CAPITAL UNIVERSITY OF SCIENCE AND TECHNOLOGY, ISLAMABAD

Pharmacological Evaluation of Pyrazolone Derivative in Animal Models of Acute Inflammatory Pain

by

Ahmad Hassan Mahmood

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I dedicate this humble work to Almighty Allah, whose endless blessings, guidance, and mercy have enabled me to accomplish this milestone. To the Holy Prophet Muhammad , who illuminated the path of knowledge and humanity. To my beloved parents, whose constant prayers, unconditional love, sacrifices, and encouragement have been the cornerstone of my success. To my teachers and mentors, for their invaluable guidance, patience, and unwavering support throughout my academic journey. Moreover, to my siblings, friends, and all well-wishers, whose words of motivation and belief in my abilities have always lifted my spirits. This achievement is as much yours as it is mine.

CERTIFICATE OF APPROVAL

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Ahmad Hassan Mahmood

(Registration No: MPH233016)

THESIS EXAMINING COMMITTEE

S. No.	Examiner	Name	Organization
(a)	External Examiner	Dr. Sagheer Ahmed	STMU, Islamabad
(b)	Internal Examiner	Dr. Fazlullah Khan	CUST, Islamabad

Dr. Muzaffar Abbas
Thesis Supervisor
October, 2025

Dr. Fazlullah Khan
Head
Department of Pharmacology
October, 2025

Dr. Muzaffar Abbas
Dean
Faculty of Pharmacy
October, 2025

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Abstract

Acute inflammatory pain presents a significant clinical challenge, demanding the development of safer and more effective treatment modalities. Drawing interest for their diverse pharmacological properties, including analgesic and anti-inflammatory effects, pyrazolone derivatives were investigated in this study for their potential in treating acute inflammatory pain. Acute inflammation was induced in adult mice via a carrageenan injection into the paw. Nociceptive and inflammatory responses were assessed through time-course behavioral tests, including measurements of paw withdrawal latency, mechanical withdrawal threshold, paw edema, and licking frequency by using Formalin test was identified . Biochemical analyses were conducted to evaluate inflammatory mediators and oxidative stress indicators, while histopathological examination of spinal cord tissues assessed protective effects at the tissue level. The results demonstrated that the pyrazolone derivative significantly mitigated hyperalgesia, edema, and oxidative stress compared to control groups. Specifically, treatment increased paw withdrawal latency up to 21 seconds and the mechanical withdrawal threshold up to 2 grams , alongside a significant reduction in paw volume and a marked decrease in licking frequency. Furthermore, the Pyrazolone derivative significantly suppressed pro-inflammatory cytokines and improved the histological architecture of inflamed tissues. These findings suggest that the pyrazolone compound possesses potent anti-inflammatory and analgesic properties, likely mediated by modulating inflammatory and oxidative stress pathways. The study concludes by highlighting the promising therapeutic potential of the pyrazolone derivative as a viable option for treating acute inflammatory pain, warranting further research into its clinical suitability.

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Abbreviations

AAALAC	Association for Assessment and Accreditation of Laboratory Animal Care International
CGRP	Calcitonin Gene-related Peptide
DAMPs	Damage-Associated Molecular Patterns
ELISA	Enzyme-linked Immunosorbent Assay
GFAP	Glial Fibrillary Acidic Protein
IHC	Immunohistochemistry
NLRP3	NOD-like Receptor Protein 3
NSAIDs	Non-steroidal Anti-Inflammatory Drugs
PAG	Periaqueductal Gray
PYZ	Pyrazolone
QSAR	Quantitative Structure–Activity Relationships
RVM	Rostral Ventromedial Medulla

Chapter 1

Introduction

1.1 Introduction

Pain is widely recognized not only as a sensory perception but also as a deeply personal, emotional experience [1]. In 2020, the International Association for the Study of Pain (IASP) reaffirmed their definition, describing pain as "an unpleasant sensory and emotional experience associated with, or resembling that associated with, actual or potential tissue damage" [2]. Immune cell, neurotransmitter, TNF-α, IL-6, and IL-1β are examples of proinflammatory cytokines that directly involve to the development and maintenance of pain by promoting central sensitization and hyperalgesia. traditional painkillers, such as opioids, sodium channel blockers, NSAIDS anticonvulsants, and antidepressants, have restricted pharmaceutical efficacy linked to multiple central nervous system related side effects in managing inflammatory pain. respiratory depression, constipation, urinary retention, vomiting, nausea and high incidence of dose-dependent side effects of opioid drugs are common drawback of using them. With NSAIDs, pain can be efficiently managed, both acute and chronic. But using these medications is always fraught with safety issues, including notably Severe damage to the kidneys, heart, and gastrointestinal tract. the dosage, timing, and mode of administration should be determined in accordance with each unique instance and drug type. This revised definition emphasizes that pain is more than just nociception—it incorporates emotional,

cognitive, and social dimensions, affirming the concept that pain is intrinsically subjective and influenced by individual life experiences [2, 3]. When functioning normally, acute pain serves an important biological and protective role, alerting the body to injury and promoting behavior that avoids further harm [4].

Pyrazole, a five-membered heterocycle featuring two adjacent nitrogen atoms and three carbon atoms, constitutes a privileged scaffold in medicinal chemistry due to its versatile biological [5]. When this core is modified with a keto group at the 4- or 5-position, the structure becomes pyrazolone, which has been historically recognized in analgesic and antipyretic compounds like antipyrine (phenazone) and metamizole (dipyrone).

Typically lasting up to three months, acute pain prompts healing and withdrawal responses. However, when pain persists beyond the expected recovery period—defined as chronic pain—it may no longer serve a protective purpose and instead becomes a primary health burden [2]. Chronic pain, often defined as pain that continues or recurs for more than three months, affects a significant proportion of individuals globally [6, 7].

One widely cited international systematic review reports that around 30% of people experience chronic pain, with estimates ranging from 10% to over 50%, depending on the region and methodology [8-10]. In the United States specifically, roughly 20% of adults—approximately 50 million individuals—report living with chronic pain, and about 8% suffer from "high-impact" chronic pain, meaning it [8]. The consequences of chronic pain are far-reaching. Individuals with persistent pain frequently report reduced mobility, diminished activities of daily living, and lower overall quality of life [9, 10].

They are also at greater risk for mental health issues, including depression and anxiety. From a societal perspective, chronic pain incurs a substantial economic toll. In the United States alone, annual costs associated with chronic pain—spanning direct medical expenses, lost productivity, and disability—are estimated between $560 billion and 635 billion, eclipsing expenditures on heart disease, cancer, or diabetes [11]. The recognition of chronic pain as a major public health issue has

been growing. In 2019, the CDC reported that well over 20% of U.S. Adults experience chronic pain, reinforcing the need for comprehensive pain assessment and management strategies [9, 12, 13]. Internationally, chronic pain is also a leading cause of disability and burden of disease, particularly musculoskeletal forms such as low back pain, which affects nearly 9-12% of the global population at any given time [6, 14, 15]. In sum, pain—particularly chronic pain—is a multidimensional, emotionally potent, and increasingly prevalent condition that creates widespread individual suffering and societal cost [19]. As such, a detailed exploration of its definitions, types, underlying mechanisms, and especially the role of neuroinflammation is both timely and essential in advancing our understanding and treatment of pain [16, 17].

Pain is mechanistically classified into three primary categories: nociceptive, neuropathic, and nociplastic pain. This classification is essential for guiding diagnosis, research, and treatment strategies by recognizing distinct underlying mechanisms [18, 19].

Nociceptive pain arises from the activation of specialized sensory neurons known as nociceptors, which are located throughout the skin, muscles, joints, and viscera [20, 21]. These receptors are stimulated by mechanical pressure, extreme temperatures, or chemical irritants in the context of tissue injury or inflammation [22, 23]. Nociceptive pain is typically protective, indicating potential or ongoing harm. It is subclassified into somatic pain, which is localized and associated with skin, bone, or muscle injury (e.g., post-surgical pain), and visceral pain, which is diffuse and originates from internal organs, often presenting as deep, dull, or cramping sensations [21, 24, 25]. This type of pain is transmitted through intact neural pathways and tends to resolve with healing, making it responsive to NSAIDs and opioids.

In contrast, neuropathic pain results from injury or disease affecting the somatosensory system. It can be peripheral (e.g., diabetic peripheral neuropathy) or central (e.g., post-stroke pain), and is often associated with maladaptive neuroplastic changes, such as ectopic nerve discharges and central sensitization [26, 27]. Patients typically report sensations such as burning, stabbing, or electric shock-like pain. These features are often accompanied by allodynia (pain from non-painful

stimuli) or hyperalgesia (increased pain response) [28, 29]. The management of neuropathic pain is particularly challenging, as conventional analgesics are often ineffective; instead, treatment may involve gabapentinoids, serotonin-norepinephrine reuptake inhibitors (SNRIs), or tricyclic antidepressants [30, 31].

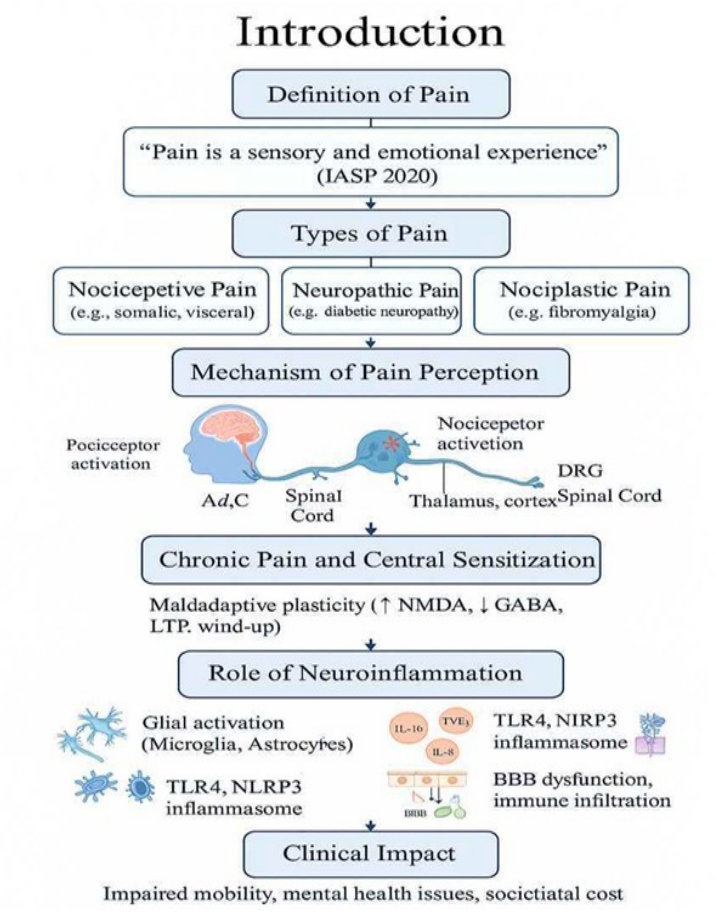


FIGURE 1.1: Classification and Mechanisms of Pain Perception

The categorization and neurological causes of pain are depicted in this conceptual flowchart. The International Association for the Study of Pain (IASP) definition is presented first in the figure, which then divides pain into three categories: nociceptive, neuropathic, and nociplastic. It also shows the journey from nociceptor activation to neuroinflammation and the ensuing clinical effects. Role of neuroinflammatory agents are described in the diagram.

Nociplastic pain is a recently defined third category introduced by the International Association for the Study of Pain (IASP) to describe pain arising from altered nociceptive function without evidence of actual tissue damage or nerve injury

[32, 33]. This type is commonly observed in conditions like fibromyalgia, irritable bowel syndrome, and certain chronic low back pain syndromes. Neuroimaging and experimental studies suggest that patients with nociplastic pain exhibit central amplification of pain signals, altered pain modulation, and widespread hyperalgesia [33, 34]. These features reflect dysfunction in descending inhibitory pathways and increased central sensitization, distinguishing nociplastic pain from both nociceptive and neuropathic types. Pharmacological approaches often include SNRIs or cognitive behavioral therapy, while NSAIDs are usually ineffective.

A complex neural network that processes and modifies information throughout the central and peripheral nerve systems controls how pain is experienced. The two main parts of this complex system are the descending pathways, which regulate pain signals, and the ascending pathways, which send pain signals to the brain. These channels combine to create a sophisticated feedback loop. They make sure that pain is not just a reflexive reaction but rather a highly controlled and flexible experience that affects both how we perceive it consciously and how our bodies react physiologically [35].

While these categories provide a useful framework, many clinical conditions involve overlapping mechanisms. For example, a patient with osteoarthritis may experience nociceptive pain from joint damage, neuropathic pain from nerve involvement, and nociplastic changes due to prolonged central sensitization. Recognizing such mixed pain phenotypes is essential for developing individualized and mechanism-targeted treatment approaches [35].

Chapter 2

Literature Review

The perception of pain is a complex neurophysiological process involving a dynamic interplay between the peripheral and central nervous systems [36]. It begins with the activation of nociceptors, which are free nerve endings located in peripheral tissues such as skin, muscles, joints, and viscera [37, 38].

These receptors are sensitive to mechanical, thermal, and chemical noxious stimuli and initiate transduction—the conversion of a harmful stimulus into an electrical signal [39, 40]. Two primary types of afferent fibers mediate this transmission: Aδ fibers, which are myelinated and convey sharp, well-localized pain, and C fibers, which are unmyelinated and carry dull, diffuse, and burning pain sensations [41, 42].

A noxious input is transported from the body's periphery to the brain for processing via the ascending pathways. A chain of three neurons, each with a unique function, can be used to understand this process. Transduction is the initial stage, during which a damaging stimulus—like pressure or heat—is transformed into an electrical signal. This happens at nociceptors, which are specialized sensory receptors found in the skin, muscles, joints, and organs. These nociceptors are first-order neurons' peripheral terminals. The dorsal root ganglion (DRG) contains the cell bodies of these neurons, which carry the electrical signal from the periphery to the spinal cord.

The signal enters the dorsal horn after it has passed through the spinal cord. Second-order neurons, the subsequent link in the transmission chain, synapse with the first-order neurons in this instance.

Because they instantly switch to the other side of the spinal cord, second-order neurons are essential. The spinothalamic tract, the primary pain transmission pathway, is formed as they ascend in the direction of the brain.

The electrical signals generated in nociceptors travel through the dorsal root ganglia (DRG) to synapse in the dorsal horn of the spinal cord, particularly in laminae I and II. Here, neurotransmitters such as glutamate, substance P, and calcitonin gene-related peptide (CGRP) are released to activate second-order neurons. These neurons then project through ascending pathways—primarily the spinothalamic tract—to supraspinal structures including the thalamus, somatosensory cortex, insula, amygdala, and anterior cingulate cortex, where the sensory-discriminative and emotional aspects of pain are processed, The limbic system is also served by other ascending routes, such as the cingulate cortex and amygdala. Because of this, pain is more than simply a physical experience; it's also a strong emotional and mental one that frequently comes with dread and fear. [43][48].

The body's endogenous pain-control system is made up of the descending pathways. This system functions as an inherent analgesic by enabling the brain to exercise top-down control over pain signals at the spinal cord level. Certain areas of the brainstem are the source of these potent modulatory circuits. The rostral ventromedial medulla (RVM) and the midbrain's periaqueductal Gray (PAG) serves as the main hubs for this descending regulation. To reach the dorsal horn, neurons from the PAG and RVM extend down the spinal cord. To affect the activity of the ascending pain neurons, they release a range of neurotransmitters at these synapses [44].

These neurotransmitters include norepinephrine, serotonin, and endogenous opioids (such as endorphins). Their release can prevent the first-order neurons from sending the pain signal to the second-order neurons. Before the pain signal even reaches the brain, this inhibitory action successfully weakens it. It explains why

a person may not experience severe harm in a "fight or flight" scenario. On the other hand, under some conditions, the descending pathways may also promote or facilitate pain signals. This effect is frequently observed when the modulation system malfunctions due to chronic pain situations. [45]

In cases of chronic pain, maladaptive changes occur within the central nervous system—a phenomenon known as central sensitization [46]. This state is characterized by an increased responsiveness of nociceptive neurons in the CNS to normal or subthreshold input and contributes to clinical features such as hyperalgesia and allodynia. Central sensitization involves mechanisms such as long-term potentiation (LTP) in spinal nociceptive circuits, increased NMDA receptor activity, and reduced inhibitory neurotransmission [47, 48]. Additionally, glial cells, including microglia and astrocytes, become activated and release pro-inflammatory cytokines like IL-1β, TNF-α, and IL-6, further amplifying neuronal excitability and sustaining pain [49, 50].

Neuroinflammatory responses also contribute to altered pain processing by modulating synaptic plasticity and promoting the release of secondary messengers, such as nitric oxide and prostaglandins, which facilitate central hyperexcitability [51]. Dysregulation of descending pain modulatory pathways, especially those involving the periaqueductal gray (PAG) and rostral ventromedial medulla (RVM), further compromises endogenous analgesic mechanisms and perpetuates chronic pain states [52, 53].

