ANTIINFLAMMATORY EFFECTS OF CRATEROSTIGMA PLANTAGINEUM EXTRACTS ON FORMALIN INDUCED INFLAMMATION AND PAIN IN MALE ALBINO RATS RATTUS NORVEGICUS

BY

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DECLARATION

I declare that the work contained in this thesis is my original work and has not been presented for a degree in any other university.

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ABSTRACT

Pain is a symptom of many diseases that affect humanity while inflammation is a physiological mechanism for repair of tissues after injury. *Craterostigma plantagineum hoscht* has been used by herbalists for treating pain. However, its efficacy and adverse effects are not scientifically validated. The broad objective of this study was to evaluate the antinociceptive and anti-inflammatory effects of qualitatively screened aqueous and organic extracts of *C. plantagineum hoscht.* Using modified resource formula. A total of 24 male albino rats aged 8-12 weeks and weighing 200-250 grams were used. Male rats were used to avoid data variability caused by estrous cycle. They were randomly assigned into 8 groups of 3 rats each. These included three aqueous extract treatment groups of different doses (25mg/Kgbwt, 50mg/Kgbwt and 100mg/Kgbwt), three ethanolic extract treatment groups of similar doses, 1 positive control group receiving 15mg/kg P.o. diclofenac, and a negative control group. Phytochemical analysis was carried out according to Harbone 1973 protocol. Pain and inflammation was induced by injecting 50microliter of 5% formalin into the sub plantar of hind left paw 60 minutes after administration of the herbal extract or positive control. The duration the rat spent lifting, flicking and licking the injected paw was observed in two phases and recorded. First phase was in the first 0-5 minutes while the second phase from 15^{th} -30^{th}minutes.Diameter of the paw was measured just before formalin injection and every 30 minutes for four hours using a digital Vernier calipers. Blood was drawn through intracardiac puncture and evaluated for AST and ALT levels at 28^{th} day. Animals were euthanized using dose sufficient chloroform. Left hind paws were harvested and preserved in 10% formaldehyde. Fixed Tissues were stained with haematoxylin and eosin for histology examination using Leica M205C stereomicroscope. Data was entered in MS Excel spread sheet and analyzed using SPSS v26.0. Statistical analysis was done using one way ANOVA and Scheffe's post hoc test at 5% significance level (α = 0.05. Qualitatively flavonoids, sterols, Saponins, tannins, terpenoids, cardiac glycosides, phenols and anthraquinones were detected. Both aqueous and ethanolic plant extracts did not demonstrate antinociceptive effects ($p>0.05$). Aqueous plant extract 100 mg/kgbwt, ethanol plant extract 25 mg/kgbwt, ethanol plant extract 50 mg/kgbwt and ethanol plant extract 100mg/kgbwt had potent paw antiedema effect ($p<0.05$) from 30^{th} minute of inflammation induction. Both aqueous and ethanolic plant extracts did not show hepatotoxic effect ($p>0.05$). Aqueous plant extract 100 mg/kgbwt, ethanol plant extract 25 mg/kgbwt, ethanol plant extract 50 mg/kgbwt and ethanol plant extract 100mg/kgbwt had potent reduction of inflammatory cells (lymphocytes) ($p<0.05$) observed from paw tissue histology.

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LIST OF ACRONYMS AND ABBREVIATIONS

%	-	Percentage
5HT	-	5 hydroxytryptamine
AA	-	Arachidonic acid
AC	-	Adenylyl cyclase
ACC	-	Anterior cingulate cortex
ALT	-	Alanine aminotransferase
ANOVA	-	Analysis of variance
AQ	-	Aqueous
AST	-	Alanine aminotransferase
BC	-	Before Christ
cAMP	-	Cyclic adenosine monophosphate
CGRP	-	Calcitonin gene related peptide
Cm	-	Centimeter
CNS	-	Central nervous system
COX-1	-	Cyclooxygenase-1
COX-2	-	Cyclooxygenase-2
CPE	-	Craterostigma plantagineum extract
CYP450	-	Cytochrome P 450
DAMPS	-	Danger associated molecular patterns
DCM	-	Dichloromethane
DICLO	-	Diclofenac sodium
DMSO	-	Dimethyl Sulfoxide
DOR	-	Delta opioid receptor
DRG	-	Dorsal root ganglia

ED - Effective dose

ETOH - Ethanol

GABA - Gamma aminobuteric acid

GILP - Glucocorticoid induced leucin zipper

GnRH - Gonadotropin releasing hormone

GR - Glucocorticoid receptor

HATS - Histioneacetyltransferases

HETEs - Hydroxyeicosatetraenoic acid

HSP90 - Heat shock protein 90

I.p - Intra peritoneal

IC - Insular cortex

ICAM-1 - Intercellular adhesion molecule 1 and 2

ICAM-2 - Intercellular adhesion molecule 1 and 2

IL-1β - Interlukin -1 beta

IL-6 - Interlukin -6

JAKSTAT - Junase kinase signal transducers and activators of transcription

Kg - Kilogram

KOR - Kappa opioid receptor

LFA-1 - Lymphocyte function associated antigen- 1

LPAM-1 - Lymphocyte peyer's patch adhesion molecule- 1

MAdCAM-1 - Mucosal vascular address in cell adhesion molecule- 1

MAP kinase - Mannose activator protein kinase

MELM - Metenkephalin like material

Mg - Milligram

MIN - Minute

MM - Millimeter

MORP - Miu opioid receptor

mRNA - Messenger ribonucleic acid

NDHN - Nociceptive dorsal horn neuron

NMDA - N-Methyl-D-Aspartate

NOR - Nociception opioid receptors

NSAIDs - Nonsteroidal anti-inflammatory drugs

NF-κB - Nuclear factor kappa B

OIH - Opioid induced hyperalgesia

PAG - Periaqueductal gyrus

PAMPS - Pathogen recognition molecular patterns

PG - Prostaglandin

PI3K - Phosphoinositide 3-kinase

PLA2 - Phospholipase A2

PLC - Phospholipase C

PNS - Peripheral nervous system

P.O - Per oral

rACC - Rostral Anterior cingulate

RVM - Rostral Ventro medial

SEM - Standard errors of the mean

SEC - Second

SGOT - Serum glutamic-oxaloacetic transaminase

SGPT - Serum glutamate pyruvate transaminase

SSA - Sub Sahara Africa

STATA - Signal transducer and transcripition activator

STC	-	Spino-thalamo-cortical
TF	-	Transcription factor
TNF-α	-	Tumor necrosis factor –alpha
TRPA1	-	Transient receptor potential cation channel subfamily A1
TRPM3	-	Transient receptor potential cation channel subfamily M3
TRPM8	-	Transient receptor potential cation channel subfamily M8
TRPV1	-	Transient receptor potential cation channel vallinoid subtype 1
TX	-	Thromboxane
USA	-	United States of America
VCAM	-	Vascular cell adhesion molecule
VlPAG	-	Ventro-lateral periaqueductal gray
VLA-4	-	Very late activation antigen 4
VPL	-	Ventro-postero-lateral
VPM	-	Ventro-postero-medial
WHO	-	World health organization

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CHAPTER ONE
INTRODUCTION

1.1 Background of the Study

"Pain is an unpleasant sensory and emotional experience associated with actual or potential tissue damage" (Crofford, 2015).Pain is considered to be of acute onset if it occurs for the first time in a patient life or if it occurs in a patient who has been free of pain for a duration of six months and lasts less than six weeks (Casser, Seddigh & Rauschmann, 2016) . On the other hand pain is considered to be chronic if present for more than six months. Chronic pain is a cause of worry for health care providers as it is a symptom that brings many patients to the hospital and may give the health care providers a feeling of helplessness as healers (Crofford, 2015).

Effective and timely pain management enhances early healing, reduces duration of hospitalization, reduces chances of progression to chronic pain, lowers cost of treatment and lowers morbidity and mortality rate among the victims (Ahmadi et al., 2016).Besides conventional approaches to pain treatment, there are complementary methods of pain management which include use of acupuncturists, chiropractors, massage therapists, relaxational and meditative techniques like yoga and tai chi, as well as use of herbal medicine.30-40% of United States of America dwellers use these approaches every year (Nahin *et al.,* 2016).

Studies have reported that *Craterostigma plantagineum hoscht* leaves and roots are concocted and taken by mouth to treat liver and diarrheal diseases(Belayneh & Bussa, 2014). The plant has been used by the Oromo community living in Harla and Dengengo valleys in Ethiopia for treatment of Liver diseases and Diarrhea (Belayneh & Bussa, 2014). The Maasai community living in Maasai Maras' Sekenani valley use it for treatment of pain in muscles and joints (Bussmann *et al.,* 2006). No published studies have been done on its antinociceptive and anti-

inflammatory activity to validate its reported effectiveness in treatment of pain. However, *Craterostigma pumilum,* a plant belonging to the same family as *plantagineum hoscht* possessed potent antiinflammatory activity at doses of 25,50 and 100mg/kgbwt, though did not have analgesic effect. Therefore the present study was carried out to investigate the effects of ethanolic and aqueous extract of *Craterostigma plantagineum hoscht* on pain behavior and antiinflammatory response as well as its effect on liver function through analysis of serum alanine and aspartate aminotransferases.

1.2 Statement of the Problem

Pain and inflammation leads to poor quality of life, loss of ability to work leading to losing income, depression and may also cause death. Different classes of painkillers and anti-inflammatory drugs are used in management of pain and inflammation. Nonetheless, some are associated with lack of efficacy, depending on the type of pain, dependence and fatal side effects (Stein *et al.*, 2009).The commonly used pain killers or analgesics confer side effects such as peptic ulcers, kidney damage and bleeding time prolongation (Monteiro-Steagall, Steagall and Lascelles, 2013). *C. plantagineum hoscht* is used in treatment of pain, however studies showing their effectiveness and safety are lacking (Bussmann *et al.*, 2006). The use of its natural extracts may offer a better pharmacological profile becoming a better substitute to the current medications in use. Therefore scientific knowledge on *C. plantagineum* hoscht will provide cognizance into alternative therapy for pain management.

1.3 Study Justification

Utilization of herbal medicine has been continuing despite the availability of modern medicine and this has been done either by choice or due to economic hardships. *Craterostigma plantagineum hoscht* is a natural herb purported to have medicinal value. The antinociceptive effects of the plant are claimed by the Maasai of Sekenani valley, Maasai Mara who use it for treatment of joint and muscle pain by boiling it and drinking the water

(Bussmann *et al.*, 2006).Since majority of analgesics and antiinflammatory medicines are associated with some side effects, natural extract from craterostigma plantagineum may offer a better pharmacological profile becoming a better alternative to the current medications in use. There is absolute need to scientifically study and validate the herbs' purported analgesic and antiiflammatory activity, as well as its safety so as to generate data for dissemination to medical practitioners and other stake holders so as to give the community applicable advice on the herbs medicinal value.

1.4 Significance of the Study

This research will help determine whether *Craterostigma plantagineum hoscht* possess antinociceptive effects as claimed by the Maasai of Sekenani valley and will help in advising the community from scientific perspective. Its publication will also increase in the pool of scientific knowledge and form a basis for further researches.

1.5 Objectives of the Study

1.5.1 General Objective

i. To assess the analgesic and antiinflammatory properties of *Craterostigma plantagineum hoscht* extracts in male albino rats

1.6 Specific Objective

i. To determine the phytochemical constituents of *Craterostigma plantagineum hoscht* extracts using ethanol and aqueous extraction.
ii. To determine pain behavior in formalin induced pain in male albino rats treated with aqueous and ethanolic extracts of *Craterostigma plantagineum hoscht*.
iii. To determine the effect of aqueous and ethanolic extract of *Craterostigma plantagineum hoscht* on formalin induced paw edema in male albino rats.

iv. To determine histologic changes in formalin injected paw tissue in male albino rats following treatment with aqueous and ethanolic extracts of *Craterostigma plantagineum hoscht*

v. To determine the effect of Craterostigma plantagineum hoscht aqueous and ethanolic extracts on serum alanine aminotransferase and aspartate aminotransferase levels.

H0: Ethanolic and aqueous extracts of *Craterostigma plantagineum hoscht* have no effect on pain behavior, inflammatory response and liver function in male albino rats.

H1: Ethanolic and aqueous extracts of *Craterostigma plantagineum hoscht* have effect on pain behavior, inflammatory response and liver function in male rats.

CHAPTER TWO
LITERATURE REVIEW

2.1 Pain

Pain is a personal and subjective feeling which is also unique to an individual and multidimensional. It is an unpleasant sensory, psychological/affective and appercipient cognoscible experience, accompanied by definite or possible tissue damage or described in terms of such damage. Gender, culture, age, previous pain experiences and emotional factors are some of the factors that affect pain(Communication & Vaajoki, 2013). Women have a lesser pain threshold and thus report more pain in comparison to the male gender. In view of age there is varying information regarding pain perception in that some studies have found that pain susceptivity decreases with age while others have found that pain responsiveness is higher in the older age groups (Wandner et al., 2012).Equally, culture affects pain perception in that it defines what behavior is observed as normal or abnormal and how people should behave in regard to this even in the event of pain. For example some cultures will anticipate immoderate behavior expression during pain while others forbearance and underplaying pain is a value to hold (Peacock & Patel, 2008).

2.1.1 Pain behavior

Humans express pain sensation in various forms. First they may express how they are perceiving the pain by describing. However, the expression of pain can be via manners suggestive of pain such as vocalizations, facial contortions, massaging or rubbing the affected area, hobble or even curling (Visser, 2009).

Since pain studies in humans have various limitations such as being subjective and as well as having ethical restraints, animals are therefore normally used for these studies. In animals pain cannot be measured directly and as such conducts that are “painlike” are assessed. Pain conducts in rats include lifting of the paw, licking of the paw, flinching of the paw,

withdrawing the part of the body from the painful stimuli, heightened grooming of the painful area as well as giving vocal sounds (Deuis et al., 2017)

2.1.2 Types of pain

The ability to respond to a stimuli that is potentially harmful is a basic sensory ability (Sneddon *et al.*, 2014). Nociceptive pain is as a result of normal body physiology that helps to appreciate noxious stimuli as being painful. It occurs when tissue damage develops sufficient noxious stimuli to activate free nerve endings (Sneddon *et al.*, 2014).

Acute pain occurs after tissue damage. It has a definite onset and a short duration. This pain wanes off as healing goes on (Brynelson, 2011). Acute pain arises from stimulation of nociceptors as a result of mechanical, thermal or chemical injury to the tissue leading to liberation of products including nerve growth factor, substance P, calcitonin gene related peptide (CGRP) and histamine. These substances activate the Transient receptor potential channels leading to development of receptor potentials and eventual development of an action potential in the nerve fiber. The action potential relayed through A-delta afferent fibers to the dorsal root and the brain is fast, and give rise to sharp pain that is highly localized. Contrary to A fiber, transmission of noxious stimuli through afferent C fibers is slow and this leads to sensation of slow pain that is not well localized (Chen et al., 2022)

However chronic pain continues beyond the anticipated healing time, thus being referred to as persistent pain. Chronic pain arises slowly, its often obdurate to treatment and has no useful purpose (Brynelson, 2011).It is usually associated with other underlying diseases such ad diabetes mellitus, arthritis as well as malignancies. These diseases enhance prolonged inflammation in the tissues and eventually may change the integrity of peripheral nerves leading to rise of neuropathic pain (Chen et al., 2022).

