ and $p<0.01$ respectively in comparison to the treatment with standard drug Pregabalin group. **r** denotes statistical significance at $p<0.001$ in comparison to the treatment with test drug PM post administration group (100mg/kg).

Fig: 11. Effect of PM on sciatic functional index in vincristine model



All the data's are expressed as mean ± SD n=6 rats per group. One way ANOVA followed by post hoc Tukey's multiple comparison test.**c** denotes statistical significance at $p < 0.001$ in comparison to the control group**.** **z** denotes statistical significance at $p< 0.001$ in comparison to the vincristine treated group. **m** denotes statistical significance at $p < 0.001$ in comparison to the treatment with standard drug Pregabalin group. **r** denotes statistical significance at $p < 0.001$ in comparison to the treatment with test drug PM post administration group(100mg/kg).**f** denotes statistical significance at $p < 0.001$ in comparison to the treatment with test drug PM post administration group(200mg/kg). **v** denotes statistical significance at $p < 0.001$ respectively in comparison to the treatment with test drug PM co-administration group(100mg/kg).

Effect of PM on excitatory neurotransmitters in rat brain

Fig: 12. 3D Value of glutamate and aspartate

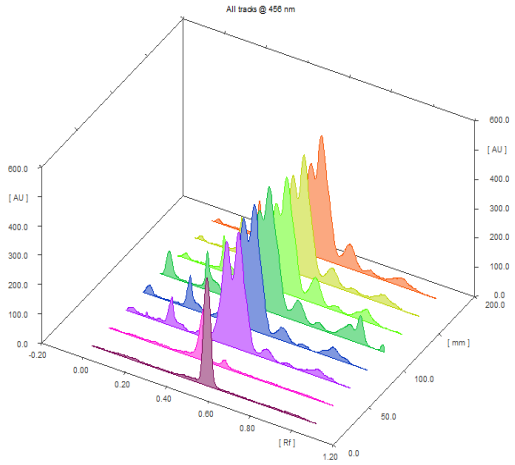


Fig: 13. Chromatogram for standard Glutamate

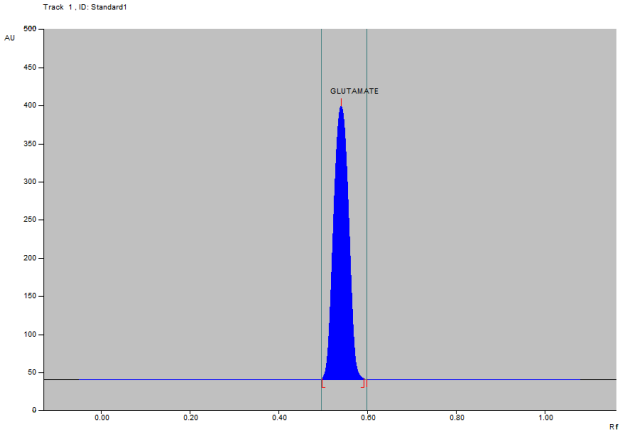


Fig: 14. Chromatogram for standard Aspartate

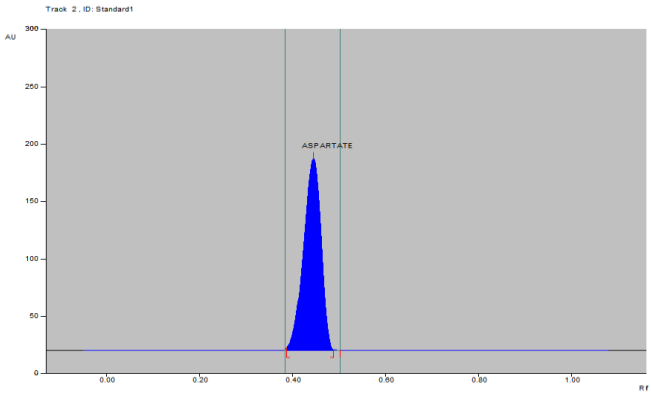
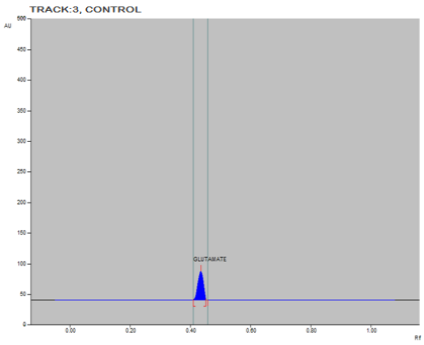
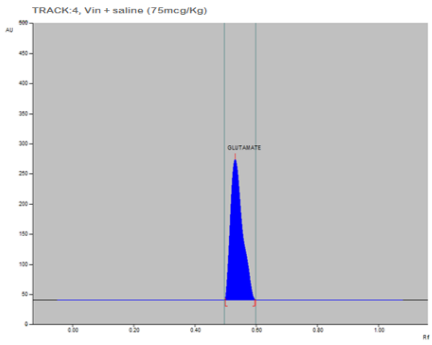


