EFFECTS OF ROLL-ON CONTAINING CANNABIS EXTRACT AND ISOLATED CANNABIDIOL ON NOCICEPTION IN MICE

NUTTHAKAN SEEJA

A Thesis Submitted to the Graduate School of Naresuan University
in Partial Fulfillment of the Requirements
for the Master of Science in Physiology
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By Nutthakan Seeja

has been approved by the Graduate School as partial fulfillment of the requirements

for the Master of Science in Physiology of Naresuan University

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Title	EFFECTS OF ROLL-ON CONTAINING CANNABIS EXTRACT AND ISOLATED CANNABIDIOL ON NOCICEPTION IN MICE
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Academic Paper	M.S. Thesis in Physiology, Naresuan University, 2023
Keywords	Muscle pain Cannabidiol Cannabis extract Analgesic effect Anti-inflammatory effect

ABSTRACT

Inflammation is a common cause of pain, associated with injuries, infections, diseases, or other health problem which declines in quality of life. Cannabis contains biological active compounds such as delta-9-tetrahydrocannabinol (Δ-9-THC) and cannabidiol (CBD). CBD has many beneficial pharmacological effects, including antinociception and anti-inflammation. This study aimed to investigate the antinociceptive and anti-inflammatory effects of the roll-on containing cannabis extract and isolated CBD on muscle pain models induced by carrageenan in male mice. Mice were applied the roll-on isolated CBD or cannabis extracts (0.5% and 1%) on gastrocnemius muscle for 19 hr. Then 3% carrageenan was used to induce muscle pain before evaluating nociceptive behavior elicited by heat and mechanical insults by using the hotplate test and von Frey filament test, respectively. Inflammatory markers (TNFα and IL-1β) and structural changes in muscle were measured by specific ELISA and Crystal violet staining, respectively. The results showed that mice treated with 1% roll-on isolated CBD, and 1% roll-on cannabis extract decreased mechanical (von Frey test) and thermal hyperalgesia (hot plate test) as well as reduced inflammatory markers TNF-α, IL-1β, and inflammatory cells. These results demonstrated that 1% roll-on cannabis extract and CBD reduced pain by modulating the inflammatory cytokines consequently alleviate the inflammatory cells.

ACKNOWLEDGEMENTS

Firstly, I would like to express my gratitude to my thesis advisor, Associate Professor Dr. Onrawee Khongsombat, and co-advisor, Associate Professor Dr. Neti Waranuch, for the supervision, guidance, and support throughout this study.

I am also grateful Associate Professor Dr. Aree Wanasuntronwong for assistance with technique and equipment and I would like to thank for their comments, guidance, and suggestion in my thesis defense.

I would like to thank Assistance Professor Dr. Narawadee Choompoo an internal examiner, for comments, guidance, and suggestion in my thesis defense.

I am also grateful to Mr. Eakkaluk Wongwad for his recommendation about the roll-on solution and for suggesting calculating the roll-on concentration solution and I am grateful to OK lab members for helping, suggesting, and teaching the particular methodology. That is important to make the thesis smooth.

For the laboratory facility and their kindness, I would like to thank the scientists and staff at the Department of Physiology, Medical Science Academic Centre, Faculty of Medical Science, Naresuan University.

Furthermore, I am also thankful to the Center of Excellence for Innovation in Chemistry (PERCH-CIC), the Office of the Higher Education Commission and the Faculty of Pharmaceutical Sciences, Naresuan University for supporting facilities. I also thank my colleagues in my laboratory unit for their assistance.

Lastly, I greatly appreciate and thank my family for their encouragement, support, and care throughout my life.

Nutthakan Seeja

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ABBREVIATION

2-AG	=	2-arachidonoylglycerol
AEA	=	Anandamide
ASICs	=	Acid-sensing ion channels
ATP	=	Adenosine triphosphate
BK	=	Bradykinin
CB1R	=	Cannabinoid receptor 1
CB2R	=	Cannabinoid receptor 2
CBD	=	Cannabidiol
CGRP	=	Calcitonin-gene-related peptide
CNS	=	Central Nervous System
COX	=	cyclo-oxygenase
DAGL	=	Diacylglycerol lipase
DRG	=	Dorsal root ganglion
ECS	=	Endocannabinoid system
ELISA	=	Enzyme-linked immunosorbent assay
FAAH	=	Fatty Acid Amide Hydrolase
IASP	=	International Association for the Study of Pain
IL-1β	=	Interleukin 1 beta
MPE	=	Maximum possible effect
NSAIDs	=	Nonsteroidal anti-inflammatory drugs
9-THC	=	9-tetrahydrocannabinol
PGE2	=	Prostaglandin E2
PNS	=	Peripheral Nervous System
SP	=	Substance P
TNFα	=	Tumor necrosis factor alpha
TRP	=	Transient receptor potential
TRPA1	=	Transient receptor potential ankyrin 1
TRPV1	=	Transient receptor potential vanilloi

CHAPTER I

INTRODUCTION

Cannabis has the ability to relieve the ailments of many major symptoms. Muscle pain is associated with injuries, infections, diseases, or other health problem. The aforementioned issues are associated with a significant decline in the quality of life (Alvarez et al., 2021; Luis F. Queme, 2017; Salaffi et al., 2005). Muscle pain starts with the activation of nociceptors by the proinflammatory cytokines TNFα and IL-1β. They develop simultaneously at the site of the inflammation, resulting in local changes (Chen et al., 2018; Zhang & An, 2007). Also, neuropeptide, a calcitonin-gene-related peptide (CGRP), is released which causes local edema and increases permeability. Second-order neurons carry nociceptive information from the spinal cord to the central nervous system, where it is processed and interpreted as pain (Basbaum et al., 2009). Inflammation sensitizes the sensory pathways, enhancing the pain response to a noxious stimulus (hyperalgesia) (Loram et al., 2007). In addition, the inflammatory response activates endogenous analgesic systems which included the endocannabinoid systems (Atalay et al., 2019; Goncalves et al., 2021; Piomelli, 2013). The endocannabinoid system also drives pain by decreasing nociceptors excitability through activating CB1 or CB2 receptors. Nonsteroidal anti-inflammatory drugs (NSAIDs), opioid analgesics, and antidepressant pharmaceuticals are currently the most often utilized medications to relieve pain (Botting, 1998; Grinspoon, 2020). It is commonly used as a first-line drug for defense of pain (Richard D. Blondell, 2013); however, many pain killers have side effects, and negative impacts on the body systems. Many complications may include: gastrointestinal irritation, liver and kidney toxicity, constrained in breathing, nausea, or vomiting (Botting, 2000; Ković et al., 2016). In a previous study, phytocannabinoids such as Cannabis *(Cannabis sativa L.)* were essential for pain relief.

Cannabis is a plant that is used for both recreational and medicinal purposes (Atalay et al., 2019; Starowicz & Finn, 2017). Cannabidiol (CBD) and Δ-9-tetrahydrocannabinol (Δ^9-THC) are two biologically active compounds found in cannabis (Mlost et al., 2020). The cannabis herb contains several pharmacological benefits which includes antinociceptive, anti-inflammatory, antioxidant, and neuroprotective properties that improve neuromodulator effects (Atalay et al., 2019; Comelli et al., 2008; Crivelaro do Nascimento et al., 2020; Menezes et al., 2021; Mense, 2008).

Chronic muscle pain is sign of a major medical problem. NSAIDs are drugs that relieve pain, but they have a risk of side effects. Recently, researchers have discovered that certain components of cannabis contain CBDs, which is a non-psychoactive compound. However, there is no reported research that examines the potential of roll-ons containing a cannabis extract or the isolated CBD used on an inflammatory muscle to alleviate pain. This scientific breakthrough relating to these potential alternative roll-ons as pain relievers prompted the discovery of an analgesic drugs that were drawn from natural herbal resources.

Objective

1. General objective

This study aims to investigate the antinociceptive and anti-inflammatory effects of roll-on containing cannabis extract or isolated CBD on muscle pain models in male mice.

2. Specific objectives

2.1. To study the antinociceptive and anti-inflammatory effects of roll-on containing isolated CBD or cannabis extracts as a topical application in muscle pain models in mice.

2.2. To study the effect of roll-on containing isolated CBD and cannabis extracts on inflammatory markers in muscle pain models mice (TNFα and IL-1β).

2.3. To study the effect of roll-on containing isolated CBD or cannabis extracts on histological changes of muscle in male mice

Scope of the Study

This study used the mice model of inflammatory muscle pain induced by carrageenan 3% concentration to investigate the anti-inflammatory and analgesic effects of isolated CBD and cannabis extracts. We applied the roll-on isolated CBD or cannabis extracts (0.5% and 1%) for 19 h before evaluating nociceptive behavior elicited by heat and mechanical insults by using the hotplate test and Von Frey filament test, respectively. Specific ELISA measured inflammatory markers (TNFα and IL-1β) for cytokines, and structural changes of muscle inflammation were evaluated by using hematoxylin and eosin.

Hypothesis

The roll-on isolated CBD or cannabis extracts (dose 0.5% and 1%) may relieve pain and inflammation in a model of muscular inflammation. Moreover, the inflammatory markers were also decreased in isolated CBD and cannabis extracts-treated mice groups.

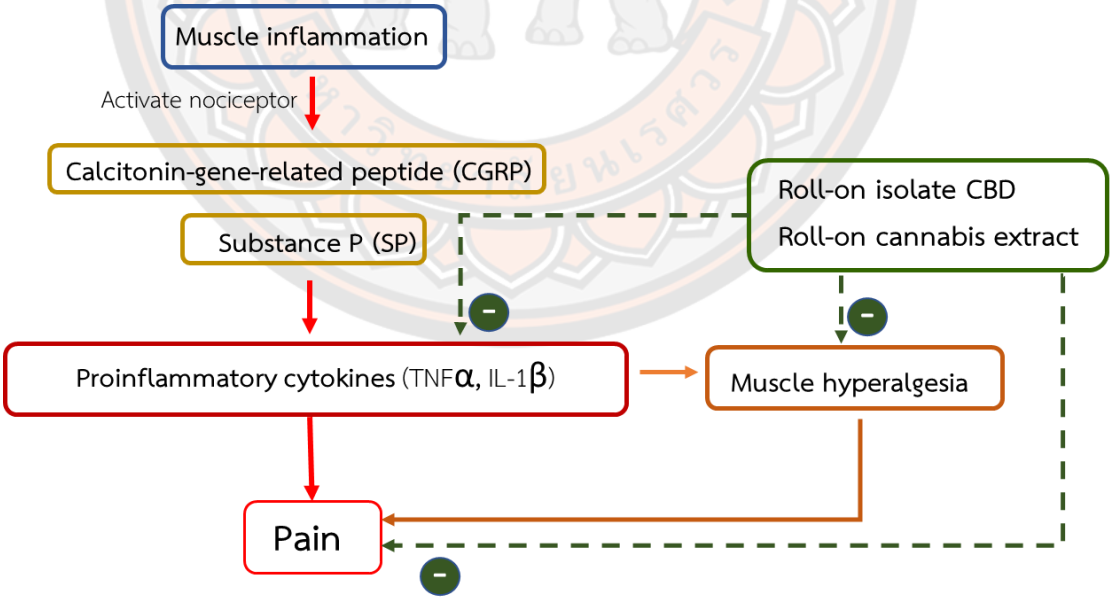

Figure 1 Research scope of effects of roll on containing cannabis extract and isolated cannabidiol.

Keywords

Muscle pain, Cannabidiol, Cannabis extract, Analgesic effect, Anti-inflammatory effect

CHAPTER II

LITERATURE REVIEW

Pain

Being in pain is a complex process because it involves more than just a physical sensation; it also has an emotional component that is influenced by one's beliefs, expectations, culture, and past experiences (Fong & Schug, 2014). "An unpleasant sensory and emotional experience associated with actual or potential tissue damage or described in terms of such damage," according to the International Association for the Study of Pain (IASP). Pain protects the body and prevents tissue damage as it recovers, but it may also become maladaptive and remain chronic pain (Stannard, 2012). The two most common types of pain are pathological pain (neuropathic and CNS dysfunctional pain) and physiological pain (nociceptive and inflammatory pain) (Woolf, 2010).

Physiological Pain

1. Nociceptive pain is the body's physiological reaction to noxious stimuli such as heat, cold, pressure, and chemical irritants to lessen or prevent injury.

2. Inflammatory pain occurs when the immune system, which includes mast cells, macrophages, neutrophils, and granulocytes, is activated due to tissue damage or infection (inflammatory soup) (Woolf, 2010).

3. Nociceptive and inflammatory pain are the most common areas in the musculoskeletal system (Dresden, 2017).

Pathological Pain

1. A lesion or illness of the somatosensory nervous system causes neuropathic pain (e.g., painful peripheral neuropathy, poststroke, and multiple sclerosis).

2. There is no such damage or inflammation in CNS dysfunctional pain (e.g., fibromyalgia, irritable bowel syndrome, and interstitial cystitis) (Fong & Schug, 2014).

Pain pathway

Nociceptors detect pain impulses, which are detached nerve endings in tissues and organs. They have high thresholds and, under typical conditions, only react to unpleasant stimuli. The pain mechanism goes through four steps when noxious stimuli are present: transduction, transmission, modulation, and perception (McEntire et al., 2016).

1. Transduction occurs when a stimulus, such as pressure, thermal energy, or chemical irritation, is converted into a nerve signal or action potential. Nociceptors are free nerve endings that are widely dispersed throughout the body and spread between epidermal cells, the musculoskeletal system, and the viscera. Nociceptors detect external stimuli like heat, cold, mechanical force, or chemical irritants and convert them into electrical signals that can be perceived as pain. Without transduction, the human body would not be able to taste, touch, hear, see, or experience pain. Transduction is a crucial step in external processing stimuli (McEntire et al., 2016). There are two types of nociceptors.

1.1. The A fibers are heavily myelinated and fast conduction velocity. They well-localized acute or pricking sensation that lasts as long as high threshold mechanoreceptors stimulate the stimuli.