Neuropathic pain, unlike nociceptive pain, occurs as a result of abnormal processing of pain stimulus from either or both the PNS and CNS (Brynelson, 2011). The nociceptors become abnormally hypersensitive to stimuli that are not noxious (Cavalli et al., 2019). Neuropathic pain precedes a primary lesion leading to malfunctioning of the nerve. This primary lesion results from multiple etiologies like trauma to the nerve, chronic diseases such as diabetes mellitus, and treatments such as chemotherapy. These eventually change the integrity and function of the peripheral and central nervous system(Chen et al., 2022). Some of the changes that happen include abnormally enhanced rate of firing of the nerves resulting to rise of spontaneous pain and pain from innocuous stimuli. Other mechanisms involve impairment of pain inhibitory interneurons in the brain or at the dorsal root leading to uncontested transmission of pain. Moreover presence of chronic pain may lead to development of pain memory in the central nervous system pain processing pathways making them sensitized. This involves sensitization of the second and third order neurons. All these pathomechanisms may exist individually or in concert.

Referred pain is the kind of pain that is spread and felt to somatic areas away from the primary location of the noxious stimuli. Theory of convergence phenomenon has been upheld for long (Jin et al., 2023). Convergence theory describes the neuroanatomical arrangement of the nerve fibers where nociceptive fibers converge on the same second order neurons with other sensory neurons making the brain not able to clearly delineate where exactly the pain signal is coming from (Murray, 2009). Another theory explaining referred pain is that of nociceptors releasing a sensitizing substance at the dorsal root that eventually leads to opening of latent synapses that connect to the neurons that transmit pain signals from the area of pain referral (Jin et al., 2023).

Visceral pain is the kind of pain that originates from the internal body organs when they are inflamed or diseased. This pain is normally referred to other body regions making it to be

poorly localized (Collett, 2013). It is also associated with heightened motor and autonomic reflexes and this is because of absence of isolate visceral sensory tracts in the spinal cord and brain, together with the fact that the number of visceral ascending nerve fibers is quite low in comparison to those of somatic emergence. All these makes this pain to be imperfectly localized, to be referred to other areas and to be associated with heightened motor and autonomic reflexes as opposed to somatic pain (Cervero, 2014).

2.2 Inflammation

Inflammation is a broad scope of content of physiological response to an antigen such as pathogens, dust particles and viruses (Arulselvan *et al.*, 2016). It's the body's biological response to injury and infection and include biological response of the somatosensory, immune, autonomic and vascular systems. Neurogenic inflammation arises from nerve activation with subsequent neuropeptide release that leads to rapid plasma extravasation and edema (Matsuda, Huh and Ji, 2019).

There are three pathways through which inflammation is triggered and include Nuclear Factor Kappa B, MAPK and the JAK-STAT. These pathways are actuated by attachment of cytokines to certain cytokine receptors associated with these proteins(Chen *et al.*, 2018).Actuation of the named pathways results to a crosstalk between them that leads to cytosolic formation of transcription factors that translocate to the nucleus of the cell to regulate and enhance expression of proinflammatory cytokines such as interleukins and TNF-alpha (Ibrahim & Huttunen, 2021).

Inflammation can be triggered by infectious and non infectious agents commonly referred to as pathogen associated molecular patterns (PAMPs). Infectious agents comprise of viruses, bacteria, as well as fungi (Picchianti-Diamanti et al., 2018).However, Noninfectious inflammatory process is initiated by sterile molecules known as danger associated molecular

patterns (DAMPs) or alarmists or cell death associated molecules. Examples of DAMPS include mitochondria DNA, nucleus DNA, histones and heat shock proteins. PAMPs and DAMPs are recognized by pattern recognition receptors (PRR) during tissue injury and they trigger cells of the immune system with resultant rise in inflammatory molecules (Rai et al., 2022; Chen *et al.*, 2018). Additionally, cytokines such as Interleukin-6 and TNF-alpha stimulate synthesis of cyclooxygenase 2, an enzyme necessary for breakdown of arachidonic acid to produce more mediators of inflammation.

Inflammation is classified into two types, an acute and chronic type of inflammation. Acute inflammation proceeds from minutes to a few days and serves to deliver leukocytes and other plasma mediators of inflammation to the site of injury. This is achieved by changes in vascular blood flow and change in diameter of small blood vessels resulting in extravasation of plasma containing neutrophils. This is associated with redness, swelling, hotness, pain, loss of function of the affected body organ and shift of acid base balance to acidosis. Transmigration of leukocytes and other plasma mediators to the injured area through the blood vessels is the primary purpose of acute inflammation (Arulselvan *et al.*, 2016).

Endothelial P-Selectin family proteins trigger the attachment of polymorphonuclear neutrophils and platelets to the vascular endothelium during the early phase of inflammation while in later phases depend on L-Selectin(Ivetic, Green & Hart, 2019).Endothelium expression of E-Selectin is activated by IL-1, endotoxin, or TNF-alpha and this leads to leukocyte rolling albeit in a slow manner. Rolling is then followed by rapid leukocytes activation of their integrin proteins (LFA-1, VLA-4, LPAM-1) (Park & Jeen, 2018).These integrin proteins then interact with adhesion proteins of the immunoglobulin family (ICAM-1 and 2, VCAM-1, MAdCAM-1),leading to firm adhesion to the endothelium (de Vries *et al.*, 2017). This is then followed by leukocytes transmigration and the release of mediators of

inflammation such as cytokines, lymphocytes and histamine. These pro-inflammatory agents stimulate corresponding nociceptors causing hyperalgesia(Serhan & Levy, 2018).

However, chronic inflammation is a sequelae of acute inflammation if not arrested. Its characterized by immunopathological changes like invasion of inflammatory cells, chronic over expression of mRNAs for proinflammatory cytokines and aberrant regulation of cellular signaling (Bruni *et al.*, 2018).

2.3 Burden of Pain

Pain is a common widespread problem experienced by large population across the globe. In United States of America over 50 million Americans are affected by pain, costing the states more than 70 billion US dollars yearly in providing medical care. It is also responsible for more than 80% of all physician visits(Gatchel *et al.*, 2007). The American Geriatrics Society Panel on Persistent Pain in Older Persons approximates that about 45% - 80% of patients in nursing homes have significant pain. Lack of proper pain management leads to deprivation of sleep, depression, malnutrition, anxiety, disquiet and the general life quality is lowered (Reisman, 2007).Pain is psychologically significant. It brings about worry; often worry of not what was but worry of what might be. This is a worry of future. Thus chronic pain can lead to even more suffering and disability(Eccleston & Crombez, 2007).Pain management includes use of such drugs as opiates and Nonsteroidal Anti-inflammatory Drugs. Opiates and opioids have bad side effects such as constipation requiring co-prescription with another drug to counter this, gastrointestinal bleeding which is also majorly associated with NSAIDs, cognitive impairment including drowsiness and even death, depression of the respiratory system, endocrine abnormalities majorly hypogonadism due to suppression of GnRH, opioid induced hyperalgesia (OIH) due to neuroplasticity effects, brain volume changes that include some parts of brain getting bigger and others smaller, tolerance, withdrawal effects, addiction

and finally opioids are responsible for more deaths than any other medication with over 16,000 people dying annually from opioid overdose(Teater, 2015).

2.4 Nociception

Nociception is the mechanism by which the body detects injurious damage from intense thermal, chemical or mechanical stimuli by use of specialized receptors (Basbaum *et al.*, 2009).This detection is followed by reflex withdrawal and other nocifensive behavior. All over the body there are specialized sensory nerve endings that transduce painful stimuli. These are called nociceptors. Nociceptors differ in their selectivity (Sneddon, 2018).Types of nociceptors include mechanical, thermal, chemically sensitive and polymodal nociceptors. Nociceptors axons are the A delta fast fibres accountable for the exquisite pain, and the slow unmyelinated C fibers responsible for the persistent dull, burning pain. They innervate the skin, most visceral organs, blood vessels and the heart. However they lack in brain despite being there in meninges (Boron, W. F., &Boulpaep, E. L.2020). 68% of the cutaneous mammalian types of fibers are nociceptive and the rest 32% are involved in touch and pressure. Out of the 68% nociceptive, 12% are A delta, 30% are polymodal C fibers, 20% are mechanothermal C fibers and 5% are silent C fibers. While out of the 32% touch and pressure fibers, 2% are A beta, 6% are non-nociceptive A delta and 5% are touch C fibers of low-threshold (Sneddon, 2018).

2.4.1 Transduction and Transmission of Pain

This is the mechanism by which noxious stimuli is coded to electrical signal, and this happens in the nociceptors. The noxious stimuli could be thermal, mechanical or chemical(Vanderah, 2007). The stimuli will lead to release of pronociceptive inflammatory molecules such as prostaglandins, serotonin, adenosine triphosphate, potassium, bradykinins, interferons, substance P, CGRP and TNF from different cells in the blood like the

polymorphonuclear cells, platelets and mast cells. Pronociceptive inflammatory mediators sensitizes the nociceptors and leads to primary hyperalgesia(Marchand, 2008).

Nociceptors fibers send the signal to the dorsal horn of the spinal cord, the fiber synapses with a second order neuron, then to the thalamus and lastly to the cerebral cortex (Dubin & Patapoutian, 2010).A delta fibers synapse majorly at the level of the superficial laminae I and II but they as well have collateral fibers to laminae V,VI,VII and X (Gauriau & Bernard, 2002).At the dorsal horn the peripheral nociceptors fibers release specific neurotransmitters such as glutamate and substance P. Glutamate binds to N-methyl-D-aspartate (NMDA) as well as Non-NMDA excitatory amid acid receptors on the nociceptive dorsal horn neurons receptors. Substance P binds to tackykinin receptor family of G-protein coupled receptors on the nociceptive dorsal horn neurons (NDHN) (Vanderah, 2007). Nociceptive ascending fibers also synapse at the spinal cord with inhibitory and excitatory interneurons, which are responsible for modulating the nociceptive signal at the second order neuron before it ascends to the brain. Second order neurons climb to the brain through two major pathways namely the spinothalamic tract and the spinoreticulothalamic tracts(Basbaum *et al.*, 2009). Nonetheless, there is also a third pathway located at the dorsal column medial aspect which also conducts nociceptive signals from the viscera (Marchand, 2008).Spinothalamic tracts pathway is more involved in sensing point of the stimuli in the body and its intensity (sensory discriminative pain aspect),while the spinoreticulothalamic does not localize pain well (Basbaum *et al.*, 2009).

Spinothalamic tract: These are responsible for conducting rapid pain. They ascend to the contralateral lateral nuclei of the ventrobasal thalamus (Ventroposterolateral and Ventroposteromedial) and the centromedian nuclei (Vania, 2021) .

Affective spinoreticular tract: They travel up to the medial thalamic nuclei and to the periaqueductalgrey (PAG) and the nucleus raphe magnus(NRM), which are brainstem structures that are involved in pain modulation. Tertially neurons from Ventrobasal nuclei project to the primary and secondary somatosensory cortices (SI, SII) while those of centromedian nucleus terminate in the limbic system (Boron & Boulpaep, 2020). Four cortical structures have been documented to be key for pain discernment. They are the somatosensory cortex SI which is located in the parietal lobe, secondary somatosensory cortex SII located in the parietal operculum, the anterior cingulate cortex situated superior to corpus callosum circumvolution and finally the insular cortex (IC) located inferior to the frontal and temporal lobes at the sylvian fissure level. Sensory discriminative aspect of pain is majorly a function of SI and SII, while ACC and IC are necessary for affective element of pain (Marchand, 2008).

2.4.2 Pain Perception

Pain arises from well-programmed stimulation of several brain areas together known as a pain matrix. Pain matrix is made of multiple connected networks which receives sensory input from the ascending afferent nociceptive pathways (Garcia-Larrea & Bastuji, 2018). Functional imaging studies do not show a single pain centre but a set of brain structures (pain matrix) that become activated to stimuli causing pain with conscious perception of pain taking less than one second. The spinothalamic and extrathalamic sensory tracts first send nociceptive signals to sensory, motor and amygdala in a parallel and concurrent manner. Afterwards there is stimulation and activation of other cortical structures which leads to conscious awareness of pain(Bastuji *et al.,* 2016).The two main pathways for nociception signal from the spinal cord to cerebrum are the spino-thalamo-cortical(STC) and the spino-parabrachial-amygdalar pathways. From thalamus, STC sends input to posterior insula, medial parietal operculum and mid cingulate cortex which are cortical regions that plays part

in sensorimotor integration and attentive drive.Spino-brachial pathway sends input to the limbic system through the amygdala complex, to stimulate autonomic responses and affective element of pain. Events of STC and spino-parabrachial-amygdala pathways happen in a simultaneous fashion(Bastuji *et al.*, 2018). Pain is an experience that is conscious. It results from a nociceptive signal to the brain whose interpretation is determined by memories, emotions as well as pathological, genetic and cognitive factors. Pain can also be reported in absence of primary nociceptive stimuli. Pain is highly subjective as an individual's response to painful stimulation is determined by what is suitable and or feasible in that particular situation. Its therefore not easy to evaluate, analyse and treat pain (Tracey & Mantyh, 2007)

2.5 Modulation of Pain

H.K. Beecher, a physician for the American soldiers in world war II observed as many as three quarters of severely wounded soldiers who presented with less pain inconsistent with the degree of the injuries they were experiencing. He made a conclusion that "strong emotions" inhibit pain (Beecher, 1991). This inhibition is conferred by the endogenous pain modulating mechanisms with signals arising from hypothalamus, amygdala and rostral anterior cingulate cortex (rACC) to stimulate the periaqueductal gray (PAG).rACC mediates Placebo and expectations analgesia by stimulating PAG (Ossipov, Dussor & Porreca, 2014).

Neuronal signals output from PAG feed the neurons in the nucleus raphe magnus and the nucleus reticularisparagigantocellularis (both pars alpha and cellularislateralis) in the rostral ventro-medial medulla (RVM). RVM also receives inputs from thalamus, parabrachial nucleus, and locus coeruleus. Neurons from RVM, which includes GABAergic, glycinergic and serotonergic, project to the spinal dorsal horns and to the trigeminal nucleus caudalis, to increase or decrease traffic of signals from nociceptive fibers. Studies have shown that serotonin is the main neurotransmitter released from this RVM projection at the spinal cord and stimulate 5-Hydroxytryptamine receptors (5-HT). Stimulation of 5-Hydroxytryptamine

type 1A, type 1B, type 1D and type 7 produces antinociception, however stimulation of 5-HT2A and 5-HT3 enhance nociception (Ossipov, Morimura and Porreca, 2014) .

2.6 Analgesic drugs

These are also known as painkillers and include various classes such as nonsteroidal antiinflammatory drugs (NSAIDs) that inhibit cyclooxygenase pathways of arachdonic acid metabolism, opioids that bind to opioid receptors , cannabinoids that bind to cannabinoid receptors, and flupirtine which is an inward potassium current rectifier (Oertel & Lötsch, 2013). Analgesic benefit is also conferred by steroidal antiiflammatory drugs,such as prednisolone (Del et al., 2018)

2.6.1 Mechanism of Action of Analgesic Drugs

2.6.1.1 Steroidal Anti-Inflammatory Drugs

The chemical structure of anti-inflammatory steroids relate in a close way to the 17-hydrocorticosterone (cortisol/hydrocortisone), which is a hormone of adrenocortical origin. Progesterone, estrogen, testosterone and aldosterone are also adrenocortical steroids but they lack anti-inflammatory effects. Steroidal anti-inflammatory drugs bind to cytosolic receptors -just like biologic cortisol – in different organs of the body (Jasani, 1979).

