ANTINOCICEPTIVE ACTIVITIES OF EXTRACTS OF *CROTON MEGALOCARPUS HUTCH (Eurphobiaceae)* USING ANIMAL MODELS

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Abbreviations

5HT2 receptors: 5-Hydroxytrptamine 2 Receptors

ANOVA: Analysis of Variance

CNS: Central Nervous System

COX Enzyme: Cyclooxygenase Enzyme

CME: *Croton megalocarpus* Extract

DMSO: Dimethyl Sulphoxide

IASP: International Association for the Study of Pain

LOX: Lipoxygenase Enzyme

NDHN: Nociceptive Dorsal Horn Neurons

NIH: National Center for Health Statistics

NSAID: Nonsteroidal Anti-inflammatory Drug

PG: Prostaglandin

PG E_2: Prostaglandin E_2

$PGF_{2\alpha}$: Prostaglandin $F_{2\alpha}$

SEM: Standard Error of Mean

WHO: World Health Organization

DECLARATION

The thesis is my original work and has not been submitted for a degree in any other university.

Signature ……..date ----------

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ABSTRACT

The use of plant parts for therapeutic purposes has been widely practiced in Africa. One of the trees used for fever and analgesia is *Croton megalocarpus.* However, animal studies have not been done to evaluate this claimed antinociceptive activity. The objective of the study was to investigate the antinociceptive activity of *Croton megalocarpus* using animal models of pain. The nociceptive tests used in this experiment were the Writhing, Tail Flick and the Formalin tests. Swiss albino mice of both sexes were used in a randomized design. In the writhing test, the mice were injected intraperitoneally with doses of the Extract, Aspirin and the Vehicle. Sixty minutes later, they were injected with 0.1 ml of 0.6% acetic acid and the number of writhes observed. In the tail flick test, the mice were injected intraperitoneally with doses of the Extract, Morphine and the Vehicle. An hour later, a light beam was focused on the animal's tail and a timer started. When the animal flicked its tail, the timer was stopped and the time recorded. The same was repeated after thirty, sixty and ninety minutes. In the formalin test, the mice were injected with doses of the Extract, Morphine, Aspirin and the Vehicle. An hour later, they were injected with 0.1 ml of 5% formalin in the sub plantar region of the hind paw. The time spent in pain behavior was then recorded. In the writhing test, all the doses of the extract exhibited significant ($p < 0.05$) antinociceptive effects compared to the vehicle. In the tail flick test the 50, 100 and 200 mg / kg doses of the extract exhibited significant ($p < 0.05$) antinociceptive effects compared to the vehicle. In the formalin test, the 50 mg / Kg dose of the extract did not exhibit significant antinociceptive effect whereas the 100 and 200 mg / Kg doses exhibited significant effects ($p < 0.05$) in the early phase compared to the vehicle. In the late phase, all the doses of the extract exhibited significant ($p < 0.05$) antinociceptive effects compared to the vehicle. These results showed that the extracts of *C. megalocarpus* exhibited peripheral, chronic and central antinociceptive activity hence it probably contains phytochemicals that may be of value in development of a novel drug for analgesia. However, further studies need to be done to elucidate nature and mechanism(s) of action of these metabolites

CHAPTER ONE

1. INTRODUCTION AND LITERATURE REVIEW

1.1 Croton megalocarpus

Plants have been used for medicinal purposes long before recorded history. Egyptian papyrus writings (Hallmann-Mikolajczak, A., 2004) describe medicinal uses for plants as early as 3,000 BC. Indigenous cultures such as African and Native American used herbs in their healing rituals, while others developed traditional medicines in which herbal therapies were used (http://umm.edu/health/medical/altmed/treatment/herbal-medicine, 12thJune 2014). In the written record, the study of herbs dates back 5,000 years to the ancient Sumerians, who described well-established medicinal uses for plants. Ancient Egyptian medicine of 1000 BC are known to have used various herbs as medicine (Hallmann-Mikolajczak, A., 2004).

In the early 19th century, when chemical analysis first became available, scientists began to extract and modify the active ingredients from plants. Later, chemists began making their own version of plant compounds and, over time, the use of herbal medicines declined in favor of synthetic drugs (http://umm.edu/health/medical/altmed/treatment/herbal-medicine, 12th June 2014). In some Asian and African countries, up to 80 % of the population still relies on traditional medicine for their primary health care needs (WHO Fact sheet N° 134 Revised December 2008).

Traditional medicine is widely practiced in Kenya and about 400 plant species have been recorded to be used in traditional remedies (Kokwaro, J. O., 2003). In the rural areas, reliance on traditional medicine is high and is attributed to both economic and cultural factors (Aketch, C. A., 1992). From an economic point of view, the high cost of imported conventional drugs and/or inaccessibility to western health care facilities implies that traditional mode of health care is the only form of health care that is affordable and available to the rural people (Matu and Staden, 2003). On the other hand, even when western health facilities are available, traditional medicine is viewed as an efficient and an acceptable system from a cultural perspective (Munguti, 1997) and as a result, traditional medicine usually exists side by side with western forms of health care (Sindiga *et. al.*, 1995).

Medicinal plants, which form the backbone of traditional medicine, have in the last few decades been the subject of very intense pharmacological studies (Unny *et. al*., 2003). This has been brought about by the acknowledgement of the value of medicinal plants as potential sources of new compounds of therapeutic value and as sources of lead compounds in drug development (Matu and Staden, 2003). Almost one fourth of pharmaceutical drugs are derived from botanicals. Besides, there also exists a very large market of minimally processed medicinal plant parts especially in Europe and America, which are usually dispensed as over-the-counter medication (Loew and Kaszkin, 2002). In developing countries, it is estimated that about 80 % of the population rely on traditional medicine for their primary health care according to the WHO, (1999).

Croton is a large genus of Euphorbiaceae, comprising around 1,300 species of trees, shrubs and herbs distributed in tropical and subtropical regions of both hemispheres (Salatino *et. al*., 2007). The genus is rich in constituents with biological activities, chiefly diterpenoids such as phorbol esters, clerodane, labdane, kaurane, trachylobane, pimarane among others (Block *et. al.*, 2004). *Croton* is also rich in active alkaloids (Amaral *et. al*., 1998, Milanowski *et. al.,* 2002). Several species of the genus are aromatic, indicating the presence of volatile oil constituents (Oliveira, *et. al*., 2001, Rodriguez *et. al.*, 2012). As most Euphorbiaceae, *Croton* species may contain latex, which is red-colored in some species, a characteristic usually associated with medicinal properties (Sandoval *et. al.,* 2002, Risco *et. al*., 2002).

Croton chemistry is considerably diverse. Terpenoids are the predominant secondary metabolite constituents in the genus, chiefly diterpenoids, which may belong to the cembranoid, clerodane, neoclerodane, halimane, isopimarane, kaurane, secokaurane, labdane, phorbol and trachylobane skeletal types. Triterpenoids, either pentacyclic or steroidal, have frequently been reported for *Croton* species. Volatile oils containing mono and sesquiterpenoids, and sometimes also shikimate-derived compounds are not rare in the genus. Several species have been reported as sources of different classes of alkaloids, a fact that

enhances considerably the importance of the genus from the medicinal point of view. Phenolic substances have frequently been reported, among which flavonoids, lignoids and proanthocyanidins predominate (Salatino *et. al*., 2007)

Crude leaf extracts of *C. cajucara* exhibited significant antinociceptive effect in rats (Campos *et. al*., 2002). The red latex of *C. lechleri* revealed strong anti-inflammatory activity (Risco *et. al*., 2003). Orally administered, the volatile oil of *C. nepetaefolius* promoted a dose-dependent antinociceptive effect in hot-plate test (Abdon *et. al*., 2002). The aqueous extract of the aerial parts of *C. cuneatus* had significant activity against plantar inflammation induced by bovine serum albumin (Pereira *et. al*., 1999). Oral administration of the volatile oil of *C. sonderianus* has antinociceptive effect (Santos *et. al*., 2005). An antinociceptive effect of the volatile oil of *C. zehntneri* was evidenced, most likely associated with anti-inflammatory activity (Oliveira *et. al*., 2001). The aqueous extract of *C. malambo* bark administered intraperitoneally showed antinociceptive and anti-inflammatory effects, comparable to acetylsalicylic acid and sodium diclofenac (Suárez *et. al*., 2003). *C. celtidifolius* bark has anti-inflammatory activity (Nardi *et. al*., 2003). Other croton species have found wide spread traditional uses in control management of pain and inflammation. Extracts from the root of *C. polytrichus* have been used to treat headaches and labour pains, *C. mubango* bark pulp has been used to treat toothache and joint pains, the vapor of a deconcotion of the leaves of *C. steenkampiunus* has been used to treat general pains while *C. scheffleri* root soaked in water has been used for fever and malaria (Protabase–Plant resources of Tropical Africa, http://www.prota.org, 3rd September 2016). *C. urucurana* Baill is employed in traditional medicine due to its analgesic and anti-inflammatory effects (Peres *et. al.,* 1998).