1.2. The C fibers are unmyelinated and have a small diameter and slowly conduction. Thermal and chemical stimuli, such as hydrogen ions, potassium ions, bradykinin, serotonin, adenosine triphosphate, and prostaglandins, could stimulate nociceptors (Botting, 2000).

A painful physical or chemical stimulus is transformed into a signal that can be transmitted to the brain and interpreted as pain. Pain is transmitted by specific ion channels, including TRP, ASIC, and voltage-gated sodium channels (McEntire et al., 2016)

2. Transmission is the process that transmits information of pain through neurotransmitters at the axon terminal. Voltage-gated calcium channels are located on the presynaptic membrane and are essential in neurotransmitter release at the dorsal horn and are also present in nociceptors (Taylor, 2009). A-fibers are fast conducting and transmit the first sharp pain on activation. They mainly terminate in lamina I, but some fibers also go to the dorsal horn's lamina V, where they synapse with second-

order neurons. L-glutamate is a neurotransmitter found in them. C fibers are unmyelinated slow conducting fibers that convey a continuous aching pain that is less well targeted and lasts after the original stimulus has passed. They come to an end at the dorsal horn's lamina II. The spinothalamic tract is responsible for transmission signaling. It is a sensory tract that conveys nociceptive, temperature, rough touch, and pressure information from our skin to the thalamus's somatosensory region. Two anterior and lateral routes are responsible for our fast withdrawal reaction to a painful stimulus. The sensory information concerning crude touch and temperature is carried through the anterior spinothalamic tract. The lateral spinothalamic tract transmits pain and temperature signals (Gupta.v, 2021).

3. Modulation refers to the process by which the body modifies a pain signal as it travels down the pain pathway. It explains why the activation of pain neurons and the subjective experience of pain do not always coincide (Kirkpatrick et al., 2015). "Gate Control Theory" by Melzack and Wall from 1965 discussed pain modulation. The substantia gelatinosa (lamina II) of the dorsal horn functions as a "gate" in the spinal cord, with its opening and shutting controlled by several neural pathways (figure 2A). For instance, when the c fibers are activated, the inhibitory interneuron is suppressed, which decreases the probability that the projection neuron will fire. By "opening the gate" activates the pain pathways. In contrast, figure 2B demonstrates that stimulating the A fibers activated the inhibitory interneuron, reducing excitation and increasing inhibition Cluett, 2019).

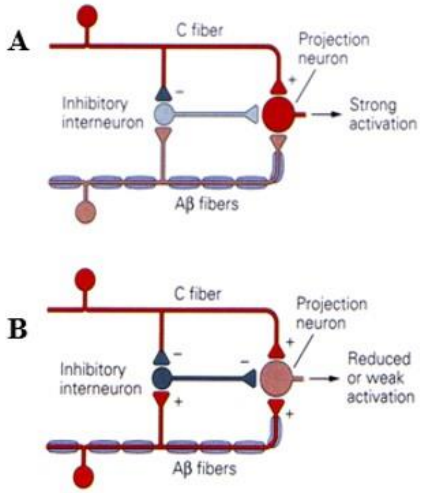

Figure 2 Gate Control Theory

(A) The activation of pain pathways; (B) The weak activation of pain pathways

Source: Cluett (2019)

4. Perception, the brain causes pain by receiving information for processing and action via the activation of the sensory cortex.

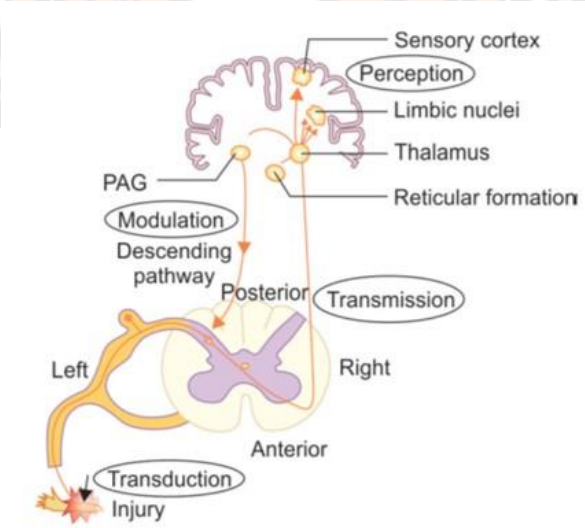

Figure 3 Pain pathway

Source: Das and Sharma (2018)

Peripheral and central sensitization are brought on by the local action of inflammatory mediators such as prostaglandins, histamine, bradykinin, potassium, hydrogen ions, 5-hydroxytryptamine, adenosine triphosphate, and nitric oxide produced by injured and immunological cells. Terminal membrane ion channels and nociceptors are phosphorylated, lowering the threshold and increasing neuronal excitability. Primary hyperalgesia is a kind of hypersensitivity that reduces the stimulus intensity required to activate nociceptors at the site of inflammation. As a consequence of central sensitization, there is an increase in excitability, which causes pain amplification. Noxious peripheral stimuli induce a significant number of action potentials. Consequently, synaptic activity increases in the dorsal horn (Botting, 2000; Das & Sharma, 2018).

Muscle pain

Muscle pain is a primary medical concern (60 percent to 85 percent). Its pain is among the most prevalent among humans. Because it is a leading cause of disability and lost productivity in the workplace, it is a clinical condition that places a significant financial burden on patients and society. Muscle discomfort is induced by repeated, sustained contractions (de Azambuja et al., 2020; Mense, 2008).

Muscle pain is induced by activating receptors that detect stimuli that might cause tissue damage and are subjectively seen as unpleasant. They consist of nerve terminals that are not myelinated and connect to the central nervous system (CNS). Strong mechanical stresses, such as trauma or mechanical overloading, may activate inflammatory mediators such as bradykinin (BK), serotonin, and prostaglandin E2 (PGE2), which are endogenous. ATP and protons (H+ ions) are chemical substances that stimulate nerve endings by binding to receptor molecules in the membrane of the nerve ending. ATP stimulates muscle nociceptors through binding to the P2X3 receptor molecule, while H+ activates transient receptor potential vanilloid 1 (TRPV1) and acid-sensing ion channels (ASICs) (Luis F. Queme, 2017; Noma et al., 2013). These receptor molecules are nerve ending channel proteins that allow Na^+ ions to enter the neuron. The ions Na^+ cause neuronal excitement. When biological tissues of any kind are harmed, ATP is released from all cells in the body.

Muscle nociceptors are also activated by weakly acidic solutions (pH 6 to 5). One of the major activators of peripheral nociceptors is a reduction in pH because

NGF is generated in muscle and stimulates muscle nociceptors, which is linked to muscular pain. It is also raised when a muscle is inflamed.

The calcitonin gene-related peptide (CGRP) and substance P (SP) are produced by nerve terminal stimulation. These generated local edema and increased permeability. A nociceptor may alter the microcirculation in its immediate proximity by releasing neuropeptides. Muscle lesions generate endogenous compounds such as BK, produced from plasma proteins by the enzyme kallikrein, and prostaglandins E2, produced from arachidonic acid by the enzyme cyclooxygenase. These two stimulating substances increase the sensitivity of nociceptors to external stimuli (peripheral sensitization) (Basbaum et al., 2009; Mense, 2008).

The excitability of posterior horn neurons is increased more by muscle nociceptors in the spinal cord than cutaneous nociceptors. As a result, the sensory neurons in the spinal cord are overexcited, which contributes to the spread of excitement. In the membranes of spinal neurons, glutamate affects NMDA (N-methyl-D-aspartate) receptors, while substance P affects neurokinin 1NK1 () receptors (central sensitization). Overexcitability of spinal nociceptive neurons is caused by two major processes (Mense, 2008).

Inflammation

Inflammation is a localized physical condition in which a bodily part becomes red, swollen, heated, and often painful. The inflammatory response, the body's response to infection or damage, is a highly effective defense and healing mechanism generated and sustained by the interaction of chemical mediators and cells. Blood artery dilation and increased permeability produce heat, redness, and swelling, enabling white blood cells and reparative proteins to collect in the wounded area. Damaged tissue and immune cells emit substances that cause pain when they are activated (Botting, 2000).

When inflammation happens, pro- and anti-inflammatory mediators grow concurrently, resulting in local alterations. Chemicals from your body's white blood cells enter your blood or tissues to protect your body from attackers. The chemical mediators released vasoactive amines such as histamine, serotonin, kinins, lipid mediators, prostaglandins, and the chemotactic leukotriene B4 (LTB4). These caused

local vasodilation and increased vascular permeability. The white blood cells accumulate locally released substances that amplify pathological changes and cause pain. Mast cells and resident tissue macrophages produce tumor necrosis factor- (TNF) during the acute phase of inflammation. This cytokine stimulates the production of the interleukins IL-1, IL-6, and IL-8 (Chen et al., 2018; Zhang & An, 2007). They induce vascular endothelial cells to create adhesion receptors that bind to endothelial cell surface glycoproteins and activate receptors on the leucocyte membrane, producing potent adhesion molecules that firmly attach the cell to the endothelial cell surface. When receptors are triggered, intracellular second messenger systems are stimulated, which causes cell migration into extravascular tissue. Chemokines are a class of cytokines that induce an influx of white blood cells in areas of inflammation. However, monocyte chemotactic protein-1 (MCP-1) and macrophage inflammatory protein-1 (MIP-1) stimulate the development of monocytic cells in the later chronic phase. TNF and other cytokines sustain the inflammatory process, and kinins play a crucial role in both the initiation and maintenance of inflammation. Kinins induce pain and vasodilation by increasing capillary permeability and releasing prostaglandins through the ubiquitous B2 receptor (Mlost et al., 2020; Zhang & An, 2007).

Pro-inflammatory cytokines

TNF regulates apoptotic pathways, activates NF-kB, and activates stress-activated protein kinases (SAPKs) through various signaling pathways, including binding receptors, TNFR1 and TNFR2. TNF-α receptors can be found in neurons and glia. Both inflammatory and neuropathic hyperalgesia has been linked to TNF-α.

IL-1β is produced mainly by monocytes and macrophages but also by non-immune cells; it is expressed in nociceptive DRG neurons and is increased in microglia and astrocytes in the central nervous system following peripheral nerve crush damage and trauma (CNS). Hyperalgesia is a side effect. Furthermore, in neuronal and glial cells, IL-1β was observed to stimulate the synthesis of the substance P and prostaglandin E2 (PGE2). IL-1ra, an antagonist of the IL-1 receptor, binds to the same receptor as IL-1β but does not transmit a cellular signal.

The neuronal response to nerve damage has been demonstrated to be dominated by IL-6. In addition, IL-6 is also involved in the activation of microglia and astrocytes and controlling neuropeptide production in neurons (Urban, 2001).

Hyperalgesia

Activating adenylate cyclase and phospholipase C by activating prostanoid receptors on sensory afferents results in the activation of protein kinase A and protein kinase C. These protein kinases modify membrane ion channels by blocking outward K+ currents and lowering the activation threshold of tetrodotoxin-resistant Na+ channels, hence increasing cell excitability (Botting, 2000).

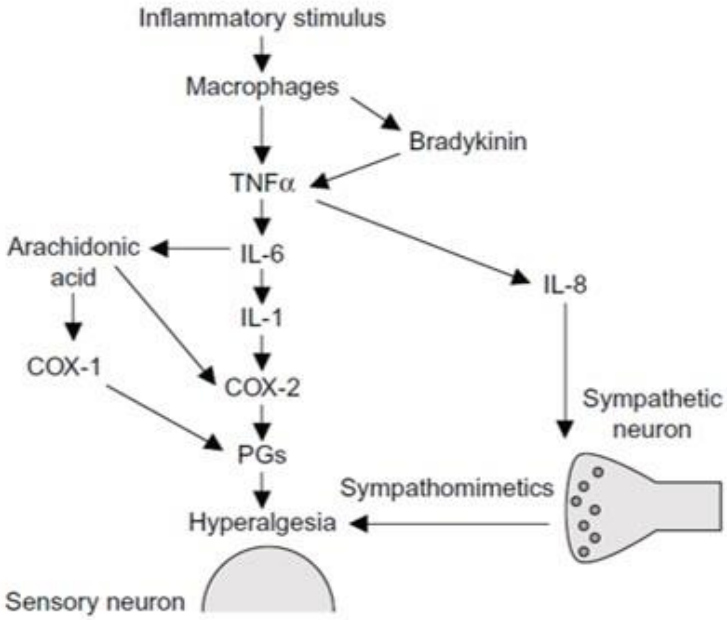

Figure 4 Development of hyperalgesia after inflammation

COX = cyclo-oxygenase; IL = interleukin; PGs = prostaglandins; TNFα = tumor necrosis factor-α.

Source: Botting (2000)

Treatment of pain

Nonsteroidal Anti-Inflammatory Drugs (NSAIDs)

NSAIDs act by inhibiting COX enzymes and reducing the production of prostaglandins. COX-2 produces pro-inflammatory prostaglandins that cause inflammatory mediators pain and edema. COX-1 generates the prostanoids thromboxane (TX) A2, which induces platelet aggregation, prostaglandin (PG) I2 inhibits intravascular aggregation and is cytoprotective for the gastric mucosa, and PGE2, which maintains renal blood flow. Although nonselective COX inhibition shows the relevance of prostaglandins in inflammatory hyperalgesia, therapeutic utility is limited due to strong gastrointestinal side effects. The activities of prostaglandins can be significantly decreased by selective receptor blockage to avoid difficulties associated with COX. The most promising strategy employs EP receptor antagonists, which are found in sensory neurons and are triggered by PGE2. Once activated by the inflammatory prostanoid PGI2, IP receptors may potentially contribute to the development of inflammatory hyperalgesia. These medications cause negative effects on a variety of physiological systems, including gastrointestinal irritation, liver and kidney damage, constricted breathing, nausea, and vomiting (Botting, 1998; Urban, 2001).