Fig: 15. Chromatogram for Glutamate in different groups

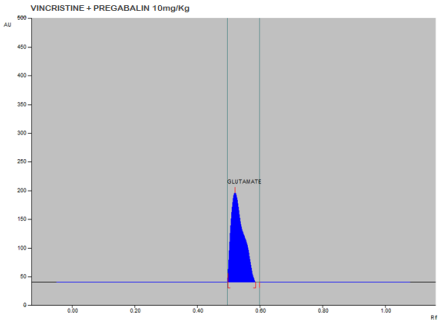
Control



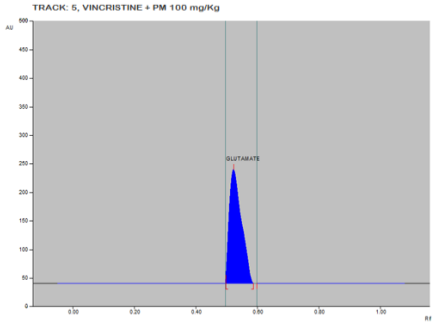
Vin+saline(75µg/kg)



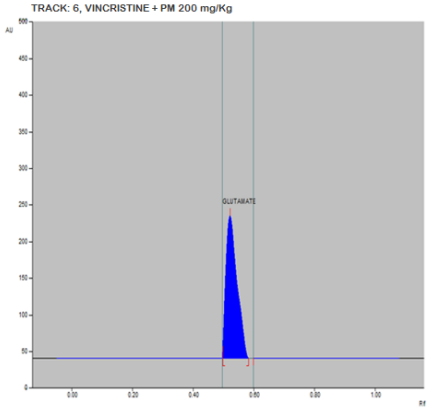
Vin +Pregabalin(10mg/kg)



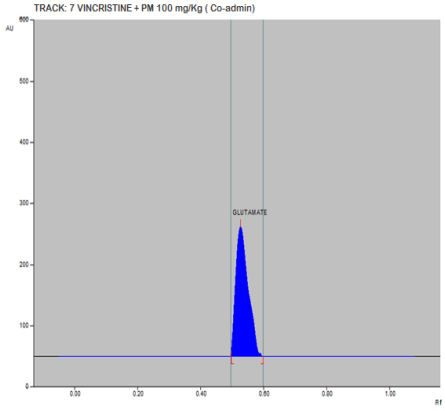
Vin+PM(100mg/kg) (post admin)



Vin+PM(200mg/kg) (post admin)



Vin+PM(100mg//kg)(co-admin)



Vin+PM(200mg//kg)(co-admin)

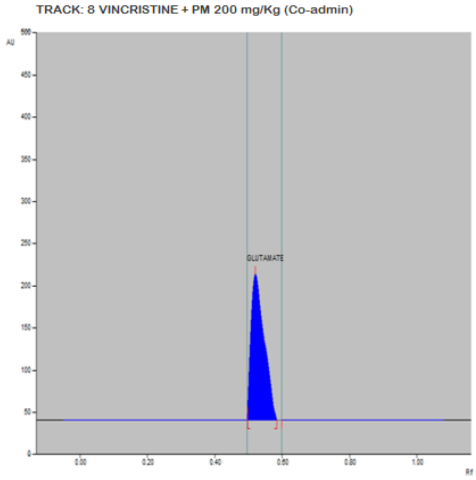
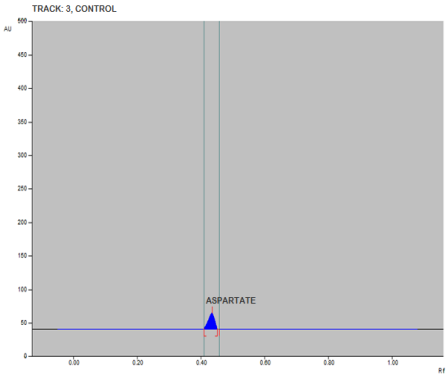
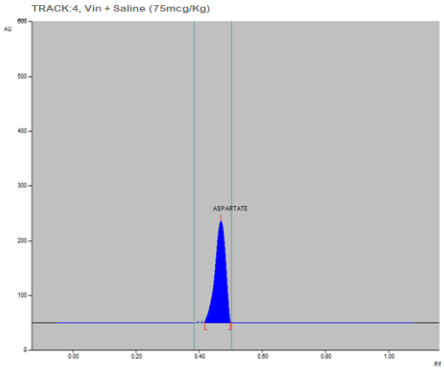


Fig: 16. Chromatogram for Aspartate in different groups

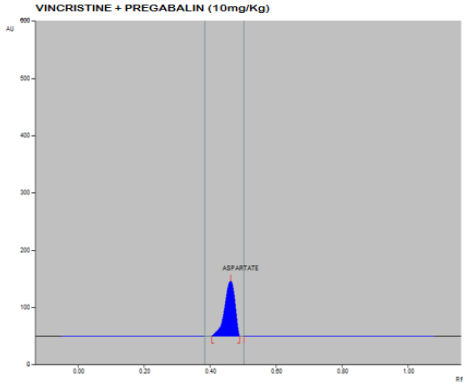
Control



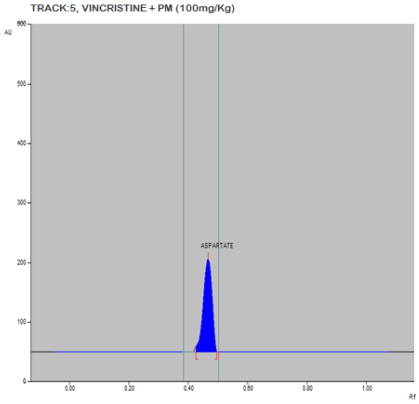
Vin+saline(75µg/kg)