A detailed diagram showing the descending and ascending pain pathways. When a nociceptor is activated by an unpleasant stimulus, the ascending route (shown on the left) starts. This signal passes via synapses in the spinal cord's dorsal horn, the dorsal root ganglion (DRG), and a first-order neuron. The sensory and emotional aspects of pain are processed by a second-order neuron that crosses to the other side and ascends via the spinothalamic tract to the thalamus and other brain regions, such as the amygdala and somatosensory cortex. The body's mechanism for regulating pain is the descending route, which is depicted on the right. It comes from the brainstem's rostral ventromedial medulla (RVM) and periaqueductal gray (PAG). In order to block the pain signal before it reaches the brain,

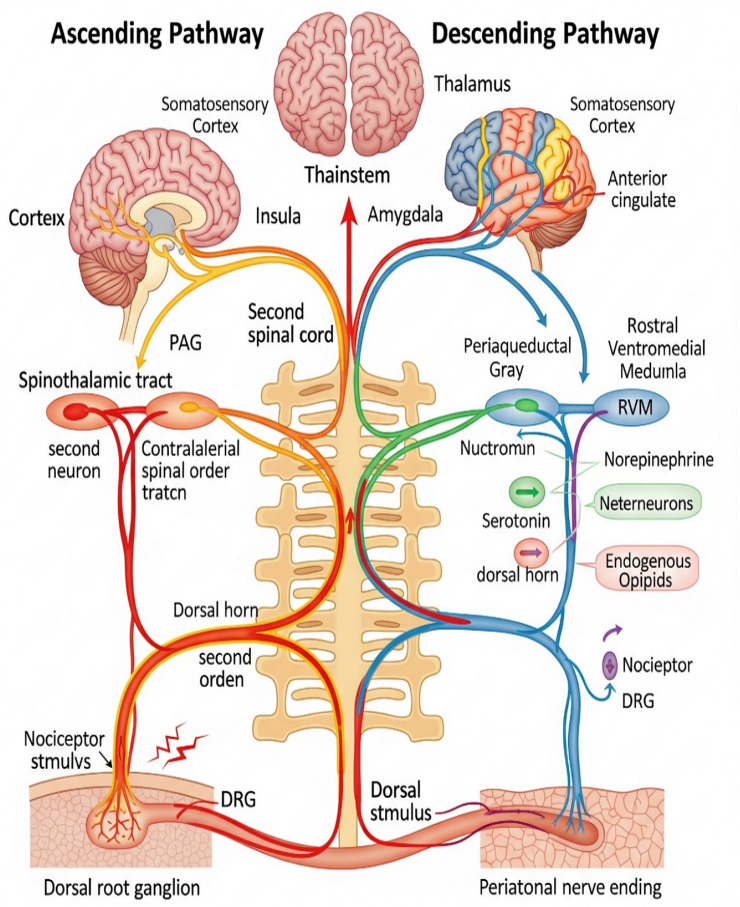


FIGURE 2.1: A Comprehensive Schematic Illustrating Both the Ascending and Descending Pain Pathways.

these neurons descend to the dorsal horn, where they release neurotransmitters like serotonin, norepinephrine, and endogenous opioids. The body can control its own pain response thanks to this top-down control.

Neuroinflammation refers to the immune-mediated response within the central nervous system (CNS), primarily involving glial cell activation and the release of pro-inflammatory mediators [54, 55]. Though traditionally considered a hallmark of neurodegenerative disorders such as Alzheimer's and Parkinson's diseases, a growing body of evidence implicates neuroinflammation as a key contributor to the pathogenesis and maintenance of chronic pain syndromes [56, 57]. This recognition marks a shift from a purely neuron-centric view of pain to one that integrates glial and immune mechanisms in both peripheral and central sensitization.

The CNS houses resident immune cells, chiefly microglia and astrocytes, which are essential for maintaining neural homeostasis [58, 59]. In response to injury,

infection, or sustained noxious stimulation, these glial cells become activated, resulting in morphological and functional changes that influence surrounding neurons [52, 60].

Upon activation, microglia transition from a surveillant to a reactive phenotype, releasing a variety of inflammatory mediators such as interleukin-1β (IL-1β), tumor necrosis factor-alpha (TNF-α), and interleukin-6 (IL-6) [67-69]. These cytokines sensitize dorsal horn neurons by modulating ion channels, increasing glutamatergic transmission, and inhibiting GABAergic tone, thus enhancing nociceptive signal propagation [51, 61].

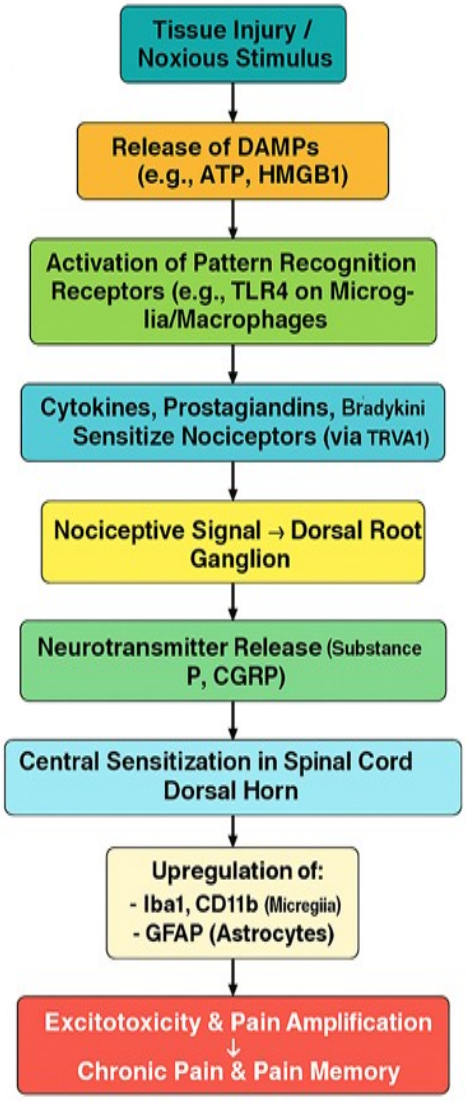


FIGURE 2.2: Conceptual Framework of Pain Perception and Chronic Pain Development

A comprehensive model of pain is shown in that image, including its clinical implications and the IASP 2020 definition. The flow chart shows the sequential process of recognizing pain and illustrates the three types of pain: nociceptive, neuropathic, and nociplastic.

Citing important molecular actors including NMDA and GABA receptors, as well as the TLR4 and NLRP3 inflammasomes, it further highlights the crucial roles that neuroinflammation and maladaptive plasticity (central sensitization) play in the development of acute to chronic pain. The final stage emphasizes the societal burden and clinical consequences.

Astrocytes, which outnumber neurons in several brain regions, also undergo activation during persistent pain states. They contribute to neuroinflammation by releasing chemokines, prostaglandins, and matrix metalloproteinases (MMPs) that amplify neuronal excitability and further recruit immune cells [62, 63]. Moreover, astrocytes participate in the maintenance of the tripartite synapse, and their dysregulation can impair synaptic clearance of glutamate, leading to excitotoxicity—a key feature of chronic pain conditions such as neuropathic pain and fibromyalgia [64, 65].

One of the pivotal pathways in glia-mediated neuroinflammation is the Toll-like receptor 4 (TLR4) signaling cascade. Expressed predominantly on microglia, TLR4 is activated by both pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs) released during tissue injury [66].

Activation of TLR4 leads to nuclear factor-kappa B (NF-κB) translocation and subsequent transcription of inflammatory genes [67, 68]. This promotes a sustained inflammatory milieu within the spinal cord dorsal horn, contributing to central sensitization, where nociceptive neurons exhibit heightened excitability and a lower threshold for activation.

Another significant player in the neuroinflammatory response is the NOD-like receptor protein 3 (NLRP3) inflammasome, a cytosolic multiprotein complex activated by cellular stress or mitochondrial dysfunction [69, 70]. NLRP3 facilitates the cleavage of pro-caspase-1 to active caspase-1, which in turn cleaves pro-IL-1β

and pro-IL-18 into their active forms [80]. The release of IL-1β has been strongly correlated with the persistence of mechanical allodynia and thermal hyperalgesia in rodent models of neuropathic pain [71, 72]. Blocking NLRP3 activation has shown promising analgesic effects, further underscoring its importance in pain regulation.

The role of peripheral immune cell infiltration into the CNS is also gaining attention. After nerve injury, the blood-brain barrier (BBB) and blood-spinal cord barrier (BSCB) become compromised, allowing monocytes, T cells, and macrophages to infiltrate the CNS [73, 74].

These cells release additional cytokines and interact with glia, exacerbating neuroinflammatory cascades. The presence of CD4+ T lymphocytes in the spinal cord has been shown to influence pain thresholds and the development of long-lasting pain [75, 76]. Furthermore, meningeal immune cells have been implicated in migraine pathophysiology, further linking CNS immune responses to pain disorders.

Gender differences in neuroimmune responses have also been reported. For instance, microglial activation is a primary driver of mechanical allodynia in male rodents, whereas female rodents show pain sensitivity via T cells and adaptive immune mechanisms [77, 78]. These findings highlight the complexity of neuroinflammatory pathways and the necessity for gender-specific pain therapeutics.

Beyond pain amplification, neuroinflammation also affects emotional and cognitive domains, thereby contributing to the multidimensional nature of chronic pain [79].

Prolonged activation of glia and release of inflammatory mediators can alter synaptic plasticity in the prefrontal cortex, amygdala, and hippocampus, areas responsible for affective and memory-related processing of pain [80]. This provides a mechanistic basis for the high comorbidity of chronic pain with depression and anxiety, further complicating treatment strategies.

Emerging evidence also supports the role of oxidative stress in neuroinflammation-related pain. Reactive oxygen species (ROS), primarily generated by activated microglia and dysfunctional mitochondria, enhance pro-inflammatory signaling and

sensitize nociceptive pathways [81]. Antioxidants targeting ROS and mitochondrial dysfunction, such as N-acetylcysteine (NAC) and coenzyme Q10, have shown efficacy in preclinical pain models [82, 83].

The interplay between neuroinflammation and pain is not unidirectional. Chronic nociceptive activity can, in turn, sustain or even exacerbate glial activation, creating a self-perpetuating cycle of pain and inflammation [84, 85]. Therefore, therapeutic strategies that target both neuronal and glial elements hold promise for effective and sustained pain relief.

Currently, several anti-inflammatory and immunomodulatory approaches are being explored to manage neuroinflammation-driven pain. These include glial inhibitors (e.g., minocycline), cytokine antagonists (e.g., IL-1 receptor antagonists), TLR4 antagonists (e.g., TAK-242), and inflammasome inhibitors. Although many of these strategies are still in preclinical or early clinical stages, they represent a promising shift toward mechanism-based pain therapy.

The intricate interplay between the immune system and nervous system during neuroinflammation involves numerous cellular and molecular mediators [86, 87]. Among these, pro-inflammatory cytokines, glial activation markers, and transcriptional regulators play pivotal roles in initiating and maintaining chronic pain states [88, 89]. Understanding these markers not only elucidates the pathophysiology of neuroinflammation-driven pain but also unveils potential targets for therapeutic intervention.

Cytokines are essential modulators of immune signaling and are rapidly upregulated following nerve injury, tissue inflammation, or persistent nociceptive input. The most well-characterized among these in the context of pain are tumor necrosis factor-alpha (TNF-α), interleukin-1 beta (IL-1β), and interleukin-6 (IL-6) [90].

TNF-α is an early response cytokine released by activated microglia, astrocytes, macrophages, and even neurons. It sensitizes nociceptors by upregulating sodium and calcium channels and enhances synaptic transmission by increasing presynaptic glutamate release and postsynaptic receptor sensitivity [91, 92]. TNF-α also stimulates the release of other pro-inflammatory mediators, including IL-1β

and prostaglandins, through the activation of the nuclear factor kappa B (NF-κB) pathway [93, 94].

IL-1β is another potent pro-inflammatory cytokine implicated in both peripheral and central sensitization. In animal models, intrathecal administration of IL-1β induces hyperalgesia, whereas blockade of its receptor alleviates pain behaviors [89, 95]. IL-1β increases the excitability of dorsal horn neurons by modulating glutamate receptor phosphorylation and enhancing the release of neuropeptides like substance P and CGRP.

IL-6, though often associated with systemic inflammation, is also produced within the CNS during neuroinflammatory states. IL-6 facilitates pain via Janus kinase/signal transducer and activator of transcription (JAK/STAT) signaling, leading to increased neuronal excitability and inhibition of GABAergic inhibition [96]. Elevated IL-6 levels have been observed in the cerebrospinal fluid of patients with neuropathic pain and fibromyalgia [97].

Microglia are the principal immune cells of the CNS and rapidly respond to damage or pathological stimuli. Upon activation, microglia undergo morphological transformation from a ramified to an amoeboid form and upregulate surface and intracellular proteins indicative of their reactive state.

Ionized calcium-binding adapter molecule 1 (Iba1) is one of the most reliable markers for microglial activation [98, 99]. Iba1 is involved in membrane ruffling and phagocytosis and is significantly upregulated following nerve injury or inflammatory insult [100, 101]. Immunohistochemical staining of Iba1 has become a standard method to evaluate microglial proliferation and activation in pain models [102].

Another key marker is CD11b, a component of the complement receptor 3 (CR3) complex. CD11b expression increases dramatically in activated microglia and contributes to microglia-neuron interactions through binding with ICAM-1, thus facilitating immune cell adhesion and migration [103, 104]. Elevated CD11b expression has been correlated with mechanical allodynia and thermal hyperalgesia in rodent models of neuropathic and inflammatory pain [105].

The activation of glial cells, which are the nervous system's non-neuronal cells, has a significant impact on central sensitization, which is not just a neuronal process. Excitotoxicity and pain amplification are the results of this state of central sensitization and chronic neuroinflammation. Pain amplification is the increased experience of pain, whereas excitotoxicity is the overstimulation of neurons to the point of destruction.

The shift from an acute injury-related pain response to a chronic state of pain and pain memory is cemented by these processes, in which the neural system has undergone fundamental changes to maintain the pain signal long after the initial damage has healed. Astrocytes, the most abundant glial cell type in the CNS, also play a critical role in neuroinflammation and pain modulation. Upon stimulation by pro-inflammatory cytokines or neurotransmitters, astrocytes shift to a reactive phenotype and begin to release chemokines (e.g., CCL2), ATP, and glutamate [106].

The most widely used marker for astrocyte activation is glial fibrillary acidic protein (GFAP), an intermediate filament protein whose expression is markedly increased under conditions of CNS stress, injury, or inflammation [107]. Elevated GFAP levels have been found in the spinal cords of rodents with chronic constriction injury (CCI), correlating with pain behaviors such as tactile allodynia [108, 109] .

Reactive astrocytes also disrupt normal neuronal-glial homeostasis by impairing glutamate uptake through downregulation of glutamate transporter-1 (GLT-1) and GLAST, contributing to excitotoxicity and central sensitization [110, 111]. This astrocyte-driven dysregulation sustains pain even in the absence of ongoing peripheral input.

At the molecular level, several intracellular signaling cascades mediate inflammatory gene expression and glial reactivity. Among these, the nuclear factor kappa B (NF-κB) and mitogen-activated protein kinases (MAPKs) pathways are prominent [112, 113]. NF-κB is a transcription factor activated in both microglia and astrocytes under inflammatory conditions. In its inactive form, it is sequestered

in the cytoplasm by IκB proteins. Upon stimulation by TNF-α or IL-1β, IκB is phosphorylated and degraded, allowing NF-κB to translocate into the nucleus and initiate transcription of genes encoding cytokines, chemokines, and adhesion molecule [114, 115]. Inhibiting NF-κB activation has been shown to attenuate both inflammatory and neuropathic pain behaviors in rodent models [116, 117].

MAPKs, including extracellular signal-regulated kinase (ERK), p38, and c-Jun N-terminal kinase (JNK), are activated in response to a variety of stressors, including cytokines, ATP, and ROS. These kinases modulate gene expression and post-translational modifications of receptors and ion channels involved in nociceptive signaling [118, 119].

For instance, p38 MAPK is predominantly activated in spinal microglia following nerve injury and contributes to the upregulation of pro-inflammatory mediators like TNF-α and IL-1β [92, 120].

In contrast, ERK activation in neurons has been linked to activity-dependent plasticity underlying central sensitization and chronic pain memory [121].

The intricate bidirectional communication between the nervous and immune systems is now recognized as a key driver in the initiation and persistence of chronic pain [122, 123]. This crosstalk, particularly through neuroinflammation, transforms the traditional understanding of pain from being a purely neuronal process to a complex neuroimmune disorder. At the heart of this phenomenon lies a cascade of events initiated by peripheral injury, immune activation, and disruption of the blood-brain barrier (BBB), which culminates in central sensitization and long-term pain hypersensitivity [124, 125]. The process begins at the site of injury or infection, where damage-associated molecular patterns (DAMPs) such as ATP, HMGB1, and HSPs are released from damaged cells. These molecules bind to pattern recognition receptors (PRRs) like Toll-like receptors (TLRs) on immune cells, initiating an inflammatory cascade [126]. This early innate immune activation leads to the secretion of pro-inflammatory cytokines such as TNF-α, IL-1β, and IL-6, which are critical in the sensitization of peripheral nociceptors and in the recruitment of circulating immune cells to the injury site [127, 128].

These cytokines not only contribute to peripheral sensitization but can signal to the CNS by crossing the blood-brain barrier or by activating endothelial cells and perivascular macrophages to secrete additional mediators [129, 130]. Chronic exposure to these cytokines results in maladaptive responses, ultimately triggering central neuroinflammation [131].

The BBB serves as a critical gatekeeper, maintaining CNS immune privilege. However, under pathological conditions like systemic inflammation or nerve injury, the barrier can become permeable. Cytokine-induced disruption of tight junction proteins such as claudins and occludins allows immune cells and circulating cytokines to infiltrate the CNS [132, 133]. This breach facilitates direct exposure of the brain parenchyma to peripheral inflammatory signals and contributes to the activation of resident glial cells.

The upregulation of matrix metalloproteinases (MMPs), particularly MMP-9, in response to TNF-α and IL-1β, has been shown to degrade BBB components and further exacerbate CNS infiltration [125]. Moreover, monocytes and T cells, once recruited into the CNS, can perpetuate the inflammatory environment by releasing interferon-gamma (IFN-) and other chemokines, thereby amplifying the nociceptive response [134, 135]. Once the inflammatory signal reaches the CNS, microglia—the resident immune cells of the brain and spinal cord—become rapidly activated.

Activated microglia adopt a reactive phenotype characterized by increased expression of CD11b and Iba1, and the release of cytokines such as IL-1β, TNF-α, and IL-18 [136]. This pro-inflammatory milieu alters synaptic plasticity in the dorsal horn of the spinal cord, facilitating central sensitization and hyperexcitability of second-order neurons [137, 138]. Importantly, the NOD-like receptor protein 3 (NLRP3) inflammasome within microglia plays a central role in amplifying this process. Activation of NLRP3 leads to the cleavage of pro-caspase-1 into its active form, which subsequently converts pro-IL-1β into mature IL-1β—a cytokine directly implicated in enhancing pain transmission [139, 140]. Inhibiting NLRP3 signaling in animal models has demonstrated reduced glial activation and alleviation of pain behaviors [141].