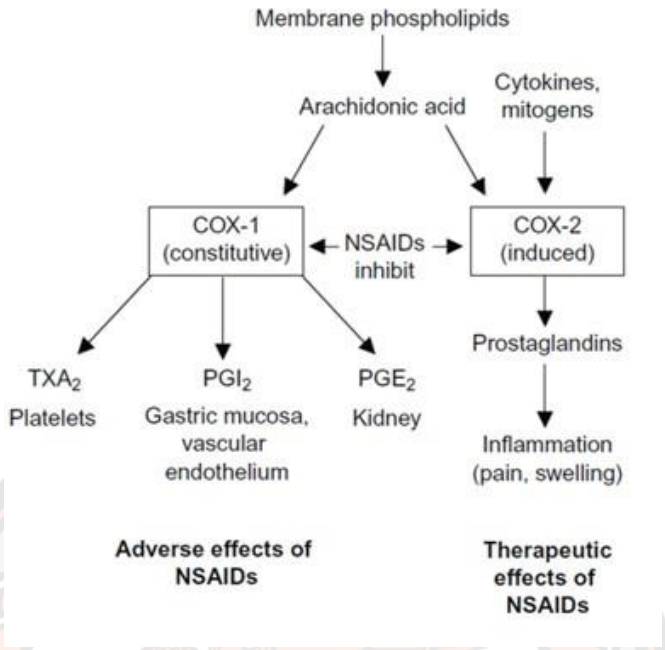

Figure 5 Mechanism of NSAID

Source: Urban (2001) (Urban, 2001)

Opioid drugs

Immune cells produce opiates, and opioid receptors are found on the membranes of neuronal cells in peripheral organs. The three primary varieties are Delta opioid receptors, Kappa opioid receptors, and Mu opioid receptors. Primary sensory neurons deliver pain signals to the brain when pain is triggered by prospective or actual tissue injury. Opiates act by binding to brain receptors and inhibiting the release of neurotransmitters from the spinal cord's main afferent terminals. This produces a pain-relieving feeling and a calming and sedative effect (Grinspoon, 2020).

Endocannabinoid

The endocannabinoid system (ECS) is comprised of the endogenous cannabis-like ligands. There are anandamide (AEA) and 2-arachidonoylglycerol (2-AG), which are derived from arachidonic acid and bind to a family of G-protein-

coupled receptors called cannabinoid receptor 1 (CB1R) and cannabinoid receptor 2 (CB2R). The CB1R receptor is widespread in the brain and is associated with motor control, emotional responses, motivated behavior, and energy balance. In peripheral adipose tissue, pancreas, liver, GI tract, skeletal muscles, heart, and reproductive organs, the receptor suppresses the production of pro-inflammatory mediators, lowering inflammatory responses. CB2R is primarily present in the immune system, modulating its functions. On demand, endocannabinoids are generated and released in a receptor-dependent way. In GABAergic and glutamatergic synapses, they act as retrograde signaling messengers and modulators of postsynaptic transmission. Fatty acid amide hydrolase (FAAH) and monoacylglycerol lipase (MAGL) degrade endocannabinoids, which are transported into cells through a specific uptake mechanism. The ECS is engaged in mammalian reproductive, cardiovascular, gastrointestinal, immunological, behavioral, and antiproliferative processes (Mouslech & Valla, 2009).

Endocannabinoid synthesis

Even though AEA and 2-AG include arachidonic acid, their generation and breakdown routes are almost similar. AEA is mainly derived from N-arachidonoyl phosphatidyl ethanol (NAPE), while 2-AG is derived from 2-arachidonoyl-containing phospholipids, such as arachidonoyl-containing phosphatidyl inositol bis-phosphate (PIP2). In addition to acting as an endogenous ligand for cannabinoid receptors, 2-AG is a significant metabolic intermediate in lipid synthesis and a major source of arachidonic acid in prostaglandin formation essential for 2-AG biology (Barrie & Manolios, 2017; Lu & Mackie, 2016).

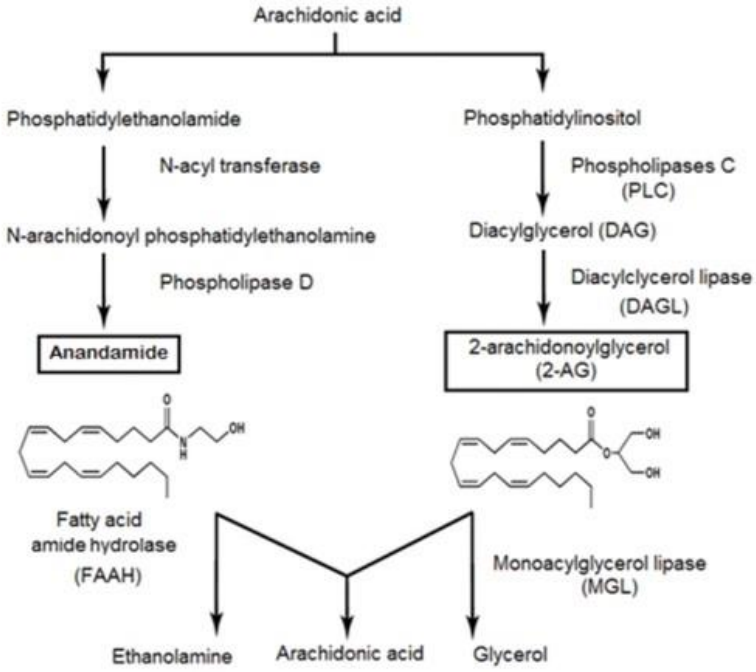

Figure 6 Synthetic and degradative pathways for anandamide and 2-AG

(Barrie & Manolios, 2017)

Source: Barrie and Manolios (2017)

These endocannabinoids are characterized by the presence of their precursors in lipid membranes. Endocannabinoids are liberated into the extracellular environment by one or two rapid enzyme reactions. On the other hand, classic neurotransmitters are produced beforehand and stored in synaptic vesicles. 2-AG is a high-efficacy agonist for CB1 and CB2 receptors, while anandamide is a low-efficacy agonist for CB1 receptors and a very low-efficacy agonist for CB2 receptors (Lu & Mackie, 2016).

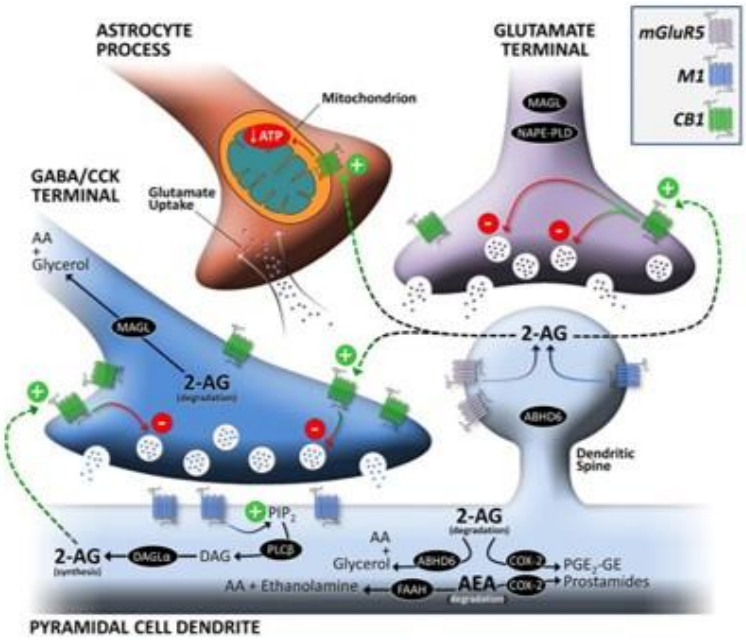

Figure 7 Synaptic localization of endocannabinoid system components
CCK, cholecystokinin; COX-2, cyclooxygenase-2; ABHD6, alpha/beta domain-containing hydrolase 6; DAGLα, diacylglycerol lipase α; M1, M1 muscarinic receptor; MAGL, monoacylglycerol lipase; mGluR5, metabotropic glutamate receptor 5; NAPE-PLD, N-arachidonoyl phosphatidyl ethanolamine-preferring phospholipase D; PLCβ, phospholipase C β.

Source: Lu and Mackie (2016)

Endocannabinoid Signaling

On presynaptic neurons, cannabinoid receptors govern the creation and release of neurotransmitters to the synapse. Neurotransmitters are released into vesicles when presynaptic neurons generate action potentials. The binding of neurotransmitters to postsynaptic receptors results in a depolarization of the postsynaptic membrane and a buildup of Ca2+ in the cytoplasm, stimulating the calcium-dependent enzymes responsible for endocannabinoid production phospholipase (PL) and diacylglycerol lipase (DAGL). After synthesis, endogenous cannabis ligands are released and diffuse inside the synapse, acting as retrograde messengers to regulate the release of

presynaptic messengers locally. CB receptor binding suppresses presynaptic Ca2+ influx via voltage-gated Ca2+ channels in a G-protein-dependent way. Binding endocannabinoids activate the enzymes PI3 kinase, sphingomyelinase, and phospholipase. The outcome is hyperpolarization of the presynaptic membrane, which modifies neurotransmitter release and synaptic transmission. Activation of CB1 receptors regulates both the amplitude and duration of synaptic transmission. It has been proven that CB2 activation inhibits inflammatory mediator activation, cell motility, and immune cell secretion. Endocannabinoids are selective transporters broken down by specialized enzymes (FAAH, MAGL) (Flores et al., 2013).

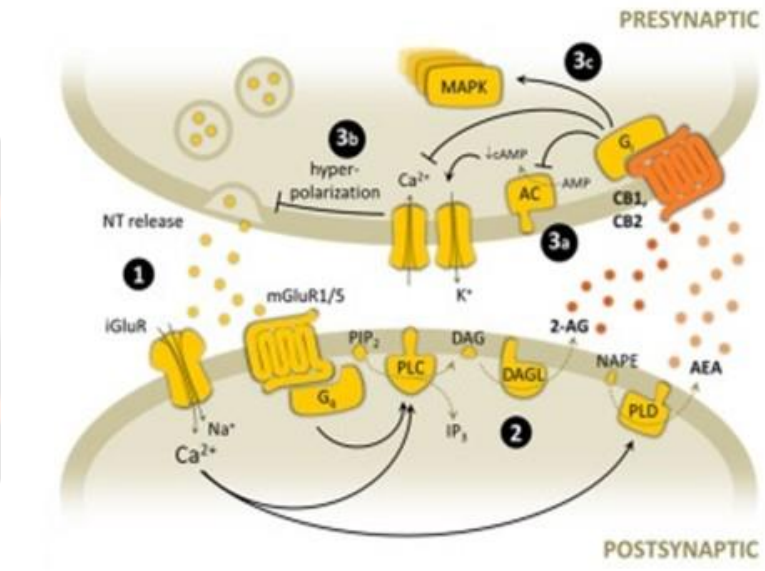

Figure 8 The synaptic signaling mediated by endocannabinoids.

(1) Presynaptic glutamate is released. (2) High Ca2+ concentrations increase endocannabinoid synthesis through PLC and PLD. 2-AG synthesis also involves Gq-protein activation (3) CB1 and CB2 presynaptic receptors are activated when endocannabinoids are released into the synaptic cleft by: (3a) inhibition of AC activity; (3b) hyperpolarization of the membrane after manipulation of the K+ and Ca2+ channels; and (3c) activation of protein kinase pathways such as the MAPK pathway.

Source: Flores, Maldonado and Berrendero (2013)

Cannabinoid Receptor 1

Numerous portions of the brain and supraspinal areas involved in nociceptive transmission include a high concentration of receptors for the central nervous system (CNS) (Tang et al., 2021) , which area in the brain, basal ganglia, hippocampus, dorsal root ganglion (DRG), spinal cord, thalamus, periaqueductal gray (PAG), and amygdala. Recently, it was shown that CB1 is abundant in the frontolimbic brain, which is implicated in pain's affective and emotional elements. Furthermore, it has been shown that CB1 inhibition of ascending nociceptive transmission, which mainly occurs in the thalamus, influences emotional pain components in limbic and cortical regions. CB1 inhibits GABA production in the paraamygdala and rostral ventral medulla, inhibiting descending input to the spinal cord nociceptive system, hence acting in the supraspinal descending inhibitory pathway. In addition, CB1 is widely expressed in the presynaptic terminals of primary afferents and excitatory neurons in the spinal cord, where it controls the transmission of unpleasant stimuli to the brain and inhibits neurotransmitter release.

Cannabinoid Receptor 2

Immune cells include many receptors, making them a suitable starting point for influencing how inflammatory pain is processed (Tang et al., 2021). CB2 receptor agonists decrease the local release of pro-inflammatory chemicals by non-neural cells in several inflammatory and nociceptive pain models, hence achieving anti-nociception. For example, in neuropathic pain or inflammatory hyperalgesia, activation of peripheral CB2 receptors causes antinociceptive responses by acting locally on peripheral immune cells and CNS microglia.

Cannabis *(Cannabis sativa L.)*

Cannabis sativa L. is a dioicous Cannabaceae plant that is extensively dispersed worldwide. It has been used as a psychoactive substance and a component of traditional medicine (Pellati et al., 2018). Delta-9-tetrahydrocannabinol (Δ-9-THC), the plant's main psychoactive and toxicant ingredient, and cannabidiol (CBD), a nonpsychoactive chemical, make up a broad group of physiologically active substances present in cannabis sativa (Andre et al., 2016; Mlost et al., 2020).

