ANTINOCICEPTIVE ACTIVITIES OF EXTRACTS FROM SELECTED MEDICINAL PLANTS USING ANIMAL MODELS

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DECLARATION

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

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF APPENDICES

ABBREVIATIONS

5-HT	5-Hydroxyhyptamine (serotonin)
Ach	Acetylcholine
AMPA	Alpha-amino-hydroxy-5-methyl-4-isoxazolepropnoniac acid
ANOVA	Analysis of variance
CGRP	Calcitonin gene-related peptide
CNS	Central Nervous System
GABA	Gamma-amino butyric acid
i. p.	Intraperitoneal
IASP	International Association for the Study of Pain
mGluR	Metabotropic glutamate receptor
NK-A	Neurokinin A
NMDA	N-methyl-D-aspartate
NO	Nitric oxide
NMR	Nuclear Magnetic Resonance
NRM	Nucleus Raphe Magnus
NS	Nociceptive specific
NSAID's	Non Steroidal Anti Inflammatory Drug
PAG	Periaqueductal gray
S. E. M	Standard Error of the Mean
SI	Primary somatosensory cortex
SII	Secondary somatosensory cortex
PG	Prostaglandins
SNI	Spared nerve injury
SNL	Spinal nerve ligation

SP	Substance P
TLC	Thin Layer Chromatography
WDR	Wide Dynamic Range
WHO	World Health Organization

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ABSTRACT

This study reports the findings of the scientific evaluation of selected indigenous plants used by Kenyan communities for the management of pain and painful conditions.

Pain is associated with high morbidity and socioeconomic burden. It is the key symptom for the diagnosis of several disease conditions and is widely accepted as one of the most important determinants of quality of life.

Plants have been claimed to have analgesic or antinociceptive effects by several communities in East Africa and a great number of people use plants for management of painful conditions. Nine plants namely, *Toddalia asiatica*, *Senna singueana, Rhus natalensis, Teclea simplicifolia, Clausena anisata, Warburgia ugandensis, Sapium ellipticum, Albizia anthelmintica* and *Psiadia punctulata* were assessed for antinociceptive properties using standard nociception animal models that target thermal and chemical stimuli.

All the test mice used in the study were initially assessed for quality neurological and motor function. The selected mice were used to assess the sensorimotor effects of the nine plants at dose 200 and 100 mg / kg. *Albizia anthelmintica* and *Psiadia punctulata* induced impaired motor coordination in mice at the tested doses and therefore were disqualified for further investigations.

Assessment of the central nociception effects of the remaining seven plants using the tail flick test, confirmed significant antinociceptive effects of root extracts of *Toddalia asiatica* ($p < 0.001$), *Senna singueana* ($p < 0.05$) and *Rhus natalensis* ($p < 0.01$)at dose 200mg / kg. Only *T. asiatica* exhibited significant ($p < 0.01$) effect at 100mg / kg dose compared to the negative controls.

Further tests using the hot plate assay (thermal stimuli) and the acetic acid induced writhing test (chemical stimuli) confirmed *T. asiatica* root extract as having superior antinociceptive affects compared to the other plants. T. asiatica was also found to have antinociceptive effects in pain caused by inflammatory chemicals using the formalin test.

Isolation of compounds from the root extract of *T. asiatica* yielded seven compounds, four alkaloids and three coumarins that were further characterized by NMR. The coumarins, Isopimpinellin and 6-(3-methylbut-2enyloxy)-8-methoxy2h-chromen-2-one did not induce any significant antinociceptive activity in the tail flick assay while 6, 7-dimethoxy-5- (3-methyl-2-oxobutyl)-2H-chromen-2-one, 8-Acetonyldihydrochelerythrine and 6-(2, 3-dihydroxy-3-methylbutyl)-5,7-dimethoxy-2H-chromen-2-one) induced significant ($p < 0.05$) antinociceptive effect compared to the negative controls. The alkaloids, 8-oxochelerythrine and dihydrochlerythrine exhibited significant ($p < 0.001$) antinociceptive effects with dihydrochlerythrine showing the highest activity.

Dihydrochlerythrine was isolated from the root extract of *T. asiatica* and assayed for antinociception for the first time in this study. More antinociceptive tests need to be done to ascertain the antinociceptive activities in chronic pain models as well comparative studies with standard analgesic drugs. Safety screening of the crude extract as well as of the isolated compounds is also recommended.

This study authenticated the analgesic use of the root extracts of *T. asiatica* for the management of pain and hence supports the folklore use of the herb.

Key words: Antinociception, *Toddalia asiatica,* mice, alkaloid, dihydrochlerythrine

CHAPTER ONE

1.0 INTRODUCTION AND LITERATURE REVIEW

1.1 Pain Definition and Terms

The ability to experience pain is vital for the survival of all mammals. Pain arises as an organism is being injured and serves as a warning signal to make an escape or evasive action possible, and as a signal to avoid strain on the injured body part. Since the days of Aristotle, there have been attempts to define pain. Studies on pain started as early as 18th century but researchers of the time found it dificu1t to define the term pain. To most of them pain represented a bodily need, like hunger or nausea, with no proper stimulus (Weber, 1978). It was not until Muller, (1842) came up with the theory of specific nerve energies, which demonstrated pain spots were in the skin (Muller, 1842). Subsequent to this pain was viewed as a special sensation, served by its own apparatus. The anatomical basis for this view has been clarified and supported by subsequent studies (Price, 1999).

Pain is a universal experience and everybody knows what it meant to feel pain. The difficulty in defining the term has been largely due to the fact that pain is a physical as well as an emotional event. The earlier researchers had suggested an operational definition of pain, where criteria such as the subject's statement, a cry or other reflexes were employed to denote the presence or absence of pain (Hudspith *et al.,* 2005). It was thought that pain refers to an experience but not to behavior produced by it. This and other several unsatisfactory attempts to define pain indicated that pain is a complex physiological phenomenon which is hard to define satisfactorily in humans (Hudspith *et al.*, 2005). It is also difficult to recognize

and interpret pain in animals. Most researchers, however, agree that pain is a perception, not a physical entity and that perception of pain depends on a functioning cerebral cortex.

The International Association for the Study of Pain (IASP. 1979) has defined pain, in humans as "An unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage." This definition of pain indicates that pain can arise from two different stimuli i.e. the actual damage and the potential damage. The association between an experience of unpleasant sensation and actual or potential tissue damage has been challenged by Dubner (1999) and instead he defined pain as "a somatic perception containing a bodily sensation with qualities like those reported during tissue-damaging stimulation, an experienced threat associated with this sensation and a feeling of unpleasantness or other negative emotion based on this experienced threat." This definition does not require that an association be made between sensation and tissue damage.

In animals pain has been defined as "an aversive sensory experience caused by actual or potential injury that elicits protective motor and vegetative reactions, results in learned avoidance and may modify species behavior, including social behavior". These stimuli have different origins and potential implications for the animal (Dennis and Melzack, 1983). They are also mediated and modulated differently by the central nervous system. Tissue injury results in a powerful and persistent signal being sent to the nervous system to activate the nociceptive elements activated from the damaged region which alters the animal behavior and motivational state. This effect results in promoting behavior conducive to healing and restorative processes whereas behavior that might exacerbate the trauma is suppressed. Pain that arises from the threat of tissue damage leads to circumstances and behavioral implications that lead the animal to prevent or greatly reduce the damage by initiating some

actions (Dennis and Melzack, 1983). The time interval between the initial contact of a potentially damaging stimulus and the onset of tissue damage is crucial. The system senses the threat and reduces the damage and that sensation is perceived as pain (Dennis and Melzack, 1983).

Other pain terminology commonly used in the field of pain research includes analgesia, hypoalgesia, pain tolerance level, pain threshold, peraesthesia, hyperalgesia and allodynia. Analgesia is the absence of pain in response to a painful or noxious stimulation, while hypoalgesia is diminished pain response to normally painful stimulus. Pain tolerance is the greatest level of pain, a subject can tolerate. Hyperalgesia is an increased responsiveness to painful stimuli and is usually associated with inflammation and / or tissue injury (Merskey *et al.,* 1986). Hyperalgesia can be induced by heat, exposure to ultraviolet radiation, or injection into the skin of hyperalgesic agents such as prostaglandins, histamines, bradykinins, capsaicin etc. (Nakamura-Craig and Smith, 1989). Hyperalgesia may also occur as a result of opioid tolerance or in some cases following acute administration of opioids (Kanui and Hole, 1990: Towett and Kanui, 1993, 1995).

Cutaneous injury due to heat and mechanical stimuli elicits hyperalgesia that occurs at the site of injury and is referred to primary hyperalgesia while the hyperalgesia felt in the area surrounding the injury site is referred to as secondary hyperalgesia (Hardy *et al.,* 1950: LaMotte *et al.,* 1982, Raja *et al.,* 1984). Primary hyperalgesia due to heat stimuli is believed to be as a result of peripheral sensitization of A-delta and C nociceptors (Meyer and Campell, 1981; LaMotte *et al.,* 1982; Torebjork *et al.,* 1992). Secondary hyperalgesia is due to central sensitization of neurons in the spinal cord caused by discharges of nocicíceptors (LaMotte *et al.,* 1991). When nociceptors are stimulated they release excitatory amino acids (EAAs) and

peptides like substance P (SP), neurokinin-A, vasoactive intestinal peptide (VIP) and calcitonin gene-related peptide (CGRP) in the CNS (Gamse *et al.,* 1979: Sorkin and McAdoo, 1993). These agents have a sensitizing effect on nociceptors and can cause hyperalgesia.

Hyperalgesia can also occur in the viscera and can exist in three forms (Giamberardino, 2000). Visceral hyperalgesia can be a form of primary hyperalgesia which involves the site of injury; it can be caused by inflammation or excess stimulation of the visceral structures or it can be referred hyperalgesia whereby the pain from the viscera is referred to somatic tissues. Viscerovisceral hyperalgesia is hyperalgesia of one visceral organ that manifest clinically on another visceral organ whose segmental afferent innervations partially overlaps (Giamberardino, 2000).

The N-methyl-D-aspartate (NMDA) receptors are essential for the development of central hyperalgesia (Haley *et al.,* 1990 b; Coderre *et al.,* 1993; Woolf and Chong, 1993; Dolan *et al.,* 2000). NMDA receptor activation results in the production of a number of intracellular messengers, including nitric oxide and prostaglandins, which are also implicated in the development of hyperalgesia (Meller et *al.*. 1994; Dolan and Nolan, 1999).

Pain threshold in man is defined as the first barely perceptible pain to appear in an instructed subject under a given condition of stimulation (Beecher, 1957; Dennis and Melzack, 1983). It is usually revealed by a verbal statement and is measured in terms of the lowest intensity of stimulus that will evoke it. In animals, reflex signs of reaction to presumed pain are used to measure pain threshold. These include the more obvious signs: such as lameness or biting and scratching, an irritation site or obscure signs, such as lassitude and dysuria. Most of the pain-related responses are autonomic, somatic motor and motivational affective (Melzack and

Casey, 1968).Pain threshold can be influenced by a number of factors, including race, sex, age, circulatory change, skin temperature, trauma, anxiety and fear and diurnal variation (Beecher, 1957; Hole and Tjolsen, 1993). Analgesic agents are also capable of altering pain threshold in animals and humans. Sweating, hyperalgesia, fatigue and high partial pressure of carbon dioxide do also influence pain threshold in both humans and animals (Beecher, 1957).

1.2 General Introduction and History of Pain

Pain in human beings is a conscious perception of suffering. It is one of the foremost causes of suffering in both humans and animals. Thousands of years ago, people attributed pain to spirits and treated it with mysticism and incantations. Today, scientists understand a great deal about the causes and mechanisms of pain leading to great improvements in the diagnosis and management of a number of painful conditions.

Before the discovery of neurons and their role in pain and different body functions, there were several theories of pain proposed among the ancient Greeks: Aristotle believed that pain was due to evil spirits entering the body through injury (Melzack and Katz, 2004), and Hippocrates believed that it was due to an imbalance in vital fluids (Linton, 2005). In the 11th century, Avicenna theorized that there were a number of senses including pain, touch and titillation (Dallenbach, 1939), but prior to the scientific Renaissance in Europe pain was not well-understood, and it was thought that it originated outside the body, perhaps as a punishment from God (Meldrum, 2003).

René Descartes in1644proposed that pain was a disturbance that passed down along nerve fibers until the disturbance reached the brain (Dallenbach, 1939; Melzack and Katz, 2004), a development that transformed the perception of pain from a spiritual, mystical experience to a

physical, mechanical sensation. Descartes's work, along with Avicenna's, prefigured the 19th-century development of specificity theory which saw pain as "a specific sensation, with its own sensory apparatus independent of touch and other senses"(Melzack and Wall, 1965). Another explanation came to prominence in the 18th and 19th centuries that pain was conceived as a unique sensory modality, as well an emotional state produced by stronger than normal stimuli such as intense light, pressure or temperature (Finger, 2001). The physiologists and physicians backed the specificity theory by the 1890s, and the intensive theory was mostly backed by psychologists. However, after a series of clinical observations by Henry Head and experiments by Max von Frey, the specificity theory was accepted and most textbooks on physiology and psychology were presenting pain specificity as a fact (Dallenbach, 1939; Bonica, 1990).

In 1955, Sinclair and Weddell developed "peripheral pattern theory" which was based on an earlier suggestion by John Paul Nafe. This theory proposed that all skin fiber endings (with the exception of those innervating hair cells) are identical, and that pain is produced by intense stimulation of these fibers (Bonica, 1990). Twentieth century saw the entry of the “gate control" theory, by Ronald Melzack and Patrick Wall (Melzack and Wall, 1965). The authors proposed that both thin (pain) and large diameter (touch, pressure, vibration) nerve fibers carry information from the site of injury to two destinations in the dorsal horn of the spinal cord, and that the more the large fiber activity dominates relative to thin fiber activity at the inhibitory cell, the less pain is felt (Meldrum,2003).

The purpose of physiological pain is protection. The International Association for the Study of Pain (IASP, 1979), defines pain as “unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage”.

There are two basic types of pain, the acute and chronic pain depending on the duration. Acute pain results from disease, inflammation or injury to tissues. This type of pain generally comes on suddenly and especially after a trauma or surgery and may be accompanied by anxiety or emotional distress. The cause of acute pain can be easily diagnosed and treated. Acute pain is self-limiting and is confined to a given period of time and severity. Acute pain therefore acts as a warning sign that something is not quite right and that one could take action like withdrawal from the tissue damaging stimulus, take medication or consult a doctor (Hudspith *et al.,* 2005).

Clinically, acute pain is pain that lasts less than 30 days, chronic pain is pain of more than six months duration, and sub acute pain lasts from one to six months (Thienhaus and Cole, 2002). A popular alternative definition of *chronic pain*, involving no arbitrarily fixed durations is “pain that extends beyond the expected period of healing" (Turk and Okifuji, 2001). It causes unnecessary suffering and lowers productivity. Chronic pain presents a disease itself and environmental and psychological factors tend to worsen it and the pain becomes difficult to treat (Hudspith *et al.,* 2005).

The need to treat pain cannot be over emphasized. Analgesics and local anaesthetics were developed from compounds isolated from higher plants. Opioids are the most potent analgesics in clinical use today. Morphine was isolated from *papaver somniferum* by a German pharmacist in 1803. It works through the body's natural pain-killing mechanisms, preventing pain messages from reaching the brain (Stein *et al.,* 2009).

The willow bark was used in traditional medicine for the relief of mild pain and fever for several years. The active ingredient, salicin, is hydrolysed to salicylic acid from which

aspirin, a non-steroidal anti-inflammatory drug (NSAID) is developed. It works by inhibiting cyclo-oxygenase, an enzyme that hydrolyses arachidonic acid into endoperoxides. This is the rate limiting step in formation of pro-inflammatory mediators especially prostaglandins (Mattison *et al.,* 1998).

The available analgesic drugs in the market are often associated with several adverse effects and are either too potent or too weak. Opiods are known to cause side effects which includes; sedation, respiratory depression, potential for addiction and tolerance whereas NSAIDS are known to cause gastric irritation that may lead to gastric bleeding. There is need for new analgesic compounds (Mattison *et al.,* 1998).

Traditional medicines provide fertile ground for modern drug development, but first they must pass along a pathway of discovery, isolation, and mechanism of action studies before clinical trials. They continue to provide front-line pharmacotherapy for many millions of people worldwide. Although their application is often viewed with skepticism by the western medical establishment, they are a rich source of therapeutic leads for future drug development.

Plants of medicinal value in ethnopharmacology are an important source of natural products with potential therapeutic effects (Blumenthal, 2000; Bisset, 2001). Study of plant species that are used in traditional herbal medicine as pain killers therefore form a logical search strategy for new analgesic drugs (Farnsworth, 1989; Mattison *et al.,* 1998). Compounds derived from medicinal extracts are appealing for several reasons; they are often stereo chemically complex, multi- or macro cyclic molecules with limited likelihood of prior chemical synthesis, and they tend to have interesting biological properties. But perhaps most

importantly, parent extracts have been "clinically" tested in their traditional milieu, in some cases over millennia (Schmidt *et al.*, 2007)

There have been claims on the effectiveness of herbal medicines for treatment of painful conditions in East Africa Kenya (Minja, 1999; Kokwaro, 2002). There is no scientific research information available from literature search concerning the antinociceptive activities of most of the plants claimed to have analgesic activities.

Scientific validation of the claims involves several steps which must be executed. One of the most popular and important procedure for drug discovery is bio-guided fractionation of the extract (Pieters and Vlietinck, 2005). This procedure involves fractionation of active extract and fractions until pure active ingredient are obtained. In this project the plant with the highest antinociceptive activity was fractionated for the pure active ingredient.

1.3 Pain Mechanisms

1.3.1 Nociception

While pain is defined as a subjective experience of noxious stimuli, the physiological and pharmacological activities that lead to a painful sensation are denominated nociception.

The nociceptive pathway can be described as a three- neuron chain that transmits nociceptive information from the periphery to the cerebral cortex (Besson and Chaouch, 1987; Almeida et al., 2004). The *first order neurons* have hair cell bodies in the dorsal root ganglion from where two axons project, one to the peripheral tissues and the other one to the dorsal horn of the spinal cord. The *second order neurons* originate from the spinal cord and ascend to the

thalamus or the regions of the brainstem. From the thalamus the *third order neurons* project to the cerebrocortex (Besson and Chaouch, 1987; Abelson, 2005).

1.3.2 Peripheral Pain Mechanisms

Lewis (1942) described two types of pain, superficial and deep pain. Intense stimulation of the skin resulted in superficial pain which can also be well localized (Besson and Chaouch, 1987). Deep pain that arises from tendons, skeletal muscles, periosteum and joints is poorly localized. It is well established that a single nociceptive stimulus of short duration, no matter the nature of the stimulus, mechanical, thermal or electrical applied to the skin, gives rise to a double sensation of pain, usually the second pain is more diffuse and of a burning type. It was proposed that first and second pain is produced by activation of Aδ and C fibers respectively (Price and Mayer, 1974; Besson and Chaouch, 1987).

Skin, muscle or visceral nociceptors are seen to terminate as free nerve endings. There is no obvious structural distinction between the endings of various nociceptors. The naked nerve endings can be directly activated by strong mechanical, thermal or chemical stimulation (Giordano, 2005). They can also be activated after being sensitized during tissue injury, inflammation, ischemia or low pH (Riedel and Neeck, 2001). The sensitization is mediated by second messenger systems resulting in production and release of prostaglandins, bradykinin, serotonin and histamines in the injured area. Receptors for these chemicals are present on the surface of most nociceptive afferents together with receptor for opiates, γ-amino butyric acid (GABA) and capsaicin (Willis and Westlund, 1997; Giordano, 2005).

The nociceptors are associated with first order neurons, the Aδ and C fibres. The aδ fibres are myelinated with a diameter of 2-6 μm and a conduction velocity of 30-100m/sec. The c fibers are small unmyelinated and thereby thinner with a diameter of 0.4-1.2 μm and a conduction velocity of 12-30m/sec; (Besson and Chaouch, 1987; Almeida *et al.,* 2004). Stimulation of

cutaneuos Aδ –fibres result in pricking pain, while activation of c fibres in the skin will give rise to a dull, burning pain sensation (Besson and Chaouch, 1987). Both fibres are present in the muscular and articular tissue often referred to as type III or IV (Willis and Westlund, 1997). In the muscle noxious stimulation gives rise to an aching and less localized pain, irrespective of the fibre type. In joints, silent nociceptors are common. These are not activated under normal condition but are sensitized during inflammation and then respond to noxious stimulation. Both Aδ and c fibres have been described in viscera but how visceral nociception arises is yet to be discovered (Besson and Chaouch, 1987; Mense, 1993; Willis and Westlund, 1997; Almeida *et al.,* 2004; Giordano, 2005).