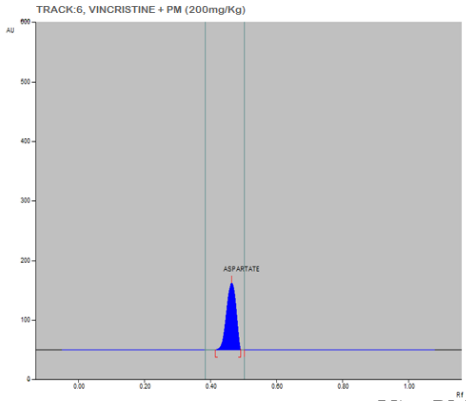
Vin +Pregabalin(10mg/kg)



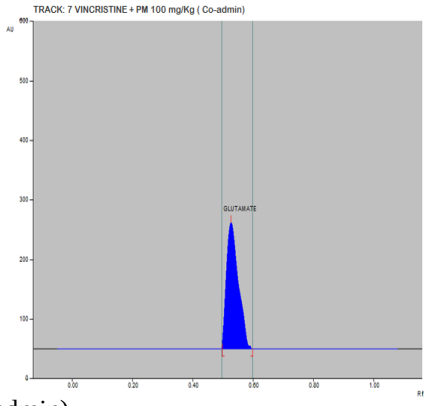
Vin+PM(100mg/kg) (post admin)



Vin+PM(200mg/kg) (post admin)



Vin+PM(100mg//kg)(co-admin)



Vin+PM(200mg/kg)(co-admin)

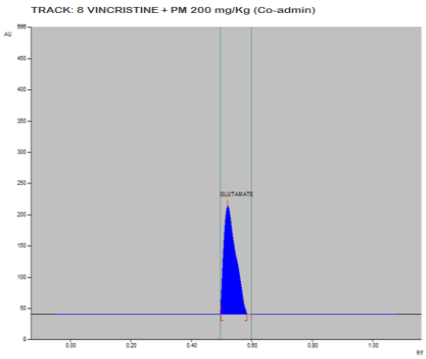
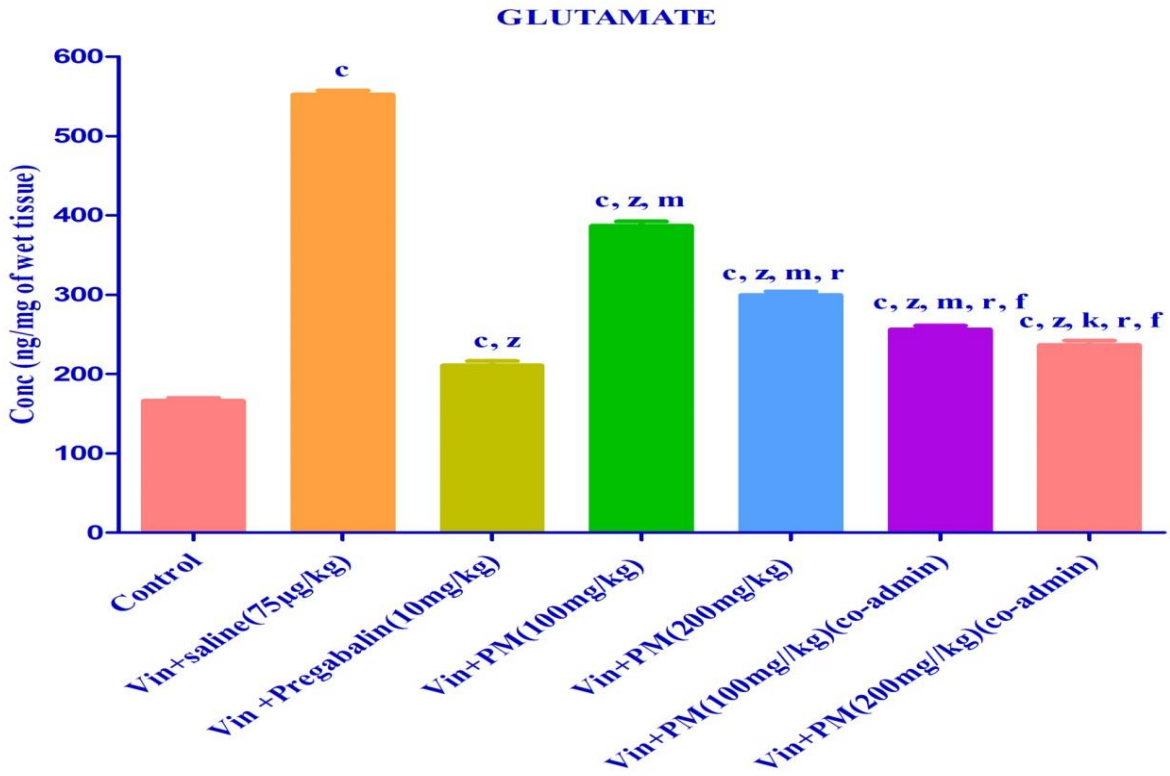