CBD is a phytocannabinoid that has pharmacological activity. It is nonpsychoactive but has several pharmacological benefits, including antinociceptive, anti-inflammatory, antioxidant, neuroprotective, and neuromodulator properties (Atalay et al., 2019; Comelli et al., 2008; Crivelaro do Nascimento et al., 2020; Menezes et al., 2021; Mense, 2008). CBD acted as an agonist on transient receptor potential vanilloid-1 (TRPV1) channels which desensitization of TRPV1 (Philpott et al., 2017) and CBD-binding cannabinoid receptor agonists inhibit nociception in inflammatory pain. Also, CBD has a low affinity for both CB1 and CB2 receptors and is a powerful antagonist of CB1 and CB2 receptor agonists. CBD's anti-inflammatory benefits may be the result of its function as an inverse agonist of the CB2 receptor. CBD has a negative allosteric modulating effect on both CB receptors. CBD may act as an agonist of transient receptor potential channels (TRPA1, TRPV1, TRPV2, and TRPV3), the peroxisome proliferator-activated receptor (PPAR), the 5-HT1A (serotonin 1A) receptor, and the adenosine A1 and A2 receptors, and as an antagonist of G-protein-coupled receptors (GPR55 and GPR18) and 5-HT3 receptor A. CBD is also a G-protein-coupled receptor inverse agonist (GPR3, GPR6, and GPR12) (Kopustinskiene et al., 2022).

THC has a strong affinity for the CB1 and CB2 cannabinoid receptors and is a powerful partial agonist. THC's analgesic and primary psychoactive actions are both mediated by CB1 receptors. The immunomodulatory effects of THC are due to its interaction with CB2 receptors. THC can also act as an antagonist of the transient receptor potential channel TRPM8 and 5-HT3 receptor A, as well as an agonist of the G-protein-coupled receptors GPR55 and GPR18, the peroxisome proliferator-activated receptor (PPAR), TRPA1, TRPV2, TRPV3, and TRPV4. It can also raise anandamide and adenosine levels (Almogi-Hazan & Or, 2020; Maccarrone et al., 2015). THC is the primary intoxicating component of cannabis, and its capacity to do so creates a surge of dopamine in the brain, resulting in the "high." THC intoxication has been proven to increase blood flow to the prefrontal cortex, which is essential for decision-making, attention, motor abilities, and other processes in the brain. The precise nature of THC's impact on these functions will differ from one individual to the next. THC can cause sensations of euphoria when it binds to CB1 receptors in the

brain reward system. THC reduces pain primarily due to its interaction with midbrain CB1 receptors (Romero-Sandoval et al., 2017).

The CB1 and CB2 are the two kinds of cannabinoid receptors. CB1 is found in central and peripheral neurons, whereas CB2 is found in immune cells and has a non-neuronal origin. The CB1 receptor is negatively linked to adenylate cyclase, preventing excitability and primary afferent activation. The activation of the CB2 receptor may have antinociceptive effects by inhibiting immune cell activity, as discussed in the next section (Mlost et al., 2020; Mouslech & Valla, 2009). THC is another pharmacologically active phytocannabinoid that has a different effect on the endocannabinoid system than CBD.

Previous research delivered CBD gel to animals for four consecutive days. The rats received daily dosages of 0.6, 3.1, 6.2, or 62.3 milligrams (mg). According to the researchers, CBD reduced inflammation and overall pain in the rats diseased joints with no discernible side effects. Low-dose CBD of 0.6 or 3.1 mg/day cannot reduce pain. The researchers observed that 6.2 mg/day reduced pain and edema in rats effectively. In addition, animals receiving 62.3 mg/day had outcomes comparable to rats receiving 6.2 mg/day. They did not feel less pain after obtaining a much larger dose. People with Arthritis may benefit from CBD gel's anti-inflammatory and pain-relieving qualities. However, further human research is necessary (Hammell et al., 2016).

Moreover, the antihyperalgesic efficacy of a Cannabis sativa extract is investigated by Comelli et al. (2008). In a neuropathic pain model, a standardized extract of Cannabis sativa has a high concentration of CBD, the plant's nonpsychoactive component, and a tiny concentration of THC and other minor cannabic and non-cannabic components totally alleviated thermal hyperalgesia (Comelli et al., 2008).

Shebaby et al. utilizing carrageenan and formalin-induced paw edema rat models, researchers evaluate the phytochemical and anti-inflammatory properties of cannabis oil. The results indicate that cannabis oil extract effectively inhibited TNF-release in Lipopolysaccharide-stimulated rat monocytes and Western blot analysis demonstrated a reduction in COX-2 levels (Shebaby et al., 2021). Also, the anti-inflammatory properties of Cannabis sativa leaf extracts on in vitro synovitis were

examined by Duangnin et al. Cannabis Sativa leaf extracts were shown to reduce the production of i-NOS, COX-2, and IL-1 in the synovial fibroblast cell line SW982 (Duangnin et al., 2017).

Carrageenan

Carrageenan is a sulfated polysaccharide produced from seaweed. Nonsteroidal anti-inflammatory medications and selective COX1-2 inhibitors have both benefited from its usage. Carrageenan is divided into three types: lambda- (λ), kappa- (κ), and iota- (ι) (Guay et al., 2004; J. Necas, 2013). Lambda has traditionally been a stimulant in vivo model of local inflammatory responses. Localized edema, infiltration of white blood cells, and elevated levels of local PGE2 occur after injection (Myers et al., 2019). After carrageenan injection, the early phase peaks after 3 hours, while the delayed phase peaks at 48 hours (Dzoyem et al., 2017; Huang et al., 2011). Neutrophophil invasion, the creation of neutrophil-derived free radicals, and the release of additional neutrophil-derived mediators are all part of the Carrageenan-induced inflammatory response. According to a prior study, the inflammatory impact generated by carrageenan is linked to free radicals. Following Carrageenan injection, free radicals, prostaglandins, and NO will be released. Free radicals assault the plasma membrane, resulting in MDA formation. As a result of the inflammatory effects, MDA levels would rise. GSH is a scavenger of oxyradicals. MDA is reduced when GSH levels are increased in favor. Carrageenan-induced local inflammation is combated in part by endogenous GSH. These ROS have been postulated to mediate cell damage independent methods in numerous pathophysiological circumstances linked with inflammation or oxidative stress, including the beginning of lipid peroxidation, the inactivation of several antioxidant enzymes, and glutathione depletion. Inducible iNOS and COX-2 produce pro-inflammatory mediators NO and PGE2 during inflammatory events. INOS is not seen in normal cells but is produced by various stimuli such as LPS, TNF-, and IL-1. COX-2, on the other hand, is increased in cells in inflamed regions in vivo by pro-inflammatory stimuli such as LPS and cytokines. Furthermore, in inflammation models, COX-2 is the isoform responsible for the generation of pro-inflammatory prostaglandins (PGs). The actions of iNOS and COX-2. Macrophages and neutrophils in the inflammatory response

release mediators (eicosanoids, oxidants, cytokines, and lytic enzymes) that are responsible for the development and persistence of acute and chronic inflammation. The most significant of these mediators is NO, which is generated by COX-2 and iNOS in macrophages. COXs are pro-inflammatory enzymes that play a role in arachidonic acid metabolism and impact biological processes, including tissue repair and immunological responses linked to inflammation. The rate-limiting enzymes in the creation of PGE2 are COX-1 and COX-2. COX-1 is a constitutively produced enzyme that plays a role in the acute inflammatory response, whereas COX-2 is only found in specific cells (i.e., macrophages, monocytes, and neutrophils). The transcription factor NF-B is recognized for regulating the production of pro-inflammatory enzymes and cytokines such as iNOS, COX-2, and TNF-α. NO is also responsible for edema production at the site of inflammation, vasodilation, and increased vascular permeability. A broad spectrum of hazardous oxidative processes causes tissue harm, including NO, superoxide, and the products of their interaction (Guay et al., 2004; Huang et al., 2011).

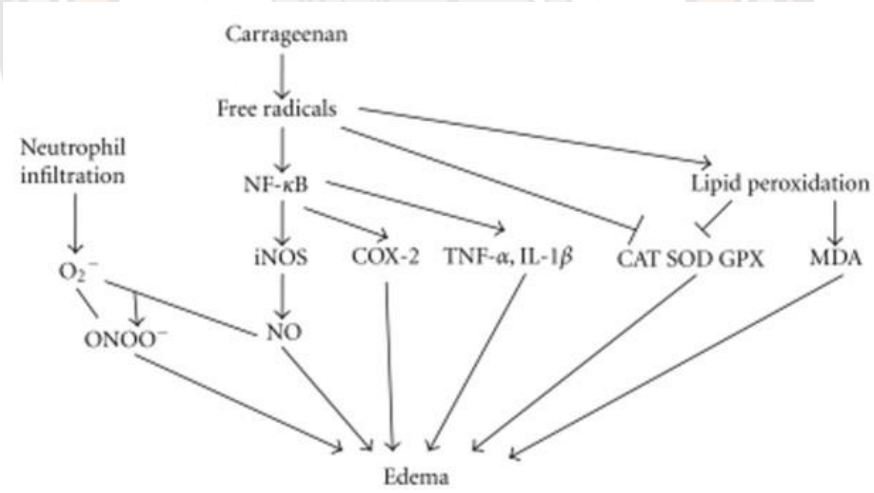

Figure 9 The mechanism of carrageenan

Source: Huang et al. (2011)

Voltaren

Voltaren active component is diclofenac sodium, a nonsteroidal anti-inflammatory medication created by selecting the right physicochemical and steric qualities. NSAIDs are helpful therapeutic drugs because they suppress prostaglandin production at COX enzymes, which reduces pain, fever, and inflammation (Delgado-Charro, 2022; Dietrich W. Scholer, 1986). COX enzymes come in two varieties: COX-1 is expressed in all cells by default, but COX-2 is typically increased at the site of tissue damage, yet the two have unique physiological functions. COX-1 enzymes regulate GI cytoprotection, platelet function, and renal function by producing equal quantities of thromboxane (TXA2) and prostacyclin (PGI2) to maintain a balance. On the other hand, COX-2 enzymes are involved in inflammation, pain, and fever, making them ideal targets for pain management and prostaglandin production. COX-2 is activated by cytokine release in response to injury and inflammation. However, it is also found in the brain, kidneys, and blood arteries, all vulnerable to thrombotic events (Atkinson & Fudin, 2020).

Transdermal CBD or cannabis extract

CBD's poor species-specific oral bioavailability hinders the development of medicines. Possible causes include substantial first-pass hepatic metabolism, instability in the acidic stomach pH, and poor water solubility, which might result in insufficient absorption. Transdermal administration, a medication that overcomes these disadvantages, might thus be a suitable option. In addition, the transdermal route of administration has high patient compliance since it is non-invasive and the time between administrations is lengthy. Transdermal delivery also allows for the maintenance of steady systemic medication concentrations. CBD, however, is a highly lipophilic molecule (Consroe et al., 1991; Lodzki et al., 2003). For example, the previous study used CBD transdermal. Using ethosomal carriers, Lodzki et al. created a transdermal CBD delivery system.

In a model of acute inflammation generated by carrageenan, the pharmacodynamic impact of the compound was evaluated. The results demonstrated that the ethosomal carrier transported CBD systemically to the injured organ and permitted therapeutic anti-inflammatory concentrations at the location. This research indicates that

transdermal CBD may be beneficial for treating chronic inflammatory disorders (Lodzki et al., 2003). A prior study administered CBD gel for four consecutive days to mice. Rats were given daily doses of 0.6, 3.1, 6.2, or 62.3 milligrams of a compound (mg). According to the researchers, CBD decreased inflammation and overall discomfort in the damaged joints of rats, with no noticeable adverse effects. The pain ratings of rats were unaffected by dosages as low as 0.6 or 3.1 mg. Researchers found that 6.2 mg/day successfully decreased discomfort and edema in rats. In addition, the results of animals receiving 62.3 mg/day were similar to those of rats receiving 6.2 mg/day. They did not experience reduced discomfort after receiving a much bigger dosage. Arthritis patients may benefit from the anti-inflammatory and pain-relieving properties of CBD gel. However, further human research is required (Hammell et al., 2016).

Structure of the Skeletal muscle

Skeletal muscle is an excitable, contractile tissue that, together with the appendicular and axial bones, is responsible for maintaining posture and moving the orbits. Tendons connect it to the bones and the orbits. Through electrical impulses, excitable tissue reacts to stimuli. Contractile tissue is capable of producing forceful strain. Each skeletal muscle is surrounded by three layers of connective tissue that give the muscle structure and compartmentalize the muscle fibers. Each muscle is surrounded by a sheath of thick, irregular connective tissue known as the epimysium, which enables the muscle to contract and move forcefully while retaining its structural integrity. Within every skeletal muscle, muscle fibers are arranged into fascicles and surrounded by a layer of connective tissue known as the perimysium. Finally, each muscle fiber is enclosed in the endomysium inside each fascicle, a thin connective tissue layer composed of collagen and reticular fibers (Carnes & Pins, 2020).

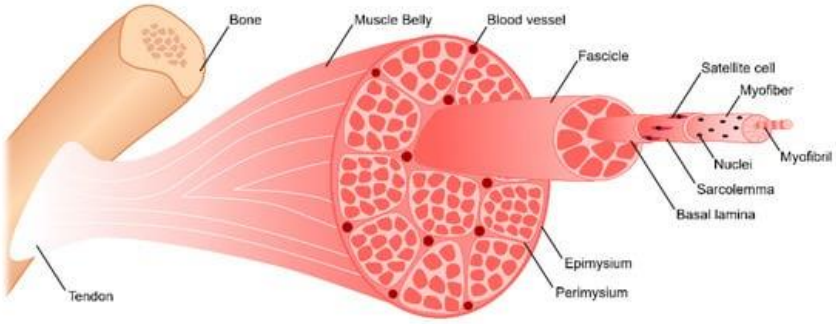

Figure 10 The skeletal muscle anatomy

Source: Carnes & Pins (2020)

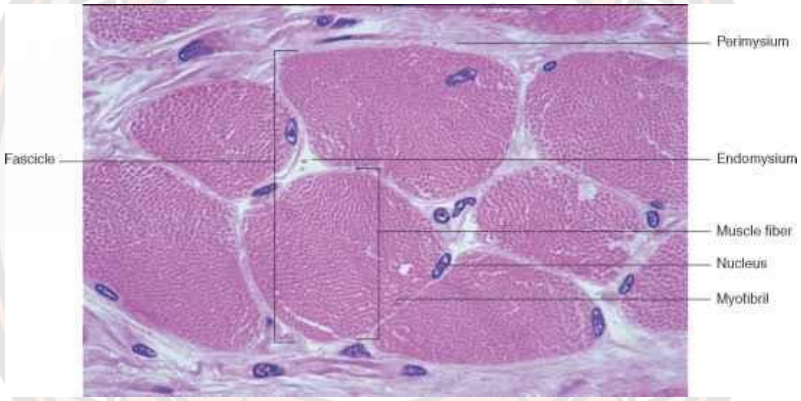

Figure 11 Histology of skeletal muscle

Source: GUWS Medical (2022)

CHAPTER III

RESEARCH METHODOLOGY

1. Study design: The effects of a roll-on comprising cannabis extract and isolate cannabidiol on nociception and inflammation in mice were examined in this study. In addition, carrageenan was used to generate inflammation in mice.