Peripheral Sensitization / Inflammation

Thermal, mechanical, and chemical stimuli activate high-threshold nociceptors that signal information to the first relay in the spinal cord. Signal transduction mechanisms include the vanilloid receptor VR1 (now TRPV1), a nonselective cation channel activated by both noxious heat, and capsaicin, the active constituent of chili peppers; acid-sensing receptors (ASIC) respond to the low pH associated with ischemia and inflammation with increased Na+ conductance; similar but as yet uncharacterized receptors are proposed to transduce noxious mechanical stimuli (Giordano, 2005).

Pain arising from direct activation or sensitization of primary afferent neurons, especially c fiber polymodal nociceptors is a dynamic process. Nociceptor activation sets in train processes that modify responses to further stimuli; for example, a relatively benign noxious stimulus such as a scratch to the skin initiates peripheral inflammation that reduces the threshold for response of the nociceptor to subsequent sensory stimuli (Giamberardino, 2000). It is essential to appreciate that surgical or traumatic noxious stimuli are usually

prolonged and associated with tissue damage of variable degrees. Clinical pain is therefore almost universally associated with peripheral sensitization (Cao *et al.,* 1998; Siddall and Cousins, 1998; Giordano, 2005).

Part of the inflammatory response is the release of intracellular contents from damaged cells and inflammatory cells such as macrophages, lymphocytes, and mast cells. Nociceptive stimulation also results in a neurogenic inflammatory response, with the release of compounds such as substance P, neurokinin A, and CGRP from the peripheral terminals of nociceptive afferent fibers. These peptides modify the excitability of sensory and sympathetic nerve fibers, induce vasodilatation and extravasation of plasma proteins, and promote the release of further chemical mediators by inflammatory cells (Siddall and Cousins, 1998). These interactions result in a 'soup' of inflammatory mediators, including K^+ and H^+, serotonin, bradykinin, substance P, histamine, cytokines, nitric oxide, and products from the cyclo-oxygenase and lipoxygenase pathways of arachidonic acid metabolism (Hudspith *et al.,* 2005). These chemicals then act to sensitize high-threshold nociceptors and produce the phenomenon of peripheral sensitization. Following sensitization, low-intensity mechanical stimuli that would not normally cause pain are now perceived as painful (Siddall and Cousins, 1998). There is also an increased responsiveness to thermal stimuli at the site of injury. This zone of 'primary hyperalgesia' surrounding the site of injury is a consequence of peripheral changes and is commonly observed following surgery and other forms of trauma (Giordano, 2005).

Peripheral sensitization may include the sympathetic nervous system, and there is evidence that sympathetic nerve terminals may themselves release prostanoids and products of arachidonic acid metabolism after peripheral injury. This provides a potential link between the peripheral sympathetic efferent and the peripheral nociceptor in complex regional pain

syndrome (CRPS), where pain complaints may vary with sympathetic efferent activity (Bach *et al.,* 1988; Siddall and Cousins, 1998).

Role of nerve growth factor in peripheral sensitization

There is a central role for nerve growth factor (NGF) in the etiology of inflammatory pain. Nerve growth factor belongs to the family of neurotropic peptides, including brain-derived neurotropic factor (BDNF), and neurotropins 3, 4, 5, and 6, which specify the phenotypic development of central and peripheral neurons. Neurotropins interact with a low affinity p75 receptor, which may modulate the expression and function of specific high-affinity tyrosine kinase (Trk) receptors for each neurotropin. The biologic effects of NGF are mediated via the TrkA receptor. The TrkA receptor is expressed on small unmyelinated nociceptive afferents that coexpress the peptide CGRP and innervate a wide variety of peripheral tissues (Fields and Bausbaum, 1999). Within these tissues there is constitutive expression of NGF at low levels by cell types such as fibroblasts, keratinocytes, immune cells, and Schwann cells. This constitutive production of NGF may determine nociceptor phenotype: the 'neurotropic hypothesis' (Siddall and Cousins, 1998). Inflammation via the cytokines IL-1β and TNF-αis associated with increased NGF expression and has been demonstrated in both animal models of inflammation and human disease, including arthritis and cystitis. The rapid onset of hyperalgesia following experimental subcutaneous administration of NGF strongly suggests a direct peripheral action mediating peripheral sensitization. Tyrosine kinase A receptors are not restricted to nociceptive afferents but are expressed by both mast cells and postganglionic efferents (Giordano, 2005). Nerve growth factor plays a central role in peripheral sensitization mediated by both direct and indirect actions of inflammatory mediators on nociceptive afferents. Furthermore, growth factors may mediate upregulation of various types

of Na^+ channel that are more likely to fire spontaneously, but also are more sensitive to Na^+ channel blockers such as lidocaine (Al-Chaer and Traub, 2002). The development of such Na^+ channels may contribute to the features of spontaneous pain and extreme mechanosensitivity seen in many pain states. Axonal transport of NGF taken up by nerve terminals has tropic effects within the spinal cord dorsal horn, contributing to central sensitization (Siddall and Cousins, 1998; Fields and Bausbaum, 1999).

Silent nociceptors

Silent or 'sleeping' nociceptors are inactive under most circumstances but become active following inflammation and sensitization by NGF. They have been identified in joint capsules and the walls of viscera. Following sensitization they become responsive and discharge vigorously, even during ordinary movement or visceral distension within the physiological range; they also display changes in receptive fields (Fields and Bausbaum, 1999). This class of nociceptor may contribute to the mechanical allodynia and hyperalgesia associated with peripheral inflammation in arthritis and visceral pain states, such as cystitis or inflammatory bowel disease (Willis and Coggeshall, 1978; Al-Chaer and Traub, 2002).

1.3.3 Neurotransmitters and Neuromodulators

The dorsal horn contains a host of peptide and amino acid neurotransmitters, neuromodulators, and their respective receptors. Neurotransmission within the dorsal horn encompasses; Excitatory transmitters released from the central terminals of primary afferent nociceptors (Hudspith *et al.,* 2005), Excitatorytransmission between neurons of the spinal cord (Hudspith *et al.,* 2005), Inhibitory transmitters released by interneurons within the spinal cord, Inhibitory transmitters released from supraspinal sources. The concept of a single neuron releasing a single transmitter within the synaptic cleft clearly does not apply to the

dorsal horn. Although exocytotic release of individual peptide or amino acid transmitters may occur, experimental data suggest that this rarely happens under physiological conditions, and two or more compounds are commonly released at the same time. Differing ratios of cotransmitter release may occur, depending on the intensity of the stimulus (Besson and Chouch, 1987; Hudspith *et al.,* 2005). Neurotransmitters may be released in close proximity to pre- or postsynaptic receptors in the dorsal horn; however, it is clear that 'volume transmission' also occurs within the dorsal horn, where spatially distant receptors may be activated by transmitters outside a classic synapse (Siddall and Cousins, 1998; Hudspith *et al.,* 2005).

I. Excitatory amino acids

Glutamate is the main CNS neurotransmitter and plays a major role in nociceptive transmission in the dorsal horn. Glutamate acts at α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors, N-methyl-D-aspartate (NMDA) receptors,kainate (KA), and metabotropic glutamate receptors (Meller *et al.,* 1994; Dalan *et al.,* 2000).

AMPA receptors are ligand-operated ion channels; the channel is not voltage dependent and permits the selective entry of Na^+ under physiologic conditions. The result is a short-latency excitatory postsynaptic potential (EPSP), and AMPA receptors are responsible for 'fast' transmission of impulses in nociceptive and nonnociceptive pathways. Such information may encode the onset, offset, and intensity of a noxious stimulus. AMPA receptors are not selectively localized to regions of the nervous system involved in nociception, and antagonists at the AMPA receptor may therefore have limited use as analgesics because of their widespread presence and function in the CNS (Hudspith *et al.,* 2005). AMPA receptors may mediate responses in the 'physiologic' processing of sensory information. However, prolonged release of glutamate or concurrent activation of neurokinin receptors results in

sustained activation of AMPA and / or neurokinin receptors. This appears to be crucial in the development of abnormal responses to further sensory stimuli by priming the NMDA receptor so that it reaches a state ready for activation (Siddall and Cousins, 1998; Hudspith *et al.,* 2005).

The NMDA receptor complex is a multimeric channel permeable to Na^+ and Ca^{2+} that is both voltage and ligand gated. At a normal resting potential (–70 mV), Mg^{2+} blocks the ionophore of the NMDA receptor, and binding of glutamate in the presence of its coagonist glycine does not result in channel opening. Priming of the NMDA receptor occurs with depolarization of the membrane to –30 mV, which enables Mg^{2+} to leave the channel. This degree of depolarization occurs when glutamate and peptides are co-released after intense afferent activation and act on AMPA and neurokinin receptors in the dorsal horn. Activation of the NMDA receptor causes large and prolonged depolarization associated with Ca^{2+} mobilization in neurons that are already partly depolarized. Activation of NMDA receptors at pre- and postsynaptic loci initiates processes that contribute to the medium- or long-term changes observed in chronic pain states, including central sensitization, changes in peripheral receptive fields, induction of gene transcription, and long-term potentiation (LTP) (Siddall and Cousins, 1998). The last refers to the changes in synaptic efficacy identified as a synaptic correlate of memory in the hippocampus and cerebral cortex, and may play a role in the development of a cellular 'memory' for pain or enhanced responsiveness to noxious inputs (Siddall and Cousins, 1998; Hudspith *et al.,* 2005).

Metabotropic glutamate receptors comprise three groups (I–III) and at least eight subtypes, mGluR1–mGluR8. Group I are coupled to G proteins linked to phosphoinositide hydrolysis and protein kinase C activation, whereas groups II and III couple negatively to adenylyl cyclase and cAMP signaling pathways through Gi/Go proteins. Their role in nociception is

currently incompletely defined and they do not appear to be involved in acute 'physiologic' pain (Giordano, 2005). However, there is now compelling evidence that spinal group I mGluRs play a modulatory role in nociceptive processing, central sensitization, and pain behavior. The role of group II and III mGluR is less clear (Giordano, 2005; Hudspith *et al.,* 2005).

Peptides

Small-diameter nociceptive primary afferent fibers are characterized by a variety of peptide transmitters, including substance P, neurokinin A, and CGRP. The release of substance P, which coexists in primary afferents with glutamate, occurs following cutaneous thermal, mechanical, or chemical noxious stimuli and is potentiated by peripheral inflammation. Although historically substance P was considered the major neurotransmitter involved in spinal mechanisms of nociception, experimental data from animals lacking the substance P receptor (NK-1 receptor 'knockout' mice) demonstrate that acute nociception persists in animals lacking substance P-mediated neurotransmission. Substance P has been shown to play a modulatory role in nociception, modifying the gain in afferent transmission. Animal data demonstrate that substance P may play an important role in the transmission of prolonged or highly noxious stimuli. The actions of substance P may be potentiated by neurokinin A and CGRP within the dorsal horn, although the role of these peptides is less well understood (Siddall and Cousins, 1998; Hudspith *et al.,* 2005). Disruption of the preprotachykinin A gene, which encodes for substance P and neurokinin A, significantly reduces the response to moderate-to-intense pain and abolishes neurogenic inflammation without affecting responses to mild pain. The release of these tachykinins from primary afferent nociceptors is therefore required to produce intense pain (Giordano, 2005; Hudspith *et al.,* 2005).

1.3.4 Pain Modulation at the Spinal Cord Level

- **The gate theory**

The transmission of nociceptive information is subject to modulation at all levels of the neuraxis, from the dorsal horn rostrally. Afferent impulses arriving in the dorsal horn initiate inhibitory mechanisms that limit the effect of subsequent impulses. Inhibition occurs through local inhibitory interneurons and descending pathways from the brain. A model of how this interaction occurs in relation to pain processing was proposed by Melzack and Wall in 1965, and has been termed the 'gate theory' of pain (Besson and Chaouch, 1987; Giordano, 2005; Hudspith *et al.,* 2005).

Gate theory proposes that transmission or T cells located in the dorsal horn project to the brain; that the output from these cells depends on information entering the dorsal horn in different types of primary afferents; and that such cell could be activated by noxious input from small-diameter primary afferents and by nonnoxious information in large-diameter primary afferents. The output from transmission cells is regulated or modulated by inhibitory cells in the substantia gelatinosa, which also receive information from the primary afferents, but the effect on the inhibitory cell is dependent on whether it is non-noxious information in large-diameter afferents or noxious information in small-diameter afferents. Nonnoxious input along large-diameter afferents primarily activates inhibitory cells, and therefore reduces output from transmission neurons. Noxious input along small-diameter afferents primarily inhibits the inhibitory cells, and therefore increases the output from transmission cells. Thus, the output from transmission cells to the brain is determined by the relative balance of activity in small and large diameter fiber afferents arriving at the dorsal horn. A further level of modulation in the gate theory is that descending pathways from the brain can also act to

inhibit transmission of information by transmission cells ((Besson and Chaouch, 1987; Giordano, 2005; Hudspith *et al.,* 2005).

The gate theory has had a significant impact on concepts of pain and has helped to explain why pain may occur in some conditions and why some treatments such as transcutaneous nerve stimulation and dorsal column stimulation may be effective. However, it has been difficult experimentally to demonstrate some of the specific circuitry suggested in the original proposal. Although descending inhibitory controls have been demonstrated, most cells in the spinal cord respond to noxious and nonnoxious stimuli and do not fit the proposed characteristics of transmission cells. Clinically, selective large-fiber loss often results in contradiction to that predicted by the gate theory. The theory also fails to explain why some people have pain after complete loss of afferent input, as occurs, for example, following complete spinal cord transection. Although it is an important and helpful advance in our understanding of pain, the gate theory does not completely resolve the specific mechanisms responsible for pain processing (Hudspith *et al.,* 2005).

Both GABAergic and glycinergic interneurons are involved in tonic inhibition of nociceptive input. Down regulation or loss of these neurons can result in features of neuropathic pain, such as allodynia. Although both $GABA_A$ and $GABA_B$ receptors have been implicated at both pre- and postsynaptic sites, $GABA_A$ receptor-mediated inhibition occurs through largely postsynaptic mechanisms. In contrast, $GABA_B$ mechanisms may be preferentially involved in presynaptic inhibition by suppressing excitatory amino acid release from primary afferent terminals (Hudspith *et al.,* 2005).

1.3.5 Ascending Systems that Transmit Information from Nociceptors

Ascending tracts

The pain impulses entering the spinal cord are conveyed after synaptic relay in the dorsal horn by the second order neurons to the supraspinal tract structures (Hudspith *et al.,* 2005), the spinal cervical and the spinal reticular tracts (Price and Mayer, 1974; Hudspith *et al.,* 2005). They have their cell bodies in the dorsal horn and their axons termination in the brain. The neurons are mainly two types; wide dynamic range (WDR) which responds to non-noxious and noxious stimuli, and the nociceptive specific (NS) which respond solely to noxious stimuli. The second order neurons reach the brain via several afferent pathways (Cross, 1994; Almeida *et al.,* 2004).

The spinal thalamic tract ascends in the anterolateral quandrant of the spinal cord and terminates in the contralateral thalamus via two projections. The lateral projection with axons originating in laminae I and IV and terminate in the ventral posterior lateral nucleus and ventral posterior inferior part of the lateral thalamus. In the medial projections the axons originate in the deeper parts of the dorsal horn and the ventral horn and they terminate in the central lateral locus. The thalamus is considered the most important relay for reception and processing of nociceptive information at the supraspinal level. The lateral part of the thalamus is thought to be involved in the sensory –discriminative component of pain, while the medial part is involved in the motivational –affective aspects of pain (Cross, 1994; Willis and Westlund, 1997; Almeida *et al.,* 2004; Hudspith *et al.,* 2005).Electron microscopy studies and degeneration studies of the spinothalamic tract have revealed that the ascending axons appear to be myelinated and only very few unmyelinated fibres have been identified (Hudspith *et al.,* 2005).

The fibers forming the tract cross shortly after their origin and the crossing is completed within the limit of the segment above the entrance of the dorsal root fibre (Kandel and Schwartz, 2005). Ascending fibers in the spinothalamic tract are arranged in a segment and are added superficially to those coming from caudal segments. This somato-topical arrangement is retained during their course through their medulla and pons. The termination sites in the thalamus for the tracts include the ventrobasal complex, the posterior nucleus group, the intralaminar nucleus and nucleus centralis lateralis (Kandel and Schwartz, 2005).

Some spinothalamic cells respond only to noxious stimuli and these are located in LI (Price and Mayer, 1974; 1975) although high threshold driven cells are also found deeper in the dorsal horn. Other spinothalamic cells can be activated by low threshold stimulation and also by noxious stimulation (Price and Mayer, 1974; Applebaum *et al.,* 1975). Spinothalamic tract is not the sole tract involved in pain transmission, Cadwalader and Sweet, (1912) reported that dogs whose ventrolateral tracts have been sectioned respond slowly to pain and extreme heat, selective transaction of spinothalamic pathway results in temporary interruption of pain impulses to the brain from levels below the transaction. This operation has been performed in several chronic pain causes. The draw back is that although the pain disappears immediately post operation, it usually resumes within months and may worsen. The recurrence of the pain shows that other pain pathways exist (Willis and Coggeshall, 1978; Kandel and Schwartz, 2005).

The spinomesencephalic tracts project to different areas in the midbrain. The axons originate from the laminae I and IV –VI and some have their origin in laminae X or the ventral horn. They terminate in the periaquiductal gray (PAG), nucleus cunieformis, intercolliculus nucleus, deep layers of the superior colliculus and the anterior and posterior pretectal nuclei. The different components of the tract have different functions. The PAG projection

contributes to the aversive behavior as well as to the activation of descending pain modulation. The deep layers of superior colliculus are likely of importance for orientation (Willis and Westlund, 1997; Almeida *et al.,* 2004).

The spinoreticular tract originates from the laminae VI of the dorsal horn and VII of the ventral horn ascends through the anterolateral quadrant and terminates in reticular formation of the brain stem. One part of the tract terminates in several nuclei in the pons and medulla such as in the nucleus gigantocellaris and nucleus reticularis pontis caudalis and oralis, nucleus paragigantocelllaris, and nucleus subcoeruleus. There is also major termination to the parabrachial region including the locus coeruleus and the parabrachial nuclei (Cross, 1994; Willis and Westlund, 1997). The spinal reticular tract is important for the motivational – affective aspects of pain, and for the descending modulatory mechanisms (Millan, 1999; Almeida *et al.,* 2004).

Several other nociceptive pathways have also been described, the spinolimbic tract which consists of the spinoreticulothalamic, the spinoamygdalar and the spinohypothalamic pathways. Pathways in the dorsal quadrant such as the spinocervicothalamic pathway and the postsynaptic dorsal column pathway, are also present (Willis and Westlund, 1997; Millan, 1999; Almeida *et al.,* 2004).

Cortical structures

The nociceptive information is transmitted from the thalamus to the cerebral cortex by third order neurons. Depending on their origin the neurons terminate in different parts of the cortex. Neurons from the lateral thalamic nuclei project to the primary somatosensory cortex (SI), where a conscious localization and characterization of the pain occurs. Neurons from the medial nuclei are projected to the anterior cingulate gyrus, which is involved in the perception of suffering (Cross, 1994; Riedel and Neeck, 2001; Casey, 2004; Abelson, 2005).

1.4 Pain Modulation

1.4.1 Antinociception

The ability to experience pain is vital and essential for the survival of all mammals, it is also essential for the organism to be able to control and modulate the pain sensation. Pain control is termed as *antinociception.* The transmission of noceptive information is rigorously controlled and modulated at most levels in the central nervous system. The modulation is hierarchically organized in the descending pain modulatory system.

Projections from the frontal and insular cortex, the thalamus, the amygdala and hypothalamus to the PAG in the mesenchephalon are mainly opioid neurons. The PAG plays an essential role in the modulation of nociceptive information at the supraspinal level, as it serves as a relay station for transmission of antinociceptive information to the lower brainstem. It is likely that antinociception can be from all regions of the PAG. However, the ventral portion (dorsal raphe nucleus) is the most effective (Harris, 1996; Siddall and Cousins, 1998).

From the PAG, neurons project to various areas in the reticular formation of the medulla. This includes the medullary nucleus, nucleus raphe magnus, reticularis magnocellularis, nucleus paragigantocellularis and noradrenergic medullary cell groups. From the PAG to the reticular formation the interneurons are GABAergic. In the reticular areas, the antinocieption is again relayed and neurons project directly or via interneurons to the dorsal horn of the spinal cord. The projections to the spinal cord dorsal horn consist of serotonergic and noradrenergic neurons. The spinal cord dorsal horn appears to be the level where the strongest antinociception occurs (Harris, 1996; Millan, 1999; Riedel and Neeck, 2001).