Glucocorticoids confer their anti-inflammatory activity by suppressing immunity through production of anti-inflammatory cytokines. They bind to glucocorticoid receptors, GR, found in the cytosol(Tashkin, Lipworth & Brattsand, 2019). GR is a member of nuclear receptor superfamily which are encoded in chromosome 5q31-31(Heming *et al.*, 2018). Free GR is bound to a chaperon heat shock protein 90 (Hsp90)(Tashkin, Lipworth and Brattsand, 2019),heat shock proteins 70 and immunophilin (Heming *et al.*, 2018).Binding of glucocorticoid to GR causes dissociation from the chaperon and formation of Glucocorticoid-GR complex which is a transcription factor that translocate into the nucleus of the cell to bind to the DNA(Tashkin, Lipworth & Brattsand, 2019). Glucocorticoid-GR

complex binds to glucocorticoid responsive elements of the target gene regulatory region. The complex inhibits formation of histone acetyltransferases (HATs) enhancing tightening of the histone and making DNA inaccessible to DNA polymerase for transcription of pro-inflammatory genes. Transcription factor (TF) NF-κβ activates HATs. Glucocorticoids increase transcription of 1κβα protein, which binds to NF-κβ inhibiting its activity. Glucocorticoids also bind to NF-κβ in the cell cytosol sequestering it. Glucocorticoids also confers its immune modulation by enhancing expression of genes coding for glucocorticoid-induced leucin zipper (GILZ) which impedes NF-κβ, promoting annexin 1 protein formation that inhibits expression of phospholipase A2 necessary for arachidonic acid catabolism and by inhibiting Mitogen activated protein kinase phosphatase 1. MAP kinase phosphatase 1 is responsible for translocation of GATA-3 which leads to expression of genes responsible for type 2 T-helper cell (Th2) inflammatory molecules expression, that is IL-4,-5,-9 & -13).Therefore absence of MAPK phosphatase 1, GATA-3 doesn't translocate into the nucleus and as such there is no enhanced cytokine release Dinarello, 2010; Heming *et al.*,2018; Hwang et al., 2010).

2.6.1.2 Non-Steroidal Anti-Inflammatory Drugs

NSAIDs like ibuprofen, diclofenac and naproxen are the mostly used drugs for treatment of mild to moderate inflammatory pain. This is because of their non-addictive nature (Grosser, Ricciotti & FitzGerald, 2017). Arachidonic acid (AA) is a phospholipid that is released from stressed cell membrane by the enzymes phospholipase A2 (PLA2) and Phospholipase C (PLC). AA is then broken down into proinflammatory mediators via three pathways: Cyclooxygenase pathway; lipooxygenase pathway; and cytochrome P450 (CYP450) pathway (Wang *et al.*, 2019). NSAIDs inhibit cyclooxygenase 1 and 2 (COX1 and COX2) enzymes which are responsible for breakdown of arachidonic acid (eicosa-5, 8,11,14-tetraenoic acid) to its metabolites that are responsible for promoting inflammation (Oladosu, Tu & Hellman,

2018).COX isoenzymes breaks down arachidonic acid to prostanoids (prostaglandin [PG] E2, D2, F2,PGI2) and thromboxane A2 (Altman *et al.*, 2015).At superphysiologic concentrations ,NSAIDs inhibit pain through other mechanisms such as proteasome activity inhibition, inhibition of NF-κβ and activation of stress kinase (Smith *et al.*, 2017).

Diclofenac sodium, (2-[2,6-dichloranilino] phenylacetic acid), is a derivative of phenylacetic acid and possess anti-inflammatory, antipyretic and antinociceptive effects(Gan, 2010). Diclofenac reduces synthesis of prostaglandins, mediators of inflammation through inhibition of cyclooxygenase enzyme. Besides, it also enhances uptake of arachidonic acid into triglycerides pool. Thus it reduces the amount of arachidonic acid available for metabolism through the 5-lipoxygenase pathways for formation of leukotrienes (Ku *et al.*, 1985)

Reduced leukotrienes levels have been associated with reduction in release of substance P, a potent neurotransmitter that promotes nociception (Zieglgänsberger, 2019). Other pathways of mechanism of action of diclofenac have been hypothesized, such as diclofenac is reported to possess analgesic benefit by increasing peripheral and central levels of cyclic guanosine monophosphate (cGMP) through the L-arginine-nitric-oxide-cGMP pathway. The effect of this is to down regulate the peripheral pain receptors that are sensitized.

In one animal study it was found that administering low dose of phosphodiesterase 5 – inhibitor elevated the analgesic effect of sub therapeutic dose of diclofenac. There is also possibility that diclofenac also has its analgesic activity through opioid receptors as administration of naloxone reversed its analgesic effect (Gan, 2010).

There is growing affirmation for disconnection between analgesic and anti-inflammatory mechanisms of action of diclofenac. Antinociceptive effect of diclofenac in the brain and the spinal cord has been associated with diclofenacs' increased levels of Kynurenate, which is an endogenous antagonist of N-MDA receptor. Intense pain is coupled with heightened release

of glutamate in the spinal cord and diencephalon that activate NMDA receptors promoting nociception. However kynurenate competitively binds for the same receptors dampening nociception(Edwards *et al.*, 2000).

2.6.2 Mechanism of Action of Opioids

Opioids are analgesics for treating moderate - severe pain, both acute and chronic. This is especially for patients suffering pain from cancer or surgery (Owusu , Hamadeh & Smith, 2017).

They mediate both peripheral and central pain analgesic mechanisms. Opioids bind to receptors coupled to G protein (GPCR) namely delta (δ) opioid receptors (DOR), kappa (κ) opioid receptors (KOR), mu (μ) opioid receptors (MORs) and nociception receptors (NOR)(Corder *et al.*, 2018)..

Opioid receptors are found in the the spinal cord, at the dorsal root, and in the brain. Opioid stimulate opioid receptors coupled to Gαi/Gα0 that inhibits adenylyl cyclase (AC) with subsequent decrease in cAMP. The Gβγ subunit inhibits P/Q-, N-, and L type voltage gated calcium channels at the presynaptic terminus. This inhibits calcium dependent trafficking and docking of synaptic vessels and subsequent decrease in exocytosis of the excitatory neurotransmitter, like glutamate, into the synaptic cleft with resultant decrease in nociceptive signals (Corder *et al.*, 2018).

Stimulation of opioid receptors at dorsal root ganglia produces antinociception. However, some studies have shown that stimulation of MOPRs in DRG nociceptors may be associated with adverse drug effects associated with prolonged use of opioids which are tolerance and opioid induced hyperalgesia (OIH) (Stein *et al.*, 2009).

Central and peripheral binding of opioid receptors leads to increased conductance of potassium (efflux) and decreased conductance of calcium (influx) thus decreasing excitability of the neuron (Rogers & Henderson, 1990).

In the CNS opioids exert analgesic activity through descending pain regulatory pathway that composes of ventrolateral periaqueductal gray (vlPAG), rostral ventromedial medulla (RVM), and the spinal cord (Basbaum & Fields, 1984).Signals from these areas exert antinociceptive activity by inhibiting "on" cells and disinhibiting "off" cells, and also by projecting to the spinal cord DRG to modulate pain at the nociceptive and trigeminal dorsal root ganglia(Corder *et al.,* 2018).

Activation of MOR leads to suppression of GABA interneurons in restromedial tegmental nucleus, suppression of GABA inputs from the nucleus accumbens-D2 expressing neurons and also suppression of GABA neurons in PAG and raphe magnus. This inhibition results to opioid analgesic activity(Valentino & Volkow, 2018).

2.7 Hepatocellular Injury

There are various ways in which destruction of liver can occur and include among others direct disruption of hepatocytes, activation of T cell that now functions as an immunogen, stimulation of apoptotic pathways and reduction of adenosine triphosphate (ATP) levels through disordered functioning of mitochondria (Fatima *et al.,* 2016).

Hepatotoxicity is dose dependent or when the herb is prepared in an incorrect way or used for incorrect purposes (Phua, Zosel & Heard, 2009). It is important to note that the degree of liver grave harmfulness is determined by evaluation of appropriate liver biochemical parameters such as ALT and AST (Venkataraman et al., 2010).

2.7.1 Alanine Aminotransferase (ALT)

Alanine aminotransferase (ALT) enzyme was earlier referred by the name serum glutamate-pyruvate transaminase. Arthur Karmen et al were the first scientists to describe this enzyme in 1950's. Serum ALT and Aspartate aminotransferase (AST) blood levels are measured as biological markers for liver health(Metón *et al.,* 2015). ALT is formed and stored in the cytosol of the hepatocytes therefore making it more specific indicator of liver inflammation.

This is unlike AST which is found in hepatocytes mitochondria and red blood cells cytosol(Agbafor *et al.,* 2017).The normal ALT blood levels ranges from 5 to 35 u/l with its blood level being directly proportional to the degree of liver damage. ALT levels can rise up to 50x the normal (Huang *et al.,* 2006); Wang *et al.,* 2016). Nonetheless, different kits have varying reference range.

ALT measurement is relatively a readily available and fairly non-expensive test used to evaluate for liver inflammation. A couple of factors affect its activity and its plasma levels. These factors include among others Muscular diseases, ethnicity, age, gender, the analysis method used in the laboratory, and diurnal variation, infections such as viral hepatitis, various medications including herbal and alcohol use (Liu *et al.,* 2014).

2.7.2 Aspartate Aminotransferase (AST)

AST was formerly known as serum glutamic oxaloacetic transaminase (SGOT) with its gene located in chromosome 16. It maintains nicotinamide adenine dinucleotide /reduce nicotinamide adenine dinucleotide ratio in cells through its role in malate aspartate shuttle (Ndrepepa, 2021).Though found mainly in the liver, it is also present in other organs just like ALT (Huang *et al.,* 2006).However, its nonspecific marker for liver inflammation compared to ALT (Metón *et al.,* 2015).The normal AST serum levels is a range of 0-50u/l.

2.7.3 Craterostigma plantagineum hoscht for pain management

Craterostigma plantagineum hoscht is one of the herbs whose leaves and roots are concocted and taken by mouth to treat liver and diarrhea diseases(Belayneh & Bussa, 2014). Maasai community living in Sekenani valley has used it for treating pain arising from muscles and joints (Bussmann *et al.,* 2006).Some of the phytochemicals in *Craterostigma plantagineum* include flavonoids. Flavonoids were documented to exhibit anti-inflammatory activity by inhibiting arachidonic acid (eicosa-5, 8,11,14-tetraenoic acid) metabolism (Mwonjoria *et al.,* 2016).Products of arachidonic acid metabolism include prostaglandins, thromboxanes,

leukotrienes, and hydroxyeicosatetraenoic acids (HETEs) which stimulate nociceptors to cause inflammation and pain sensation (Wang et al., 2019).Thus *Craterostigma plantagineum* antinociceptive and anti-inflammatory role could possibly be secondary to flavonoids hampering of arachidonic acid metabolism.

2.7.4 Biology of Craterostigma plantagineum hoscht

Craterostigma plantagineum belong to a group of plants called the resurrection plants. These plants can bring to life from an air dried condition because they are homoiochlorophyllous and they keep hold of their chlorophyll together with complete photosynthetic structures. Thus they dry without senescence and easily recover from desiccation (Bartels, 2005).The process by which these plants acquire senescence and how desiccation pathways are controlled is not clear. However different genes are regulated during dehydration and rehydration (Griffiths, Gaff & Neale., 2014). Craterostigma plantagineum has high amounts of 8-carbon sugar octulose that is utilized as carbohydrate supply in states of dehydration. They also contain various sugar alcohols and sugar acids which work conjointly to steady proteins and macromolecules, cushioning them from destruction by reactive oxygen species (Gechev *et al.*, 2014).

2.8 Phytochemicals

These are substances that have biological effects and are found in plants, fruits, grains and vegetables. According to a study by Passon *et al.* (2021), there was high phenolics extraction in craterostigma plantagineum hoscht. However, there is scarcity of literature regarding phytochemicals present in Craterostigma plantagineum hoscht. Some of the more than 10,000 established phytochemicals are the saponins, tannins, steroids, flavones, alkaloids, triterpenoids, polyphenols and cardiac glycosides. Though there still exists a large number of phytochemicals that are not yet identified (Zhang *et al.*, 2015). Phytochemicals are classified broadly into six groups: alkaloids, phenolics, organosulphurs, carbohydrates and the nonnutritive proteins and lipids. Phenolics include the tannins, stilbenes, napthoquinones,

lignans, and flavonoids.carbohydrates consist of phytates while lipids consist of carotenoids, sterols and chlorophyls (Bolling *et al.*, 2011). Phenolics are available in large quantities and are the most known to have antioxidant and anti-inflammatory effects. Other phytochemicals with antioxidant/anti-inflammatory effects include the carotenoids, terpenoids, polysaccharides and Saponins (Yu *et al.*, 2021; Manganaris *et al.*, 2018). Saponins possess anti-inflammatory effects as well as controls blood sugar and cholesterol levels. However they cause hemolysis when given intravenously while in some cases they lead to loss of function of the liver and the kidneys (Marrelli *et al.*, 2016).

Several studies have linked phytochemicals with potent anti-inflammatory activities. This activity is mediated through epigenetics where inflammatory genes are methylated in white blood cells in peripheral circulation suppressing their expression. This happens with subsequent expression of genes that are anti-inflammatory (Manganaris *et al.*, 2018)

Different solvents are used for extraction of phytochemicals with the polarity of the solvent being the determinant of what type and how much of the particular phytochemical will be extracted. High polarity solvents such as methanol and water extractions yield more phytochemicals compared to ethanol, chloroform, acetone or dichloromethane extraction. In a study done by Truong *et al.*(2019), Methanol yielded more phenolic extraction 13.36mg/GAE/g DW compared to water extraction at 5.95mg/GAE/g DW, with flavonoids, alkaloids and terpenoids yields being slightly lower in water extraction compared to methanol extraction. In a study done on phytochemical extraction from Bucidabuceras and Phoradendroncalifornicum using different solvents, methanol extraction yielded the highest phenolic compounds followed by aqueous extraction. However it's important to note that polyphenols degree of solubility is determined by the presence and position of its hydroxyl groups, as well as the size of the polyphenol molecule, the polarity of the solvent and solute solvent ratio (Do *et al.*, 2014).

2.9 Experimental Rat Biology

Rats are used as models in medical research studies because of their genetic and physiologic similarity to people. They are also social, reproduce quickly and their small size makes it possible to store them in laboratories easily. Their relative bigger size than mice make them easier to handle (Papadimitriou *et al.,* 2008).They are white in colour and the eyes are pink. Males become sexually mature at the age of 6-7 weeks and live for 2-3 years.

2.10 Nociceptive Tests

2.10.1 Tail Flick Test

This test was first described and used by D'amour and Smith in 1941.They used heat rays from a Mazda 1184, and reflected it on the tip of the rat tail using 6-8 volt bulb with a reflector. The tail of the rat was placed 6 inches above a groove where the heat emanated (D'amour & Smith, 1941). The time was then noted between the exposure to heat to when the rat twitched its tail suddenly an indication of feeling pain. This latency period is a measure of pain threshold. The tail flick response is a spinal reflex which is used to test for nociception. The test is greatly influenced by the skin temperature in an inverse relationship and any drug that lowers the skin temperature extends the tail flick latency period (Yam *et al.,* 2020). Animals respond to painful stimuli by simple reflexes such as withdrawal from the source followed by vocalization and then either licking, lifting or scratching the stimulated body part (Baumans *et al.,* 1994).