2. Instruments and Materials

2.1 Instruments

2.1.1 Digital calipers (SmartReloader® Electronic)

2.1.2 Hotplate test

2.1.3 Light Microscope (Olympus, Japan)

2.1.4 Microplate reader (Winoaski, Bio-Tek Instrument)

2.1.5 Microtome (Leica Biosystems, Germany)

2.1.6 Microcentrifuge tube (Hycon)

2.1.7 Plexiglas cubides

2.1.8 Spectrophotometer (Model CE1010, CECIL)

2.1.9 Tissue embedding machine (kunz instruments WD-4, Shandom company)

2.1.10 Tissue processing machine (Citadel 1000, Shandom company)

2.1.11 Video Camera (Logitech, Switzerland)

2.1.12 Von Frey filament (Biosed, USA)

2.1.13 Vortex (Votex-genie 2, Scientific)

2.1.14 Water bath (WB22, Memmert)

2.1.15 Weighing machine (WB22, Memmert)

2.2 Materials

2.2.1 Acetic acid solution (Sigma-Aldrich, USA)

2.2.2 Carrageenan (Sigma-Aldrich, USA)

2.2.3 Crystal Violet (Panreac)

2.2.4 Diclofenac Diethylammonium (Volttaren Emulgel, Thailand)

2.2.5 ELISA kits (Thermo Fisher Scientific)

2.2.6 Ethanol (Sigma-Aldrich, USA)

2.2.7 Formalin (Sigma-Aldrich, USA)

2.2.8 Liquid nitrogen

2.2.9 Normal saline (A.N.B laboratories, Thailand)

2.2.10 Paraplast (Leica, Germany)

2.2.11 Roll-on isolate CBD 0.5% (CosNat, Faculty of Pharmacy, Naresuan University)

2.2.12 Roll-on isolate CBD 1% (CosNat, Faculty of Pharmacy, Naresuan University)

2.2.13 Roll-on extract 0.5% (CosNat, Faculty of Pharmacy, Naresuan University)

2.2.14 Roll-on extract 1% (CosNat, Faculty of Pharmacy, Naresuan University)

2.2.15 Roll-on base (CosNat, Faculty of Pharmacy, Naresuan University)

2.2.16 Xylene (Sigma-Aldrich, USA)

3. Methods

3.1 Animal and grouping

Male ICR mice aged 6-8 weeks, weighing 25-40 g, were purchased from the animal center, Nomura Siam International, Bangkok, Thailand. A maximum of five mice were housed in a rodent cage where food and water were free to access. The animal room was maintained at 22±1 ºC with a 12-hour light cycle starting at 06:00 am. After adapting to the new environment for at least 7 days, the mice were randomly divided into eight groups (5 mice/group) as follows:

1. Control group: Mice were injected with normal saline 20 µl on gastrocnemius muscle.

2. Carrageenan group: Mice were injected with 3% carrageenan 20 µl on gastrocnemius muscle.

3. Roll-on base group: Mice were injected with 3% carrageenan 20 µl and applied roll-on solution base 6.4 µl on gastrocnemius muscle.

4. 0.5% roll-on isolate CBD group: Mice were injected with 3% carrageenan 20 µl and applied 0.5% Roll-on isolate CBD 6.4 µl on gastrocnemius muscle.

5. 1% roll-on isolate CBD group: Mice were injected with 3% carrageenan 20 µl and applied 1% Roll-on isolate CBD 6.4 µl on gastrocnemius muscle.

6. 0.5% roll-on cannabis extract group: Mice were injected with 3% carrageenan 20 µl and applied 0.5% Roll-on isolate CBD 6.4 µl on gastrocnemius muscle.

7. 1% roll-on cannabis extract group: Mice were injected with 3% carrageenan 20 µl and applied 1% Roll-on isolate CBD 6.4 µl on gastrocnemius muscle.

8. Voltaren group: Mice were injected with 3% carrageenan 20 µl on gastrocnemius muscle and applied Voltaren 6.4 µl (Benchmark product).

All experiments reported herein follow the Guide for the Care and Use of Laboratory Animals, and the Committee on Animal Care at Naresuan University approved all procedures.

3.2 Experimental schedule

As shown in figures 15 and 16, animals were trained in habituation with each instrument for 5 minutes/mice/testing 24 h before treatment. The next day, at 0 h, applied roll-on solution isolate CBD, roll-on solution cannabis extract, and Voltaren on gastrocnemius muscle on the left leg of mice. After 18 h, animals were tested in a Hot plate, Von Frey filament, and measured the diameter on the gastrocnemius muscle for baseline. After applying 19 h, the researcher injected a gastrocnemius muscle of 20 µl of normal saline in the control group, and 20 µl of concentration 3 % carrageenan in the carrageenan group, Roll-on base group, 0.5% Roll-on isolate CBD group, 1% Roll-on isolate CBD group, 0.5% Roll-on cannabis extract group, 1% Roll-on cannabis extract group and Voltaren group. After the research injected carrageenan and normal saline into the gastrocnemius muscle at 4, 8, and 24 h, all animals were tested the Hot plate, Von Frey filament, and measured the diameter on the gastrocnemius muscle.

After the behavioral test, all groups of mice were terminated by perfusion and collected gastrocnemius muscle for biochemical analysis and histological study shown in figure 12.

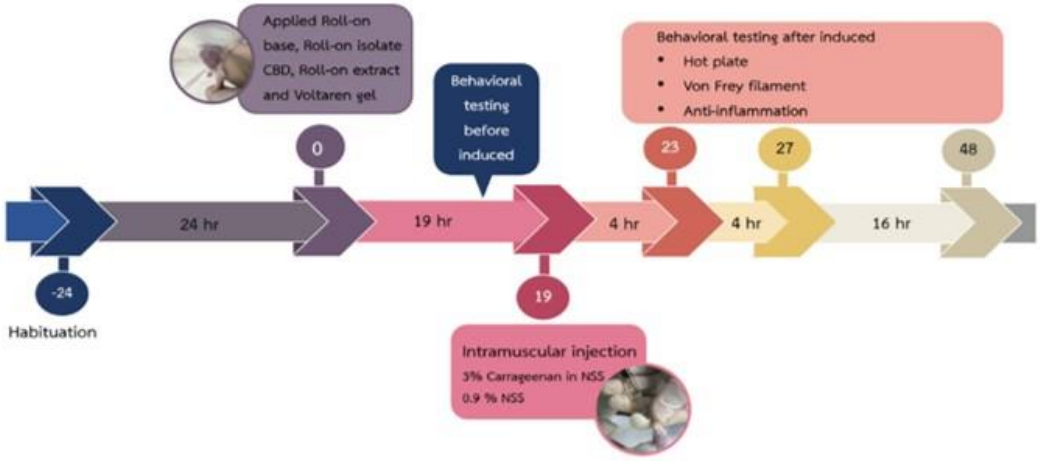

Figure 12 Experimental schedule

3.3 Herbal extract

Roll-on solution isolates CBD and roll-on solution cannabis extract from Cosmetics & Natural products Research Center (CosNat), Faculty of Pharmacy, Naresuan University, and the roll-on isolate CBD concentration of 0.5% and 1 %. Moreover, roll-on extract from CosNat concentration of 0.5% and 1 %.

3.4 Application of Roll-on solution isolate CBD and Roll-on solution cannabis extract on gastrocnemius muscle

All animals were applied with 6.4 µl roll-on solution isolate CBD, roll-on solution cannabis extract (0.5% and 1% roll-on isolate CBD and roll-on extract), and Voltaren (Benchmark product). First, 0.5x0.5 cm2 was applied on the gastrocnemius muscle (Hammell et al., 2016). After, the researchers observed the area of local reactions on gastrocnemius muscle, such as redness and swelling.

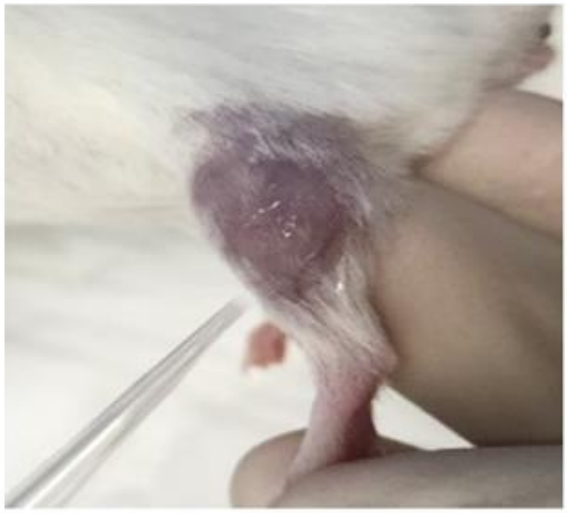

Figure 13 Application of the roll-on solution

3.5 Intramuscular injection into the gastrocnemius muscle

We chose the gastrocnemius muscle for generated inflammation in this investigation because we knew that a roll-on containing cannabis extract and isolated cannabidiol might help with muscular pain. This location could be easily assessed without causing the mice unnecessary stress during manipulation. Furthermore, the earlier selection of a specific area for intramuscular injection was used to ensure that our processes were repeatable. Crystal violet was injected into the gastrocnemius muscle of mice to ensure that the location caused muscular inflammation.

After 19 h applied, the research was injected carrageenan 3% volume 20 µl by 1ml Syringe and needle No.27 on gastrocnemius muscle of mice, all groups excepted control group it was injected normal saline 20 µl. Next, we were behavioral test after injected the carrageenan and normal saline for 4 h (Rajan Radhakrishnan & Sluka, 2004)

Figure 14 Injection of carrageenan on gastrocnemius muscle

3.6 Behavioral testing

Before the behavioral test, all animals were habituated to each instrument test for 5 minutes.

3.6.1 Hot plate test

The hot plate test recorded the delay for either removing the paw off the hot plate or licking the paw. Supraspinal structures were elevated to escape the hotplate (Deuis et al., 2017; Gregory et al., 2013).

Test procedure of paw withdrawal latency (PWL) to the heat stimulus

1. The research placed mice in 20 x 20 x 30 cm Plexiglas cubicles on a glass-top platform and was given 5 minutes to adapt to a quiet environment.

2. After turning on the radiant heat source beneath one of the paws, focus the radiant heat on the paw's mid-plantar aspect.

3. the researcher turned the heating element to 50±2 ºC and placed the animal in the heat stimulus, which started a timer.

4. We keep records of the paw's motions. The timer was stopped when the animal pulled its paw back abruptly in response to thermal stimulation and picked up the mice from the heat source.

5. As the PWL, keep track of the time from when the animal was lowered to when the heat stimulus was applied and when the paw was withdrawn.

6. To avoid tissue injury, the heat treatment should be stopped after 20 seconds.

7. Picking up the animal outward from the heat source and recording the PWL reading as 20 sec if the animal does not retract the paw within 20 seconds.

8. Repeat steps 3–5 for each animal in the test group. Allow 5 minutes after the previous.

9. Repeat the test cycle three times and then use the average of three PWLs as the baseline result for each animal (Rajan Radhakrishnan & Sluka, 2004).

After the animals were injected with carrageenan for 4, 8, and 24 hours, we repeated steps 3 to 5 for all of the animals in the test group. Repeat the test procedure once for each animal and note the time duration as the PWL after 4, 8, or 24 hours. The PWL was calculated are %MPE (Mean Percent Maximum possible effect) by the following equation (Berrendero et al., 2005):

$$\%MPE = [(TL-CL) / (\text{cut-off time} - CL)] \times 100$$

TL: Test latency

CL: Control latency

Figure 15 A procedure of PWL (De
uis et al., 2

3.6.2 Von Frey test

In rodents, the paw with von Frey filaments is commonly used as a mechanical withdrawal threshold. Examining the response frequency to repeated administration of a single von Frey filament is an alternate method of determining thresholds. Von Frey filament testing is particularly beneficial for simulating clinical diseases with increased cutaneous sensitivity, such as neuropathic pain, postoperative pain, and inflammation since it measures cutaneous hyperalgesia (Deuis et al., 2017; Gregory et al., 2013).

Test procedure threshold to mechanical stimulus (Tang et al., 2021)

1. The animals were allowed to adapt for 5 minutes in a quiet environment, with one animal in each Plexiglas cubicle area 10x8x8 cm platform.

2. Then, apply the von Frey filament (1, 1.4, 2, 4, 6, 8, 10, and 15 g) to the mid plantar aspect of the hind limb five times, at least 1 sec apart, with the lowest bending force (1 g).

3. the researcher observed outside mice a paw withdrawal. If there is no response after five applications, try the next higher force filament.

4. We applied three applications, and a paw withdrawal developed. As the mechanical withdrawal threshold, record the value of the force filament that causes

the paw to retract. In rats, a cut-off of 15 g is usually used. This method's dependability has been confirmed by testing and retesting.

5. Using the same technique, determine the threshold of the opposite paw.

6. Steps 2–4 were repeated for all animals, and the mechanical thresholds should be recorded as the baseline value (Rajan Radhakrishnan & Sluka, 2004).