Croton megalocarpus Hutch is a tree of the Euphorbiaceous family, Euphorbiaceae subfamily, Crotoneae tribe and the genus croton. It is found widely distributed in East Africa. Although trees are usually not browsed by livestock, leafy twigs may serve as forage for goats. Seeds are used as poultry feed and seed oil is used as bio-fuel. The flowers provide nectar for honey bees. *Croton megalocarpus* is planted in hedges, live fences, shelterbelts and windbreaks, and as an ornamental shade (Lemmens *et. al.,* 2012). It is a pioneer species and is found growing in

cleared parts of natural forests, forest margins or as a canopy tree. It is native in Burundi, Democratic Republic of Congo, Kenya, Malawi, Mozambique, Rwanda, Tanzania and Uganda. The seed is incorporated in poultry feeds, as its protein content is high (50%). Well-dried nuts are reportedly used in some areas together with charcoal in cooking stoves. The tree is also utilized for firewood (Lemmens *et. al*., 2012). It grows at attitudes between 1200 and 2450 meters above sea level requiring a mean annual rainfall of 800 – 1900 mm. Croton megalocarpus grows to 15-35 m; it has distinctive layering of branches and a rather flat crown. The bark is dark grey, rough, and crackling. Hardy and fast growing, leaves are variable, long, oval and pointed to about 12 cm. The dull green upper surface contrasts with the pale, silvery underside. Flowers are conspicuous but very short-lived; yellow white, inserted in many flowered, silver-budded racemes, up to 30 cm long; a few female flowers towards the base, the remainder male. Fruit turns from green to greyish-brown as it matures. The endocarp is hard and woody. Each fruit contains 3 ellipsoid-ovoid or oblong-ellipsoid seeds, 2.2-2.4 cm long and 1.2-1.4 cm wide. Seeds are white when immature, grey brown when mature, with a minute caruncle. After pollination by insects, fruit development takes 5 months and mature fruits can be collected from the ground. In Kenya, seeds mature during October-November in central regions, and from January to March in western regions. *C. megalocarpus* is monoecious but occasionally dioecious.

The Suiei Ndorobo of northern Kenya have used the bark soaked in water as medicine for fever (http://130.54.103.36/aflora.nsf, 14th June 2014). The Samburu have used the roots to treat malaria, fever, chest pains, pneumonia and abdominal pains (Bussmann, 2006). The Kikuyus have used the bark for severe colds and pneumonia (Gacathi, 1989) and the root and bark to treat fever and malaria (Njoroge and Bussmann, 2006). The Batemi of Ngorongoro, Tanzania use the powdered bark to treat malaria and other fevers, as well as to treat abdominal problems associated with gall bladder and spleen problems (Johns *et. al*., 1994). It has also been used for diarrhea management in central Kenya (Njoroge and Kibunja, 2007). The sap from the leaves has been used to treat wounds and cuts (Njoroge and Bussmann, 2007). The Masai have used extract from the boiled bark to treat east coast fever and anthrax (Bussmann, 2006).

Croton megalocarpus organic extracts have very mild antibacterial activity (Matu and Staden, 2003). Both the aqueous and organic extracts of the roots and leaves were found to have high inhibitory activity of up to 80 % the activity of Indomethacin on cyclooxygenase activity (Matu and Staden, 2003). A phytochemical analysis of the extracts of *Croton megalocarpus* showed the presence of alkaloids, glycosides, terpenoids, flavonoids, flavones, reducing sugars and saponins in the extracts (Waiganjo *et. al*., 2013).

1.2. Pain

International Association for the Study of Pain (IASP) and the World Health Organization define pain as "an unpleasant sensory and emotional experience associated with actual or potential tissue damage or described in terms of such damage (IASP, 1979).

Acute pain, also known as "warning pain", is type of pain which comes on suddenly and signals that something is wrong inside the body (IASP, 1986). It is a physiological response that warns us of danger and is the physical suffering associated with a bodily disorder (such as a disease or injury) and accompanied by mental or emotional distress. Acute pain can sometimes be eliminated by treating the underlying cause (IASP, 1986). A person may respond to acute pain with fear, anxiety, and restlessness. If the underlying cause is untreatable, the pain may develop into chronic pain. Chronic pain is persistent and sometimes debilitating. This type of pain is often associated with a long-term or life-threatening illness. Pain is associated with a wide range of injury and disease, and is sometimes the disease itself. Some conditions may have pain and associated symptoms arising from a discrete cause, such as postoperative pain or pain associated with a malignancy, or may be conditions in which pain constitutes the primary problem, such as neuropathic pains or headaches (IASP, 1986).

1.2.1. Problem of Pain

Millions suffer from acute or chronic pain every year and the effects of pain exact a tremendous cost on our country in health care costs, rehabilitation and lost worker productivity, as well as the emotional and financial burden it places on patients and their families (http://www.painmed.org/patientcenter/facts_on_pain, 12thJuly 2014). The costs of unrelieved

pain can result in longer hospital stays, increased rates of re-hospitalization, increased outpatient visits, and decreased ability to function fully leading to lost income. Pain affects more Americans than diabetes, heart disease and cancer combined (http://www.painmed.org/patientcenter/facts_on_pain, 12thJuly 2014). Pain can be a chronic disease, a barrier to cancer treatment, and can occur alongside other diseases and conditions (e.g. depression, post-traumatic stress disorder, traumatic brain injury). It is one of the most common reasons for patients to seek medical attention and one of the most prevalent medical complaints in the US (Watkins *et. al*., 2008). It is considered to be one of the most important symptoms associated with inflammatory diseases and affects a large portion of the population, diminishing their quality of life (White *et. al*., 2005).The use of anti-inflammatory drugs has been required to inhibit the mediators of the inflammation, preventing the acute response and the development of the chronic process (Bovill, J.G., *1997).* Therefore, search for new natural products from medicinal plants with analgesic and anti-inflammatory properties has been encouraged (Vázquez *et. al*., 2011).

1.2.2. Nociception

There are four basic processes involved in nociception (McCaffery and Pasero, 1999). These are transduction, transmission, perception and modulation

1.2.2.1. Transduction of Pain

Transduction begins when the free nerve endings (nociceptors) of C fibers and A-δ fibers of primary afferent neurons respond to noxious stimuli.

The nociceptors are distributed in the Somatic structures (skin, muscles, connective tissue, bones, joints), visceral structures (visceral organs such as liver, gastro-intestinal tract). The C and A-δ fibers are associated with different qualities of pain.

1.2.2.1.1. Noxious Stimuli and Responses

There are three categories of noxious stimuli which are mechanical, thermal and chemical. The cause of stimulation may be internal, such as pressure exerted by a tumor or external, for

example, a burn. This noxious stimulation causes a release of chemical mediators from the damaged cells including: prostaglandin, bradykinin, serotonin, substance P, potassium ions and /or histamine. These chemical mediators activate and/or sensitize the nociceptors to the noxious stimuli (Basbaum and Jessell, 2000).

1.2.2.2. Transmission of Pain

The transmission process occurs in three stages. The pain impulse is transmitted from the site of transduction along the nociceptor fibers to the dorsal horn in the spinal cord. From the spinal cord it is transmitted to the brain stem and then through connections between the thalamus, cortex and higher levels of the brain.

The C and A- δ fibers terminate in the dorsal horn of the spinal cord. There is a synaptic cleft between the terminal ends of the C and A-δ fibers and the nociceptive dorsal horn neurons (NDHN) (Basbaum and Jessell, 2000). In order for the pain impulses to be transmitted across the synaptic cleft to the NDHN, excitatory neurotransmitters are released, which bind to specific receptors in the NDHN. These neurotransmitters are adenosine triphosphate, glutamate, Calcitonin gene-related peptide, bradykinin, nitrous oxide and substance P.