2.10.2 Writhing Test

This test is chemically induced and was first described by Siegmund et al (1957). Intraperitoneal phenylquinone were injected into mice which induced "writhing syndrome" characterized by abdominal contractions, extension of hind limbs, and twisting and turning of the trunk. Other chemicals can be injected intraperitoneally such as acetic acid and typical nociceptive behavior observed include arching of the back, stretching of the body and forelimbs extension (Kotoda *et al.,* 2019).Writhing is an open response to intense pain

secondary to chemical induced irritation, and cause increased production of prostaglandins and substance P which increase nociceptors sensitivity (Gawade, 2012). Ability of a drug or herb extract to reduce chemically induced writhing test indicates a process of antinociception that involve inhibition of COX pathways thus reducing Prostaglandin synthesis. The specific prostaglandins involved in pain are PGE2 and PGF2α. Suppression of PG synthesis is a peripheral way of pain modulation (Muhammad, Saeed & Khan, 2012).

2.10.3 Formalin Test

This test was first described for rats and cats by Dubuisson and Dennis (1977). This test involves injecting of formalin into the paw of the animal to stimulate pain. It is widely used for pain in research and produces two waves of pain; an early phase and a late phase. Both phases are characterized by behavior suggestive of pain such a licking, biting, shaking and lifting of the injected animal paw. The first phase of pain involves direct stimulation of nociceptors while the subsequent second phase involves central sensitization (Lopes *et al.*, 2019).The first phase of pain is made of the first 5 minutes after formalin injection. From 5-15 minutes is the interphase. And the second phase of pain is from the fifteenth minute to the end of the sixtieth minute. A visual score is given as follows:0-if the rat doesn't show any unusual behaviour; 1-the rats claw is on the floor but doesn't bear weight; 2-the rat strikes the floor with the claw or lifts the claw to the surface of the abdomen; and 3-if the rat licks or bites the injection area of the claw (Rahimi, Sajedianfard & Owji, 2019).

Injection at hind paw is important because the forepaw are used for grooming purposes and it is easier to inject the soft tissues of the hind paw as opposed to the fore paw (Capone & Aloisi, 2004).Subcutaneous injection of formalin produces acute pain that lasted about 5 minutes (initial or first phase of pain),then vanish completely for about 5 minutes, and then reappeared lasting about 20 minutes with a peak occurring between 15-20 minutes (second phase of pain) (Ohkubo et al., 2012). Subcutaneous formalin injection in the paw induced

activation of rat's spinal cord enkephalinergic neurons by release of metenkephalin like material (MELM) in the spinal cord CSF at the lumbar region. MELM release occurred 5-10 minutes after formalin injection and lasted for 5-10 minutes. This is responsible for the transient reduced nociception observed 5-10 minutes after formalin injection (Capone & Aloisi, 2004; Le & Gazariuand Cadden, 2001).

Scoring by licking of the hind paw is more reliable as it is consistent, easy to observe and to quantify. Opioids acts on CNS and inhibits both phases of nociception equally while NSAIDS/steroids work peripherally and suppress the second episode of nociception (Ferreira *et al.*, 2013).

2.10.4 Experimental use of DMSO

DMSO exhibits amphipathic nature thus able to dissolve hydrophobic drug molecules. It's used as a vehicle for biological compounds which have weak solubility in water. It generally shows low toxicity and this is normally seen in chronic use, that is over 6 months at concentrations above >10%.

CHAPTER THREE
MATERIALS AND METHODS

3.1 Study Area

This study was conducted at University of Eldoret, a public university located in Eldoret Township along the Eldoret Ziwa road, approximately 9km from Eldoret Town in Uasin Gishu County, Kenya. The university offers both scientific and arts courses. All experiments that pertain to handling and administration of craterostigma plantagineum hoscht extract, Diclofenac sodium, DMSO and harvesting of paw tissues were done in the department of Zoology because of the established animal house where the rats were kept. Tissue preparation was done in the same university, while processing for histology study and analysis was done at the Jomo Kenyatta University of Agriculture and Technology (JKUAT) School of medicine department of human anatomy, histology laboratory. The photomicrographs were taken at the same department.

3.2 Study design

This was a post-test only true experimental study design in which an intervention was implemented to compare results against the control group.

3.3 Experimental animals

This study was conducted on male albino rats of the species of *Rattus norvegicus* that were purely bred from department of zoology whereby the animal house can hold 150-200 rats in a given research period. At the time of this study the animal house had 160 animals. The albino rats were bred in cages which can hold 5-10 rats per cage. The rats have a white coat with pink eyes. They weigh an average of 200-450grams and become sexually mature at about 6-7 weeks in males and 4-5 weeks in females. They have a lifespan of two to three years. Male rats were used to avoid data variability caused by estrous cycle.

3.4 Sample size determination

The sample size was calculated using the modified resource equation method, which is an alternative to the power analysis approach as it is difficult to get prerequisites such as standard deviation (Arifin and Zahiruddin, 2017).

$$n = DF/k + 1$$

n= animal number per group

k= group number

DF= error of degree of freedom

DF range from 10 to 20 is used as a way of obtaining the maximum and minimum number of animals per each group.

Therefore, sample size per group is calculated as

$$\frac{20}{8} is\ 2.5 + 1 = 3.5$$

Since the highest degree of freedom is used, then the number of animals per group is round down to 3

Therefore the total number of subjects:

$$N = k\ X\ n.$$

$$N = 3x8 = 24$$

3.5 Sampling method

Systematic Simple random sampling (SSRS) method was used whereby the animals were given numbers 1-90 and every 3rd rat was picked into the study until the 24 rats were

randomly picked from the 90 male rats that had attained the weight of 200-250 grams. They were assigned to the two major groups as either experimental or control using simple random sampling.

3.6 Selection criteria.

3.6.1Inclusion criteria

- Male albino rats weighing 200-250 grams.

3.6.2 Exclusion criteria

- Sick animals.
- Animals that had not attained the desired weight

3.7 Grouping of animals

Animals were grouped into two major groups, the control and the experimental group. Simple random sampling was used to allocate the 24 animals into their appropriate groups. The control group had 6 animals that were further classified into two;3 animals in positive control group (diclofenac sodium) and 3 animals in negative control group. The experimental group had 18 animals that were further categorized into two; the ethanolic extract and the aqueous extract treatment groups with each having three different dosages of 25mg/Kgbwt, 50mg/Kgbwt and 100mg/Kgbwt with 3 experimental animals in each dosage.

Table 3.1: Grouping and dosing of study animals

	Extract doses	**Aqueous Extract group**	**Ethanol extract group**	**Positive control group (Diclofenac sodium 15mg/Kgbwt/day)**	**Negative Control group**
No.Of animals.	25mg/Kgbwt	3	3	3	3
	50mg/Kgbwt	3	3		
	100mg/Kgbwt	3	3		

3.8 Feeding of the rats

The animals were fed on rodent pellets that were obtained from Eldoret Parkers in Eldoret town and water *ad libitum.* Animals were fed in the morning inside the cages that were

spacious and as they were allowed to stay in the cage for two weeks to acclimatize. They were housed in a 12-hour dark light cycle. Overnight fasting of the animals was done prior to initiation of treatment.

3.9 Handling of animals

Cleaning of cages, animals head count to ascertain their numbers per group as well as feeding were done every morning throughout the study period by the researcher. The researcher further ensured that the safety measures on occupational health were adhered to through consistent donning on laboratory coats, wearing eye glasses, gloves and closed shoes, and ensuring hand hygiene was observed before and after entry into the animal house.

3.10 Materials

3.10.1 Plant parts

The plants were collected from the Sekenani valley of Maasai Mara and were identified and authenticated at the herbarium school of biological sciences of JKUAT. A voucher specimen was reserved for reference at the same herbarium.

Figure 3.1: An image of *Craterostigma plantagineum hoscht p*lant

3.10.2 Drugs and Chemicals

The drugs and chemicals used in this study included ethanol, distilled water and 5% dimethyl Sulfoxide (DMSO), Sulphuric acid, ammonia, Mayer's reagent, 5% lead acetate solution, 0.1% ferric chloride, 10% sodium chloride, 1% gelatin, water, magnesium turnings. While the drugs included 50mg, oral Diclofenac sodium tablets. Dissolving diclofenac sodium tabs in normal saline made the positive control. While the negative control composed of the group that was subjected to formalin injection with no treatment given.

3.11Extraction of the Plant Materials

The plants parts were air dried and powdered by grinding using a micro-mill ® grinder motor 115v. The aqueous and organic constituents of the powder were obtained as follows:

3.11.1 Aqueous extract

Aqueous extraction was done by putting 500grams of ground powder in a flask with distilled water. It was then set in a shaker for 48hours and filtered twice using a filter paper. The filtrate was then frozen in a deep freezer for 2 days and then taken for freeze drying in a freeze drier. The product was removed from freeze drier after 2-3 days when all water had been removed. The product was then weighed (56g) and preserved at 4 degrees Celsius. During the experiment, 500mg of the product was reconstituted in 2.5ml of 5% DMSO. Normal saline was then added to make up 25ml.This made up a solution strength of 20mg/ml of CPE in 5% DMSO in NS. This is what was taken as 25,50 and 100mg/kg bodyweight in the experimental mice.

3.11.2 Ethanol extract

500grams of Ground powder of Craterostigma plantagineum was soaked in 70% ethanol for 48 hours. The compound was then sieved two times; initially using a muslin cloth followed by a filter paper. Ethanol in the filtrate was allowed to vaporize for 48 hours utilizing a rotary evaporator. The product was preserved in the refrigerator at 4 degrees Celsius. During the experiment, 500mg of the product was reconstituted in 2.5ml of 5% DMSO. Normal saline

was then added to make up 25ml.This made up solution strength of 20mg/ml of CPE in 5% DMSO in NS. This is what was given to the experimental group as 25mg/kg, 50mg/kg and 100mg/kg bodyweight. DMSO was used because it is able to solvate polar and non-polar substances.

3.12 Sensorimotor Test

This test was done to investigate for probable imprecise muscle relaxant or depressant effects of the plant extracts. This test was done before giving the extract, drug or the negative control and also one hour after these treatments. A sensorimotor apparatus was used which consisted of 3 vertical rods with diameter of 2.5cm, heights of 20,32 and 64 cm. Animals were gently positioned on top of every rod for 20 seconds per rod.20 seconds was used as the cut off time per rod.

3.13 Phytochemicals Screening

These tests were used to evaluate for presence or absence of some plant specific chemicals. The reagents were sourced from Kobian Kenya Limited Mombasa road, Nairobi, Kenya.

Test for anthraquinone-5ml of the extract solution will be hydrolyzed Sulphuric acid (H2SO4) extracted with benzene.1 ml of diluted ammonia will then be added. Presence of rose pink colour will be indicative of anthraquinone presence

Test for alkaloids –1ml of the extract was tested with Mayer's reagent. Appearance of opalescence or yellow precipitate indicated presence of alkaloids

Test for polyphenols-3 drops of 5% lead acetate solution was added to the extract. Yellow precipitates indicated a positive test.

Test for tannins – 1ml of the extract was dissolved in water followed by addition of 1% gelatin and 10% sodium chloride. Blackish blue or greenish black discoloration indicated presence of tannins.

Test for Saponins – 1ml of the extract was put into a test tube then followed by addition of 50ml distilled water. The mixture was then shaken vigorously. Presence of Saponins was indicated by a blue, green or red color with an associated pink ring.

Test for flavonoids- 1ml of the extract was put into a test tube. Then 4 drops of hydrochloric acid and 0.5 grams of magnesium turnings were added. Positive result was indicated by a pink or magenta red colour.

Test for cardiac glycosides – 50 mg of *Craterostigma plantagineum hoscht* extract was mixed with 2 milliliters ferric chloride. Appearance of a pink precipitate confirmed existence of glycosides.

Test for sterols and steroids-1ml of the extract was put into a test tube. This was followed by addition of 0.5ml Sulphuric acid,0.5ml acetic anhydride and 0.5ml chloroform. Red coloration will indicate presence of sterols while steroids will be indicated by a green coloration.

3.14 Administration of the drug and the extracts

The animals were picked gently by the tail and placed on the bench. Diclofenac sodium 15Mg/Kgbwt was administered orally to the positive control group. The drug was bought at Supreme pharmacy in Nakuru town. The experimental groups were orally administered various doses of the extract. Administration of the drug and the extracts was done via oral gavage. This was then followed with the nociceptive test that was done one hour after the compounds administration.

3.15 Parameters indicative of pain

Lifting, biting, flinching and licking of the formalin administered paw was regarded to indicate pain. Cessation of the same was an indication that pain had resolved.

3.16 Antinociceptive assay using Formalin Test

The left hind paw was used for the injection of formalin and was marked with a permanent marker pen. Pain was produced by injecting 50microliter of 5% formalin in the paw sub plantar region by using a 30-gauge needle. The rats received different doses of treatment as follows: Oral extract of aqueous and ethanolic extract of CP at 25, 50 and 100mg/kg; p.o. diclofenac at 15mg/kg. All the treatment was administered one hour before injection with formalin.

Then the animals were singly put in transparent Plexiglas cage for observation. The total time duration that was spent raising, biting, withdrawing and licking the injected paw was considered to indicate pain and was documented for 30 minutes post injection with formalin. Phase 1 nociception was measured from 0-5th minute while second phase was between 15th to 30th minute following injection with formalin. First phase of pain represented neurogenic pain while second phase indicated inflammatory pain as well as central sensitization.

3.17 Assessment of Anti-inflammatory Activity

Inflammation was triggered by injecting 50microliter of 5% formalin into the sub plantar of hind left paw, one hour after treatment with Oral extract of aqueous and ethanolic extract of CP at 25, 50 and 100mg/kg; and per oral (P.o.) diclofenac at 15mg/kg. Paw diameter was measured just prior to formalin injection and half hourly for four hours using a digital caliper.

3.18 Humane killing of the albino rats and harvesting the paw

3.18.1 Materials

Chloroform, cotton wool, specimen collection bottles, fixatives, formalin solution, scalpel, scalpel holder, mounting board, mounting pins, hypodermic needle gauge 20, surgical gloves, pair of scissors, pair of toothed forceps.

3.18.2 The procedure for anaesthetizing albino rats

For humane killing of animals, dose sufficient chloroform was used, cotton doused with chloroform was put into jar that had a tight fit so as to prevent it from leaking out into the air, Albino rats were put into the jar for 3-5 minutes to euthanize and after euthanasia the rats were mounted on wooden board with pins in supine position. An incision cut was made from xiphoid process to pubic symphysis using a scalpel and forceps. Blood for liver biochemistry was drawn from intracardiac puncture and the hind left paw harvested and preserved in 10%formalin for histology studies.

3.19 Sample collection of blood for liver function test

Various sites of blood collection in a rat include the maxillary, jugular and saphenous veins, and the heart (cardiac puncture). Cardiac puncture facilitates large volume of blood collection, as much as 10ml of blood being collected from a rat weighing about 150g (Beeton*et al* 2007).

3.19.1 The procedure

Confirmation for deep anesthesia was done by checking for lack of spontaneous movements or response to stimuli. The rat was placed on its back and the four libs pinned on the wooden board. An incision was made along midline abdomen using a scalpel and forceps and the heart exposed. A 5 ml syringe with a gauge 23 needle was used to perform an intracardiac puncture with an average of 3- 5 ml of blood being collected into a purple top vacutainer.

