Pharmacological Evaluation of 25-MHA Underlying Anti-nociceptive Properties in Animals Models

M.Phil. Thesis

by

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Pharmacological Evaluation of 25-MHA Underlying Anti-nociceptive Properties in Animals Models

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DECLARATION

I hereby declare that the thesis titled "**Pharmacological evaluation of 25-MHA underlying anti-nociceptive properties in animals models**" submitted at Department of Pharmacy, Faculty of Biological Sciences, Quaid-i-Azam University Islamabad for the award of degree of Master of Philosophy in Pharmacy (Pharmacology) is the result of research work carried out by me under the supervision of Dr. Salman Khan during the period 2016-2018. I further declare that the results presented in this thesis have not been submitted for the award of any other degree or fellowship. I am aware of the terms copyright and plagiarism. I will be responsible for any copyright violation found in this work.

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List of Abbreviations

IL	Interleukins
NF-kB	Nuclear factor kinase B
CNS	Central Nervous System
Nrf2	Clear factor (erythroid-derived 2)-like 2
HO-1	Heame oxygenase-1
SOD2	Sulphur oxide dismutase 2
NIK	Nuclear factor-κB inducible kinase
GI tract	Gastro Intestinal Tract
DRG	Dorsal root ganglia
SP	Substance P
CGRP	Calcitonin gene related proteins
ATP	Adenosine triphosphate
NGF	Neuronal growth factor
5-HT	5-hydroxy tryptamine
GABA	GABA amino butyric acid
NSAIDs	Non-steroidal anti-inflammatory drugs
CFA	Complete freunds adjuvants
25-MHA	25-methoxy hispidol A
ALT	Alanine amino transferase
AST	Aspartate amino transferase

TNF	Tumor necrosis factor
SOM	Somatostatin
VIP	Vasoactive intestinal peptide
PAG	Periequadecatal grey area
RVM	Rostroventromedial medulla
COX	Cyclooxygenase
DLPC	Dorsolateral pontine tegmentum
NO	Nitric oxide
DNA	Deoxyribo nucleic acid
PAMPS	Pathogen associated molecular patterns
PPR	Pattern recognition molecules
TLR	Toll-like receptor
NMDA	N-methyl-D-aspartate
ASICs	Acid sensing ion channels
AMPA	Amino-3-hydroxy-5-methylisoxazole-4- propionic acid
MOPR	Mμ-opioids receptors
KOPR	Kappa-opioids receptors
DOP	Delta-opioids receptors
PKA	Protein kinase A
cAMP	Cyclic adenosine mono phosphate
DNIC	Diffuse noxious inhibitory control

Abstract

The 25-MHA obtained from *Poncirus trifoliate* have several traditional applications such as anti-allergic, anti-cancer, anti-platelet and against GIT ulcers. The 25-MHA is currently evaluated against the CFA, Carrageenan, formalin and acetic acid induced nociception. The 25-MHA was evaluated against the both acute and chronic CF-induced mechanical hyperalgesia, mechanical allodynia, and thermal hyperalgesia. The pre-treatment of 25-MHA significantly reduced the mechanical hyperalgesia, allodynia and thermal hyperalgesia in both acute and chronic doses. Similarly, the 25-MHA also exhibited remarkable inhibition on the Carrageenan-induced hyperalgesia compared to the negative control. Formalin-induced nociception is an acute inflammatory pain model and served as screening tool to investigate the anti-nociceptive activity of new drug. The 25-MHA treatment significantly reduced the formalin-induced biphasic nociceptive responses i.e. inflammatory and neurogenic. The intraperitoneal administration of acetic acid is associated with the production of writhing response characterized by the contraction of abdominal muscles and extension of hind limbs and serve as acute visceral pain model to screen the anti-nociceptive potential of new drug entity. The intraperitoneal administration of 25-MHA significantly inhibited the acetic acid induced writhing response when compared to the negative control. The inflammatory cytokines are associated with sensitization of nociceptors and reducing the pain threshold. During inflammatory process several inflammatory cytokines are released, however, the most important cytokines that are implicated in most painful conditions are IL-1β, IL-6, tumour necrosis factor-α (TNF-α) and growth factor like vascular endothelial growth factor (VEGF). The 25-MHA treatment significantly reduced the mRNA expression levels of these inflammatory cytokines compared to the treatment with negative control. Furthermore, the mRNA expression level of anti-oxidant enzyme were also assessed using Quantitative Real Time polymerase chain reaction (qRT-PCR). The most important anti-oxidant enzymes implicated in the various oxidative stress conditions are nuclear factor erythroid 2 (NFE2)-related factor 2 (Nrf2), Heme Oxygenase-1 (HO-1) and Sulphur oxide dismutase (SOD2). The mRNA expression levels of these anti-oxidant enzymes were significantly increased by the 25-MHA treatment when compared to the negative control. The Nitric oxide (NO) is an important mediators of inflammation and its level in significantly increased in many inflammatory

conditions. The NO production was assessed in the plasma of animals in different treatment groups. The 25-MHA treated group significantly inhibited the NO production when compared to the negative control, thus, indicates the strong anti-inflammatory activity of 25-MHA. Many available analgesics like NSAIDs and opioids are associated with various intractable side effects such as GIT ulceration and addiction respectively. The 25-MHA was also assessed for any potential side effects against the liver, kidney damage and any toxic effect on the muscle strength and co-ordination. The 25-MHA treatment did not exhibited any side effect on the liver, kidney and muscle strength when compared to the negative control. The 25-MHA mechanism by which it produced analgesia was also studied in the current study.

Key words: Mechanical hyperalgesia, Mechanical allodynia, CFA, Carrageenan, Nociception

CHAPTER 1

INTRODUCTION

1. INTRODUCTION

Pain is an unpleasant feeling comprises of sensory and emotional component, and results from the activation of sensory nerve endings in response to noxious stimuli (Montes et al., 2017; Moratalla et al., 2017). The pain responses acts as an alarm to activate suitable protective mechanism against the real or potential threat ((Montes et al., 2017). However, the pain encompass beyond its protection and rather becomes distressing and unbearable. The sensation of pain is perceived differently by different individuals because of their emotional state, gender, ethnicity, anxiety level, early experiences and memories (Montes et al., 2017). The pain produced during the process of inflammation is triggered by various mediators which sensitize the nociceptive nerve endings. The activation of the primary sensory neurons is the main triggering factor in inflammatory pain which leads to hyperalgesia (meaning the state of increased response to a stimulus that is painful in nature) and allodynia (which means that the state of increased response to the stimulus that is non-painful in nature) (de Lima et al., 2011; Quintão et al., 2012; Khan et al., 2013b).

The management of pain can improve the quality of life of an individual and depends on the class of drugs used to treat it. The various classes of drugs which are used to treat the chronic pain such as non-steroidal anti-inflammatory drugs (NSAID's) Paracetamol and aspirin, Narcotic analgesic like opioids and analgesic adjuvants like Gabapentin and Pregabalin have their own pros and cons (Ahmad et al., 2017). These current available therapies are unable to successfully ameliorate the pain and are associated with the various intractable adverse effects (Ahmad et al., 2017). The NSAID's use are commonly associated with the gastrointestinal disturbances such as stomach ulcer, increase the tendency of bleeding, and they are mostly contraindicated in child. Thus, to avoid these side effect selective COX-2 inhibitors were developed such as celecoxib but these are implicated in various cardiovascular abnormalities (Onasanwo et al., 2017). Additionally, the opioids are strong analgesic and used to treat various painful conditions such as cancer pain, biliary colic, and chest pain. However, these opioids are associated with various side effects such as central nervous system (CNS) and respiratory depression, tolerance and addiction (Ahmad et al., 2017). The analgesic adjuvants consist of several drugs groups such as Pregabalin, Gabapentin are employed primarily for other condition rather than the analgesic,

however, these drugs are currently been employed as analgesics in specific circumstances (Ahmad et al., 2017). The appropriate use of these adjuvants analgesic along with other available analgesic have been tried along to counter the painful conditions effectively. The NSAIDs, opioids and analgesic adjuvants are associated with various unbearable side effect which necessitate the development and discovery of new and safer drug for the stated condition (Ahmad et al., 2017).

The role of cytokines has been well established as mediators between the cell injuries and the process of inflammation and have been implicated in hyperalgesia, fever and edema (Shin et al., 2010; Khan et al., 2014). The mediators produced during the process of inflammation modulates the perception of pain. These pro-inflammatory cytokines such as IL-1β, IL-6, TNF-α and growth factors like VEGF are associated with the increased hyperalgesia and allodynia (Khan et al., 2013b). The role of these cytokines are well established in inflammation and nociception in a sense that the use of NSAID's are associated with the reduce production of these inflammatory cytokines and hence, inflammation and nociception (Khan et al., 2013b).

In order to search new drugs for attenuation and ailments of various painful condition, many researchers have focused their attention on the natural products that have proven fruit full over the long time in various clinical condition (Shin et al., 2010). Similarly, these natural products are also associated with minimum side effects. One of such natural product is 25-MHA, isolated form the immature fruit of *Poncirus trifoliate*. It has been traditionally tried as medicine for the treatment of ulcers, gastritis and other inflammatory diseases (Xu et al., 2008; Shin et al., 2010). Due to the potent anti-inflammatory property, it was hypothesized to investigate the role of 25-MHA in modulating the nociceptive sensitization and mediators of inflammation, with the intention to understand the possible mechanism of anti-nociception of 25-MHA. Furthermore, the toxicity associated with the 25-MHA daily oral administration was also evaluated.

1.1. Types of Pain

1.1.1. Nociceptive pain

The perception of pain is actually consist of three things and many physician mostly fail to make proper distinction (Daniel et al., 2008). The first important thing is that it acts as an early alarming sign for the body to initiate the appropriate protective

mechanism to detect and minimize the contact of the body with the tissue damaging noxious stimuli (Daniel et al., 2008). This is the sensation of pain we feel when one touches something very cold, hot or sharp. As this pain is concerned with sensing the noxious stimuli that's why it is called as nociceptive pain. This pain is of high threshold and only activated in the presence of intense stimuli (Martin et al., 2008). The neurobiological system that is associated with perception of nociception is most primitive nervous system evolutionary to potential or actual tissue damaging stimuli. In order to confer protection this system needs quick attention and action which occur via withdrawal reflex it activates, the unpleasantness sensation it produce and the emotional components it activates (Martin et al., 2008). The nociceptive pain present itself as something that need to be avoided as it overrule many other neuronal mechanism (Martin et al., 2008).

1.1.2. Inflammatory pain

The second type of pain that is adaptive and protective pain which occur following tissue damaging response. Inflammatory pain assist in the healing of the injury via creating an environment that inhibit the physical contact and movement of the injured tissue (Nassar et al., 2004). The hypersensitivity or tenderness associated with the inflammatory pain reduces further risk of damage and promote recovery of the damaged tissue as in the case of surgical wound or inflamed joint where normally innocuous stimuli may elicit an unpleasant response (Jarvis et al., 2007). The inflammatory pain is associated with the activation of immune system and involves the infiltration of immune cells and is therefore called as inflammatory pain. Pain is considered as one of the cardinal feature of inflammation (Jarvis et al., 2007). However, the inflammatory pain is of adoptive and protective nature but still need still debilitating and needs to be reduced such as in the case of rheumatoid arthritis and severe injured conditions (Jarvis et al., 2007).

1.1.3. Neuropathic pain

The third type of pain is not protective in nature but maladaptive and result from the dysfunction of nervous system (Duehmke et al., 2006). The pathological pain is not the symptom of the any disease but rather a disease of the nervous system in which there is pathological dysfunction in the nervous without any inflammation or any damage and is called as neuropathic pain (Duehmke et al., 2006). The situation that are associated with the provoking of dysfunctional pain are fibromyalgia, irritable

bowel syndrome, tension headache, tempro-mandibular joint disease, interstitial cystitis and other syndromes in which there is pain but involve no noxious stimuli and having no peripheral inflammatory disease (Finnerup et al., 2010). Clinically the pathological pain is largely the consequence of amplified signals in the CNS and is of low threshold pain (Finnerup et al., 2010). Analogically, if there is an alarm of fire than there will be the activation of nociceptive type of pain to take an appropriate measures, if there is an alarm of warm temperature there will be an activation of inflammatory pain, and pathological pain is activated by the an alarm that is false in nature and caused by the malfunction of the system itself (Finnerup et al., 2010). In all the cases the net effect is the perception of pain. However, as the mechanism by which these pathway are activated are quite different and treatment must be directed against the distinct mechanism responsible (Finnerup et al., 2010).

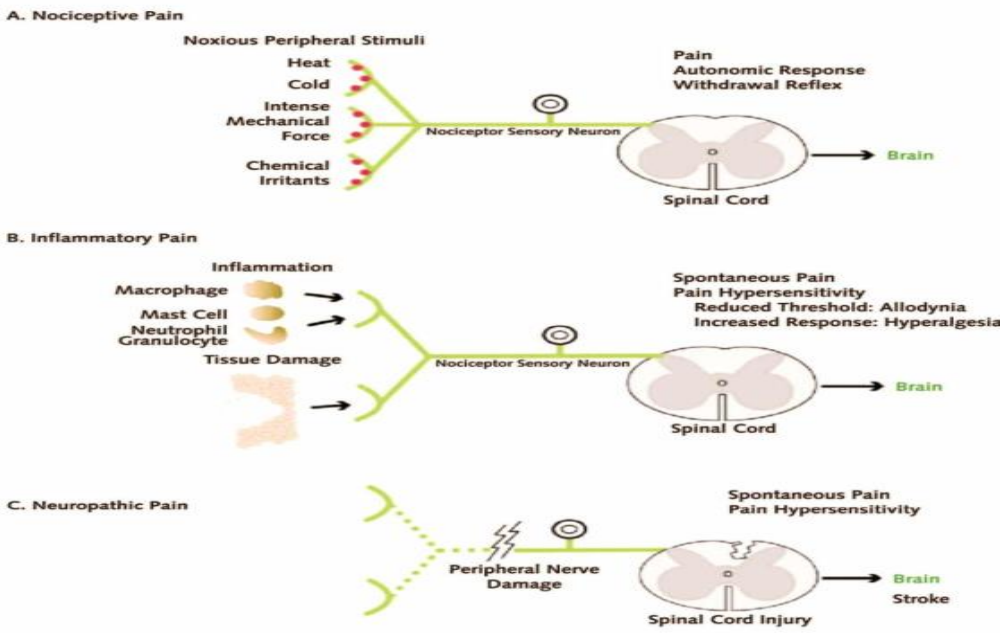

Figure 1.1: Types of pain: The nociceptive pain (A) is produced when there is any noxious stimuli such as heat, cold, mechanical and irritant stimulus, than there is sensitization of nociceptors which are activated and the information are transmitted to CNS. The inflammatory pain (B) is produced when there is any inflammatory insult or tissue damage and hence release of various inflammatory cytokines. The neuropathic pain (C) is produced when there is any damage to peripheral nerves and the sensation of pain are produced and transmitted to CNS. (Adopted from Clifford J. Woolf et al., 2010).

1.2. The various Mechanism Underlying the Perception of Pain Stimuli

1.2.1. Peripheral mechanism of pain

The sensory system is continuously tasting the external environment and keeping the central nervous system updated (Baron et al., 2010). The afferent neurons of the somatosensory system continuously respond in a coordinated fashion to noxious stimulus in order to develop an integrated response to the external stimuli and to retain the homeostatic integrity of the human being and restrain any tissue damaging response (Baron et al., 2010). The nociception is the main component of the somatosensory nervous system whose main purpose is to keep the body updated regarding the exteroception, proprioception and interoception (North, 2004). The exteroception functions includes mechanical, heat and nociception transmission. The proprioception keeps the CNS informed about the relative position of the body joints, muscles and tendon. While, the interoception informed the CNS about the visceral organs. The primary sensory neurons having cell bodied in the dorsal root ganglia (DRG) are classified according to their size, diameters of axon, conduction speed, degree of myelination, neurochemistry, and their inherited ability to respond to the neurotropic factors (NTFs) (North, 2004).

1.2.1.1. Classification on the basis of size

a. A-fibres

The A-fibres are myelinated, having large cell body diameters and are further classified into Aα, Aβ and A-δ fibres. Aα fibres are concerned with appreciation of muscle spindles and Golgi tendons organs and transmit the information of position of the body to CNS. AB fibres are associated with the mechanoception and are of low threshold, cutaneous and slowly adopting. A-delta fibres are mechanical and thermal receptors (Ringkamp et al., 2011).

b. C-fibres

These fibres are unmyelinated or thinly myelinated, small diameter and constitutes 65-70% of sensory nerves entering the spinal cord and are concerned with the perception of nociception (Ringkamp et al., 2011).

1.2.1.2. Classification on the basis of neurochemistry

The sensory neurons are also classified according to the neurotransmitter such as C-fibres are mainly classified as whether they are peptidergic or non-peptidergic (Ringkamp et al., 2011). Fifty percent of the C-fibres express calcitonin gene related peptides (CGRP), substance P (SP), somatostatin (SOM), vasoactive intestinal peptide (VIP) and galanin (Ringkamp et al., 2011). While, the remaining C-fibres are express cell surface glycol-conjugates having binding affinity to IB4 and some of these fibres also respond to ATP via expressing purine receptors (P2X3) (Ringkamp et al., 2011).

1.2.1.3. Classification on the basis of response to the stimuli

a. Mechanoceptors

These receptors are concerned with the detection of tactile non painful stimuli. The mechanoceptors are divided into either rapidly adopting (respond at the onset and offset of the response) or slowly adopting (respond to the stimuli throughout the duration of stimulus) (Seal et al., 2009). The mechanoceptors are located either on the hairy skin (low threshold, and rapidly adopting) and Glaboruous skin (rapidly adopting (Meissiner capsule) and slowly adopting (Merkel disc) receptors)(Seal et al., 2009).

b. Mechano-heat sensitive receptors (CMH and AMH)

CMH are polymodal fibres as they respond to mechanical, heat, cold and chemical stimuli (Chen et al., 2002). The activation of these receptors causing the characteristics burning sensation in the affected area (Chen et al., 2002). They are slow conducting and transmit the sensation of late pain. AMH activation produce harp, pricking and aching sensation having high conduction velocity and transmit the sensation of first pain (Chen et al., 2002).

1.2.2. The mechanism underlying the transmission and perception of nociception

Nociception is a neuronal mechanism by which the body is updated about the possible or actual tissue damaging stimuli (Rogoz et al., 2012). The perception of nociception is takes place through the multiple events.

1.2.2.1. Transduction

The transduction is the conversion of energy from one form to other form. The transduction process occurs along the nociceptive pathway via several stages such as stimulation of nerve fibres by chemical agent which is then converted to electrical signals by the neurons (Rogoz et al., 2012).

1.2.2.2. Transmission

Once the chemical stimulus is converted to the electrical energy it is transmitted between the synaptic cleft and then via neuronal circuit to the CNS (Rogoz et al., 2012).

1.2.2.3. Modulation

The electrical activity generated in 1st order neurons are than modulated at various point such as spinal cord, thalamus and then at the cortical level and the information are generated at these level to produce appropriate response (Rogoz et al., 2012).