Table: 4. Effect of PM on Glutamate levels in vincristine model

Sl. No	Treatment	Conc (ng/mg of wet tissue)
1.	Control (saline)	173.687
2.	Vincristine +saline(75µg/kg)	551.186
3.	Vincristine +Pregabalin(10mg/kg)	213.776
4.	Vincristine +PM(100mg/kg) (post admin)	394.770
5.	Vincristine +PM(200mg/kg) (post admin)	303.497
6.	Vincristine +PM(100mg//kg) (co-admin)	261.977
7.	Vincristine +PM(200mg//kg) (co-admin)	231.434

Fig: 17. Effect of PM on Glutamate levels in vincristine model

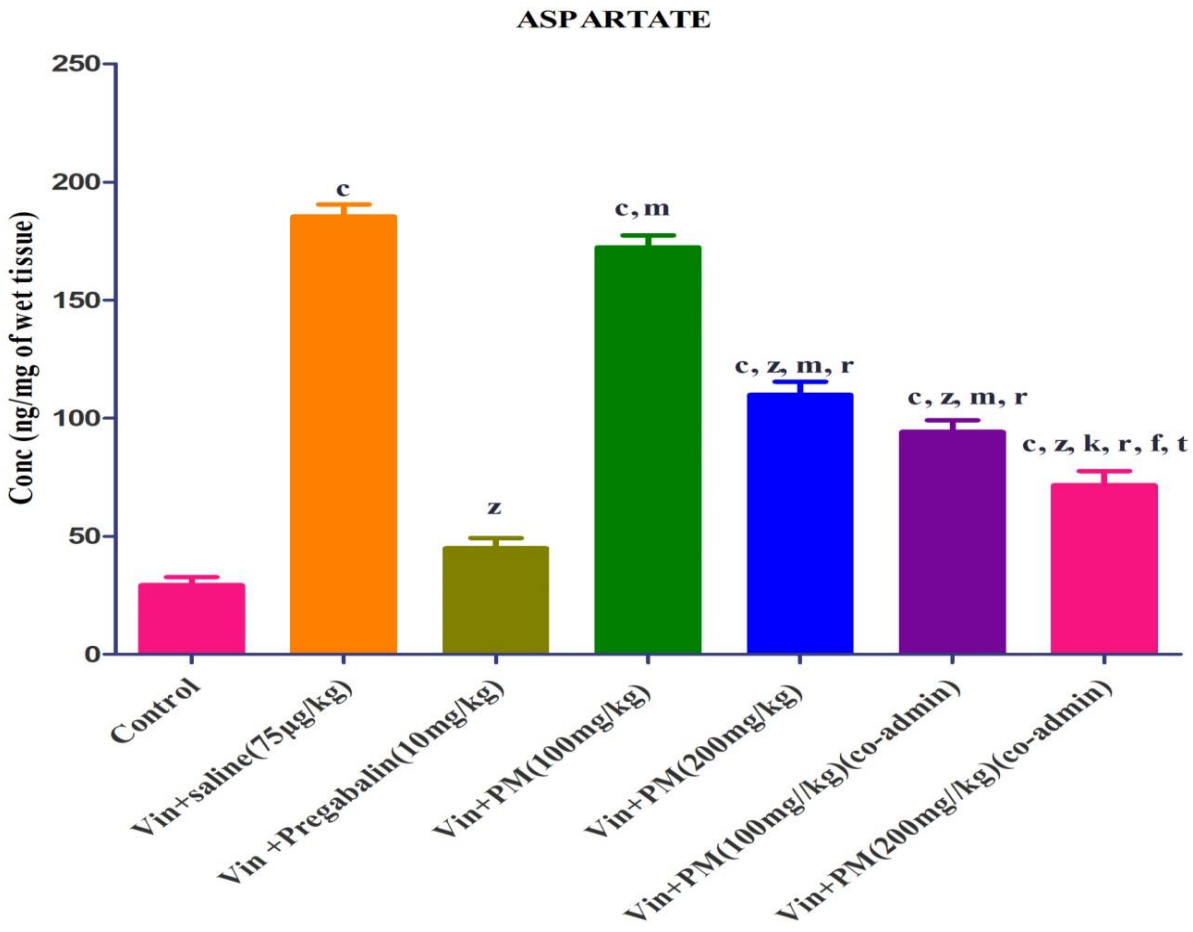


All the data's are expressed as mean ± SD n=6 rats per group. One way ANOVA followed by post hoc Tukey's multiple comparison test.**c** denotes statistical significance at $p < 0.001$ in comparison to the control group**. z** denotes statistical significance at $p< 0.001$ in comparison to the vincristine treated group. **k**,**m** denotes statistical significance at $p < 0.05$, $p < 0.001$ in comparison to the treatment with standard drug Pregabalin group. **r** denotes statistical significance at $p < 0.001$ in comparison to the treatment with test drug PM post administration group(100mg/kg).**f** denotes statistical significance at $p < 0.001$ in comparison to the treatment with test drug PM post administration group(200mg/kg).