On entering the spinal cord, the pain signals take two pathways to the brain, the neospinothalamic tract and the paleospinothalamic tract (Basbaum and Jessell, 2000). The neospinothalamic tract transmits pain mainly from the fast A-δ pain fibers. This fibers, which transmit mainly mechanical and acute thermal pain, terminate mainly in lamina I (lamina marginalis) of the dorsal horns and there excite second-order neurons of the neospinothalamic tract. These give rise to long fibers that cross immediately to the opposite side of the cord through the anterior commissure and then turn upward, passing to the brain in the anterolateral columns. A few fibers of the neospinothalamic tract terminate in the reticular areas of the brain stem, but most pass all the way to the thalamus without interruption, terminating in the ventrobasal complex. A few fibers also terminate in the posterior nuclear group of the thalamus. From these thalamic areas, the signals are transmitted to other basal areas of the brain as well as to the somatosensory cortex (Basbaum and Jessell, 2000).The

paleospinothalamic pathway transmits pain mainly from the peripheral slow-chronic type C pain fibers, although it does transmit some signals from type A-δ fibers as well. In this pathway, the peripheral fibers terminate in the spinal cord almost entirely in laminae II and III of the dorsal horns. Most of the signals then pass through one or more additional short fiber neurons within the dorsal horns themselves before entering mainly lamina V, also in the dorsal horn. Here the last neurons in the series give rise to long axons that mostly join the fibers from the fast pain pathway; passing first through the anterior commissure to the opposite side of the cord, then upward to the brain in the anterolateral pathway. The paleospinothalamic pathway terminates widely in the brain stem. Most terminate in one of three areas: (1) the reticular nuclei of the medulla, pons, and mesencephalon; (2) the tectal area of the mesencephalon deep to the superior and inferior colliculi; or (3) the periaqueductal gray region surrounding the aqueduct of Sylvius. These lower regions of the brain appear to be important for feeling the suffering types of pain, because animals whose brains have been sectioned above the mesencephalon to block pain signals from reaching the cerebrum still evidence undeniable suffering when any part of the body is traumatized (Allan *et. al*., 2010; Basbaum and Jessell, 2000). From the brain stem pain areas, multiple short-fiber neurons relay the pain signals upward into the intralaminar and ventrolateral nuclei of the thalamus and into certain portions of the hypothalamus and other basal regions of the brain (Almeida *et. al*., 2004; Allan *et. al*., 2010)

1.2.2.3. Perception of Pain

Perception of pain is the end result of the neuronal activity of pain transmission where pain becomes a conscious multidimensional experience. The latter has affective-motivational, sensory-discriminative, emotional and behavioral components. When the painful stimuli are transmitted to the brain stem and thalamus, multiple cortical areas are activated and responses are elicited (Abelson, 2005; Almeida *et. al*., 2004; Apkarian *et. al*., 2005; Casey, 1999; Cross, 1994; Davis *et. al*., 1997; Riedel and Neck, 2001)

These areas are:

- The reticular system: This is responsible for the autonomic and motor response to pain and for warning the individual to do something, for example, automatically removing a hand when it touches a hot saucepan. It also has a role in the affective-motivational response to pain such as looking at and assessing the injury to the hand once it has been removed from the hot saucepan.
- Somatosensory cortex: This is involved with the perception and interpretation of sensations. It identifies the intensity, type and location of the pain sensation and relates the sensation to past experiences, memory and cognitive activities. It identifies the nature of the stimulus before it triggers a response, for example, where the pain is, how strong it is and what it feels like.
- Limbic system: This is responsible for the emotional and behavioral responses to pain for example, attention, mood, and motivation, as well as processing pain and past experiences of pain.

1.2.2.4. Modulation of Pain

The degree to which a person reacts to pain varies tremendously. This results partly from a capability of the brain itself to suppress input of pain signals to the nervous system by activating a pain control system, called an analgesia system. The analgesia system consists of three major components. The periaqueductal gray, periventricular areas of the mesencephalon and upper pons which surround the aqueduct of Sylvius and portions of the third and fourth ventricles. Neurons from these areas send signals to the raphe magnus nucleus; a thin midline nucleus located in the lower pons and upper medulla, and the nucleus reticularis paragigantocellularis, located laterally in the medulla. From these nuclei, second-order signals are transmitted down the dorsolateral columns in the spinal cord to a pain inhibitory complex located in the dorsal horns of the spinal cord (Basbaum and Fields, 1984; Gebhart G. F, 2004; Hudspith *et. al*., 2005; Ossipov *et. al*., 2004). At this point, the analgesia signals can block the pain before it is relayed to the brain. Electrical stimulation either in the periaqueductal gray area or in the raphe magnus nucleus can suppress many strong pain signals entering by way of the dorsal spinal roots. Also, stimulation of areas at still higher levels of the brain that excite the periaqueductal gray area can also suppress pain. Some of these areas are the periventricular

nuclei in the hypothalamus and to a lesser extent, the medial forebrain bundle, also in the hypothalamus.

Several transmitter substances are involved in the analgesia system especially encephalin and serotonin (Azami *et. al*., 1982; Basbaum and Fields, 1984; Gebhart G. F, 2004; Harris and Westbrook, 1994; Hudspith *et. al*., 2005; Millan, 2002; Ossipov *et. al*., 2004). Many nerve fibers derived from the periventricular nuclei and from the periaqueductal gray area secrete encephalin at their endings. Thus, the endings of many fibers in the raphe magnus nucleus release enkephalin when stimulated. Fibers originating in this area send signals to the dorsal horns of the spinal cord to secrete serotonin at their endings. The serotonin causes local cord neurons to secrete enkephalin as well. The enkephalin is believed to cause both presynaptic and postsynaptic inhibition of incoming type C and type A-δ pain fibers where they synapse in the dorsal horns. Thus, the analgesia system can block pain signals at the initial entry point to the spinal cord (Basbaum and Fields, 1984; Gebhart G. F., 2004; Harris and Westbrook, 1994; Hudspith *et. al*., 2005; Millan, 2002). In fact, it can also block many local cord reflexes that result from pain signals, especially withdrawal reflexes. Injection of minute quantities of morphine either into the periventricular nucleus or into the periaqueductal gray area of the brain stem causes an extreme degree of analgesia. Morphine-like agents, mainly the opiates, also act at many other points in the analgesia system, including the dorsal horns of the spinal cord (Azami *et. al*., 1982; Ossipov *et. al*., 2004). About a dozen such opiate-like substances have now been found at different points of the nervous system; all of which are breakdown products of pro-opiomelanocortin, proenkephalin, and prodynorphin. Among the more important of these opiate-like substances are β-endorphin, met-enkephalin, leu-enkephalin and dynorphin. The two enkephalins are found in the brain stem and spinal cord, and β-endorphin in both the hypothalamus and the pituitary gland. Dynorphin is found mainly in the same areas as the enkephalins but in much lower quantities (Besson and Chaouch, 1987; Hudspith *et. al*., 2005).

Thus, although the fine details of the brain's opiate system are not understood, activation of the analgesia system by nervous signals entering the periaqueductal gray and periventricular areas, or inactivation of pain pathways by morphine-like drugs, can almost totally suppress many pain signals entering through the peripheral nerves. This multiple, complex pathways

involved in the modulation of pain are referred to as the descending modulatory pain pathways (DMPP) and can lead to either an increase in the transmission of pain impulses (excitatory) or a decrease in transmission (inhibition). Descending inhibition involves the release of inhibitory neurotransmitters that block or partially block the transmission of pain impulses, and therefore produce analgesia (Akil *et. al*., 1984). Inhibitory neurotransmitters involved with the modulation of pain includes endogenous opioids such as enkephalins and endorphins, serotonin, norepinephrine, Gamma-amino butyric acid (GABA), neurotensin, acetylcholine and oxytocin. Endogenous pain modulation helps to explain the wide variations in the perception of pain in different people as individuals produce different amounts of inhibitory neurotransmitters (Basbaum and Fields, 1984; Basbaum and Jessell, 2000; Hudspith *et. al*., 2005; McCaffery M, Pasero C., 1999).

1.2.3. Pain Management

The World Health Organization recommends a pain ladder for managing analgesia (WHO, 2009). It was first described for use in cancer pain, but it can be used by medical professionals as a general principle when dealing with analgesia for any type of pain. In the treatment of chronic pain, whether due to malignant or benign processes, the three-step WHO analgesic ladder provides guidelines for selecting the kind and stepping up the amount of analgesia. The exact medications recommended will vary with the country and the individual treatment center, but the following gives an example of the WHO approach to treating chronic pain with medications. If, at any point, treatment fails to provide adequate pain relief, then the doctor and patient move onto the next step. For mild pain, centrally acting analgesics such as paracetamol can be used. In mild to moderate pain, paracetamol or non-steroidal anti-inflammatory products are used. Non-steroidal anti-inflammatory products are widely used in management of pain. They work by inhibiting the cyclooxygenase enzyme which is involved in synthesis of prostaglandins which are strong mediators of pain (Smith *et. al*., 2000). They are however associated with side effects such as gastric and duodenal ulceration, (Botting, R. M., 2006). Due to these side effects, their use in management of chronic pain is usually discouraged. Their use in severe hypovolemia will normally result in decrease in renal blood flow due to their depression of prostaglandin synthesis, which is beneficial to the kidneys in

such conditions. Because currently available anti-inflammatory drugs have considerable side effects that inhibit their clinical use, many studies are currently underway to develop new treatments for inflammatory diseases (Shukla *et. al*., 2010).