In parallel, astrocytes, the most abundant glial cells in the CNS, are also recruited to the inflammatory response. Upon activation, they upregulate glial fibrillary acidic protein (GFAP) and release chemokines (e.g., CCL2) and ATP, further activating microglia and sensitizing neurons [142]. Reactive astrocytes also reduce the expression of glutamate transporters like GLT-1, leading to glutamate accumulation and excitotoxicity, a key mechanism in central pain amplification [143, 144].

The release of pro-inflammatory cytokines within the CNS profoundly alters synaptic transmission. TNF-α enhances AMPA receptor trafficking to the neuronal membrane, increasing excitatory post-synaptic potentials, while IL-1β promotes NMDA receptor phosphorylation, reducing the threshold for pain signal transmission [145, 146]. Simultaneously, inhibitory interneurons become dysfunctional due to cytokine-mediated downregulation of GABA and glycine receptor activity [51]. The result is a state of disinhibition, in which the spinal cord becomes hyperresponsive to peripheral input.

This heightened state of responsiveness, known as central sensitization, is the neurophysiological basis of chronic pain conditions such as fibromyalgia, complex regional pain syndrome, and neuropathic pain [45, 147]. The involvement of immune mediators in this process signifies a paradigm shift from neuron-only theories to integrated neuroimmune mechanisms of pain chronification.

The interplay between immune cells and neurons is not a transient phenomenon but is sustained by a positive feedback loop. For example, IL-1β released by glia stimulates further glial activation, while glutamate from overactive neurons exacerbates astrocyte and microglial reactivity [148, 149].

This cytokine-glutamate cycle locks the CNS in a pro-nociceptive state, reinforcing pain perception even in the absence of ongoing tissue damage. Moreover, persistent activation of signaling pathways such as NF-κB and MAPKs (e.g., ERK, JNK, and p38) leads to the transcription of genes encoding inflammatory mediators and pain-associated proteins [150]. These pathways also contribute to the formation of "pain memory" within the CNS, wherein repeated nociceptive input results

in long-lasting changes in gene expression and synaptic structure, mirroring the molecular underpinnings of learning and memory [151, 152].

A critical consequence of sustained neuroinflammation is the inhibition of endogenous pain modulatory systems. Normally, descending inhibitory pathways from the periaqueductal gray (PAG) and rostral ventromedial medulla (RVM) utilize serotonin (5-HT) and norepinephrine (NE) to suppress spinal nociceptive transmission [53]. However, neuroinflammatory cytokines interfere with these systems by reducing receptor sensitivity and neurotransmitter availability, thereby weakening natural analgesia.

In fact, IL-6 has been shown to disrupt serotonergic transmission in the spinal cord, while TNF-α decreases opioid receptor expression, reducing the effectiveness of both endogenous and exogenous opioids [153]. This not only contributes to pain persistence but also to opioid tolerance, complicating pharmacological management.

NSAIDs—including ibuprofen, naproxen, and diclofenac—alleviate pain by inhibiting cyclooxygenase enzymes COX-1 and COX-2, thereby reducing the production of pro-inflammatory prostaglandins [154, 155]. They are effective for mild to moderate nociceptive pain linked to peripheral inflammation. However, in neuroinflammatory or neuropathic pain conditions, their benefit is limited. Moreover, long-term NSAID use can result in gastrointestinal ulceration, renal impairment, and heightened cardiovascular risk [156, 157].

Morphine, oxycodone, and other opioids are powerful analgesics that act primarily via μ-opioid receptors to inhibit nociceptive signaling [158]. Although effective in the short term, opioids pose serious concerns: tolerance, physical dependence, immune suppression, and opioid-induced hyperalgesia driven by neuroinflammatory responses [159]. Their long-term utility in chronic pain is thus limited.

Drugs like gabapentin and pregabalin modulate the $\alpha\delta$ subunit of voltage-dependent calcium channels, reducing excitatory neurotransmitter release. They are considered first-line agents for neuropathic pain [160, 161]. While they can indirectly reduce neuroinflammatory signaling by dampening neuronal activity, they lack

specificity for glial mechanisms and are often limited by side effects such as sedation, dizziness, and edema.

SNRIs (e.g., duloxetine) and tricyclic antidepressants (e.g., amitriptyline) enhance descending inhibitory pathways by increasing synaptic serotonin and norepinephrine concentrations [162, 163]. They are approved for certain neuropathic and nociplastic pain syndromes. However, they provide limited neuroimmune modulation directly and are associated with side effects like dry mouth, orthostatic hypotension, and cardiac conduction issues.

Minocycline, a tetracycline antibiotic capable of crossing the blood-brain barrier, inhibits microglial activation through multiple mechanisms—including suppression of NF-κB signaling, inhibition of iNOS and COX-2, and reduction of oxidative stress [164, 165]. In animal models, it attenuates pain behaviors, cytokine release, and glial activation. Human studies, however, present mixed outcomes [89]. A systematic review reported consistent benefits of minocycline in diabetic and leprotic neuropathy, as well as chemotherapy-induced neuropathy, while results were null for radicular and postoperative pain [166, 167]. Safety profiles are generally favorable, with adverse events limited to mild gastrointestinal symptoms and rare skin discoloration; serious immune-mediated reactions are uncommon [168]. Ongoing trials, such as randomized controlled studies in chronic low back pain, continue to investigate minocycline's efficacy and mechanism in humans.

Targeted biologics—including anti-TNF agents (infliximab, etanercept) and anti-IL-6 receptor antibodies—have shown efficacy in reducing neuroinflammation [183]. In preclinical models, IL-6 blockade alleviated pain behaviors post-spinal cord injury. Similarly, TNF inhibitors have provided relief in radiculopathy cases, though data remain preliminary [169].

Limitations include restricted CNS penetration, high cost, and potential systemic immunosuppression leading to infections. Thus, while biologics offer targeted mechanisms, their practical use in chronic pain is still limited [170].

SCS delivers electrical impulses to the dorsal columns of the spinal cord to modulate pain signaling. It is effective in complex regional pain syndrome and failed

back surgery syndrome [186]. Evidence suggests SCS may reduce neuroinflammatory markers in the spinal cord, although precise glial modulation is not fully understood.

TMS applies magnetic pulses to cortical areas—often including the motor cortex—to enhance descending inhibitory pathways and opioid release [171, 172]. Studies in fibromyalgia and neuropathic pain suggest TMS may also lower peripheral neuroinflammatory markers, though larger trials are needed to confirm these findings.

Interest is growing in phytochemicals like curcumin and resveratrol for their anti-inflammatory and neuroprotective effects.

Curcumin, from turmeric, inhibits NF-κB, MAPKs, and NLRP3 inflammasome activation. It reduces spinal cytokine levels and glial activation in preclinical diabetic neuropathy models [173]. However, its therapeutic use is hampered by poor oral bioavailability. Nano formulations and adjunct agents like peperino can improve CNS delivery and efficacy.

Resveratrol, a polyphenol found in grapes and berries, modulates JAK/STAT signaling, suppresses TNF-α, IL-1β, and IL-6, and strengthens BBB integrity in preclinical models [174, 175]. Though studied mostly in oncology, emerging pain models—such as spinal cord injury—show reduced allodynia and neuroinflammation following resveratrol therapy [176].

Future strategies should emphasize precision medicine, combining mechanistic diagnosis with individualized treatment [177]. For example, patient-selected regimens combining CNS-penetrant glial inhibitors with neuromodulation and optimized phytochemical formulations may yield synergistic benefits. Well-designed clinical trials and advances in drug delivery—such as nanoparticles and BBB-targeted carriers—are essential.

Pyrazole, a five-membered heterocycle featuring two adjacent nitrogen atoms and three carbon atoms, constitutes a privileged scaffold in medicinal chemistry due to its versatile biological [5]. When this core is modified with a keto group at the 4-

or 5-position, the structure becomes pyrazolone, which has been historically recognized in analgesic and antipyretic compounds like antipyrine (phenazone) and metamizole (dipyrone). Pyrazolone derivative have attracted research attention for their anti-inflammatory and analgesic potential [178]. Preclinical studies report that novel pyrazolone–pyridazine conjugates significantly reduced carrageenan-induced paw inflammation and formalin-induced hyperalgesia in rodents, while also inhibiting COX-1/2 with favorable safety profiles compared to indomethacin [179]. Similarly, systematic synthetic efforts have yielded pyrazolone analogues with potent dual inhibitory activity on COX-2 and 5-LOX—key inflammatory enzymes—with effective reduction of PGE in vivo and insignificant gastrointestinal toxicity [180, 181].

A broader review of pyrazolone derivative revealed several analogs demonstrating strong anti-inflammatory properties via COX-1/2 and 5-LOX inhibition, analgesic efficacy, and excellent quantitative structure–activity relationships (QSAR). Colony-forming single compounds exhibited superior potency in animal models compared to standard NSAIDs, alongside reduced ulcerogenic risk. These findings illustrate how structural modifications—such as chlorophenyl or benzene sulfonamide substituents—optimize both efficacy and safety. modern pyrazalone analgesics (e.g., dipyrone, propyphenazone, and antipyrine) have been shown to antagonize the TRPA1 channel on nociceptors. In rodent models, these pyrazolones block TRPA1-mediated calcium signaling and mechanical allodynia without affecting prostaglandin levels, suggesting a dual mechanism distinct from classic COX inhibition.

Numerous new pain receptor targets that go beyond the conventional opioid pathways have been discovered as a result of the search for efficient pain management. Ion channel receptors stand out among these due to their potential for transducing and conveying pain signals. For example, the voltage-gated sodium channel Nav1.7, which is nearly exclusively present on nociceptors, is a prime target since blocking it selectively may provide strong pain relief without the systemic adverse effects of other sodium channel inhibitors. Similar to this, researchers are looking into blocking particular pain sensations by targeting Transient Receptor Potential

(TRP) channels, such as TRPV1 and TRPA1, which function as sensory transducers for heat, cold, and inflammatory chemicals. Furthermore, the potential of G protein-coupled receptors (GPCRs) has been brought to light by a better knowledge of neuroinflammation. Antagonism against the P2X7 receptor on glial cells may break the neuroinflammatory feedback loop because it stimulates the release of inflammatory cytokines when triggered by ATP produced during injury.

Furthermore, the CB2 cannabinoid receptor, which is mostly found on immune cells, offers a way to produce analgesic and anti-inflammatory effects without the psychoactive side effects that the CB1 receptor mediates. When taken as a whole, these new objectives mark a dramatic change in thinking about creating extremely targeted, non-addictive pain treatments.

2.1 Aims and Objectives

To evaluate the analgesic and anti-inflammatory effects of selected pyrazolone derivative using the formalin and carrageenan-induced paw edema models.

This objective focuses on using well-established in vivo models to assess the therapeutic efficacy of pyrazolone compounds. The formalin test will help distinguish between neurogenic and inflammatory pain responses, while the carrageenan-induced paw edema model will assess the compound ability to suppress acute inflammation. Behavioral and physical parameters such as paw thickness, flinching, and licking will be recorded and compared with standard drugs.

To quantify the impact of pyrazolone derivative on key inflammatory mediators through biochemical assays.

In this phase, biochemical markers of inflammation—including tumor necrosis factor-alpha (TNF-α), interleukin-1 beta (IL-1β) will be quantified using enzyme-linked immunosorbent assay (ELISA). This will validate the anti-inflammatory potential of the tested compounds at the molecular level, confirming whether systemic or localized cytokine levels are downregulated after treatment.

To explore the effects of pyrazolone derivative on cellular inflammation via immunohistochemistry (IHC)

This objective aims to investigate cellular responses in tissues affected by inflammation. Spinal cord will be stained for markers such as Iba1 (microglial activation) and GFAP (astrocyte activation). Reduction in glial marker expression would indicate a dampening of the neuroinflammatory cascade, supporting the therapeutic potential of the compounds.

To conduct molecular docking studies to predict binding affinity and interaction profiles of pyrazolone derivative with inflammation-related targets

Using computational modeling, pyrazolone compounds will be docked with biological targets. These in silico results will support experimental findings by showing how structural features of the compounds may contribute to their biological effects.

To integrate behavioral, biochemical, and computational results to identify the efficacy of pyrazolone compounds.

Chapter 3

Methodology

3.1 Animals

The Association for Assessment and Accreditation of Laboratory Animal Care International's (AAALAC) recommendations were followed in all aspects of animal care and handling for the current investigations.

The Capital University of Science and Technology (CUST) Ethics Committee accepted the experimental protocols (Approval No. [REC/FoP/F2024/17], fully adhering to national and international guidelines for the moral use of lab animals. Five groups (n=6) of male Balb/c mice, weighing 25g–30g and obtained from the CUST animal house, were randomly assigned.

One group was given normal saline as a control, another was given inflammation (carrageenan) to act as a negative control, and a third was given a standard medication following inflammation induction to act as a positive control. After the induction of inflammation, the other two groups were given different therapies.

The mice had unrestricted access to food and water and were kept in cages with six mice each, with a 12-hour light/dark cycle (lights on from 8:00 to 20:00). All animals were handled with extreme care. Special care was made for temperature and humidity, as these conditions directly effect the health of animal

FIGURE 3.1: BALB/c Mice Animal Handling

These mice were selected for animal study. All ethical consideration were carried out as required. (Photograph was captured at Fop, Capital University of Science & Technology, Islamabad). animal requires some time to adjust with the new environment. The animals are provided care so that they do not become aggressive.

3.2 Food Composition

In order to suit their dietary needs, mice involved in studies were usually provided a balanced pellet diet. Frequently used ingredients included bran, flour, fish, and powdered milk. Reliable experimental results and the health of the mice were guaranteed by consistent and standardized diets.

TABLE 3.1: Composition of Animal's Food

Food item	**2.5 Kg**	**5.0 Kg**	**10.0 Kg**	**20.0 Kg**
Wheat Flour	1.615 Kg	3.25 Kg	6.5 Kg	13 Kg
Chokar	375 g	750 g	1. 5 Kg	3.0 Kg
Fish	375 g	750 g	1. 5 Kg	3.0 Kg
Dry Skimmed Milk	100 g	200 g	400 g	800 g
Vitamins/Minerals*	37.5 g	75 g	100 g	200 g

3.3 Mice Handling

The Capital University of Science and Technology (CUST), Islamabad's Ethics Committee authorized all experimental methods involving mice, which were carried out strictly in compliance with globally recognized ethical standards for the care and use of laboratory animals. For efficient and trustworthy results, mice were acclimated for seven days before the trial began. All mice were given seven days to get used to the conditions of the animal facility before the experiment started. Animals were handled gently to reduce fear and acclimate them to human interaction during this time, and they were observed every day for indications of stress, disease, or unusual behavior.

Mice were recognized by identifying their cages and using non-invasive marking methods including tail marking. To lessen bias, animals were divided into experimental and control groups at random using a straightforward randomization technique. Before beginning handling, gloves were put on and aseptic procedures were followed. Mice were placed on the wire cage lid and held at the base of the tail for gentle but firm restraint. Keeping the tail under control, the thumb and forefinger of the other hand were placed on the lower back. The slack skin at the nape of the neck was carefully pinched and lifted to provide a firm but humane hold, and the little finger was used to move the tail toward the wrist and fasten it. Stable handling was made possible by this technique, which also ensured proper ventilation and prevented escape while reducing stress.

Animals were observed every day for clinical indications of pain, suffering, or disease during the trial period. Important metrics were noted, including body weight, food and drink consumption, grooming habits, and overall activity level. Following institutional protocols, any animal displaying symptoms of extreme discomfort or bad health was mercifully put down via cervical dislocation.

The above figure depicts the careful handling of a laboratory mouse with sterile latex gloves. Before the trial began, the mice were given seven days to become used to the new surroundings. Accurate experimental results depend on the animals' ability to adjust to housing settings during this acclimatization period, which

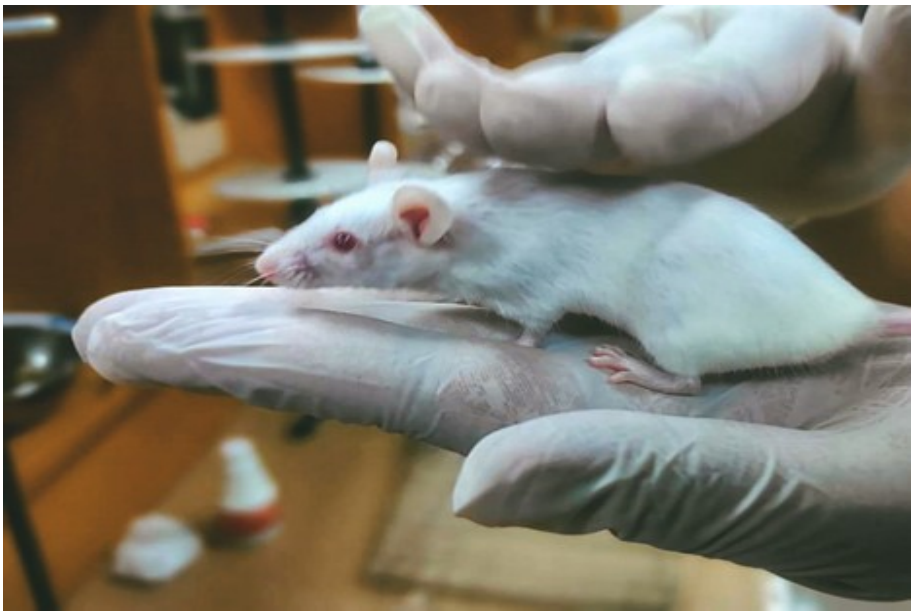


FIGURE 3.2: Handling of Animal (Mice)

reduces stress and stabilizes physiological parameters. Mice must be handled carefully and frequently after acclimation, employing proper methods such as cupping with gloved hands. By reducing stress and fear responses, this method improves the animal's ability to adjust to human interaction.

The quality and reproducibility of behavioural and drug research results are enhanced by routine and compassionate handling, which also preserves animal welfare (picture taken at the Faculty of Pharmacy, Capital University of Science and Technology, Islamabad).

3.4 Mice Bedding

Mice were housed on bedding materials that provide comfort, absorb moisture, and reduce odor. Common bedding types include wood shavings (such as aspen) was used. Animal bedding was made with consideration of its potential impact on animal health, behavior, and the reliability of experimental outcomes. Bedding was changed on daily basis to provide them non-toxic, dust and stress-free environment for the mice.