3.19.2 Assessment of Liver Function Test

Five milliliters of blood was collected using a 23-gauge needle from cardiac puncture into red top vacutainers which are plain glass tubes that allow for clot formation which forms between 15-30 minutes. This was then centrifuged at 1000 revolutions per minute for 10 minutes using 2016 Medsor impex centrifuge machine made in India. Serum was then collected using a Pasteur pipette into micro vacutainers. Biochemical studies for

hepatocellular injury were done by evaluating serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels at 28^{th} day of the study using rat chemiluminescence immunoassay (CLIA) ALT/AST kit and automatic chemiluminescence analyzer from Roche diagnostics, Switzerland.

3.20 Processing of paw tissues for light microscopy

Fixation of tissues was done using the formaldehyde solution for 24 hours, dehydration of samples was done using ethanol that had been prepared in an ascending concentration to a maximum of 100% with each concentration lasting one hour, Clearance of paw sample were done using xylene. The sample tissues were then infiltrated with wax for twelve hours at 56^0c and Orientation of tissues was then done by making longitudinal cuts from the apex to base. At this point, the samples were embedded in paraffin wax where they had been put on the wooden blocks, to properly expose the whole paw tissue, all the excess wax was chopped off from the blocks, rotary microtome was utilized to cut prepared sections into 5µm thick longitudinal sections and to properly spread the tissue, the cut sections were allowed to float in water at thirty-seven degrees. The sections were then put on top of the glass slides and a thin film was applied over it by a micro- dropper, using an oven, the slides were dried at thirty-seven degrees for 24 hours, slides were then taken through the procedural staining with Hematoxylin and eosin (H&E) and selection of slides for viewing under the light microscope was done through simple random sampling method.

3.20.1Materials for staining

Paw tissue, specimen bottles, Zenker solution, distilled water, acetic acid, DPX mountant, glass slides, cover clips, glass staining square jar, hematoxylin, eosin, wax paraffin, knives, rotary microtome, slide holders, heater, water bath container, formaldehyde 40% concentration, xylene, isopropyl alcohol, glass ware for preparing dilutions, wood blocks, beakers, dropper and toluidine solution.

3.20.2 Staining of paw tissue slides

The procedure of staining paw tissue sections was done using the hematoxylin and eosin solutions as described by Ghosh *et al*., (2014).

3.20.3 Procedure for staining with hematoxylin and eosin

Place the glass slides with paraffin sections on staining racks, clean the paraffin from samples using xylene by dipping the slides in it three times at two minutes on every dip, the paw tissue samples were Hydrated using the following steps, slides were transferred through three steps of 100% ethanol whereby at each step lasted at least two minutes, they were then transferred to 95% ethanol for at least 2 minutes and after the two minutes of staying in 95% ethanol, the slides were then transferred to 70% ethanol for two minutes. The slides were washed in running tap water at room temperature for two minutes.

Thereafter, hematoxylin solution was used by dipping the paw sample slides for three minutes, they were then washed with running water for 5 minutes at a room temperature. Then sample slides were stained using eosin Y solution for two minutes, the eosin Y-stained slides were then taken through the dehydration process as follows; The slides were put in a 70% concentrated ethanol for a total of twenty minutes, they were transferred to 95% ethanol beaker for two minutes, It was then taken through two steps of 100% ethanol with each step lasting for two minutes, the samples were then washed three times with xylene for 2 minutes per every single turn, Place a drop of Per mount over a tissue placed on each slide and a coverslip was added to ensure that the tissue was firmly held on the glass slide and they were then dried and therefore deemed ready to be viewed using a light microscope.

3.21 Materials and procedure used in microphotography

3.21.1 Acquisition of photomicrograph

After staining with Hematoxylin and Eosin, the slides were mounted on a Labomed light microscope that was mounted with IVU 3100 camera using a total magnification of x 400 and the images were captured.

3.22 Data analysis

Primary data from each set was entered coded and cleaned using statistical package for social sciences version 26.0 (SPSS 26.0), analyzed then presented as the mean ± standard errors of the mean (SEM), median and mode. Further, data was analyzed using analysis of variance (ANOVA) on antinociceptive/antiinflammation /paw tissue edema and liver biochemical analysis between the treatment groups and negative control group. Posthoc analysis was done using Scheffe's post hoc test at 5% significance level ($\alpha = 0.05$). P value < 0.05 ($p<0.05$) was regarded statistically significant.

3.23 Ethical Approval

After proposal was approved by the school of medicine the document was forwarded to the school of postgraduate studies for their consideration. The approved document was then forwarded to East Africa University of Baraton committee of animal ethics for further approval (Approval number UEAB/ISERC/10/04/2023) as there is no animal ethics committee for Maseno University. The document was finally forwarded to NACOSTI and the study was approved (License No: NACOSTI/P/23/25645). The study animals were finally sacrificed in a humane way based on prescribed protocol

CHAPTER FOUR

PRESENTATION OF RESEARCH FINDINGS

4.1Qualitative yield of phytochemicals in aqueous and ethanol extracts

The percent yield of the ethanol extract: Yield (%)=weight of solvent free extract (g) x 100/ dried extract weight. Ethanol extraction yield= 16.6g x 100/ 500 = 3.32 % yield. Aqueous % yield: 56gx100/500= 11.2% yield. Qualitative phytochemicals were established using screening test as per the protocols. Alkaloids and terpenoids were absent in ethanol extract but present in aqueous extract. Phenols and Anthraquinones were absent in aqueous extract but present in ethanol extract. Flavonoids, Saponins, tannins and cardiac glycosides were higher in ethanol extract as compared to aqueous extract. Sterols were higher in aqueous extract (Table 4.1).

Table 4.1: Phytochemicals in aqueous and ethanol extracts

Phytochemical	Ethanolic extract	Aqueous extract
Alkaloids	Absent	Trace
Flavonoids	+++	++
Sterols	Trace	++
Saponins	+++	+
Tannins	+++	+
Terpenoids	Absent	++
Cardiac glycosides	+++	++
Phenols	+++	Absent
Anthraquinones	+	Absent

Key: +++ = Highly present; ++ = moderately present; += mildly present; Absent =not detected

4.2 Pain behavior in male rats treated with aqueous and ethanolic extracts of *Craterostigma plantagineum hoscht*

Pain behavior was observed and timed in two phases. Phase 1 (0-5 mins) indicating neurogenic pain secondary to direct activation of nociceptors. Phase 2 (15-30 mins) indicating inflammatory pain secondary to the effect of inflammatory mediators and activation of the dorsal horns of the spinal cord (Table 4.2)

Table 4.2*:* Effect of Aqueous extract of *Craterostigma plantagineum hoscht* on the duration Spent in Pain

Dose	CONTROL	AQ 25	AQ 50	AQ100	DIC 15MG/KG	F-Value	*P*-value
0-5 MIN	300.0±0.0	300.0±0.0	300.0±0.0	300.0±0.0	112.0±8.0*	552.25	0.000
P Value		1.000	1.000	1.000	0.000		
15-30 MIN	900.0±0.0	900.0±0.0	900.0±0.0	900.0±0.0	800.0±52.9*	3.57	0.047
P Value		1.000	1.000	1.000	0.038		

Key: * =statistically significant (p<0.05); AQ-aqueous; ETOH- Ethanol; DIC-Diclofenac;MG/KG-Milligram per Kilogram body weight; F-F value; MIN-Minute

The duration of pain observed after administering aqueous extracts of craterostigma plantagineum hoscht 25 mg/kgbwt,50 mg/kgbwt and 100 mg/kgbwt to the rats had no significant statistical difference when compared to the negative control=1.000, *p*=1.000 and *p*=1.000 respectively in both 0-5th and 15th -30th minutes. The duration of pain observed after administering diclofenac 15 mg/kgbwt to the rats (0-5mins) was statistically significantly different (*p*=0.000) when compared to the negative control group. Equally, the duration of pain observed after administering diclofenac 15 mg/kgbwt to the rats (15-30 min) showed statistically significant difference (*p*=0.038) when compared to the negative control group (Table 4.2).

0-5 min

The duration of pain observed in Aqueous treatment groups of 25 mg/kgbwt (*p*=1.000), 50 mg/kgbwt (*p*=1.000) and 100 mg/kgbwt (*p*=1.000) had no statistical significant difference compared to the negative control group in phase 1 of pain. The animals spent the entire 300 seconds (5minutes) in pain. However, the duration of pain observed in positive control group in comparison to the negative control group was statistically significantly different in that the former spent 112.0±8.0 seconds in pain (*p*0.000) (Table 4.2).

15-30 min

The duration of pain observed in Aqueous treatment groups of 25 mg/kgbwt (p=1.000), 50 mg/kgbwt (p=1.000) and 100 mg/kgbwt (p=1.000) was not significant different when compared to the negative control group in the late phase of pain. The animals spent the entire 900 seconds (15minutes) in pain. In comparison to the negative control group, the duration of pain observed in diclofenac control group was statistically significantly different (p=0.038) (Table 4.2).

Table 4.3: Effect of Ethanolic extract of Craterostigma plantagineum hoscht on the duration Spent in Pain

Dose	NEGATIVE CONTROL	ETOH 25 MG/KG	ETOH 50 MG/KG	ETOH 100MG/KG	DIC 15MG/KG	F	*p*-value
0-5 MIN	300.0±0.0	300.0±0.0	300.0±0.0	300.0±0.0	112.0±8.0*	552.25	0.000
P Value		1.000	1.000	1.000	0.000		
15-30 MIN	900.0±0.0	900.0±0.0	900.0±0.0	900.0±0.0	800.0±52.9*	3.57	0.047
P Value		1.000	1.000	1.000	0.038		

Key: * = statistically significant (p<0.05); AQ-aqueous; ETOH- Ethanol; DIC-Diclofenac; MG/KG-Milligram per Kilogram body weight; F-F value; MIN-Minute

The duration of pain observed after administering ethanol extracts of *Craterostigma plantagineum hoscht* to the rats did not indicate significant statistical difference when compared to the negative control. The duration of pain observed after administering diclofenac 15 mg/kgbwt to the rats (0-5mins) was significantly different (p=0.000) compared to the negative control group. Similarly, the duration of pain observed after administering diclofenac 15 mg/kgbwt to the rats (15-30 min) showed statistically significant difference (p=0.038) compared to the negative control group. (Table 4.3).

0-5 min

The duration of pain observed in ethanol 25 mg/kgbwt (p=1.000), ethanol 50 mg/kgbwt (p=1.000) and ethanol 100 mg/kgbwt (p=1.000) had no significant difference compared to the negative control group in phase 1 of pain. However, the duration spent in pain in the diclofenac group compared to the negative control was statistically significant different in that the former spent 112.0±8.0 seconds in pain (p=0.000) (Table 4.3).

15-30 min

The duration of pain observed in ethanol25 mg/kgbwt (p=1.000), ethanol 50 mg/kgbwt (p=1.000) and ethanol 100 mg/kgbwt (p=1.000) was not significant different from the control group in phase 2 of pain. However, there was statistically significant difference in the duration of pain observed in diclofenac group in comparison with the negative control (p=0.038) (Table 4.3).The information was further illustrated below.

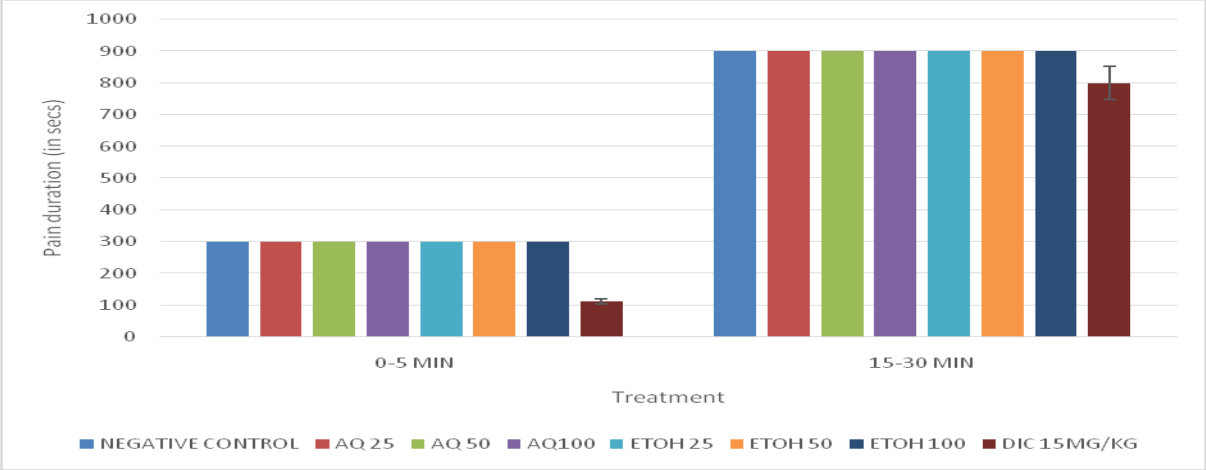

Figure 4.1: The effect of aqueous and ethanolic extract of Craterostigma plantagineum hoscht on formalin induced pain.

Key: AQ-aqueous; ETOH- Ethanol; DIC-diclofenac; MG/KG-Milligram per Kilogram body weight; secs-seconds; MIN- Minutes

The animals in aqueous and ethanol extract treatment groups did not show statistical significant difference in the duration of pain in both phases compared to the control group

(p>0.05). Nonetheless, there was statistical significant difference in the duration of pain in the positive control group (0-5 mins) in comparison to the negative control group (p=0.000), and in the late phase of pain (15-30mins) (p=0.038)(Table 4.3 and Figure 4.1)

4.3 Effect of aqueous and ethanolic extract of *Craterostigma plantagineum hoscht* on formalin induced paw edema in male rats as a function of time

The results were presented as mean ± standard error (SEM). Significance was determined using ANOVA with Scheffe's post hoc test at 5% significance level (α = 0.05). Results were considered significant when the probability value was less than 0.05 (p<0.05). The results are shown below (Table 4.4).