Table: 5. Effect of PM on Aspartate levels in vincristine model

Sl.no	Treatment	Conc (ng/mg of wet tissue)
1.	Control (saline)	34.571
2.	Vincristine +saline(75µg/kg)	191.337
3.	Vincristine +Pregabalin(10mg/kg)	46.588
4.	Vincristine +PM(100mg/kg) (post admin)	171.859
5.	Vincristine +PM(200mg/kg) (post admin)	121.358
6.	Vincristine +PM(100mg//kg) (co-admin)	95.911
7.	Vincristine +PM(200mg//kg) s(co-admin)	70.700

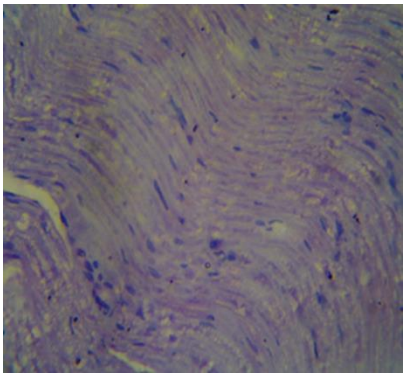
Fig: 18. Effect of PM on Aspartate levels in vincristine model



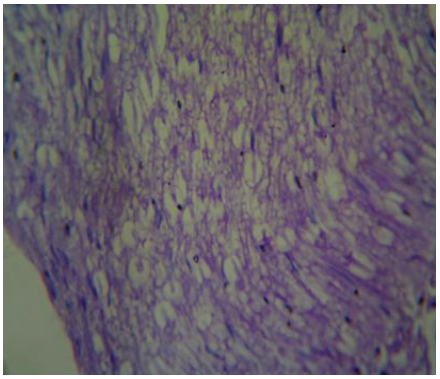
All the data's are expressed as mean ± SD n=6 rats per group. One way ANOVA followed by post hoc Tukey's multiple comparison test.**c** denotes statistical significance at $p < 0.001$ in comparison to the control group**.** **z** denotes statistical significance at $p< 0.001$ in comparison to the vincristine treated group. **k**,**m** denotes statistical significance at $p<0.05$, $p<0.001$ in comparison to the treatment with standard drug Pregabalin group. **r** denotes statistical significance at $p < 0.001$ in comparison to the treatment with test drug PM post administration group(100mg/kg).**f** denotes statistical significance at $p < 0.001$ in comparison to the treatment with test drug PM post administration group(200mg/kg). **t** denotes statistical significance at $p<0.05$ in comparison to the treatment with test drug PM co-administration group(100mg/kg).

Fig: 19. Histopathology of sciatic nerve

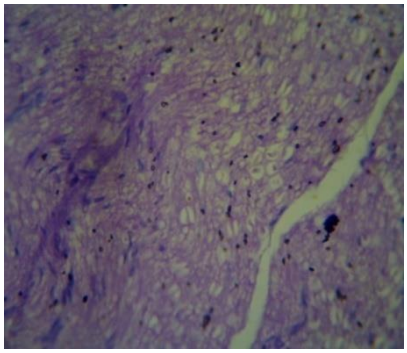
Control



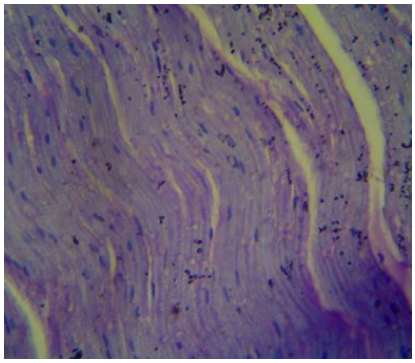
Vin+saline(75µg/kg)



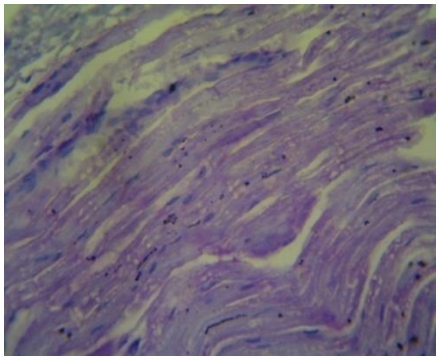
Vin +Pregabalin(10mg/kg)



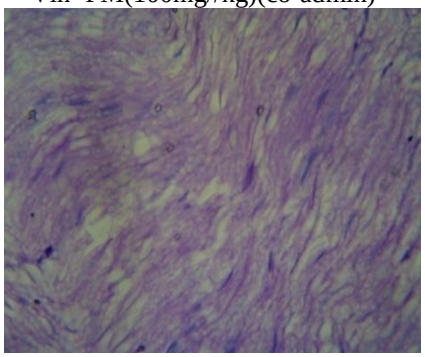
Vin+PM(100mg/kg)(post admin)