1.4.2 Descending Antinociceptive Systems Acting on the Dorsal Horn

Inputs from neurons located at various sites in the brain are also received at the dorsal horn. The descending system include corticospinal, the raphe-spinal and the reticulo-spinal systems (Giordano, 2005; Hudspith *et al.,* 2005). The corticospinal tract cells terminate in the LIII-LVI or even in the LVII in the Cat and are absent in LI and LII. The origin of corticospinal tract was demonstrated to be from cytoarchitectonic regional 4, 3a, 3b, 1, 2, and5 (Wall, 1967). Influence of the corticospinal pathway upon dorsal horn interneurones has no effect on LIV neurons, prominent inhibition in LV and excitation in LVI (Wall, 1967). Fetz (1968) reported more inhibition dorsally and excitation ventrally. Reports of inhibition by both Wall (1967) and Fertz (1968) are in agreement with earlier reports of afferent depolarization (Anderson *et al.,* 1964), indicating the operation of pre-synaptic inhibition of cutaneous and group 1b and II muscle afferents and inhibition and excitation of various dorsal horn neurons including those giving rise to ascending pathways .

The raphe spinal system arise from midline raphe nuclei of the brain stem and consists of the dorsal lateral funiculi and terminate in LI,LII LV and medial parts of LVI and VII. The parts of the dorsal horn which receive inputs from the raphe nuclei are those parts concerned with nociceptors and which give rise to spinothalamic and spinoreticular tracts (Basbaum and Fields, 1977).

Noradrenaline and serotonin containing neurones have been demonstrated in the raphe spinal fibres and administration of serotonin on the dorsal horn neurons inhibit both spontaneous and evoked activities of the neurons. Electrical stimulation of the nucleus raphe magnus leads to primary afferent depolarization indicating presynaptic inhibition of the dorsal horn neurone in L1, Vand V1 that receive noxious mechanical input (Willis and Coggeshall, 1978).

The reticulo-spinal system is related to the motor system and Basbaum and Fields (Basbaum and Fields, 1977) showed by means of autoradiography that fibers from nucleus reticularis magnocellularis descend in the ipsilateral dorsolateral part of the spinal cord and terminate in LI, II, V, and VII in the dorsal horn. Using radioactive leucine, nucleus reticularis gigantocelularis was shown to terminate ipsilaterally in LVII and LVIII and contralaterally in LII in the ventral horn. These parts are related to the motor system, thus even pathways that are generally regarded as part of the motor system produce action at the spinal cord level that has consequences for sensations (Basbaum and Fields, 1977).

There are also other descending systems capable of influencing the dorsal horn directly or indirectly. Stimulation of the vestibular nerve has been shown to excite interneurones in both dorsal and ventral horn and this could be mediated by way of either vestibulospinal or reticulospinal tracts (Willis and Coggeshall, 1978).

1.4.3 Neurotransmitters and Neuromodulators in the Descending System

- **Gamma –amino-butyric acid**

In the spinal cord dorsal horn, nociception can be inhibited in various ways. γ-amino- butyric acid (GABA) is a major inhibitory transmitter. Binding sites for GABA and GABA containing neurons have been localized in almost all structures in the spinal cord, including interneurons and synaptic terminals. The highest concentration of GABA is the dorsal horn, especially laminae I-III. There are two main GABA receptor subtypes: the ligand gated chloride channel $GABA_A$, and the GTP-binding protein coupled receptor $GABA_B$. Both types are important in antinociception at the spinal level. Action of the GABAergic interneurons reduces the release of glutamate, SP and CGRP from the primary nociceptive afferents (Furst, 1999; Abelson, 2005).

- **Nitric oxide**

NO is known to be involved in transmission of nociceptive information but there is also evidence of its involvement in antinociception. It has been shown to contribute to the antinociceptive actions of morphine as well as of adrenergic and cholinergic agonists at both spinal and supraspinal levels (Xu *et al.,* 1997).

- **Serotonin**

Studies using florescence histochemistry have shown serotonin (5-HT) containing cell bodies and neuronal projections to be associated with brain stem raphe nuclei (Besson and Chaouch, 1987).In the dorsal horn the 5-HT is increased by stimulation of the nucleus raphe magnus (NRM) and ventromedial medulla (VMM). Behavioral studies indicate that the analgesic effects of VMM stimulation are reduced by intrathecal injection of 5-HT antagonist. Depletion of 5-HT by P-chlorophenylalanine reduced stimulation induced analgesia and administration of 5-HT precursor, 5-hydroxytrytophan, restored the effect. Thus descending 5-HT neurons may be involved in the tonic regulation of nociception and a tonic inhibitory influence mediated by descending 5-HT has been suggested (Besson and Chaouch, 1987).

The site of action of 5-HT is unclear. Descending 5-HT containing nerve terminal synapse largely with dorsal horn neurons forming few, if any, direct contacts with sensory terminals (Besson and Chaouch, 1987). It is at these synapses where endogenous serotonin is released to inhibit nociceptive transmission. Serotonin receptor classes 5HT1, 5HT2, 5HT3 and especially 5HT1 and 5HT3 have been shown to play a role in antinociception although some sub types seem to facilitate nociceptive transmission (Furst, 1999).

- **Noradrenergic and catecholaminergic system**

Noradrenergic (NA) pathways participate in spinal modulation of nociceptive information at the spinal cord level. Activity following stimulation of the brain stem is reversed to some extent by intrathecal administration of noradrenalin antagonists. The analgesics effects of intrathecal injection of NA are mediated by activation of adreno-receptors. The inhibitory effects have been demonstrated both in the superficial and deep laminae of the dorsal horn and are generally more marked and more selective than those of 5-HT. The descending noradrenergic system arises mostly from the locus coeruleus, the subcoeruleus and the A5 group of the cerebral cortex and terminates in the marginal layer, LII, IV, VI and the ventral horn. Their effects on spinal cord nociceptive transmission could involve pre and post synaptic mechanism (Besson and Chaouch, 1987).

The adrenergic receptors involved in antinociception are generally α2-adrenergic receptors, which consists of three subtypes α2a, α2b, and α2c. Stimulation of α2- receptor results in a very potent antinociception, as seen after intrathecal administration of α2-adrenergic agonists (Saunders and Limbird, 1999).

Lesions of descending catecholaminergic pathways have been reported to alter responses to noxious stimuli in the hot plate and formalin tests. These pathways tonically inhibit nociceptive sensitivity recorded with hot plate test, but tonically enhanced the behavioral responses to pain induced by formalin. This indicates that mechanisms involved in spinal modulation of nociception, by catecholaminergic system may be different for different types of pain. There is tonic regulation of nociceptive sensitivity by spinal catecholaminergic pathways (Fasmer *et al.,* 1986).

- **Acetylcholinergic system**

Several studies have investigated the role of acetylcholine system in antinociception both at the spinal cord level and also at the supraspinal level (Hudspith *et al.,* 2005). Systemic as well as intrathecal administration of muscarinic agonists produces potent antinociception in several species. Different pharmacological studies have attempted to determine the spinal muscarinic subtypes relevant for the antinociceptive effect suggesting the involvement of the M1, M2, M3, and M4 subtypes (Abelson, 2005). Davies et al. (1932) reported the antinociception of nicotine. This has been supported by other studies (Iwamoto, 1991; Sahley and Berston, 1979). Neuronal nicotinic receptors are a promising target in pain treatment. Nicotinic agonists administered into the spinal cord produces nociceptive as well as antinociceptive effects (Abelson, 2005).

The acetylcholine receptor system has been shown to interact with most other receptor systems in the spinal cord. Muscarinic receptors have been shown to be involved in spinal antinociceptive mechanisms mediated by the opioid GABAergic and adrenergic receptor systems (Hudspith *et al.,* 2005). Nicotinic receptors are also involved in modulation of nociceptive information by the serotonergic andadrenergic systems and the antinociceptive mechanism of nitrous oxide in the spinal cord level. The mechanism of action is still unknown. One conceivable explanation could be that stimulation of the acetylcholine system releases acetylcholine in the spinal cord which in turn inhibits the nociceptive system. In several studies, the intrathecal administration of neostigmine revealed that part of the antinociceptive effect of this substance, both in man and animals, is mediated at the spinal cord level (Abelson, 2005). Since neostigmine prevents the biodegradation of acetylcholine in the synaptic cleft, the amount of acetylcholine is increased strengthening the theory that endogenous acetylcholine contribute to the inhibition of nociceptive information at the spinal

cord. Cholinergic agonists applied at the spinal level can reduce the release of substance P and glutamate a plausible explanation is that both muscarinic and nicotinic receptor are present on the first order and second order neurons. When the acetylcholine release increases, acetylcholine binds to the receptors of the nociceptive neurons followed by a reduction in the activity of these cells. Muscarinic M2 and M4 subtypes are known G-coupled receptors that reduce the nerve activity when stimulated. Nicotinic receptors can also reduce the cellular activity when stimulated depending on the subtype. Other explanation could be that the released acetylcholine binds to receptors present on other interneurons that in turn release the inhibitory transmitters or further stimulate descending neurons, which eventually lead to a decreased nociceptive transmission (Abelson, 2005).

- **Opioidergic system**

Endogenous opioids are involved in antinociceptive mechanisms both at the spinal cord and suprasinal regions (Hudspith *et al.,* 2005). From the original in vitro observations, a model was proposed whereby enkephalinergic interneurones found locally in region of primary afferent synapses provided axo-axonic terminals on SP –containing primary afferents and provided an inhibitory system for selective blockade of nociceptive information by opioids. The circulating opioids cross into the spinal cord selectively depressing SP release (Hudspith *et al.,* 2005).

Enkephalins have been demonstrated in the periaqueductal gray matter (PAG) and nucleus raphe magnus (NRM). β endorphin is linked with major neuronal system in the brain originally in the arcuate nucleus of the hypothalamus and both enkaphalins and β endorphins are present in supraspinal sites allied to nociception. Injection of β endorphin or enkephalin analogue leads to profound antinociception. Regions around the PAG are among the most sensitive of all sites to elicit antinociceptive effects, suggesting the participation of opioid

peptides in the descending spinal inhibitory system (Besson and Chaouch, 1987; Hudspith *et al.,* 2005).

Opioid receptors are important in supraspinal as well as spinal antinociceptive mechanisms. The three major receptor types are μ-, κ- and δ- receptors. The μ- is generally considered the most essential in antinociceptive mechanisms, but the κ- and δ- receptors have also been shown to mediate antinociception. The fourth type ε- receptor is thought to mediate β-endorphin –induced analgesia, but the existence of such a receptor is still controversial. The endogenous ligands for opioid receptors can be divided into three different families of opioid peptides; endorphins, enkephalins and dynorphins (Furst, 1999). Opioids can exert their antinociceptive activity through various mechanisms. Activation of opioid receptors can inhibit Ca^{2+} channels specifically on primary afferent C-fibres and thereby inhibit their spinal activity. Opioid receptors are also present on interneurons and cell bodies of second -order neurons, where the nociceptive information can be blocked. The opioid system also interacts with NMDA receptors which might contribute to the antinociceptive actions of opioids but also to the development of tolerance to and dependence of opioid agonists. The opioids may act by modulating the NMDA receptor-mediated electrophysiological events or by interacting at an intracellular level (Ossipov *et al.,* 2004).

Opioid peptides play a role as neuormodulators by modulating changes produced by other neurotransmitters e.g. 5HT, and NA. Many neurochemical studies have implicated descending 5-HT and NA in morphine induced analgesia and a reduction in the antinociceptive effects of morphine after lesioning of 5-HT and NA descending pathways (Mao, 2004).

1.5 Nociceptive Tests

1.5.1 Tail Flick Test

The test was first introduced by D'amour and smith (D'amour and Smith, 1941). This phasic pain test uses radiant heat focused on the tip of the tail. It measures the latency before the rat "flicks" its tail out of the beam as a sign of nociception. The tail flick reflex is a spinally integrated reflex not disrupted by spinalization. This test is used for screening drugs as well as for nociceptive tests. The test also allows repeated testing without conditioning effects. There is minimal individual variation in this test. Its potency ranking of opiate analgesics correlated well with accepted clinical ratings. The skin temperature greatly influences the test and if an analgesic drug lowers the skin temperature, this tends to prolong the tail flick latency (LeBars *et al.,* 2001).

1.5.2 Hot Plate Test

It was developed by Wolfe and Macdonald (Wolfe and MacDonald, 1944) and is one of the most commonly used tests of nociception in rodents. Originally the test measured nociceptive response (kicking and dancing, licking the hind paw, fore paw or both) of mice placed on a hot plate at temperature varying from 55-70 degrees Celsius. After modification, a constant hot plate temperature of 55 degrees Celsius was maintained. The nociceptive responses measured were; shaking of the foot, holding the foot tightly against the body, and licking of the fore paw, hind paw or both. A modified hot plate test for use in mice and rats has been developed. The temperature is slowly increased from non –noxious levels up to the end point which is the temperature when the first hind paw lick occurs. If no hind paw lick is observed, the test is terminated at 52 degrees Celsius (cut of value). The modified model is more sensitive and gives more consistent, valid and reliable results. In the hot plate test, the licking

of hind paw or fore paw is what is commonly recorded (LeBars *et al.,* 2001; Hunskaar *et al.,* 1986b).

1.5.3 Chemically Induced Writhing

The test was first introduced by Siegmund *et al*. (1957). It is a phasic pain test. The animal was injected with phenylquinone intraperitoneally which induced "writhing" syndrome where the animal exhibited abdominal contractions, twisting and turning of the trunk and extension of the hind limbs. Other chemicals that have also been used to induce writhing are acetic acid (Koster *et al.,* 1959; Emele and Shanaman, 1963) and acetylcholine (Collier *et al.,* 1968). This test is simple to perform and is possible to quantify the response and to correlate the variable with the stimulus intensity within a reasonable range; as such it has been employed as a screening method. However it lacks specificity as many drugs without analgesic effects in man can effectively inhibit the writhing response in laboratory animals. The mechanism of the syndrome is not known and mediators like prostagladins have been proposed (LeBars *et al.,* 2001).

1.5.4 Formalin Test

The formalin test is based on experiments which were performed using small amounts of hypertonic solution injected under the skin of human subjects which produced brief intense pain. This test was first described for rats and cats by Dubuisson and Dennis (1977).

Subcutaneous injections of 0.1ml and 0.05 ml of 5% formalin into the fore paw induced pain in rats and cats. The pain produced had a biphasic response. Pain intensity was rated according to a visual analog scale and given numerical values. ''O'' indicated that the injected paw pressed firmly on the floor and bore the animals weight and there was no discernible difference in how the two fore paws were used during locomotion. ''1'' indicated

that the paw rests lightly on the floor or wall and is definitely in contact with it, but little or no weight is placed on it and during locomotion there was a definite limp. ‘’2’’ the injected paw is elevated from the ground and not in contact with any surface and ‘’3’’ the animal licked, bite or shook the affected paw. The test has been modified in subsequent studies (Hunskaar *et al.,* 1985; Shibata *et al.,* 1989; Takahashi *et al.,* 1984) and only one behavioral response (licking of the hind paw) has since been monitored. Licking of the hind paw is easy to observe and to quantify and is consistent. Scoring the hind paw licks gives reliable results because it has less interference with the rearing and grooming habit.

There are several advantages that the formalin test has over other tests. The pain stimulus bears a resemblance to most clinical pain. There is little or no restraint during the experiment and the animals are not stressed. Stress can alter the animals’ behavior. The pain elicited by the stimulus is continuous and enables a temporal nociceptive profile to be measured (LeBars *et al.,* 2001). Intradermal injection of formalin produces a biphasic response with an early and a late phase during which there is high licking activity. The two phases represent different types of pain. The early phase is thought to be caused by direct stimulation of the nociceptors by formalin and central release of substance P. The late phase is caused by inflammation (Alreja *et al.,* 1984; Takahashi *et al.,* 1984; Hunskaar *et al.,* 1985, 1986a; Hunskaar and Hole 1987; Shibata *et al.,* 1989; Tjolsen *et al.,* 1992). The late phase is caused by mediators of inflammation like bradykinin, prostaglandins, histamine and serotonin (Shibata *et al.,* 1989).

The mechanisms of action of formalin are still obscure. Subcutaneous injection of formalin in the hind paw induced a transient activation of enkephalinergic neurons segmentally in the spinal cord of rats. The release of the met-enkephalin like material (MELM) in the celebrospinal fluid at the lumbar level took 5-10 minutes after formalin injection and was of short duration (5-10 mins). In the formalin test there is a transient decrease of nociception 5-

10 minutes after formalin injection in mice which is concomitant with the enhancement of the release of the MELM. This may explain the reduction in nociception during that period of time. Following formalin injection an increase in the excitability of the dorsal horn cells has been demonstrated using electrophysiological studies and the central changes induced in the early phase may contribute to the development of the late phase. This suggests that mechanisms other than inflammation may also be involved (LeBars *et al.,* 2001; Capone and Aloisi, 2004).

Immunoreactive SP is increased in dorsal horn after one hour of formalin injection into the hind paw. The increase of SP-like immunoreactivity may be due to an increase in SP release from the primary afferent neurons. The early and the late phase in the formalin test may be modulated differently in the central nervous system (LeBars *et al.,* 2001).

The involvement of NMDA receptors in the second phase of formalin test has been suggested. NMDA receptors play a major role in the development of central analgesia. Formalin injection causes a cascade of events leading to the release of excitatory amino acid glutamate which activates the NMDA receptors in the spinal cord. The release of glutamate requires nitric oxide which is believed to play a role during prolonged nociception such as that caused by formalin injection ((LeBars *et al.,* 2001; Capone and Aloisi, 2004)

The formalin test is a very useful test in studies of pain mechanism and in the evaluation of analgesic drugs. Dubuisson and Dennis (1977) evaluated the analgesic effects of morphine and pethidine in cats and dogs in the early and the late phases of the formalin test. They demonstrated that intraperitoneal injections of morphine (0.8 mg / kg) produced analgesia in both phases in the cat in about 10-15 minutes with no return to pain. Pethidine (8mg / kg) produced analgesia in both phases but showed greater individual variations in cats. In rats, morphine (2mg / kg) produced slight analgesia in both phases. Morphine (6mg / kg) produced

clear analgesia while pethidine (25mg / kg) produced analgesia in the early and late phase, though of shorter duration than that of morphine. The rats fell into a stupor 10-20 minutes after drug administration (Dubuisson and Dennis, 1977). Cats were injected with 0.1 ml of 5% formalin and rats with 0.05 ml of 5% formalin. In the modified formalin test 20 µl of 1% or 5% formalin is injected into the hind paw of mice (Hunskaar *et al.,* 1985; 1986a; Hunskaar and Hole, 1987). Nociceptive behavior in the early phase (0-5 minutes) and late phase (20-30 minutes) was scored as the amount of time spent licking the injected paw i.e. the dorsal surface of the paw, the toes or the leg. Morphine (2.5 -10 mg / kg) caused dose dependant antinociception in both phases (Hunskaar and Hole, 1987). In a study using 20 µl of 0.5% formalin in mice morphine (1, 3, 6 mg / kg) caused a dose dependent antinociception (Shibata *et al.,* 1989; Capone and Aloisi, 1999).

The effect of different formalin concentrations in mice has been studied. Using 0.02% - 0.2% formalin only the early phase was observed. 1% or more induced both the early and the late phase. Using very low concentration (0.2%) repeated testing on the same paw could be performed at different intervals of 1 week without any significant damage to the tissue or change in response. It was concluded that very low concentration of formalin should be used to minimize animal suffering. The early phase can be studied using concentration of 0.05% - 0.2% whereas 1% or higher is recommended for the late phase (Rosland *et al.,* 1990).

Ambient temperature has been shown to influence the licking response in the late phase. An increase in the temperature causes an increase in the intensity and duration of licking in the late phase. It is recommended that temperature in the testing chamber should be carefully controlled for reliable results. The ambient temperature when using mice should preferably be maintained at above 23 degrees Celsius for an optimum behavioral response (Rosland, 1991; Hole and Tjolsen, 1993).

1.6 Mechanism of Action of Analgesic Drugs

1.6.1 Steroidal Anti-inflammatory Drugs

Steroids are so far the most potent anti-inflammatory agents. They are capable of suppressing the cardinal signs of inflammation regardless of the cause. These drugs exert their effects on inflammation in several ways (Hunskaar and Hole, 1987). They inhibit phospholipase A2 activity, which is necessary for the release of arachidonic acid (Terenius, 1981; Vane and Botting, 1987). Anti -inflammatory steroids inhibit phosphiolipase A2 indirectly by causing the release of an inhibitory protein, which has been variously termed macrocortin, lipomodulin, renocortin, or lipocortin (Vane and Botting, 1987). The steroids also stimulate both the release and the re-synthesis of lipocortin by binding onto specific membrane receptors. Lipocortin neutralizes both the cyclooxygenase and the lipo-oxygenase pathways by inhibiting phosholipase A2 activity.