In management of moderate to severe pain, the type of the pain, whether acute or chronic, needs to be considered. The type of pain can result in different medications being prescribed. Certain medications may work better for acute pain, others for chronic pain, and some may work equally well on both. Acute pain medication is for rapid onset of pain such as from an inflicted trauma or to treat post-operative pain. Chronic pain medication is for alleviating long-lasting, ongoing pain and the opiates are recommended with morphine being the gold standard. Although opioids are strong analgesics, they do not provide complete analgesia regardless of whether the pain is acute or chronic in origin (Specialist pain international, 2013). Opioids are efficacious analgesics in chronic malignant pain and modestly effective in non-malignant pain management. However, there are associated with adverse effects, especially during the commencement or change in dose. When opioids are used for prolonged periods, drug tolerance, chemical dependency, diversion and addiction may occur which limits their use (Howard, L. Fields, 2011).

Many inflammatory diseases are associated with the synthesis of prostaglandins, which are responsible for a sensation of pain. The primary enzyme responsible for prostaglandins synthesis is the membrane-associated cyclooxygenase enzyme, (COX), which occurs in two isoforms, COX-1 and COX-2 (Vane and Botting, 1996). COX-1 is constitutively expressed while COX-2 is induced in the inflamed tissue. Modulation of the activity of the enzyme implies that the inflammation process can be modified (Smith *et. al., 2000)*. At present, although synthetic drugs are dominating the market, the element of toxicity that these drugs entail, cannot be ruled out. Their prolonged use may cause severe adverse effects the most common being gastro-intestinal bleeding and peptic ulcers (Corley *et. al*., 2003)

Opiates have been used for centuries and remain to this day the most potent and reliable analgesic agents (Pasternak, 2011). They are used routinely and effectively for the treatment of acute severe pain following trauma, extensive burns or surgery. They are also used for patients

with painful terminal diseases such as cancer. In these time-limited situations the efficacy of opiates is extensively documented and broadly accepted. In fact, their use has recently grown, in part because providing adequate pain relief is now considered an important standard of care and is required by law in some states. Beyond potent analgesia, opiates reduce anxiety and produce mild sedation and a palpable sense of well-being, often to the point of euphoria. These are an unmitigated benefit for patients who would otherwise have to endure the pain and suffering of acute or terminal medical conditions. The problem is that the most powerful opiate analgesics are also the most liable to cause abuse and addiction (Howard, L. Fields., 2011). Opioids are defined by their actions at one of the family of opioid G-protein coupled receptors (GPCRs). There are four known opioid receptors (mu (μ), delta (δ), kappa and the nociceptinorphanin peptide receptor. However, only μ receptor agonists consistently produce potent analgesia and drugs activating them such as heroin, morphine and oxycodone are also the most commonly abused (Koob and Le Moal, 2006; Pasternak, 2011). Despite decades of research, pharmaceutical companies have been unable to design opioid ligands that retain high analgesic potency but with reduced abuse potential. Furthermore, there are currently no non-opioid analgesics with either the broad range of analgesic efficacy or the potency of μ agonists. The inability to uncouple powerful analgesia from addictive potential is a barrier to resolving the current dilemma about opiate use for chronic pain. In some ways, dissolving the bond between potent analgesia and addiction is the holy grail of pain research. One could argue that if a drug were found that was potent across a broad range of painful conditions, was not addicting and to which patients did not develop tolerance, pain would cease to be a significant medical problem. The decision about long term opiate prescribing is further complicated by the substantial increase in people diverting and abusing prescription opiate analgesics (Howard, L. Fields., 2011). Data from the Substance Abuse and Mental Health Administration raise significant red flags, for example, “In 2009, an estimated 3.1 million persons aged twelve years or older used an illicit drug for the first time within the past 12 months.” In addition to their addictive potential, high doses of potent opiate analgesics cause profound respiratory depression, the leading cause of death from these drugs. Although opiate analgesics are potent for a variety of time limited painful conditions, the duration of their efficacy has only been

established for up to two months. Animal studies indicate that tolerance and dependence are common with repeated opioid use and both animal and human studies indicate that long term administration of opiate analgesics can actually worsen pain (Howard, L., Fields, 2011).

1.2.4. Nociceptive Tests

1.2.4.1. Tail Flick Test

The test was first introduced by D'amour and Smith (1941). A radiant heat is applied to the tail of a rodent and the response latency to remove its tail from the heat recorded as a sign of nociception. The tail flick reflex is a spinal reflex and is used for screening drugs as well as for nociceptive tests. It also allows repeated tests without conditioning effects and there is minimal individual variation in the test. The skin temperature greatly influences the test and if an analgesic drug lowers the skin temperature, this tends to prolong the tail flick latency (Irwin *et. al.,* 1951; Berg *et. al*., 1980; Le Bars *et. al.,* 2001). Most commonly, a light beam is focused on the animal's tail and a timer started. When the animal flicks its tail, the timer is stopped, the recorded time (latency) being a measure of the pain threshold. Researchers testing the effectiveness of drugs on the pain threshold often use this test to measure the extent to which the drug being tested has reduced the amount of pain felt by the model organism. Both laboratory mice and rats are a common model organism for these tests.

1.2.4.2. Writhing Test

Writhing test is a chemical method used to induce pain of peripheral origin by injection of irritant principles like phenylquinone or acetic acid in mice. Analgesic activity of the test compound is inferred from decrease in the frequency of writhes. The manifestations of abdominal writhing in mice were first described by Sigmund *et. al.,* (1957) as an arching of back, extension of hind limbs and contraction of abdominal musculature. This is a typical model for inflammatory pain and has been used as a tool to screening for analgesic or anti-inflammatory properties of new agents and in most cases as a model to study the peripheral antinociceptive effect of extracts/compounds. It represents stimulation of the cyclooxygenase (COX) and lipooxygenase (LOX) (Ikeda *et. al*., 2001) and indirectly leads to the release of endogenous nociceptive mediators (PGE_2, $PGF_{2\alpha}$, serotonin, histamine, cytokines and eicosanoids) as well as

other LOX products in peritoneal fluids that can induce various nociceptive neurons sensitive to NSAIDs within the peritoneal cavity (Ikeda *et. al*., 2001; Vasudevan *et. al*., 2006). Ability of extract to attenuate the acetic acid induced abdominal constriction test will suggest that its antinociceptive mechanism involves, in part, its ability to inhibit COX and LOX in the peripheral tissues leading to decrease in PGEs synthesis and blockage of the pain transduction in primary afferent nociceptors (Mohd *et. al.,*2012).

1.2.4.3. Formalin Test

The most predictive of the models for acute pain is the Formalin Test. It was first described for rats and cats by Dubuisson and Dennis, (1977). The formalin test is carried out as described by Zakaria *et. al.,* (2001). A 5% solution of formaldehyde is injected subcutaneously to the mouse or rat paw to produce a biphasic pain response over a test period of sixty minutes. The initial pain response occurs at one minute following sub-plantar injection of formalin and results from direct stimulation of nociceptors. The second phase of pain response occurs after a period of sensitization (quiescent period) during which inflammatory phenomena take place (Dubuisson and Dennis, 1977; Alreja *et. al., 1984*; Hunskkar and Hole., 1987; Shibata *et. al.,* 1989). Pain response scoring includes counts per unit of time of reactions to the pain stimulus by licks, twitches, raising or shaking of the injected paw. The formalin injection into the paw causes an immediate and intense increase in the spontaneous activity of C fiber afferents and evokes a distinct quantifiable behavior indicative of pain (i.e., licking of injected paw) (Heapy *et. al*., 1987). This test, which represents a model of prolonged pain, is very useful in studies of pain mechanism and in the evaluation of analgesic drugs (Shibata *et. al*., 1989). The early phase, classified as neurogenic pain, is an acute response observed immediately after the administration of formalin and persists for 10 minutes (0-10min) as a result of direct action of injected formalin on nociceptors (Dennis and Dubuisson, 1977; Alreja *et. al.,* 1984; Hunskaar *et. al*., 1985 a, 1986; Hole and Hunskaar, 1987; Shibata *et. al*., 1989). The late phase, classified as inflammatory pain, is a tonic response resulting from the inflammatory processes generated by the release of inflammatory mediators such as histamine, serotonin, prostaglandins, bradykinin (Verma *et. al*., 2005) and activation of the dorsal horns of the spinal cord (Hunskaar and Hole, 1987; Tang *et.al*., 2007). It appears between 15 and 60 minutes after administration of formalin

(Dubuisson and Dennis, 1977; Alreja *et. al.,* 1984; Hole and Hunskkaar, 1987; Shibata *et. al*., 1989). Centrally acting drugs (e.g. opioids) inhibit both phases while peripherally acting drugs (e.g., NSAIDs) inhibit only the late phase (Mohd *et. al*., 2012).