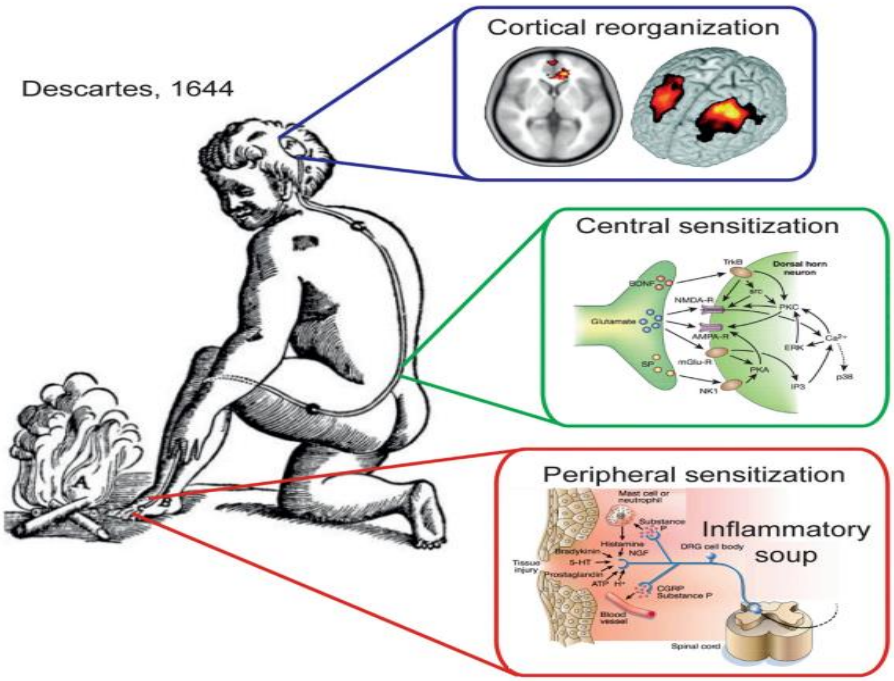

Figure 1.2: The transduction, transmission and modulation of pain at different level. Whenever there is any painful sensation, this painful stimuli is transformed to electrical energy, and this energy is transmitted to the spinal cord and brain, where it is modulated. (Adopted from Marwan N. Baliki1 et al., 2015).

1.2.3. The central sensitization of pain and modulation of pain at different level

The pain perception is processed at several level such as spinal cord and CNS (Neugebauer, 2002). The transmission of nociception information depends on the balance of inhibitory and facilitatory influence that occur along the neuronal circuits of the somatosensory system (Neugebauer, 2002). These sensation of pain are integrated at multiple level in the CNS such as spinal cord, brain stem and at several cortical level (Neugebauer, 2002).

1.2.3.1. Modulation of nociceptive sensation at the level of spinal cord

The dorsal horn of the spinal is an important site for the integration and processing of information coming via first order neurons, local internitial neurons, and coming from descending neurons brain (Neugebauer, 2002). The somatosensory information from the ascending output depends upon the sensory input coming from the first order sensory neurons (Neugebauer, 2002). Several researchers proposed that the modulation of nociception occur at several level. One of such theory "Gate control theory of pain" (Neugebauer, 2002). This theory suggest that activity in the low threshold myelinated sensory neurons inhibit the ascending nociceptive neurons by activating the inhibitory internitial neurons (Neugebauer, 2002). This inhibitory internitial neurons inhibits the ascending nociceptive neurons normally. Once there is any noxious stimuli the unmyelinated C-fibres tends to inhibit the internitial neurons and enhance the nociceptive threshold (Neugebauer, 2002). Furthermore, the A-myelinated fibres tends to activate the internitial neurons and reduce the sensation of nociception (Kline and Wiley, 2008). The inhibitory neurotransmitter within the dorsal root ganglia are GABA, serotonin (5-hydroxy tryptamine), adenosine, endogenous cannabinoids and the endogenous opioids (Kline and Wiley, 2008). Another modulatory mechanism within the dorsal root ganglia is endogenous opioids system playing crucial role in the transmission and modulation of nociception (Kline and Wiley, 2008). The opioids system comprises of three receptors types such as μ-opioids receptors (MOP), δ-opioids receptors (DOP) and k-opioids receptors (KOP) (Kline and Wiley, 2008). The dorsal horn (DH) has considerable concentration of endogenous peptides such as enkaphalin and endorphin which are released by the internitial neurons (Kline and Wiley, 2008). These opioids receptors are expressed on the pre-synaptic neurons of the 1st order neurons and on the post synaptic dendrites of the second order neurons (Kline and Wiley, 2008). The endogenous opioids once acts

on the receptors tends to reduce the concentration of the nociceptive neurotransmitters from the presynaptic nerves and thus reduce the depolarization of the post-synaptic neurons and hence decrease the sensation of pain (Kline and Wiley, 2008). The third pain modulatory mechanism is known as "diffuse noxious inhibitory control"(Kline and Wiley, 2008). According to this hypothesis the perception of pain in one part of the body can be reduced by the application of noxious stimuli to other part of the body (Yarnitsky, 2010). The idea that "pain inhibit pain" is the back bone of this hypothesis by applying counter irritation (Yarnitsky, 2010). The response to harmful stimuli is reduced by applying another stimuli outside that respective field and neuro-physiologically basis is provided to the diffuse noxious inhibitory control (DNIC) (Yarnitsky, 2010).

1.2.3.2. Supra spinal modulation of the pain

The descending pathway primarily on the DH of the spinal cord and inhibit the transmission of noxious information to the central nervous system (McGaraughty et al., 2003). The evidence to the supra-spinal inhibitory descending pathway is provided by the electrical stimulation of analgesia centre (McGaraughty et al., 2003). The electrical activation of grey matter that surrounds the periequadecatal grey area (PAG) and the grey matter of fourth ventricle produce profound analgesia (McGaraughty et al., 2003). This has also been proved by the placing electrodes in the human reduced the sensation of pain, however, the sensation of tactile and thermal are unchanged (McGaraughty et al., 2003). The PAG received information from the multiple higher centre such as amygdala, frontal lobe and hypothalamus. PAG also receive information from the ascending nociceptive pathway (DH) (McGaraughty et al., 2003). The PAG control the nociceptive information received from the DH by sending information to the rostroventromedial medulla (RVM) and to the dorsolateral pontine tegmentum (DLPT) (McGaraughty et al., 2003). The endogenous peptides and their receptors are heavily concentrated along this pathway (Flores et al., 2004). The opioids action is not limited to the modulation of pain in the DH of the spinal cord but the opioids agonist also stimulate the PAG and RVM to activate the descending analgesia system (Flores et al., 2004). Other neurotransmitters like 5-HT and nor-epinephrine are also implicated in the PAG and RVM projecting neurons to the DH (Flores et al., 2004). The evidence to the analgesic effect of these neurotransmitters are provided by the direct application of these neurotransmitters

produce analgesia while the destruction of these neurons block the action of systemically administered opioids (Flores et al., 2004). Recently, the role of endogenous cannabinoids "anandamide" have been focused for the analgesic effect via interacting with the CB1 receptor on the DH neurons. The anandamide anti-nociceptive action are also have been implicated in PAG and RVM (Flores et al., 2004). Some of their action are mediated by acting on the opioids receptors and some action of endogenous cannabinoids are independent of the opioids action (Flores et al., 2004).

1.2.3.3. Higher cognitive processing and the pain matrix

The development of various imaging techniques have made it possible to determine the various vital functions of the higher centre including perception of pain. One of the key finding of these key techniques are that multiple regions of the brain are activated by the application of painful stimuli (Millan, 2002). The region of pain matrix includes the thalamus, 1st order neurons, 2nd order neurons, somatosensory cortex, the insular cortex, anterior cingulate gyrus and motor regions such as the regions of pre-motor cortex and cerebellum (Millan, 2002). Pain is composed of two component sensory (concerned with the location and intensity of pain) and emotional component. According to several psychophysiological studies different neuronal substrates encodes different information of the experience of pain (Millan, 2002). The activation of primary and secondary somatosensory cortex is associated with the perception of sensory component while the sensitization of the insular cortex and anterior cingulate gyrus is associated with affective or emotional component (Millan, 2002). Some part of the brain such as pre-motor cortex and cerebellum associated with the generation of skilled movement are also implicated in the perception of pain (Millan, 2002). This means that the pain is not associated with the sensory processing but also includes appropriate motor response as well (Millan, 2002).

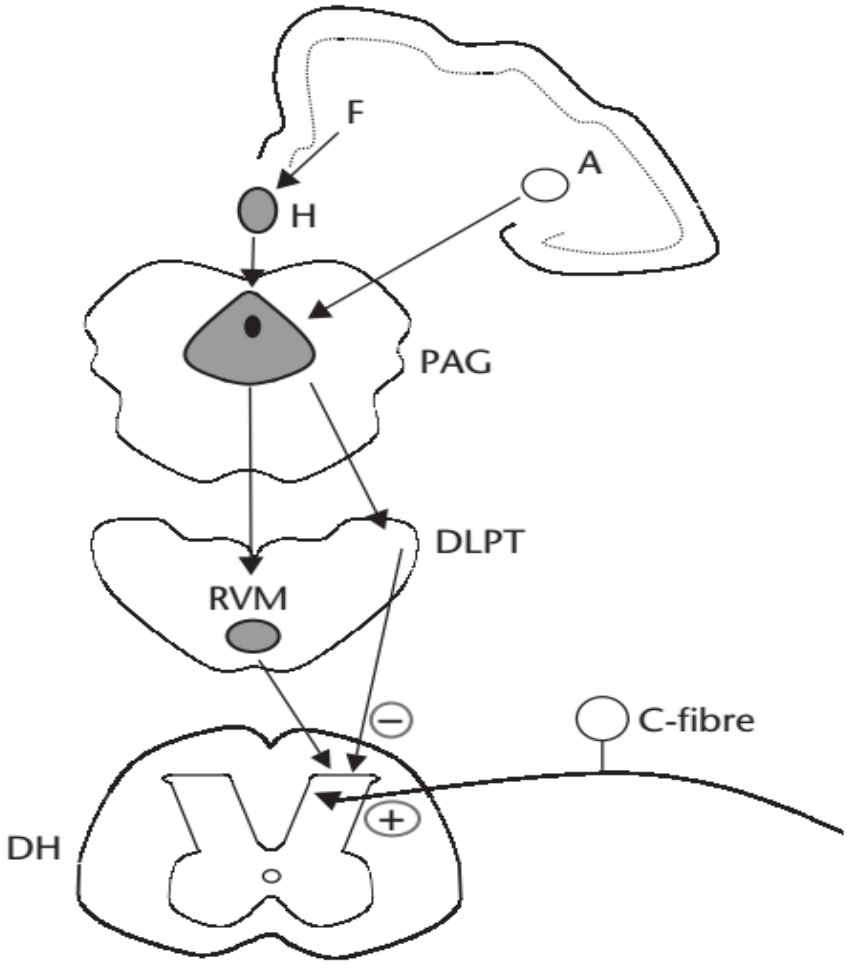

Figure1.3: The role of PAG in transmission and modulation of pain. The PAG and RVM are involved in the modulation of painful sensory information and are descending modulatory pathway. Adopted from Core topics in pain, Cambridge university press.

1.3. Mediators Released During the Nociception

1.3.1. Glutamate

Glutamate is an important neurotransmitter implicate in the transmission of noxious information (Bleakman et al., 2006). In the CNS it is co-located with the opioids system. The glutamate have been reported to be the most important mediator, which are released from the central sensory terminal neurons along the ascending pain pathway (Bleakman et al., 2006). The glutamate neurons are originated form the DRG, sensory cortex and cerebellum interact with other brain areas such as thalamus where it modulate the 2nd order neuronal transmission (Bleakman et al., 2006). The

other neurotransmitters co-released with the glutamate are NKA, SP and CGRP (Bleakman et al., 2006). The glutamate thus released interact with the two major receptors such as inotropic receptors like Amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA), NMDA, Kainate and metabotropic receptors (G-protein coupled receptors) (Bleakman et al., 2006). The AMPA receptors are associated with synaptic current mostly and mediate baseline activity (Tao et al., 2005).. While, the stimulation of NMDA trigger windup and is associated with the long term plastic alterations (Tao et al., 2005).. The Kainate receptors has also been implicated in the noxious stimuli transmission. Functional NMDA receptors exist as heteromeric combinations of various subunits (Tao et al., 2005). This NMDA receptor includes an (NR) 1 subunit plus one or more of NR2A, NR2B, NR2C and NR2D (Tao et al., 2005). These receptors have binding affinity with the glutamate, glycine, zinc, magnesium and phencyclidine (Tao et al., 2005). However, the activation of these receptors are induced once the stimuli is above the threshold and extracellular magnesium pore blockade. However, activation only occurs when the noxious input is above threshold level and pore blockade is removed (Tao et al., 2005). Concurrently, the glutamate must interact with the NMDA and stimuli must induce the depolarisation of the postsynaptic membrane (Tao et al., 2005). The stimulation of NMDA receptors causes increased calcium level within the neurons and downstream activation of several pathways such as protein kinases, protein phosphatases and immediate early genes post-synaptically (Tao et al., 2005). Recently it has been postulated t that nitric oxide (NO) and prostanoids can also induce the activation of NMDA receptors (Tao et al., 2005).

1.3.2. Substance P (SP)

SP is one of the critical mediator responds to the noxious potential stimuli which can harbour the integrity of the biological system (Hill, 2000). SP is considered as an important mediators which provide immediate defence, repair, stress and the survival system (Hill, 2000). The SP is associated with the expression of most the well-known inflammatory mediators and these released mediators in turn increase the induction of SP and NK1 receptors (Hill, 2000). SP and other neuropeptides release are also occur from the peripheral sensory nerves of the skin, muscles and joints (Hill, 2000). It is suggested that this release of SP trigger the neurogenic inflammation, which is a local inflammatory reaction to certain types of inflammatory insults (Hill, 2000). Similarly,

the preclinical data augments the notion that SP is a critical factor in pain perception (Hill, 2000). The sensory function of SP is related to the sensitization of the sensory nerves to the transmission of pain information into the central nervous system (Greco et al., 2008). SP release is occurred along with the excitatory neuro-transmitter glutamate from the primary sensory nerves that results in the production of painful stimulation (Greco et al., 2008). Unfortunately, the NK1RAs (NK1-receptor antagonists) have failed as efficacious anti-nociceptive drugs in clinical studies and its exact role in the pain perception and transmission yet to be elucidated (Greco et al., 2008).

1.3.3. Calcitonin Gene Related Peptide (CGRP)

CGRP is protein neuropeptide in nature having high distribution within the nervous system. In most of the sensory neurons (about 50% of the polymodal C-fibres, 33% of A-fibres and 20% of the A/A fibres release CGRP) are released in the DRG after their synthesis in the stated neurons (Greco et al., 2008). Its release in the peripheral nervous system augments the effect of other excitatory neurotransmitters such as SP and increase the intracellular release of the calcium ions. Various types of the CGRP receptors have been identified along with the calcitonin like receptors (Greco et al., 2008). These receptors are coupled with the G-protein and are mostly expressed in the nucleus accumbens which indicates the important role of CGRP in pain transmission (Ren and Dubner, 2008). Similarly, the CGRP role in the migraine has also been established where alpha-CGRP subtype is predominantly found and is presumed to be involved in the development of migraine pain (Ren and Dubner, 2008). Furthermore, its beta form have also been identified keratinocytes of the epidermis. Where its expression is enhanced several times in certain chronic painful conditions (Ren and Dubner, 2008). Correspondingly, the level of beta CGRP are also implicated to increase in the animals model of pain (Ren and Dubner, 2008). Although, very little is known about the functional differences amongst these two isoforms. However, several study also confirms that beta-CGRP has also been increased in the enteric nervous system, where it is associated with initiation of visceral nociception (Ren and Dubner, 2008).

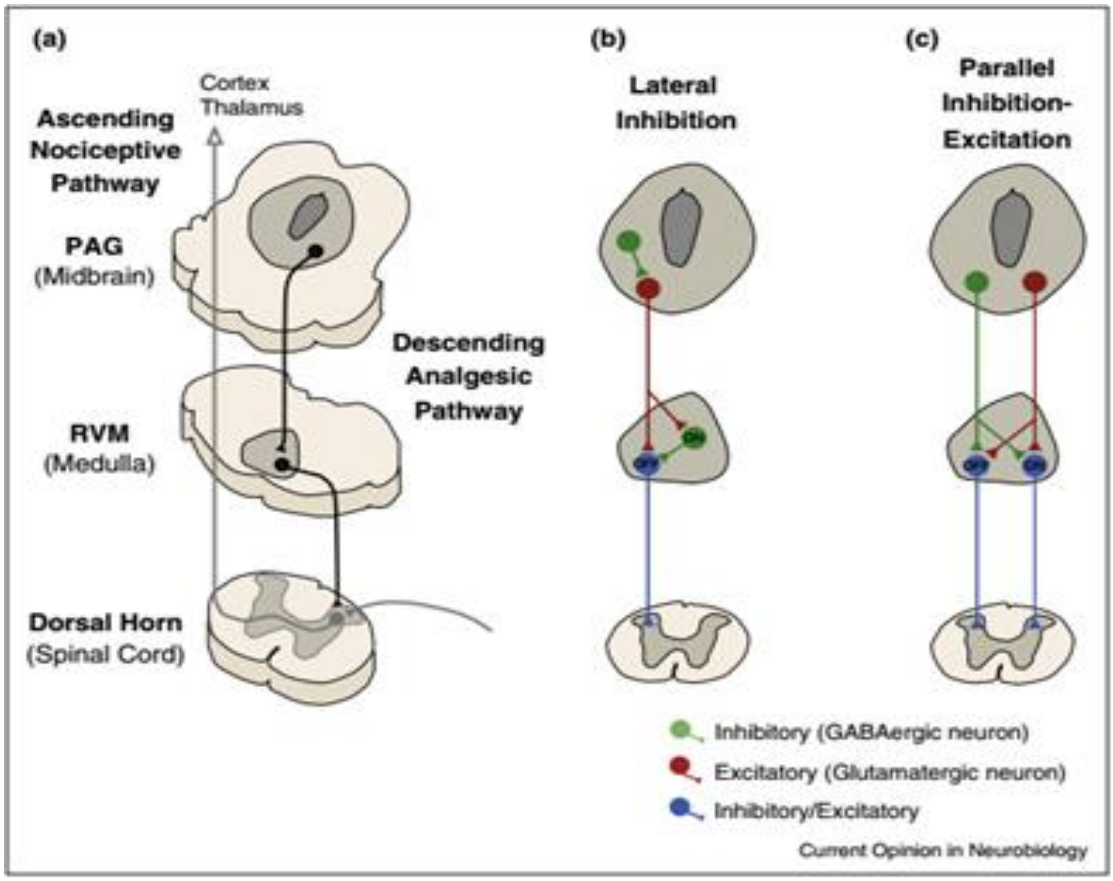

Figure 1.4: Role of inhibitory and excitatory mediators. GABA disinhibition in the descending PAG-RVM pathway. (a) Schematic of an ascending pain pathway (left) and descending analgesic pathway (right). A particular projects via the PAG and RVM to modulate the ascending pain pathway at the spinal cord level. (b). Lateral inhibition model-according to this predominant model, local GABAergic interneurons tonically modulate the activity of outputs neurons that constitute the anti-nociceptive pathway. Opioids and cannabinoids indirectly excite output neurons by suppressing the inhibitory influence of neighbouring GABAergic interneurons. This leads to disinhibition of the descending PAG-RVM pathway, thereby inhibiting nociceptive transmission at the spinal cord level. (c). Parallel inhibition-excitation model-according to this alternative model, inhibitory and excitatory neurons form two distinct, parallel pathways. Opioids and cannabinoids act independently on these excitatory/inhibitory neurons to mediate pronociception and antinocicpetion. Adopted from Benjamin K Lau et al., 2014).