Table 4.4: The effect of aqueous and ethanolic extract of *Craterostigma plantagineum hoscht* on formalin induced paw edema in male rats as a function of time

Treatment	Control	AQ 100	ETOH 25	ETOH 50	ETOH 100	DIC 15MG/KG	F -Value	*P*-value
0 MIN	2.70±0.17	2.73±0.15	2.83±0.17	2.70±0.12	2.57±0.09	2.47±0.03	2.41	0.069
P VALUE		0.730	0.999	1.000	0.999	0.964		
30 MIN	6.23±0.19	5.73±0.22	**5.00±0.26***	**4.80±0.06***	**4.60±0.06***	**4.33±0.09***	24.07	0.000
P VALUE		0.643	**0.006**	**0.001**	**0.000**	**0.000**		
60 MIN	6.10±0.15	**5.10±0.10***	**4.63±0.07***	**4.57±0.03***	**4.50±0.06***	**4.27±0.12***	58.64	0.000
P VALUE		**0.001**	**0.000**	**0.000**	**0.000**	**0.000**		
90 MIN	5.83±0.09	**4.90±0.12***	**4.73±0.15***	**4.60±0.12***	**4.40±0.15***	**3.97±0.09***	32.30	0.000
P VALUE		**0.007**	**0.001**	**0.000**	**0.000**	**0.000**		
120 MIN	5.47±0.22	**4.53±0.12***	**4.33±0.09***	**4.23±0.07***	**4.00±0.06***	**3.87±0.12***	22.97	0.000
P VALUE		**0.015**	**0.003**	**0.001**	**0.000**	**0.000**		
150 MIN	4.97±0.09	**4.13±0.03***	**4.10±0.06***	**3.87±0.19***	**3.83±0.19***	**3.77±0.09***	27.71	0.000
P VALUE		**0.004**	**0.003**	**0.000**	**0.000**	**0.000**		
180 MIN	4.77±0.03	**3.83±0.03***	**3.77±0.09***	**3.70±0.12***	**3.53±0.12***	**3.47±0.07***	35.75	0.000
P VALUE		**0.000**	**0.000**	**0.000**	**0.000**	**0.000**		
210 MIN	4.47±0.09	**3.57±0.09***	**3.53±0.20***	**3.27±0.18***	**3.13±0.20***	**2.93±0.15***	15.76	0.000
P VALUE		**0.043**	**0.033**	**0.004**	**0.001**	**0.000**		
240 MIN	4.20±0.06	**3.33±0.03***	**3.30±0.06***	**3.17±0.03***	**2.93±0.09***	**2.57±0.07***	84.34	0.000
P VALUE		**0.000**	**0.000**	**0.000**	**0.000**	**0.000**		

Key: *=statistical significant difference from the negative Control (P<0.05)AQ- Aqueous; ETO-Ethanol; DIC-Diclofenac sodium; MG/KG- Milligrams per kilogram bodyweight; F-F value; MIN- Minute

There was significant difference in the size of paw edema observed in, ethanol 25 mg/kgbwt (p=0.006), 50 mg/kgbwt (p=0.001), 100 mg/kgbwt (p=0.000) and diclofenac 15 mg/kgbwt (p=0.000) observed from 30th minute, while aqueous 100 mg/kgbwt (p= 0.001) significant difference was observed from 60th minute compared to the negative control group. There was no statistical significant difference observed in the size of paw edema in aqueous 25 mg/kgbwt and 50 mg/kgbwt treatment groups in comparison to the negative control group (Table 4.4).

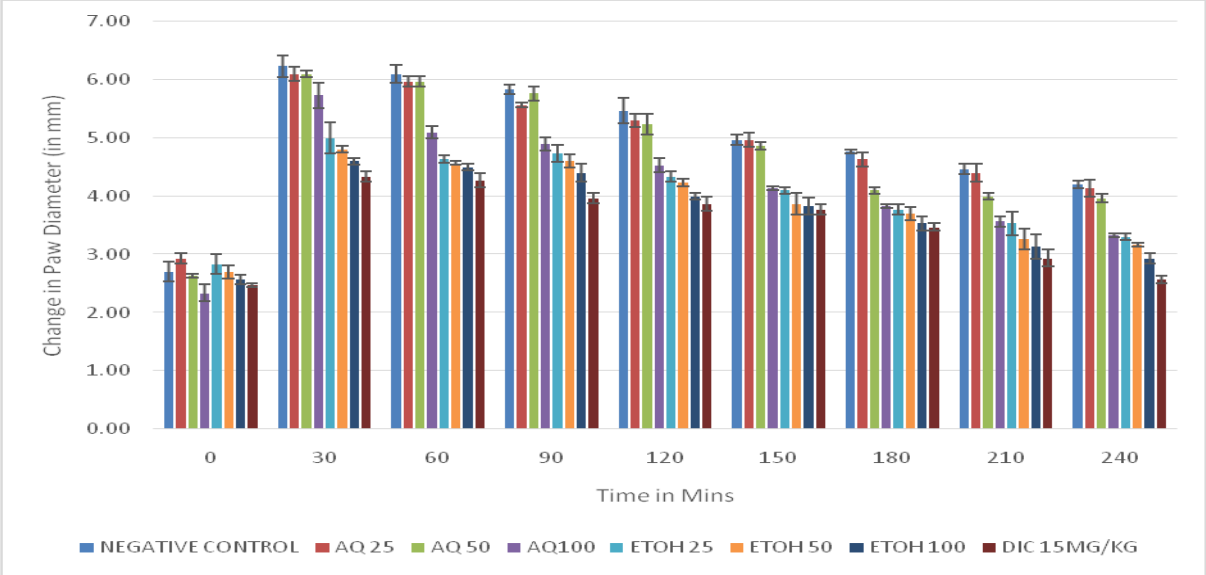

Figure 4.2: The effect of aqueous and ethanolic extract of *Craterostigma plantagineum hoscht* on formalin induced paw edema in male rats as a function of time.

Key: AQ-aqueous; ETOH- Ethanol; DIC-Diclofenac;MG/KG-Milligram per Kilogram body weight; mm-millimeter

There was a significant difference in the size of paw edema observed in, ethanol 25 mg/kgbwt (p=0.006), 50 mg/kgbwt (p=0.001), 100 mg/kgbwt (p=0.000) and diclofenac 15 mg/kgbwt (p=0.000) observed from 30th minute, while aqueous 100 mg/kgbwt (p= 0.001) significant difference was observed from 60th minute compared to the negative control group. There was no statistical significant difference observed in the size of paw edema in aqueous 25 mg/kgbwt and 50 mg/kgbwt treatment groups in comparison to the negative control group (Figure 4.2, Table 4.4).

Paw edema as a function of time (N^{th} minute)

Zero (O) minute

There was no significant difference in the size of the paw edema observed at baseline across all the treatment groups (p>0.05). Similar observation was recorded in the aqueous 25 and 50 mg/kgbwt extract treatment groups across the entire study time of 240 minutes unlike in the rest of the treatment groups (Table 4.4).

30^{th} minute

There was a significant difference in the size of paw edema observed among ethanol 50 mg/kgbwt (p=0.001), ethanol 100 mg/kgbwt (p=0.000) and diclofenac 15 mg/kgbwt (p=0.000) in comparison to the negative control (Table 4.4).The statistical significant difference was observed upto the 240^{th} minute (Table 4.4).

60^{th} minute

There was statistical significant difference in the size of paw edema observed among aqueous 100 mg/kgbwt (p=0.001), ethanol 50 mg/kgbwt (p=0.000), ethanol 100 mg/kgbwt (p=0.000) and diclofenac 15 mg/kgbwt (p=0.000) in comparison to the negative control (Table 4.4). The statistical significant difference in the aqueous 100mg/kgbwt extract treatment group was observed upto the 240^{th} minute (Table 4.4)

4.4 Histology changes in formalin injected paw tissue in male rats following treatment with aqueous and ethanolic extracts of *Craterostigma plantagineum hoscht.*

The epidermis is made of keratinocytes at different stages of development with the basal layer being made of a single layer of cuboidal cells with a deeply staining nucleus. The stratum spinosum composes of several layers with cells varying in shape with those closer to the basal layer almost cuboidal and those closer to stratum granulosum being fusiform. The nuclei of these cells stain with Haematoxylin with different intensities whereby cells closer to the basal layer picks the dye more in comparison to the cells closer to stratum granulosum.

Stratum granulosum is made up of 2-3 layers of keratinocytes demonstrating a basophilic cytoplasm. The most superficial layer, the cornified layer demonstrates a thin layer of anucleate keratinocytes. The papillary dermis demonstrates the skin appendages and several cells including fibroblasts and lymphocytes. The fibroblasts are fusiform with a deeply staining nucleus and have cytoplasmic extensions whereas the lymphocytes have a rim of cytoplasm with a greater percentage of cytoplasmic volume being occupied by a single nucleus. Qualitatively, the relative ratio of fibroblasts to lymphocytes is high i.e. more fibroblasts than lymphocytes. Collagen fibres have no particular orientation and are thin with lots of ground substance surrounding the fibres and the cells. The reticular dermis shows an abundance of fibroblasts with thick collagen fibres and very little extracellular matrix.

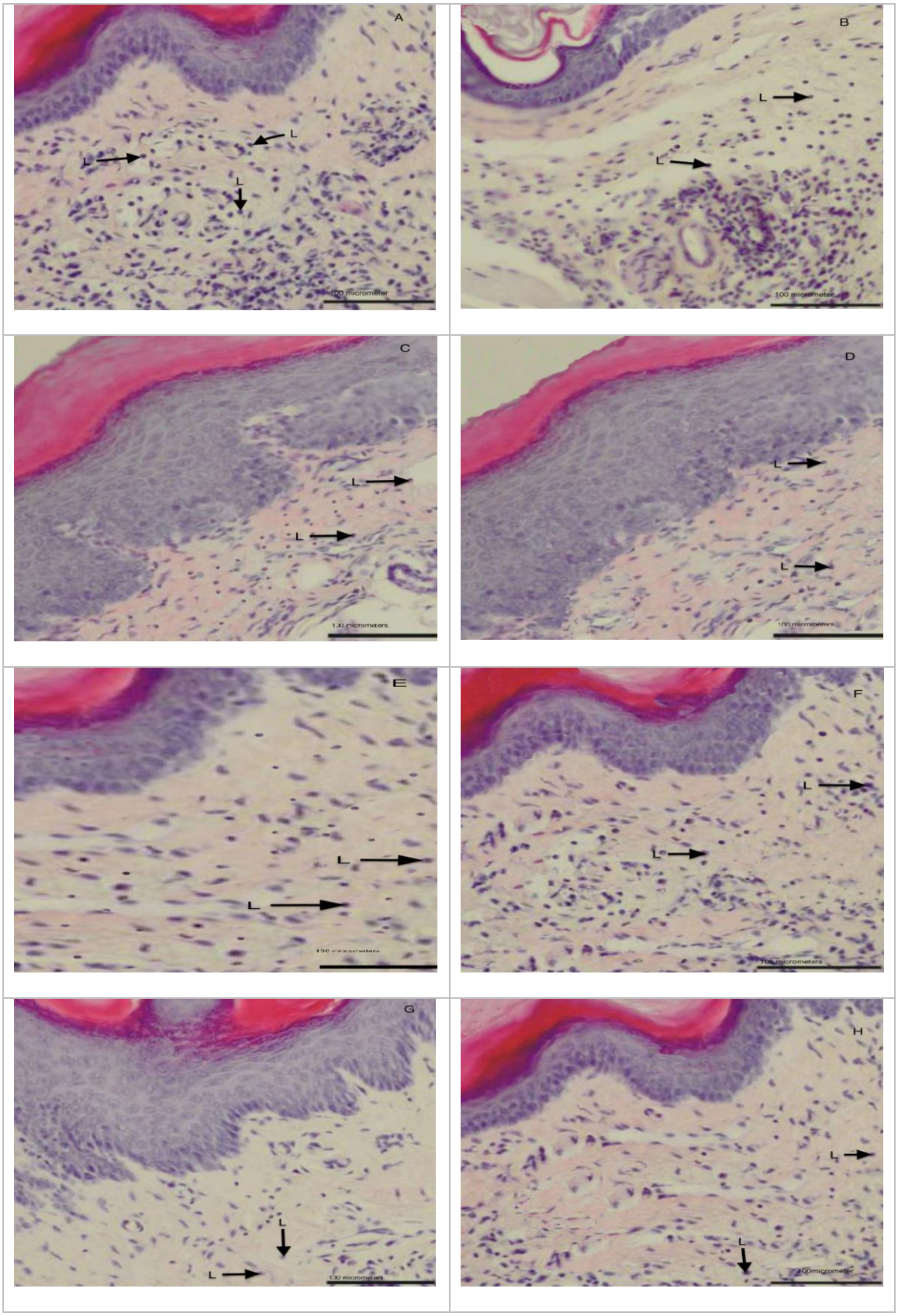

Figure 4.3: Photomicrograph of the paw tissue (A, B, C, D, E, F, G, H) for the various experimental groups.

Key: L=lymphocytes in the papillary dermis, A=negative control group, B=aqueous 25 Mg/Kgbwt, C=aqueous 50 mg/kgbwt, D=aqueous 100 mg/kgbwt, E= ethanol 25 mg/kgbwt, F= ethanol 50 mg/kgbwt, G=ethanol 100 mg/kgbwt and H=diclofenac 15 mg/kgbwt treatment groups.

A: Negative control paw tissue. There was increased number of inflammatory cells observed in papillary dermis in comparison to Aq 100 mg/kgbwt (p=0.001), ethanol 25 mg/kgbwt (p=0.000), ethanol 50 mg/kgbwt (p=0.000), ethanol 100 mg/kgbwt (p=0.000) and diclofenac 15 mg/kgbwt (p=0.000) (Figure 4.3).

B: Aqueous 25 mg/kgbwt group. The number of lymphocytes observed in the papillary dermis was not significantly different compared to the negative control group (p=1.000) (Figure 4.3).

C: Aqueous 50 mg/kgbwt group. The number of lymphocytes observed in the papillary dermis was not significantly different when compared to the negative control group (p= 1.000) (Figure 4.3).

D: Aqueous 100 mg/kgbwt group. The number of lymphocytes observed in the papillary dermis was significantly different when compared to the negative control group (p= 0.001)

E: Ethanol 25 mg/kgbwt group. The number of lymphocytes observed in the papillary dermis was significantly different compared to the negative control group (p= 0.000) (Figure 4.3).

F: Ethanol 50 mg/kgbwt group. The number of lymphocytes observed in the papillary dermis was significantly different compared to the negative control group (p= 0.000) (Figure 4.3).

G: Ethanol 100 mg/kgbwt group. Number of lymphocytes observed in the papillary dermis was significantly different compared to the negative control group (p= 0.000) (Figure 4.3).

H: Diclofenac 15 mg/kgbwt group. There were few number of lymphocytes observed in the papillary dermis which was significantly different compared to the negative control group (p= 0.000) (Figure 4.3).

The results were presented as mean ± standard error (SEM). Significance was determined using one-way analysis of variance with Scheffe's post hoc test at 5% significance level (α = 0.05). Results were considered significant when the probability value was less than 0.05 (p<0.05). The results are shown below (Table 4.5)

Comparison of inflammatory cells was done across the various experimental groups through ANOVA with scheffe post hoc test at 5% significance level (α = 0.05).The total number of inflammatory cells (lymphocytes) was significantly higher in the negative control group (23.33±0.33) in comparison to the treatment groups aqueous 100 mg/kgbwt (16.33±0.88), ethanol 25 mg/kgbwt (15.67±0.67),ethanol 50 mg/kgbwt (14.00±1.15),ethanol 100 mg/kgbwt (10.67±0.33) and diclofenac 15 mg/kgbwt (7.67±0.33)which were significantly lower. The total number of lymphocytes in the experimental groups Aq 25 mg/kgbwt (23.67±0.88) and 50 mg/kgbwt (22.67±0.88) were not statistically different in comparison to the negative control group (23.33±0.33), p=1.000, 1.000 respectively.