1.3.1. Statement of the Research Problem

Herbal medicines have been used for control of pain in many communities. There is no one particular compound which is very effective for treatment of pain and majority of products available have side effects. Research on plants with traditional medicinal claims has led to development of new compounds of therapeutic value as well as lead compounds in drug development. It is therefore important to scientifically validate the claimed analgesic effects since they could have less side effects or more analgesic activity than the existing drugs.

Croton megalocarpus has been used for relief of pain in some communities. These claims have not been scientifically validated.

1.3.2. Justification

Croton megalocarpus is a natural product with traditional claims of medicinal effects (Gacathi, 1989; Johns *et. al.,* 1994; Bussman, 2006; Njoroge and Bussmann, 2006*)*. It has been used by the Samburu in treatment of chest and abdominal pains (Bussmann, 2006).There is need to provide scientific data on this claimed antinociceptive effects. Experimental studies found extracts of the roots and leaves to high inhibition activity on cyclooxygenase activity (Matu and Staden, 2003). There is need to investigate whether the experimental cyclooxygenase enzyme inhibition activity can be reproduced in animal models of pain testing. The aim of this study was to investigate the antinociceptive effects of *Croton megalocarpus* extracts using animal models.

1.4. OBJECTIVES OF THE STUDY

1.4.1. Overall Objective

To investigate the antinociceptive activity of *Croton megalocarpus* using animal models of nociception

1.4.2. Specific Objective

To investigate the antinociceptive effects of the root, leaf and bark extracts of *Croton megalocarpus* using different animal models of nociception

1.4.3. Null Hypothesis: Roots, Leaves and Bark extracts of *Croton megalocarpus* have no antinociceptive effects

CHAPTER TWO

2.0. MATERIALS AND METHODS

2.1. Materials

Plant parts: Roots, Bark and leaves of *Croton megalocarpus.*

The plant parts were collected from natural habitat in Gatundu area, about 45 km from Nairobi. Identification and authentication was done by the University of Nairobi, School of Biological Sciences Herbarium and a voucher specimen WGG2014/01 reserved for reference by the same. The bark for the roots and stems were skimmed off and dried under shade for two weeks. The leaves were dried separately under shade for three weeks at room temperature. The dried samples, in equal ratios for the bark, root and leaves was then ground into powder in the chemistry department, University of Nairobi.

2.2. Drugs and Chemicals

The following reagents were used:

Solvents: Methanol, Dichloromethane and Dimethyl Sulfoxide (DMSO).

Chemicals: Acetic Acid and Formalin

Drugs: Morphine and Acetyl Salicylic Acid.

The positive controls were made by dissolving the drugs, morphine and acetyl salicylic acid in normal saline. The *Croton megalocarpus* extract (CME) was prepared by dissolving known

weight in 10% DMSO in normal saline just before use. The vehicle used was 10% DMSO in normal saline as the negative control.

2.3. Experimental Animals

Swiss albino mice of both sexes weighing between 20 and 25 g were used. They were obtained from the department of medical physiology animal house. They were housed in cages at room temperature and humidity on a 12-hour light- darkness cycle. They were supplied with mice pellets from Unga Feeds and water a*d-libitium* up to the start of the experiment. The animal house was maintained on a 12/12 hour light/dark cycle and room temperature of 22 degrees centigrade. The "Principle of Laboratory Animal Care" (NIH publication No. 85-23)
Guidelines and procedures were followed in this study (NIH publication revised 1985). The experiments were carried out between 8 am and 6 pm to minimize the effects of environmental changes.

2.4. Experimental Design

The experiment was a randomized design. The animals were randomly picked for the experiment. A preliminary test to determine dose-response was done. Three levels of the dosage of extract were used. Each dose level comprised of eight experimental animals and a positive and negative control with the same number of animals (n = 8). The doses used were 50mg / kg, 100mg / Kg and 200mg / kg body weight.

2.5. Extraction

The aqueous and organic components of the powder were obtained by using methanol and dichloromethane as the solvents in a (1:1) ratio. One hundred fifty grams of the powder was weighed and then soaked in 200 ml of a 1:1 ratio mixture of methanol and dichloromethane and left overnight. The supernatant was then filtered using Whatman no. 1 filter paper. The residue was subjected to same procedure another two times. Supernatant from each extraction were pooled together and then subjected to evaporation using rotary drying and the residue weighed. About 8.56 grams was obtained. The extract was then constituted in 10% Dimethly Sulfoxide in normal saline prior to experimentation. 500 mg of the powder was dissolved in 2.5ml of dimethyl sulfoxide (DMSO). Normal saline is then added to make up to 25mls. This

resulted in a solution containing 20 mg / ml of CME extract in 10 % DMSO in Normal Saline. The volumes of this solution to be injected for 50mg / kg, 100 mg / kg and 200 mg / kg body weight is 0.0625ml, 0.125ml and 0.25ml respectively.

2.6. Drug Administration

The animal was carefully picked by the tail and placed on the bench. Using the left hand, the loose skin on the dorsal side of the neck was held and the animal placed on the palm of the left hand while the tail was held with the little finger of the left hand. The ventral side of the animal was then exposed and the drug or vehicle injected intraperitoneally using a 1 ml syringe. The drug was injected 1 cm to the left of the midline and in the lower abdomen. The drug was given one hour prior to the nociceptive testing.

2.7. Sensorimotor Test

To evaluate possible nonspecific muscle relaxant or sedative effects of the plant extracts, a sensorimotor apparatus which consists of 3 vertical rods, diameter 2.5 cm, with the height of 20, 32, and 64 cm was used. This test was done prior to extract, drug or negative control (10% dimethyl sulfoxide in normal saline) injection and also one hour after the animals were treated with the extract, control drug or negative control. Animals were placed on top of each rod for 20 seconds to test their sensorimotor function. The cut-off time used was 20 seconds per rod.

2.8. Nociceptive tests

2.8.1. Writhing Test

The mice were allowed to adapt to the observation chamber for fifteen minutes prior to the test. They were then injected with 10% DMSO in normal saline (negative control), 10 mg / kg ASA (positive control) and CME (50, 100 and 200 mg / kg). Sixty minutes after respective test solution administration, the mice were then injected intraperitonially with 0.1 ml solution of 0.6% acetic acid. The animals were then immediately placed individually into Perspex boxes of dimensions 30 cm x 30 cm x 30 cm. The abdominal constriction resulting from injection of acetic acid consisted of a contraction of the abdomen together with a stretching of at least one hind limb. The number of abdominal constrictions produced in these animals were then counted in blocks of five minutes cumulatively for twenty five minutes. Antinociceptive activity,

indicated by reduction in the mean number of contractions compared with the control group, was calculated as the percentage inhibition of abdominal constrictions (percentage inhibitory level) using the formula: (mean of (control – test group)/control group x 100).

2.8.2. Tail flick Test

Tail-flick test was used in the present work as described by D'Amour and Smith (1941) using radiant heat An IITC Inc. Model 33 tail-flick analgesiometer. The mice were injected intraperitonally with 10% DMSO (negative control), 5 mg / kg morphine (positive control), or CME 50, 100, and 200 mg / kg. Sixty minutes after the respective test solution administration, the tail of the mouse was placed on a level surface. A radiant heat was then applied to the tail and the response latency to flick its tail from the heat recorded. The maximum time of heat exposure ("cut-off" time) to avoid tissue damage was 20 sec. The prolongation of the latency times was then compared with the values of the controls and used for statistical comparison.

2.8.3. Formalin Test

The formalin test was carried out as described by Zakaria *et. al.,* (2003) but with slight modifications. The animals were allowed to adapt to the observation chamber, a Perspex observation box of dimensions 30cm x 30 cm x 30cm, for 20 minutes. Pain was induced by injecting 0.1ml of 5 % formalin in 0.9 % sodium chloride solution in the sub-plantar region of the right hind paw. The mice were pretreated by intraperitoneal injection with 10 % DMSO (negative control), 100 mg / kg ASA (positive control), 5 mg / Kg morphine (positive control) or CME 50 mg / Kg, 100 mg / Kg and 200 mg / Kg, 60 minutes prior to the formalin injection. Immediately after the formalin administration, the mice were individually placed in the Perspex observation box. The amount of time (in seconds) spent licking and or lifting the injected paw, considered as an indicator of pain, was then recorded using a stop watch in 5 minute blocks for a period of 30 minutes in two phases, the early (0-10 min) and late (15-30 min) phases. The ED50 was then computed using nonlinear regression with least estimation and the sigmoid function was fitted where response was plotted against log of dose. The analysis of data was done using STATA version 13 software.