1.3.4. Adenosine Triphosphate (ATP)

ATP act as an important messenger within the cell, however, its role has also been recognised extracellularly (Souslova et al., 2000). Once it is released form the storage vesicle or from the lysed cells, it interact with the ion channel to modulate its activity. The receptors for the ATP found peripherally are expressed on the skin while centrally it is expressed on 2nd order neurons of the DH (Souslova et al., 2000). Peripherally its effect can be augmented by histamine, bradykinin and SP. ATP

undergo metabolism to adenosine by the action of surface-located enzymes called as ectonucleotidases (Souslova et al., 2000). This adenosine thus formed acts upon the P1 (A1- or A2-types) receptors and, hence, further alter the sensation of pain peripherally as well as centrally (Souslova et al., 2000). Adenosine interact either with purinergic G-protein-coupled (P2Y) or inotropic (P2X) receptor subtypes (Souslova et al., 2000). The P2X (P2X1–P2X7) receptors seven subtypes have been identified so far, of whom six receptors (P2X1–P2X6) are expressed on the sensory fibres. These six receptors interact to form functional heteromeric receptors (Souslova et al., 2000). However, most recently trimeric complexes of identical subunits which form a model P2X3 structural channel are identified (Souslova et al., 2000). P2X3 is of great interest because it is expressed on the small C-fibres mostly. It has also been postulated recently that the expression of P2X3 channel are increased during inflammation probably due to the hypersensitivity of the receptors (Souslova et al., 2000). Furthermore, these receptors are reported to be involved in the processing of the majority of noxious information from the peripheral ATP released (Zhao et al., 2008). The ATP can interact both pre and post synaptically at P2X3 receptors, these both receptors are distinct in nature with apparent different sub-unit population. In the spinal cord the ATP is released along with the GABA. ATP also activate the release of nociceptive glutamate (Zhao et al., 2008).

1.3.5. Cannabinoids

The *Cannabis sativa* has many constituents however, the 9-tetrahydrocannabinol (9-THC) is one of the most important reported psychoactive constituent (Fox et al., 2001). It mediates its action by interacting with the Gi-protein coupled receptors. The activation of these receptors results in the inhibition of adenalyle cyclase, activation of K-efflux and the closure of the voltage-gated calcium ion channel in the same way as the activation of opioids receptors (Fox et al., 2001). The G-protein coupled receptor with whom the 9-THC interact are of two types, these are cannabinoid receptor type 1(CB1) and CB2, however, the third type (CB-1a) has also been identified which is of less importance (Fox et al., 2001). The CB1 is mainly expressed on the neuronal cells both pre- and post-synaptically in many areas of the spinal cord and brain. The CB2 receptors are located mainly on the immune cells (Karst et al., 2010). The low level of respiratory system depression associated with the cannabinoids might be due to the lack of cannabinoids receptors in the brain stem

(Fox et al., 2001). The skin tissue are also associated with the increase expression of the cannabinoids receptors, which mediate anti-nociceptive responses by modulating the activity of NO, SP and particularly inhibiting the release of CGRP (Karst et al., 2010). The mRNA level of CB1 receptors expressed in the A-fibres are directly associated with anti-nociceptive mechanism mediated by the cannabinoids. The cannabinoids modulate the sensation of pain at all level of the spinal cord and brain (Fox et al., 2001).

1.3.6. Gamma-amino-butyric-acid (GABA)

The GABA is considered one of the most important inhibitory neurotransmitter distribute in the central nervous system (Rea et al., 2007). The neurons which are associated with the expression of the GABA receptors are highly concentrated in the cerebellum, neo-cortex and in the internitial neurons (Rea et al., 2007). The GABAergic neurons are mainly present in the brain having no nociceptive modulatory role in the spinal cord. The GABergic neurons were found to have long projections towards striatum, substantia nigra, and also between the forebrain and hypothalamus (Enna and McCarson, 2006). However, some GABergic neurons were also found in the spinal cord which are associated with the release of peptides. The GABergic system is mainly associated with the decrease in the nociceptive pain and nociceptive sensation (Enna and McCarson, 2006).

1.3.7. Serotonin (5-HT)

The serotonin is an indolamine mediator and associated with increase in the sensation of hyperalgesia following nerve injury or trauma (Bardin, 2011). The serotonin produced by the mat cells, enterochromafin cells and the brain cells. The serotonergic pathway are present in parallel with the nor-epinephrine in the raphe nuclei of the brain (Marks et al., 2009). Similarly, the NGF are considered an important mediator associated with the inhibition of the mast cells degranulation, and thus it is proposed that serotonin has important role in the hyperalgesia mediated by the NGF (Wei et al., 2010). The most important serotonin receptor expressed in the DH of the rats are mainly on the small fibres are 5-HT2A and 5-HT3. These receptors play vital role in serotonin mediated nociceptive responses (Wei et al., 2010). Furthermore, the mRNA of the serotonin is co-localized with mRNA of the CGRP indicating that serotonin

belongs to TrkA and NGF dependent group of neurons concerned with the sensation of nociception (Wei et al., 2010).

1.3.8. Neurotropic growth factor (NGF)

The NGF are released by number of cells like fibroblast and trigger important inflammatory once released. The NGF released during the inflammatory process is related with the increase sensation of the hyperalgesia in both humans and animals model of inflammatory states (Kerr et al., 2001). Once NGF is released it interact with the its receptor tyrosine kinase A (TrkA), and increase the sensitization of NGF dependent subsets of nociceptors along with the increase release of other pro-inflammatory mediators such as bradykinin. The increase release of the NGF has also been implicated to increase the sensitivity of the nociceptors to heat and capsaicin by interacting with the relative receptor such as TRPV1 (Kerr et al., 2001). Furthermore, the NGF are associated with the degranulation of mast cells and thus increasing the release of further mediators (NGF and others) and amplifying the inflammatory signals (McMahon, 1996). The release of these mediators provokes the influx of neutrophils and thus further release the sensitizing agents like lipooxygenase (LOX) enzyme products, and hence maintain the state of hyperalgesia (McMahon, 1996). This state of increase hyperalgesia was inhibited by neutralization of NGF. In animals model the exogenous administration of NGF increase the state of hyperalgesia and the degranulation of mast cells. NGF is well established mediator of inflammation and persistent pain (McMahon, 1996).

1.3.9. Capsaicin

Chemically, the capsaicin is 8-methyl-***N***-vanillyl-6-noneamide and is a lipid vanniloid molecule and whose botanical source is of *Capsicum chilli* paper and induce the sensation of burning hot pain upon exposure (Lee et al., 2007). The capsaicin acts via their specific ligand-gated cation channel, expressed on the surface of small and medium nociceptors. Vanniloid receptors type 1 have been recognized on the dorsal root of C and A fibres (Lee et al., 2007). The VR1 activation is facilitated by the capsaicin, acidification and temperature above 43^0C. The several inflammatory mediators such as BK and NGF enhance the sensitivity to the capsaicin and heat (Magerl et al., 2001). This sensitivity is induced by the stimulation of PLC (phospholipase C) probably via reducing PIP2 (phosphoinisotol-diphosphate)

inhibition on the receptors (Magerl et al., 2001). The VR1 protein is member of transient receptor potential TRP) superfamily ligand gated ion channel, whose main transmembrane structure resemble that of voltage gated K^+ ion-channel (Magerl et al., 2001). This VRI exhibits high permeability to Ca-ion and most of the action are executed via this mechanism observed for capsaicin (Magerl et al., 2001). The other member of VR family recognized is VR2 and is activated by the noxious heat stimuli that is near about 52^0C. A VR-like (VRL-1) receptor existence has also been proposed on the nociceptors. The endo-cannabinoids like anadamide share structure with the capsaicin has also been postulated to act on the VR1. AEA induces the vasodilation by VR1 followed by the release of CGRP (Magerl et al., 2001).

1.3.10. Protons

The tissue damage induce the release of numerous chemical entities from the cells (Vellani et al., 2001; Chen et al., 2002). The numerous mediator released during the inflammatory process includes one of the most important mediator is proton released from the cell during tissue damage along with the common neurotransmitter serotonin, having direct action on the sensory afferent neurons (Vellani et al., 2001; Chen et al., 2002). This protons are associated with the enhancing the permeability mechanism that have similar characteristics with the noxious stimulation of nociceptors by the capsaicin. Exposure of C-fibers and A-fibres to the PH below the 6 can activate acid sensing ion channel (Vellani et al., 2001; Chen et al., 2002). A lowered PH during the inflammation process also enhance the activity of others mediators as well (Vellani et al., 2001; Chen et al., 2002). Furthermore, the local production of heat acts upon heat activated-ion channel (which have many features of the vanniloid receptor the transient receptors 1(TRPV1) formally known as VR1 at which capsaicin acts) and may contribute to the increased hyperalgesia (Vellani et al., 2001; Chen et al., 2002).

1.3.11. Prostaglandins (PGs)

The PGs are not associated directly with the sensation of pain but tends to enhance the effect of other mediators like serotonin and bradykinin via G_S-proteins. Interestingly, the BK may induce the expression of PG release thus acting as self-sensitizing effect. Other eicosanoids and Prostacyclin may induced the pain but the evidence regarding their effect is still need to be elucidated (Ochroch et al., 2003). The product of COX

and LOX metabolism prostanoids are synthesized by the enzymatic activity of cyclooxygenase and lipooxygenase on the arachidonic acid and performs several inflammatory functions (Ochroch et al., 2003). The arachidonic acid inflammatory role have been evident from the use of corticosteroid as it inhibits the arachidonic acid and thus ameliorate the inflammation. The corticosteroids inhibits the phospholipase A2 and thus prevent the formation AA an important mediator of inflammation (Ochroch et al., 2003). During the process of inflammation several prostaglandins are produced but the PGE2 is the most important one, whose synthesis is regulated by the COX2 isoform and associated with the direct activation and sensitization of nociceptors by acting on the PGE2 EP receptors (Sinatra, 2002). PG also enhance the effects of BK and induce the release of neuropeptides (including substance P (SP) and calcitonin gene related peptides (CGRP). The most important class of analgesic NSAIDs acts by inhibiting the COX and decrease the synthesis of sensitizing PGs (Sinatra, 2002). Current evidence suggest that PGs might be involved in the initiating the inflammation induced secondary hypersensitivity in the central nervous system, implicating a novel central role of NSAIDs in the reducing the secondary hypersensitivity in CNS (Sinatra, 2002). The NSAIDs act by inhibiting the COX and inhibits the synthesis of sensory PGs (Sinatra, 2002). Furthermore, the immune cells in CNS such as microglia tends to release the same nociceptive mediators in the spinal cord much like peripheral immune cells (Sinatra, 2002).

1.3.12. Leukotriene B4 and LOX

The leukotriene B4 produced as a result of activation of 5-LOX pathway from the immune cells, which sensitize nociceptors by increasing the cyclic AMP level in the cell. The arachidonic acid is produced by the action of phospholipase 2 on the PI2P, which is associated with activation of calmodolin pathway (Luan and Xu, 2006). The leukotriene, thus, produced acts on the cell and increase the level of cAMP within the cell by acting on the adenyle cyclase, which further activates PKA downstream. The increase level of cAMP are thought to be the basis for the sensitization of primary nociceptors in many etiological conditions (Luan and Xu, 2006). The leukotriene is an important chemo-attractant in nature and facilitate the recruitment of the various immune cells into the site of inflammation(Levy, 2009). Once these immune cells infiltrate the site of inflammation it than further facilitate the release LTB4 and there metabolites like (such as the sensitizing substance 8(R), 15(S)-dihydroxy

eicosatetraenoic acid) (Levy, 2009). Of particular the LOX metabolites produced by the neutrophils have been implicated to acts on the TRPV1 receptors and might be culprit for the link between neutrophils and inflammatory hyperalgesia. This has also been proved by the animals study as the inflammation induced neutrophils accumulation is linked with the increase LTB4 and both the neutrophils accumulation and LTB4 are recued in the mast cells deficient animals. This indicates that NGF-mast cells-LTB4-neutrophils axis is of huge importance (Levy, 2009).

1.3.13. Kinins

The kinins are peptides in nature and are activated at the site of injury following the cleavage from the circulating proteins (Levy, 2009). The typical kinins, bradykinin is formed form the high molecular weight kinonogen by the action of kaliikrein and is found in high concentration in inflamed tissue. The BK thus formed induces the activation and sensitization of nociceptors (Levy, 2009). The BK action are executed by the interacting with G-protein-coupled BK1 and B2 receptors and hence trigger the activation of PKC. The BK is associated with the synergistic action of other inflammatory mediators such as prostaglandins, nerve growth factors and can activate the release of other inflammatory mediators (Levy, 2009).

1.4. Role of Cytokines in the Nociception

The inflammatory cytokines are acts as signalling molecule that are released from various immune cells such as T helper cells, macrophages and several other cells to promote the process of inflammation (Ren and Torres, 2009). Inflammatory mediators are extensively produced during the process of inflammation and tends to enhance the process of inflammation (Ren and Torres, 2009). The excessive production of inflammatory cytokines for long time contribute to the various pathological states such as atherosclerosis and cancer (Ren and Torres, 2009). The balance release between the pro-inflammatory and anti-inflammatory mediators are critical for the maintenance for the homeostatic environment of the body. The release of inflammatory mediators during the process of inflammation are important to mount immune response against the invading pathogens by mobilization of innate immune response. Similarly, certain (Ren and Torres, 2009). The various cytokines implicated in inflammation and nociception are described here.

1.4.1. Interleukin-1β

IL-1β is an important mediator associated with various inflammatory responses, and implicated in numerous cellular activities such as cell proliferation, differentiation, and apoptosis. Within the CNS the activation of cyclooxygenase-2 (PTGS2/COX2) by the IL-1 might be the contributory factor to the inflammatory pain hypersensitivity (Kay and Calabrese, 2004). IL-1β injection into the peripheral tissue is associated with the increase sensation of mechanical and thermal hyperalgesia (Kay and Calabrese, 2004). The level of IL-1β is increased significantly in the DRG and following the intra-plantar injection of CFA, Carrageenan and lipopolysaccharide (LPS) bacterial endotoxin mediate, which strongly sensitize the nerves for thermal, mechanical hyperalgesia (Kay and Calabrese, 2004). The other mechanism by which the IL-1β produce the nociception is the up-regulation of pro-nociceptive mediators and the administration of IL-1β receptor antagonist remarkably inhibited the mechanical hyperalgesia produced by a CFA i.p injection as well as CFA mediated up-regulation of Nerve Growth Factor (NGF), which is a neurotropic factor having critical role in a various acute and chronic painful conditions. Interestingly, the NGF antagonists inhibited the CFA mediated hyperalgesia, however, not inhibited the level of IL-1β suggesting that NGF indirectly bring changes in the behavior (Mika, 2008). This increase in the NGF level by the IL-1β can occur both transcriptional and post-transcriptionally. IL-1β signaling mechanism is also associated with the increase in other nociceptive molecules such as Prostaglandin, Interleukin-6, Substance-P, and MMP9 (Mika, 2008).

1.4.2. IL-6

Interleukin 6 (IL-6) is a secretory inflammatory protein and is produced by various cells such as T cells and macrophages to initiate the immune response against the infection and trauma, particularly against the burns or several other tissue damaging reaction condition to set inflammatory condition (Brenn et al., 2007). Additionally, IL-6 is an important mediator regulating the fever and released during acute phase responses. Its increase the body temperature by crossing the blood brain barrier and tends to synthesize the PGE2 in the hypothalamus and change the body set temperature (Brenn et al., 2007). IL-6 is also produced by the macrophages against the microbe specific molecules which is called as pathogen associated molecular patterns (PAMPs). The PAMPs than interact with the important group of molecule of

the immune system referred to as pattern recognition molecules (PRRs) like Toll like receptors (TLRs). The PRRs are expressed either on the surface or intracellular compartment and tends to induce the intracellular signalling pathways leading to the synthesis of the inflammatory cytokine (De Jongh et al., 2003). Interestingly, IL-6 administration into right hind paw 1 week after the CFA injection produced immediate analgesia (5min), and was reversed by the naloxone. It was later revealed that IL-6 injection ipsilaterally following CFA injection produce local opioids from the immune cells, which attributes to the IL-6 mediated analgesia (De Jongh et al., 2003). However, in the IL-6 knockout mice the nociceptive responses to mechanical hyperalgesia and thermal hyperalgesia was lowered compared to the responses in the wild type of mice having IL-6 gene intact (De Jongh et al., 2003). In the rats inflammatory pain models such as chronic constriction injury. Axotomy, and crushed injury have a direct co-relation was found between the postoperative mechanical allodynia and the number of IL-6 positive cells in the sciatic nerve determined after the 14 days of surgery (De Jongh et al., 2003). While, the rats without evident allodynia and sham-operated rats had the smallest number of IL-6-upregulated cells (De Jongh et al., 2003).

1.4.3. Tumor necrosis factor (TNF-α)

TNF-α is produced chiefly by activated macrophages mainly, however, it can also be produced by many other cell types such as $CD4^+$ lymphocytes, NK cells, neutrophils, mast cells, eosinophil and neurons (Zhang et al., 2011). TNF-α is main inflammatory cytokine secreted not only in the immune system but also in the peripheral and central nervous system in various pathological conditions. Numerous report suggest the key role of TNF-α in the pathogenesis of various painful conditions such as neuropathic pain, inflammatory pain, and cancer pain (Zhang et al., 2011). The activation of the peripheral nerves by the TNF-α is well documented. The TNF-α injection either intra-plantar, intradermal, endoneurial, or intramuscular provokes thermal hyperalgesia and mechanical allodynia (Sommer and Kress, 2004). TNF-α also alters the activity of multiple ion channels such as capsaicin receptor (TRPV1), Na^+ , Ca^{2+} , and K^+ channels and also produce spontaneous stimulation of the primary sensory fibers (Sommer and Kress, 2004). Various studies proposed that TNF-α is associated with the central sensitization such as by enhancing the synaptic transmission and hyperexcitability in DH neurons (Sommer and Kress, 2004). The

TNF-α expression significantly increased in spinal cord glial cells in various chronic pain models. Similarly, the intrathecal injection of TNF-α is associated with the increase sensation of thermal hyperalgesia and mechanical allodynia (Sommer and Kress, 2004). While the intrathecal injection of TNF-α inhibitor like etanercept reduces the chronic pain sensation (Sommer and Kress, 2004). The perfusion of spinal cord slices with TNF-α enhances the spontaneous excitatory postsynaptic currents (sEPSCs) and enhances NMDA-induced currents in lamina II neurons of DRG (Sommer and Kress, 2004). Furthermore, the injection of TNF-α is related with the increased increases the trafficking and surface expression of α-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA) receptors (AMPARs), thus leading to the enhancement of the synaptic transmission in various region of the brain especially in the hippocampal neurons (Sommer and Kress, 2004). Following the injury of spinal cord, TNF-α tends to increase the trafficking of GluR2-lacking AMPARs to the plasma membrane, leading to cell death of spinal cord motor neurons (Sommer and Kress, 2004). Recently, it was also reported that inflammation also encourages a TNF-α-dependent surface trafficking of GluR1 AMPARs in the DH neurons (Sommer and Kress, 2004). TNF-α executes its activity by interacting with the TNFR1 and TNFR2 receptors and the activation of these receptors regulates various painful conditions including neuropathic pain and cancer pain (Sommer and Kress, 2004). Additionally, TNFR1 regulates all phases of pain mediated by the inflammatory process, while a unique role of TNFR2 in mediating early-phase inflammatory pain has also been reported (Sommer and Kress, 2004).