There are reports suggesting that steroid antinflammatory drugs may cause potent analgesia. For instance, in the formalin test, the analgesic effect of hydrocortisone and dexamethasone has been demonstrated in the late phase (Hunskaar and Hole, 1987). It is postulated that the analgesic effect of steroids can be attributed to their anti-inflammatory effects and subsequently reduced symptoms of inflammation (Hunskaar and Hole, 1987). However, studies have demonstrated the centrally mediated analgesic effects of steroids (Deutsch, 1992). This is a reserve of the pregnane derived steroids and therefore depicting a structural activity relationship in the way that it binds to the receptors. The CNS $GABA_A$ receptors have been shown to consist of four domains with a pentameric arrangement and the steroids bind to one of the domains and enhance the inhibitory effects of GABA. This is achieved by prolonging the duration in which chloride ion channel remains open, leading to increased chloride ion influx hence sustained hyperpolarisation (Majewska, 1987; Burg *et al.,* 2003).

1.6.2 Non-Steroidal Anti-inflammatory Drugs

Non -steroidal anti-inflammatory drugs (NSAIDs) are the most widely used analgesic, anti-rheumatic and antipyretic drugs. Some of the several different hypotheses that have been put forward to explain the actions of NSAlDs include an interference with oxidative phosphorylation, the displacement of endogenous anti-inflammatory peptide from plasma protein, interference with migration of leucocytes, inhibition of leucocytic-phagocytosis, stabilization of lysosomal membranes, inhibition of the generation of lipoperoxides, and hyperpolarization of neuronal membranes (Ferreira and Vane, 1974). Most of these hypotheses have been discounted on the basis that they could not explain the analgesic effects of NSAIDs. Vane (1971) reported the mechanism of action of NSAIDs is the inhibition of prostaglandin biosynthesis and since then their mechanism of action has been linked to it (Ferreira, 1972; Ferreira and Vane, 1974; Ferreira *et al.,* 1978).

Following tissue injury algogens such as bradykinin, histamines, serotonin, dopamine, acetylcholine, acids, and prostaglandins are released (Ferreira and Vane, 1974). Systemic administration of these algogens has been shown to produce pain-related behavior in animals (Guzman and Lim, 1968). Prostaglandins (PGs) are synthesized from arachidonic acid through a cascade of reactions controlled by enzymes. These enzymes are phospholipase A2 and cyclooxygenase I and 11. NSAIDs are said to prevent PG biosynthesis by inhibiting cyclic endoperoxides. Thus, PGs which are potent inflammatory mediators (Vane and Botting, 1987) will not be produced. This effect can therefore explain the anti-inflammatory actions of NSAIDs (Ferreira, 1972).

Although PGs are potent inflammatory mediators, they also cause pain on intraperitoneal or intradermal injection (Ferreira, 1972*).* However, the nociceptive response induced by PGs

when administered alone is small, suggesting that they have no effects on pain receptors. PGs appear to facilitate the response to other stimuli affecting nociceptors (Ferreira, 1972; Handwerker, 1976). It has been postulated that NSAIDs may exert their peripheral analgesic effect by preventing the sensitizing action of PGs on the pain receptors (Ferreira, 1972).

NSAIDS may also exert their analgesic effect centrally (Ferreira *et al.,* 1978; Hunskaar *et al.,* 1985). Whereas the peripheral analgesic effect of NSAIDs is linked to inhibition of prostanglandin biosynthesis, the mechanisms of their central action is not clear. However, PGs are at least in part implicated. This is based on the fact that PGs are also widely distributed in the central nervous system, and may be released following painful peripheral stimulation (Ramwell and Shaw, 1966; Ramwell *et al.,* 1966). Several PG biosynthesis inhibitors are capable of counteracting hyperalgesia induced by intrathecal administration of PGs (Ferreira *et al.,* 1978, Ferreira, 1983). This finding led to the suggestion that peripheral and central analgesic effect of NSAIDs is due to cyclooxygenase inhibition (Ferreira *et al.,* 1978). However, in a study where substance P was administered intrathecally, pre-treatment with intraperitoneal aspirin and paracetamol was reported to reduce the behavior caused by substance P (Hunskaar *et al.,* 1985). It has therefore been postulated that the analgesic effect of NSAIDs cannot be entirely linked to inhibition of PG biosynthesis. It has also been shown that the serotonergic systems may play a role in the analgesic effect of paracetamol in mice (Tjolsen *et al.,* 1992). The difference in potency of NSAIDs suggests that they have other modes of action in addition to their common effect on cyclooxygenase enzyme.

1.6.3 Opioid Drugs

Opioids consist of the most effective class of analgesics in clinical use today. The endogenous opioid peptides and the exogenous opiates cause analgesia by acting on the same

systems in the body. The endogenous opioid systems have been described in a wide range of species and phyla of invertebrates (Kavaliers, 1988).

The endogenous opioid systems play an important role in the regulation of pain. There are more than a dozen endogenous opioids which are classified into 3 main groups; enkephalins, dynorphins, and endorphins (Yaksh, 1987). They exert their antinociceptive effects by binding to opioid receptors which are all G-protein linked receptors. Three types of opioid receptors have been isolated; μ opioid receptors (MORs), δ opioid (DOR) and κ opioid (KOR) receptors (Simonin *et al.,* 1995). The opioid receptors show a high degree of structural homology in their transmembrane and intracellular domains. They however differ widely in their extracellular domains. These differences explain the difference in ligand-sensitivity between the three receptor types (Akil *et al.,* 1998). The interactions between the opioids and their receptors are very complex with a lot of cross-sensitivity (Mansour *et al.,* 1995).

Activation of all the three types of opioid receptors results in the inhibition of adenyl cyclase. The net result of opioid binding is a reduction in neuronal excitability (Stein *et al.,* 2009). Various intracellular mechanisms are involved in this reduction of neuronal excitability. The activation of the three types of opioid receptor results in the suppression of the activity of the various types of Calcium ion channels (N, T and P/Q) found on the presynaptic membrane of the primary afferent (Childers, 1991; Kiefer, 1995; Stein *et al.,* 2009). This results in a reduction in the Calcium ion influx into the presynaptic neuron. Consequently this leads to a reduction in the excitation and /or neurotransmitter release in many neuronal systems. A prominent example of this is the inhibition of substance P from central and peripheral terminals of sensory neurons (Kondo *et.* al., 2005).

Opioid receptors mediate hyperpolarization at the postsynaptic membrane by causing the opening of potassium ion rectifier channel. This has the net effect of reducing neuronal excitability thereby preventing excitation and / or propagation of action potentials (Zollner and Stein, 2007). There is also evidence that these opioid receptors may also be coupled to other second messenger systems (Gutstein *et al.,* 1997).

The analgesic effects of the opioids are exerted both peripherally and centrally. Opioid receptors are expressed peripherally on small, medium size and large-diameter dorsal root ganglion neurons (Wang and Wessendorf, 2001; Silbert *et al.,* 2003; Rau *et al.,* 2005; Gendron *et al.,* 2006). The peripheral antinociceptive effects of opioids are mediated by peripheral opioid receptors (Bartho *et al.,* 1990). The peripheral opioid receptors are often co-expressed with neuropeptides such as substance P (SP) and calcitonin-gene related peptide (CGRP) (Minami *et al.,* 1995; *Li et al.,* 1998; Zhang *et al,* 1998; Stander *et al.,* 2002; Mousa *et al.,* 2007) similarly to the central opioid receptors. As a result of this co-expression, opioid agonists can attenuate inflammation induced by increase in the excitability of primary afferent neurons and the release of proinflammatory neuropeptides (SP and GCRP) from central and peripheral terminals (Junger *et al.,* 2002). These events lead to antinociceptive and anti-infalmmatory effects particularly within the injured tissue.

The expression of these peripheral opioid receptors is up regulated in the presence of tissue inflammation as well as in the presence of neural damage (Ji *et al.,* 1995; Zhang *et al.,* 1998; Mousa *et al.,* 2002; Ballet *et al.,* 2003; Troung *et al.,* 2003; Zollner *et al.,* 2003; Puehler *et al.,* 2004; Shaqura *et al.,* 2004; Walczak *et al.,* 2005; Kabli and Cahill, 2007). The up regulation in receptor expression occurs acutely i.e., within minutes or hours after tissue damage / neural damage. The molecular mechanisms that underlie this up regulation are

varied and include increased receptor trafficking to the cell membrane as well as increased receptor protein synthesis among others (Stein *et al.,* 2009). It can therefore be seen that these peripheral opioid receptors mediate the early peripheral antinociceptive response which in most cases precedes the midbrain mediated descending analgesia.

The central opiodergic analgesic effects arise from the activation of the descending inhibitory systems arising from the mid brain structures (Mansour *et al.,* 1995; Gutstein *et al.,* 1997). The first evidence for an endogenous opioid system came from studies showing that microinjections of morphine into the Pariaqueductal Gray Area (PAG) caused analgesia (Yaksh *et al.,* 1988). The analgesic effect could be reversed by the administration of naloxone (Akil *et al.,* 1976). Opioids also exert forebrain mechanisms of analgesia in addition to the dorsal horn and mid brain mechanisms described above (Franklin and Mathies, 1992). It should be noted that all the three types of opioid receptors participate in the mediation of the descending opioid analgesia in contrast to earlier beliefs which tended to ascribe these effects solely to the μ receptors.

1.7 Use of Medicinal Plants in Pain Management

World Health Organisation (WHO) estimates that approximately 80% of the world population relies on traditional healers for their day to day health care needs. The traditional healers use herbal remedies in managing the health conditions of their communities. Approximately 70 % of the people of sub Saharan Africa are reported to be using herbal medicine for treatment of various medical conditions (W.H.O., 2002). Medicinal plants are an important and treasured local resource of the people of East Africa. The use of the plants

in the indigenous cultures are multiple and diverse; they are used as food, medicine, firewood, construction material, dyes or ornaments or even for ritual paraphernalia. The same plants may be used by different communities for different purposes or even as medicine for treatment of different ailments (Schlage *et al.,* 1999). Based on anecdotal evidence, herbal remedies used in most communities are claimed to be effective. There is need to scientifically evaluate their effectiveness for the benefits of the general population.

From the first recorded account, over 7000 year ago, various forms of plant preparations have been utilized to treat pain disorders. Prototypical examples include the opium poppy (*Papaver somniferum*) and the bark of the willow tree (*Saalix* ssp). In the 19th century individual compounds were isolated from these plants and were shown to possess the desired effects. Analgesic substances have been purified from plants resulting in the identification of novel structures with known mechanism of actions. Natural products still hold a great promise for the future of drug discovery in the treatment of pain disorders (McCurdy and Scully, 2005). A study done in the Washambaa community of Tanzania reported twenty two plants as being routinely used by the community for the treatment of pain and inflammation (Schlage *et al.,* 1999). Most of these plants are also available in Kenya and are used for the same purpose.

1.7.1 *Teclea simplicifolia (Vepris simplicifolia)*

It belongs to the family Rutaceae. It is a large tree found in evergreen forest, riverine forest and woodland. The bark is smooth or grey; branchlets glabrous. Leaves are trifolialate and the fruit is yellow, orange or red, round or ellipsoid. *T. simplifolia* is commonly associated with colonizing forests, thickets, forest edges and mixed forest from low land to lower mountain areas. It is geographic distribution in Ethiopia, Kenya, Tanzania, Uganda (Kokwaro, 2009). The fruit is edible. It has essential oils that are used as medicine. The leaf

or root decoction mixed with honey is used against pneumonia in Kenya. The roots are used as an anthelminthic. The steam inhalation of the leaves reportedly cures fever. The leaves are used by the local communities for the treatment of painful conditions (Mascolo *et al.,* 1988; 2009).

1.7.2 *Clausena anisata*

It belongs to the family Rutaceae. The plant is a small, neat and attractive tree. Crushed leaves give off a strong aniseed-like scent which is considered by many to be unpleasant. The ripening fruits turn from red to black. It is found in all the tropical and sub-tropical parts of Africa and Asia. It is found in the undergrowth and on the margins of evergreen to semi-evergreen forests and woodlands. In Kenya it is found in the Ngong forest among others.

The leaves, bark, wood and root are used extensively across Africa and Asia for a myriad of ailments including the treatment of fever, pneumonia, headache, sore throat and sinusitis, wounds, aching teeth, sores, abscesses, burns, haemorrhoids, whooping cough, malaria, syphilis, kidney troubles, diabetes and as an insect repellent. Leaf infusion is used as a steam bath deodorant, to cleanse the body internally, to cure rheumatism and to strengthen the heart (Kokwaro, 2009). It is known to contain carbazole alkaloids, coumarins and limonoids (Schlage *et al.,* 1999).

1.7.3 *Warburgia ugandensis*

It belongs to the family Canellaceae. It is a spreading evergreen tree 4.5-30 m tall with berry fruits which are initially green and ellipsoidal, later subspherical and turning purplish, The genus is named after Dr. Otto Warburg (1859-1938), born in Hamburg, lecturer in botany at the University of Berlin and author of numerous botanical papers. It is found in lowland rainforest, upland dry evergreen forest and its relicts in secondary bush land and grassland;

also on territorial in swamp forest. In Kenya it is found in the Ngong forest among others. The bark of this plant has been shown to yield sesquiterpene dialdehydes, warburganal and polygodial and is used for the treatment of pain and inflammation. It has also been shown to have anti-fungal activities (Schlage *et al.,* 1999; Kokwaro, 2009). The dried bark is commonly chewed and the juice swallowed as a remedy for stomach-ache, constipation, toothache, cough, fever, muscle pains, weak joints and general body pains. It is also effective in powdered form for treating the same diseases. Fresh root are boiled and mixed with soup for the prevention of diarrhea. Leaf decoction baths are used as a cure for several skin diseases. The inner bark is reddish, bitter and peppery and has a variety of applications. It provides treatment for the common cold; dried and ground to a snuff, it is used to clear sinuses; and it is chewed, or smoke from the burning bark inhaled as a remedy for chest complaints. The bark, root or leaves can be boiled in water and the decoction drunk to treat malaria, but this causes violent vomiting (Schlage *et al.,* 1999; Kokwaro, 2009).

1.7. 4 *Sapium ellipticum*

It belongs to the family Euphorbiaceae**.** *Sapium ellipticum* is a small to medium-sized, deciduous or semi-deciduous tree up to 12 m in height, occasionally reaching 20-25 m (max. 35).The Bark is light brown to very dark almost black. It is common on the outskirts of evergreen forest and in wooded ravines. It is a tree of the afromontane rainforest and undifferentiated afromontane forest (mixed podocarpus forest), often in clearings, riverine forest also in secondary montane evergreen bush land and closed lowland forest. In Kenya it is found in the Ngong forest among others (Beentje, 1994). Leaves and root are used to treat mumps and for management of painful conditions (Schlage *et al.,* 1999; Kokwaro, 2009).

1.7.5 *Senna singueana*

It belongs to the family Caesalpinioideae. *Senna singueana* is a shrub or small tree 1-15 m high; branchlets glabrous to densely pubescent crown open; bark reddish, becoming grey-brown and rough with age. It is a species of the drier tropical Africa regions and is often found in thickets, deciduous woodland, and savannah. It is frequently associated with termite mounds, in luggas or riverine. In Kenya it is a component of the mid- and highland dry evergreen forests (Beentje, 1994; Kokwaro, 2009). The root bark is used for management of convulsions, gonorrhoea, bilharzia, heartburn, stomach-ache, constipation, wounds and snake bites. The ash from the burnt root mixed with porridge provides a remedy for stomach pains (Schlage *et al.,* 1999; Kokwaro, 2009).

1.7.6 *Albizia anthelmintica*

It belongs to the family Mimosoiceae. It is a thorny/spiny, deciduous, multi-stemmed, medium canopied tree growing to about 8m. The bark is smooth, gray to brown. Young branches are glabrous and the twigs are often spine-tipped. *A. anthelmintica* commonly occurs in deciduous or evergreen bush land and scrubland especially along seasonal rivers and on termite- mould clump thickets (Beentje, 1994; Kokwaro, 2009). The stem bark is widely used as a purgative and anthelmintic. The tree is said to provide a cure for gonorrhea and painful conditions. The twigs are used as toothbrushes for oral hygiene (Schlage *et al.,* 1999; Kokwaro, 2009).

1.7.7 *Rhus natalensis*

It belongs to the family Anacardiaceae. *Rhus natalensis* is a shrub 2-3 m high or a small tree up to 8 m tall; bark of the branchlets greyish or white and older ones dull grey, lenticillate and rough. *R. natalensis* is normally found in deciduous and evergreen bush land and

woodland, riverine associations, forest edges, often on well drained slopes. It is also common in coastal bush, thickets and forest (Beentje, 1994; Kokwaro, 2009). In Kenya it is found in Kiserian area. The branchlets of this tree are used as toothbrushes. The root decoctions are taken orally to stop diarrhoea. Branch decoctions administered orally for stomach upset. Leaves are used in treating coughs and stomachaches. The root decoction also forms part of a medicine for hookworms. Leaves are boiled and given to cattle as a pain killer or used as infusion in preparing a cough mixture (Maundu *et al.,* 1999; Schlage *et al.,* 1999; Kokwaro, 2009).

1.7.8 *Psiadia punctulata*

It belongs to the family Asteraceae. It is shrub or woody herb 0.5 to 2.5 m high. Stems are dark grayish-brown and the leaves are shiny and sticky. It is mainly found in Masai districts, in grassland areas, bushed grassland and evergreen-bush land in the dry forest edges. In Kenya it is found in the Ngong forest. *P. punctulata* is used in treatment of cough. An infusion of the plant is taken for treatment of colds and as a cough mixture. It is also used externally for removal of ectoparasites from cattle. In Kenya the infusion is used by adults for treatment of fever (Maundu *et al.,* 1999; Schlage *et al.,* 1999; Kokwaro, 2009).

1.7.9 *Toddalia asiatica*

It belongs to the family Rutaceae. It is a flowering plant in the citrus family containing the single species *Toddalia asiatica* commonly known as orange climber (Orwa *et al.,* 2008). Botanical synonyms of *Toddalia asiatica (L.) Lam.* Include *Paullinia asiatica, Toddalia aculeate, Toddalia effusa* and *Toddalia ambigua*. It is a liana with woody, corky, thorny stems that climb on trees, reaching up to 10 meters in length. It has yellow-green flowers,

shiny green citrus-scented leaves, and orange fruits about half a centimeter in diameter that tastes like orange peel. The seeds are dispersed by birds and monkeys that eat the fruits within the forests (Bussmann, *et al.,* 2006). *T. asiatica*, is native to many countries in Africa and Asia and it grows in forested riparian habitat with high rainfall forest (Beentje, 1994; Nabwami, *et al.,* 2007; Kokwaro, 2009). In Kenya it is found in the Ngong forest. It is used as a folklore remedy for various ailments in many areas of the world. *T. asiatica* is used in treatment of several diseases that includes malaria (Orwa, *et al.,* 2008; Jeruto, *et al.,* 2011), stomach ache, chest pains, sore throat, food poisoning, (Orwa, *et al.,* 2008), cough, indigestion, nasal and bronchial pains (Kokwaro, 2009).

1.8 Principles of Extraction of Phytochemicals

Extraction refers to the initial step of separating a part from the whole plant materials. An important factor governing the general and specific method used in an extraction is the type of chemical class that one is aiming to extract (Peter and Amala, 1998). Phytochemicals are natural chemicals that are produced by the plant. They have no nutritional value but play an important role in plant defense against diseases and pathogens. They possess anti-oxidant, anti-inflammatory, anti-cancer and anti-bacterial properties among others. The main phytochemical groups of compound include fixed oils, fat and waxes, volatile or essential oils, anthraquinones, flavonoids, saponins, carotenoids, alkaloids, glycosides, phenolic compounds, polysaccharides and proteins (Sarker *et al.,* 2007). It is therefore important to subject an extract to phytochemical tests in order to confirm the phytochemical groups present since this forms the basis of a subsequent extraction method.

Phytochemical extraction technique follows a more or less standard protocol which can be modified in order to extract targeted specific phytochemical group of interest. Solvent extraction is the most scientifically preferred method of extraction as it is simple and easy to perform.

Different solvents are used depending on the phytochemical groups that are targeted for extraction. Solvents differ in polarity just like phytochemicals do. There are three polarity strengths of solvents; polar, medium-polar and non-polar. Polar solvents extract polar compounds while non-polar solvents extract non-polar compounds. Polar solvents include methanol, ethanol and water. Medium-polar solvents include ethyl acetate, acetone and dichloromethane whereas non-polar solvents include diethylether, toluene, chlorofom *and* hexane (Peter and Amala, 1998). Thus in extracting crude plant materials, solvents can be mixed or they can be used in sequence in the same sample material.

Different solvent extraction methods exist. The simplest and easiest use of these methods is maceration, hydro-distillation using steam and soxhlet extraction (Jones and Kinghorn, 2005).