2.9. STATISTICAL ANALYSIS

The data for each set was pooled and expressed as the mean ± standard errors of the mean (S.E.M). To determine the level of significance, p, one way analysis of variance (ANOVA) followed by Tukey's Honest Significance *post hoc* test to compare test and control group values. The difference in test values against negative control values was considered to be statistically significant at $p \leq 0.05$.

CHAPTER 3

3. RESULTS, DISCUSSION AND CONCLUSION

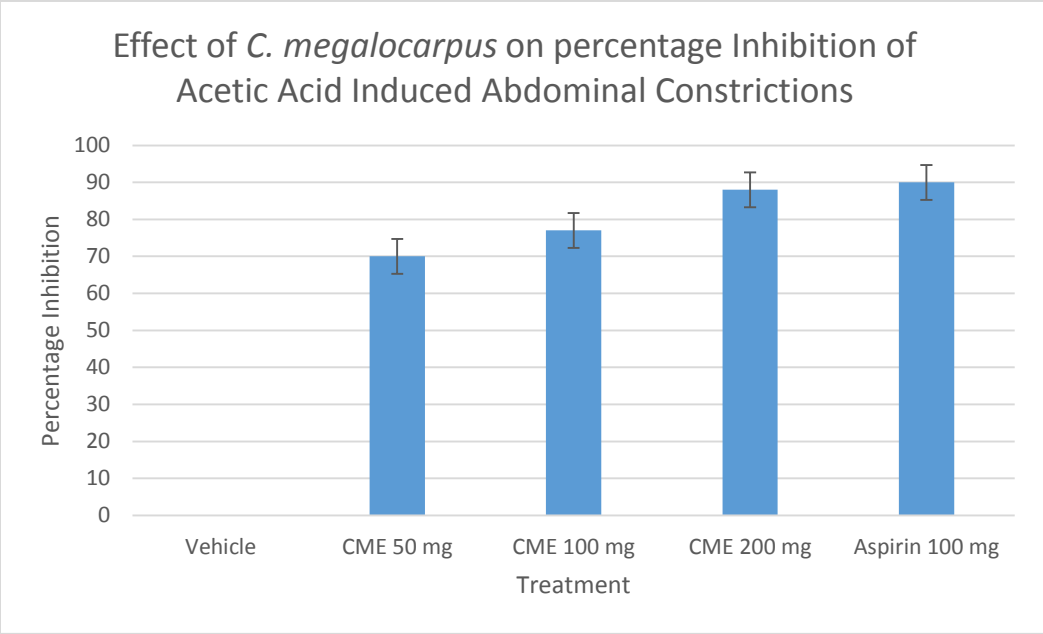
3.1. Writhing test

In the writhing test three doses, 50 mg / Kg, 100 mg / Kg and 200 mg / Kg of the extract were used (Figure 1 and Table 1). Aspirin 100 mg / kg was used as the positive control and 10% dimethyl sulfoxide (DMSO) used as the negative control. The antinociceptive activity, indicated by reduction in the mean number of contractions compared with the control group, was calculated as the percentage inhibition of abdominal constrictions (percentage inhibitory level) using the formula: (mean of (control – test group)/control group x 100). The percentage inhibition to writhing was 70%, 77% and 88% respectively for the 50 mg / Kg, 100 mg / Kg and 200 mg / Kg of the extract respectively. All the doses of the *Croton megalocarpus* extract caused highly significant ($p < 0.01$) antinociceptive effects compared with the vehicle. Aspirin also exhibited highly significant ($p < 0.01$) antinociceptive effects compared with the vehicle. There was a significance difference ($p < 0.05$) between the 50 mg / Kg dose of the extract compared with that of aspirin treated animals. However, there was no significance difference for the 100 and 200 mg / Kg doses of extract compared with aspirin.

Table 1: Effect of *C. megalocarpus* on Writhing Test

Treatment (n = 8)	Writhes	% Inhibition	p values
Vehicle	21.87 ± 0.47		
CME 50 mg / Kg	6.5 ± 0.98	70	p < 0.01
CME 100 mg / Kg	4.87 ± 1.34	77	p < 0.01
CME 200 mg / Kg	2.625 ± 0.37	88	p < 0.01
Aspirin 100mg / Kg	2.12 ± 0.22	90	p < 0.01

Figure 1**: Effect of *C. megalocarpus* on percentage inhibition on Writhing Test**



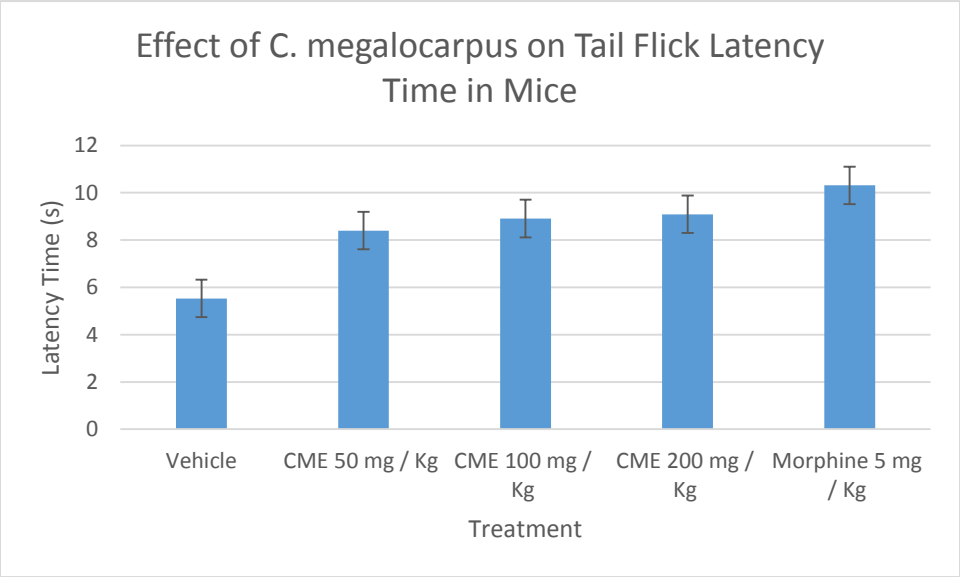
3.2. Tail Flick Test

In the Tail Flick Test, three doses, 50 mg / Kg, 100 mg / Kg and 200 mg / Kg of the extract were used (Figure 2 and Table 2). Morphine 5 mg / kg was used as the positive control and 10% Dimethyl Sulphoxide (DMSO) in normal saline as the negative control. The positive control induced highly significant antinociceptive effects ($p < 0.01$) as compared to negative control. All doses of the extract exhibited highly significant antinociceptive effects ($p < 0.01$) compared to negative control. There was significant difference in effect between the 50 mg / Kg dose compared to morphine ($p < 0.05$) as compared to the other doses where the difference in antinociceptive effects was insignificant between 100 mg / Kg and 200 mg / Kg doses compared to morphine. It is evident from the study that increase in dosage increases the latency period for the tail flick from noxious stimuli.