1.4.4. VEGF (Vascular endothelial growth factor)

The VEGF plays various physiological and pathological roles in angiogenesis, embryogenesis, wound healing and remodeling, as well as in malignancy and inflammatory conditions (Kamei et al., 2004). In addition to inducing the endothelial cell proliferation and migration, it's also regulates the chemotaxis of the monocytes to injury and modulates the process of coagulation (Kamei et al., 2004). Furthermore, the VEGF has also been reported to acts as important factor for the ossification (fusion of bone with other bone) of the endocardium of the cartilage and to transform the cartilage into the bone (Kamei et al., 2004). The VEGF facilitate the angiogenesis process into the cartilage tissue and the recruitment of cells involved in the cartilage resorption and bone deposition (Kamei et al., 2004). Normally during bone

morphogenesis the chondrocytes of the epiphyseal plate become hypertrophic, induce the angiogenesis and then finally undergo the process of apoptosis (Kamei et al., 2004). This important angiogenic factor VEGF produced only in the chondrocytes when become hypertrophic and not produce during the resting or proliferating stage (Kamei et al., 2004). Furthermore, VEGF couples angiogenesis with hypertrophic cartilage remodeling and ossification (fusion of bone with other bone) during endochondral bone formation (Kamei et al., 2004). Specially, the program cell death (apoptosis) of the terminal hypertrophic chondrocytes is hindered, and the enrolment of chondroclasts, the monocyte-derived cells that are associated with the cartilage resorption, is reduced after the hindrance of VEGF activity by facilitating the interaction of protein to a soluble receptor (Sellam and Berenbaum, 2010). The crucial role of VEGF in the embryonic formation of bone by cartilage contrasts with its lack of expression in the adult (Sellam and Berenbaum, 2010). However, the genes expressed during fetal development often reemerge during the disease state (Sellam and Berenbaum, 2010).

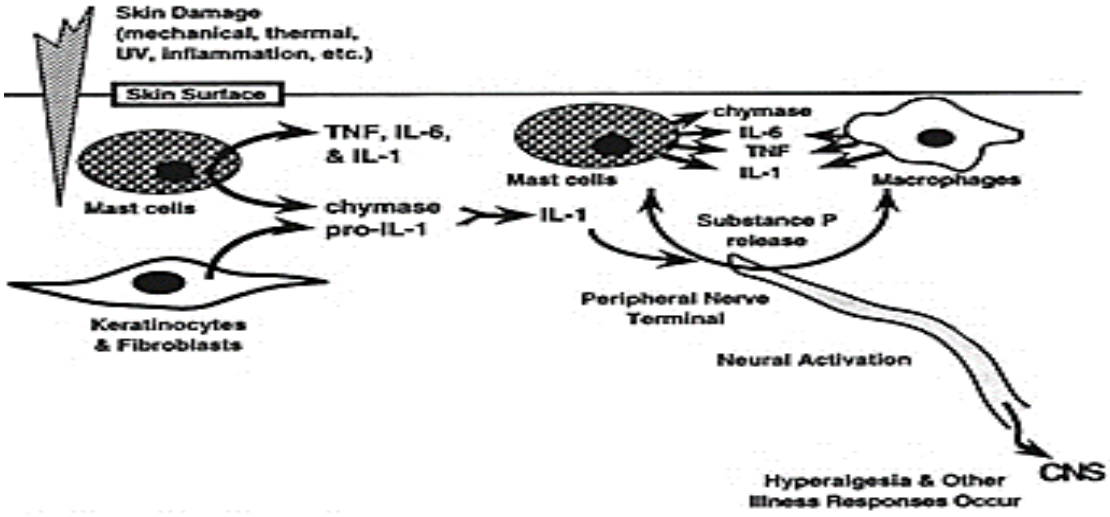

Figure 1.5: The role of cytokines in hyperalgesic response. Keratinocytes and fibrinocytes in the skin tonically make, store and release a precursor form of IL-1β (pro-IL-1β). Upon damage of the skin by the any of the mechanism including, burn, trauma, mechanical and inflammatory insults, mast cells that reside in skin are joined by additional mast cells migrating into the area of trauma. These mast cells release cocktail of substances including TNF, IL-1β, IL-6 and chymase. Adopted from **(**Linda R. Watkins et al., 1995).

1.5. Aims and Objectives

The non-steroidal anti-inflammatory drugs **(**NSAIDs) are most widely used analgesic and anti-inflammatory class of drugs and works by reducing the concentration of PGs by inhibiting the COX enzyme. The non-selective inhibition of the COX are responsible for the side effect associated with the use of NSAIDs (Vane, 2000). Furthermore, the opioids derivative offer one of the most important and potent class of the analgesic and employed to treat various painful conditions such as cancer pain, post-operative surgery, cardiac asthma and other analgesia. In spite of being very potent analgesic in nature however, are associated with various intractable side effects such as dependence and CNS depression (Grady et al., 2011). Natural products offer one of the most important source of new drug candidates to deal with various pathological conditions and are associated with less side effects and have better tolerability. One of such natural product is 25-MHA obtained from *Puerara lobata* (family Rutaceae).

The aims and objectives of the current study are following.

- Whether the 25-MHA shows anti-nociceptive properties or not?
- What will be the effect of 25-MHA on disease linked with inflammation?
- What will be the thorough mechanism of 25-MHA exhibits anti-hyperalgesic and anti-allodynic properties?
- What happened to the mediators of inflammation and pain during the treatment?
- Whether the 25-MHA exhibited any side effect/adverse effect in the entire study?

CHAPTER 2

METHODOLOGY

2. Materials and Methods

2.1. Chemical and reagents

The various chemical used in the study includes dexamethasone (Shaigan pharmaceuticals, Pakistan), complete freunds adjuvants (CFA) (provided by national research foundation NRF, Republic of South Korea), Carrageenan (provided by national research foundation NRF, Republic of South Korea), normal saline (Shaigan pharmaceuticals, Pakistan), DMSO (Shaigan pharmaceuticals, Pakistan), Piroxicam (Shaigan pharmaceuticals, Pakistan), gabapentin (Shaigan pharmaceuticals, Pakistan), tramadol (Shaigan pharmaceuticals, Pakistan), and 25-MHA (provided by national research foundation NRF, Republic of South Korea). Similarly, acetic acid and formalin was also used. The quantitative real time polymerase chain reaction was performed for the determination of the expression level of various cytokines such as IL-1, IL-6, and TNF-α and the expression of anti-oxidant enzyme level such as Nrf2, HO-1 and SOD2.

2.2. Animals

In the current study male albino mice were used, having the age of 3-4 weeks and weighing 30-35 grams. All animal studies were performed in the pathogen-free barrier zone of the Quaid-i-Azam University, Islamabad, Pakistan, accordingly to the instruction and guidelines set by the Guide for the care and use of Laboratory animals (Quaid-i-Azam University, Islamabad) and ethical committee approval certificate NO: (**No. #BEC-FBS-QAU2017-3**). The animals were kept at a temperature of 23+0.5 ^{0}C with 12 h light and dark cycle and at the humidity of 10%. The international association for the study of pain (IASP) guidelines were followed for animals care and handling for the use of animals in pain research (Zimmermann, 1983). All the behaviour experiments were performed without knowing that which group is receiving which treatment, thus followed double blind control. All animals study were performed between 8:00 am and 8:00 pm and the experimental animals were used only once. In order to minimize the discomfort and harm to the animals the minimum no of animals were used in the study. The animals were divided into 4 groups, the normal control received the normal saline dissolved in 2% DMSO, the negative control were subjected to the treatment with either CFA (20 µl/paw) or Carrageenan (100 µg/paw), positive control were treated with either dexamethasone 5mg/kg or

Piroxicam 5mg/kg and the treat group received 25-MHA (10mg/kg). The drugs were dissolved in the 2% DMSO before the administration into the animals. Each treatment group consist of 5-7 animals. The drugs were either injected into the animal intra-peritoneal or intra-plantar routes.

2.3. Formalin assay

The formalin test is extensively used to evaluate the inflammatory pain and this model have been tried in mice, rats and dogs to investigate the painful sensory behaviour of the animals (de Oliveira et al., 2011). The formalin administration produce characteristics nociceptive behaviour consisting of lifting, biting and flinching of the hind paw to whom the formalin was injected. Following the administration of formalin, the animals display nociceptive behaviour in two phases. The formalin induced nociception phase 1 extends from 5-10 min and phase 2 extends to 30-40 min following the injection of formalin (de Oliveira et al., 2011). The formalin mediated phase 1 nociception is due to the activation of local neurons and this phase is called as neurogenic phase. The phase 2 is called as inflammatory phase because formalin injection produce inflammation at the site of injection (de Oliveira et al., 2011). Formalin injection produces a biphasic nociceptive behaviour i.e. phase 1 (about 5–10 min) followed by a quiescent period (about 5 min) and then phase 2 (about 30–40 min). It is well established that formalin injection is associated with activation of dorsal horn neurons. The sensitization of the C-fibres by the formalin injection in phase 1 induces the inflammatory response and central sensitization in the phase 2 (de Oliveira et al., 2011). Furthermore, there is also continuing activity in the peripheral nerve during phase 2 and the administration of formalin (5%) into the dorsal horn produce hyperalgesia after 1-3 days and lasts for 3-6 weeks. While lower concentration of formalin (1-2%) are still associated with the initiation of long-lasting secondary hyperalgesia and allodynia and mechanism underlying this long lasting nociception is still unknown. However, the expression of activating transcription factor 3 (ATF3) in DGR, 3 days following the injection of formalin (2-5%) may be the possible mechanism by which produces the hypersensitivity similar to the rats exposed to spinal nerve injury. Additionally, the formalin injection associated with alteration in the expression of ATF3 and calcium channel α2δ-1 in DRG that is produced by the nerve injury (de Oliveira et al., 2011).

In the current study, anti-nociceptive potential of the 25-MHA (10mg/kg) was evaluated using formalin test in mice. Formalin-induced nociception was used as preliminary screening tool for the investigation of anti-inflammatory and analgesic potential of new drug candidate (de Oliveira et al., 2011). Forty min prior to the administration of formalin, normal saline (2% DMSO) was administered to the normal control, dexamethasone was administered (5mg/kg) to the positive control and 25-MHA (10mg/kg) was administered to the treatment group. The animals were placed in an open transparent glass box for 30 min to get acclimatize with the surrounding and then subjected to formalin administration. The animals were handle carefully and 20µl of 2.5% of formalin (1:100 dilution of stock formalin solution, 37% formaldehyde in 0.9% saline) was injected to the plantar surface of right hind paw via 30 gauge needle. After the administration of formalin into the mice they were transferred to observation chamber for 60 min. The formalin produce charchterteristics flinching response in two phases and the total time taken was noted.

2.4. Writhing Test

Acetic acid induced writhing test is used as a screening tool to investigate the anti-nociceptive potential of new drug. Following the intra-peritoneal administration of acetic acid there is writhing response characterized by abdominal contraction and extension of the hind limbs (de Oliveira et al., 2011). Writhing test is a common method of pain induction in the animals by the injection of acetic acid into peritoneal cavity. Acetic acid induced writhing response is used as screening tool to investigate the anti-inflammatory and anti-nociceptive potential of new drug (de Oliveira et al., 2011). The acetic induced writhing response is characterized by the contraction of abdominal muscles, arching of back and extension of hind limbs. The acetic acid induced writhing response is acute visceral nociception animal model (de Oliveira et al., 2011). In order to evaluate the anti-nociceptive potential of 25-MHA against the acetic acid induced writhing response, the whole study were divided into three groups. The negative control received only acetic acid, positive control received dexamethasone, and the treatment received 25-MHA. The analgesic potential of a drug is inferred by decreasing the activity of writhing movements.

2.5. Cold Acetone Test

The anti-nociceptive potential of the 25-MHA against the cold pain was also evaluated. The animals were subjected to CFA injection into the right hind paw as described previously. Following the intra-plantar injection of CFA into the right hind paw, the animals were transferred into the cubicle containers of small Plexiglas having metal mesh floor. To perform the cold acetone assay the animals were divided into four groups. The normal control received the normal saline dissolved in 2% DMSO, negative control were administered CFA only, the positive control were subjected to the treatment with dexamethasone (5mg/kg) and the treatment group were administered 25-MHA (10mg/kg). After the administration of CFA, the drop of acetone was applied to the right hind paw of the animals and the reading were recorded. The 25-MHA was investigated against the cold acetone pain for both acute and chronic effect. The acute anti- nociceptive effect of 25-MHA against the cold stimulus was recorded 6 h after the administration of CFA, while the chronic anti-nociceptive effect of 25-MHA (10mg/kg) was determined at the day 6 of the experiment. The time taken by the mice while flinching, licking and biting the acetone (25µl) exposed paw during one minute of the application of acetone.

2.6. Thermal hyperalgesia (hot plate)

The 25-MHA (10mg/kg) was also evaluated against the hot plate evoked thermal hyperalgesia. The thermal hyperalgesia was investigated against the hot plate having temperature of 60 ^{0}C by placing mice on that. Once the animals were kept on the hot plate, the licking, flinching, and biting of the paw were taken as positive response and the total time during which the paw were biting and licking was recorded. However, to avoid any harmful effect on the mice the cutoff time was set as 60 sec. In order to investigate the potential anti-nociceptive potential of the all the animals were divided into four groups. The normal control were administered normal saline dissolved in 2% DMSO, the negative control just received CFA, positive control were subjected to the treatment with the dexamethasone (5mg/kg), and treatment group was administered 25-MHA (10mg/kg). The 25-MHA (10mg/kg) was evaluated against both acute and chronic thermal hyperalgesia. The acute effect of 25-MHA (20mg/kg) was determined 2 h after the administration of CFA, three times for with duration of 2 h. The chronic effect against the thermal hyperalgesia was appraised form 0-6 days while skipping the dose at day 5 for elucidate the possible tolerance effect.

2.7. Mechanical Hyperalgesia Evaluation

Randall-Selitto was used to evaluate the mechanical hyperalgesia against the persistent inflammatory hyperalgesia and nociception according to the previously described methods. Before the application of Randall-Selitto animals were acclimatize for 15-30 min in quite room prior to the initiation of the test. The digital Randall-Selitto works on the principle of applying force on the right hind paw with a handful force transducer (Digital paw pressure Randall-Selitto meter ,IITC Life Science Inc., Woodland Hills, CA). All the experimental animals were classified into 4 groups. the normal control were administered with normal saline dissolved in 2% DMSO, the negative control group were treated with the CFA injected into the right hind paw, positive control were subjected to the treatment of dexamethasone (5mg/kg), and the treatment group animals received the 25-MHA (10mg/kg). Following the administration of different groups with different treatment, the Randall Selitto test was initiated. The mechanical hyperalgesia was evaluated using the Digital Randall-Selitto by applying the tip to the right hind paw center by gradual increase in the force. The paw withdrawal is considered the end point followed by clear movement of the animals. In order to investigate the therapeutic effect of 25-MHA (10mg/kg) animals were subjected to intra-plantar injection of CFA (20µl/paw) or Carrageenan (100µg/paw). Forty min before and one h before the injection of Carrageenan, animals received different treatment as described above. To observe the acute effect of the CFA treatment the mechanical hyperalgesia was investigated 2 h after the administration of CFA for 6 h with the interval of 2 h. Similarly, to determine the chronic anti-nociceptive effect of the 25-MHA (10mg/kg) was investigated for 0-6 days with interval of day 5 to observe the tolerance effect as described previously. Furthermore, the 25-MHA (10mg/kg) was also evaluated against the Carrageenan and the reading was noted 2 h after the administration of Carrageenan for 6 h with the interval of 2 h. The animals were tested before and after the treatment of the different groups. The reading were repeated three time and there average were calculated.

2.8. Mechanical allodynia

The Von Frey filament were used to analyse the mechanical allodynia as described previously (Khan et al., 2013a). For the investigation of mechanical allodynia animals were transferred to the transparent plastic box with a mesh floor in order to allow the access of the filaments into dorsal surface of the right hind paw. Prior to the

investigation of the mechanical allodynia animals were acclimatized to the testing environment for at least 35 min. The mechanical allodynia was analysed via calibrated Von Frey (Stoelting, USA) using double blinded assignment. The filaments were applied five times in the ascending order force. The withdrawal reflex three times out of five by the application of filaments is considered as positive response. To investigate the inhibitory effect of the 25-MHA (10mg/kg) on the CFA and carrageenan-induced hyperalgesia following the administration into the right hind paw. The animals were divided into 4 groups, the normal control received normal saline, negative control were treated with CFA, positive control were subjected to the treatment with dexamethasone and the treatment group received 25-MHA (10mg/kg). Following the intra-plantar injection of CFA (20μl/paw) the acute effect was determined at the interval of 2, 4 and 6 h following the administration of CFA. Similarly, to investigate the chronic anti-nociceptive effect of 25-MHA against the CFA, the reading was taken from 0-6 day, while, skipping the dose at day five to observe the possible tolerance effect. Moreover, the Carrageenan induced allodynia was evaluated at the interval of 2, 4 and 6 h.

2.9. Paw Edema Test in Mice

The paw edema test was used to observe the inhibitory effect of 25-MHA (10mg/kg) on the CFA and Carrageenan-induced inflammation in right hind paw. All the experimental animals were divided into 4 groups. The normal control received the normal saline into peritoneal, the negative control was subjected to the treatment of CFA only, positive control was administered dexamethasone (5mg/kg), and the treatment group received the treatment of 25-MHA (10mg/kg) intra-peritoneal. CFA were administered 40 min after the administration of different drugs. Following the administration of CFA the paw edema was measured via Dial Thickness Gauge meter (No. 2046 F, Mitutoyo, Kawasaki, japan). The 25-MHA (10mg/kg) was evaluated against CFA-induced both acute and chronic inflammation. For acute effect, the data was taken 2 h after the administration of 25-MHA (10mg/kg) for 6 h with the interval of 2 h. The effect of 25-MHA (10mg/kg) on chronic CFA-induced inflammation was evaluated from 0-6 days, while skipping the dose at day 5 to determine the tolerance effect of the 25-MHA as described previously (Khan et al., 2013a).