In maceration, the homogenized plant sample is soaked in solvent in a closed container and left at room temperature for a period of at least 3 days with frequent agitation until the soluble matter has dissolved. The solvent is then decanted and filtered to remove debris. In hydro-distillation, plant sample which is either dry or wet is placed in a flask and immersed in water. The flask is then connected to a condenser and heated. The distillate is collected in a tube that is connected to the condenser. It comes out as a mixture of oils and water. They are

collected separately. Hydro-distillation is good for extraction of volatile phytochemicals (Jones and Kinghorn, 2005).

Soxhlet process is useful for the exhaustive extraction of plant material with a particular solvent e.g. for defatting. It is also useful where exhaustive sequential extraction with a series of solvents of increasing polarity is desired e.g. hexane then dichloromethane then methanol then water (Peter and Amala, 1998). However, it is necessary to dry the plant material in between changes of solvent to prevent carry-over of traces of the previous solvent into the next one.

Other methods of extraction includes percolation, supercritical fluid extraction and use of liquefied gases under moderate pressure (phytosol® extraction). The choice of method depends on several factors that includes; volatility, flammability and boiling point, toxicity, reactivity and the cost of solvent (Jones and Kinghorn, 2005).

1.9 Nuclear Magnetic Resonance Spectroscopy

Nuclear magnetic resonance (NMR) spectroscopy is a research technique that exploits the magnetic properties of certain atomic nuclei to determines the physical and chemical properties of atoms or the molecules in which they are contained. NMR relies on the phenomenon of nuclear magnetic resonance and can provide detailed information about the structure, dynamics, reaction state, and chemical environment of molecules (Wemmer, 2000).

NMR spectroscopy is used to investigate the properties of organic molecules, although it is applicable to any kind of sample that contains nuclei possessing spin. Suitable samples range from small compounds analyzed with 1-dimensional proton or carbon-13 NMR spectroscopy

to large proteins or nucleic acids using 3 or 4-dimensional techniques. The impact of NMR spectroscopy gives a range of information and the diversity of samples (Keeler, 2007).

The NMR sample is prepared in a thin-walled glass tube - a NMR tube and then placed in a magnetic field. NMR active nuclei (such as ^{1}H or ^{13}C) absorb electromagnetic radiation at a frequency characteristic of the isotope (Shah *et al.,* 2006). The resonant frequency, energy of the absorption, and the intensity of the signal are proportional to the strength of the magnetic field.

In NMR spectroscopy, the chemical shift is the resonant frequency of a nucleus relative to a standard. The position and number of chemical shifts are diagnostic of the structure of a molecule. The chemical shift provides information about the structure of the molecule. Important factors influencing chemical shift are electron density, electronegativity of neighboring groups and induced magnetic field effects (Hornak, 1999).

A spinning charge generates a magnetic field that results in a magnetic moment proportional to the spin. In the presence of an external magnetic field, two spin states: one spin up and one spin down, where one aligns with the magnetic field and the other oppose it. The difference in energy between the two spin states increases as the strength of the field increases, but this difference is usually very small, leading to the requirement for strong NMR magnets (1-20 T for modern NMR instruments). Irradiation of the sample with energy corresponding to the exact spin state separation of a specific set of nuclei will cause excitation of those set of nuclei in the lower energy state to the higher energy state (Hornak, 1999).

The energy differences between two spin states at a given magnetic field strength are proportional to their magnetic moments. However, even if all protons have the same magnetic moments, they do not give resonant signals at the same field/frequency values. This

difference arises from the differing electronic environments of the proton. Upon application of an external magnetic field, these electrons move in response to the field and generate local magnetic fields that oppose the much stronger applied field. This local field thus "shields" the proton from the applied magnetic field, which must therefore be increased in order to achieve resonance. Such increments are very small, usually in parts per million (ppm). However a frequency scale is commonly used to designate the NMR signals, even though the spectrometer may operate by sweeping the magnetic field (Shah *et al.,* 2006).

The location of different NMR signals is dependent on the external magnetic field strength and the reference frequency. The signals are usually reported relative to a reference signal, usually that of TMS (tetramethylsilane). Additionally, since the distribution of NMR signals is field dependent, these frequencies are divided by the spectrometer frequency and the resulting number is too small, thus it is multiplied by a million. This operation therefore gives a locator number called the "chemical shift" with units of parts per million (Keeler, 2007). To detect such small frequency differences the applied magnetic field must be constant throughout the sample volume. Chemical shifts for protons are highly predictable since the shifts are primarily determined by simpler shielding effects (electron density), but the chemical shifts for many heavier nuclei are more strongly influenced by other factors including excited states such as "paramagnetic" contribution to shielding tensor (Shah *et al.,* 2006).

Some of the most useful information for structure determination in a one-dimensional NMR spectrum comes from J-coupling, a special case of spin-spin coupling between NMR active nuclei. This coupling arises from the interaction of different spin states through the chemical bonds of a molecule and results in the splitting of NMR signals. This coupling provides detailed insight into the connectivity of atoms in a molecule (Shah *et al.,* 2006). Coupling

combined with the chemical shift (and the integration for protons) gives information about the chemical environment of the nuclei and also the number of neighboring NMR active nuclei within the molecule (Shah *et al.,* 2006).

Software allows analysis of signal intensity of peaks, which under conditions of optimal relaxation, correlate with the number of protons of that type. Analysis of signal intensity is done by integration. The peak height as well as the width are measured since the size is dependent on its area not its height (Shah *et al.,* 2006).

1.10 Justification for the Study

Pain is always subjective and is one of the foremost causes of suffering in human beings as well as in animals and there is need to develop an analgesic devoid of the side effects associated with opioids and NSAIDs. Approximately 80% of the world's population consults traditional healers who use herbal medicine. Herbal medicines hold the promise of future drug discovery with idealistic pharmacological profile. Several plants are listed as being widely used in the treatment of pain and pain related ailments in many parts of the world including East Africa (Schlage *et al.,* 1999; Kokwaro, 2009). There is need to provide scientific data on the claimed analgesic effectiveness of these plants.

This study was therefore undertaken to evaluate the analgesic activities of nine plants with the aim of identifying the most potent plant using animal models of nociception.

1.11 OBJECTIVES

1.11.1 Overall Objective

To evaluate the antinociceptive activities of nine selected indigenous plants used by Kenyan communities for the management of pain using animal models of nociception.

1.11.2 Specific Objectives

The specific objectives for the study were:-

1. To establish the antinociceptive activities of nine plants using animal models of nociception for preliminary assays and to identify the plant with the highest antinociceptive activity.
2. To isolate, characterize and establish the antinociceptive activities of pure compounds obtained from the plant with the highest nociceptive activity

1.11.3 Null Hypothesis

The plants / herb extracts do not have antinociceptive activity.

CHAPTER TWO

2.0 MATERIALS AND METHODS

2. 1. Materials

2.1.1. Plant Parts

The plant part was collected from Ngong forest and Kiserian area in the outskirts of Nairobi (Table 1), identified and authenticated at the herbarium, (specimen numbers) School of Biological Sciences University of Nairobi and a voucher specimen reserved for reference at the same herbarium. The skeels were peeled off and debarked while they were still fresh, cut into small pieces and dried in the shade for a period of three weeks. The leaves were also prepared and dried separately in the shade for a period of three weeks. The plant part was then be pulverized to powder using a mechanical grinder. The plant parts used and the voucher number are presented in table 1.

Table 1: Plant Parts and the Voucher Numbers

Plant	Plant part	Place of collection	Voucher no.
Albizia anthelmintica	Root	Ngong forest	2008/ UONHNK/01
Clausena anisata	Root	Ngong forest	2008/ UONHNK/02
Psiadia punctulata	Root	Ngong forest	2008/ UONHNK/ 03
Rhus natalensis (Searsia nataleneis)	Root	Kiserian area	2008/ UONHNK/ 04
Sapium ellipticum	Root	Ngong forest	2008/ UONHNK /05
Senna singueana	Root	Ngong forest	2008/ UONHNK/ 06
Toddalia asiatica	Root	Ngong forest	2008/ UONHNK/ 07
Teclea simlicifolia	Leaves	Ngong forest	2008/ UONHNK /08
Warburgia ugandensis	Stem/bark	Ngong forest	2008/ UONHNK /09

2.1.2 Experimental Animals

Adult Swiss albino mice of both sexes weighing 20–26 g were used. The breeds were obtained from the stock in the Department of Medical Physiology animal house, University of Nairobi. The animals were housed in cages with food and water *ad libitum.* The animal house was maintained at a temperature (20 -22 ˚C) and with controlled lighting (12 h light/dark cycle). Habituation to the equipment was done 24 hours, before the commencement of the experiments. The "Principle of Laboratory Animal Care" (NIH publication No. 85-23) guidelines and procedures were followed in this study (NIH publication revised 1985). All the tests were carried out during the daytime in a quiet laboratory setting with ambient illumination and temperature similar to those of the animal house. Animals were allowed to

acclimatize to the test laboratory setting for 1 hour before the experiments began. The animals were allowed 24 hours acclimatization in the experimental laboratory before the commencement of the experiment. Each experimental unit comprised of a treated group of eight animals and a control group with similar number of animals. All effort was made to minimize animal suffering and to reduce the number of animals used. All the test mice used in the study were initially assessed for quality neurological and motor function.

2.2. Methods

2. 2.1. Extractions of Plant Materials

Air dried and powdered plant parts were extracted with dichloromethane and methanol in the ratio of 1:1. The extracts were then concentrated under vacuum in a rotary evaporator to give a residue which was weighed and a percentage weight of the dried powdered plant materials was calculated. The extracts were then reconstituted in 5% dimethylsulfoxide (DMSO) in normal saline to achieve the desired working concentrations of 50mg /kg, 100mg/ kg and 200mg /kg body weight. From this stock solution, various concentrations were prepared by serial dilution. 5% dimetylsulfoxide (DMSO) in normal saline was used as the negative control.

2. 2.2. Experimental Design

A randomized design was used. The animals and the treatment were randomly assigned to an experimental unit. Each experimental unit comprised of a treated group and a control group. The experimenter was blind to the extract or negative control to be administered. In all the experiments each animal was used once. The test materials were prepared in clearly labeled sample bottles and the samples coded by an independent person to ensure blinding of the experimenter. The coding was broken after data analysis.

2. 2.3. Drug Administration

The animal were carefully picked by the tail and placed on the bench. All injections were administered intraperitoneally (i.p.) in volumes of 20ml. Aspiration was performed prior to injection to ensure that the drug is not injected into the intestines. The drug was injected 1cm to the left of the midline and in the lower abdomen. The drug and /or herb extract administration was performed one hour prior to the nociceptive tests. Room temperature and rectal temperature were also recorded.

2. 2.4 Sensorimotor Test

To evaluate possible nonspecific muscle relaxant or sedative effects of the plant extracts was done using sensorimotor apparatus which consists of 3 vertical rods, diameter 2.5 cm, with the height of 20, 32, and 64 cm. This test was done prior to extract, drug or negative control (5% dimetylsulfoxide (DMSO) 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 was20 seconds per rod.

2. 2.5. Nociceptive Tests

2. 2.5.1 Tail Flick Test

A radiant heat An IITC Inc. Model 33 tail-flick analgesiometer was used to measure response latencies according to the method described previously (D'Amour and Smith, 1941; Corrêa *et al.*, 1996). Animals responded to a focused heat-stimulus by flicking or removing their inflicted tail exposing a photocell in the apparatus immediately below the tail. Animals were selected 24 hours previously on the basis of their reactivity in the test. Each animal was tested twice before administration of drugs to determine the baseline.

The reaction time was recorded for the animals pretreated with negative control (5% DMSO in normal saline), Morphine hydrochloride (Martindale Pharma) 5mg /kg, Disprin® (acetylsalicyclic acid) (Reckitt Benckiser) 100 mg /kg or plant extract 1 hr before testing. An automatic 20 seconds cut-off was used to minimize tissue damage.

2. 2.5.2 Hot Plate Test

An IITC Inc. Model 35D analgesiometer was used to perform the thermal test. The temperature was maintained at 50 ± 1 °C. This temperature has been shown to cause clear quantifiable nociceptive responses in mice. The copper plate (27 X 29 cm) was enclosed by a 30 X 30 X 30 cm perspex box. The surface of the plate was always kept clean and the temperature continually monitored with the aid of a digital thermometer (Thermoketrol, a/s, Type 2105 serial no. 1296; sensor Cu/Ni). Before the start of the experiment the animals were placed on the cold plate for 10 minutes per day for a period of three days. This procedure was meant to acclimatize the animals to the analgesiometer and also to the physical handling by hands. The mouse was gently placed on the hot plate and the latency (in seconds) to the stepping or lifting of the hind paw (hot plate response latency) was recorded. The reaction time was recorded for the animals pretreated with negative control (5% DMSO in normal saline), Morphine hydrochloride (Martindale Pharma) 5mg /kg, Disprin® (acetylsalicyclic acid) (Reckitt Benckiser) 100 mg / kg or plant extract i.p. 1 hr before testing. Any animal failing to respond by 20 seconds were removed from the plate and assigned a response latency of 20 seconds. The experiments were performed in room temperature of 20– 22 °C with adequate light and devoid of unnecessary noise or any other form of disturbance.

2. 2.5.3 Acetic Acid Writhing Test

Mice were allowed to adapt to the observation chamber for 15 minutes before the commencement of the test. Injection of 0.5 ml of 10% glacial acetic acid (Riedel, Seelze, Germany, Analar) i. p. was done and the animal placed in an observation chamber measuring 30 X 30 X 30 cm and scoring started immediately. Abdominal constrictions were defined as a mild constriction and elongation passing caudally along the abdominal wall accompanied by a slight twisting of the trunk followed by bilateral extension of the hind limbs. The cumulative time spent in such behaviour and the number of abdominal constrictions and trunk elongations were recorded in blocks of five minutes for 15minutes. The reaction time was recorded for the animals pretreated with negative control (5% DMSO in normal saline), Morphine hydrochloride (Martindale Pharma) 5mg /kg, Disprin® (acetylsalicylic acid) (Reckitt Benckiser) 100 mg /kg or plant extract i.p. 1 hr before testing. The experiments were performed in room temperature of 20– 22 °C with adequate light and devoid of unnecessary noise or any other form of disturbance.

2.2.5.4 Formalin Test

The modified formalin test was used (Shibata *et al.* 1989). The mice were allowed to adapt to the observation chamber (Perspex box, 30cm X 30cm X 30cm) for 20 minutes prior to formalin injection. The test was done after the animals pretreated with negative control (5% DMSO in normal saline), Morphine hydrochloride (Martindale Pharma) 5mg /kg, Disprin® (acetylsalicylic acid) (Reckitt Benckiser) 100 mg /kg or plant extract was injected i.p. 1 hr before testing. Using a microliter syringe and a 26 gauge needle, 20µl of 1% formalin (Sigma) in 0.9% NaCl was injected subcutaneously into the dorsal side of the right hind paw of each animal. The animal was then immediately returned to the observation chamber and the observation period started. The amount of time (in seconds) the animal spends licking

and/or lifting the injected paw was recorded using a stop watch in 5 minutes blocks for a period of thirty minutes. The animals' behaviour was also recorded in terms of whether it was active, quiet or asleep for the whole period of the experiment.

2. 2.6 Experimental Protocol

2. 2.5.1. Experiment 1: Antinoceptive assay of the Crude Extracts of the selected Nine Plants

The plant extracts used in these experiments were made from the parts of the plant commonly used by the communities in management of pain. Eight animals were used for each dose of the plant extract.

Sensorimotor test: This test is scientifically used to .to evaluate possible nonspecific muscle relaxant or sedative effect using the three rods apparatus... The mice were injected with negative control (5% dimethylsulfoxide (DMSO) in normal saline), extract or positive control drugs one hour prior to the test. .Two doses of each plant extract were used.

Tail flick: This nociceptive test was used to evaluate the central nociceptive actions specifically spinal cord intergrated actions of the plant extracts that passed the sensorimotor test.

Hot plate test: The three most potent plant extracts identified from the results of tail flickwere further screened usingthe hot plate test to evaluate peripheral as well as central nociceptive effects.

Acetic acid writhing test: This assay was used to evaluate the visceral pain antinociception of the most active .plant extract following the results of tail flick and hot plate and to ascertain which plant part is more potent.

Formalin test: The test was used to evaluate the antinociceptive effects in the moderate, continuous pain that is generated by tissue damage. This was done with the extract of the part of the plant that showed the overall highest antinociceptive activity in the above assay methods.

2.2.5.1 Experiment 2: Extraction, Separation and Purification of *Toddalia Asiatica Root E*xtract

The root extract of *Toddalia asiatica* (500 g) was extracted using dichloromethane: methanol (1:1) by cold percolation to give 200ml of crude extract. It was then subjected to fractionation on silica gel (150 g) eluting with n-hexane (fraction I), n-hexane-dichloromethane, 1:1 (Fraction II), dichloromethane (fraction III), dichloromethane-methanol, 9:1 (fraction IV), dichloromethane-methanol, 4:1 (fraction V), and dichloromethane-methanol, 1:1 (fraction VI).

Fraction I was washed several times with n-hexane and gave a white crystal of HK3 (15 Mg). Purification of fraction II using Sephadex LH-20 (dichloromethane-methanol, 1:1) gave white amorphous solids of HK 3 (34 mg) and HK5 (10 mg).

Fraction III was subjected to column chromatography on silica gel (80 g) eluting with n-hexane containing increasing percentage of ethyl acetate. A total of 180 fractions totaling 300 ml were collected and combined into 23 fractions based on their Thin Layer Chromotograhpy (TLC) profile. Fraction VI (1% of ethyl acetate in n-hexane) yielded a white amorphous solid of HK3 (15 mg). Purification of fraction 13-18 (2% ethyl acetate) yielded HK5 (50 mg) amorphous solid. Fractions 26-30 (3% of ethyl acetate) were purified and crystallized from dichloromethane to give white crystals of HK7 (25 mg). The mother liquour was separated on Sephadex LH-20 (eluted with dichloromethane-methanol, 1:1) to give HK-13 (20 mg).The

combined fractions of 91-100 (30% ethyl acetate) gave a brown solid of HK15 (20 mg). Purification of fractions 121-130 (50% ethyl acetate) yielded a white amorphous solid of HK18 (50 mg). Subsequent crystallization gave compounds of approximately 50 mg each. The compounds were then subjected to nuclear magnetic resonance spectroscopy for structure elucidation. Fraction IV has similar TLC profile with Fraction III and was not further purified. The purification of Fraction V by using Sephadex LH-20 yielded 5 fractions. TLC profile of the fractions showed similar spots with Fraction III. The pure compounds were assayed for central antinociceptive activity using the tail flick test.

2.2.5.1. Experiment 3: Structure Elucidation of compounds isolated from *Toddalia asiatica* Using NMR

^{1}H NMR (200 MHz) and ^{13}C NMR (50 MHz) of the isolated compounds from *Toddalia asiatica* were obtained on Varian spectrometer (200 MHz) using TMS as internal standard.

2. 3. Statistical Analysis

The primaary data was analysed and presented as the mean +/- standard error of the mean (s. e. m). To determine the level of significance the data was further analyzed using the one way analysis of variance (ANOVA) followed by Scheffe's *post hoc* test for multiple comparisons. The difference in the test values versus the negative control values was considered to be statistically significant at $p \leq 0.05$. The primary data on values of the time spent in pain behavior as recorded were used in statistical calculation. All data was analysed using Statview version 6 software.

CHAPTER THREE

3.0 RESULTS

3.1 Screening for Neurological and Motor Effects of Extracts from Nine Selected Plant

Evaluation of possible nonspecific muscle relaxant or sedative effect (Sensorimotor effects) of the plant extracts was done using the stationary rods. The negative control consists of 5% dimethylsulfoxide (DMSO) in normal saline. Extracts from seven of the nine plants were tested and passed while extracts from the other two failed (Table 2).

Table 2: Evaluation of Neurological and Motor Effects of the Extracts from Nine Selected Plants (n= 8)

Plants /dose	Plant part extract	100mg /kg			200mg /kg		
Rod height		20cm	32cm	64cm	20cm	32cm	64cm
Negative control		X	X	X	X	X	X
Albizia anthelmintica	Root	O	O	O	O	O	O
Clausena anisata	Root	X	X	X	X	X	X
Psiadia punctulata	Root	O	O	O	O	O	O
Rhus Natalensis	Root	X	X	X	X	X	X
Sapinum ellipticum	Root	X	X	X	X	X	X
Senna singueana	Root	X	X	X	X	X	X
Toddalia asiatica	Root	X	X	X	X	X	X
Teclea simlicifolia	Leaves	X	X	X	X	X	X
Warburgia ugandensis	Bark	X	X	X	X	X	X

X= Plant extract that passed the sensorimotor test;

O = Plant extract that failed the sensirimotor test.