FIGURE 3.3: Standard Housing Condition for Laboratory Animals

Corn cob bedding, a popular substrate in experimental research because of its superior moisture absorption, efficient odour control, and capacity to provide a hygienic, comfortable environment that is crucial for preserving animal health and well-being, was used to line the cages in which the mice were kept. To maintain cleanliness, reduce ammonia accumulation, and avoid microbiological contamination, bedding was changed every day. In addition to lowering stress levels and encouraging natural nesting and exploratory activities, this arrangement also improved the consistency and dependability of behavioural and physiological results. Additionally, regular cage care and proper bedding reduced respiratory discomfort from dust, prevented skin lesions, and fostered a stable, healthy study environment. (Photo courtesy of Capital University of Science and Technology, Islamabad, Faculty of Pharmacy).

3.5 Animal Gender

The distance between the anal and genital orifices is used as a measure to differentiate between male and female, mouse and rat. This distance is greater in case of

male while female has small distance between anal and genital orifices compared to male.

3.6 Randomization

Animals were randomly assigned before the trial started in order to guarantee fair distribution and eliminate data skew among test groups. Animals were permanently marked on the tail using non-toxic ink to facilitate identification during the trial. To maintain the patterns' clarity, the marking procedure involved applying circular bands in varying widths and numbers with little to no space between them. Particularly, Animal 1 was tagged with one small circular band around the tail, whereas Animal 2 was tagged with two small circular bands spaced evenly apart. There were three little circular rings on Animal 3 and four on Animal 4. One thick circular band served as a distinguishing feature for animal 5. Lastly, Animal 6 has one broad circular ring and one thin one, separated by a tiny space. Throughout the trial, this identifying technique made it possible to precisely gather data on each animal and conduct routine monitoring.

3.7 Euthanizing Methods

3.7.1 Dislocation

The AAALAC Panel on Euthanasia authorized cervical dislocation as a method of ending a mouse's life in 1972. The AAALAC limits the treatment's use to tiny rodents (less than 200g) handled by experienced persons and requires that the procedure put an animal comatose in 15 seconds with no discomfort or distress. Since then, this treatment has been given using a variety of techniques, each with a unique set of steps to guarantee a quick and painless demise. In the hemostat-assisted cervical dislocation technique, a big hemostat is placed precisely beneath the base of the skull, and the operator pulls the mouse's tail sharply backward

to dislocate the cervical spine. The cervical spine can be manually dislocated using a similar concept, but without the need of equipment. The operator applies pressure with their thumb and forefinger at the base of the head, and then pulls sharply on the tail to achieve dislocation. To dislocate the skull from the vertebrae without causing skin damage, anterograde cervical dislocation involves holding the tail firmly in one hand while using the other to drive the top cervical spine forward and downward, occasionally twisting. In a second form known as thoracic dislocation, the thorax is gripped at the caudal end of the rib cage with the thumb and forefinger to secure the chest. The thoracic vertebrae are then

FIGURE 3.4: Cervical Dislocation Method

forced apart by a quick pull on the tail. Dislocation physiologically breaks the connections between the brainstem and the spinal cord, deactivating the reticular activating system (which is in charge of consciousness) and stopping pain perception in less than 0.3 seconds. Diaphragmatic paralysis results in respiratory arrest and loss of vasomotor tone, which causes circulatory collapse and death. An additional technique for confirming death After the operation, bilateral pneumothorax is required. Validation studies verify sustained AAALAC recommendations, the absence of pain-associated behaviors (vocalization, limbic activity), and isoelectric

EEG readings within 0.5–2 seconds. In keeping with ethical animal care, all of these methods are meant to induce rapid unconsciousness and death with the least amount of pain and suffering.

Laboratory mice are humanely put to death by cervical dislocation with a wooden spatula. In order to separate the cervical vertebrae, the mouse is gently restrained and the wooden spatula is pressed firmly on top of the base of the skull while the tail is rapidly pulled.

This method provides for a speedy and least painful treatment while causing instant death and loss of consciousness (picture taken at the Faculty of Pharmacy, Capital University of Science and Technology, Islamabad).

3.7.2 Concussion

Concussion is a method of euthanasia used on small lab animals. It involves a forceful blow to the skull, intended to cause immediate unconsciousness and brain hemorrhage. The sudden acceleration and deceleration of the head causes the brain to hit the inside of the skull, leading to a rapid change in pressure and damage to neural tissue. This disrupts the brain's ability to control consciousness, breathing, and heart function. The goal is to induce instant death, or at least a state of deep unconsciousness, by mechanically disrupting the brain's vital functions.

This technique is not simple; it requires great skill and precision. The person performing it must have a detailed understanding of the animal's anatomy to hit the correct spot with sufficient force. A strike that is too weak or poorly aimed can fail to cause unconsciousness, leading to unnecessary pain and distress for the animal, which is a serious ethical violation.

Concussion alone is not a humane method of euthanasia. Leading animal welfare guidelines, such as those from AAALAC, consider it only a stunning technique because its effects can be temporary. To ensure an animal's death is humane and irreversible, a secondary method, such as cervical dislocation or decapitation, must immediately follow the concussion.

In scientific contexts, concussion is typically only used when chemical euthanasia would interfere with the study's results (e.g., in certain biochemical analyses). Even then, its use must be ethically justified and performed only by highly trained and qualified personnel.

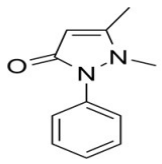
The procedure must always be part of a two-step process to ensure the animal does not regain consciousness and that death is instantaneous and humane.

3.8 Chemicals and Drugs

TABLE 3.2: List of Chemicals and Drugs Used in the Study Along With Their Sources and Suppliers

Sr.	**Chemical/Substance**	**Source**
1	Carrageenan	Sigma-Aldrich
2	Diclofenac Sodium	Shaigan Pharmaceuticals Pvt LTD
3	Formaldehyde	Shaigan Pharmaceuticals Pvt LTD
4	Pyrazolone	Synthesized in lab / precursor from Sigma-Aldrich

TABLE 3.3: Physicochemical properties of PYR-1

Code	**Structure**	**Name**	**Colour**	**Molecular Weight**	**Log $P_{o/w}$**
PYR-1		1,2-Dihydro-1,5-dimethyl-2-phenyl-3H-pyrazol-3-one	White to slightly yellowish crystals or powder	188.23 g/-mol	0.81

3.9 Acclimatization

Mice were acclimatized to the laboratory environment for a period of seven days before the initiation of the experimental procedures. This acclimatization period allowed the animals to adjust to their new surroundings, minimizing stress and ensuring physiological and behavioral stability, which is essential for obtaining reliable and reproducible experimental results.

3.9.1 Grouping of Animals

Experimental animals were randomly assigned into five groups (n = 6 per group) based on their body weights to ensure uniform distribution. The treatment groups were organized as follows:

Group I (saline control group): Vehicle only.

Group II (negative control): carrageenan at a dose 25 μL of a 1% carrageenan solution

Group III (positive control): carrageenan at dose 25 μL of a 1% carrageenan solution + diclofenac sodium at a dose of 5 mg/Kg.

Group IV (test group I): carrageenan at a dose 25 μL of a 1% carrageenan solution + diclofenac sodium at a dose of 5 mg/Kg.

Group V (test group II): carrageenan at a dose 25 μL of a 1% carrageenan solution + diclofenac sodium at a dose of 10 mg/Kg.

3.9.2 Drug Administration

Before drug administration, each mouse was individually weighed to ensure accurate dose calculations for Carrageenan and pyrazolone. Fresh solutions of all drugs were prepared regularly to maintain stability and efficacy.

The mice were randomly divided into five groups as follows:

Group 1 (control): Received an equivalent volume of vehicle (i.p.) and served as the control group.

Group 2 (carrageenan-only): carrageenan at a dose 25 μL of a 1% carrageenan solution into subplantar region

Group 3 (carrageenan + diclofenac sodium): carrageenan at a dose 25 μL of a 1% carrageenan solution into subplantar region, after 30 mint administration of standard drug (intraperitoneal)

Group 4 (pyrazolone low dose): carrageenan at a dose 25 μL of a 1% carrageenan solution into subplantar region, after 30 mint administration of test drug pyrazolone (intraperitoneal)

Group 5 (pyrazolone high dose): carrageenan at a dose 25 μL of a 1% carrageenan solution into subplantar region, after 30 mint administration of test drug pyrazolone (intraperitoneal.).

FIGURE 3.5: Intraperitoneal (i.p) Drug Administration in Mice.

In the illustration above, an injection is administered intraperitoneally (i.p.) to a laboratory mouse in the lower right quadrant of the abdomen. To reduce the possibility of harm, this site is selected to exclude important organs such as the bladder, cecum, and intestines. To deliver the test ingredient accurately and with the least amount of disruption, a sterile insulin syringe (typically 1 mL with a

26–30 gauge needle) is used. The needle is inserted into the right lower quadrant of the abdomen at a shallow angle (15–30°) while the mouse is being gently kept in place. To guarantee correct insertion, the syringe is examined for the presence of blood or fluid before to injection. This method is frequently used for drug delivery because of its fast systemic absorption, and should be carried out under strict aseptic to facilitate animal welfare and reliability of experimental results (photography taken at Faculty of Pharmacy, Capital University of Science and Technology, Islamabad).

3.9.3 Drug Solubility

As the test compound was poorly soluble in water, an initial attempt was made to dissolve it in normal saline with vortexing. However, due to limited solubility, a co-solvent approach was employed. Specifically, 40μL of dimethyl sulfoxide (DMSO) was added to an eppendorf tube containing the compound, and the mixture was vortexed thoroughly to ensure complete dissolution. Following this, 950μL of normal saline was added to the solution and vortexed again to obtain a final volume of 1mL. This preparation ensured a homogenous and stable drug solution suitable for intraperitoneal administration.

3.9.4 Dose Preparation

For the preparation of a 5 mg/Kg drug solution (1 mL), 0.5 mg of the drug was initially dissolved in 40 μL of dimethyl sulfoxide (DMSO) in a sterile Eppendorf tube. Subsequently, 950 μL of normal saline was added, and the solution was vortexed thoroughly to ensure complete dissolution. The drug was fully soluble in DMSO before dilution. To prepare a 10 mg/Kg drug solution (1 mL), 1 mg of the drug was first dissolved in 40 μL of DMSO, followed by the addition of 950 μL of normal saline. The mixture was then vortexed to obtain a clear and homogeneous solution, confirming complete dissolution of the drug in DMSO before dilution. For carrageenan solution, 0.1 mg of carrageenan was accurately weighed and dissolved

in 1 mL of distilled water. The solution was stirred properly to ensure complete dissolution before administration.

3.10 Formalin Induced Neuropathic Tonic Pain

The formalin test, first described by Dubuisson and Dennis in 1977, has become an indispensable model for studying persistent pain that involves both peripheral and central sensitization mechanisms [182]. Unlike acute reflex tests, it offers a biphasic response that closely models neuropathic and inflammatory pain observed in humans [183, 184].

Following a subcutaneous injection of dilute formalin into the rodent hind paw, behavioral pain responses emerge in two distinct phases [185, 186].

Phase I occurs immediately and lasts approximately 5–10 minutes; it reflects the direct activation of C-fiber nociceptors, causing acute nociceptive behaviors like licking, lifting [21]. A quiescent "interphase" of roughly 5–10 minutes follows, during which behaviors subside [182, 187].

Then, Phase II arises from approximately 15 minutes post-injection and can persist up to 60–90 minutes [188]. This late phase involves inflammatory processes at the injection site and central sensitization within the dorsal horn of the spinal cord.

3.10.1 Mechanisms Underlying Phase I

The initial, neurogenic, Phase I is driven by direct activation of nociceptive fibers—purely peripheral in origin. Formalin stimulates transient receptor potential ankyrin 1 (TRPA1) channels on nociceptors, rapidly depolarizing C-fibers and triggering immediate pain behaviors [189, 190]. This acute input is independent of immune or inflammatory mediators and is responsive to opioids that act centrally and peripherally.

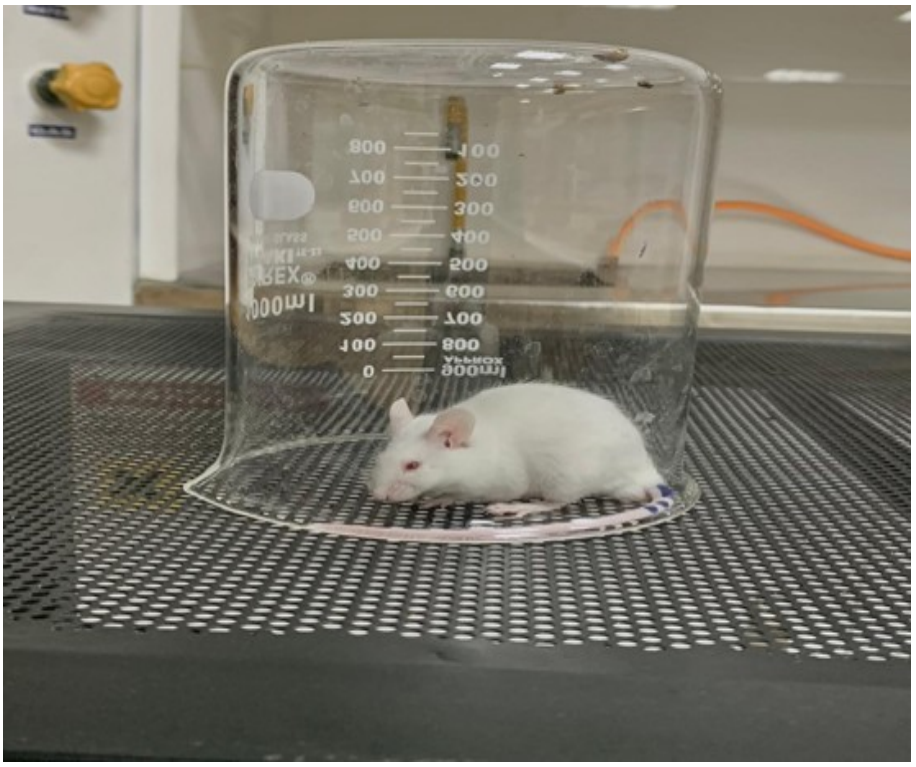


FIGURE 3.6: The Acute Neurogenic Pain Response is Demonstrated in Phase 1 of the Formalin Test.

In the initial minutes following formalin delivery, the animal vigorously licks, chews, and shakes the injected paw. Phase 1 of formalin model is the initial phase in which the mice experience moderate pain showing the responses. The response are licking, biting of the infectious paw. photography taken at Faculty of Pharmacy, Capital University of Science and Technology, Islamabad).

3.10.2 Mechanisms Underlying Phase II

Phase II is more complex, mirroring neuropathic pain through active inflammatory signaling and synaptic plasticity. Peripheral tissue damage triggers release of pro-inflammatory mediators—such as bradykinin, prostaglandins, and cytokines—creating a continuous barrage of input into the spinal cord [84, 191]. This repeated activation drives central sensitization, evidenced by increased excitability and prolonged discharge of dorsal horn neurons, often referred to as "wind-up" [192]. Electrophysiological studies confirm enhanced firing in lamina V wide dynamic range neurons post-formalin injection. Consequently, even subthreshold

stimulation of the affected region can evoke amplified pain responses. It results in showing of pain responses.

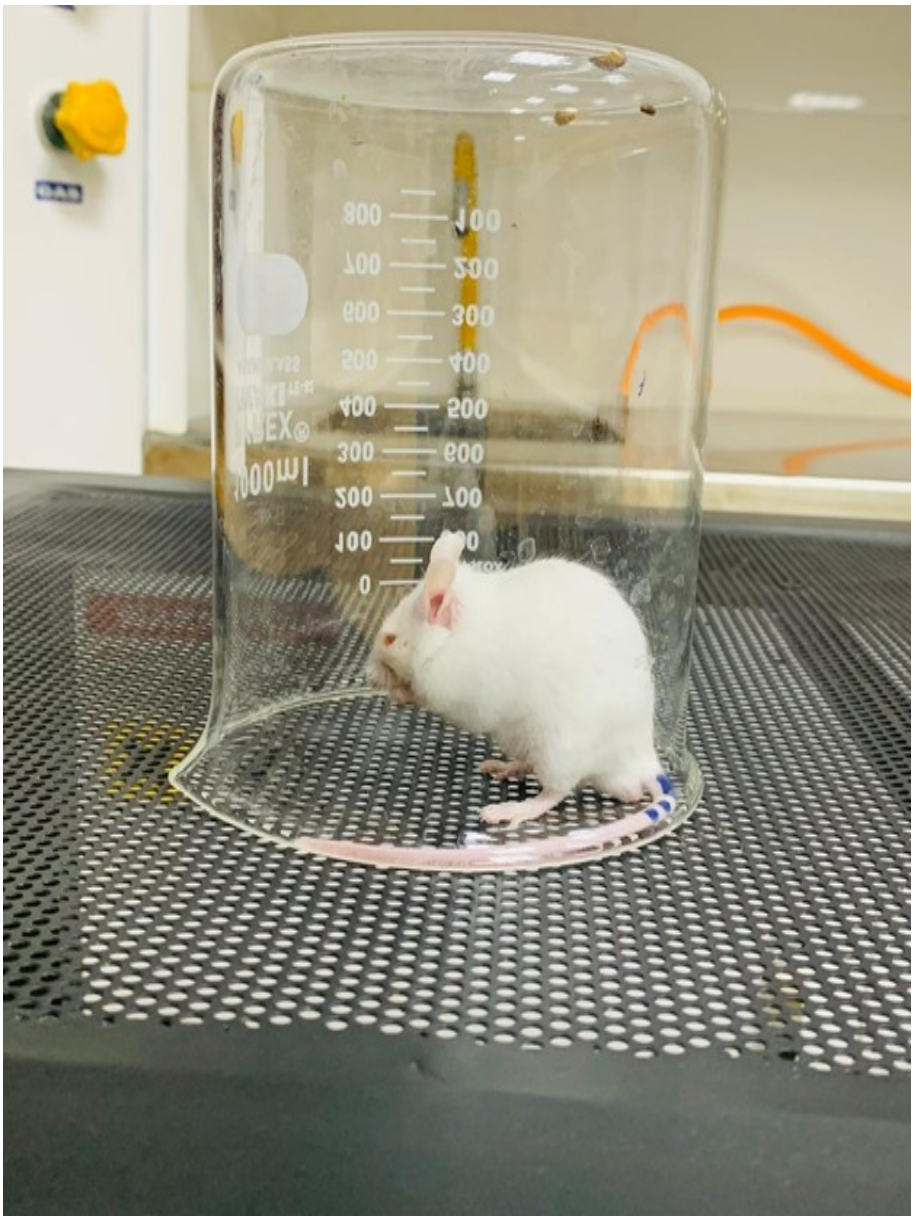


FIGURE 3.7: The Formalin Test's Second Phase

Figure illustrates the persistent chronic inflammatory pain response. Because of central sensitization and inflammation, this phase, which begins around 15 minutes after injection, and it last for 40 minute. the identification is marked by protracted licking, biting, and lifting of the injected paw. photography taken at Faculty of Pharmacy, Capital University of Science and Technology, Islamabad).