2.10. NO Determination in Plasma

NO is an important mediator of inflammation and its concentration is directly associated with degree of inflammation (Khan et al., 2013a). In the current study the level of NO was also determined in the plasma of mice to observe the effect of 25-MHA on the NO production. All the animal belonging to various groups such as normal control, negative control, positive control and 25-MHA treated group were sacrificed at day 6 of the experiment and the blood was collected in the EDTA tubes. The collected blood was subjected to the process of centrifugation at 5000 rpm at 4 ^{0}C and the temperature of 4 ^{0}C. The plasma was collected and separated from the blood cells and stored in the separate tubes. The NO concentration was determined in the plasma according to the method described previously. The 25µl of the plasma were mixed with equal volume of saline and then mixed with the equal volume of Griess reagent. The mixture stored at room temperature for 30 min. The concentration of the NO was determined from the micro plat reader. The concentration of NO in different groups were compared and plotted.

2.11. Analysis of LFT's, RFT's and Histopathology of GIT

Most of the analgesics are associated with various side effects upon chronic use. In current study, the cytotoxic effect associated with 25-MHA on the liver and kidney was assessed. The biochemical test such as alanine amino transferase (ALT), aspartate amino transferase (AST) were performed to explore the effect of 25-MHA on the concentration of these live functions test. Similarly, to assess the effect of chronic treatment on the kidney function serum creatinine test was performed. All the animals were weighted and divided into 4 treatment groups. The normal control received the only normal saline, the negative control were subjected to the treatment with CFA, positive control were exposed to the treatment with dexamethasone and the treatment group were administered 25-MHA. The treatment group was treated daily with 25-MHA (10mg/kg) for 6 days with the interval of day five. The normal control group received the normal saline. At the end of the experiment i.e. day six the animals were sacrificed and blood sample was collected in the EDTA tubes. The blood cells and plasma were separated at 500rpm for 5 min and the biochemical analysis were performed on the plasma. Furthermore, the possible toxicity of the 25-MHA on gastric mucosa was evaluated following daily administration. The animals were divided into three groups, normal control, Positive control (Piroxicam 10mg/kg) and

treatment control (25-MHA 10mg/kg). The Hemotoxylin and Eosin staining was performed following three days of treatment as described previously (Brzozowski et al., 2000; de Lima et al., 2011).

2.12. Histological Analysis of Mice Paw Tissue

The effect of 25-MHA treatment on histopathology was also investigated in the current study. Following daily treatment with 25-MHA (10mg/kg) the histo-pathological analysis of the paw tissue were performed. At day six of the experiment the mice were sacrificed using CO2 and the inflamed paw removed. After the removal of the paw, it was washed with normal saline, fixed in the solution of formalin (10%), undergone the process of rinsing, dehydrated and embedded in paraffin solution as described previously. The removed paw tissue blocks were sectioned at 4 μm thickness and subjected to the process of haematoxylin-eosin staining. The stained tissue were observed by microscopy (40x). The stained tissue were characterized for the infiltration of the immune cells such as neutrophils, macrophages, monocytes and osteoclast.

2.13. Radiological Analysis of Mice Paw

The anti-inflammatory role of 25-MHA was also investigated using X-ray analysis to determine the extent of tissue inflammation and bone resorption. The effect of daily treatment on the paw inflammation was evaluated and the X-ray analysis was performed at day 6. The animals were divided into 4 groups, the normal control received the normal saline, the negative control was treated with the only CFA, positive control was subjected to the treatment of dexamethasone, and the treatment group was treated with the 25-MHA. At day 6 X-ray was performed on all treated groups to assess the degree of swelling and inflammation. The animals were anesthetized with the chloroform at the day six and the X-ray film of paw were obtained as described previously. The animals were anesthetized with the chloroform, and placed on radiographic box, and the X-ray of the hind paw were obtained.

2.14. The Measurement of Cytokines Level in Paw Tissue

The tissue samples from all the treated groups such as normal control, CFA control, positive control and treatment control were prepared for the determination of the expression level of various cytokines such as IL-1β, IL-6, TNF-α and growth factor like VEGF according to the previously described method (Khan et al., 2014; Khan et

al., 2016). Briefly, skin tissues were removed from the paw and the protein were extracted using Trizol reagents. The Trizol Reagent was used to isolate the total RNA from the mouse paw tissue according to the instruction of the manufacturer (Invitrogen Life Technologies, Carlsbad, CA, USA) as described (Khan et al., 2014; Khan et al., 2016). Briefly, Applied Biosystem (AB) detection instrument and software was used to determine the expression of various target genes mRNA (TNF-α, IL-1β, IL-6, Nrf2, HO-1, SOD2, VEGF, GAPDH/β-actin) via RT-PCR. SYBR green DNA binding dye was used for the quantitative analysis of mRNA expression. Using standard procedure the melting point, optimal conditions and the specificity of the reaction were determined first. The working stock solution of SYBR Green was 1:100 (Bio-Rad). Quantitative PCR was performed in a 48-well plate with 10pmol sense and anti-sense primers, and the working solution SYBR green, using a PCR master mix, under the following conditions: 95 ^{0}C for 5 min, followed by 40 cycles at 95 ^{0}C for 1 min, 55 ^{0}C for 45 sec and 72 ^{0}C for 30 sec. A housekeeping gene GAPDH/β-actin was used as an internal standard to control for variability in amplification because of differences in starting mRNA expression. The copy number of each manuscript was calculated as the relative copy number normalized by GAPDH/β-actin copy number. The sequences of the primers (forward and reverse primers) are listed in the table 1.

2.15. Measurement of Cytokines (IL-1β and TNF-α) Production in Paw Tissue

The production of these inflammatory cytokines was determined in the paw tissue. The skin tissue were removed from the paw tissue and tissue proteins were extracted using 100mg tissue/ml PBS and to this solution 0.05% Tween 20, 0.4 M NaCl and the protease inhibitors were added. The samples were subjected to the process of centrifugation for 10 min at 3000g and supernatant obtained were frozen at -80 ^{0}C as described previously (Khan et al., 2013a). The production of these inflammatory cytokines were determined using commercially available ELISA kit (eBioscience, Inc., San Diego, CA).

2.16. Muscle strength and co-ordination

To investigate the non-specific effect of 25-MHA (10mg/kg) on locomotor activity, the mice were treated with the 25-MHA (10mg/kg). The locomotor activity of animal were assessed before the treatment and after the treatment with 25-MHA (10mg/kg).

The 25-MHA (10mg/kg) was evaluated for both acute and chronic muscle strength and co-ordination 6 h after the treatment of 25-MHA (10mg/kg) for acute toxicity and after 6 days of the treatment for the chronic toxicity. The different animals groups received different treatment. The normal control group received normal saline (2%DMSO), positive control received dexamethasone (5mg/kg), negative control received CFA (20µl/paw) or Carrageenan (100µg/paw) and the treatment group received 25-MHA (10mg/kg). The various groups are subjected to chain weight lifting test having different weights. Similarly, the muscle coordination were assessed by using the inverted mesh table and the time taken by animals to attach itself with the inverted mesh was noticed. The weight lifting test and inverted mesh screen test are used to assess the animals muscle strength and co-ordination.

2.17. The Elucidation of Possible Mechanism of Action

To determine the possible mechanism of action underlying the anti-nociceptive properties of 25-MHA (10mg/kg) were subjected to the Carrageenan-induction according to previously described method (Khan et al., 2014; Khan et al., 2016). To evaluate the involvement of calcium channel on the anti-nociceptive response of 25-MHA (10mg/kg), one group of animals received only Carrageenan, the second group received Carrageenan + 25-MHA (10mg/kg), and third group received Carrageenan + 25-MHA (10mg/kg) + Gabapentin (50mg/kg). Similarly, to investigate the involvement of opioid receptors, norepinephrine and serotonin in the 25-MHA (10mg/kg) mediated anti-nociceptive response another set of experiments were performed. In this experiments the same protocol was followed as discussed above. Finally, the involvement of cyclooxygenase involvement was assessed by the use of Piroxicam (5mg/kg) with another set of experiments and the same protocol was followed as described above (Quintão et al., 2012). The Carrageenan was injected to the right hind paw of the animals 40 min after the administration of the different drugs. Four h following the injection of Carrageenan, the mechanical hyperalgesia, mechanical allodynia and thermal hyperalgesia were assessed.

2.18. Molecular Docking

Molecular docking studies of compound 25-MHA (10mg/kg) was performed against NF-κB-inducing Kinase (NIK) using AutoDock Tool 1.5.6 and AutoDock4.2 (Morris et al., 2009). Crystal Structure of NF-κB-inducing Kinase (NIK) was downloaded

from RCSB using PDB ID: 4DN5 having resolution of 2.5 Å. Crystal Structure of NF-κB-inducing Kinase (NIK) was found to be co-crystalized ligand ATP-gamma-S; adenosine 5'-(3-thiotriphosphate) within the active site of target (Liu et al., 2012). 3D Structure generation as well as energy minimization of 25-MHA was carried out using ChemDraw suit (O'Boyle et al., 2011). Before docking calculation of 25-MHA, first target as well as co-crystal ligand molecule were re-docked to optimize the docking protocol. After optimizations of docking protocol, compound 25-MHA was docked within the active site of NF-κB-inducing kinase using AutoGrid program. Parameters for grid generation were of 60 × 60 × 60 dimension, x= -8.171419 y= 30.22, z= -4.762645 in xyz direction and 0.375 spacing centered around co-crystalized ligand. While Lamarckian genetic algorithm (LGA) was used as docking search parameters, in which total GA runs and number of maximum evaluation were set to 50 and 5×10^6 respectively. The final selection of docked pose was made conferring to the estimated size of clusters and free binding energy delta G. After carefully analyzing overall docking calculation, docked pose with the lowest value of free binding energy was considered as the most stable one and best binding mode. Openable was utilized for cross conversion of pdbqt files into pdb files to visualize in Discovery Studio (DS) Visualizer program. DS was used for visualizing the binding interactions of 25-MHA (10mg/kg) within the active site of target (Spassov and Yan, 2013).

2.19. Effect of 25-MHA on NF-κB (p65) Production in Paw Tissue

The NF-κB signalling is an important pathway involved in the pathogenesis of various diseases such as inflammation and cancer (Bremner and Heinrich, 2002). In the current study the effect of 25-MHA (10mg/kg) was evaluated against the NF-κB (p65) production using ELISA kit (Caymen chemical) as described previously (Khan et al., 2014).

2.20. Statistical Analysis

The results are expressed as mean ± standard deviation (S.D) unless otherwise stated. One way analysis of variance (ANOVA) was applied to the data followed by Dunnett's t test to determine the statistical significance of the differences between the various study groups (SPSS Version 20.0®). For the statistical significance a value of $P < 0.05$ was chosen as the criterion.

CHAPTER: 3

RESULTS

3. Results

3.1. 25-MHA Inhibited Formalin Induced Nociception

The formalin induces the nociceptive behaviour in animals in two phases i.e. early phase and the late phase. The early response in thought to be due to the activation local of sensory neurons, while, the late phase is due to the initiation of local inflammatory response, characterized by the infiltration of immune cells. The formalin induced phase lasts for 10 min while the late phase of formalin induced nociception phase 2 lasts for 10 to 30 min. Forty min after the administration of 25-MHA (10mg/kg), formalin was administered. The 25-MHA administration reduced the paw licking and biting response in phase significantly. The 25-MHA also significantly inhibited formalin induced paw biting and licking response in the mice significantly Figure 3.1. The negative control treated with formalin only exhibited no reduction in the nociceptive response in both early and late phase. However, the positive control treated with the Piroxicam significantly inhibited the formalin induced nociception in early as well as late phase of formalin induced nociception figure 3.1.

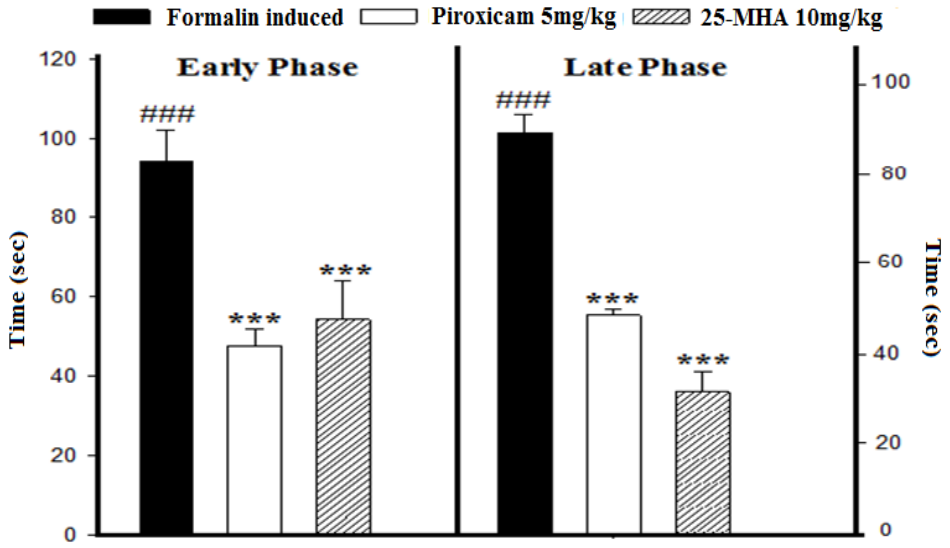

Figure 3.1: The effect of 25-MHA (10mg/kg) on the formalin-induced biphasic nociception was investigated. The results indicates that the 25-MHA significantly ($p<.001$) inhibited both early and late phase nociception.

3.2. Writhing Response

The analgesic potential of any drug is evaluated using acetic acid induced writhing response. The acetic acid administration into the peritoneum produce typical response characterized by contraction of abdominal muscles, and extension of the hind limbs. Forty minutes after the administration of 25-MHA acetic acid was administered intra-peritoneally. The 25-MHA treated group significantly Figure 3.2 inhibited the writhing response. Similarly the positive control treated with the Piroxicam also significantly inhibited the writhing response. While the negative control received only acetic acid exhibited no anti-nociceptive response. Thus, on the basis of these results 25-MHA exhibited significant anti-nociceptive response against the acetic acid-induced writhing response.

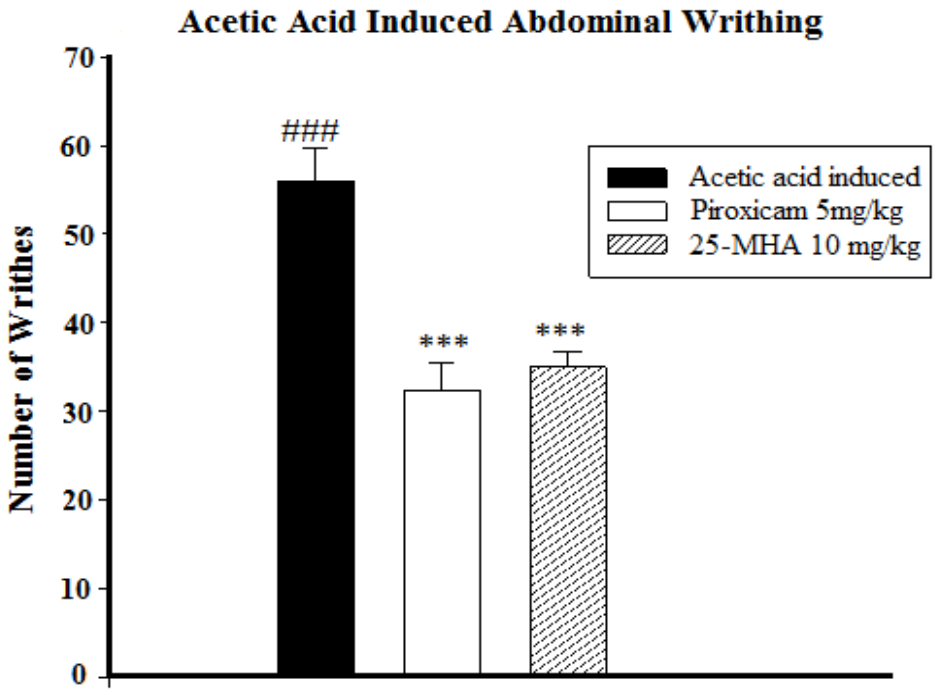

Figure 3.2: The effect of 25-MHA (10mg/kg) against the acetic acid-induced writhing response was investigated. The 25-MHA exhibited significant ($P < 0.001$) reduction in the writhing responses compared to the negative control.

3.3. 25-MHA Inhibited Mechanical Hyperalgesia

The 25-MHA was also investigated against the mechanical hyperalgesia induced by the intra-plantar injection of CFA. The Randall Selitto was used to measure the mechanical hyperalgesia. One h after the administration of the 25-MHA, CFA was injected into the right hind paw. Following the administration of CFA the reading was noted 2 h after the CFA injection with 2 h interval for 6 h for acute anti-nociceptive effect. To evaluate the chronic effect of 25-MHA on the anti-nociceptive properties the reading were taken from day 0-6, with the interval of day 5 to observe the tolerance effect. The 25-MHA significantly inhibited the acute and chronic nociceptive response. Similarly, the positive control received the dexamethasone also significantly reduced the sensation of pain in both acute and chronic CFA-induced nociception. The normal control treated with the normal saline (2% DMSO) exhibited no response. While the negative control showed no anti-nociceptive response Figure 3.3. Similarly, 25-MHA (10mg/kg) was also investigated against the Carrageenan induced mechanical hyperalgesia. Following the administration of 25-MHA, forty min later the Carrageenan was administered intra-plantar. The mechanical hyperalgesia was assessed 2, 4 and 6 h after the administration of Carrageenan Figure 3.4**.** The 25-MHA exhibit significant reduction in the mechanical hyperalgesia compared to the negative control. Similarly, the Piroxicam treated group also significantly reduced the mechanical hyperalgesia as compared to the negative control.

3.4. 25-MHA Inhibited Mechanical Allodynia

The 25-MHA (10mg/kg) was also evaluated against the mechanical allodynia induced by CFA and Carrageenan. The 25-MHA (10mg/kg) administration, followed by the intra-planter CFA injection was evaluated against the both acute and chronic response. The acute CFA-induced nociception was evaluated at 2, 4 and 6 h after the administration of CFA. While the chronic effect was evaluated from day 0-6 with the interval of day 5 to observe the tolerance effect associated with the treatment of 25-MHA. The 25-MHA treatment significantly reduced the mechanical allodynia in mice in both acute and chronic model compared to the negative control Figure 3.3. Similarly, the animals treated with the dexamethasone also remarkably inhibited the mechanical allodynia compared to the negative control. While the normal control received normal saline did not exhibited any analgesic response. In order to observe

the tolerance effect as described previously the dose was withhold at day 5. However, skipping the dose at day 5 re-established the mechanical allodynia. Thus indicate that to achieve continuous analgesic effect the 25-MHA must be administered daily. The Carrageenan-induced nociception was determined at the interval of 2, 4 and 6 h following the administration of Carrageenan. The 25-MHA significantly reduced the mechanical allodynia compared to the negative control Figure 3.4. Similarly, the dexamethasone treated group also exhibited significant reduction in the mechanical allodynia compared to the negative control. However, the normal control treated with the Normal saline did not exhibited any analgesic response.