We repeated the testing steps 2 to 4 for all the animals in the test group after induced carrageenan at 4, 8, and 24 h and recorded this paw withdrawal. Then, the paw withdrawal was calculated by the following equation (Liang et al., 2007):

%reversal = 100 x (ipsilateral postdrug threshold- ipsilateral predrug threshold) / (cut-off (15 g) – ipsilateral predrug threshold)

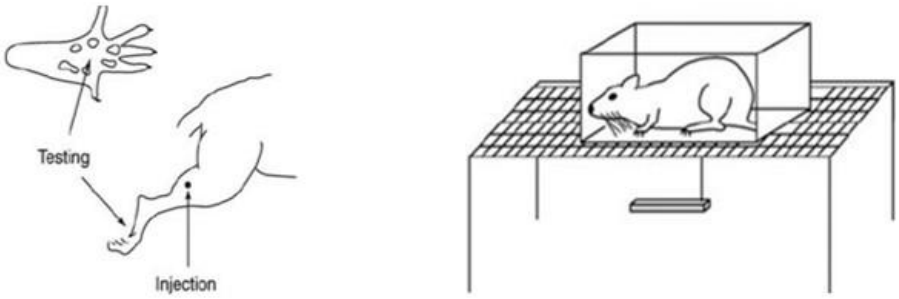

Figure 16 A procedure of mechanical withdrawal thresholds (Rajan Radhakrishnan & Sluka, 2

Source: Rajan Radhakrishnan and Sluka (2004)

3.6.3 Antiinflammation test

The inflammation on the gastrocnemius muscle was measured with a digital caliper to be used as the baseline of muscle size.

Test procedure Antiinflammation

1. For The right gastrocnemius muscle, we used a digital caliper to measure it twice per side on the vast and thick sides.

2. As the baseline value for the right gastrocnemius muscle, record the average diameter.

3. Repeat steps 1 to 2 for the left side

We repeated steps 1–3 for all the test group animals after being exposed to induced carrageenan for 4, 8, and 24 hours. In addition, we measured the diameter of both sides of the gastrocnemius muscle. The percentage inhibition of muscle swelling between the treatment and control groups measures anti-inflammatory activity. The anti-inflammation is calculated using the equation below (Annie George, 2014):

% Inhibition of edema = 100 x (1-Vt/Vc)

Vt: The edema volume measured in the test groups

Vc: The edema volume observed in the control

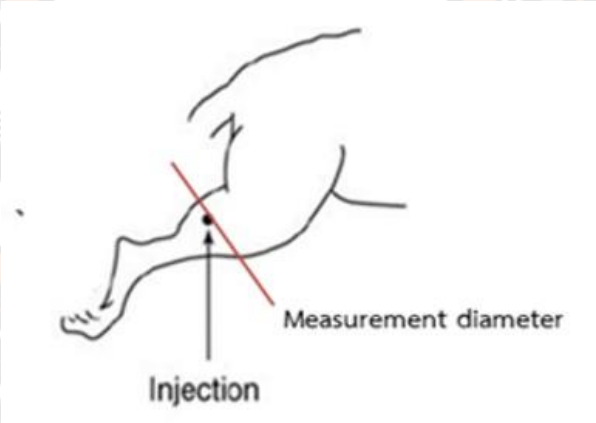

Figure 17A procedure of muscle diameter measurement

Source: Rajan Radhakrishnan and Sluka (2004)

3.7 Biochemical analysis

Measurements of inflammatory marker (TNF-α and IL-1β)

The procedure was performed after euthanasia, mice were deeply anesthetized, and transcardial perfusion was performed using PBS 0.1 M, pH 7.4. The tissue extract was used to measure cytokines in this study of gastrocnemius muscle. An enzyme-linked immunosorbent test was used to evaluate cytokine concentrations. We used ELISA or enzyme-linked immunosorbent assay. It is a type of immunological test used to quantify antibodies, antigens, proteins, and glycoproteins in biological samples. Measurements of cytokines are one example. ELISA tests are often performed in 96-well plates, allowing for the measurement of numerous samples in a single experiment. These plates must be exceptionally absorbent to guarantee that the antibody or antigen clings to the surface. Each ELISA tests for a different antigen. The absorbance was measured using a microplate ELISA reader (Spectrophotometer) at 450 nm. The muscle was homogenized in 0.1 M PBS (pH 7.4) and centrifuged at 9,000 x g for 20 min at 4˚C, the tissue was extracted and used to measure cytokines. An enzyme-linked immunosorbent assay (ELISA) was used to evaluate the cytokine concentrations TNF-α (Elabscience, cat NO. E-EL-M3063), and IL-1β (Abcam, cat NO. ab100705) followed by protocol of each kit

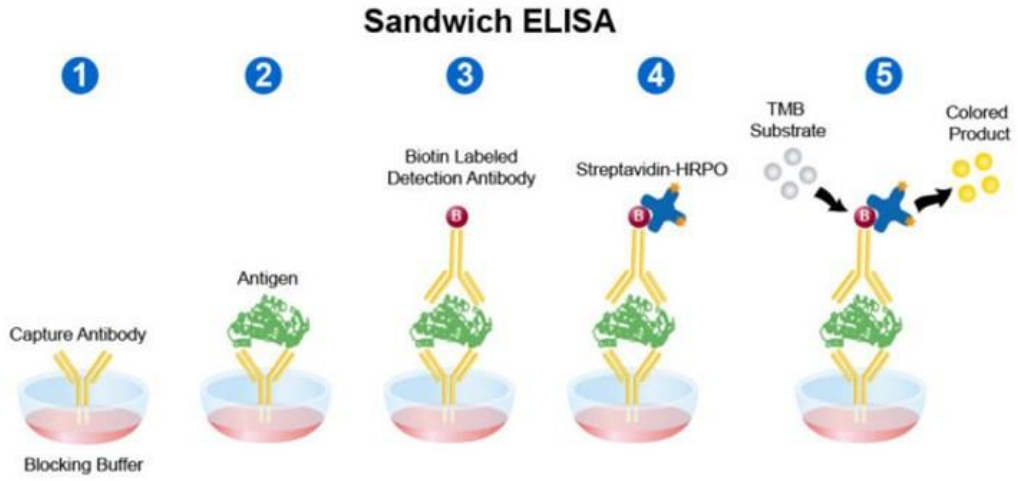

Figure 18 A sandwich ELISA is seen above, with the stages of the test numbered in sequence 1-5

Source: Meefro (2020)

3.8 Histological analysis

The procedure was performed after euthanasia, mice were deeply anesthetized, and transcardial perfusion was performed using PBS 0.1 M, pH 7.4. Then, the gastrocnemius muscle was removed and fixed in 10% paraformaldehyde before being embedded in paraffin. Muscle samples were then sectioned at a width of about 5 µm. We kept 4 section/1 mice, and the continuous segment was put on slides like a ribbon. Finally, H&E will be used to stain the samples. The presence of neutrophils in tissue samples will be used to test for inflammation. The sections were visualized under a light microscope which H&E staining. We chose the center of the section area for the capture part and counted the inflammation cell all over the area under 10X magnification using ImageJ software. The section was counted for four sections of each mice.

4. Statistical analysis

Data were expressed as the mean S.E.M. T-test was used to analyze the difference between the control and carrageenan groups. Comparisons between groups were performed by one-way ANOVA, followed by Tukey's multiple comparisons test. Statistical significance was set as $P<0.05$, and all graphs and analyses were performed with GraphPad Prism 9 software (GraphPad Software Inc., San Diego, CA, USA).

CHAPTER IV

RESULTS

Effect of roll-on applicators containing cannabis extract or isolated CBD on mechanical allodynia

The somatic sensitivity was examined under the condition group and treatment group (n=5/group), measured the mechanical threshold used by the von Frey filament test before being induced for 0 h (baseline) and after being induced for 4, 8, and 24 h. As a complementary analysis, we evaluated the percentage of reversal using the following parameters: 100 x (ipsilateral post-drug threshold- ipsilateral pre-drug threshold) / (cut-off (15 g) – ipsilateral pre-drug threshold). The data was calculated to show the area of analgesia (% reversal/h). A T-test was performed to verify the data mean±SEM of the condition group (control vs. carrageenan). Afterward, the treatment group carrageenan differences between the roll-on applicators containing cannabis extract (0.5%, 1%) and isolated CBD (0.5%, 1%) solution groups were calculated.

The result also showed that the condition of carrageenan group had an increased in hyperalgesia induced by inflammation at a significant difference $P < 0.001$ when compared with the control group. In the treatment group Voltaren, roll-on isolated CBD solution, 1%; and roll-on extract solution, 1% decreased mechanical allodynia a significant difference $P < 0.05$, $P < 0.01$, $P < 0.001$ when compared with carrageenan group. In contrast, 0.5% roll-on isolated CBD and 0.5% roll-on extract solution was not able to reverse mechanical allodynia as shown in Table 1-2 and Fig. 19.

Table 1 Carrageenan induced mechanical allodynia

Group	Area of analgesia (%reversal/h)
Control	0.000±0.000
Carrageenan	-277.5±52.86***

Note: ****P* <0.001 compared with control group.

Table 2 Effect of roll-on applicators containing cannabis extract or isolated CBD on mechanical allodynia

Group	Area of analgesia (%reversal/h)
Carrageenan	-277.5±52.86
Voltaren	245.8±114.0*
Roll-on Base	-104.3±46.73
0.5% roll-on isolated CBD	-138.0±70.79
1% roll-on isolated CBD	285.7±175.8**
0.5% roll-on cannabis extract	117.8±77.68
1% roll-on cannabis extract	324.7±115.2**

Note: **P* <0.05, ***P* <0.01 compared with carrageenan group.

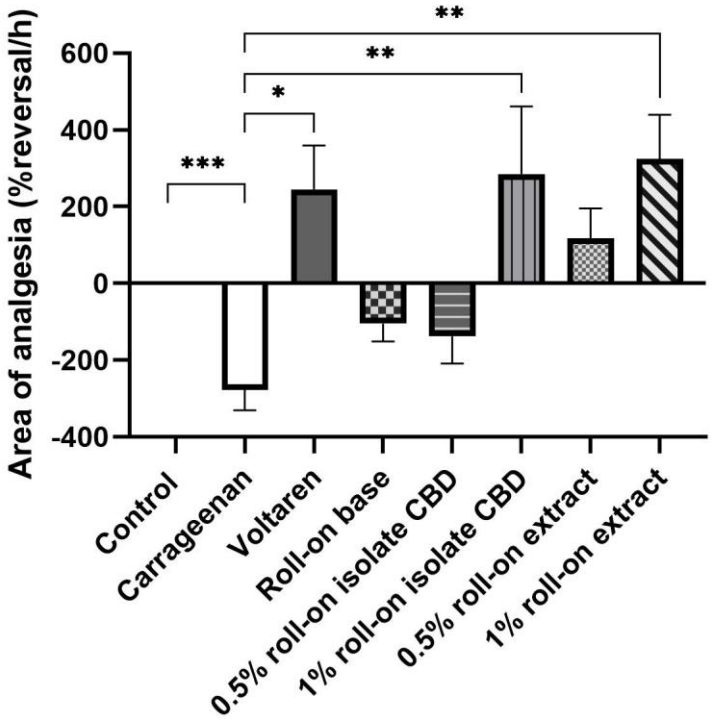

Figure 19 Roll-on applicators containing 1% concentration of a cannabis extract and an isolated CBD solution reduced mechanical allodynia.

The significantly increased the area of analgesia compare with carrageenan group ****P* **<0.05, *****P* **<0.01, ******P* **<0.001.

Effects of roll-on containing cannabis extract and isolated CBD on mechanical hyperalgesia

The hot plate test was used to establish a thermal threshold analysis that was essential to assess heat sensitivity; the mechanical thermal test was calibrated at 0 h (baseline) and afterwards manually activated at 4, 8, and 24 h. As a complementary analysis, the researcher evaluated the percentage of maximum possible effect (MPE) using the following parameters percent of MPE= [Test latency (TL) minus Control latency (CL) / (cut-off time (30s) – CL)] x100. The data was calculated and showed the area of analgesia was a percentage of MPE/h and the T-test analysis repeated measures to the data mean±SEM, and the condition group. Furthermore, the treatment group was calculated to show that in the area of analgesia (%MPE/h), the Tukey's multiple comparisons test for repeated measures was applied to the data mean±SEM.

The result showed that the condition group carrageenan increased the hyperalgesia triggered by inflammation, a significant difference P <0.01 when compared with the control group. In the treatment group Voltaren group, roll-on isolated CBD 1%, roll-on extract 0.5% and roll-on extract 1% decreased thermal hyperalgesia, a significant difference P <0.001, P <0.01, P <0.001, and P <0.001 when compared with carrageenan group. In contrast, roll-on isolated CBD 0.5% was not able to decrease thermal hyperalgesia as shown in Table 3-4 and Fig. 20.

Table 3 Carrageenan induced mechanical hyperalgesia

Group	Area of analgesia Area of analgesia (%MPE/h)
Control	29.99±22.32
Carrageenan	-226.8±58.85**

Note: ***P* <0.01 compared with control group.

Table 4 Effect of roll-on applicators containing cannabis extract or isolated CBD on mechanical hyperalgesia

Group	Area of analgesia Area of analgesia (%MPE/h)
Carrageenan	-226.8±58.85
Voltaren	306.1±104.9***
Roll-on Base	-303.2±61.27
0.5% roll-on isolated CBD	-117.5±88.50
1% roll-on isolated CBD	278.3±83.32**
0.5% roll-on cannabis extract	339.1±58.72***
1% roll-on cannabis extract	484.5±102.0***

Note: ***P* <0.01, ****P* <0.001 compared with carrageenan group.