Table 4.5: The effect of aqueous and ethanolic extract of *Craterostigma plantagineum hoscht* on the number of lymphocytes observed in each rat's paw

Treatment	Number of lymphocytes	P -value
Negative control	23.33±0.33	
AQ 25 mg/kgbwt	23.67±0.88	1.000
AQ 50 mg/kgbwt	22.67±0.88	1.000
AQ 100 mg/kgbwt	16.33±0.88*	0.001
ETOH 25 mg/kgbwt	15.67±0.67*	0.000
ETOH 50 mg/kgbwt	14.00±1.15*	0.000
ETOH 100 mg/kgbwt	10.67±0.33*	0.000
DIC 15 mg/kgbwt	7.67±0.33*	0.000
F-value (*P*-value)	65.47 (0.000)	

Key:* = statistically significant from the Negative Control (P<0.05) AQ- Aqueous; ETO-Ethanol; DIC-Diclofenac sodium; MG/KG- Milligrams per kilogram bodyweight

There was statistical significant difference in the number of lymphocytes observed in the paw tissue in aqueous 100 mg/kgbwt (p=0.001), ethanol 25 mg/kgbwt (p=0.000), ethanol 50 mg/kgbwt (p=0.000), ethanol100 mg/kgbwt (p=0.000) and diclofenac 15 mg/kgbwt

(p=0.000) compared to the negative control group. There was no significant difference in the number of lymphocytes observed in the paw tissue in aqueous 25 mg/kgbwt (p=1.000) and 50 mg/kgbwt (p=1.000) compared to the negative control group (Table 4.5)

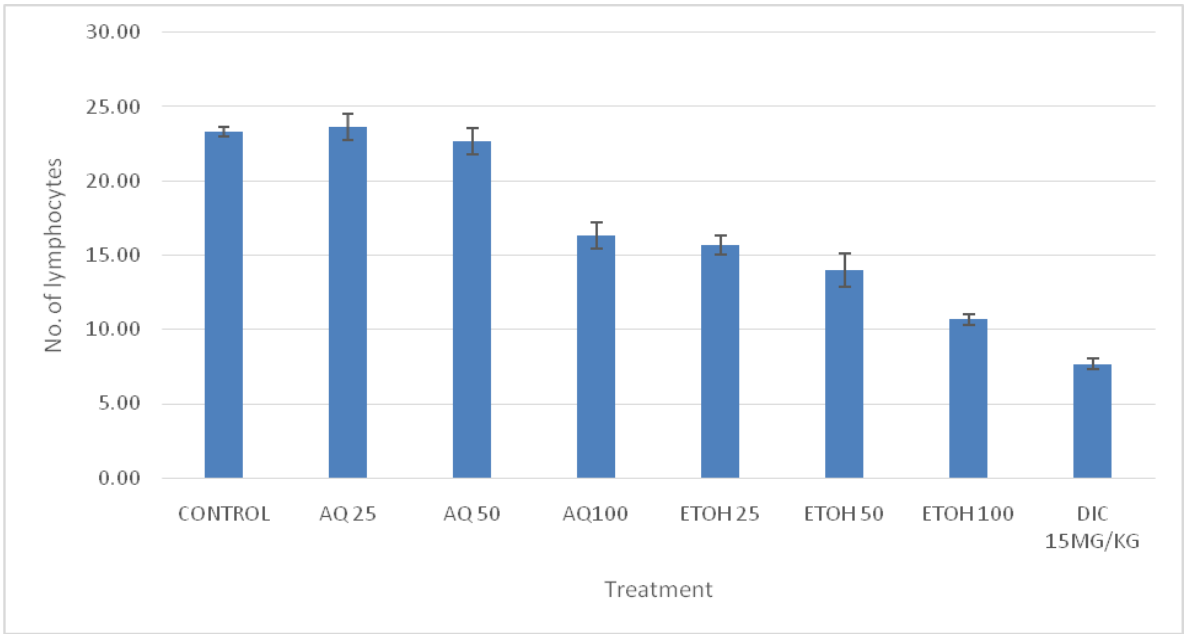

Figure 4.4: The effect of aqueous and ethanolic extract of Craterostigma plantagineum hoscht on the number of lymphocytes observed in each rat's paw.

Key: AQ- Aqueous; ETO-Ethanol; DIC-Diclofenac sodium; MG/KG- Milligrams per kilogram bodyweight; No.-Number

A significant difference in the number of lymphocytes was observed in the paw tissue in aqueous 100 mg/kgbwt (p=0.001), ethanol 25 mg/kgbwt (p=0.000), ethanol 50 mg/kgbwt (p=0.000), ethanol 100 mg/kgbwt (p=0.000) and diclofenac 15 mg/kgbwt (p=0.000) compared to the negative control group. However, no significant difference was observed in the number of lymphocytes in the paw tissue in aqueous 25 mg/kgbwt (P=1.000) and 50 mg/kgbwt (p=1.000) compared to the negative control group (Figure 4.4).

4.5 Liver function of male rats following treatment with aqueous and ethanolic extracts of Craterostigma plantagineum hoscht

AST and ALT levels at Day 28

Table 4.6: Effect of aqueous and ethanolic extract of *Craterostigma plantagineum hoscht* on AST and ALT levels at Day 28

Dose	NEG CONT	AQ 25	AQ 50	AQ100	ETOH 25	ETOH 50	ETOH 100	DIC 15MG/KGBWT	F	*P*-Value
AST (U/L) Day 28	22.30±1.37	21.37±4.86	24.73±4.10	21.37±0.49	19.93±0.55	22.40±7.88	23.57±4.50	19.63±2.88	0.29	0.947
P-val		1.000	1.000	1.000	1.000	1.000	1.000	1.000		
ALT (U/L) Day 28	25.77±1.69	24.73±7.02	19.93±6.43	39.37±6.47	21.90±3.19	30.10±3.95	18.87±1.77	18.87±3.10	2.26	0.084
P-val		1.000	0.997	0.741	1.000	0.999	0.991	0.991		

Key: * =statistically significant (p<0.05); AQ- Aqueous; ETO-Ethanol; DIC-Diclofenac sodium; MG/KG-Milligrams per kilogram bodyweight; F-F value; ALT-Alanine aminotransferase; AST-Aspartate aminotransferase;U/L-Units per liter

There was no statistical significant difference in the level of AST and ALT among the various doses of aqueous *Craterostigma plantagineum hoscht* extract treatment groups in comparison to the negative control group (Table 4.6)

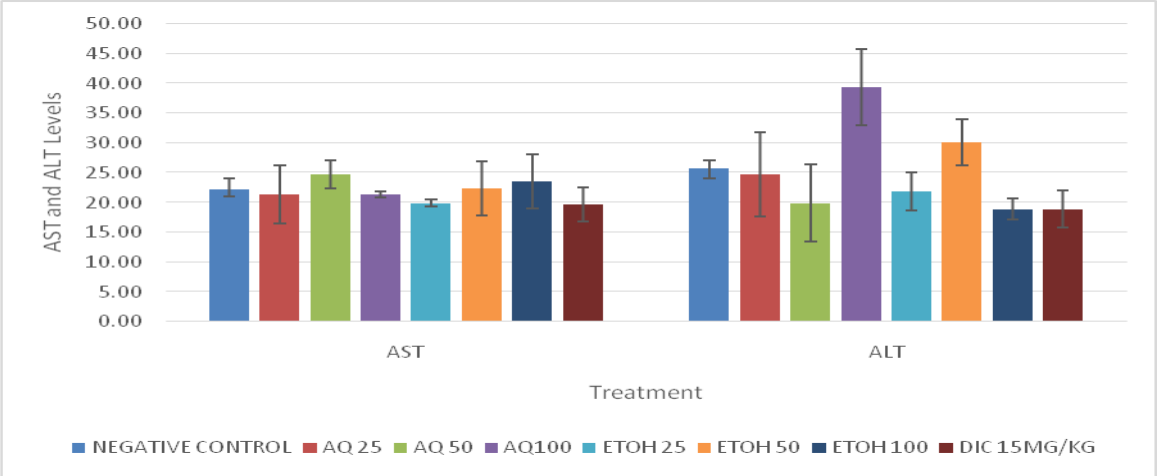

Figure 4.5: The effect of aqueous and ethanolic extract of Craterostigma plantagineum hoscht on AST and ALT levels (units/ liter) at Day 28

Key: AQ-aqueous; ETOH- Ethanol; DIC-Diclofenac sodium; MG/KG-Milligram per Kilogram body weight; AST-Aspartate aminotransferase; ALT-Alanine aminotransferase

There was no statistically significant difference in the level of AST and ALT among the various doses of aqueous *Craterostigma plantagineum hoscht* extract treatment groups in comparison to the negative control group (Figure 4.5).

CHAPTER FIVE
DISCUSSION

5.1Phytochemicals in aqueous and ethanolic extract of Craterostigma plantagineum hoscht

The phytochemical constituents qualitatively detected in craterostigma plantagineum hoscht in aqueous and ethanolic extraction included flavonoids, phenols and anthraquinones, sterols, Saponins, tannins, alkaloids and cardiac glycosides.

Phenols and tannins were highly extracted in ethanol while alkaloids were extracted in water though in trace amounts. According to a study done by Plaskova and Mlcek (2023) phenols and tannins have poor solubility in water and have better solubility in organic solvents. This explains reason as to why phenols and tannins concentrations were higher in ethanolic extraction as compared to aqueous extraction. Alkaloids on the other hand are water soluble and best extracted in water**.**

According to a study by Passon *et al.* (2021) there was high phenolics extraction in *Craterostigma plantagineum hoscht*, which is a similar finding in this study (Table 4.1).

The plant shares family with craterostigma pumilum, which is a member of resurrection plants. These plants can loose as much as 98% of their water during dry season and yet get back to normal metabolism once rehydrated. According to a study done by Mwonjoria et al. (2016) on craterostigma pumilum, qualitative phytochemical yield contained flavonoids in high levels, moderate amounts of tannins, mild amounts of Saponins, terpenes, phenolics and glycosides. However, alkaloids were not detected. These phytochemicals were extracted in water and dichloromethane, however in their article "Anti-inflammatory activity of craterostigma pumilum (hoscht) is associated with hyperalgesia" the researcher did not categorize these phytochemicals as to whether were extracted in water or dichloromethane. With exception to alkaloids, which were absent in their extract, all other phytochemicals

extracted were similar to those detected in this study. Though, alkaloids were detected in trace amounts.

Alkaloids were detected in aqueous extract in moderate range unlike in craterostigma pumilum where they were not detected at all. Flavonoids have been documented to disappear during rehydration of the resurrection plants perhaps due to inability of the plant to synthesize the phytochemicals at that state of their desiccation tolerance (Xu *et al.*, 2021). There is possibility that craterostigma pumilum did not have alkaloids in entirety, however there still exists a possibility that the phytochemicals were equally affected by the different states of the resurrection plants desiccation tolerance (dehydration and rehydration cycle).

Flavonoids are the phytochemicals that give plants their characteristic flavor and colour. They possess potent anti-inflammatory effect as well as ability to modulate the immune system (Yahfoufi et al., 2018**).** According to Chaves *et al.* (2020) different flavonoids have been identified and include the following groups: isoflavones, flavones, anthocyanidins, chalcones and flavonols, and several scholars have documented that flavonoids are better extracted in organic compounds. This concurs with the findings of this study in that ethanolic extraction yielded more flavonoids compared to aqueous extraction.

In the current study, cardiac glycosides were highly present in ethanol extract. Extraction of desired amounts of cardiac glycosides is best done using ethanol or methanol. This is because waters' ingress and egress into and out of the plant respectively is restricted thus not able to extract cardiac glycosides efficiently. An alternative extraction solvent is use of water-alcohol mixture. Generally, cardiac glycosides occur in low amounts in the plant and this may affect their isolation from the plant (Morsy and Morsy, 2017)

Acetone has been identified as a solvent that best yield Saponins (Chua *et al.*, 2019). According to Ngo *et al.* (2017) , the best solvent for extraction of Saponins is 50% acetone or

ethanol or mixture of water with 50% of these solvents. This agrees with this study in that Saponins were highly present in ethanol extract in comparison to the aqueous extract.

In this study, terpenoids were absent in aqueous extract but present in ethanolic extract. Extraction of Terpenoids using ethanol leads to significant Terpenoids loss and this is because ethanol leads to its significant volatilization as opposed to aqueous or weak nonpolar organic solvent such as hexane(Jiang, Kempinski & Chappell, 2016). Terpenoids yield in this study was consistent with this literature. There were high levels of phenolic in this study. This is in agreement with a study by Passon *et al.* (2021) whereby there was high phenolics extraction in *Craterostigma plantagineum hoscht*. Studies have shown that sterols are absent in aqueous extraction as they are water insoluble and better extracted in solvents that are less polar (Bugaets et al., 2020).

5.2 Assessment of analgesic and anti-inflammatory effect

In this study, both aqueous and ethanolic extracts of Craterostigma plantagineum hoscht did not possess analgesic effect in both phase 1 and 2 of pain. However, diclofenac sodium possessed statistically significant analgesic outcome in both phase 1 and 2 of pain (Table 4.2 and 4.3). During the first 0-5 minutes of pain behavior observation, diclofenac sodium in comparison to the negative control group showed statistically significant analgesic effect (p=0.000), with similar antinociceptive findings during the 15^{th}-30^{th} minute (p=0.038) (Table 4.2).

Alkaloids like morphine exerts their analgesic mechanism through binding to opioid receptors especially mu-opioid receptors in both central and peripheral nervous system. Through this alkaloids inhibit peripheral nervous system nociceptive afferent fibers as well as activation of the central nervous system descending inhibitory pathways (Ku *et al.*, 1985).Contrary to this literature, alkaloids in this study did not possess similar analgesic

effects. It is important to note that alkaloid levels were detectable in trace amounts thus possibly not able to reach therapeutic plasma levels to effect the analgesic benefit.

Terpenes analgesic effects are documented to be exerted through binding to opioid receptors, modulating N-Methyl-D-aspartate ion channels and transient receptor potentials with ultimate analgesic effect. They as well suppress production of proinflammatory cytokines and decrease nociceptive transmission across the synaptic cleft. However, terpenes are non-selective to their receptor targets with potential to activate anti or pro-inflammatory pathways with resultant decrease or increase in transmission of nociceptive signal across the synapse (Zieglgänsberger, 2019).

According to a study done by Harris et al. (2019) isolated terpenes did not confer analgesic benefits on rats observed pain behavior. Indeed, terpenes increased observed duration of pain. It is possible that terpenes enhanced nociceptive signals thus dampening the analgesic effects of other phytochemicals. According to Mwonjoria et al. (2016) craterostigma pumilum extract contained terpenes among other phytochemicals known to have analgesic effects yet the extract was associated with hyperalgesia.

Flavonoids act through several pathways for both analgesic and anti-inflammatory effect. They inhibit nuclear factor kappa B (NF-κB) pathway with resultant decrease in cyclooxygenase-2 (COX-2), inhibit junase kinase signal transducers and activators of transcription (JAKSTAT) pathways with resultant decrease in interlukin -1 beta (IL-1β), tumor necrosis factor alpha (TNF-α)&interlukin-6 (IL-6), and also inhibit phosphoinositide 3-kinase(PI3K) “plasma membrane –associated lipid kinases” with resultant decrease in IL-1β, TNF-α& IL-6.They also inhibit transient receptor potential cation channel vallinoid subtype 1 (TRPV1), transient receptor potential cation channel subfamily A1(TRPA1), transient receptor potential cation channel subfamily M3 &M8 (TRPM3 & TRPM8) and nav

1.8 in spinal cord & dorsal root ganglia thus reducing the pain (Edwards *et al.*, 2000).However, pain sensation is dampened through other several pathways that include increasing brain levels of kynurenate that competes with glutamate for N-methyl-D-aspartate receptor(N-MDA)binding, increasing peripheral and central nervous system amounts of cyclic Guanosine Monophosphate and blocking of opioid receptors (Edwards *et al.*, 2000,Gan, 2010)). There is possibility that flavonoids in this study lacked capacity to reduce pain because they were unable to exert the physiologic effect through these other pathways. It is also probable that terpenes reduced the analgesic benefits of flavonoids since they are known to enhance pain sensation.