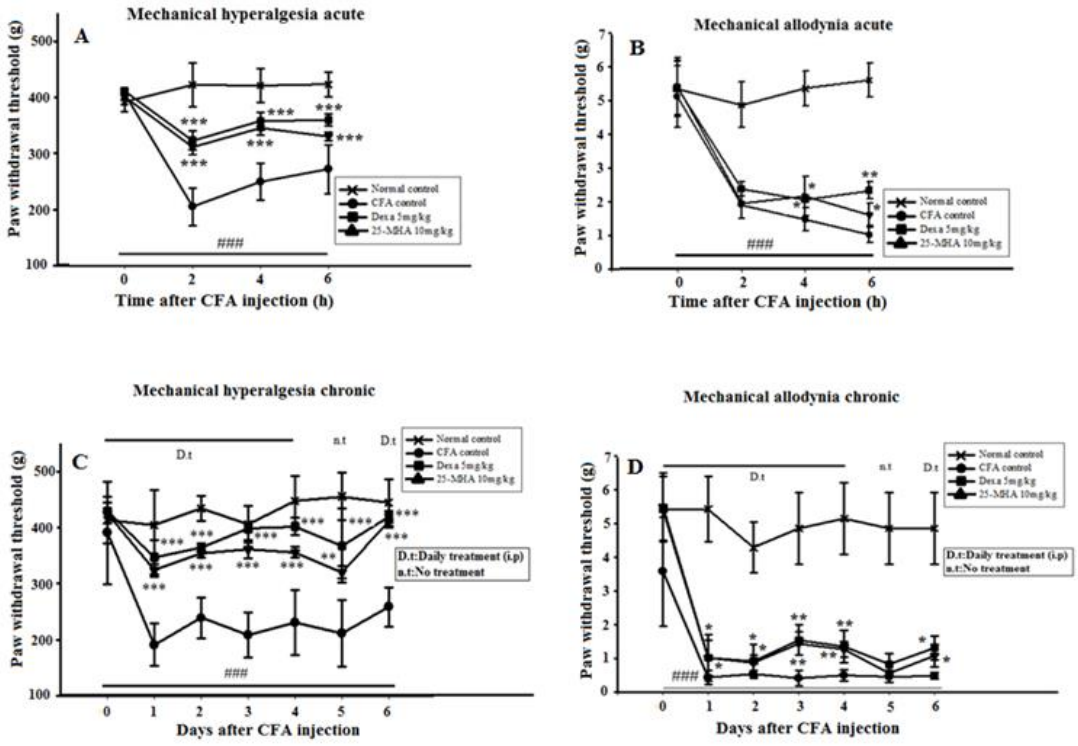

Figure 3.3: The effect of the 25-MHA on the CFA-induced mechanical hyperalgesia and mechanical allaodynia. The 25-MHA significantly inhibited the mechanical hyperalgesia and allodynia as described in material and methods.

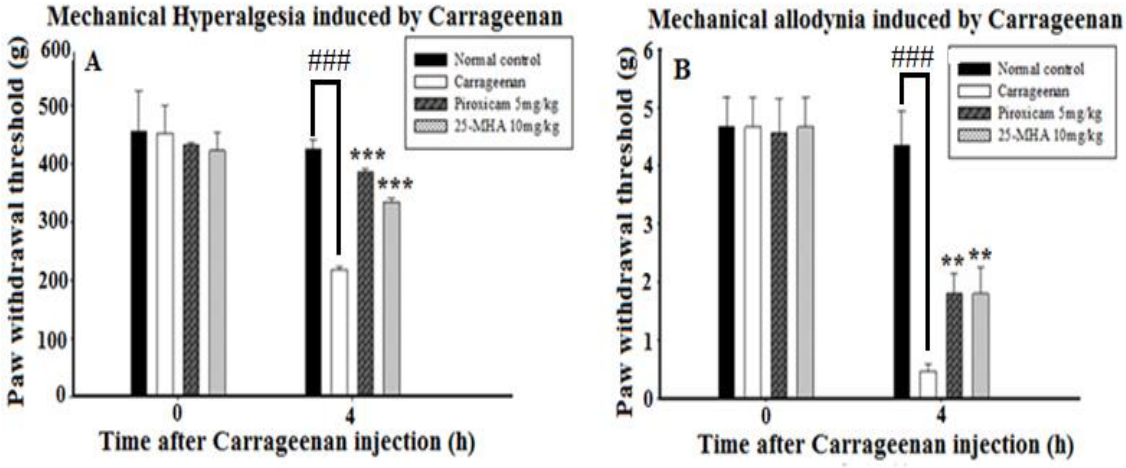

Figure 3.4: The effect of 25-MHA on the Carrageenan-induced mechanical hyperalgesia and allodynia was evaluated. The 25-MHA significantly inhibited the mechanical hyperalgesia and allodynia as described in the materials and methods section.

3.5. 25-MHA inhibited Carrageenan and CFA-induced thermal hyperalgesia (hot plate) in mice

The effect of 25-MHA was investigated against both CFA and Carrageenan-induced thermal hyperalgesia. The 25-MHA (10mg/kg) exhibited significant analgesia at 2, 4, and 6 h after CFA induced hyperalgesia Figure 3.5 and exhibited significant anti-nociceptive activity at 4 h following Carrageenan-induced hyperalgesia Figure 3.6. Similarly, in order to investigate the long term effect of 25-MHA (10mg/kg) on thermal hyperalgesia, the animal were daily administered with 25-MHA (10mg/kg) for 6 days. The 25-MHA shows significant inhibition of thermal hyperalgesia. Furthermore the positive control received dexamethasone also significantly inhibited the thermal hyperalgesia. The normal control received the vehicle (2% DMSO) but showed no significant response.

3.6. 25-MHA inhibited CFA-induced (acute and chronic) cold pain in mice

The 25-MHA (10mg/kg) was evaluated for the sensory behaviour against the cold acetone stimulus. The response was evaluated for acute as well as chronic cold stimulus. The 25-MHA (10mg/kg) administration 40 min prior to the acetone stimulus showed significant activity against acetone induced nociception at 4 h of the acetone induction Figure 3.5. Similarly, the dexamethasone also exhibited significant anti-nociception against the cold acetone stimuli. To evaluate the chronic sensory

behaviour against the cold pain, 25-MHA (10mg/kg) was administered 40 min prior to the administration of acetone. The daily administration of 25-MHA (10mg/kg) shows promising activity against the cold pain at day 4 compared with the vehicle control.

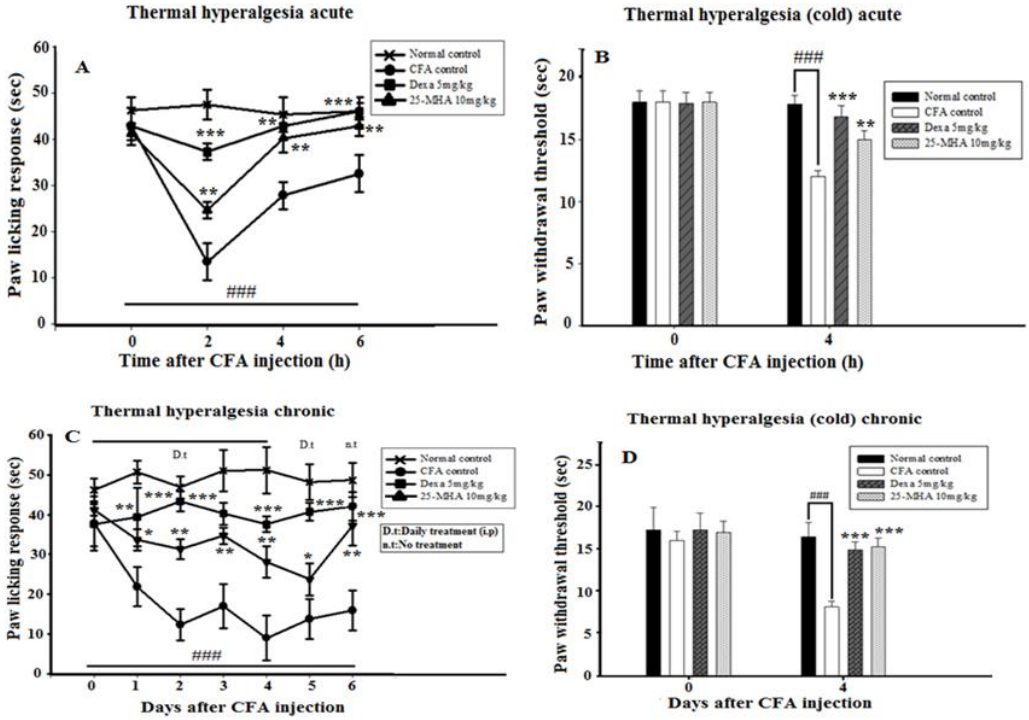

Figure 3.5: The effect of 25-MHA on thermal hyperalgesia, acute (A and B) thermal hyperalgesia and chromic (C and D). the 25-MHA significantly inhibited (P<.05)both acute and chronic thermal hyperalgesia.

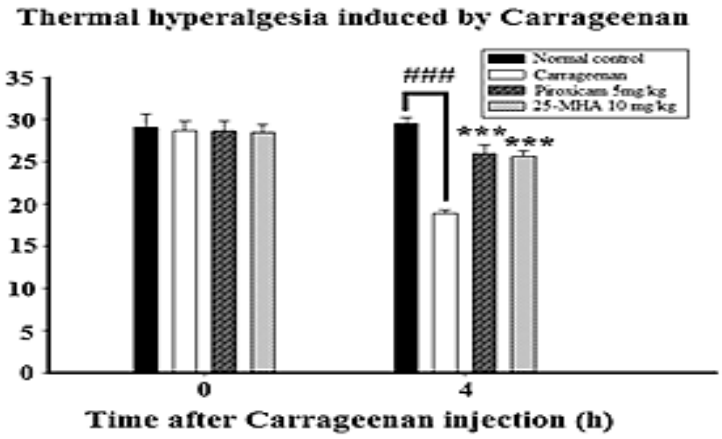

Figure 3.6: Effect of 25-MHA on carrageenan induced thermal hyperalgesia. The 25-MHA significantly inhibited the thermal hyperalgesia as described in materials and methods"

3.7. Effect of 25-MHA on the paw edema

The effect of 25-MHA was also investigated on the paw edema induced by CFA. Forty minutes after the administration of the 25-MHA, intra-plantar administration of CFA was performed. The 25-MHA was evaluated for both acute and chronic paw edema. The acute effect of 25-MHA was investigated at 2, 4 and 6 h after the administration of CFA. For chronic effect the 25-MHA was administered daily from day 0 to day 6 while skipping the dose at day 5. The paw edema was measured using Dial Paw Thickness Gauge meter. The 25-MHA administration significantly inhibited the paw edema in acute case when compared to the negative control. Similarly, the 25-MHA was also associated with the dramatic reduction in chronic paw edema when compared to the negative control group. Similarly, the dexamethasone treated group also inhibited the paw edema both acute and chronic when compared to the negative control group only challenged with CFA treatment. The normal control treated with normal saline did not reduced the paw edema Figure 3.7. The tolerance effect was also investigated however, the 25-MHA did not exhibit persistent anti-nociceptive effect and the paw edema re–established after the withholding the treatment at day 5. Thus it is anticipated to achieve the persistent therapeutic effect 25-MHA must be administered daily.

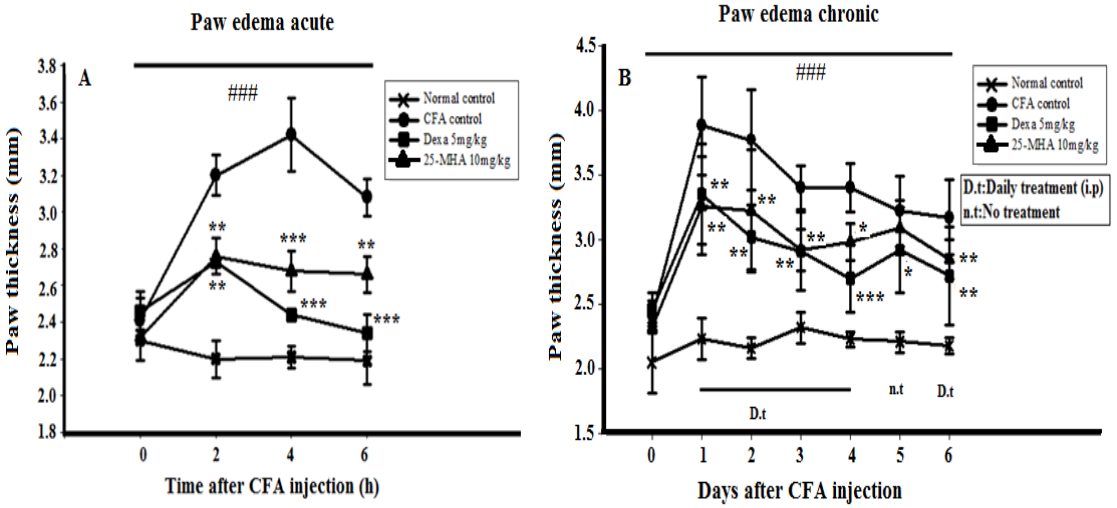

Figure 3.7: The effect of 25-MHA on the paw edema induced by CFA. The 25-MHA significantly inhibited the paw edema in both acute (A) and chronic (B) paw edema compared to the negative control as described in material and methods.

3.8. Effect of NO Production

NO is an important mediator of inflammation and its concentration is implicated in various inflammatory conditions. The level of NO was also assessed in the current study in different groups treated with different drugs. Following the administration of CFA intra-plantar, the whole blood was taken at day 6 of the CFA induction. Plasma was separated from the whole blood via the process centrifugation. The level of NO was determined in the plasma as described previously. The equal amount of plasma was mixed with Griess reagent i.e. 25µl of plasma was mixed with the 25µl of Griess reagent and put in the 96 well plate. The mixture was kept at 4 ^{0}C overnight and microplate reader was used to determine the absorbance. The absorbance on the microplate reader was directly correlated with the NO production and inflammation. The 25-MHA treated group significantly reduced the level of NO production when compared to the negative control. Similarly, the positive control treated group significantly reduced the level of NO when compared to the negative control. The normal control received the normal saline did not exhibited any response Figure 3.8.

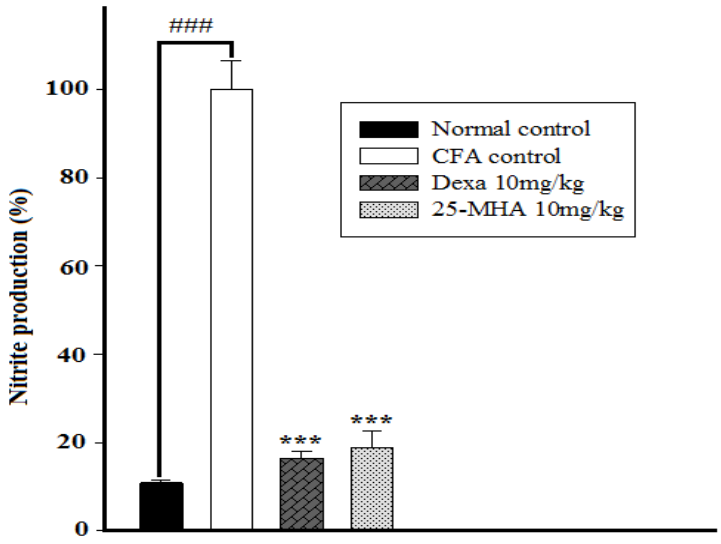

Figure 3.8: The effect of 25-MHA on the NO production in plasma. The 25-MHA significantly inhibited ($p<0.001$) the NO production as compared to the negative control.

3.9. Effect of 25-MHA on the Expression level of Cytokines

Cytokines are well known mediators of inflammation and are associated with sensitization of nociceptors. The various cytokines implicated with inflammatory environment and the development of noxious sensations are IL-1β, IL-6, TNF-α and VEGF. In current study it was also evaluated whether the use of 25-MHA is associated with the reduction in these inflammatory cytokines or not. The qRT-PCR was performed to assess the mRNA expression level of these inflammatory cytokines. For this purpose paw tissue were taken and the mRNA was converted into cDNA and the PCR was performed. The QT-PCR was performed for all groups such as normal control, negative control, positive control and treatment control. Following the administration of CFA intra-plantar into the mice, the daily treatment with the 25-MHA was investigated. Following the treatment with 25-MHA the right paw was removed using chloroform as general anaesthetic. The paw tissue were stored at -80 ^{0}C for quantification purpose. The 25-MHA treated group significantly inhibited the expression levels of IL-1β, 1L-6, TNF-α, and VEGF. Similarly the positive control treated group with dexamethasone also reduced the mRNA expression level of these inflammatory cytokines as compared to the negative control. Furthermore the normal control treated with the normal saline did not exhibited any response Figure 3.9. The sequences of primers used are listed in Table 1.

Table 1. Sequence of PCR primers

Genes	Forward primer	Reverse primer
GADPH	AACGACCCCTTCATTGAC	TCCACGACATACTCAGCAC
B-actin	CCCACTCCTAAGAGGAGGATG	AGGGAGACCAAAGCCTTCA
HO-1	CACGCATATACCCGCTACCT	CCAGAGTGTTCATTCGAGA
SOD2	GCGGTCGTGTAAACCTCAT	GGTGAGGGTGTCAGAGTGT
Nrf2	TGGGGAACCTGTGCTGAGTCACTGGAG	ACCCCTTGGACACGACTCAGTGACCTC
IL-1β	TCCAGGATGAGGACATGAGCAC	GAACGTCACCCAGCAGGTTA
IL-6	CCACTTCACAAGTCGGAGGCTTA	CCAGTTTGGTAGCATCCATCATTT C
TNF-α	GTTCTATGGCCCAGACCC TCA	GGCACCACTAGTTGGTTGTCTTTG
VEGF	TTACTGCTGTACCTCCACC	ACAGGACGGCTTGAAGATG

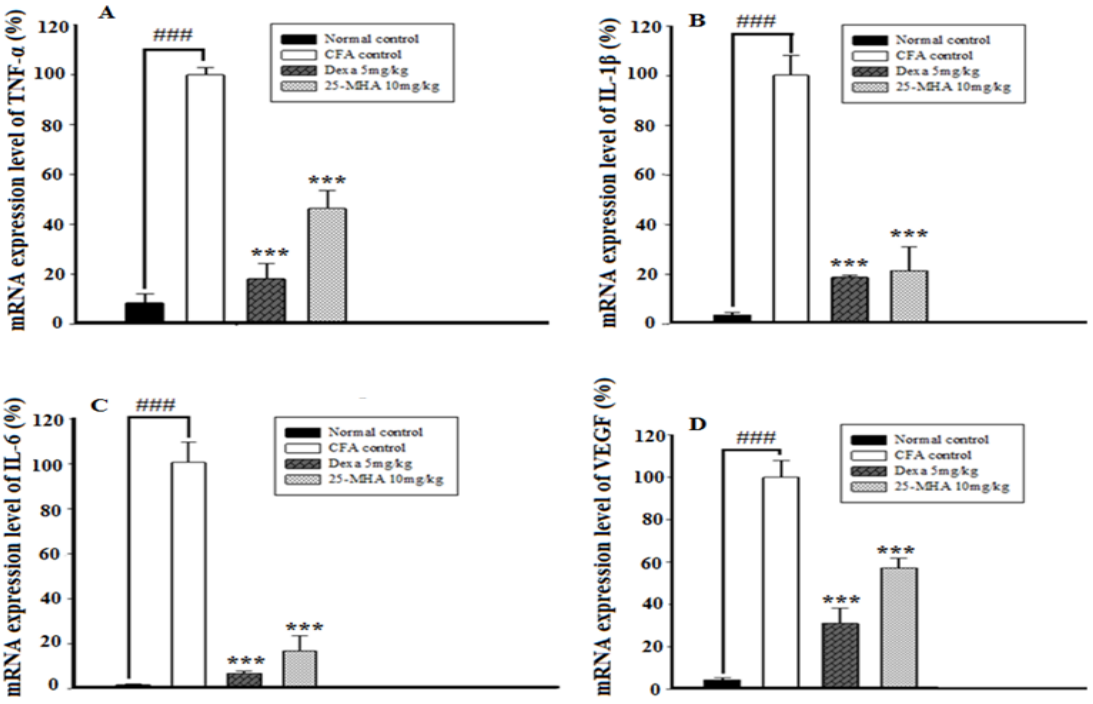

Figure 3.9: Effect of 25-MHA on the mRNA expression levels of inflammatory cytokines. The 25-MHA significantly inhibited the mRNA expression levels of IL-1β (A), IL-6 (B), TNF-α (C) and VEGF (D).