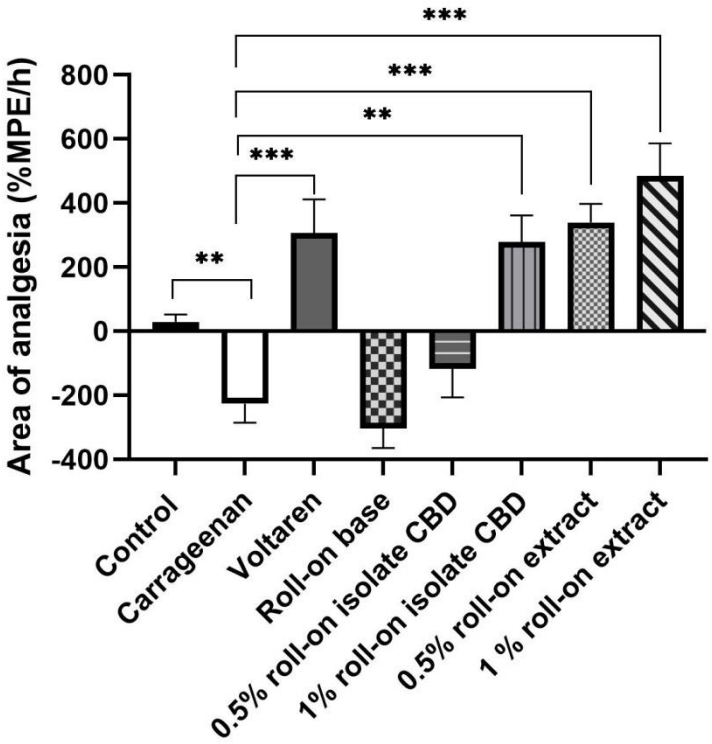

Figure 20 Roll-on applicators containing a cannabis extract and an isolated CBD solution reduced mechanical hyperalgesia.

The significantly increase the area of analgesia compare with carrageenan group *****P* **<0.01, ******P*** **<0.001.**

Effects of roll-on containing cannabis extract and isolated CBD on edema

The diameter of the gastrocnemius muscle was used to measure edema. It was used to indirectly measure anti-inflammatory. That was measured after activated inflammation at 4, 8, and 24 h. As a complementary analysis, the percentage inhibition of muscle swelling between the treatment and control groups is used to measure anti-inflammatory activity. That calculated used the equation: percent Inhibition of edema = 100 x (1- The edema volume measured in the test groups / The edema volume observed in the control). In the condition group, the carrageenan group significantly increased the diameter of the gastrocnemius muscle when compared with the control group (P <0.01). In contrast, the treatment group did not able to reduce the

diameter. The result demonstrated that carrageenan activates muscle inflammation. The treated group did not change the edema shown in Table 5-6 and Figure 21.

Table 5 Carrageenan induced muscle edema

Group	%Inhibition of edema		
	After induced 4 h	**After induced 8 h**	**After induced 24 h**
Control	0.5389±0.7107	0.4809±0.6595	0.8879±0.7606
Carrageenan	-28.87±3.199*	-34.99±5.036*	-40.49±3.229**

Note: *P<0.05 **P<0.01 compared with control group.

Table 6 Effects of roll-on containing cannabis extract and isolated CBD on edema

Group	**%Inhibition of edema**		
	After induced 4 h	**After induced 8 h**	**After induced 24 h**
Carrageenan	-28.87±3.199	-34.99±5.036	-40.49±3.229
Voltaren	-19.25±6.726	-17.73±5.821	-20.92±5.821
Roll-on Base	-18.57±4.201	-24.75±6.716	-26.26±3.950
0.5% roll-on isolated CBD	-21.03±3.471	-25.69±5.350	-24.70±7.592
1% roll-on isolated CBD	-10.73±3.045	-18.66±2.591	-13.56±2.816
0.5% roll-on cannabis extract	-27.04±1.148	-25.31±3.193	-27.79±3.338
1% roll-on cannabis extract	-29.14±2.226	-24.45±3.455	-36.38±2.790

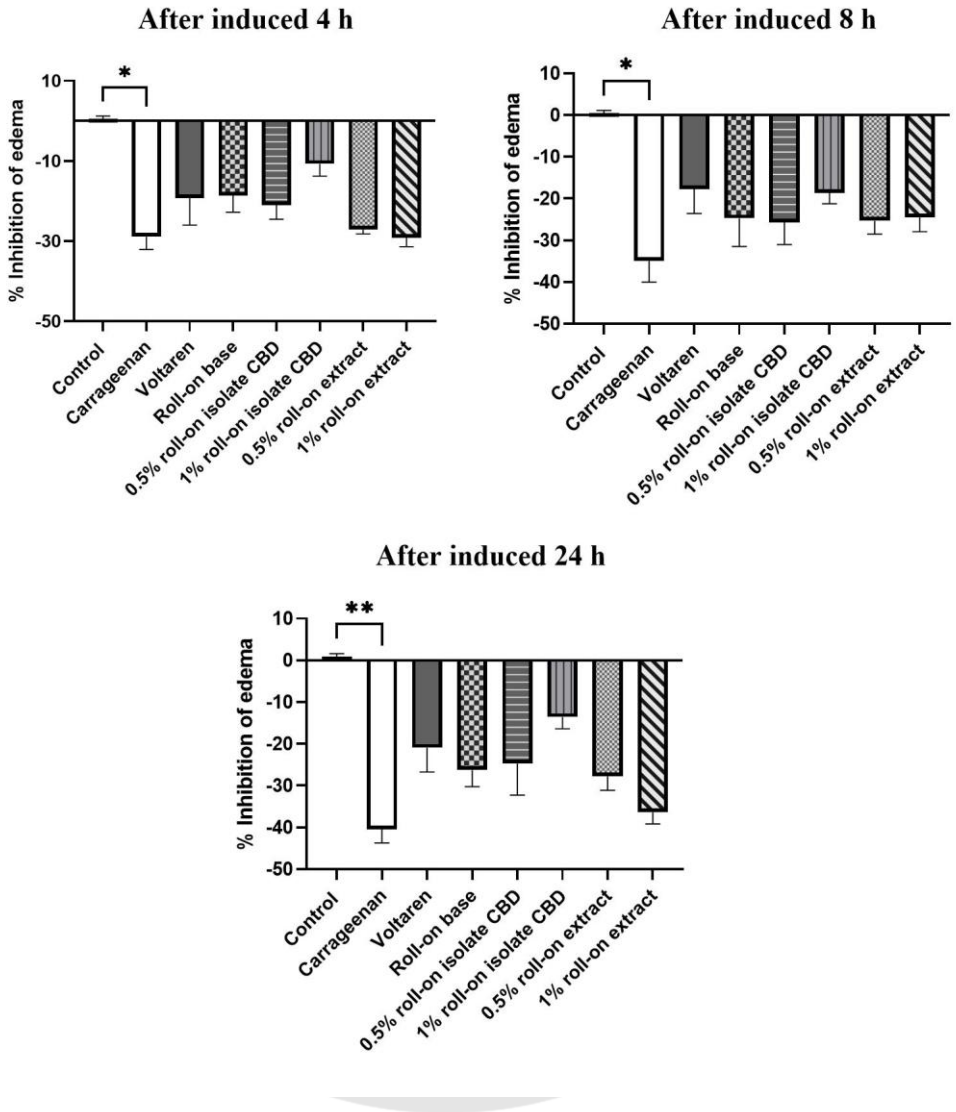

Figure 21 Effects of roll-on containing cannabis extract and isolated CBD on edema. A: percent inhibition of edema after activate inflammation 4 h B: percent inhibition of edema after activate inflammation 8 h C: percent inhibition of edema after activate inflammation 4 h.

A significan difference compare with carrageenan group ****P* **<0.05, *****P* **<0.01.

Effect of roll-on solutions containing cannabis extract and isolated CBD on an inflammatory marker (TNF-α and IL-1β)

Inflammatory cytokine TNF-α expression in the gastrocnemius muscle was measured by ELISA. The result showed a significant difference P <0.01 in the concentration of TNF-α which was higher than the control group the condition carrageenan group; as a result, the TNF-α expression in the condition group increases after induced inflammation. Afterward, the treatment group demonstrated Voltaren group, the roll-on isolated CBD 1% and roll-on extract 1% solution decreased the cytokine TNF-α with a significant difference associated with the carrageenan group P <0.01. Consequently, findings indicated that the roll-on isolated CBD and roll-on extract 1% solution concentration was able to reduce pain by deceased TNF-α, Table 7-8 and Fig.22A.

Additionally, the researcher demonstrated that the inflammatory cytokine IL-1β used the same control directive as TNF-α. The data indicate that the carrageenan group had increased IL-1β inflammation compared with the control group, a significant difference in P <0.01 Table 9-10 and fig. 22B. All groups except for the roll-on base group were able to decrease IL-1β, considerably; however, there was a significate differences P <0.05 associated with the carrageenan group. The data show that the roll-on isolated CBD and roll-on extract solution, 0.5% and 1% concentration, was able to reduce inflammation and pain by decreasing the cytokine IL-1β.

Table 7 Carrageenan induced inflammatory marker TNF-α

Group	Concentration of TNF-α (pg/ml)
Control	119.7±26.61
Carrageenan	444.5±75.96**

Note: **P <0.01 compared with control group.

Table 8 Effect of roll-on solutions containing cannabis extract and isolated CBD on an inflammatory marker TNF-α

Group	Concentration of TNF-α (pg/ml)
Carrageenan	444.5±75.96
Voltaren	191.0±13.70**
Roll-on Base	347.5±63.47
0.5% roll-on isolated CBD	322.0±51.40
1% roll-on isolated CBD	168.2±24.59**
0.5% roll-on cannabis extract	283.2±26.77
1% roll-on cannabis extract	198.0±18.25**

Note: **P <0.01 compared with carrageenan group.

Table 9 Carrageenan induced inflammatory marker IL-1β

Group	Concentration of IL-1β (pg/ml)
Control	71.13±15.35
Carrageenan	380.6±64.22**

Note: **P <0.01 compared with control group.

Table 10 Effect of roll-on solutions containing cannabis extract and isolated CBD on an inflammatory marker IL-1β

Group	Concentration of IL-1β (pg/ml)
Carrageenan	380.6±64.22
Voltaren	169.8±18.83**
Roll-on Base	310.8±47.86
0.5% roll-on isolated CBD	151.7±48.40**
1% roll-on isolated CBD	120.3±36.51***
0.5% roll-on cannabis extract	177.1±24.37*
1% roll-on cannabis extract	179.7±21.54*

Note: *P <0.05, **P <0.01, ***P<0.001 compared with carrageenan group

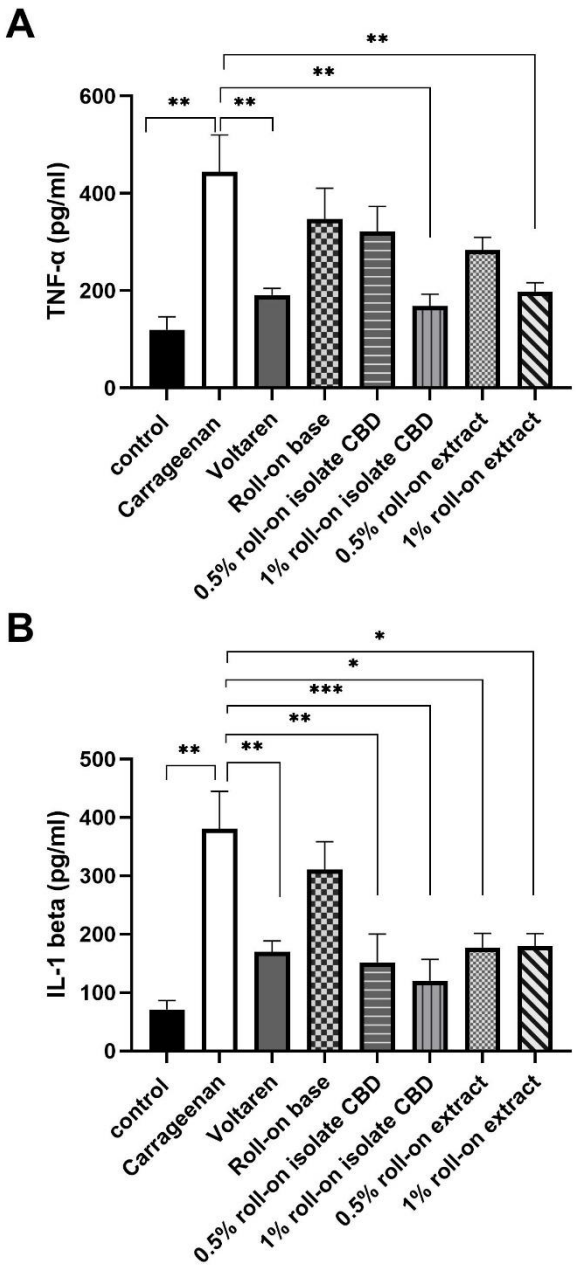

Figure 22 Effect of roll-on solutions containing cannabis extract and isolated CBD on an inflammatory marker (A) Roll-on solution containing cannabis extract and isolated CBD on an inflammatory marker significantly decrease TNF-α on the gastrocnemius muscle (B) Roll-on containing cannabis extract and isolated CBD solution applied on an inflammatory marker.

The significantly decrease IL-1β on the gastrocnemius muscle compare with carrageenan group ****P* **<0.05, *****P* **<0.01, ******P* **<0.001.

Effect of a roll-on containing cannabis extract and isolated CBD solution on an Inflammatory cells expression

Inflammatory cells on gastrocnemius muscle were measured by applying a histology H&E stain technique, the researcher evaluated by counting the cell in the space between muscle cell, which is shown in Fig.23. Subsequently, the result demonstrated inflammatory cells as the carrageenan group more than the cell in the control group was a significant difference P <0.001 which is shown in Fig.24. Later, a comparison of the inflammatory cells in another group the data show various opinion that inflammatory cells decreased in the roll-on isolated CBD 1% and roll-on extract 1% significant associated with carrageenan in contrast to the roll-on isolated CBD 0.5% and roll-on extract solution, 0.5%, which was not able to reduce cell inflammation. As the data related to the roll-on isolated CBD and roll-on extract 1% solution and concentration showed a decrease inflammatory cell on gastrocnemius muscle in the model, further study of be needed to measure the impact the roll-ons will have for other treatments.