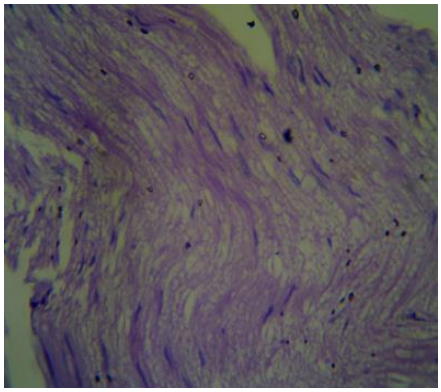
Vin+PM(200mg/kg) (post admin)



Vin+PM(100mg//kg)(co-admin)



Vin+PM(200mg/kg)(co-admin)



Chapter 7

Discussion

7. DISCUSSION

In the present study, we investigated the neuroprotective effect of *Pterocarpus marsupium*(PM) marketed capsules(100mg/kg & 200mg/kg) post and co–administration against vincristine(VCR) induced neuropathy. As demonstrated earlier, (*Balaji et al., 2015, Muthuraman et al.,2011,2008*) vincristine injected rats (75µg/ kg ,i.p for 10 days) exhibited peripheral neuropathy which was evident from the significant decrease in nociceptive threshold in behavioural models such as thermal hyperalgesia, mechanical hyperalgesia, mechanical allodynia. It increased the paw licking and paw elevation response in formalin test and also caused sciatic functional loss. It was also observed that there was neurochemical(rise in excitatory neurotransmitters such as glutamate and aspartate levels) and histopathological changes(axonal degeneration) due to vincristine administration. The behavioural, neurochemical and histopathological alterations caused by vincristine may be linked to itsneurotoxic effects in which they act directly or indirectly on the sensory neurons and causes a reduction in amplitude of action potential,slow conduction velocity and thereby inducing pain(Jain *et al*.,2012).Administration of *Pterocarpus marsupium* marketed capsules ameliorated vincristine induced behavioural, neurochemical and histopathological changes. These effects of PM may be due to its anti-inflammatory, anti –oxidative, analgesic activity which is in line with the previous studies. (Pant DR *et al*., 2017).

Vincristine has been widely employed as a chemotherapeutic agent for the management of various cancers including Hodgkin's disease (Villani F *et al*., 2008).However, its clinical application has been limited due to unavoidable painful peripheral neuropathy Effective management of chemotherapy induced peripheral neuropathy (CIPN) in the clinical setting has been an important and challenging goal to achieve. Systemic treatment with vincristine damages Schwann cells and DRG neurons. (Kiguchi N *et al*., 2009).The molecular mechanism behind vincristine induced neurotoxicity was found to involve high cellular calcium levels, free radical generation, TNF-alpha expression, and MPO activation (Jaggi *et al*., 2012, Kesik V *et al*., 2010).These events are responsible for axonal degeneration, indicating that cellular oxidant and inflammatory mediators play a key role in the pathogenesis of painful neuropathy. Earlier reports suggest that neuropathic pain has been linked with rise in neuronal calcium levels followed by enhance in the oxidative stress markers (free radicals) and inflammatory cytokines IL-6, etc.

(Jain V *et al*., 2009, Thiagarajan VR *et al.,* 2014).Other possible mechanismsbehind vincristine induced neuropathy are mitochondrial changes (Broyl A *et al*., 2010), increase in sodium ion current in DRG predisposing to paraesthesia and fasciculations (Ghelardini C *et al*., 2010), activation of calcium activated proteases calpains and caspases in DRG neurons (JosephEK *et al.,* 2004) increase in 5-HT_{2A} receptors on dorsal horn and DRG neurons (Thibault K *et al*., 2008) and dysfunction of the spinal NO/cGMP pathway (Kamei J *et al.,* 2005).

Thermal hyperalgesia (hot plate test) and mechanical hyperalgesia (Randall Sellito test) associated with vincristine treatment were thought to occur due to the hyper responsiveness of the degenerated Aδ and C-myelinated fibres (Dougherty *et al*., 2007; Xiao and Bennett, 2008). Spontaneous discharges and abnormal 'wind-up' to electrical stimuli in spinal cord neurons suggest a central sensitization after repeated vincristine treatment that may contribute to the spontaneous pain and hyperalgesia (Weng*et al*., 2003).In accordance with the previous findings, thermal and mechanical hypersensitivity can occur due to the increased expression of 5-hydroxytryptamine (5-HT) receptor 5-HT_{2A}in the dorsal horn after vincristine treatment. (Hibaultk *et al*., 2008) (Hansen, N *et al*., 2011). In addition, vincristine treatment increases the activity of T-type calcium channels and TRPV4 in sensory neurons of the DRG (Flatters *et al***.,** 2004) (Alessandri-Haber *et al*., 2004). As per the previous studies it was found that mechanical hyperalgesia and allodynia (electronic Von Frey test) can occur due to the increased expression of TRPV1 in the rat DRG (Terumasa Chiba *et al*., 2017). Mechanical allodynia is mainly caused due to the hyperresposiveness of the Aβ fibres (Thibault K *et al*., 2008) and a research report suggested that IL-6 may stimulate the invasion of macrophages in the peripheral nervous system and elicit VCR-induced mechanical allodynia (Gong SS *et al* ., 2016). According to the previous studies spinal microglia and astrocytes respond to vincristine induced peripheral neuropathy. Numerous laboratories have highlighted the importance of central glial activation in the development of hyperalgesia and allodynia in both peripheral inflammatory as well as in vincristine induced neuropathic pain model (J. Mika *et al*., 2013). These activated glial cells upregulate and secrete pronociceptive substances such as NO, prostaglandins and pro-inflammatory interleukins and TNF-α. Hence, any agent which can attenuate pro-nociceptive substances and pro-inflammatory mediators have been successful in attenuating neuropathic pain (V.A. Carozzi *et al*., 2014) (M. Sisignano *et al*., 2014). In this line, the analgesic and anti-