3.10. Inhibitory effect of 25-MHA on inflammatory cytokine (IL-1β and TNF-α) Production

The 25-MHA treatment is significantly associated with the reduction of mRNA expression levels of IL-1β, and TNF-α. However, to investigate the effect of 25-MHA on the production of these inflammatory proteins ELISA assay was performed. The 25-MHA treatment is significantly associated with the production of these inflammatory proteins compared to the negative control (Figure 3.10). Similarly, the positive control treatment also remarkably reduced the production of these inflammatory proteins.

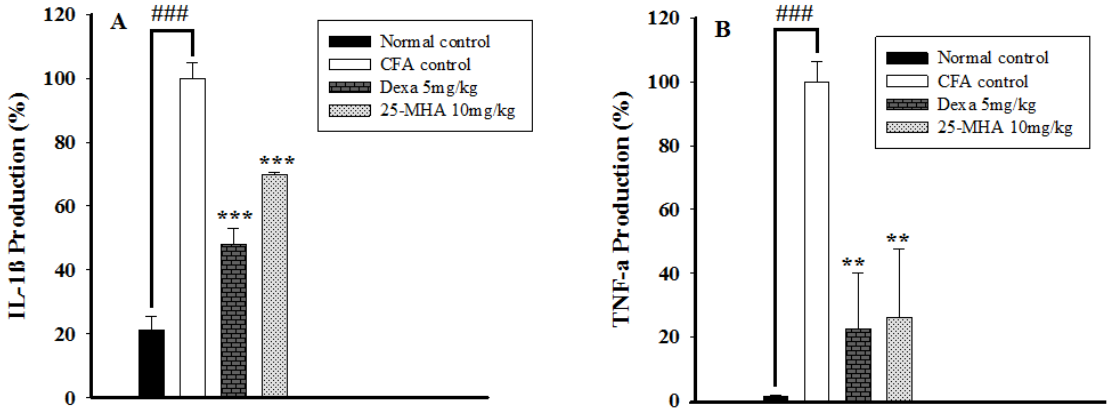

Figure 3.10: Effect of treatment with 25-MHA on the production of (A) IL-1β and TNF-α in CFA-induced paw tissue. The mice were treated with 25-MHA (10 mg/kg, i.p.), dexamethasone (5 mg/kg, i.p.) or vehicle control.

3.11. Effect of 25-MHA on the Expression Levels of Anti-Oxidant Enzymes

The effect of 25-MHA on the expression level of different anti-oxidant enzyme was investigated such as Nrf2, HO-1 and SOD2. The mRNA expression level of these anti-oxidant enzymes were assessed using qRT-PCR. The paw tissue were removed and the mRNA was extracted using triazole reagent. The mRNA thus extracted was subjected to the process of DNA polymerization. The 25-MHA treated group significantly increased the expression levels of these anti-oxidant enzymes, thus it is anticipated that 25-MHA might be having protective effect on the liver by increasing the expression level of anti-oxidant enzymes. The positive control treated group also significantly increased the expression levels of anti-oxidant enzyme as compared to the negative control. The normal control treated group did not exhibited any effect on the level of these anti-oxidant enzymes Figure 3.11**.**

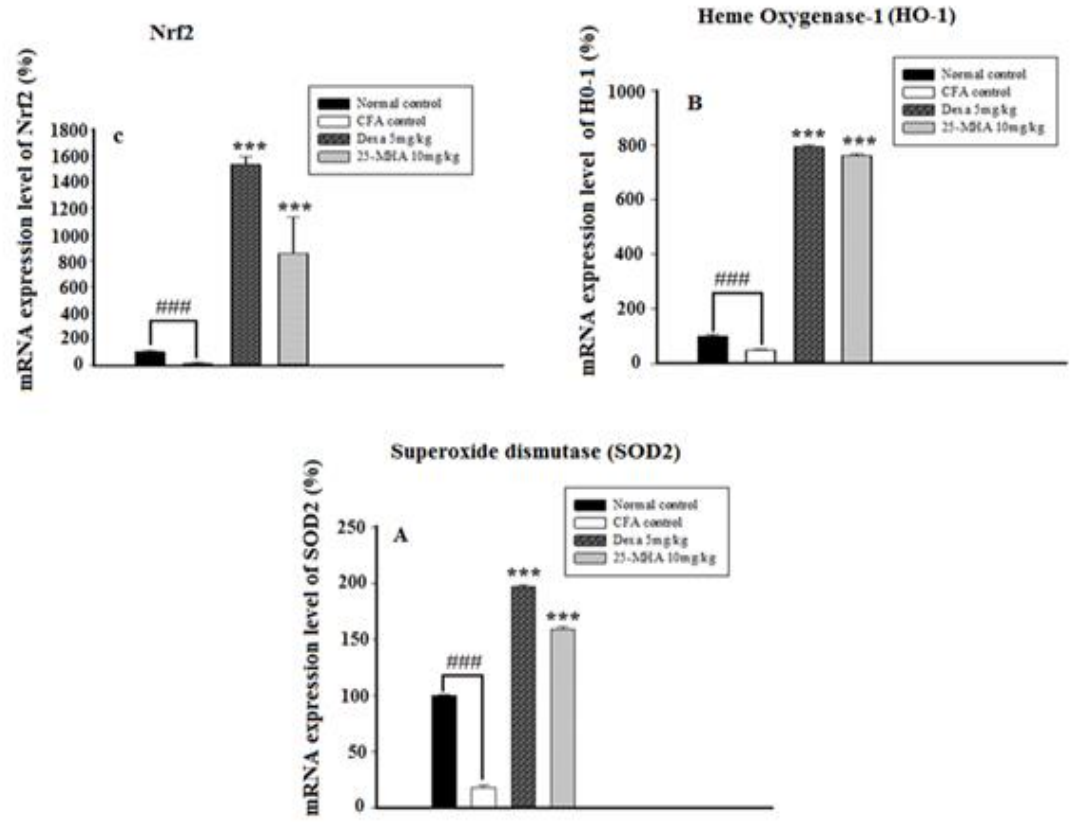

Figure 3.11: The effect of 25-MHA on the mRNA expression levels of anti-oxidant enzymes. The 25-MHA significantly expressed the mRNA levels of the anti-oxidant enzymes such as Nrf2 (A), HO-1 (B) and SOD2 (C) compared to the negative control.

3.12. Effect of 25-MHA on the Histological Study

The histological analysis was performed to assess the effect of CFA intra-plantar injection on the histpathologocal changes on the paw tissue. The effect of 25-MHA was evaluated on the on the histological changes on the paw tissue following the administration of algogenic substance. The H and E was performed for all the treatment groups. The paw tissue after removal was treated with the formalin and kept at -80C. The paw tissue was sectioned according to the method previously described. The normal group treated with the normal saline, the negative only subjected to CFA only, positive control received dexamethasone and the treatment control received 25-MHA, were subjected to the H and E process. The infiltration of the various immune cells such as neutrophils, monocytes, were inhibited by the treatment with the 25-MHA when compared to the negative control. The positive control treated with dexamethasone also inhibited the immune cell recruitment and thus decrease the

inflammation. However, the normal control administered with the normal saline did not exhibited any response Figure 3.12.

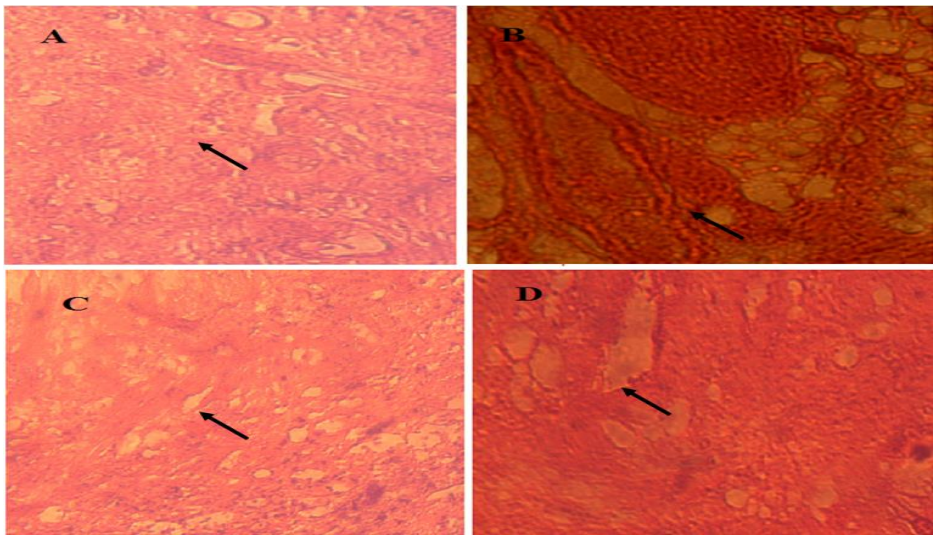

Figure 3.12: The histo-pathological analysis of the paw tissue of the mice. The normal control (A) received vehicle, negative control (B) received only CFA, positive control received (C) dexamethasone and treatment control (D) received 25-MHA. The 25-MHA significantly reduced the infiltration of the immune cells.

3.13. Effect of 25-MHA on the LFT's, RFT's and GIT Toxicity

Most of the analgesic drugs available are associated with adverse effect on the liver and kidneys. The 25-MHA was investigated whether it is associated with liver and renal toxicity or not. The 25-MHA treated group have not exhibited any toxic effect on the liver and kidney as it is evident from the ALT, AST and creatinine test. Similarly, the normal control administered with the saline also have no effect on the liver and kidney functions. Dexamethasone treated group were also not associated with any significant toxicity Table 2. Consequently, the oral administration of the 25-MHA is not associated with any toxic effect on the gastric mucosa. The Piroxicam (10mg/kg) treatment is associated significant toxicity on the gastric mucosa as the infiltration of immune cells, and the distortion of the normal texture can be seen from the H and E staining Figure 3.13.

Table 2. Effect of daily treatment with 25-MHA (10mg/kg) on the biochemical parameter

Sample	AST/GPT (UI/L)	AST/GOT (UI/L)	Creatinine (mg/dL)
Normal control	33.5±1.4	148±5.7	0.3±0.14
25-MHA (10mg/kg)	37.5±1.3	148±4.4	0.3±0.1

The values are represented as the means ± S.D for five animals per group using Student t test.

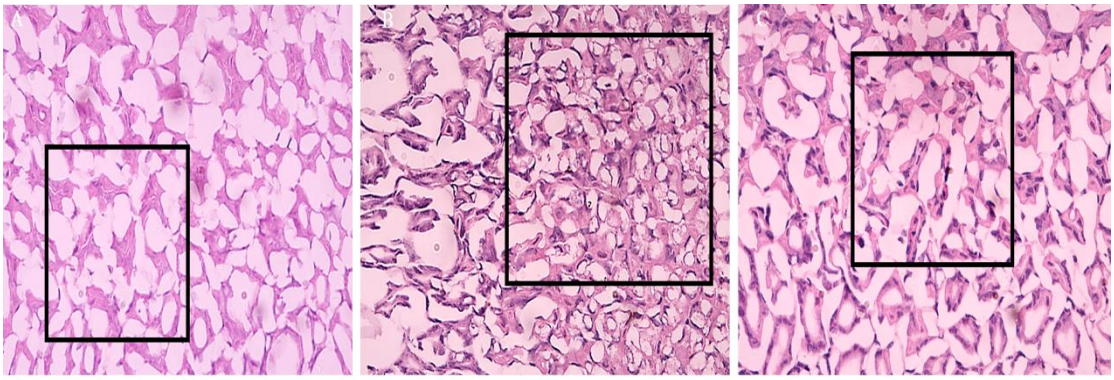

Figure 3.13: Effect of 25-MHA on GIT mucosa. The (A) Normal control, (B) the Piroxicam control and (C) 25-MHA treated group. The 25-MHA did not exhibited any toxic effect on the GIT mucosa as evident from the H and E staining.

3.14. Effect of 25-MHA Treatment on the Muscle Strength and Co-Ordination

The 25-MHA treatment was also evaluated to explore any observable harmful effect on the muscle strength and co-ordination. The acute effect was evaluated 6 h after the administration of CFA. While the chronic effect was evaluated 6 day after the administration of the CFA. The 25-MHA exhibited no observable effect on the muscle strength and co-ordination in both acute and chronic treatment. Similarly, the positive control treated with the dexamethasone also did not affect the muscle strength and co-ordination 6 hour after the CFA injection for acute effect and at day 6 for the chronic effect. The normal control was administered with normal saline did not showed any response, while the negative control challenged with CFA only significantly affected the muscle strength and co-ordination Figure 3.14.

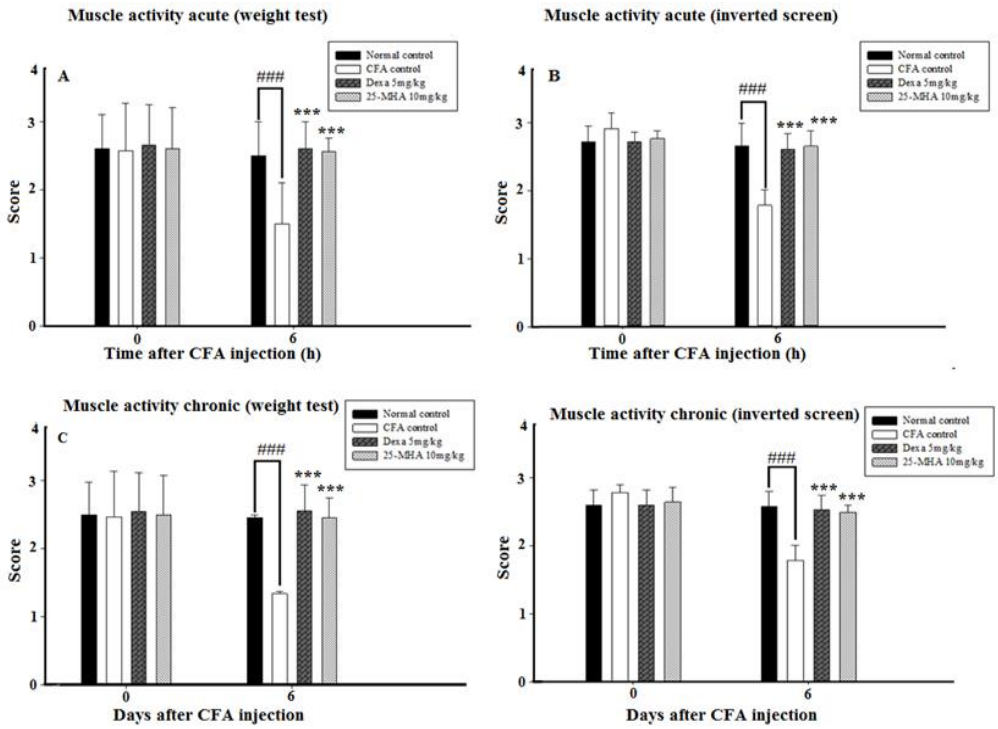

Figure 3.14: The effect of 25-MHA on the muscle strength and co-ordination in mice. The 25-MHA exhibited no significant toxicity on the muscle strength and co-ordination.

3.15. Effect of 25-MHA on the Paw Swelling as Evident from X-ray Analysis

The x-ray analysis was performed to determine the tissue swelling as well as the changes in the bone and soft tissue in different treatment group. The intra-plantar administration of CFA 40 minutes after the intra-peritoneal administration, the 25-MHA chronic effect was evaluated on day six of the CFA administration. At the day 6 of the CFA administration the paw tissue were subjected to X-ray analysis. The normal control treated with the normal saline, the negative control was subjected to the treatment with CFA, positive control received the treatment with the dexamethasone and the treatment control was challenged with the 25-MHA administration. The 25-MHA treatment significantly reduced the paw swelling and reduced bone resorption when compared to the negative control. The positive control treated group also reduced the negative changes in the paw tissue. The normal treated group did not exhibited any response Figure 3.15.

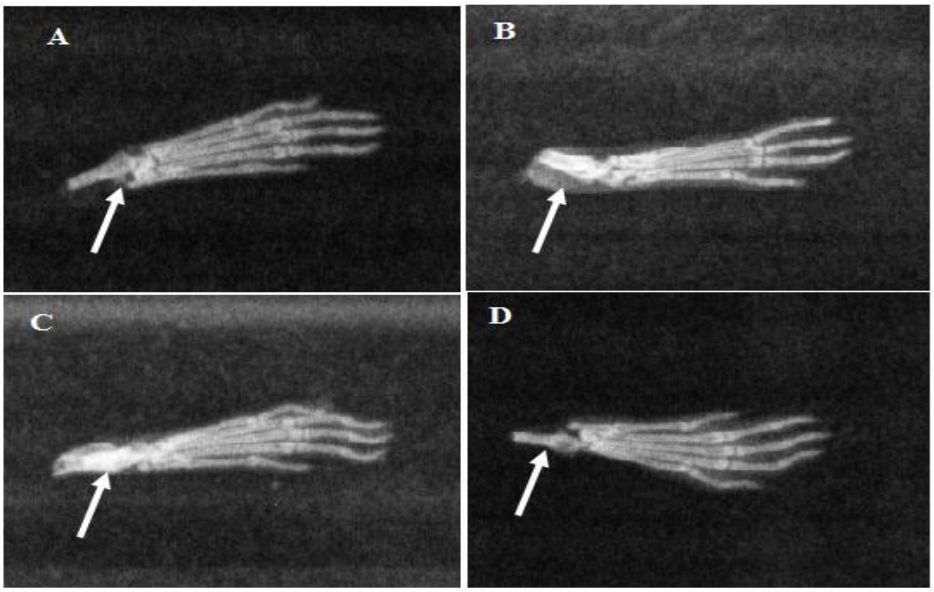

Figure 3.15: Radiological analysis of the mice paw. (A) Normal (B) negative control, (C) Positive control group and (D) 25-MHA treated group. Tissue swelling can be observed in the tibiotarsal region of CFA treated mice as compared to 25-MHA treated mice.