The extract from the seven plant .that passed the sensorimotor test were used in the tail flick test.

3.2 Central Antinociceptive Effects of Extracts from the Seven Plants Using the Tail Flick Test

The seven plant extracts that had passed the sensorimotor test were evaluated for analgesic effects using the tail flick test. Acetyl salicylic acid (ASA) and morphine were used as positive controls while of 5% dimethylsulfoxide (DMSO) in normal saline was the negative controls. The positive controls induced significant antinociceptive effects ($p < 0.001$) compared to the negative controls. Two doses (100mg /kg and 200mg /kg) of each extract and eight animals were used for each dose of extract, drug or negative controls.

Administration of extracts from the plant (100 and 200 mg/Kg i. p.) given an hour prior to the test did not elicit a significant increase in the tail flick response latency (*C. anisata* (p value 0.9 and 0.4 respectively), *S. ellipticum* (p value 0.9 and 0.3 respectively), *T. simplifolia* (p value 0.7 and 0.6 respectively) or *W. ugandensis* (p value 0.7 and 0.9 respectively) compared to the negative controls (Table 3). The root extract of *S. singueana* showed no significant antinociceptive effects at dose 100 mg /kg (6.750 ± 0.14and 6.513 ± 0.212 seconds, respectively), while *T. asiatica* (6.625 ± 0.324seconds) and *R. natalensis* (5.75 ± 0.25 seconds) at dose 100 mg /kg showed significant antinociceptive effect compared to the negative controls. At 200 mg /kg dose *S. singueana* exhibited a significant antinociceptive effect (7.25 ± 0.21 seconds) while *R. natalensis* (7.00 ± 267 seconds) exhibited significant antinociceptive effect. *T. asiatica* at 200 mg /kg dose was the most potent with significant antinociceptive effect ($p < 0.001$) compared to the negative controls (Table 3).

Table 3: Central Antinociceptive Effects of the Crude Extracts from the Seven Plants Using Tail Flick Test (Mean ± SEM, n = 8, *p < 0.05, **p < 0.01 and ***p < 0.001)

TREATMENT GROUP	**Dose**	**Latency (seconds)**	**Remarks**
Negative control	0 mg /kg	5.925 ± 0.283	
Morphine	5mg /kg	8.013 ± 0.261	***
Acetyl salicylic acid (ASA)	100mg /kg	8.575 ±0.358	***
C. anisata (root extract)	100mg /kg	7.75 ± 0.796	Not significant
	200mg /kg	9.375 ± 0.706	Not significant
S. ellipticum (root extract)	100mg /kg	9.125 ± 1.076	Not significant
	200mg /kg	10.0 ± 0. 598	Not significant
W. ugandensis (bark extract)	100mg /kg	7.375 ± 0.46	Not significant
	200mg /kg	8.375 ± 0.743	Not significant
S. singuana(root extract)	100mg /kg	6.750 ± 0.14	Not significant
	200mg /kg	7.25 ± 0.21	*
R. natalensis (root extract)	100mg /kg	5.75 ± 0.25	*
	200mg /kg	7.00 ± 267	**
*T.simplifolia (*Leaf extract)	100mg /kg	7.14 ± 0.35	Not significant
	200mg /kg	7.25 ± 0.435	Not significant
T. asiatica (root extract)	100mg /kg	6.625 ± 0.324	*
	200mg /kg	7.875± 0.398	***

3.3 Peripheral and Central Antinociceptive Effects of *Rhus natalensis, Senna singuaenae* and *Toddalia asiaticaroot* Extracts Using the Hot Plate Test

Rhus natalensis, Senna singuaenae and *Toddalia asiatica root* extracts had the most potent antinociceptive effects using the tail flick assay method. Assesment of these active extracts using the hot plate test method to assess peripheral and central antinociceptive effects showed that:

R. natalensis root extract at 100 mg /kg dose (4.8 ± 0.3seconds) and 200 mg / kg (4.0 ± 0.3 seconds) did not exhibit any antinociceptive activity in the hot plate test assay compared to the negative controls (3.43 ± 0.06 seconds). *Senna singuaenae* root extract at dose 100 mg /kg dose (4.7 ± 0.3 seconds) also did not exhibit significant antinociceptive activity in the hot plate test compared to the negative controls (3.4 ± 0.1 seconds). However *S. singuaenae* 200 mg / kg dose (6.3 ± 0.5 seconds) induced a significant antinociceptive effect ($p < 0.01$) compared to the negative controls (3.4 ± 0.1 seconds). The positive control morphine (5 mg / kg) with a latency of 5.9 ± 0.3 seconds induced significant antinociception ($p < 0.001$) while acetyl salicylic acid (100 mg / kg) with a latency of 4.9 ± 0.2 seconds also showed significant antinociceptive effects ($p < 0.05$) compared to the negative controls (3.4 ± 0.1 seconds) (Table 4).

The 100 mg / kg dose of *Toddalia asiatica* root extract with a latency of 4.75 ± 0.25 seconds failed to induce any significant antinociceptive effects compared to the negative controls (4.12 ± 0.3 seconds). However the 200 mg / kg dose (6.25 ± 0.45 seconds) induced a significant antinociceptive effect ($p < 0.001$) compared to the negative controls (4.12 ± 0.3 seconds). The positive control morphine (5 mg / kg) with a latency of 7.88 ± 0.4 seconds and acetyl salicylic acid (100 mg / kg) with a latency of 5.63 ± 0.26 seconds also induced

significant antinociception ($p < 0.001$ and $p < 0.05$ respectively) compared to the negative controls (4.12 ± 0.3 seconds) (Table 4).

Table 4: Peripheral and Central Antinociceptive Effects of *Rhus natalensis, Senna singuaenae and Toddalia asiatica* Root Extracts Using the Hot Plate Test (Mean ± SEM, n = 8, *p < 0.05, **p < 0.01 and ***p < 0.001)

TREATMENT	**Dose**	**Latency (seconds)**	**Remarks**
Negative control	0 mg /kg	3.78 ± 0.04	
Morphine	5mg /kg	6.91 ± 0.34	***
Acetyl salicylic acid	100mg /kg	5.26 ± 0.22	*
Rhus natalensis root extract	100 mg /kg	4.82 ± 0.34	Not significant
	200 mg /kg	3.96 ± 0.34	Not significant
S. singuaenae root extract	100 mg /kg	4.68 ± 0.28	Not significant
	200 mg /kg	5.53 ± 0.25	**
Toddalia asiatica root extract	100 mg /kg	4.75 ± 0.25	Not significant
	200 mg /kg	6.25 ± 0.45	***

3.4 Visceral Antinociceptive Effects of *Toddalia asiatica* Root and Leaf Extracts Using Acetic Acid-Induced Writhing

T. asciatica yielded the most potent of the plant extracts. Animals treated with the leaf and root extracts were subjected to acetic acid writhing test to determine the most potent plant part. A significant reduction in the number of acetic acid-induced abdominal contractions of the extract injected mice compared to the negative control group was taken as an indication of analgesic activity. This extract was tested at three dose levels, 50, 100, and 200 mg / kg.

Animals treated with the leaf extract of *T. asiatica* (50, and 100 mg / kg, i. p.), did not exhibit significant antinociceptive effects in the acetic acid- induced abdominal contraction. The 200 mg /kg dose assays exhibited significant antinociceptive effect ($p < 0.01$) compared to the negative controls. The root extract was more potent than the leaf extract as it induced significant antinociception ($p < 0.01$) at dose 100mg / kg and 200 mg / kg (Fig 1a).

Figure 1a: Antinociceptive Effects of *Toddalia asiatica* Leaf Extract in Visceral Pain

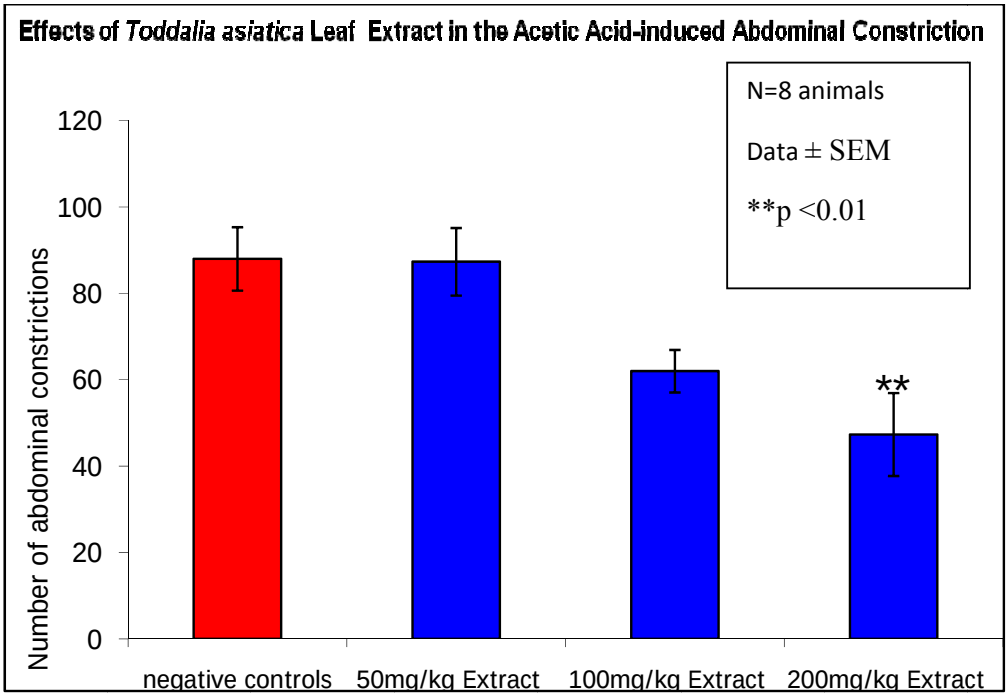
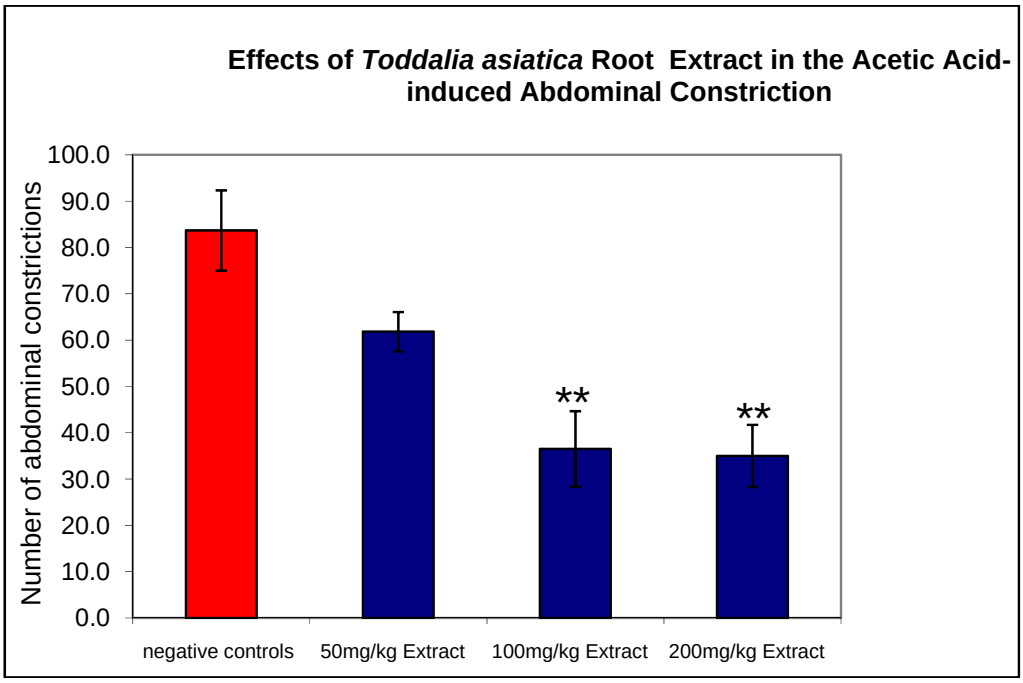


Figure 1b: Antinociceptive Effects of *Toddalia asiatica* Root Extract in Visceral Pain



3.5 Antinociceptive Effects of Root Extracts from *Toddalia asiatica* in the Moderate, Continuous Pain Using the Formalin Test

Animals treated with the root extracts from *T. asiatica* in the early phase of formalin test did not exhibit any antinociception at dose 50 mg /kg and the 100 mg /kg (207.5 ± 9.0 and 212.1 ± 7.6 seconds, respectively) compared to the negative controls (221.8 ± 10.6 sec.). The 200 mg /kg dose with a latency of 181.7 ± 4.2 seconds exhibited significant antinociceptive effect ($p < 0.01$) compared to the negative control (221.8 ± 10.6 sec.). The positive control Indomethacin 50 mg /kg (221.4 ± 6.7 sec.) showed no significant antinociceptive effects while the acetyl salicylic acid 100 mg /kg (166.7 ± 7.4) exhibited significant antinociception ($p < 0.001$) compared to the negative controls (221.8 ± 10.6 sec.) (Table 5 and Fig. 2a).

Table 5: Antinociceptive Effects of Root extract of *T. asiatica* in the Early Phase and the Late Phase of the Formalin Test (n=8, NS –not significant, **p < 0.01, ***p < 0.001)

		Time spent in pain behavior (secs.)	
Treatment Group	**Dose (mg / kg)**	Early phase(0-5min)	Late phase (15-30min)
Negative control	0	221.84 ± 10.56 NS	190.90 ± 7.47 NS
T. asiatica root extract	50	207.54 ± 9.21 NS	202.10 ± 21.29 NS
	100	212.06 ± 7.64 NS	110.58 ± 8.45***
	200	181.68 ± 4.21**	183.54 ± 11.38 NS
Indomethacin	50	221.37 ± 6.71 NS	25.33 ± 4.05***
Acetyl salicylic acid	100	166.70 ± 7.37***	153.31 ± 10.96 NS

Animals treated with 200 mg /kg dose of the root extract of *T. asiatica* one hour prior to the formalin test exhibited a significant antinociceptive effect ($p < 0.01$) in the early phase at and did not exhibit any significant antinociceptive effect in the late phase of formalin test compared to the negative controls. The 100 mg /kg dose did not exhibit any antinociceptive effect in the early phase but induced a significant antinociception ($p < 0.001$) in the late phase of formalin test compared to the negative controls (Table 5 and Fig. 2a &b).

Figure 2a: Effects of *Toddalia asiatica* RootExtract in the Early Phase of Formalin Test

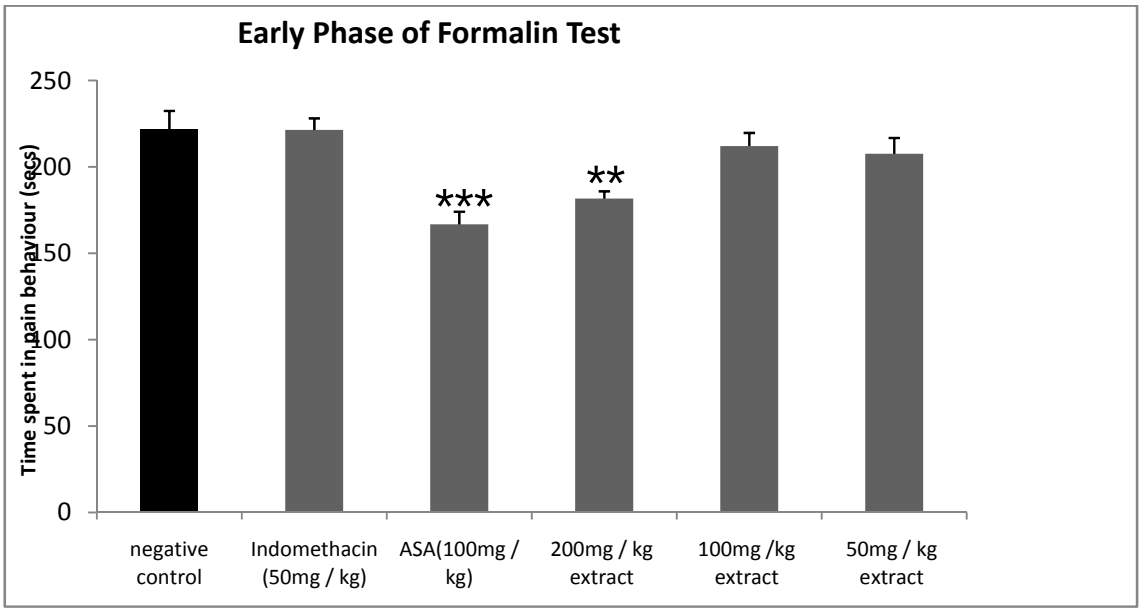
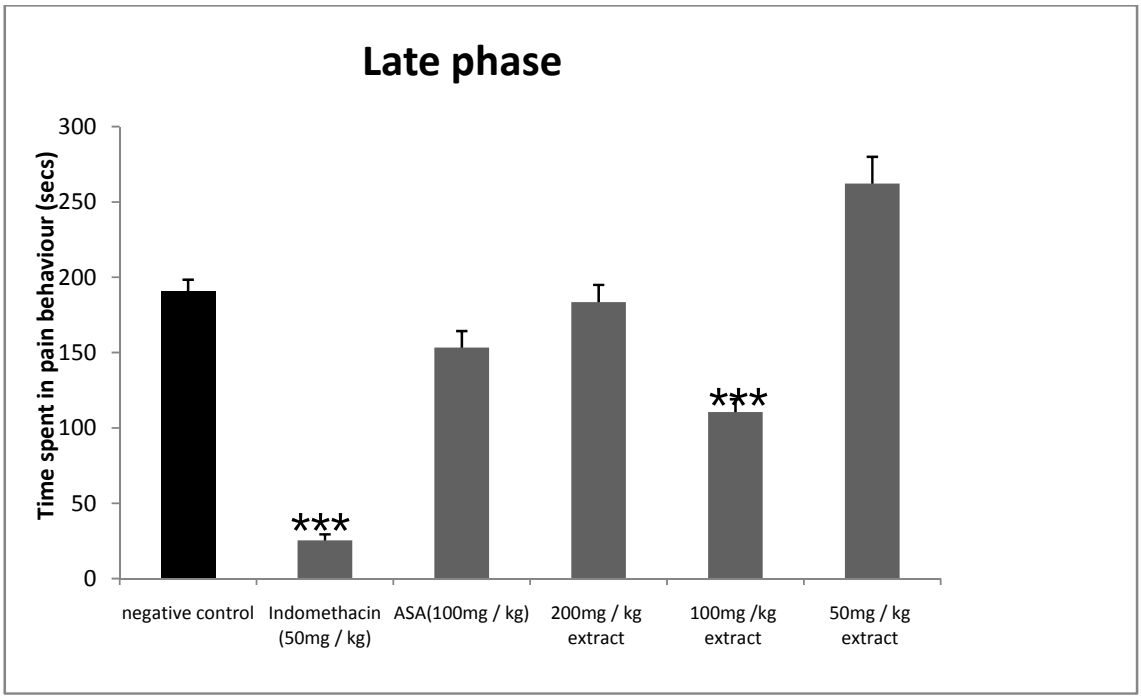


Figure 2b: Effects of *Toddalia asiatica* Root Extract in the Late Phase of Formalin Test.



3.6 Fractions from the Root Extract of *Toddalia asiatica*

The root extract of *Toddalia asiatica* was fractionated on silica gel 70/120 mesh (150 g) eluting with n-hexane-dichloromethane, 1:1 (Fraction I), dichloromethane (fraction II), dichloromethane-methanol, 9:1 (fraction III), dichloromethane-methanol, 4:1 (fraction IV), and dichloromethane-methanol, 1:1 (fraction V) Each fraction yielded approximately 400mg.

3.7 Central Antinociceptive Effects of Fractions I-V from the Root Extract of *Toddalia asiatica* Using the Tail Flick Test

Sensorimotor test: Animals were treated with the fractions I-V from the root extract of *T. asiatica* one hour prior to sensorimotor testing. All the animals exhibited good motor coordination as assessed by the level of grip on the rods.

The 50 mg /kg and the 100mg/kg doses of fraction I induced significant antinociceptive effect ($p < 0.001$) with the tail flick assay. Fraction II at 200 mg /kg and the 100mg/kg doses as well as fraction III at 50 mg /kg dose did not induce any significant antinociceptive effect ($p > 0.05$) in the tail flick assay. Fraction III at a dose 100 mg / kg exhibited significant antinociceptive ($p < 0.001$) effects in the tail flick test compared to the negative controls. The 50 mg / kg dose of fraction IV had a significant effect ($p < 0.05$) in the tail flick test while the same fraction at 100mg /kg dose induced significant antinociceptive effect ($p < 0.001$) in the tail flick assay compared to the negative controls. Fraction V at doses 50 mg / kg and 100 mg/ kg in the tail flick test assay exhibited significant antinociception ($p < 0.001$) compared to the negative controls. The level of significance is comparable to that of morphine 5 mg / kg and acetyl salicylic acid (ASA) 100 mg / kg (Table 6 and Figs 3a &3b).