3.11 Carrageenan-Induced Hind Paw Edema Model

Carrageenan, a sulfated polysaccharide extracted from red algae, has been extensively used to induce acute inflammation and nociceptive pain in rodent models due to its reproducible and robust effects [193]. When injected subcutaneously into the hind paw—typically as a 1–2% λ-carrageenan solution in a 25–100μL volume depending on the rodent species—it produces measurable edema and hyperalgesia within 1–2 hours, peaking around 3–5 hours and resolving within 24 hours [193]. The inflammatory cascade in this model occurs in multi-phasic stages. Initially (0–2h), histamine and serotonin release enhance vascular permeability [194].

This is followed by a kinin-mediated response and later by prostaglandin-driven inflammation, primarily via COX-2 and PGE synthesis, which sustains edema and pain between 3–6 hours. During these phases, neutrophil infiltration and the generation of reactive oxygen and nitrogen species (ROS/RNS) further amplify the inflammatory and nociceptive processes [195].

Assessment methods include plethysmometry to measure paw volume via fluid displacement or caliper measurement of paw thickness, with data recorded at baseline and hourly intervals up to 6 hours [196]. This allows quantification of peak edema and calculation of percentage inhibition by test compounds. The behavioral component of pain is evaluated through mechanical allodynia using von Frey filaments and thermal hyperalgesia with hot plate or Hargreaves tests.

These assessments peak around 3–5 hours post-injection, coinciding with inflammatory mediator action. At the molecular level, carrageenan-induced inflammation triggers elevated COX-2, iNOS, and elastase activity, along with increased cytokines such as TNF-α, IL-1β, and IL-6. These markers can be quantified through ELISA, Western blotting, or immunohistochemistry to correlate tissue-level effects with behavioral outcomes

An illustration of the carrageenan-induced biphasic inflammatory response. The rapid release of inflammatory mediators such as histamine, serotonin, and

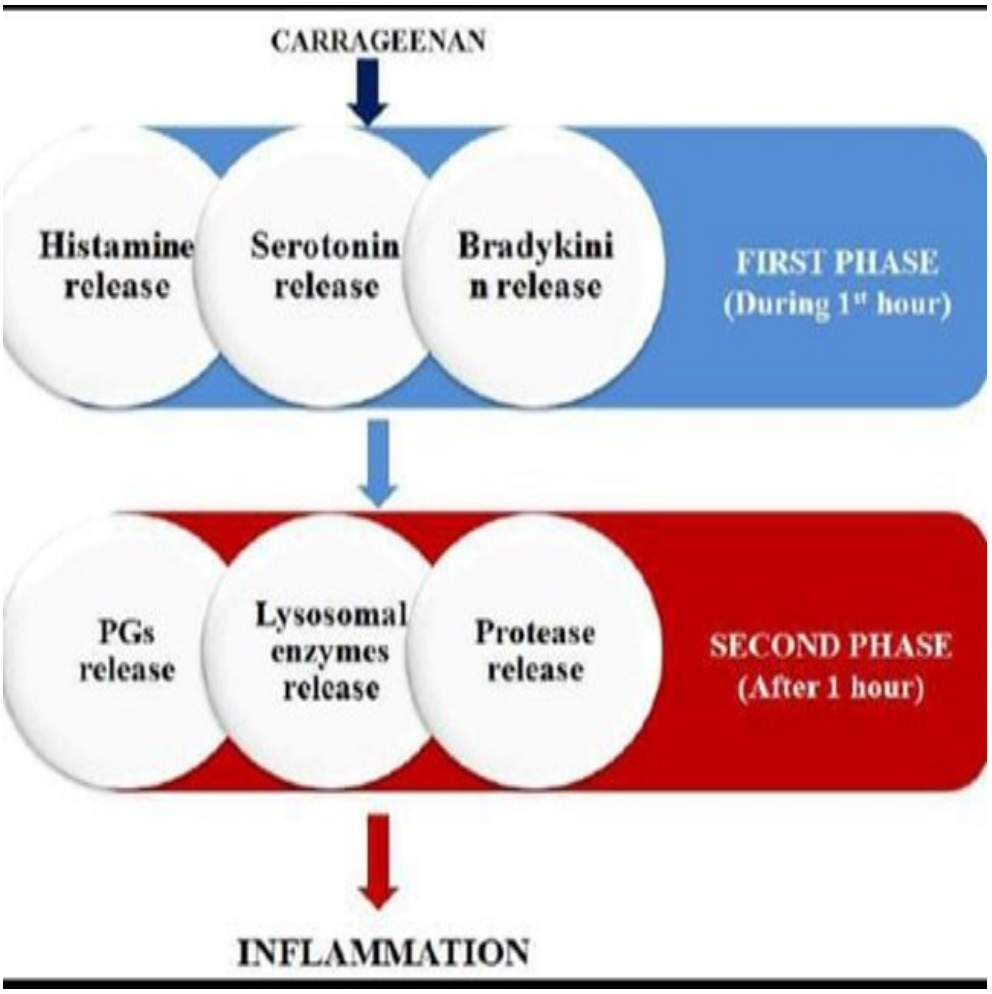


FIGURE 3.8: Carrageenan Induced Biphasic Inflammation Model

bradykinin propels the First Phase, which lasts for the first hour. A more marked and prolonged inflammatory and pain response results from the Second Phase, which happens an hour later and is marked by the prolonged production of prostaglandins (PGs), lysosomal enzymes, and proteases.

3.12 Allodynia and Thermal Hyperalgesia

Allodynia is defined as pain prompted by a stimulus that does not normally cause pain, while hyperalgesia is characterized by an exaggerated response to a typically painful stimulus [197]. These conditions often coexist in neuropathic pain models but manifest via different underlying mechanisms. We will explore mechanical (touch/pressure) and thermal (heat/cold) modalities to assess both conditions.

3.12.1 Quantitative Sensory Testing

QST is a standardized psychophysical approach used in both preclinical and clinical settings to quantify thresholds and responses to controlled stimuli, enabling differentiation between allodynia and hyperalgesia [198]. It can detect sensory gain (hyperalgesia/allodynia) and loss, utilizing modalities such as thermal, mechanical, vibration, and pressure stimuli. In animal studies—especially rodents—QST complements reflex-based nociception tests, improving translational value.

3.13 Mechanical Sensitivity Assays

3.13.1 Von Frey Filament Testing

Using calibrated filaments, incremental forces are applied to the hind paw until a withdrawal response is elicited. For allodynia, low threshold forces (e.g. 0.02–1g) are tested; withdrawal at these levels indicates tactile allodynia. Hyperalgesia is assessed by applying higher forces and comparing withdrawal thresholds to baseline controls. Filaments of different strengths are used to induce and identification of Pain. Filaments are useful to identify the level of pain that mice can bear and the intensity at which animal is showing reflexes. These are flexible filaments showing intensity to be captured by the mice

3.13.2 Randall–Selitto Paw Pressure Test

This method places increasing pressure on the paw until withdrawal or vocalization, quantifying mechanical hyperalgesia. Sudden withdrawal at lower pressures than baseline indicates hyperalgesia; withdrawal to light touch suggests allodynia [199, 200].

FIGURE 3.9: Vonfrey Filaments Used to Induce Pain in Mice Paw

3.14 Thermal Sensitivity Tests

3.14.1 Hargreaves' Test Radiant Heat

An infrared heat beam is applied beneath the paw, and withdrawal latency is recorded. Reduced latency compared to baseline indicates thermal hyperalgesia [201].

3.14.2 Hot Plate Tests

These tests subject animals to constant or ramping temperature stimuli. Thermal allodynia is inferred when innocuous temperatures provoke pain behaviors (lifting,

licking), and thermal hyperalgesia is indicated by heightened responses at noxious plate temperatures [202]. Ramp protocols effectively discriminate between cold and heat allodynia and hyperalgesia.

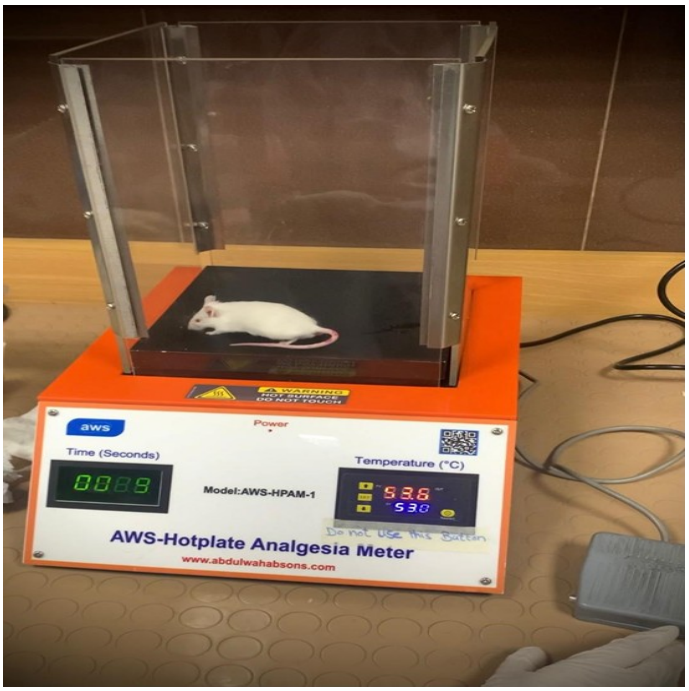


FIGURE 3.10: Hotplate Analgesia Meter

Instrument that causes pain sensation on the paw of animal. It shows the time period in seconds at which animal response. Instrument was actively used in research model. This instrument is used to detect the time in seconds at which the animal show response. Effectively different groups of mice were treated showing the latency time at which response is recorded.

3.14.3 Cold Allodynia Assays

Cold sensitivity is evaluated using acetone drops or cold plate/radiant ice ball methods. Pain behaviors following cold application to otherwise innocuous cold stimuli indicate cold allodynia, common in neuropathic conditions [203].

3.14.3.1 Behavioral Scoring

Each test records reflexive responses: withdrawal, paw licking, flinching, jumping. A cutoff threshold protects against tissue injury (e.g., 20s on hot plate). Data is typically captured using video or manual timers at defined intervals [204].

3.14.3.2 Study Design and Analysis

Animals are first acclimated in testing cages. Baseline sensory thresholds are recorded before neuropathy induction (e.g., nerve ligation). Testing is repeated at multiple post-operative time points (e.g., Days 3, 7, 14). Behavioral changes are quantified as percent change from baseline or area under the curve analyses

3.15 Enzyme-Linked Immunosorbent Assay

The sandwich ELISA represents a gold-standard technique for precise and sensitive quantification of cytokines such as IL-1β and TNF-α in biological fluids. Its design—an antibody "sandwich"—provides high specificity and low background signal, making it ideal for neuroinflammation studies [205].

3.15.1 Plate Coating with Capture Antibody

A 96-well high-binding polystyrene microplate is coated with 100μL per well of mouse-specific capture antibody (e.g. anti–IL-1β or anti–TNF-α) diluted in carbonate-bicarbonate buffer (pH9.4) at 3μg/mL. The plate is incubated for 1h at room temperature or overnight at 4°C to allow efficient adsorption. After incubation, wells are washed with PBS-Tween (0.05% Tween-20) five times, tapping out residual wash buffer each time [206].

3.15.2 Block Non-specific Binding Sites

To reduce nonspecific interactions, wells are blocked with 300μL of 1–5% BSA or casein in PBS-Tween for 1h at room temperature. Blocking prevents adsorption of extraneous proteins, which is vital for maintaining assay specificity and sensitivity.

3.15.3 Sample and Standard Incubation

Standards are prepared by serial dilutions of recombinant IL-1β or TNF-α, typically ranging from 7.8pg/mL to 500pg/mL. Samples (e.g. serum, tissue homogenate) are diluted in the same buffer. Each well receives 100μL of standard or sample, in duplicate, then incubated for 1–2h at room temperature with gentle shaking (˜500rpm). This allows the antigen to bind to the immobilized capture antibody.

3.15.4 Add Detection Antibody

After washing five times to remove unbound sample, 100μL of HRP-conjugated detection antibody specific to a different epitope from the capture antibody is added to each well. The plate is incubated for 1–2h with shaking. If a biotinylated detection antibody is used instead, a subsequent streptavidin-HRP incubation follows for signal amplification [207].

3.15.5 Signal Development with TMB Substrate

Following washes to clear unbound antibodies, 100μL of TMB substrate is added and plates are incubated in the dark for 15–30min at room temperature. The reaction is stopped by adding 50–100μL of 1M sulfuric acid or HCl, turning the solution yellow.

3.15.6 Optical Density Measurement and Data Analysis

Absorbance is measured at 450nm (with optional reference at 550nm to correct for plate artifacts) within 30min of stopping the reaction. A four-parameter logistic (4-PL) curve is fitted to standard readings using software such as SkanIt or equivalent. Cytokine concentrations in samples are interpolated from this calibration curve.

3.15.7 Ensuring Accuracy and Reliability

Duplicates/triplicates: Running each sample in at least duplicate ensures reproducibility

Controls: Negative (blank wells) and positive controls validate assay integrity.

Optimized antibodies: Using matched pairs targeting distinct epitopes avoids steric hindrance and enhances specificity Strict washing: Proper washing is essential to reduce background and increase sensitivity.

Temperature and timing consistency: Fixed incubation times and temperatures ensure experimental consistency .

3.15.8 Interpreting Results

Results are reported in pg/mL, with calculated mean±SD for each group. Data are normalized to protein content if analyzing tissue homogenates. Statistical methods (e.g. ANOVA) are used to compare cytokine levels between experimental groups.

3.16 Tissue Collection and Processing

Adult mice will be deeply anesthetized to ensure no pain perception, then perfused transcranial first with ice-cold phosphate-buffered saline (PBS) to flush out blood and reduce autofluorescence and background staining, followed by 4%

paraformaldehyde (PFA) to fix neural tissue in situ. Proper perfusion ensures rapid fixation and excellent preservation of tissue structure and antigen integrity. A post-fixation step immerses brains in 4% PFA at 4°C for 24hours—no longer, to avoid excessive crosslinking and epitope loss [226]. Fixed tissues are rinsed in PBS and stored at 4°C until further processing. Fixed brain tissues are trimmed to 3mm thickness and placed in tissue cassettes [226]. Tissues then undergo graded dehydration: 50%, 70%, 80%, 95%, and 100% ethanol (≈ 45minutes each), followed by clearing in xylene or xylene substitute to remove lipids. Finally, specimens are embedded in paraffin, which preserves morphology and enables cutting of 3–5μm sections using a microtome; thinner slices (3–4μm) improve antibody penetration and resolution. Slides are affixed to charged glass slides and dried at 50–60°C for 1hour to firmly adhere tissue sections [226]. Slides are immersed twice in xylene (5min each) to dissolve paraffin, followed by graded ethanol (100%, 95%, 80%, 70%; 2min each) and final rinse in distilled water. Inadequate deparaffinization can impair antigen access and lead to high background [208].

3.17 Histological Examination

3.17.1 Hematoxylin and Eosin Staining Protocol

The H&E method, a proven approach for overall tissue morphology, was used for histological staining in order to assess tissue architecture and pathological alterations. First, standard histological procedures were used to process tissue samples that had been formalin-fixed. To preserve morphological integrity, tissues were preserved in 10% neutral-buffered formalin for 24 to 48 hours at room temperature. Following fixation, an automated tissue processor was used to dehydrate the samples in a graded series of ethanol (70%, 80%, 95%, and 100%), clarify them in xylene, and then embed them in paraffin wax. Using a rotary microtome, tissue blocks fixed in paraffin were divided into sections that were 4-5 μm thick. Before staining, the slices were placed on sterile glass microscope slides and dried for either one hour at 60°C or overnight at 37°C to increase adhesion. The slides were

first deparaffinized for H&E staining by soaking in xylene for two changes of five minutes each. This was followed by a quick rinse in distilled water and rehydration in a decreasing order of alcohol (100%, 95%, and 70% ethanol). After staining the cell nuclei for five to seven minutes with Harris' hematoxylin solution, sections were washed under running water to remove any remaining stain. Differentiation was done, if needed, using 1% acid alcohol (1% HCl in 70% ethanol) for a few seconds to eliminate non-specific nuclear staining, and nuclear bluing was done by soaking the slides in 0.2% ammonia water or alkaline tap water for 1 minute.

After that, 1% eosin Y solution was used to counterstain the cytoplasm and extracellular material for 30 to 60 seconds. To prevent overstaining, the slides were immediately cleaned in distilled water after eosin staining.

The stained sections were cleaned in xylene (two changes, three minutes each) after being dehydrated using an escalating sequence of ethanol concentrations (70%, 95%, and 100%). Finally, the stained portions of the coverslips were fixed by mounting them in a permanent mounting media based on xylene. After allowing the stained slides to air dry completely, they were examined at various magnifications (10×, 40×) using a bright-field light microscope to evaluate tissue architecture, cellular morphology, and histopathological changes. For documentation and analysis, representative photos were collected.

3.18 Molecular Docking and Computational Analysis

To forecast the binding interactions and affinities of particular drugs with target proteins, molecular docking simulations were run. A set of software tools was used in these computational investigations to prepare the proteins and ligands, run docking simulations, and examine the complexes that were produced. Finding the compounds' most stable binding conformations and important chemical interactions at the protein's active site was the main objective in order to comprehend the possible mechanisms of action of the substances.