inflammatory effect observed by PM (100mg/kg & 200mg/kg) post and co–administration treatment in vincristine induced peripheral neuropathy can be justified.

P. marsupium showed potential analgesicand anti-inflammatory activity which is in agreement with the previous studies. These effects may be due to the presence of phytochemicals such as alkaloids, coumarins, flavonoids, glycosides, terpenoids, tannins, phenols, saponins, and steroids in the drug (Pant *et al*., 2017, Mohammed RM *et al*., 2012, Sikdar A *et al.,* 2013). It was reported that α-DHC isolated from the heartwood of *P. marsupium* is responsible for its anti-inflammatory activity.The anti-inflammatory activity is exerted by two ways a) by down-regulating MAPKs and restricting nuclear stabilization of NF-κB that ultimately inhibits the over expression of pro-inflammatory cytokines as well as inflammation mediating enzymes b) by disrupting Nrf-2–Keap-1 complex leading to induction of cytoprotective enzymes that indirectly repress the expression of inflammatory mediators (Chakraborty P *et al*., 2015).The anti-inflammatory and analgesic activity of the drug can also be linked to the PGE_2 inhibitory activity of the drug. This inhibitory activity of the drug is due to the presence of pterostilbene which exhibits a COX -2 inhibitory activity (Hougee S*et al*., 2005). In accordance with the previous studies PM decreased the elevated inflammatory cytokine, tumor necrosis factor (TNF-α) in type 2 diabetic rats which can also be taken into consideration for its anti-inflammatory activity (Kiranahalagappa *et al*., 2010).The activity of PM in vincristine induced peripheral neuropathy can also be linked to its anti-oxidant activity. Previous studies have reported the anti-oxidant activity of the drug (Mahnaaz Mohammadi *et al*., 2009).

The nociception induced by formalin is associated with injured tissue. It has been reported that this nociception resembles more closely the clinical pain in comparison to other tests that induce pain by mechanical or thermal stimuli (Lopes *et al*., 2009). Formalin test is characterized by two phases: acute and delayed. The acute phase (0-10 mins) is short-lived and initiates immediately after injection and is characterized by C-fiber activation due to peripheral stimuli. The delayed phase (20-40 mins) is a longer, persistent period caused by local tissue inflammation and also by functional changes in the dorsal horn of the spinal cord. Therefore this phase is inhibited both by opioids and analgesic agents. Substances that act primarily as central analgesics inhibit both phases while peripherally acting drugs inhibit only the delayed phase (Le bars *et al*., 2001). According to the present study it was found that PM decreased the pain responses such as paw

licking and paw elevation in acute and delayed phase of formalin test.This suggests that PM may be involved in both central and peripheral analgesic mechanisms as it inhibited both the acute and delayed phase in formalin test.

SFI is an assessment of functional loss using three variables that measure print length, intermediatory toe spread and total toe spread. Thus SFI provides a noninvasive and quantitative method to evaluate functional recovery of walking ability, the ultimate goal is the regeneration of injured sciatic nerve (A.S. Varejao *et al*., 2004.) It was found that PM improved the sciatic functional loss caused by VCR and this may be due to the neuroprotective effect of the drug.

Neuropathic pain caused by diabetic neuropathy and nerve injury is associated with increased glutamaterelease from primary afferent terminals and stimulation of AMPA receptors and metabotropic glutamate receptors, especially mGluR5, in the spinal cord (Hong-Yi Zhou *et al*., 2011). Glutamate signaling is also altered in CIPN. Although glutamate mainly acts on postsynaptic receptors to mediate excitatory neurotransmission, presynaptic NMDARs can increase the release of neurotransmitters such as substance P from the primary afferent terminals in the spinal dorsal horn. Increased *N*-methyl-D-aspartate receptor (NMDAR) activity contributes to central sensitization in certain types of neuropathic pain.

In the present study it was found that PM decreased the level of excitatory neurotransmitters such as glutamate and aspartate .The mechanism is still unclear, further investigation has to be done regarding this. VCR treatment caused axonal degeneration, schwann cell hyperplasia, damage of myelin fibrosis. This observation was in line with the previous studies (Wolfgang Boehmerle *et al*., 2014). It was found that PM reversed the histopathological changes caused by VCR.This may be due to the potential neuroprotective effect of the drug.