In this study diclofenac exhibited potent analgesic and antiinflammatory affect (table 4.2, 4.3 & 4.4; figure 4.1 & 4.2). Diclofenac analgesic effect is through reduction in production of substance P by reducing synthesis of leukotrienes. Substance P is a potent pain neurotransmitter (Zieglgänsberger, 2019). However, there is a growing affirmation for disconnection between analgesic and anti-inflammatory effects of diclofenac. This is by diclofenac working through other mechanisms to produce analgesic effects.

Diclofenac increases peripheral and central levels of cyclic guanosine monophosphate (cGMP) through the L-arginine-nitric-oxide-cGMP pathway. In the brain and the spinal cord, diclofenac increases levels of Kynurenate, which is an endogenous antagonist of N-MDA receptor (Edwards *et al.*, 2000). There is also possibility that diclofenac also has its analgesic activity through opioid receptors as administration of naloxone reversed its analgesic effect (Gan, 2010).

It is therefore plausible that the aqueous and ethanolic extracts of Craterostigma plantagineum did not possess the antinociceptive effects because they lacked capacity to work through these other pathways like diclofenac sodium. Yet, since several of these

phytochemicals are known to possess analgesic benefits in other studies, phenols (Boussouf., et al 2017), flavonoids (Ferraz et al., 2020), Tannins (Souza et al., 2007), Saponins (Tan., 2022) and alkaloids (Shoaib et al., 2016) then the most possible reason for lack of this benefit is the role of tannins enhancing pain as documented by Zieglgänsberger (2019) and Harris et al. (2019).

However, the aqueous 100 mg/kgbwt, Ethanol 25 mg/kgbwt, 50 mg/kgbwt and 100 mg/kgbwt antiinflammatory effects of the extract were comparable to diclofenac (Table 4.4).

In this study, phenols, flavonoids and tannins were highly present in the ethanolic extract as compared to the aqueous extract (Table 4.1). Phenols, flavonoids, sterols and tannins have been documented to possess anti-inflammatory and antioxidant activity (Rodriguez-Yoldi,2021). Equally, flavonoids exert anti-inflammatory activity through inhibiting cytokine release by mast cells (Ginwala *et al.*, 2019). Phenol, flavonoids and tannins having been highly extracted in ethanol, it is possible that they were responsible for the antiiflammatory effect as individual agents or in concert with other phytochemicals. However, they did not exert analgesic effects perhaps due to counteracting effects of terpenes.

The anti-inflammatory findings of the extracts of Craterostigma plantagineum increased in ascending order from aqueous 100Mg/Kg, ethanol 25 mg/kgbwt, ethanol 50 mg/kgbwt and ethanol 100 mg/kgbwt (Table 4.4 and figure 4.2) and were comparable to diclofenac. Diclofenac antiiflammatory pathways include reduced synthesis of arachdonic acid (Oladosu, Tu & Hellman., 2018) and enhanced arachdonic acid uptake (Ku et al., 1985).These reduce synthesis of mediators of inflammation. The active anti-inflammatory phytochemicals in Craterostigma plantagineum hoscht extract were comparable to diclofenac. It can be inferred that Craterostigma plantagineum hoscht contains phytochemicals with anti-inflammatory effect but no antinociceptive effects

5.3 Histology changes in the paw tissue of rats treated with Craterostigma plantagineum hoscht

Layers of the skin include the epidermis, dermis and hypodermis. The epidermis has 5 layers with cells present being the melanocytes, Langerhans', merkel's cells and keratinocytes (most abundant). While the dermis has two layers that is the papillary and the reticular layer (Yousef, Alhajj and Sharma, 2022).The major cell component of the dermis is the fibroblast, while other cells present are the macrophages, mast cells and adipocytes (Brown & Krishnamurthy, 2022)

The study findings were that negative control, aqueous 25 mg/kgbwt and 50 mg/kgbwt had similar histology findings of active infiltrate of lymphocytes compared to the rest of the treatment groups. There was no significant statistical difference between the number of lymphocytes in the mentioned treatment groups (p=1.000) (Table 4.5 & figure 4.4). This is an indication of chronic inflammation (Maciel *et al.*, 2022). In comparison to the negative control group, papillary dermis lymphocytes number kept decreasing with treatment groups of aqueous 100 mg/kgbwt (16.33±0.88, p=0.001), ethanol treatment groups of 25 mg/kgbwt (15.67±0.67, p=0.000), 50 mg/kgbwt (14.00±1.15, p=0.000) and 100 mg/kgbwt (10.67±0.33, p=0.000) while the diclofenac treatment group had the least number of lymphocytes (7.67±0.33, p=0.000) (Table 4.5 & figure 4.4). These extract doses were comparable to the effect of diclofenac sodium and therefore exhibited potent antiiflammatory benefits.

Inflammation is denoted by the presence of cells of inflammation that include macrophages, neutrophils, lymphocytes mast cells and monocytes (Ruiz et al 2019). In acute inflammation the main inflammatory cell component are the neutrophils. No neutrophils were observed in this study at 28thday an indication of absence of an ongoing acute inflammatory process.

While this is the case for acute inflammation, chronic inflammation has insidious gradual onset, less marked cardinal signs and symptoms with the main inflammatory cell component

being the lymphocytes and the monocytes/macrophages(Maciel *et al.*, 2022).Chronic inflammation arises from several etiologies. In this regard it is due to persistence of acute inflammation that never cleared in the negative control group and the aqueous 25/50 mg/kgbwt treatment groups in comparison to the ethanol treatment groups/positive control group. The Aqueous 100 mg/kgbwt treatment group had significant antiinflammatory effect (p=0.001) compared to the rest of the aqueous groups possibly due to quantitatively higher levels of active phytochemicals in the dose.

Flavonoids inhibit the NF-κB pathway leading to a low level ofcyclooxygenase 2, inhibit PI3K and JAKSTAT leading to a decrease in the quantity of proinflammatory mediators (IL-1β, TNF-α& IL-6) release (Edwards et al., 2000).Equally, Phenols, sterols and tannins have significant anti-inflammatory and antioxidant activity (Rodriguez.,2021). High levels of flavonoids, phenols and tannins were isolated in the ethanolic extract which as well exhibited significant antiiflammatory activity. These phytochemicals can be attributed to this benefit. Reduced inflammation is associated with reduction in capillary endothelial injury thus reduced extravasation of inflammatory cells (Schnoor et al., 2015; Jutila et al., 1989)

Diclofenac sodium anti-inflammatoryactivity is through its inhibition of cyclooxygenase pathway decreases release of arachdonic acid as well as boosts its uptake. All these lead to reduced synthesis of mediators of inflammation through the cyclooxygenase and the lipoxygenase pathways (Gan, 2010). Flavonoids, phenols and tannins are known to promote anti-inflammatory responses.

In this study significant anti-inflammatory activity was observed in ethanolic treatment group.It is important to note that these phytochemicals were highly present in the ethanolic extract and therefore can be attributed for the anti-inflammatory activity found in this treatment group.

5.4 Craterostigma plantagineum hoscht extract effects on liver function

In the present study the aqueous and ethanolic extract of craterostigma plantagineum hoscht did not impair alkaline aminotransferase (p=0.08) and alanine aminotransferase (p=0.94) levels (Table 4.6). This explains that the extract most likely did not damage the liver and therefore most likely safe for the liver.

The findings of this study concur with a study done on Amaranthus tricolor Linn. roots which did not show hepatotoxic effects. Infact the extract exhibited hepatoprotective effect against paracetamol induced liver damage in a dose dependent manner. The plant contained alkaloids, flavonoids, Saponins, tannins and phenolic compounds similar to those phytochemicals isolated in this study. The natural antioxidant properties in phytochemicals have ability to scavenge off free radicals that can cause tissue damage such as hepatic tissue injury (Aneja *et al.*, 2013).Similar findings have been documented in a study on hepatoprotective effects of Alhagi maurorum Boiss (Rehman, Ahsan & Khan, 2013). Phytochemicals in the herb (Alhagi maurorum Boiss) included alkaloids, Saponins, tannins and flavonoids similar to those isolated in the current study. Therefore, the hepatoprotective benefits of these compounds can be thought to have maintained the liver function within physiologic values.

Lignans like silymarin, flavonoids, sesquiterpenoids, triterpenoids, glycosides and Saponins confers antihepatotoxic benefit, while terpenoids and phenols are choleretic (Valan, Britto & Venkataraman, 2010). This relates with this study in that similar phytochemicals were isolated thus possibly conferring similar effects.

However, herbal medicines have been wrongfully considered as free of side effects and with possibility of being nontoxic. This is not true as some have been associated with severe toxicity such as hepatotoxicity (Stournaras & Tziomalos, 2015)

According to Quan, Xuan & Teschke (2020) a large number of phytochemicals have been associated with hepatotoxicity. These include classes such as glycosides, Saponins, several phenolics, and anthraquinones, with terpenoids and alkaloids being the major groups associated with hepatotoxicity through mitochondrial apoptotic pathways activation as well as depressed transporters of bile acids. This disagrees with the current study in that AST and ALT levels remained unimpaired.

It is important to note that each of these groups of phytochemicals contain varied subtypes, and of different molecular structures and interactions (Patra et al., 2021). Thus there is possibility of each effecting their activities in different ways ending up as hepatoprotective or hepatotoxic. Equally, there is also possibility of phytochemicals interacting either to work synergistically or antagonistically (Chen et al., 2022). This may account for the varied outcomes of similar group of phytochemicals on hepatic function depending on the subtypes of the phytochemicals present, as well as the molecular interaction between the different classes of phytochemicals present within the group.

5.5 Strengths of the study

The study was a posttest only true experimental study thus able to link cause and effect (internal validity).

5.6 Limitation of the study

Serum ALT and AST levels were evaluated at the end of the study (Chronic effect). Thus the study did not assess the acute effects of the extract on the serum ALT and ALT levels.

CHAPTER SIX
CONCLUSION AND RECOMMENDATIONS

6.1Conclusion

1. Phenols and anthraquinones were extracted in ethanol solvent only while alkaloids and terpenoids were extracted in aqueous only. The rest of the phytochemicals were present in both solvents though majorly more abundant in ethanol extract. Thus less polar solvents like ethanol may be a better solvent compared to more polar solvents such as aqueous for phytochemicals extraction in *C. plantagineum hoscht.*
2. *Craterostigma plantagineum hoscht lacked* capacity to offer analgesic effect. This could be attributed to counteracting effects of terpenoids and inability of the phytochemicals to reduce pain by working through other analgesic pathways such as increasing brain kynurenate levels and central/peripheral levels of cGMP.
3. *Craterostigma plantagineum hoscht* has potent antiinflammatory effect attributable to the antioxidative and antiinflammatory effects of the phytochemicals present.
4. *Craterostigma plantagineum hoscht* extract did not derange liver biochemistry on analysis of AST and ALT though more studies need to be carried out to confirm these findings since the biochemistry tests were done once at the end of the study**.**

6.2 Recommendations

1. Though several phytochemicals were extracted from Craterostigma plantagineum hoscht in ethanol and aqueous solvents, it would be important to extract in nonpolar solvent since ethanol and aqueous solvents are both polar. This would help identify and compare the yield in a polar versus non-polar solvent.

2. Further studies should be carried out to determine why the plant extract failed to confer analgesic effects. This can be achieved by isolating and assessing the analgesic effect of each individual phytochemical to rule out a possible antagonistic interaction

between the phytochemicals as would be seen with terpenoids having hyperalgesic effects.

3. It is evident from this study that *Craterostigma plantagineum hoscht* possess potent antiinflammatory activity. Antiinflammatory agents are associated with adverse side effects such as gastric erosion and renal insult for the NSAIDS while steroids are associated with hypertension, diabetes, etc. *C. plantagineum hoscht* should be evaluated for such side effects as would offer a safe alternative drug if devoid of such insults.

4. Besides plant extracts having hepatotoxic effects, nephrotoxicity is also a feature as kidneys also play a role in metabolism and excretion of these phytochemicals. As such, evaluation of renal function should be done to assess whether the plant has nephrotoxic effects.

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APPENDICES

Appendix I: Ethical Approval

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B10130432023 April 13, 2023

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Department of Physiology
Maseno University.

Dear Gichuki,

RE: Assessment of Pain Behaviour and Antiinflammatory Response in Male Rats Treat with Craterostigma Plantagineum Hoscht Extract.

This is to inform you that the Institutional Scientific Ethics Review Committee (ISERC) of the University of Eastern Africa Baraton has reviewed and approved your above research proposal. Your application approval number is UEAB/ISERC/10/04/2023. The approval period is April 13th ,2023 April 13th, 2024.

This approval is subject to compliance with the following requirements;

i. Only approved documents including (informed consents, study instruments, MTA) will be used.
ii. All changes including (amendments, deviations, and violations) are submitted for review and approval by the Institutional Scientific Ethics Review Committee (ISERC) of the University of Eastern Africa Baraton.
iii. Death and life threatening problems and serious adverse events or unexpected adverse events whether related or unrelated to the study must be reported to the Institutional Scientific Ethics Review Committee (ISERC) of the University of Eastern Africa Baraton within 72 hours of notification.
iv. Any changes, anticipated or otherwise that may increase the risks or affected safety or welfare of study participants and others or affect the integrity of the research must be reported to the Institutional Scientific Ethics Review Committee (ISERC) of the University of Eastern Africa Baraton within 72 hours.
v. Clearance for export of biological specimens must be obtained from relevant institutions.
vi. Submission of a request for renewal of approval at least 60 days prior to expiry of the approval period. Attach a comprehensive progress report to support the renewal.
vii. Submission of an executive summary report within 90 days upon completion of the study to the Institutional Scientific Ethics Review Committee (ISERC) of the University of Eastern Africa Baraton.

Prior to commencing your study, you will be expected to obtain a research license from National Commission for Science, Technology and Innovation (NACOSTI) https://oris.nacosti.go.ke and also obtain other clearances needed.

Sincerely yours,

Prof. Jackie K. Obey, PhD
Chairperson, Institutional Scientific Ethics Review Committee

University of Eastern Africa, Baraton
13 APR 2023
P. O. Box 2500-30100, ELDORET
Research Ethics Committee

A SEVENTH-DAY ADVENTIST INSTITUTION OF HIGHER LEARNING

Appendix I: Research Permit

REPUBLIC OF KENYA

NATIONAL COMMISSION FOR SCIENCE,TECHNOLOGY & INNOVATION

Ref No: **766319**

Date of Issue: **01/May/2023**

RESEARCH LICENSE

This is to Certify that Mr.. JOSEPH MAINA GICHUKI of Maseno University, has been licensed to conduct research as per the provision of the Science, Technology and Innovation Act, 2013 (Rev.2014) in Uasin-Gishu on the topic: ASSESSMENT OF PAIN BEHAVIOUR AND ANTIINFLAMMATORY RESPONSE IN MALE RATS TREATED WITH CRATEROSTIGMA PLANTAGINEUM HOSCHT EXTRACT for the period ending : 01/May/2024.

License No: **NACOSTI/P/23/25645**

766319

Applicant Identification Number

Director General
NATIONAL COMMISSION FOR SCIENCE,TECHNOLOGY & INNOVATION

Verification QR Code

NOTE: This is a computer generated License. To verify the authenticity of this document, Scan the QR Code using QR scanner application.

See overleaf for conditions