3.18.1 Ligand Preparation

The compounds' energies were reduced and their chemical structures were sketched. After preparation, the compounds were structured for simulations of docking.

3.18.2 Protein Preparation

The Protein Data Bank (PDB) was used to obtain the target proteins' three-dimensional structures. After being cleaned and their active sites located, these proteins were ready for docking.

3.18.3 Ligand Docking and Analysis

Using specialist software, the synthesized compounds were docked into the protein's active regions. The most advantageous binding positions were determined by analyzing the data using binding energy and root mean square deviation (RMSD). Following that, 2D and 3D visualizations and analyses were conducted on the particular amino acid residues implicated in these interactions. These analyses were compared to a reference standard.

3.19 Statistical Analysis

All experimental data derived from behavioral, biochemical, or histological assays will be analyzed using GraphPad Prism 6.0, a widely accepted platform for biomedical research data analysis and graphical representation. Prior to statistical testing, data distributions will be examined for normality using the Shapiro–Wilk test, a reliable method for small sample sizes ($n<50$) and standard in high-quality studies. Provided data satisfy assumptions of normality and homogeneity of variances (confirmed using Levene's test), a one-way analysis of variance (ANOVA) will be employed to compare differences across multiple treatment groups (e.g., control,

standard drug, low-dose pyrazolone, high-dose pyrazolone) . One-way ANOVA efficiently determines whether there are overall group differences in parameters such as cytokine concentrations, paw edema, or neuronal marker expression.

To identify specific group differences following a significant ANOVA result, Tukey's post hoc multiple-comparison test will be used. This test controls the family-wise error rate and allows all pairwise comparisons while maintaining overall Type I error at the set level .

For longitudinal measures (e.g., paw thickness over time), a two-way ANOVA—with factors for treatment and time—followed by Tukey's test will be used to detect interactions between treatment effects and temporal changes. Significance thresholds will be set at $P< 0.05$, in alignment with common practice in pharmacological research.

Exact P values will be reported (e.g., $P=0.032$), and results with $P<0.01$ or $P<0.001$ will be annotated with '**** or '*-****, respectively, to denote increasing levels of statistical significance and to improve interpretability. Data are represented as mean ± standard error of the mean (SEM) to indicate group variability and support inferential interpretation.

GraphPad Prism also enables visual representation of data trends through bar charts, dose–response curves, time-course plots, and box-and-whisker diagrams. In addition to ANOVA, effect sizes will be calculated—using metrics such as eta-squared (η^2) for ANOVA and Cohen's d for selected group comparisons. Reporting effect sizes provides context on practical significance and complements P values.

Data integrity will be ensured by blinding analysis, where the analyst is masked to group identities during data entry and interpretation to prevent confirmation bias. All raw data, statistical outputs, and Prism project files will be archived and made available upon request to support transparency and reproducibility.

To uphold data integrity and minimize bias, the entire data analysis process was conducted under blinded conditions, where the individual performing the analysis was unaware of the group allocations throughout data entry and interpretation.

3.20 Acute Toxicity Study

Acute toxicity study will be performed and their prospective effects will be identified at a dose of 100mg/kg.

Chapter 4

Results

4.1 Computational Studies

Molecular docking of the selected pyrazolone derivative with interleukin-1 beta (IL-1β) revealed a binding affinity of -6.9 kcal/mol, indicating a moderate to strong interaction with the target protein as shown in Fig-1. The 2D interaction diagram shows that the ligand forms multiple stabilizing interactions with key amino acid residues within the IL-1β binding pocket:

Conventional hydrogen bonds were observed with:

Serine 5 (SER A:5)

Serine 43 (SER A:43)

These interactions contribute significantly to ligand stabilization through polar bonding with the carbonyl oxygen of the pyrazolone core.

Carbon hydrogen bonds were noted with:

Tyrosine 90 (TYR A:90)

Proline 87 (PRO A:87)

Tyrosine 68 (TYR A:68)

These weaker interactions still play a role in maintaining ligand orientation within the binding cavity.

Hydrophobic interactions included:

Alkyl interactions with Proline 91 (PRO A:91)

Pi-Alkyl interactions with Valine 85 (VAL A:85) and Proline 87 (PRO A:87)

These hydrophobic contacts enhance the binding by contributing to the non-polar environment around the ligand's aromatic ring systems.

The spatial arrangement and diversity of interactions suggest that this pyrazolone derivative fits well within the IL-1β active site, showing potential for anti-inflammatory activity through inhibition of this pro-inflammatory cytokine.

The pyrazolone derivative exhibited a binding affinity of –6.3 kcal/mol with tumor necrosis factor-alpha (TNF-α), indicating a moderately strong and potentially biologically relevant interaction with the cytokine's active site.

As illustrated in the 3D and 2D interaction diagrams

Carbon hydrogen bonds were formed with:

Arginine 6 (ARG A:6)

Alanine 14 (ALA A:14)

These interactions provide polarity-based stabilization and contribute to correct ligand positioning.

Hydrophobic and aromatic interactions include:

Pi-alkyl interactions with Valine 13 (VAL A:13) and Tyrosine 59 (TYR A:59)

Alkyl and pi-sigma interactions with Proline 8 (PRO A:8), Leucine 36 (LEU A:36), and Isoleucine 155 (ILE A:155) These hydrophobic contacts enhance the affinity and snug fit of the compound within the hydrophobic pocket of TNF-α.

The docking pose revealed that the aromatic core of the pyrazolone scaffold is well accommodated within the binding cavity, surrounded by aliphatic residues, while polar groups are oriented toward surface residues enabling hydrogen bonding. The presence of both hydrogen bonding and hydrophobic interactions suggests a dual stabilization mechanism, making this derivative a promising lead for TNF-α inhibition.

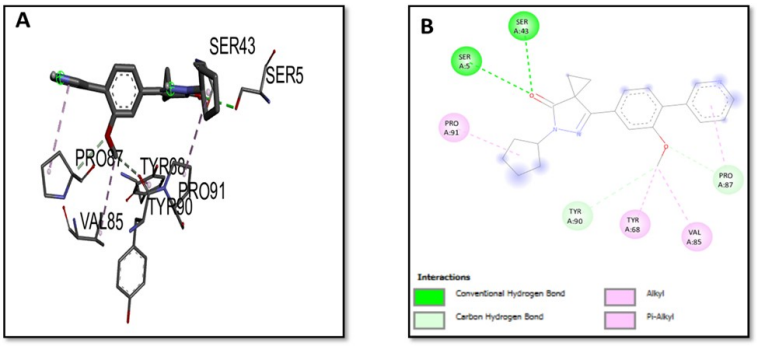


FIGURE 4.1: Molecular Docking of Pyrazolone derivative with IL1β (PDB ID:9ILB).

(A) Dimensional structural illustration of Molecular interaction between pyrazolone derivative and IL1β, (B) 2-Dimentional structural illustration of Molecular interaction between pyrazolone derivative and IL1β, (B) 2-Dimentional structural illustration of Molecular interaction between pyrazolone derivative and IL1β. Molecular docking enables to interact a relation at which site the compound must show its high affinity.

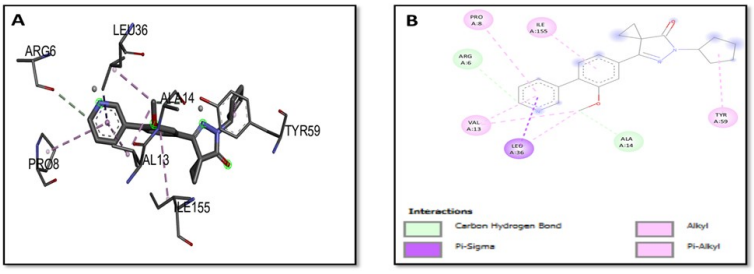


FIGURE 4.2: Molecular Docking of Pyrazolone derivative with TNF-α PDB ID:1TNF

(A) Dimensional structural illustration of Molecular interaction between pyrazolone derivative and TNF-α, (B) 2-Dimentional structural illustration of Molecular interaction between pyrazolone derivative and TNF-α. Molecular docking

enables to interact a relation at which site the compound must show its high affinity.

4.2 Effect of Pyrazolone Derivative on the Reduction of Paw Edema

The anti-inflammatory effects of the pyrazolone derivative were evaluated using the carrageenan-induced paw edema model in mice. The progression of paw edema was recorded at 0 hour (baseline), 30 minutes, 1 hour, 2 hours, 4 hours, 5 hours, and 6 hours post-carrageenan injection. Each group consisted of six animals ($n = 6$). The paw volumes of each group at different time intervals are illustrated in Figure 16.

In the saline control group, paw edema gradually increased over time from a baseline of 1.016 ± 0.02 to 1.25 ± 0.03mm at 6 hours, reflecting the natural progression of inflammation. The carrageenan-only group exhibited a significant increase in paw volume from 1.183 ± 0.04 at baseline to a peak of 1.966 ± 0.05 mm at 4 hours, confirming successful induction of acute inflammation ($p < 0.001$ vs saline at 4h).

Treatment with the standard anti-inflammatory drug, diclofenac sodium (5mg/kg) + carrageenan, demonstrated an early and sustained anti-inflammatory effect. Paw volume rose from 1.03 ± 0.02 to 2.0 ± 0.06 mm at 2 hours and declined to 1.48 ± 0.05 mm by 6 hours. Although the initial edema response was similar to the disease group, a significant reduction in paw swelling was observed at 4, 5, and 6 hours ($p < 0.01$ vs carrageenan).

The pyrazolone derivative at 5 mg/kg showed a moderate anti-inflammatory effect. The paw volume increased from 1.117 ± 0.03 at baseline to a peak of 1.933 ± 0.04 mm at 2 hours, followed by a progressive reduction to 1.267 ± 0.03 mm at 6 hours. While the reduction in edema was notable compared to the carrageenan group, statistical significance was achieved only at 6 hours ($p < 0.05$ vs carrageenan).

On the other hand, the pyrazolone derivative at 10 mg/kg showed a better anti-inflammatory profile. The paw volume peaked at 1.967 ± 0.04 mm at 2 hours, followed by a sustained decrease to 1.40 ± 0.03 mm at 6 hours. The edema inhibition was significantly greater at 5 and 6 hours compared to the disease group ($p < 0.01$ and $p < 0.001$ respectively), indicating a dose-dependent effect of the pyrazolone compound as per Fig-8.

Overall, the pyrazolone derivative demonstrated a time- and dose-dependent reduction in carrageenan-induced paw edema, with the 10 mg/kg dose exhibiting efficacy comparable to the standard diclofenac treatment.

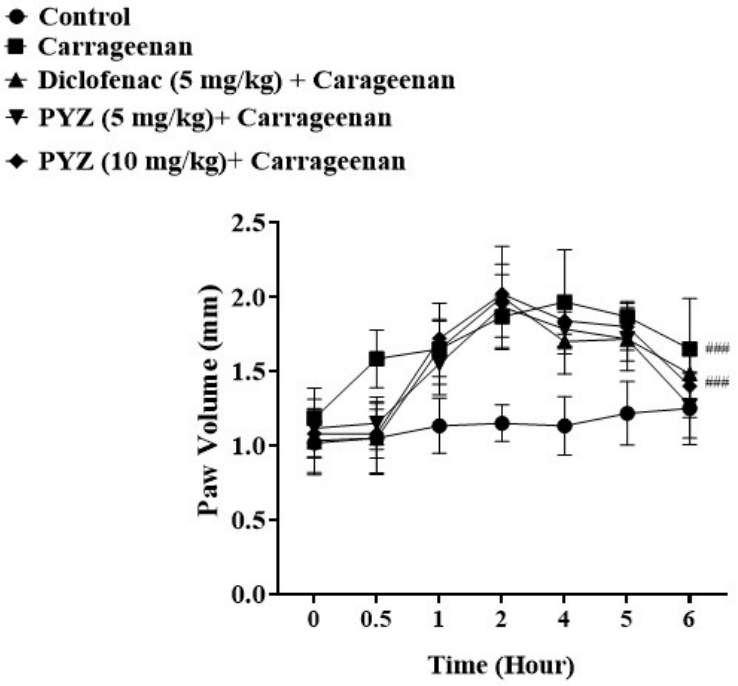


FIGURE 4.3: Effect of Pyrazolone Derivative on the Reduction of Paw Edema.

Paw size of mice was a standard that was to be observed across different groups, the main purpose of this was to identify that at which particular dose the maximum response was observed. Paw of mice was measured at normal condition, after inducing inflammation and also after administration of test drug. Significant results were observed as shown in the graph.

Symbol * Represents (Carrageenan) relative to the saline group. Symbol represents [Pyz (5mg/kg)+Carrageenan, Pyz (10mg/kg)+ Carrageenan, Diclofenac+Carrageenan] group. Symbol * which represents $p<0.05$; **, represents

p<0.01; and ***, represents p<0.001. All data are expressed as mean ± standard error of the mean ± (SEM).

4.3 Effect of Animal Response on the Hot Plate Method

The analgesic activity of the pyrazolone derivative was assessed using the hot plate test in carrageenan-induced acute inflammatory pain. Latency to paw licking or jumping (in seconds) was recorded at baseline (0 hour) and at multiple post-treatment intervals (30 minutes, 1 hour, 2 hours, 4 hours, 5 hours, and 6 hours). Each group consisted of six animals (n = 6). The mean reaction times for each group are shown in Figure 11.

The carrageenan-only (disease) group exhibited a marked reduction in pain threshold, with reaction times decreasing significantly from a baseline of 8.55 ± 0.4 s to 1.83 ± 0.2 s at 2 hours, reflecting the establishment of inflammatory hyperalgesia ($p < 0.001$ vs saline at 2h). A slight spontaneous recovery was noted by 6 hours (4.33 ± 0.3 s), but reaction times remained significantly lower compared to the saline control throughout the test. In contrast, the saline control group displayed stable latency times, with minor fluctuations from baseline (10.87 ± 0.3 s) to 12.15 ± 0.4 s at 6 hours, indicating absence of nociceptive sensitization. The standard treatment group (diclofenac + carrageenan) exhibited a significant and sustained increase in pain threshold starting from 30 minutes (11.83 ± 0.3 s) with a peak effect at 5 hours (21.45 ± 0.6 s, $p < 0.001$ vs disease), which was maintained at 6 hours (20.27 ± 0.5 s, $p < 0.001$ vs disease). This indicates a potent analgesic response.

The pyrazolone derivative at 5 mg/kg demonstrated a gradual onset of analgesia. After an initial transient drop at 1 hour (8.47 ± 0.3 s), a consistent rise in reaction time was observed, reaching 13.95 ± 0.4 s at 4 hours and 14.70 ± 0.3 s at 6 hours. These values were significantly higher than those in the disease group at later time points ($p < 0.05$ at 4h, $p < 0.01$ at 6h), suggesting moderate analgesic activity.

More pronounced effects were observed with the pyrazolone derivative at 10 mg/kg. Although a temporary decrease in latency was noted at 1 hour (7.18 ± 0.3 s), a significant increase was recorded from 4 hours onward, peaking at 17.07 ± 0.4 s at 5 hours and remaining elevated at 16.92 ± 0.4 s at 6 hours ($p < 0.001$ vs disease at both time points). This indicates a stronger and sustained analgesic effect at the higher dose as per Fig-11

Overall, both doses of the pyrazolone derivative improved pain latency compared to the carrageenan-only group in a dose- and time-dependent manner, with the 10 mg/kg dose demonstrating near-comparable efficacy to diclofenac in late-phase analgesia.

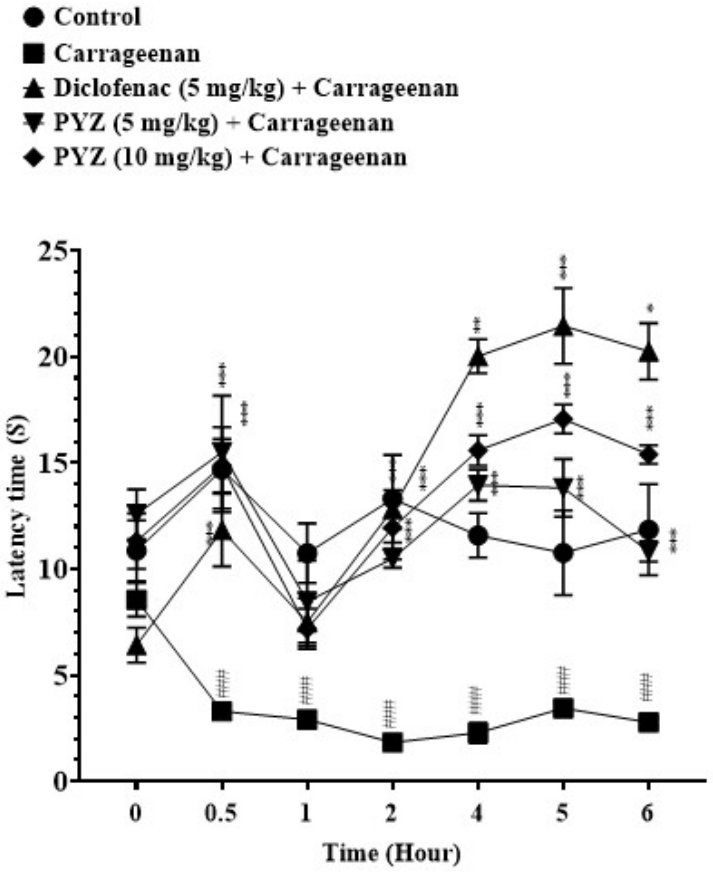


FIGURE 4.4: Effect of Animal Response on the Hot Plate Method.

Hot plate method is an important pharmacological method to identify the fluctuation of latency time period across different groups. Hot plate instrument was available at Capital University of Science and Technology. Animal from different groups were tested and results are described in the graphs

Symbol * Represents (Carrageenan) relative to the saline group. Symbol represents [Pyz (5mg/kg)+Carrageenan, Pyz (10mg/kg)+ Carrageenan, Diclofenac+Carrageenan.] symbol * which represents p¡0.05; **, represents p¡0.01; and ***, represents p¡0.001. All data are expressed as mean ± standard error of the mean ± (SEM).