Table 11 Carrageenan induced inflammatory cells expression on gastrocnemius muscle

Group	Inflammatory cells
Control	79.40± 1.080
Carrageenan	300.7±7.160***

Note: ****P* <0.001 compared with control group.

Table 12 Effect of roll-on solution cannabis extract and isolated CBD which inflammatory cells expression on gastrocnemius muscle

Group	Inflammatory cells
Carrageenan	300.7±7.160
Voltaren	100.6±2.137***
Roll-on Base	265.8±7.472
0.5% roll-on isolated CBD	267.1±2.431
1% roll-on isolated CBD	110.1±4.312***
0.5% roll-on cannabis extract	242.0±4.221
1% roll-on cannabis extract	114.8±2.594***

Note: ****P* <0.001 compared with carrageenan group.

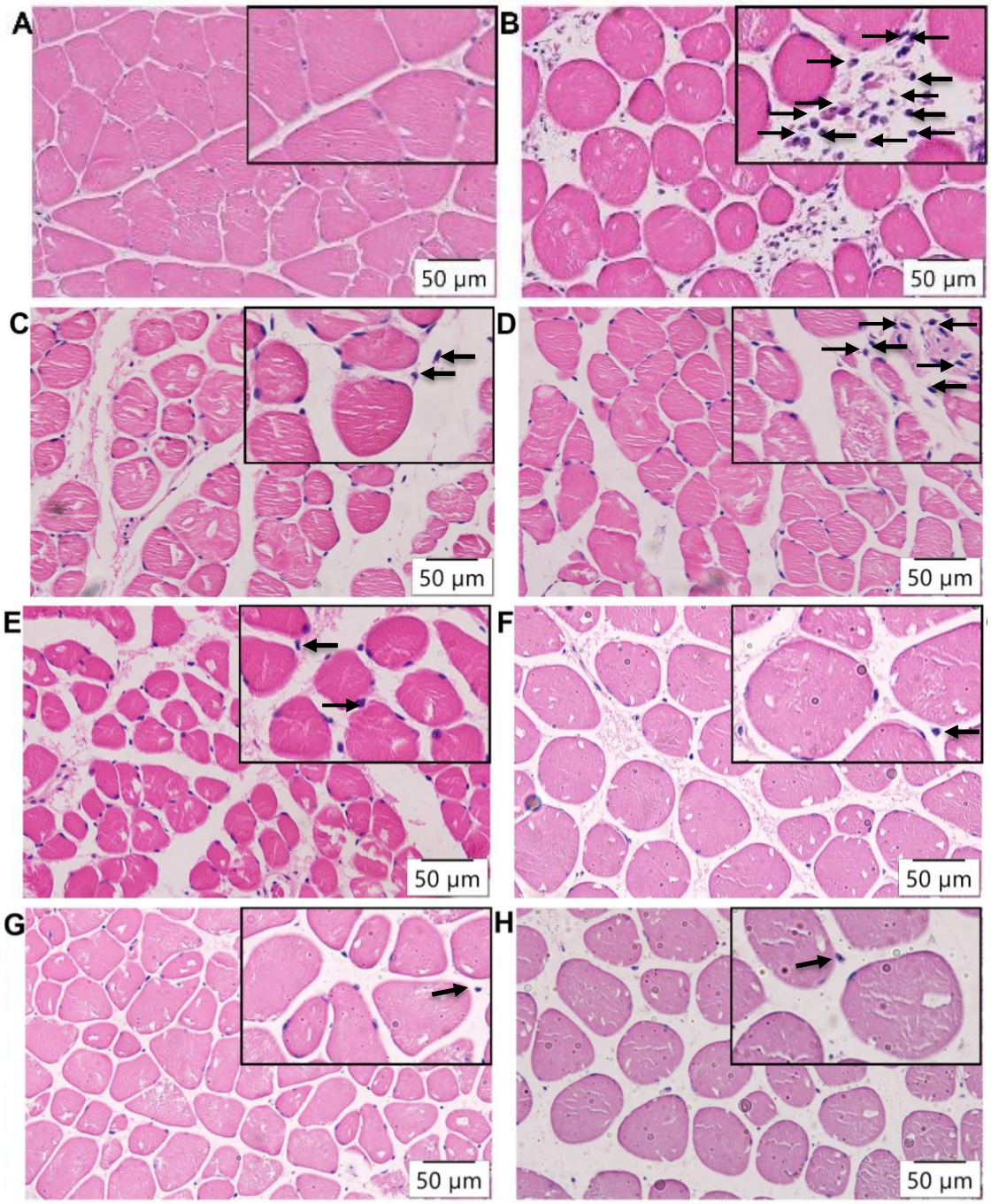

Figure 23 Microscope photograph of inflammatory cells in the transverse section gastrocnemius muscle. The images Figure was obtained with a 10x objective lens.

(A) Control group; (B) Carrageenan group; (C) Voltaren group; (D) Roll-on base group; (E) 0.5% roll-on isolated CBD; (F) 1% roll-on isolated CBD; (G) 0.5% roll-on cannabis extract; (H) 1% roll-on cannabis extract.

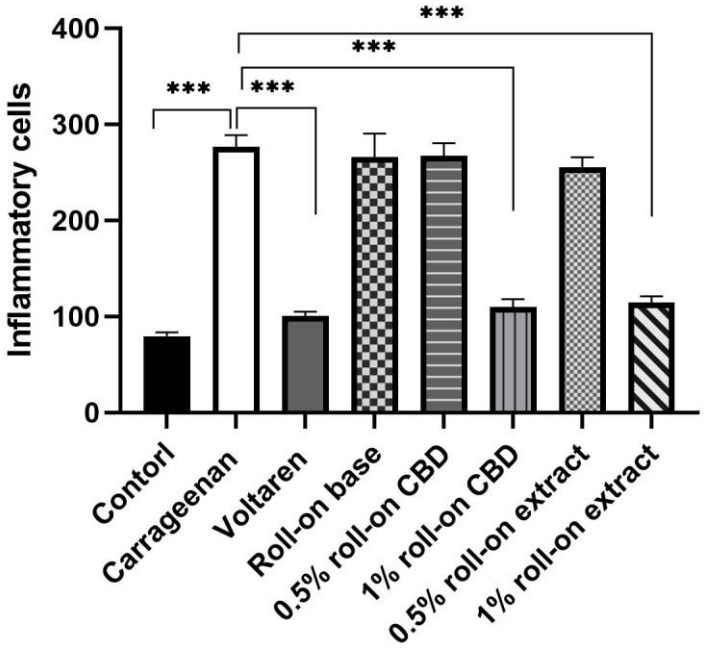

Figure 24 Roll-on solutions containing cannabis extract and isolated CBD on inflammatory cell expression on gastrocnemius muscle decreased.
The significant when compared with the carrageenan group ***P <0.001.

CHAPTER V

DISCUSSION AND CONCLUTION

This study aimed to investigate the effects of a roll-on containing cannabis extract and isolated cannabidiol solution on nociceptive and inflammatory pain. The primary emphasis of the researchers was the reversal of mechanical allodynia and heat hyperalgesia in the muscle.

At the beginning of the experiment, the researcher applied the roll-on solution to the gastrocnemius muscle of the left leg. As well as muscle pain was induced by applying a 3% carrageenan solution on the gastrocnemius muscle, which activated free radicals, prostaglandins, and NO along with produced proinflammatory mediators by a variety of stimuli such as TNF-α, and IL-1β (Guay et al., 2004; Huang et al., 2011). Subsequently, the mechanical and thermal sensitivity was observed after 4 h, 8 h, and 24 h carrageenan injections, which is able to be caused inflammation. The early phase of inflammation peaked after 3-4 hours, whereas the delayed phase peaked at 48 hours (Dzoyem et al., 2017; Huang et al., 2011). In this research, a roll-on containing 1% cannabis extract (CBD, lower THC) and 1% isolated cannabidiol (CBD) decreased mechanical and thermal sensitivity. The percentage of mechanical and thermal outcomes demonstrated enhancements, producing the greatest impact. In their trials, Hammell et al. (2016) used 1% transdermal cannabis to lower mechanical and heat sensitivity. A test including noxious heat stimuli and a hotplate determined that pain ratings were lowered. Furthermore, the previous research experiments (von Frey, Acetone and Hot plate test) (Silva-Cardoso et al., 2021) showed that the cannabidiol effects on the neuropathic pain model reduced mechanical allodynia. In the same way, the experimenter used a standardized extract of Cannabis sativa that completely relieved thermal hyperalgesia in a neuropathic pain model (Comelli et al., 2008). In previous research, Comelli et al. (2008) investigated the antihyperalgesic efficacy of a *Cannabis sativa* extract containing a high percentage of CBD (non-psychoactive component) and a small percentage of THC (psychoactive component),

which completely alleviated thermal hyperalgesia in a neuropathic pain model. After evaluating mechanical and thermal sensitivity tests, the researcher determined inflammatory markers TNF-α and IL-1β. These results showed that applying a roll-on cannabis extract and CBD solution reduced pain by modulating the inflammatory cytokines and consequently alleviated the inflammatory cells. The application of a CBD gel on lab rat specimens provides effective treatment of pain. Regarding CBD ability to treat pain, cannabidiol has a pharmacological activity which has several non-psychoactive, for they are antinociceptive, anti-inflammatory, and associated with the reduction of pain (Atalay et al., 2019; Comelli et al., 2008; Crivelaro do Nascimento et al., 2020; Menezes et al., 2021; Mense, 2008). Previous studies have displayed a positive effect of a CBD gel on rats once a day for four days. CBD decreased the inflammation and the overall discomfort in the rat's afflicted joints. No negative effects were noticeable even though *Cannabis sativa*, which contained a high percentage of CBD, was extracted. In the same way, the phytochemical and anti-inflammatory properties of cannabis oil decreased the TNF-α release in the Lipopolysaccharide-stimulated rat monocytes The previous study, Duangnin et al. (2007) investigated the anti-inflammatory effects of *Cannabis sativa* leaf extracts on synovitis *in vitro*, which were reported to lower the expression levels of IL-1β. As aforementioned, this study used the application of a roll-on containing cannabis extract and isolated CBD solution for the treatment of inflammatory muscle pain. Although the precise mechanism of action of CBD is still unknown, this is said to attenuate mechanical and thermal hypersensitivity brought on by inflammatory and neuropathic pain models (Starowicz & Finn, 2017); yet, several studies used cannabis or cannabidiol to treat pain (Boychuk et al., 2015; Lynch & Campbell, 2011). However, it has been acknowledged that CBD activates cannabinoid (CB) receptors in the endocannabinoid system, which are found in the peripheral and central nervous systems (Bourke et al., 2022). Moreover, CBD also functions as an agonist for ionotropic cannabinoid receptors, which include chemosensitive and thermosensitive TRP channel superfamily members (Fioravanti et al., 2008; Hosami et al., 2021; Mahmoudinoodezh et al., 2022). TRPA1 and TRPV1 are two extensively co-expressed ion channels discovered in CGRP-expressing peptidergic nociceptors necessary for neurogenic inflammation and inflammation-induced mechanical and

thermal hypersensitivity (Akopian, 2011; Frias & Merighi, 2016; Hammell et al., 2016). The THC which is a large part of the 1% of roll-on cannabis extract has a strong affinity for the CB1 and CB2 cannabinoid receptors and is a powerful partial agonist. THC's analgesic and primary psychoactive actions are both mediated by CB1 receptors. That reduces pain primarily due to its interaction with midbrain CB1 receptors (Romero-Sandoval et al., 2017). The immunomodulatory effects of THC are due to its interaction with CB2 receptors. THC can also act as an antagonist of the transient receptor potential channel (Almogi-Hazan & Or, 2020; Maccarrone et al., 2015). In animal models of inflammation, a lack of or suppression of TRPA1 in vivo reduces mechanical hypersensitivity. In many pain models, the absence of TRPV1 in vivo lowers inflammation-induced swelling, heat hypersensitivity, and nociceptive behaviour (Arpád Szabó 1, 2005 ; Helyes et al., 2007). Local TRPV1 expressions elevate the immunized cells after inflammation. Once activated, proinflammatory cytokines and chemokines such as IL-1β, IL-6, and TNF-α are produced, and the area of inflammation not only sensitizes primary afferents but also discharges the TNF-α. Equally important, it increases the expression of TRPV1 through an autocrine mechanism (Westlund et al., 2010). Moreover, because the peripheral release of proinflammatory cytokines is sensitizing the pain, the primary afferents are surrounded by cells that manufacture and release these molecules. Therefore, the CBD's ability to desensitize TRPV1 might halt the continuation of the pain sequence (Kochukov et al., 2009). In this study, administering cannabis extracts roll-on solution and extracted CBD reduced proinflammatory biomarkers in muscle fibres. Consequently, both the roll-on solution and the rat model demonstrated a reduction in the inflammatory markers IL-1β and TNF-α on the gastrocnemius muscle.

The application of a roll-on cannabis extract and isolated CBD solution decreased pain by modifying the inflammatory cytokines; consequently, reduced the number of inflammatory cells.

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APPENDIX

Composition of solution

1. 70% Ethanol (1000 ml)

90% Ethanol (777 ml)

Distilled water (223 ml)

2. 80% Ethanol (1000 ml)

90% Ethanol (889 ml)

Distilled water (111 ml)

3. 85% Ethanol (1000 ml)

90% Ethanol (944 ml)

Distilled water (56 ml)

4. 10% Normal Buffered Formalin (1000 ml)

37% Formaldehyde (100 ml)

Distilled water (900 ml)

NaH_2PO_4 (4g)

Na_2HPO_4 (6.5 g)

5. 10X Phosphate buffer saline (10X PBS, pH 7.4, 1000 ml)

Na2HPO4 anhydrous (10.9 g)

NaH2PO4 anhydrous (3.2 g)

NaCl (90 g)

Distilled water