Table 2: Effect of *C. megalocarpus* extract on Tail Flicking Time in mice

Treatment (n = 8)	Mean latency time (S)	p values
Vehicle	5.53 ± 0.12	
CME 50 mg / Kg	8.40 ± 0.39	$p < 0.01$
CME 100 mg / Kg	8.91 ± 0.44	$p < 0.01$
CME 200 mg / Kg	9.09 ± 0.50	$p < 0.01$
Morphine 5 mg / Kg	10.31 ± 0.65	$p < 0.01$

Figure 2: Effect of *C. megalocarpus* extracts on Tail Flicking Time in mice



3.3. Formalin Test.

In the formalin test, three doses, 50 mg / Kg, 100 mg / Kg and 200 mg / Kg of the extract were used (Figure 3 and Table 3). Aspirin 10 mg / Kg and morphine 5 mg / Kg were used as the positive controls with 10% DMSO as the negative control. In the early phase, the CME 50 mg / Kg dose did not exhibit significant antinociceptive effect compared to the vehicle whereas, the 100 and 200 mg / Kg doses exhibited significant ($p < 0.01$) effects compared to the vehicle. Aspirin exhibited significant ($p < 0.05$) antinociceptive effects and morphine very significant ($p <$

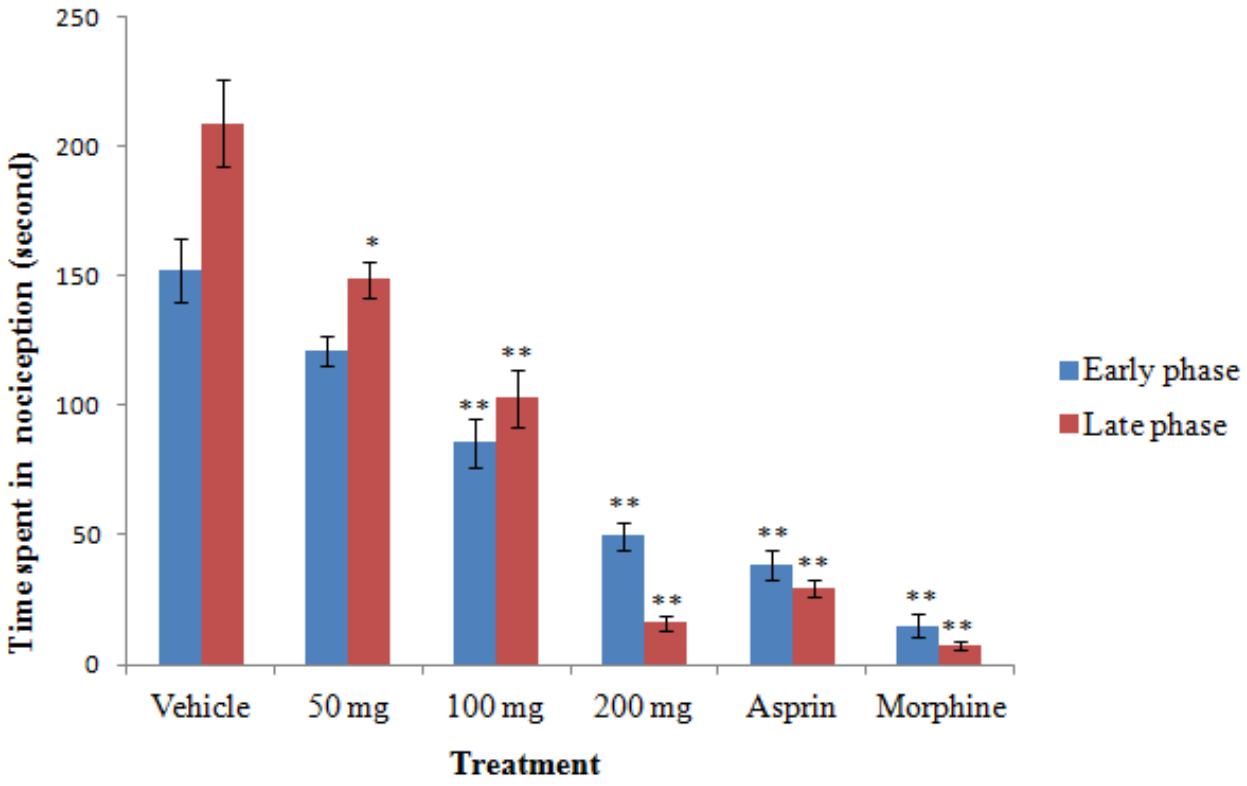
0.01) antinociceptive effects compared to the vehicle. There was significant difference between 50 mg / kg and the 100 mg / kg as compared to aspirin and morphine. However, for the 200 mg / kg, the difference with aspirin was insignificant hence the effects are comparable to aspirin. There is significant difference between this dose and aspirin.

In late phase of the formalin test, all the doses of the extract exhibited highly significant ($p < 0.01$) antinociceptive effects compared to the vehicle. The positive controls of Morphine and Aspirin exhibited highly significant ($p < 0.01$) antinociceptive effects compared to the negative control (vehicle). There was significant difference between the 50 mg / kg and the 100 mg / kg and the morphine and aspirin. However, the difference between the 200 mg / kg of CME as compared to the morphine and aspirin was insignificant hence the effects were comparable.

Table 3: Effect of *C. megalocarpus* extract on the Formalin Test

Treatment (n = 8)	**Early Phase Time (Seconds)**	**p values**	**Late Phase Time (Seconds)**	**p values**
Vehicle	152.5 ± 12.42		209.75 ± 16.74	
CME 50 mg / Kg	121.38 ± 5.87	Insignificant	149.91 ± 7.05	P < 0.01
CME 100 mg / Kg	85.81 ± 9.62	P < 0.01	103.68 ± 10.96	P < 0.01
CME 200 mg / Kg	49.83 ± 5.3	P < 0.01	16.03 ± 2.86	P < 0.01
Aspirin 100 mg / Kg	38.32 ± 5.93	P < 0.05	29.15 ± 3.31	P < 0.01
Morphine 5 mg / Kg	15.09 ± 4.5	P < 0.01	7.39 ± 1.59	P < 0.01

Figure 3: Antinociceptive effects of *C. megalocarpus* extract on the Early Phase of Formalin Test



3.4. DISCUSSION

The writhing test is a model test to study the peripheral antinociceptive effect of extracts and compounds. It represents stimulation of the cyclo-oxygenase (COX) and lipoxygenase (Ikeda *et. al*., 2001) and indirectly leads to the release of endogenous nociceptive mediators (PGE_2, $PGF_{2\alpha}$, serotonin, histamine, cytokines and eicosanoids) as well as other LOX products in peritoneal fluids that can induce various nociceptive neurons within the peritoneal cavity (Ikeda *et. al*., 2001; Vasudevan *et. al*., 2006). The ability of the extract to attenuate the acetic acid induced abdominal constriction test suggests that its antinociceptive mechanism may involve, in part, its ability to inhibit cyclo-oxygenase enzyme in the peripheral tissues leading to decrease in prostaglandin synthesis and blockage of the pain transduction in primary afferent nociceptor

(Mohd *et. al.,* 2012). Results from this test are indicative that the extract has peripheral antinociceptive effects.

The tail flick test is a spinally mediated nociceptive test commonly used to study pain mechanism. In this study the extract was found to prolong the latency withdrawal time after radiant heat was directed to the tail of the test mice. This effect was comparable to morphine at the doses of 100 and 200 mg / Kg. Since the tail flick is a spinally mediated reflex, it is likely that *Croton megalocarpus* extract acted via the central nervous system by blocking pain pathway at spinal level.

The formalin test, which represents a model of prolonged pain, is very useful in studies of pain mechanism and in the evaluation of analgesic drugs (Shibata *et. al*., 1989). The early phase, classified as neurogenic pain, is an acute response observed immediately after the administration of formalin as a result of direct action of injected formalin on nociceptors (Alreja *et. al.,* (1984)*:* Dubuisson and Dennis, 1977, 1984; Hunskaar *et. al*., 1985 a, 1986; Hunskaar and Hole, 1987, Shibata *et. al*., 1989). The late phase, classified as inflammatory pain, is a tonic response resulting from the inflammatory processes generated by the release of inflammatory mediators such as histamine, serotonin, prostaglandins, bradykinin (Verma *et. al*., 2005) and activation of the dorsal horns of the spinal cord (Hunskaar and Hole, 1987; Tang *et. al*., 2007). Centrally acting drugs (e.g., opioids) inhibit both phases while peripherally acting drugs (e.g., NSAIDs) inhibit only the late phase (Mohd *et. al*., 2012). Aspirin has been found to inhibit both phases of pain in mice (Hunskaar and Hole, 1987). This could be because of prostaglandin inhibition in CNS or increasing serotonin although it may be due to down regulation of 5-HT2 receptors that can centrally reduce pain. In this study, antinociceptive activity was observed in both phases compared to the vehicle. Results from this test suggest that the extract may be exerting its effect both through the central as well as peripheral nociceptive mechanisms. It is evident from the study that *Croton megalocarpus* exhibits significant central and peripheral antinocicetive effects in Swiss albino mice compared to the negative controls. The ED50 for early phase was found to be 71.98 (95% CI, 33.68, 110.29) and for late phase 61.72 mg / kg (95% CI 55.15, 68.29)

From the literature review, a number of the members of the croton species have been found to have traditional uses in relief of pain. Other croton species have been scientifically found to have anti nociceptive and anti-inflammatory effects. Extracts from *C. macrostachyus*, C. urucurana C. cajucara, C. crassifolius, C. guatemalensis, C. malambo and C. celtidifolius, among others have all been found to poses antinociceptive and anti-inflammatory activity in experimental animals. Essential oils from *C. cajucara, C. nepetaefolius* and *C. sonderianus* have also been found to poses antinociceptive activity. As with the other members of the croton species, croton megalocarpus was found to have significant antinociceptive effects in this experiments.