4.4 Effect of Pyrazalone Derivatives on Thermal Mechanical Allodynia

Thermal allodynia was evaluated using the Mechanical stimulus withdrawal latency method in carrageenan-induced inflammatory pain. Paw withdrawal latencies (in grams) were recorded at baseline (0 hour) and at subsequent time points (30 minutes, 1 hour, 2 hours, 4 hours, 5 hours, and 6 hours) post-treatment. Each group comprised six animals (n = 6). The results are presented in Figure 18.

In the saline control group, the latency remained relatively stable over the 6-hour observation period, with minor fluctuations from 1.6 g at baseline to 2.00g at 6 hours. This indicated a normal thermal nociceptive threshold without induced inflammation or allodynia.

The carrageenan-only (disease) group exhibited a marked and sustained reduction in withdrawal latency, reflecting the development of Mechanical allodynia. Latency decreased significantly from 1.6 g at baseline to 0.4 g at 4 hours ($p < 0.001$ vs saline), with only minimal recovery by 6 hours (1.6 g, $p < 0.001$ vs saline). This confirmed effective induction of inflammatory pain and allodynia.

Administration of diclofenac + carrageenan resulted in a rapid and sustained analgesic effect. Latency dropped to 0.6g at 2 hours but gradually increased to 1.60g by 6 hours. Compared to the disease group, significant improvements were observed at 4, 5, and 6 hours ($p < 0.01$ to $p < 0.001$), confirming the efficacy of the standard treatment. The pyrazolone derivative at 5 mg/kg showed a moderate protective effect against mechanical allodynia. Although latency sharply declined

to 0.4 g at 2 hours, a progressive increase was seen from 4 hours 1.4g to 1.6 g at 6 hours, significantly higher than the disease group ($p < 0.05$ at 5h, $p < 0.01$ at 6h). A stronger effect was observed in the pyrazolone 10 mg/kg group, where latency reached its lowest at 1 hour 0.16 g similar to the disease group. However, a steady recovery was noted afterward, with significantly increased latency values at 5 and 6 hours (1.0 g and 1.6 g respectively), demonstrating a dose-dependent analgesic effect ($p < 0.01$ vs disease) as per Fig-18

Taken together, both doses of the pyrazolone derivative attenuated carrageenan-induced mechanical allodynia, with the 10 mg/kg dose showing superior efficacy, particularly in the late phase of the response.

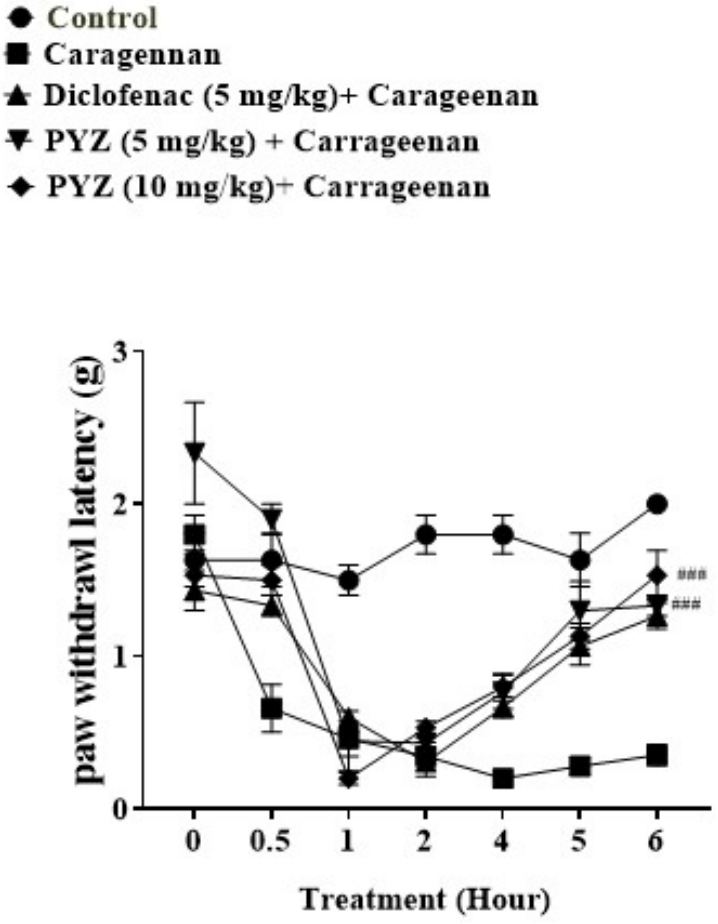


FIGURE 4.5: Effect of Pyrazalone Derivatives on Mechanical Thermal Allodynia

A behavioral test demonstrating thermal allodynia, a condition in which a normally non-painful thermal stimulus is perceived as painful. This phenomenon is often assessed by measuring the latency of a mouse to withdraw or lick its paw

in response to a mild heat source. This test is used to evaluate the effectiveness of analgesic compounds by observing their ability to reverse or reduce this hypersensitivity.

Symbol * Represents (Carrageenan) relative to the saline group. Symbol represents [Pyz (5mg/kg)+Carrageenan, Pyz (10mg/kg)+ Carrageenan, Diclofenac Carrageenan] . Symbol * which represents p<0.05; **, represents p¡0.01; and ***, represents p¡0.001. All data are expressed as mean ± standard error of the mean ± (SEM).

4.5 Effect of Pyrazolone Derivative on Quantitative Analysis of Inflammatory Marker

To assess the anti-inflammatory effects of the pyrazolone derivative, tumor necrosis factor-alpha (TNF-α) levels were quantified in serum samples using ELISA. Each group consisted of six animals (n = 6), and the average TNF-α concentrations are reported below. A marked elevation in TNF-α was observed in the carrageenan-only (disease) group, while both diclofenac and the pyrazolone treatments reduced TNF-α levels to varying degrees.

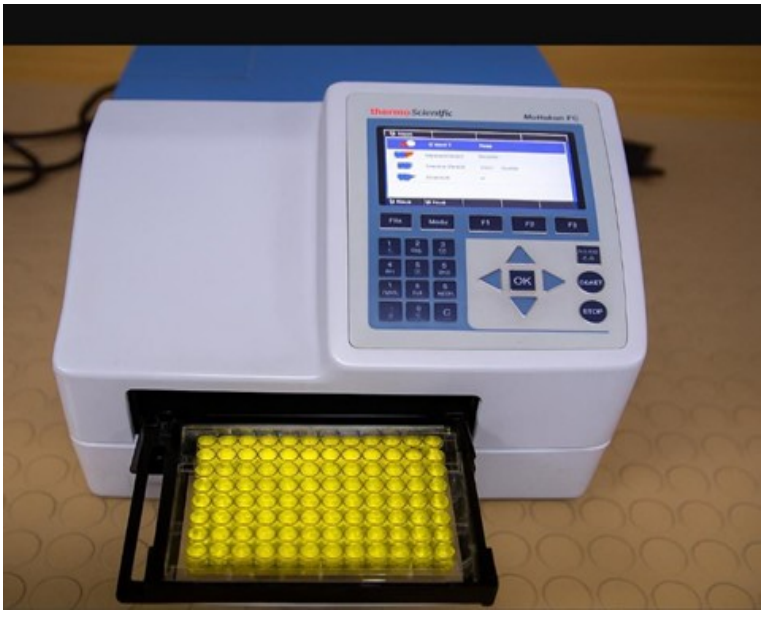


FIGURE 4.6: Primary Coated Antibody 96 Well Plates for ELISA.

The above figure shows quantitative analysis of TNF-α as measured by ELISA. Each well contains samples or standards processed to detect and quantify cytokine levels indicative of inflammatory response (photography taken at Faculty of Pharmacy, Capital University of Science and Technology,) Islamabad.

The carrageenan group exhibited a significant increase in TNF-α levels (1.588 $\pm$ 0.09 and 1.206 $\pm$ 0.08, mean 1.397 $\pm$ 0.11), confirming induction of acute inflammation ($p < 0.001$ vs saline).

The saline control group showed low TNF-α levels (0.915 $\pm$ 0.04, 1.045 $\pm$ 0.05, mean $\approx$ 0.980 $\pm$ 0.03), consistent with a non-inflammatory baseline.

Treatment with diclofenac + carrageenan produced the greatest suppression of TNF-α expression (0.310 $\pm$ 0.02, 0.104 $\pm$ 0.01, mean 0.207 $\pm$ 0.01), which was significantly lower than the carrageenan group ($p < 0.001$), demonstrating strong anti-inflammatory efficacy.

The pyrazolone 5 mg/kg group showed moderate reduction in TNF-α levels (0.510 $\pm$ 0.03, 0.956 $\pm$ 0.04, mean $\approx$ 0.733 $\pm$ 0.04), which was significantly decreased compared to the disease group ($p < 0.05$), but not as effective as diclofenac.

Interestingly, the pyrazolone 10 mg/kg group exhibited elevated TNF-α levels (1.687 $\pm$ 0.08, 1.381 $\pm$ 0.06, mean $\approx$ 1.534 $\pm$ 0.07), which were comparable or even slightly higher than the carrageenan group ($p > 0.05$ vs carrageenan). This suggests a potential pro-inflammatory effect or loss of efficacy at higher doses.

In summary, the ELISA findings indicate that the pyrazolone derivative at 5 mg/kg exhibits moderate anti-inflammatory activity by reducing TNF-α levels, while the 10 mg/kg dose failed to suppress cytokine levels and may not be therapeutically effective in this inflammatory model.

4.6 Microscopic Examination

The most popular histological method for examining tissue morphology is hematoxylin and eosin (H&E) staining. A basic pigment called hematoxylin attaches

itself to acidic materials, such the nucleic acids in the cell nucleus, and turns them blue to dark purple, making it easy to distinguish between chromatin and nucleoli. The rough endoplasmic reticulum and other basophilic components are also stained by it. In contrast, eosin is an acidic dye that produces a range of pink to red hues when it binds to basic cytoplasmic and extracellular proteins. Collagen fibers have a light pink tone, muscle fibers and cytoplasm are usually pink, and red blood cells stain bright red to orange because of the hemoglobin they contain.

A light microscope was used to view the stained slices at different magnifications, such as 10x and 40x. In the grey and white matter of the spinal cord, cellular and tissue architecture was assessed, including the existence of inflammatory cell infiltration, edema, tissue disarray, and general morphological alterations. In order to document and compare the various treatment groups, photomicrographs were taken.

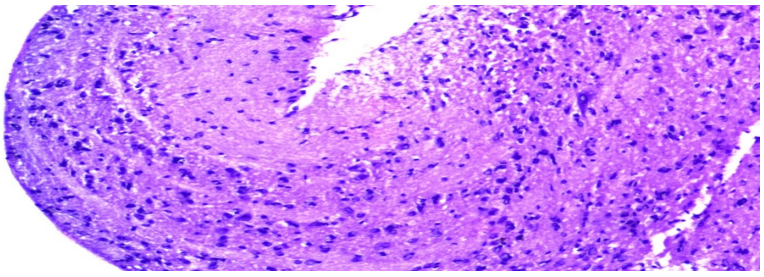


FIGURE 4.7: Histological Analysis of the Saline Control Group

At 10x magnification There are no indications of inflammation, disease, or treatment-related alterations, and the tissue displays normal cellular morphology and architecture. When comparing this image to the experimental and positive control groups, it acts as a baseline. It will provide information when its compared with other mice groups.

Spinal cord cross-section displaying a grey matter gliosis area. Reactive glial cells are densely concentrated in the image at 10x magnification, suggesting a strong inflammatory and reparative reaction to a localized injury or pathological condition. This result validates that spinal cord injury was successfully induced in the positive control group. There is a clear evidence that who inflammation was produced when inflammatory agent carrageenan was administered

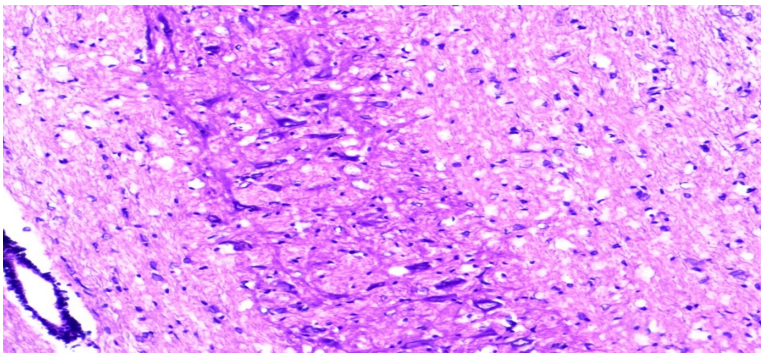


FIGURE 4.8: Positive Control Group

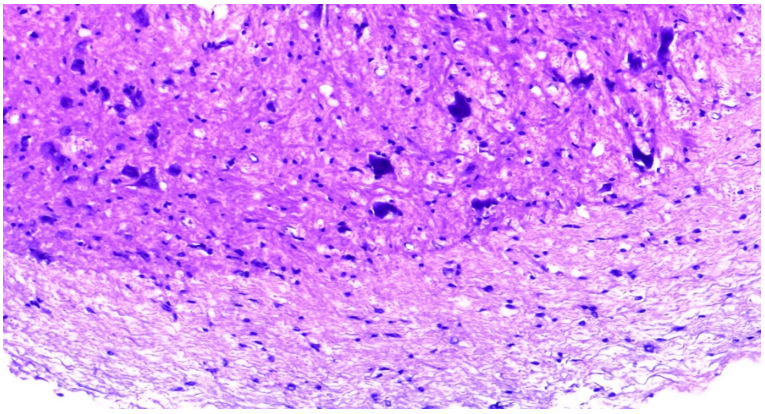


FIGURE 4.9: A spinal Word Tissue Histologically After Inflammation was Induced and Treated with the Standard Medication Diclofenac Sodium.

A decrease in gliosis, cellular infiltration, or any lingering indications of inflammation in comparison to the inflammatory control. This illustrates how diclofenac sodium affects the spinal cord tissue's inflammatory response. a clear evidence indicating the potency of diclofenac sodium as anti inflammatory drug.

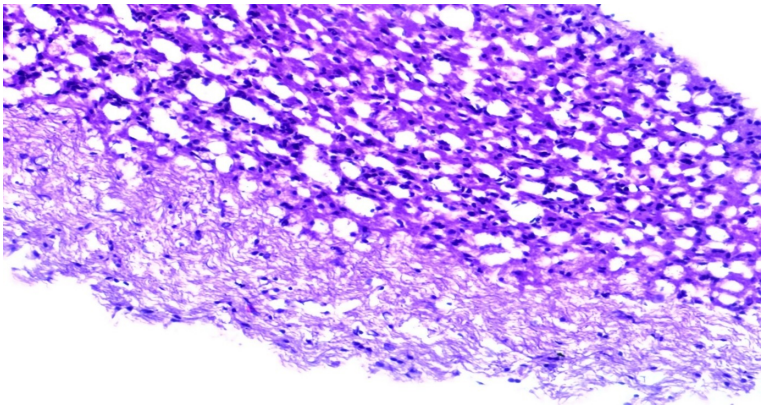


FIGURE 4.10: H&E stain, 10x Magnification, and Spinal Cord Tissue from the Treatment Group Treated with 5 mg/kg pyrazolone.

The picture displays noticeable differences from the inflammatory control, such as decreased gliosis or cellular infiltration. This suggests that pyrazolone at this dosage may have an impact on spinal cord inflammation. A typical illustration of the pyrazolone derivative's anti-inflammatory properties. The treated tissue shows a discernible decrease in gliosis and cellular infiltration when compared to the inflammatory control group, indicating that the pyrazolone chemical at this dosage can successfully modify spinal cord inflammation. Overall potency of pyrazolone compounds are noticeable.

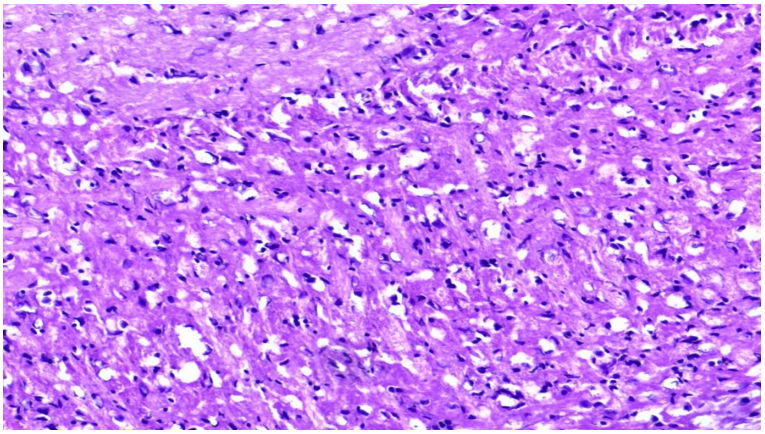


FIGURE 4.11: Spinal Cord Tissue from the Treatment Group that Received 10 mg/kg of theMedication pyrazolone

There is a notable decrease in gliosis and inflammatory cellular infiltration. The 10 mg/kg dose successfully reduced the generated inflammation and shielded the tissue from serious pathological alterations, indicating a dose-dependent therapeutic effect. Comparing tissue from the 10 mg/kg treatment group to the control, histopathological examination shows a marked decrease in inflammation. Gliosis and inflammatory cellular infiltration are both noticeably reduced in the image, suggesting that this particular dosage of the substance effectively reduced the produced inflammation and offered protection against pathological changes. This finding points to a therapeutic efficacy that is dose-dependent.

When it comes to sending neural messages from the brain to the rest of the body, the spinal cord is essential. While the white matter is made up of myelinated axons that guarantee quick signal conduction, the grey matter is made up of neuronal cell bodies that process information. Serious neurological abnormalities may result

from damage to these areas, such as from oedema, inflammation, or a disruption of the neuronal architecture.

Therefore, histopathological analysis of spinal cord tissue is a crucial method for determining the degree of damage and the effectiveness of treatment measures. Sections of the spinal cord stained with haematoxylin and eosin (H&E) were seen under light microscopy in this investigation at magnifications of 10× and 40×. Parameters such as inflammatory cell infiltration, edema, tissue disarray, and overall morphological changes in the grey and white matter were assessed across the experimental groups.

The spinal cord in the negative control group showed a well-preserved architecture with distinct white and grey matter. The neuropil seemed uniform, the axonal tracts in the white matter were neat, and the cell bodies of the neurones were undamaged.