PM post and co-administration showed comparable results to standard Pregabalin (10mg/kg) in various behavioral models,biochemical estimation as well as histopathological studies. Pregabalin (10mg/kg) is a well-known agent currently used clinically to manage neuropathic pain of various etiologies (Navarro *et al*., 2011; Plested *et al*., 2010; Pérez *et al*., 2010). Although pregabalin mediated beneficial effects are proposed to be potentially mediated via inhibition of voltage gated calcium channels. In addition pregabalin has also been shown to

possesses good anti-oxidative, anti-TNF-alpha as well as anti-inflammatory actions (Ha *et al*., 2011, 2008, Muthuramanand Singh *et al*., 2011,Beyreuther *et al*., 2007).

Thus based on the behavioral,biochemical as well as histopathologoical results it can be concluded that *Pterocarpus marsupium* marketed capsules (100mg/kg & 200mg/kg) post and co-administration) exerts a beneficial effect in vincristine induced neuropathic pain and thus it can be used as a neuroprotective agent.

Chapter 8
Summary & Conclusion

8. SUMMARY AND CONCLUSION

In our present study, we evaluated the anti-nociceptive effect of *Pterocarpus marsupium* marketed capsules (100mg/kg & 200mg/kg) post and co–administration against vincristine induced neuropathy in rat model.

- *Pterocarpus marsupium* capsules (100mg/kg & 200mg/kg) post and co–administration showed significant increase in pain threshold in thermal hyperalgesia (hot plate test), mechanical hyperalgesia (Randall Sellito test), mechanical allodynia (electronic Von Frey test).
- Neuropathic pain was induced by vincristine (75µg/kg) and the pain threshold was assessed by various behavioral studies. Vincristine caused a rise in the level of excitatory neurotransmitters such as glutamate and aspartate.
- PM exhibited both central and peripheral actions in nociception as it decreased the paw licking and elevation in both acute and delayed phase of formalin test.
- It attenuated the sciatic functional loss caused by vincristine.
- PM decreased the levels of excitatory neurotransmitters such as glutamate and aspartate in the brain.
- Histopathology of sciatic nerve also supported the neuroprotective activity of the drug as it caused axonal regeneration and reversed the histopathological changes caused by vincristine.

CONCLUSION

The study can be concluded that PM attenuated VCR induced behavioral, biochemical and histopathololological changes in rats. These effects of PM may be attributed to different actions which include anti-nociceptve, anti-inflammatory, neuroprotective and anti- oxidant activity.

Chapter 9

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9. REFERENCES

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Annexure

PSG Institute of Medical Sciences & Research
Institutional Animal Ethics Committee

Registration No. : 158 / PO / ReBi / SL / 99 / CPCSEA
POST BOX No. 1674, PEELAMEDU, COIMBATORE 641 004, TAMIL NADU, INDIA
Phone : 91 422 - 2570170, 2598822 Fax : 91 422 - 2594400 Email : psganimalethics@gmail.com

DATE: 02.01.2017

Title of the Project: Neuroprotective of Pterocarpus marsupium marketed capsules against Vincristine induced neuropathy in rats.

Proposal Number:	357/2017/ IAEC.
Name of the Applicant:	Neethi shaju.
Approval date:	02.01.2017.
Expiry date (Termination of the Project):	01.01.2018.
Methodology:	Approved.

Name of species: **Swiss albino mice/ Wistar rats/ Sprague Dawley rats/ Guinea pigs/ Newzealand White rabbits.**

Male/Female/Both sex----------animals approved. 24 Female wistar rats

Signature of Chairperson

Date: 02/1/17

Dr.M.Ramanathan

Name of the chairperson

The Chair Person, CPCSEA
IAEC of PSGIMS&R
Coimbatore-641 004.

Signature of the CPCSEA nominee

Date: 02/01/2017

Dr.C.Kathirvelan

Name of IAEC/CPCSEA nominee

Main Nominee, CPCSEA
IAEC of PSGIMS&R
Coimbatore-641 004.

PSG INSTITUTE OF MEDICAL SCIENCES AND RESEARCH

COIMBATORE - 641 004

CME Accreditation Certificate

This is to certify that Neethi

has participated in

MOLECULAR BASIS OF MALIGNANCY - CURRENT TRENDS

held at PSG IMS&R on 23-06-2016 *as a participant*

This activity has been reviewed and accepted by The Centre for Accreditation, The Tamil Nadu Dr. MGR Medical University and the University designates this educational activity for a maximum of 05 *Credit points in Category* 2

Moderator

Dr. S. Ramalingam
Dean

PSG COLLEGE OF PHARMACY, COIMBATORE

WORKSHOP ON

ROLE OF PHARMACIST IN CLINICAL RESEARCH

Certificate of Participation

This is to certify that Dr. / Mr. / Ms. Neethi Shaju

has participated as delegate in one day workshop sponsored by **Novartis Healthcare Pvt Ltd, Hyderabad** *on 24th June 2016 held at PSG College of Pharmacy, Coimbatore*

Mr. Santosh Shevade
Program Coordinator
Novartis Healthcare Pvt Ltd

Dr. M. Ramanathan
Principal
PSG College of Pharmacy