3.16. The elucidation of possible mechanism of action of 25-MHA

The 25-MHA (10mg/kg) was investigated for the mechanism by which it produces the anti-nociceptive response according to the methodology described previously with some modification (Quintão et al., 2012). To elucidate the involvement of calcium channel, the animals treated with 25-MHA (10mg/kg) + Gabapentin (50mg/kg) showed significant reduction in mechanical hyperalgesia, mechanical allodynia and thermal hyperalgesia compared to group treated with either 25-MHA (10mg/kg) or Gabapentin alone Figure 3.16. Thus, 25-MHA (10mg/kg) may involve produce anti-nociception by interfering with calcium channel. Similarly, the involvement of cyclooxygenase system was evaluated for the possible involvement in 25-MHA (10mg/kg) mediated anti-nociceptive response. The result showed that 25-MHA (10mg/kg) + Piroxicam (5mg/kg) treated group remarkably reduced the nociception compared to the 25-MHA (10mg/kg) or Piroxicam (5mg/kg) treated group. Furthermore, the involvement of opioids and monoamine reuptake system was also investigated for the involvement of 25-MHA (10mg/kg) mediated anti-nociception. The result indicates that 25-MHA (10mg/kg) + tramadol (50mg/kg) showed significant reduction in hyperalgesia, thus suggests that 25-MHA (10mg/kg) may interfere with opioids and monoamine reuptake system in reducing the hyperalgesia.

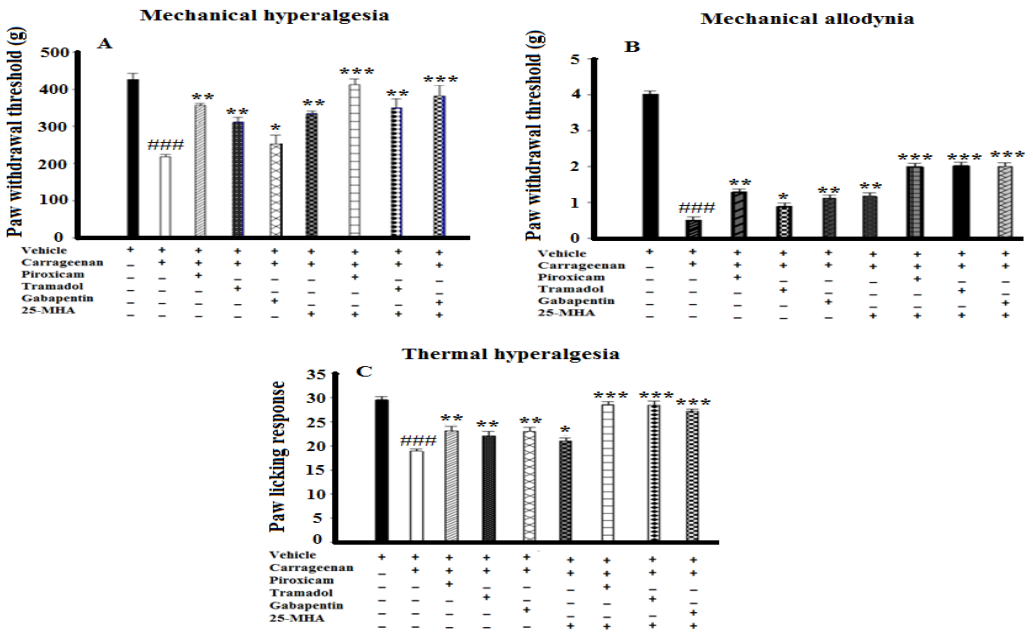

Figure 3.16: To elucidate the possible anti-nociceptive mechanism mediated by 25-MHA. The 25-MHA was co-administered with Gabapentin, Piroxicam and tramadol.

3.17. Binding Interaction of 25-MHA (10mg/kg) with NF-κB-inducing Kinase

Docking studies of 25-MHA (10mg/kg) compounds were carried out to investigate the plausible binding interaction within active site of NF-κB-inducing kinase. Optimization of docking protocol revealed good RMSD value of 1.7 Å which shows good agreement because RMSD value of < 2.0 Angstrom between a docked pose and the crystal structure is considered good. Figure 3.17A shows the default and redocked pose of co-crystalized ligand within the active pocket of target structure. Putative binding interactions of compound 25-MHA (10mg/kg) within NIK are shown in Figure 3.17B. Analysis of binding interaction of 25-MHA revealed that compounds is well docked inside the active site of target structure similar to that of co-crystalized ligand. Binding interaction of 25-MHA (10mg/kg) exhibited relative less no. of hydrogen bonds as compared to the co-crystal ligand which is might be due to the lesser number of hydrogen bond acceptor or donor atom in structure as compared to co-crystalized ligand Figure 3.17.

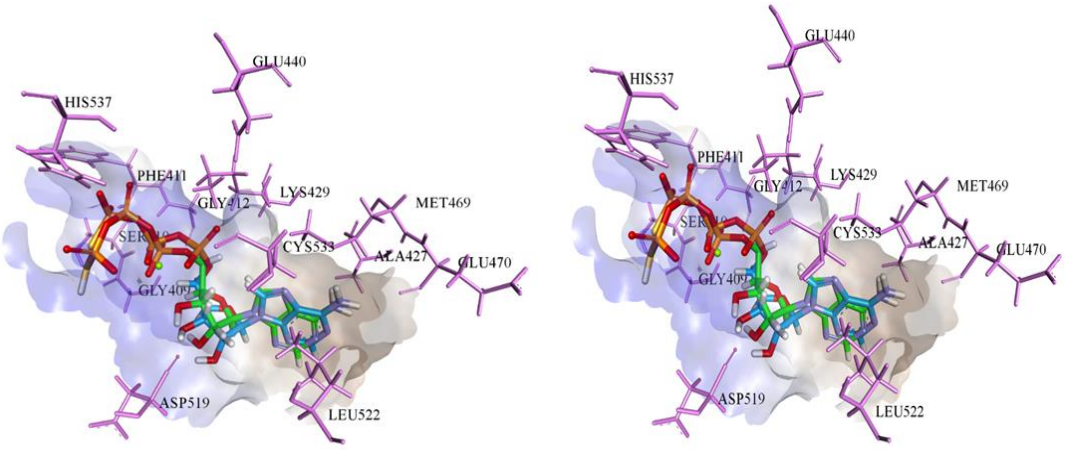

Figure 3.17A: Binding orientation of default (light blue coloured) and redocked pose (green coloured) of co-crystallised ligand within the active pocket of NF-κB-inducing kinase. Interacting amino acid of NF-κB-inducing kinase are shown in pink colour stick. **Fig 3.17B**. Binding orientation of compound 25-MHA (golden coloured) within the active pocket of NF-κB-inducing kinase. Interacting amino acid of NF-κB-inducing kinase are shown in pink colour stick.

3.18. Inhibitory effect of 25-MHA on p65 via ELISA assay

The 25-MHA treatment significantly inhibited the production of NF-κB (p65) nuclear protein using ELISA assay in paw tissue compared to the negative control. Similarly, the positive control treatment was also associated with significant inhibition of NF-κB (p65) nuclear protein production as shown in figure 3.18.

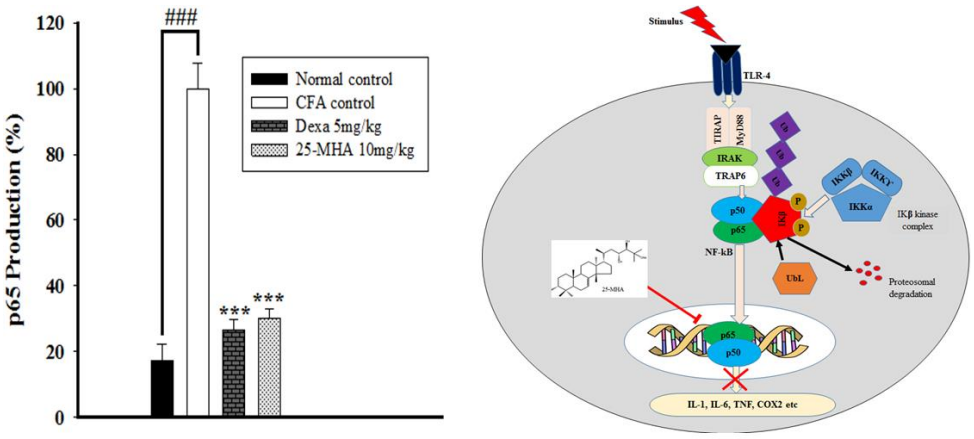

Figure 3.18: Effect of 25-MHA was evaluated against the NF-κB (p65) nuclear protein production using commercially available ELIS kit. The results indicates that 25-MHA significantly inhibited the NF-κB (p65) pathway.

Chapter 4

DISCUSSION

4. Discussion

Chronic pain is affecting about 30% of the world population and regarded as big health problem worldwide with rapid increase in the incidence proportion of new cases (Woolf and Salter, 2000; Quintão et al., 2012). Various strategies are employed to treat the chronic pain, however, these therapeutic strategies still unable to effectively control the pain. Similarly, these treatment modalities are also associated with the debilitating side effects (Woolf and Salter, 2000; Quintão et al., 2012). These therapeutic approaches includes opioids, NSAIDs and mono-amine reuptake inhibitors. Thus to search and develop new compounds with potential anti-inflammatory and anti-nociceptive activity that could be employed to treat both acute and chronic pain is a challenge (Woolf and Salter, 2000; Quintão et al., 2012).

The somatosensory sensation are transmitted by various nerve fibres such as Aα, Aβ, Aδ fibres and C-fibres. The A fibres are myelinated while the C-fibres are non-myelinated (Woolf and Salter, 2000; Quintão et al., 2012). The Aα fibres are concerned with the proprioception, the Aβ fibres are concerned with the mechanoceptors having no nociceptive role, Aδ fibres are mechanical and thermal receptors and C fibres are mainly nociceptive in nature (Woolf and Salter, 2000; Quintão et al., 2012). Furthermore, it is important to understand the central nervous system to adapt to the different stimuli (Woolf and Salter, 2000; Quintão et al., 2012). The central sensitization involve various cerebral structures that are involved in the perception of painful sensation and consist of specific interaction between the neurons and glial cells (Woolf and Salter, 2000; Quintão et al., 2012). The neuronal system become hypersensitive to the persistent painful stimuli and induce molecular modification to such high frequency inputs with resultant peripheral and central sensitization (Woolf and Salter, 2000; Quintão et al., 2012).

Natural product are the important source of new drugs development and are considered to be associated with less side effect. The 25-MHA isolated form the immature fruit of the *Poncirus trifoliate* are extensively used as traditional medicines for the cure of gastritis, allergic inflammation, gastritis and dysentery (Woolf and Salter, 2000; Quintão et al., 2012). Recently, the extract or pure compounds of these plants are also tried for other biological activities such as anti-cancer and apoptosis

induction. Furthermore, several other activities such as anti-inflammatory, anti-H-pylori, and anti-anaphylactic are also reported (Shin et al., 2010).

The most important component extracted from this specie is Poncirin a flavonoids whose anti-inflammatory activity is contributed due to the NF-κB inhibition. However, recently several novel form of triterpinoids have been reported form this species such as 21(a,b)-methylemelianodiols by LPS activated RAW cells (Zhou et al., 2007). Further, research was conducted to identify and isolate more anti-inflammatory and anti-nociceptive compounds from the *Poncirus trifoliate*, with resultant two compounds were isolated called as 25-MHA and 25-MHB. Thus keeping of the previous reported study of shin et al., 2010, the current study was proposed whether the 25-MHA exhibit any anti-nociceptive activity (Shin et al., 2010).

The CFA and Carrageenan are well known inducers of inflammation and nociception following local administration into the mice paw. The 25-MHA is administrated prior to the local injection of CFA and Carrageenan to assess the anti-nociceptive activity of the 25-MHA (Shin et al., 2010). The 25-MHA is administered daily up to six days, while skipping the drug at day five to assess the tolerant effect. While the Carrageenan anti-nociceptive response was assessed at single time point and 4 h after its administration. The 25-MHA remarkably reduced both acute and chronic mechanical hyperalgesia induced by the CFA and Carrageenan (Shin et al., 2010). Similarly, the 25-MHA also significantly reduced the mechanical allodynia against the negative control in both acute and chronic animal model. While the 25-MHA also reduced the nociceptive behaviour against the thermal hyperalgesia. Furthermore, the 25-MHA also significantly reduced the nociceptive behaviour induced by formalin and acetic acid compared to the control. The 25-MHA was also evaluated for the tolerance effect but the 25-MHA showed no tolerance effect and the hyperalgesia re-established after a period of treatment disruption. Cytokines have close relation with the sign of inflammation such as edema, hyperalgesia, permeability and cell migration. This relation has been formalized by using which are associated the inhibition of cytokines and consequently the sign of inflammation (de Oliveira et al., 2011).

Previous study demonstrated that 25-MHA isolated form the *Poncirus trifoliate* exhibited promising inhibitory effect on the production of various cytokines

such as IL-1β, IL-6 and TNF-α and growth factor like VEGF. On the basis of the previous finding the current study was hypothesized that the 25-MHA anti-nociceptive properties might be contributed by the inhibitory effect of the 25-MHA on these cytokines. The local injection of CFA and Carrageenan are associated with the chronic inflammation and release of various inflammatory and nociceptive mediators, which stimulate the primary sensory fibre for prolong time. Thus in the current study 25-MHA was investigated for the inhibitory effect on the level of cytokines in CFA induced nociception model. The data showed that 25-MHA is associated with significant reduction in production of various inflammatory cytokines following daily administration of 25-MHA for 6 days. Thus it may be hypothesized that the anti-nociceptive response mediated by 25-MHA may contributed by the inhibition of inflammatory cytokines. NF-κB is molecular signalling cascades associated with the various pathophysiological conditions including inflammation and cancer. The NF-κB is a transcriptional factor that after translocation into the nucleus induce the expression of various inflammatory cytokines. The 25-MHA administration is associated with the significant inhibition of NF-κB signalling cascade (Shin et al., 2010). This NF-κB inhibition might be involved with 25-MHA mediated reduction in inflammatory cytokines. Similarly, the CFA local injection was associated with the increased production of NO in plasma. While the pre-treatment with 25-MHA significantly reduced the NO production in plasma.

Most of the drug used to treat the chronic pain have effect on the CNS, and the effect of the drug in CNS is associated with several adverse effect such as muscle weakness and coordination. However, the 25-MHA was not associated with the development of deficit of muscle coordination and strength (Millan, 2002). Similarly, the 25-methoxy hispidol A was studied for systemic toxicity on various organs such as liver and kidney by assessing the liver function test and renal function test. However, the 25-methoxy hispidol A showed no toxicity on the stated organs compared to the control. It is well known that both prophylactic and therapeutic use of NSAIDs efficiently reduce the nociception, suggesting greater involvement of inflammatory pathway (Kroenke et al., 2009). While the anti-depressants and anticonvulsants drugs are associated with the ameliorating neuropathic pain suggesting the involvements of the central mechanism of pain control (Kroenke et al., 2009). On the basis of this discussion the involvement of these pathway were

investigated for the 25-MHA mediated anti-nociception. This study demonstrated that the co-administration with tramadol significantly interfere with the anti-nociceptive effect of 25-methoxy hispidol A, suggesting that 25-MHA may interfere with opioid system upstream and downstream to ameliorate the pain. The activation of opioids receptor in C and Aδ fibres located pre-synaptically inhibit the voltage gated calcium channel with resultant decrease in the level of cAMP, inhibiting the release of nociceptive mediators such as substance P, glutamate, and CGRP (Quintão et al., 2012). Similarly, the anti-nociceptive response of the 25-MHA also evaluated in the other acute and chronic experimental model. Gabapentin is used clinically to control the chronic pain and mediate its anti-nociceptive activity via modulating the $\alpha_2\delta$ subunit of the calcium channel (Li et al., 2006). The 25-MHA and gabapentin treated group significantly inhibited the mechanical hyperalgesia and allodynia compared to the control, thus suggesting the 25-MHA might interact with this system to produce the anti-nociceptive response. Furthermore, the involvement of inflammatory pathway in 25-MHA associated anti-nociceptive response, was evaluated using Piroxicam an anti-inflammatory drug. The pre-treatment of the animals with 25-MHA and Piroxicam significantly inhibited the hyperalgesia, allodynia and significantly reduced the paw swelling, thus reflecting the greater involvement of inflammatory pathway in 25-MHA mediated anti-nociception. Similarly, the same was confirmed using ELISA kit and the results indicates that 25-MHA treatment is associated with significant reduction in the production of NF-κB (p65) nuclear protein.

Conclusion

In the current study, the anti-nociceptive properties of 25-MHA was evaluated against various nociceptive pain models. The formalin and acetic acid-induced nociception is used as screening tool to investigate the anti-nociceptive potential of any new drug candidate. The 25-MHA exhibited significant anti-nociceptive activity against the formalin as well as acetic acid induced nociception. Furthermore, the 25-MHA was evaluated against the CFA and Carrageenan-induced nociception. The 25-MHA treatment was associated with significant inhibition of the thermal hyperalgesia (hot plate and cold acetone) in both CFA and Carrageenan-induced model. Similarly, the 25-MHA also significantly reduced mechanical hyperalgesia and mechanical allodynia in both acute Carrageenan-induced and CFA-induced models. The effect of 25-MHA was evaluated against the CFA-induced paw edema and results shows that 25-MHA treatment significantly inhibited the paw edema. The NO is an important mediator of inflammation and the level of NO production is directly related with the degree of inflammation. The 25-MHA treatment significantly inhibited the level of NO production in plasma. The cytokines have well established role in nociception and inflammation. In the current study the 25-MHA evaluated against the pro-inflammatory cytokines mediators and the results indicate that 25-MHA treatment significantly inhibited the mRNA expression levels of pro-inflammatory cytokines such as IL-1β, IL-6, and TNF-α. Similarly, the 25-MHA treatment also significantly inhibited the mRNA expression level of VEGF. The ELISA assay was performed to assess 25-MHA effect on the production of IL-1β and TNF-α production in the mice paw tissue. The 25-MHA significantly inhibited the production of IL-1β and TNF-α. The mechanism by which 25-MHA exhibited anti-nociceptive properties were also evaluated and the data suggest that may mediates its anti-nociceptive properties by inhibiting the NF-κB (p65) nuclear protein. The 25-MHA was also investigated to observe whether the daily dosing of 25-MHA is associated with any side effect? However, the results indicates that 25-MHA exhibited no side effect on the liver, kidney, GIT and muscle strength and co-ordination.

Future prospective

The 25-MHA exhibited significant anti-nociceptive activity in various animals model. In the current study 25-MHA exhibited remarkable anti-nociceptive activates against the CFA, Carrageenan, formalin and acetic acid induced inflammation. The 25-MHA also significantly reduced the mRNA expression of several important inflammatory mediators such as IL-1β, IL-6, TNF-α and growth factor like VEGF. On the basis of the current study it is anticipated that it can be develop as one of the potent analgesic agent associated with no toxic effect on the animals treated with it. However, to employ it as analgesic in clinical setting, it need further in depth evaluation especially the molecular mechanism involved in the inflammation and nociception.

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