Normal, healthy tissue was seen with no signs of oedema, degeneration, or infiltration of inflammatory cells. Significant histological alterations were evident in the positive control group, which included perivascular cuffing, significant oedema, loss of normal tissue organization, and dense infiltration of inflammatory cells inside the grey matter.

With a decrease in inflammatory infiltrates, less oedema, and a partial return to normal cytoarchitecture, the standard treatment group showed a discernible improvement, suggesting therapeutic effectiveness. Compared to the positive control, the pyrazolone low-dose group (5 mg/kg) showed a moderate histological recovery, with fewer inflammatory cells, minor oedema, and better organisation of neural structures, but some disorder remained. Similar to the negative control, the pyrazolone high-dose group (10 mg/kg) showed almost total preservation of normal spinal cord morphology, with little oedema, little inflammatory infiltration, and well-aligned axonal pathways in the white matter.

Overall, the microscopic analysis shows a distinct pattern, with the negative control showing intact architecture, the positive control showing significant pathological alterations, and the treatment groups showing incremental histological repair.

By reducing inflammation and structural damage in a dose-dependent manner, the high-dose pyrazolone treatment demonstrated the strongest neuroprotective impact, indicating its potential effectiveness in maintaining spinal cord integrity.

4.7 Acute Toxicity Study

Acute toxicity study was performed, and dose of Pyrazolone derivative at a dose of 100mg/kg was administered through the intraperitoneal route. In order to evaluate the results, it was observed that after 24 hours there was no mortality at a dose of 100 mg/kg. which reflects that this dose is stable to be used in mice for toxicity study profiles at Higher doses.

Chapter 5

Conclusion

5.1 Discussion

Acute inflammatory pain is a rapid-onset physiological response to tissue injury, infection, or noxious stimuli, characterized by redness, swelling, heat, and functional impairment at the affected site [209]. The primary purpose of this response is protective—it serves to eliminate the initial cause of cell injury, clear out necrotic cells and tissues, and establish a reparative environment. From a molecular standpoint, acute pain is initiated through the activation of nociceptors—specialized peripheral sensory neurons—that detect and transmit pain signals upon encountering pro-inflammatory mediators such as prostaglandins, bradykinin, histamine, and interleukins [210].

The pathophysiology involves complex interactions between immune cells, vascular endothelium, and peripheral neurons [211]. Upon tissue insult, immune cells like neutrophils and macrophages infiltrate the site, releasing cytokines such as tumor necrosis factor-alpha (TNF-α), interleukin-1β (IL-1β), and interleukin-6 (IL-6), which not only potentiate inflammation but also sensitize nociceptors [212]. This process is further amplified by the production of prostaglandins through the cyclooxygenase (COX) pathway, particularly COX-2, which enhances pain sensitivity by lowering the activation threshold of nociceptors.

A significant element of acute pain is the transition from peripheral to central sensitization. Prolonged peripheral input leads to heightened excitability of spinal cord neurons, particularly in the dorsal horn, a phenomenon responsible for hyperalgesia (increased sensitivity to painful stimuli) and allodynia (pain in response to normally non-painful stimuli). This central component is modulated by glial cell activation and sustained release of excitatory neurotransmitters like glutamate and substance P [213].

Given the complexity of these mechanisms, effective pharmacological intervention often necessitates targeting multiple pathways involved in pain perception and inflammation [214]. Traditional non-steroidal anti-inflammatory drugs (NSAIDs) like ibuprofen and diclofenac have been widely used due to their COX-inhibitory actions, but their long-term use is limited by gastrointestinal, renal, and cardiovascular side effects [215]. As such, the search for safer and equally efficacious alternatives has led to the exploration of other chemical classes—among them, the pyrazolones.

Pyrazolones are a class of heterocyclic compounds known for their anti-inflammatory, analgesic, antipyretic, and antioxidant properties. Their therapeutic potential has been recognized since the introduction of phenylbutazone and metamizole (also known as dipyrone), which became widely used in clinical settings across Europe and Latin America. These agents act primarily through inhibition of prostaglandin synthesis, but emerging evidence indicates that their mechanism of action may extend beyond COX inhibition to include modulation of reactive oxygen species (ROS) and influence on endogenous opioid pathways [216, 217].

One of the most well-known pyrazolones, metamizole, is unique due to its dual action: it exhibits potent peripheral analgesic effects and central antipyretic actions. It is rapidly metabolized into active metabolites that inhibit COX enzymes and may also inhibit prostaglandin synthesis in the brain. Importantly, it has been shown to cause fewer gastrointestinal complications compared to traditional NSAIDs, possibly due to its selective action and minimal direct mucosal irritation [218]. Despite controversies related to agranulocytosis risk, the incidence appears

to be rare and regionally variable, suggesting genetic or environmental factors influence susceptibility.

Recent studies have also explored novel synthetic derivatives of pyrazolones with enhanced pharmacological profiles. These include hybrid molecules combining pyrazolone rings with other pharmacophores such as thiazolidinones, quinolines, or isoxazoles to enhance both anti-inflammatory and antioxidant activities [219].

Molecular docking and in vivo studies have shown that these derivatives exhibit high affinity for inflammatory mediators and attenuate oxidative stress markers, suggesting potential in treating acute inflammatory conditions [220, 221].

Moreover, experimental models of acute inflammatory pain—such as carrageenan-induced paw edema and formalin-induced nociceptive behavior in rodents—have demonstrated that pyrazolone-based compounds significantly reduce edema formation, inhibit leukocyte infiltration, and decrease expression of inflammatory cytokines like IL-6 and TNF-α. These findings underscore the multifaceted role of pyrazolones in modulating both the early and late phases of inflammation [222].

From a mechanistic perspective, one of the crucial advantages of pyrazolones lies in their antioxidant capability. During acute inflammation, excessive ROS production exacerbates tissue damage and pain perception. Pyrazolones have been reported to scavenge free radicals and upregulate endogenous antioxidant defenses such as glutathione and superoxide dismutase (SOD), which contribute to their protective effects [223]. This dual anti-inflammatory and antioxidant mechanism makes pyrazolones particularly valuable in conditions where oxidative stress is a significant contributor to nociception.

Importantly, the structure-activity relationship (SAR) of pyrazolones has been actively investigated, leading to identification of substituents that enhance selectivity and potency. For example, substitutions at position 3 and 5 of the pyrazolone ring have been associated with improved COX-2 selectivity, thereby potentially reducing adverse effects associated with COX-1 inhibition [224]. This SAR-guided optimization aligns with the current trend in drug discovery aiming for personalized, safer anti-inflammatory therapies.

Despite these promising findings, clinical translation of newer pyrazolone derivative remains limited due to regulatory hurdles, safety concerns, and variability in pharmacokinetics. Further clinical trials are essential to validate their safety profile, determine optimal dosing regimens, and explore their potential in combination therapy with other analgesics or immunomodulators. Given the limitations of existing analgesics, especially in acute surgical or trauma-induced pain, pyrazolones could fill an important therapeutic gap.

Inflammatory pain management relies significantly on pharmacological agents that modulate the biochemical pathways involved in the inflammatory cascade. The most commonly used agents include non-steroidal anti-inflammatory drugs (NSAIDs) such as ibuprofen, diclofenac, and celecoxib. While these drugs exert their effects primarily by inhibiting the cyclooxygenase (COX) enzymes—thereby reducing prostaglandin synthesis—they are often associated with adverse effects such as gastrointestinal irritation, renal dysfunction, and cardiovascular risks, particularly with long-term use.

Pyrazolone derivative, a distinct class of heterocyclic compounds, offer an alternative approach to inflammation control. Historically used in clinical practice with drugs like phenylbutazone and dipyrone (metamizole), these agents possess analgesic, anti-inflammatory, and antipyretic activities. Their clinical utility has been overshadowed in some countries due to safety concerns, particularly agranulocytosis. However, recent pharmacological advances and structural modifications have reignited interest in pyrazolones, especially due to their broader mechanism of action and potentially safer derivatives [178, 225].

Mechanistically, pyrazolones share some commonalities with standard NSAIDs. Both inhibit COX enzymes, thereby reducing prostaglandin-mediated sensitization of nociceptors. However, newer pyrazolone derivative exhibit additional mechanisms including inhibition of reactive oxygen species (ROS), suppression of pro-inflammatory cytokines like TNF-α and IL-6, and in some cases, modulation of nitric oxide (NO) pathways [226]. These broader pharmacodynamic effects give pyrazolones a potentially superior profile in conditions where oxidative stress is a contributing factor to inflammation and pain.

Comparative animal model studies have provided evidence supporting the anti-inflammatory potency of pyrazolones. For instance, in carrageenan-induced paw edema and formalin-induced nociception models in rats, pyrazolone derivative have demonstrated effects comparable to or greater than standard NSAIDs such as diclofenac or indomethacin, especially when modified with electron-donating or bulky side groups to enhance COX-2 selectivity and free radical scavenging [227, 228]. Moreover, pyrazolone were found to exert their effects more rapidly and with fewer gastrointestinal lesions compared to diclofenac, suggesting a potentially improved safety profile.

One of the critical differences lies in the selectivity for COX isoforms. Traditional NSAIDs are often non-selective, inhibiting both COX-1 and COX-2. COX-1 inhibition, while contributing to anti-inflammatory effects, is primarily responsible for gastrointestinal side effects due to its role in maintaining gastric mucosal integrity. Selective COX-2 inhibitors like celecoxib were developed to reduce such adverse effects but carry cardiovascular risks due to COX-2's involvement in vascular homeostasis [229]. Some pyrazolone derivative, particularly those modified for COX-2 selectivity, appear to strike a better balance between efficacy and safety, though further clinical validation is needed.

A notable advantage of pyrazolone especially metamizole, is their favorable gastrointestinal tolerability. Clinical observations have shown that metamizole causes fewer cases of dyspepsia, ulcers, or bleeding compared to ibuprofen or naproxen [230]. Furthermore, pyrazolones do not appear to impair renal perfusion as significantly as traditional NSAIDs, particularly under conditions of dehydration or compromised renal function, though more comparative studies are needed.

Nevertheless, concerns over hematological toxicity—especially the rare but serious risk of agranulocytosis—have limited the widespread use of some pyrazolones. This risk, however, appears to vary by population and genetic background. For example, agranulocytosis rates are notably low in Latin American and Southern European populations, where metamizole remains in routine clinical use [231]. The development of newer pyrazolone analogues with improved safety margins may overcome this limitation.

The pharmacological activity of pyrazolone derivative in inflammatory pain models is mediated through the modulation of several molecular targets and signaling pathways implicated in both peripheral and central sensitization. These compounds, beyond their classical cyclooxygenase (COX) inhibition, appear to act through a multi-target mechanism that includes suppression of pro-inflammatory cytokines, attenuation of oxidative stress, and possible interactions with nociceptive receptors.

One of the most established targets for pyrazolones is the cyclooxygenase-2 (COX-2) enzyme. This inducible isoform of COX is upregulated in response to inflammatory stimuli and is directly involved in the biosynthesis of prostaglandins such as PGE, which sensitize peripheral nociceptors and amplify inflammatory responses. Several pyrazolone analogues have demonstrated selective inhibition of COX-2, leading to reduced PGE production and subsequent analgesic and anti-inflammatory effects without the gastrointestinal complications commonly associated with COX-1 inhibition [232].

In addition to prostaglandin modulation, pyrazolones have shown an ability to interfere with key pro-inflammatory cytokines, notably tumor necrosis factor-alpha (TNF-α) and interleukin-1 beta (IL-1β). These cytokines play a crucial role in promoting endothelial adhesion, leukocyte migration, and peripheral sensitization during acute inflammation. Pyrazolone derivative have been reported to downregulate the gene expression and protein levels of these cytokines in preclinical models, indicating possible interference with nuclear factor kappa B (NF-κB) signaling—a master regulator of inflammatory gene transcription [233].

Oxidative stress is another important contributor to inflammatory pain, as reactive oxygen species (ROS) can sensitize ion channels and disrupt redox-sensitive signaling cascades. Pyrazolones possess intrinsic antioxidant activity, which contributes to their protective effects in inflamed tissues. This includes the scavenging of free radicals and upregulation of endogenous antioxidant systems such as superoxide dismutase (SOD) and glutathione peroxidase Finally, there is emerging evidence that some pyrazolone derivative may interact with transient receptor potential (TRP) channels and endogenous opioid receptors.

5.2 Conclusion

Pain remains a pervasive clinical and societal challenge, with inflammatory pain constituting a significant subset that demands effective and targeted therapeutic interventions. Despite the availability of numerous analgesics, limitations related to side effects, tolerance, and limited efficacy in specific patient populations have necessitated the development of novel anti-inflammatory agents with better safety profiles and broader mechanisms of action. In this context, the present study was designed to explore the analgesic and anti-inflammatory potential of pyrazolone derivative using well-established in vivo models, including carrageenan-induced paw edema, hot plate test, thermal allodynia assessment, and ELISA-based cytokine profiling.

The pyrazolone derivative demonstrated compelling anti-inflammatory and analgesic properties in acute inflammation models. Its mechanism appears to be multifaceted, involving both peripheral inflammation suppression and central pain threshold modulation. The compound's efficacy, particularly its ability to suppress edema and improve nociceptive and hypersensitivity thresholds, validates its therapeutic relevance. The study concludes that this pyrazolone derivative is a highly promising candidate for the treatment of acute inflammatory pain. However, the paradoxical TNF-alpha response at the higher dose underscores a critical need for further dose-response studies and detailed investigation into the specific molecular pathways affected at varying concentrations to ensure optimal clinical suitability and safety. The current investigation successfully demonstrated that pyrazolone derivative, particularly at lower doses, exhibit notable anti-inflammatory and analgesic effects in acute inflammation models. In the carrageenan-induced paw edema assay, both 5 mg/kg and 10 mg/kg doses of the pyrazolone compound resulted in a significant reduction of paw swelling. The attenuation of edema followed a dose- and time-dependent pattern, with the 10 mg/kg dose demonstrating effects comparable to those of diclofenac sodium, the reference standard. These findings suggest that the pyrazolone scaffold, previously known for its analgesic utility in historical drugs such as dipyrone, holds renewed promise as a basis for contemporary anti-inflammatory therapy. Consistent with the edema results, the hot plate

test revealed that pyrazolone derivative extended latency to thermal nociceptive responses, indicating central analgesic activity. Both doses produced measurable improvements in withdrawal latency, with the higher dose yielding a prolonged effect similar in magnitude to that observed with diclofenac. These results support the notion that pyrazolone compounds may exert dual actions—modulating peripheral inflammation and enhancing central pain thresholds. In the thermal allodynia model, which reflects sensitization to innocuous thermal stimuli, pyrazolone treatment significantly restored withdrawal latency in rats exposed to carrageenan. Notably, the 10 mg/kg dose showed a more substantial effect in the later hours post-administration, aligning with the typical time course of central sensitization and the involvement of neuroimmune pathways. The progressive recovery in pain thresholds observed in the treated groups indicates the potential of these compounds to modulate inflammatory hypersensitivity.

From a biochemical standpoint, ELISA quantification of TNF-α levels in serum provided compelling molecular evidence of the anti-inflammatory effect of the pyrazolone derivative. TNF-α, a pivotal cytokine in the inflammatory cascade, was significantly elevated in the carrageenan-only group, as expected. Administration of pyrazolone at 5 mg/kg led to a marked reduction in TNF-α concentration, reflecting its systemic anti-inflammatory efficacy. Interestingly, the 10 mg/kg dose did not replicate this reduction and in fact showed TNF-α levels comparable to or even higher than the untreated inflammatory group. This paradoxical response suggests that higher doses may activate alternative pro-inflammatory mechanisms or fail to suppress cytokine release effectively, underscoring the importance of dose optimization in therapeutic development.

Taken together, these findings support the therapeutic relevance of pyrazolone derivative in mitigating acute inflammatory pain. The compounds demonstrated consistent anti-inflammatory activity through suppression of paw edema, improved thermal nociceptive thresholds, and modulation of cytokine expression. Furthermore, their analgesic profile was evident in both reflexive and sensitization-based models, highlighting a multifaceted mechanism of action that potentially involves both central and peripheral targets.

Mechanistically, previous literature has implicated pyrazolones in the inhibition of cyclooxygenase (COX) enzymes and possible modulation of TRPA1 ion channels. The anti-nociceptive and anti-inflammatory effects observed in this study are consistent with such dual targeting, where inhibition of prostaglandin synthesis reduces peripheral inflammation, while TRPA1 antagonism modulates sensory neuron excitability. These mechanisms may explain the broad-spectrum analgesic response noted in various behavioral assays.

The differential performance between the two tested doses further highlights the necessity for careful pharmacodynamic assessment in drug development. While the 5 mg/kg dose showed balanced anti-inflammatory and analgesic efficacy with favorable cytokine modulation, the 10 mg/kg dose, though effective in behavioral assays, failed to lower TNF-α levels, suggesting a narrow therapeutic window. Future studies should explore whether this discrepancy arises from receptor desensitization, off-target effects, or saturation kinetics at higher concentrations.

In terms of translational relevance, the current results position pyrazolone derivative as attractive candidates for further development in the treatment of acute inflammatory pain. Given their efficacy in standard models and relatively favorable performance compared to diclofenac, they warrant additional exploration through mechanistic studies, chronic pain models, and ultimately clinical trials. It will also be critical to assess long-term safety, bioavailability, and potential interactions with other analgesic pathways to determine their viability as therapeutic agents.

Moreover, this research reinforces the value of integrated experimental approaches that combine behavioral, biochemical, and molecular analyses to comprehensively evaluate drug efficacy. The consistency across multiple pain models strengthens the validity of the findings and supports the hypothesis that pyrazolone derivative operate through multiple converging mechanisms to alleviate inflammatory pain.

In conclusion, the present study underscores the analgesic and anti-inflammatory potential of pyrazolone-based compounds in a rodent model of acute inflammation. The observed efficacy, particularly at lower doses, highlights their promise as

future therapeutics in pain management. However, the dose-dependent variability observed in cytokine regulation emphasizes the need for further pharmacological profiling. Overall, these findings contribute valuable insights into the evolving landscape of pain therapeutics and pave the way for the development of safer, more effective alternatives to traditional non-steroidal anti-inflammatory drugs.

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