Table 6: Central Antinociceptive Effects of Fractions I-Vfrom the Root Extract of *Toddalia* asiaticaUsing the Tail Flick Test (Mean ± SEM, n = 8, *p < 0.05, **p < 0.01 and ***p < 0.001)

Treatment Group	**Dose**	**TAIL FLICK Latency (secs)**	**Remarks**
Negative control	0 mg /kg	3.43 ± 0.10	
Morphine	100 mg /kg	8.35 ± 0.08	***
Aspirin	200 mg /kg	6.68 ± 0.21	***
Fraction I	50mg /kg	5.33 ± 0.2	***
	100mg /kg	5.90 ± 0.26	***
Fraction II	50mg /kg	4.23 ± 0.2	Not significant
	100mg /kg	4.34 ± 0.14	Not significant
Fraction III	50mg /kg	4.65 ± 0.21	Not significant
	100mg /kg	5.48 ± 0.2	***
Fraction IV	50mg /kg	4.79 ± 0.35	*
	100mg /kg	5.4 ± 0.3	***
Fraction V	50mg /kg	5.1 ± 0.21	***
	100mg /kg	4.97 ± 0.14	**

Figure 3 a: Central Antinociceptive Effects of Fractions from the Root Extract *Toddalia asiatica* **(100 mg / kg dose)** Using the Tail Flick Test

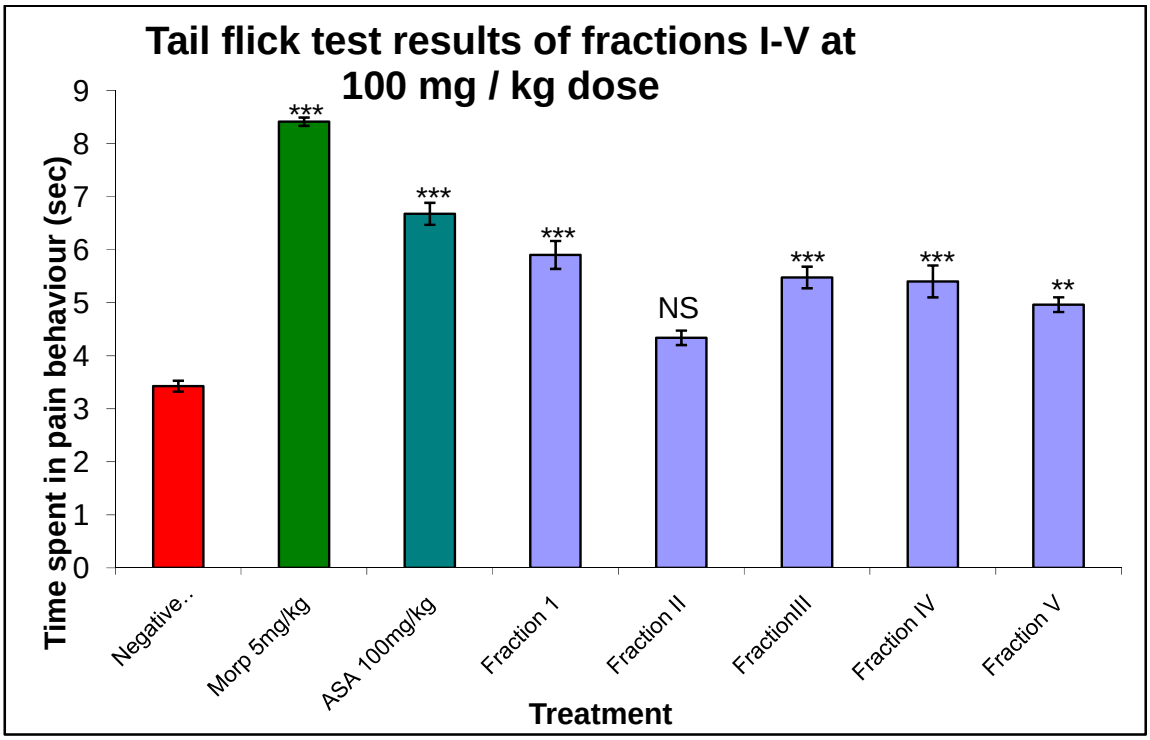
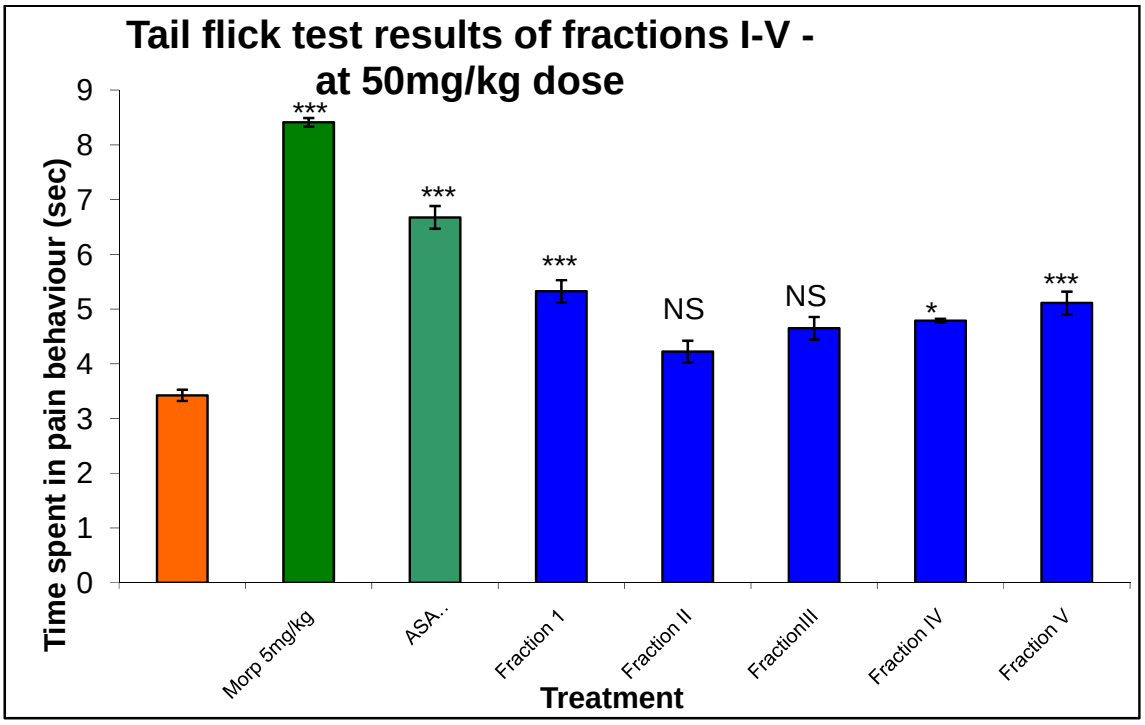


Figure 3b: Central Antinociceptive Effects of Fractions I-V of the Root Extract from *Toddalia asiatica* **(50 mg / kg dose)** Using the Tail Flick Test



3.8 Compounds Isolated from the Root Extract of *Toddalia asiatica*

The root extract of *Toddalia asiatica* (500 g) was fractionated using preparative thin layer chromatography and seven compounds isolated (Table 7).

Table 7: Compounds Isolated from the Root Extract of *Toddalia asiatica*

Compound	**HK3**	**HK5**	**HK6**	**HK7**	**HK-13**	**HK15**	**HK18**
Total Yield (mg)	199	60	37	25	20	20	50

3.9 Central Antinociceptive Effects of the Compounds Isolated from Root Extract of *Toddalia asiatica* Using the Tail Flick Test

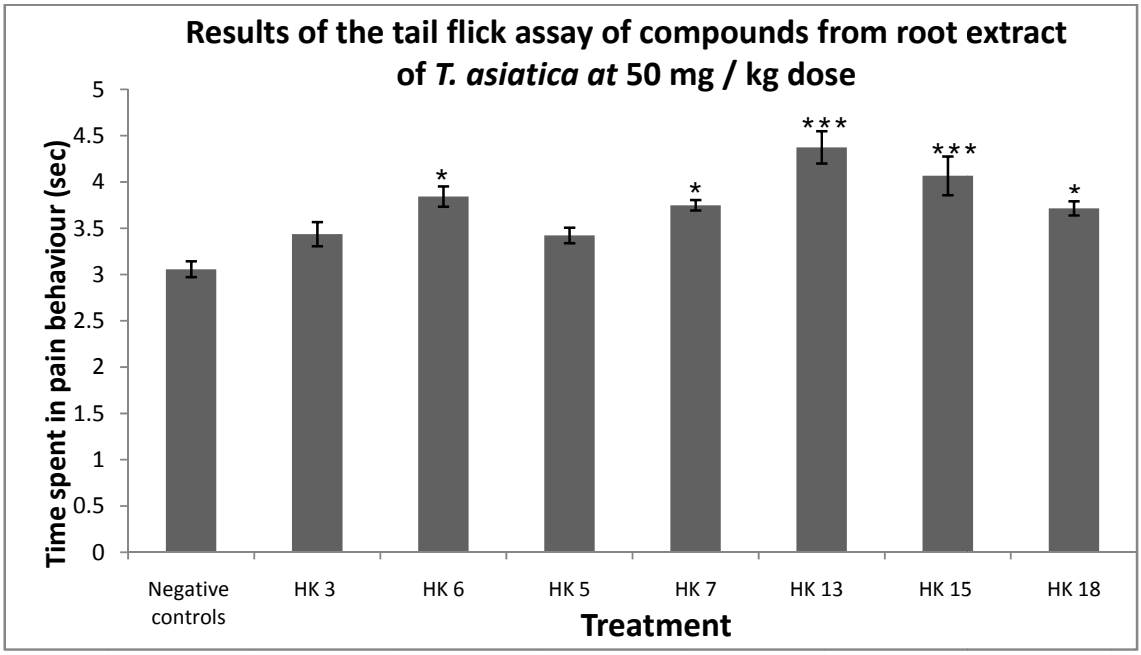
Sensorimotor test: The compounds isolated from the rooot extract of *T. asiatica* were administered to the animals one hour prior to sensorimotor testing. All the animals exhibited good motor coordination as assessed by the level of grip on the rods.

The 50 mg / kg doses of compound HK 6, HK 7 and HK 18 induced significant antinociceptive effects ($p < 0.05$) compared to the negative controls while HK15 and HK13 induced significant antinociceptive effect ($p < 0.001$) in the tail flick test. Compound HK13 was the most potent ($p < 0.001$) (Table 8 and Figure 4).

Table 8: Central Antinociceptive Effects of the Compounds Isolated from the Root Extract of *Toddalia asiatica* (Mean ± SEM, n= 8, *p < 0.05 and ***p < 0.001)

Treatment Group			**TAIL FLICK Latency (sec)**	**Remarks**
Negative control		0 mg /kg	3.058 ± 0.086	Reference
Compound isolated from the root extract of *T. asiatica*				
CODE	**NAME**	**Dose**		
HK 3	Isopimpinellin	50 mg/kg	3.436 ± 0.13	Not significant
HK5	6-(3-methylbut-2enyloxy)-8-methoxy2h-chromen-2-one)	50 mg/kg	3.422 ± 0.084	Not significant
HK 6	HK 6 is 6, 7-dimethoxy-5- (3-methyl-2-oxobutyl)-2H-chromen-2-one.	50 mg/kg	3.843 ± 0.11	*
HK 7	8-Acetonyldihydrochelerythrine	50 mg/kg	3.749 ± 0.057	*
HK 13	Dihydrochlerythrine	50 mg/kg	4.374 ± 0.175	***
HK 15	8-Oxochelerythrine	50 mg/kg	4.066 ± 0.210	***
HK 18	6-(2,3-dihydroxy-3-methylbutyl)-5,7-dimethoxy-2*H*-chromen-2-one)	50 mg/kg	3.715 ± 0.076	*

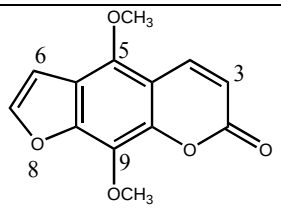
Figure 4: Central Antinociceptive Effects of the Compounds Isolated from the Root Extract of *Toddalia asiatica* Using the Tail Flick (n=8, Mean ± SEM **p < 0.01 and ***p < 0.001



3.10 Identification of Compounds Isolated from the Root Extract of *Toddalia asiatica* Using Nuclear Magnetic Resonance Spectroscopy

The chemical structures of the isolated seven compounds HK3, HK5, HK6, HK7, HK13, HK15, and HK18 were elucidated using Nuclear Magnetic Resonance (NMR) Spectroscopy. The NMR data and the proposed structures of the compounds are summarized in table 9.

Table 9a: ^{1}H and^{13}C spectroscopic data of HK-3

Position	^{1}H data	^{13}C data	
1			
2		178.0	
3	7.08	113.9	
4	7.66	140.1	
5		161.1	
6	6.39	104.9	
7	8.10	145.6	
9		150.0	
10		143.4	
11		109.6	
12		114.3	
13		144.6	
9-OCH_3	4.14	62.6	
5-OCH_3	4.03	61.4	

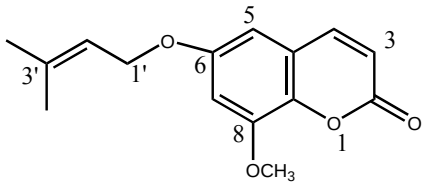
$C_{13}H_{10}O_5$

Mol. Wt.: 246.22

HK-3 (Common name: Isopimpinellin)

Class: Coumarin

Table 9b: ^{1}H and^{13}C spectroscopic data of HK-5

Position	^{1}H data	^{13}C data	
2		160.7	
3	6.25, d	113.0	
4	8.14, d	139.9	
5	6.99, s	145.3	
6		144.5	
7	7.62, s	105.2	
8		151.0	
9		139.9	
10		114.8	
1’	4.86, d	70.6	
2’	5.57, m	120.0	
3’		107.8	
8-OCH3	4.17, s	61.0	
3’-CH3	1.69, s	18.2	
3’-CH3	1.73, s	26.0	

$C_{15}H_{16}O_4$

Mol. Wt.: 260.29

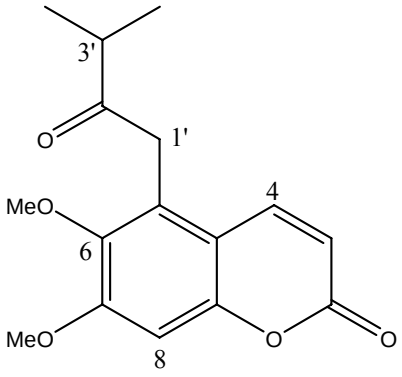
HK-5

(Common name: 6-(3-methylbut-2enyloxy)-8-methoxy2h-chromen-2-one)

Class: Coumarin

Table 9c: ^{1}H and ^{13}C spectroscopic data of HK-6

Position	1H data	13C data
1		
2		161.4
3	6.25, d	112.7
4	7.84, d	139.0
5		107.3
6		156.2
7		155.6
8	6.61, s	95.6
9		161.3
10		114.8
1’	3.79, s	40.9
2’		212.0
3’	2.78, m	35.8
3’-CH3	1.19, s	18.6
3’-CH3	1.19, s	18.6
6-OCH3	3.81, s	63.5
7-OCH3	3.76, s	56.3



$C_{16}H_{18}O_5$

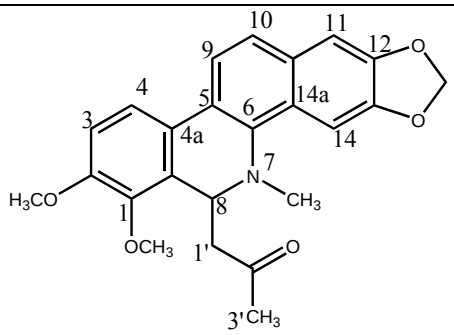
Mol. Wt.: 290.3111

HK-6 (6, 7-dimethoxy-5- (3-methyl-2-oxobutyl)-2H-chromen-2-one).

Class: Coumarin

Table 9d: ^{1}H and ^{13}C spectroscopic data of HK-7

Position	1H data	13C data
1		145.7
2		152.3
3	6.93, d	111.7
4	7.73, d	119.9
5		131.2
6		124.1
8	5.06, dd	55.1
8a		128.3
9	7.68, d	119.0
10	7.46, d	142.1
10a		123.5
11	7.10, s	104.5
12		147.8
13		148.4
14	7.52, s	100.8
14a		127.4
1’	2.30, dd	47.0
2’		207.7
3’	2.06, s	31.3
1-OCH_3	3.92, s	55.1
2-OCH_3	3.95, s	56.0
OCH_2O	6.04, s	101.2
N-CH_3	2.64, s	43.0



$C_{24}H_{23}NO_5$

Mol. Wt.: 405.44

HK-7

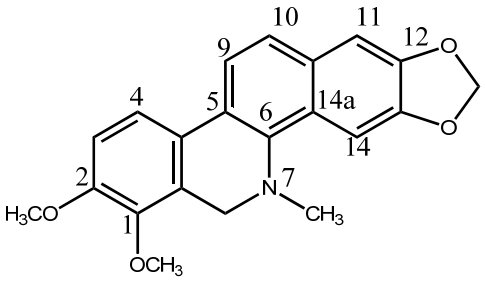
Common name:

(8-Acetonyldihydrochelerythrine),

Class: Alkaloid

Table 9e: ^{1}H and ^{13}C spectroscopic data of HK-13

Position	1H data	13C data
1	-	146.0
2	-	152.7
3	6.96, d	111.4
4	7.51, d	118.8
4a	-	126.4
5	-	
6	-	145.0
8	4.27, s	48.9
8a	-	124.6
9	7.71, d	120.4
10	7.48, d	123.9
10a	-	131.1
11	7.12, s	104.4
12		148.4
13		148.7
14	7.66, s	100.8
14a		126.4
1-OCH_3	4.11, s	61.7
2-OCH_3	4.04, s	56.8
OCH2O	6.14, s	101.7
N-CH3	2.58, s	41.4



$C_{21}H_{19}NO_4$

Mol. Wt.: 349.1314

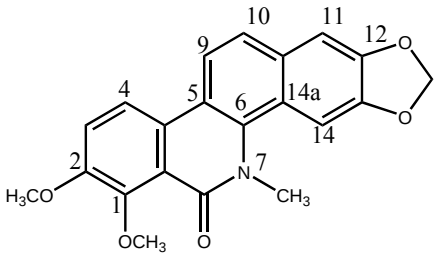
HK-13

(Common name: Dihydrochlerythrine)

Class: Alkaloid

Table 9f: ^{1}H and ^{13}C spectroscopic data of HK-15

Position	^{1}H data	^{13}C data
1		145.7
2		152.2
3	6.51, d	104.2
4	6.82, d	125.3
5		133.6
6		135.6
8		164.7
8a		146.9
9	7.28, d	127.6
10	7.74, d	127.6
10a		128.9
11	7.19, s	104.5
12		148.3
13		149.5
14	7.07, s	99.5
14a		131.4
1-OCH_3	3.90, s	61.3
2-OCH_3	3.92, s	56.0
OCH2O	6.08, s	101.7
N-CH3	2.95, s	33.2



$C_{21}H_{17}NO_5$

Mol. Wt.: 363.36

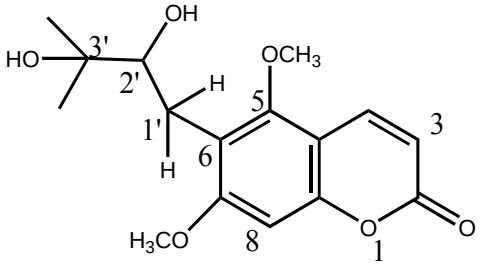
HK-15

Common name: 8-Oxochelerythrine

Class: Alkaloid

Table 9g: ^{1}H and ^{13}C spectroscopic data of HK-18

Position	^{1}H data	^{13}C data
2		161.6
3	6.25, d	113.0
4	7.82, d	138.9
5		156.2
6		107.4
7		155.2
8	6.65, s	95.9
9		161.2
10		118.0
1’	2.89, dd	26.3
	2.71, dd	
2’	3.61, dd	78.3
3’		73.1
5-OCH_3	3.89, s	56.4
7-OCH_3	3.87, s	63.4
3’-CH_3	1.29, d	23.9



$C_{16}H_{20}O_6$

Mol. Wt.: 308.33

HK-18

(Common name: 6-(2, 3-dihydroxy-3-methylbutyl)-5,7-dimethoxy-2*H*-chromen-2-one)

Class: Coumarin

CHAPTER FOUR

4.0 DISCUSSION, CONCLUSION AND RECOMMENDATIONS

4.1 DISCUSSION

Pain as a sensory modality, represents the symptom for the diagnosis of several disease conditions and physiologically has a protective function. Pain is widely accepted as one of the most important determinants of quality of life because of its widespread adverse effects, including diminishing mental health and wellbeing and impairing the individual's ability to perform daily activities. Chronic pain impacts upon a large proportion of the adult population, including the working age population, and is strongly associated with markers of social disadvantage (Blyth *et al.,* 2001). For thousands of years medicine and natural products have been closely linked prominently through the use of plant extracts. Clinical, pharmacological, and chemical researches of these traditional medicines, which are derived predominantly from plants, are the basis of many therapeutic agents. Medicinal plants contain a diversity of biologically active compounds that belong to different natural product chemical classes and man throughout history has used many different forms of medicinal plants for relief of pain. Developing treatment for pain relief has been the motivating factor behind many studies carried out in response to the demand for powerful analgesics and anti-inflammatory agents that exhibits their pharmacological response through new mechanisms of action and with fewer side effects (De Sousa, 2011).