3.5. CONCLUSION

The root, bark and leaf extracts of C*roton megalocarpus* exhibits significant antinociceptive activity on animal models. It exerts this activity on both central and peripheral mechanisms of nociception. However, further studies need to be done to elucidate nature and mechanism(s) of action of the possible active substance(s) present.

3.6. RECOMMENDATIONS

Further studies need to be carried out to determine the exact mechanism of the antinociceptive activity of the extracts. The pharmacological effects of the plant should also be investigated. The ED50, should be tabulated as well as the toxicity profile. During this studies, the phytochemicals rensponsible for this nociceptive effects should also be identified.

3.6. LIMITATIONS OF STUDY

The major limitation of the study was that in the preparation of the extract, after dissolving the extract in Dimethyl sulphoxide, the resultant solution was not fully dissolving in normal saline. A better solvent may be required to dissolve the extract in later experiments.

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APPENDIX 1

Writhing Test Raw Data

Vehicle (10% DMSO in Normal Saline)

Time	Mice 1	Mice 2	Mice 3	Mice 4	Mice 5	Mice 6	Mice 7	Mice 8
5	3	3	3	2	3	2	2	4
10	6	6	6	6	6	5	5	6
15	6	5	5	8	8	7	8	7
20	5	5	5	5	4	5	6	5
25	1	1	1	2	2	3	2	1
Total	21	20	20	23	23	22	23	23

CME 50 mg / Kg

Time	Mice 1	Mice 2	Mice 3	Mice 4	Mice 5	Mice 6	Mice 7	Mice 8
5	2	0	0	0	0	0	0	0
10	2	3	0	0	0	2	1	1
15	0	1	0	0	2	2	1	1
20	0	0	1	0	2	2	0	2
25	0	0	0	0	2	0	2	2
30	0	0	0	0	0	0	1	0

Total	4	4	1	0	6	6	5	6

CME 100 mg / Kg

Time	Mice 1	Mice 2	Mice 3	Mice 4	Mice 5	Mice 6	Mice 7	Mice 8
5	0	0	0	1	0	0	0	0
10	0	0	0	0	1	0	0	0
15	0	0	2	0	2	2	0	0
20	0	0	2	0	2	2	0	0
25	0	0	0	0	2	0	0	0
30	2	0	0	0	1	2	0	0
Total	2	0	4	1	8	6	0	0

CME 200 mg / Kg

Time	Mice 1	Mice 2	Mice 3	Mice 4	Mice 5	Mice 6	Mice 7	Mice 8
5	0	0	0	0	0	0	0	0
10	0	0	0	0	0	0	0	0
15	0	1	0	0	0	0	0	0
20	0	1	0	0	0	0	0	0
25	0	0	0	0	0	1	0	0
30	0	0	1	0	0	2	0	0
Total	0	2	1	0	0	3	0	0

ASA 100 mg / Kg

Time	Mice 1	Mice 2	Mice 3	Mice 4	Mice 5	Mice 6	Mice 7	Mice 8
5	0	0	0	0	0	0	0	0
10	0	0	0	0	0	0	0	0
15	0	1	0	0	1	0	0	1
20	0	0	0	0	0	0	0	0
25	0	0	0	0	0	0	0	0
30	0	0	1	0	0	0	0	0
Total	0	1	1	0	1	0	0	1

APPENDIX 2

Tail Flick Test Raw Data

CME 50 mg / Kg

	Mice 1	Mice 2	Mice 3	Mice 4	Mice 5	Mice 6	Mice 7	Mice 8
0	5.6	3.39	7.2	5.7	7.8	5.08	7.4	6.7
30	8.14	7.85	9.84	7.62	6.8	9.65	11.1	7.23
60	5.62	8.84	7.29	8.43	9.8	10.3	11.95	7.37
90	8.37	9.65	8.8	10.39	10.08	11.6	12.88	10.23

CME 100 mg /Kg

Time	1	2	3	4	5	6	7	8
0	6.7	8	5.34	6.6	6.6	6.34	5.9	7.14

30	7.9	8.44	7.95	7.65	7.1	12.01	9.6	7.87
60	8.96	14.65	8.7	8.33	7.7	12.56	9.1	7.21
90	9.91	11.02	15.7	11.7	10.2	8.66	8.8	8.64

CME 200 mg /Kg

Time	1	2	3	4	5	6	7	8
0	6.2	5.73	5.82	6	4	5.82	6	4.95
30	8.5	6.8	10	8	9	10	12.64	12
60	12.25	6.99	8.14	9	12	10	12	12
90	13	8.2	13	11	11.8	12	12	11.6

Vehicle (10% DMSO in Normal Saline)

Time	1	2	3	4	5	6	7	8
0	5.2	5.1	5.4	4.3	6.28	5.46	6	5.26
30	5.7	5.8	5.8	4.6	6.67	5.85	6.35	5.72
60	5.47	5.6	5.4	4.2	6.72	5.9	4.32	5.4
90	5.5	5.4	6.1	4.6	6.52	5.7	5.8	5.62

Morphine (5 mg / Kg)

Time	1	2	3	4	5	6	7	8
0	6.3	6.25	7.2	4.85	6.35	7.1	6.8	5.72
30	8.76	8.23	11.75	6.23	9.4	13	13.7	8.19
60	9.34	7	14	9.18	14.4	10.92	14.5	10.63
90	13.75	17.2	13.35	12.78	13.46	13.52	16.3	12.35

APPENDIX 3

Formalin Test Raw Data

Vehicle (10% DMSO in Normal Saline)

Time	Mice 1	Mice 2	Mice 3	Mice 4	Mice 5	Mice 6	Mice 7	Mice 8
0 - 5	65.5	68	59	52	82	84	99	64
5 - 10	102.5	60	57	56	96	92	109	74
15 - 20	98	105	92.6	49.8	48	44	59	87
20 - 25	68	69	67	39.9	61	50	48	65
25 - 30	73	87	123.4	128.3	58	77	52	28

CME 50 mg / Kg

Time	Mice 1	Mice 2	Mice 3	Mice 4	Mice 5	Mice 6	Mice 7	Mice 8
0 - 5	62	54.35	59	48	65	54.64	65.7	59.3
5 - 10	44	52.12	67.43	60.5	78	59	83	59
15 - 20	60	72	63	69	55	58	45	57
20 - 25	52	71.63	48	58	39	54	73	62
25 - 30	43	45	29	30	23	33.65	29	30

CME 100 mg / Kg

Time	Mice 1	Mice 2	Mice 3	Mice 4	Mice 5	Mice 6	Mice 7	Mice 8
0 - 5	22	39	34.4	42.6	28.2	22	40.65	62

5 - 10	33	46	55	59.34	35	38	53.35	76
15 - 20	11.8	16	17.85	14.15	5.76	23.8	36	16
20 - 25	47.8	26	36	17	8.01	35	41	32
25 - 30	52	98	58.39	28	41.51	42.44	44	81

CME 200mg / Kg

Time	Mice 1	Mice 2	Mice 3	Mice 4	Mice 5	Mice 6	Mice 7	Mice 8
0 - 5	22.5	18.4	21.18	16.46	24.6	32	26	18
5 - 10	20.5	31.4	27.5	21.74	25.4	53	20	20
15 - 20	0	2	4	1.13	12	3	2	8.3
20 - 25	4	7	7	3	12	7	4.85	12
25 - 30	4	4	5	2.8	7.2	5	6.94	4

ASA 100 mg / Kg

Time	Mice 1	Mice 2	Mice 3	Mice 4	Mice 5	Mice 6	Mice 7	Mice 8
0 - 5	26	28	10	15.2	16.15	7.8	19	23
5 - 10	40	32	16.07	12.2	15	11.2	15	20
15 - 20	35	28	4.85	6	22	18	12	7
20 - 25	23	32	14.4	12	28	36	18	17.8
25 - 30	12	10	4	7	4	4	4	7

Time	Mice 1	Mice 2	Mice 3	Mice 4	Mice 5	Mice 6	Mice 7	Mice 8
0 - 5	8	16	3.2	15.16	10.1	0	2	4
5 - 10	12.9	22.4	0	2	8.1	5.23	4.45	7.25
15 - 20	1.86	2	0	3	3	6	3.35	1.8
20 - 25	2	3.78	1.33	3.8	4	8	6	2
25 - 30	0	1	1.53	0	1	2.67	1	0

APPENDIX 4

Croton megalocarpus Images