Medicinal plants are an important local resource for most of the people living in the developing countries and WHO estimates approximately 80% of the world population relies on medicinal plants for their primary health care needs and 70% of those are in Sub-Saharan Africa (WHO, 2005). Alternative medicines have been widely used and claimed to play an important role in the healthcare as result of the high cost of medical care. Modern medicines are in inadequate supplies and have side effects associated with their uses. Most of the conventional analgesics are unsatisfactory in terms of efficacy, tolerability and toxicity (Scholz and Woolf, 2002). Some of the drugs like rofecoxib a selective Cyclooxygenase 2 enzyme (COX-2) inhibitor has been banned/ withdrawn due to its cardiotoxic effects (McGettigan and Henry, 2006). Chronic pain conditions are disabling and have an expensive socio-economic factor making the development and discovery of more efficacious drugs are priority (Apkarian *et al.,* 2009). The beliefs that plants hold the cure for many disease conditions, including painful and inflammatory conditions, are increasing and have led to a re awakening of interest in the utilization of plants and plant products.

This study was undertaken to evaluate plants that the Kenyan communities have claimed have analgesic effects and to study in more details the antinociceptive activities of the most potent plant among them. *Toddalia asiatica* was the most active and pure compounds isolated from it were assayed for antinociceptive properties using animal models.

The animals were evaluated for possible nonspecific motor or neurological effects using standard sensorimotor equipment. Root extracts of *Albizia anthelmintica* and *Psiadia punctulata* at the tested dose levels induced impaired motor function in the animals (Table 2). Extracts from the other seven plants did not affect the sensorimotor activity at the tested dose levels of the test animals indicating that they do not induce neurological deficit. Judging by

these results the use of these plants by the community will not interfere with alertness and physical activity of the user.

The Tail flick test is an easy and convenient assay method used to evaluate the antinociceptive activity of different pharmacological agents (King *et al.,* 1997).

Tail flick response is a reflex that is spinally integrated, although the response latencies have also been shown to be sensitive to pharmacological manipulation with analgesics acting at supraspinal levels (Le Bars *et al.,* 2001). In this study the tail flick test method was used as the screening test for the selected nine plant extracts as well as in assaying the fractions and the isolated compounds from root extract of *Toddalia asiatica*.

The 200 mg / kg and 100 extracts mg / kg doses of the root extracts of *C. anisata S. ellipticum W. ugandensis* and the leaf extract of *T. simplifolia* did not induce any significant central antinociceptive effect with the tested dose levels compared to the negative controls (Table 3). These extracts may be inducing antinociception at higher dose levels or may have peripheral nociceptive activity.

The root extracts of *Toddalia asiatica*, *Senna singueana* and *Rhus natalensis* induced significant central antinociceptive effects at dose 200mg / kg compared to the negative controls. *Senna singuaenae* root extract at100 mg / kg dose level did not induce any significant central antinociception. The root extract of *Rhus natalensis* at 100 and 200 mg /kg induced significant antinociception ($p < 0.01$) in the tail flick assay compared to the negative controls (Table 3). *T. asiatica* root extract was the most potent and exhibited significant antinociceptive effect ($p < 0.001$) compared to the negative controls and this was comparable

to the positive control morphine (5 mg / kg) and Acetyl Salicylic Acid (100 mg / kg). The results obtained from the tail flick assay point to central nociceptive actions of these plant extracts (Le Bars *et al.,* 2001) by acting at the spinal level as well as supra spinal level.

The hot plate test assay is used in evaluating analgesic effects of pharmacological agents in thermal nociception (Le Bars *et al.,* 2001). The response is supra spinally integrated (Bannon and Malmberg, 2001; Le Bars *et al.,* 2001). In this study, the root extract of *Rhus natalensis* at 100 and 200 mg / kg and *Senna singuaenae* 100 mg / kg did not induce any significant antinociception in the hot plate test assay compared to the negative controls. *Senna singuaenae* 200 mg /kg dose induced a significant ($p < 0.05$) antinociceptive activity compared to the negative controls (Table 4). *Toddalia asiatica* Root extract 200 mg / kg doe in the hot plate assay induced significant ($p < 0.001$) antinociceptive effect compared to the negative controls. Antinociceptive effects of *T. asiatica* were comparable to those of morphine (5mg / kg) (Table 4). Judging by these results, it can be inferred that the extracts have their antinociceptive activity supraspinally mediated (Bannon and Malmberg, 2001).

The acetic acid-induced writhing test is a simple, reproducible and sensitive method widely used for evaluating peripheral analgesic effects (Gene, *et al.,* 1998). *T. asiatica* root and leaf extract tested at 200 mg / kg induced significant ($p < 0.01$) antinociceptive effect compared to the negative controls. At 100 mg / kg dose only the root extract showed significant activity suggesting that the root extract is more potent than the leaf extract (Fig.1a and 1b). The results also indicate that *T. asiatica* has peripheral pain modulation.

The antnociceptive effects of the root extract of *Toddalia asistica* assayed using the formalin test showed significant antinociceptive activity in the early and the late phases of the formalin

test. A 200 mg / kg dose caused significant antinociceptive effects in the early phase of formalin test while the (100 mg / kg) extract caused a highly significant antinociceptive effect in the late phase. These results were comparable to those of the positive controls (indomethacin and ASA) (Table 5& Fig. 2a). The 200 mg / kg dose of the root extract did not induce any significant antinociceptive effects in the late phase of formalin test compared to the negative controls (Table 5& Fig. 2b). There was no statistically significant difference between the 200mg / kg dose of the extract and the negative control group of the late phase of formalin test. The results of the formalin test suggest that *T. asiatica* root bark extract has both peripheral and central sites of action. Since the two phases represent different types of pain, *T asiatica* compounds may be inhibiting direct stimulation of the nociceptors by the noxious stimuli as well as having anti-inflammatory effects (Le Bar *et al.*, 2001). Animal models of nociception and inflammatory reactions have been used to demonstrate various analgesic and anti-inflammatory effects of phytomedicines used in the traditional healthcare system for the management of pain and inflammation (Hollander *et. al.,* 2003).

The results of the present study indicate that the root extract of *T. asiatica* possess antinociceptive activity in chemical, thermal, and inflammatory models of pain and that the effects of the extract showed dose-dependent antinociceptive effects. The effects were less than those of reference drug ASA in the early phase, and comparable to indomethacin in the late phase of formalin test. The observed antinociceptive effects of *T. asiatica* root extract are due to the presence of biologically active chemical compounds in the extract.

Fractionation of the root extract of *T. asiatica* using silica gel 70/120-mesh column chromatography yielded five fractions. The least polar compounds were eluted with hexane: dichloromethane (1:1) while the most polar with dichloromethane: methanol (1:1). The tail flick test results obtained with the non-polar fractions showed significant antinociceptive

effects ($p < 0.001$) using the 50 mg / kg and the 100 mg / kg doses compared to the negative controls. These effects were comparable to those of positive controls (acetyl salicylic acid and morphine (Table 6). The polar fractions also induced significant antinociceptive effects with the 50mg / kg ($p < 0.001$) (Table 6 and Fig. 3a) and 100 mg/kg dose ($p < 0.01$) (Table 6 and Fig. 3b) compared to the negative controls. The fractions with compounds that have moderate polarity did not induce significant antinociceptive effect compared to the negative controls. From these results it can be inferred that the polar and non-polar compounds of *T. asiatica* have antinoceptive activity but those with moderate polarity do not (Table 6).

Fraction from the root extract of *T. asiatica* were purified using Saphradex LH20 based on molecular size separation as well as thin layer chromatography yielding seven compounds, four alkaloids and three coumarins (Table 7). The coumarins HK 3 (Isopimpinellin) and HK5 (6-(3-methylbut-2enyloxy)-8-methoxy2h-chromen-2-one), did not induce any significant antinociceptive activity in the tail flick test. Compound HK 6 is 6, 7-dimethoxy-5-(3-methyl-2-oxobutyl)-2H-chromen-2-one, HK7 (8-Acetonyldihydrochelerythrine) and HK 18 (6-(2, 3-dihydroxy-3-methylbutyl)-5,7-dimethoxy-2H-chromen-2-one) induced significant ($p < 0.05$) antinociceptive effects compared to the negative controls (Table 8). The alkaloids HK 15 (8-Oxochelerythrine) and HK 13 (dihydrochlerythrine) induced significant ($p < 0.001$) antinociceptive effects compared to the negative controls with compound HK 13 exhibiting the highest antinociceptive activity (Fig. 4).

Phytochemical studies done on *Toddalia asiatica* yielded coumarins and alkaloids. Nuclear magnetic resonance spectrometry (NMR) of the compounds revealed that the coumarins HK3 and HK5 have molecular weight of 246 and 260 respectively. Alkaloids HK 7 and HK 15 have molecular weight of 405 and 363 respectively (Table 9).

T. asiatica has been documented to possess antinociceptive / analgesic and anti-inflammatory effects (Kavimani, *et al.,* 1996; Hao *et al.,* 2004; Balasubramaniam, *et al.,* 2012). Administration of alkaloids of *Toddalia asiatica* were shown to inhibit the auricle swelling caused by xylol and joint swelling caused by agar as well leucocyte migration and decreasing the body-distortion of the rats (Hao, *et al.,* 2004) confirming a peripheral mode of action. Results of the present work have elucidated a central antinociceptive mode of action of the alkaloids isolated from the root extract of *T. asiatica*. By virtue of these findings the powerful antinociceptive activity of *T. asiatica* is scientifically supported. These findings support the widespread use of the T. asiatica in the management of pain by the communities as documented in folklore.

The proposed structure of compound HK 6 is 6, 7-dimethoxy-5- (3-methyl-2-oxobutyl)-2H-chromen-2-one. There is no documented literature on previous isolation of this compound from *T. asiatica*. This work therefore reports this structure for the first time. Additional structure elucidation work is needed to fully confirm the structure.

Alkaloid dihydrochlerythrine (HK13) was isolated from the root extract of *T. asiatica* and assayed for antinociception for the first time in this study. The alkaloid at dose 50 mg / kg, exhibited significant ($p < 0.001$) antinociception when compared to the negative controls using the tail flick assay. Judging from these results, HK13 has central antinociceptive activity.

Safety screening studies on HK 13 on various organs and body systems would be essential in establishing the value of the compound in management of pain.

4.2 CONCLUSION

Nine plants (*Toddalia asiatica*, *Senna singueana, Rhus natalensis, Teclea simplicifolia, Clausena anisata, Warburgia ugandensis, Sapium ellipticum, Albizia anthelmintica* and *Psiadia punctulata)* were evaluated for motor and / or neurological effect. Two of them (*Albizia anthelmintica* and *Psiadia punctulata)* were found to cause the sensorimotor deficit at the doses tested and were therefore not followed further.

The remaining seven were assayed for antinociception. *Teclea simplicifolia, Clausena anisata, Warburgia ugandensis, Sapium ellipticum,* did not induce significant antinociceptive effect at the doses tested and were therefore not followed further.

The remaining three plants, *Toddalia asiatica*, *Senna singueana* and *Rhus natalensis* showed significant peripheral and central antinociceptive effects in mice and did not cause motor or neurological deficits and were therefore good candidates for further work.

Toddalia asiatica was the plant with most potent antinociceptive effect compared to all the others. The root extract of *T. asiatica* exhibited significant antinociceptive effects compared to the leaf extract. *T. asiatica* root extract exhibited peripheral and central antinociceptive effects and the antinociception was comparable with that of morphine and acetyl salicylic acid.

The alkaloids were found to be more active than coumarins in antinociception and showed significant central antinociception. Coumarin HK 6 is 6, 7-dimethoxy-5- (3-methyl-2-oxobutyl)-2H-chromen-2-one, was isolated and the proposed structure elucidated using NMR. The compound induced significant antinociception compared to the negative controls.

Alkaloid dihydrochlerythrine (HK 13) exhibited significant central antinoception and did not cause any neurological or motor deficit. This is the first time this compound has been isolated from *T. asiatica* and assayed for antinociception.

The present study scientifically supports the anecdotal use of *T. asiatica* in the management of pain and identifies some of the compounds that contribute to this activity.

4.3 STUDY LIMITATIONS

The yields of the pure compounds and especially dihydrochlerythrine (HK13) were inadequate for comprehensive nociceptive screening including comparative studies with the standard analgesic drugs. Except for the known compounds reported in literature, the chemical structure of HK 6 is proposed to be 6, 7-dimethoxy-5-3-methyl-2-oxobutyl)-2H-chromen-2-one but further elucidation techniques need to be applied to confirm the structure.

4.4 RECOMMENDATION

More research needs to be done with lower doses on the plants that caused sensorimotor deficit to ascertain if they have any antinociceptive activity. Studies with higher doses are also recommended for the plants that did not exhibit any antinociception at the tested dose level, as the results may have been dose related. Future work can build and enhance the current phytochemical work as well as screen for safety of the crude extract as herbalists are still using these plants to manage pain.

The present work confirms the antinociceptive properties of the crude extract as well as the isolated compound from *T. asiatica*. More antinociceptive tests need to be done to ascertain the antinociceptive activities in chronic pain models as well comparative studies with standard analgesic drugs. Safety screening of the compounds is also recommended.

4.5 REFERENCES

Abelson, K. (2005). Acetylcholine in spinal pain modulation: An *in vivo* study in the rat. Digital Comprehensive Summaries of Upsulla Dissertations from the faculty of Medicine. 19. 55pp. Uppsula. ISBN 91-554-6178-6.

Akil, H., Mayer, D.J. and Liebeskind, J.C. (1976). Antagonism of stimulation-produced analgesia by naloxone, a narcotic antagonist. *Science,* 96: 1-2.

Al-Chaer, E. D. and Traub, R. J. (2002). Biological basis of visceral pain: recent developments. *Pain,* 96:221–225.

Almeida, T. I., Roizenblatl, S. and Tufik, S. (2004). Afferent pain pathways: a neuroanatomical review. *Brain Research,* 1000:40-56.

Alreja, M., Mutalik, P., Nayar, U., Manchanda, S.K. (1984). The formalin test: a tonic pain model in the primate.*Pain,* 20(1):97-105.

Anderson, P., Eccles, J.C. and Sears, T. A. (1964). Cortically evoked depolarization of primary fibers in the spinal cord*. Journal of Neurophysiology*, 27 issue 1: 63-77.

Apkarian, AV., Baliki, M.N., Geha, PY. (2009).pain. Progressive *Neurobiology*, 87(2):81-97.

Applebaum, A. E., Beall, K.E., Foreman, R.D. and Willis, W. D. (1975). Organization and receptive fields of primate spinothalamic tract neurons. *Journal of Neurophysiology,* 38: 572-586.

Bach, S, Noreng, M.F. and Tjellden, N.U. (1988). Phantom pain in amputees during the first 12 months following limb amputation. *Pain,* 33: 297–301.

Balasubramaniam, A., Manivannan, R., Paramaguru, R., Vijayakumar, M. (2011). Evaluation of anti- inflammatory an anti-oxidant activities of stem bark of *Toddalia asiatica* (L) Lam. Using different animal models. *African Journal of Basic and Applied Sciences, 3* (2): 39-44

Ballet, S., Conrath, M., Fiscer, J., Kaneko, T., Hamon, M. and Cesselin, F. (2003). Expression of G-protein coupling of mu-opioid receptors in the spinal cord and dorsal root ganglia of polyathritic rats. *Neuropeptides,* 37(4): 1-9.

Bannon, A., Malmberg, A. (2001). Models of Nociception: Hot-Plate, Tail-Flick, and Formalin Tests in Rodents. *Current Protocols in Neuroscience,* 41, 8.9.1-16

Bartho, L., Stein, C. and Herz, A. (1990). Involvement of capsaicin- sensitive neurons in hyperalgesia and enhanced opioid antinociception in inflammation. *Archives of pharmacology*, 342 (6): 666-670.

Bausbaum, A. I. and Fields, H.L. (1977). The dorsal lateral funiculus of the spinal cord. A major route for descending brain stem control. *Neuroscience,* 3: 499.

Beecher, H. K. (1957). The measurement of pain. *Pharmacology Reviews*, 9:59-209.

Beentje H. J. (1994). Kenya trees, shrubs and lianas. National Museums of Kenya.

Besson, J. M. and Chaouch, A. (1987). Peripheral and spinal mechanisms of Nociception. *Physiology Reviews*, 67: 67 – 186.

Bisset, N. G. (2001). Herbal Drugs and Phytopharmaceuticals, 2nd ed. CRS Press, New York, pp 342-344

Blumenthal, M. (2000). Herbal Medicines. Intergrative Medicines Communications, Austin, pp 419-423.

Blyth, F. M, March, L. M., Brnabic, A. J., Jorm, L. R., Williamson, M., Cousins, M. J. (2001). Chronic Pain in Australia: A Prevalence Study. *Pain,* 89 (2-3):127-34.

Bonica, J. J. (1990). The management of pain. 2 ed. Vol. 1. London: Lea &Febiger; 1990. History of Pain Concepts and Therapies. p. 7.

Burg, M., Heinemann, U. and Schmitz, I. (2003). Neuroactive Steroids Induce GABAA receptor mediated depolarizing post synaptic potentials in hippocampal CAI pyramidal cells of the rat. *European Journal of Neuroscience*, 9: 2880-2886.

Bussmann, R. W., Gilbreath, G. G., Solio, J., Lutura, M., Lutuluo, R., Kunguru, K., Wood, N., Simon, G. Mathenge, S. G. (2006). Plant use of the Maasai of Sekenani Valley, Maasai Mara, Kenya.*Journal of Ethnobiology Ethnomedicine, (*2) 22.

Cadwalader, W. B. and Sweet, J. E. (1912). Experimental work on the function of anterolateral column of the spinal cord. *Journal of American Medical Association*, 58: 1490 – 1493.

Cao, Y. Q., Mantyh, P. W. and Carlson, E. J. (1998). Primary afferent tachykinins are required to experience moderate to intense pain. *Nature*, 392: 390–400.

Capone, F. and Aloisi, A. M. (2004). Refinement of pain evaluation technique. The formalin test. *Annali dell'Istituto Superiori di Sanita*, 40 (2): 223-229.

Casey, K. L. X. (2004). Forebrain mechanisms of nociception and pain Analysis through imaging. *Proceedings of the National Academy of Science*, 96: 7668-7674.

Childers, S. R. (1991). Opioid receptor coupled second messenger systems. *Life Science,* 48 (21): 1991-2003.

Coderre, T. J. and Melzack, R., (1992). The contribution of excitatory amino acids to central sensitization and persistent nociception after formalin-induced tissue injury. *Journal of Neuroscience.* 12, 3665-3670.

Coderre, T.J., Katz, J., Vaccarino, A.L. and Melzack R. (1993). Contribution of central neuroplasticity to pathological pain: A review of clinical and experimental evidence. *Pain,* 52: 259-285.

Collier, H. O. J., Dineen, L. C., Johnson, C. A. and Schneider, C. (1968). The abdominal constriction response and its suppression by analgesic drugs in the mouse. *British Journal of Pharmacological Chemistry,* 32: 295 – 310.

Corrêa, C.R., Kyle, D. J., Chakraverty, S. and Calixto, J. B. (1996). Antinociceptive profile of the pseudopeptide B_2 bradykinin receptor antagonist NPC 18688 in mice. *British Journal of Pharmacology,*117:552–558.

Cross, S., A. (1994). Pathophysiology of Pain. *Mayo Clinic Proceedings*, 69: 375-383.

D'amour, F. E. and Smith, D. L. (1941).A method for determining loss of pain sensation. *Journal of Pharmacology and Experimental Therapeutics*, 72 (1): 74-79.

Dallenbach, K. M. (1939). Pain: History and present status. *American Journal of Psychology*, 52: 331–347.

Davis, L., Pollock, L. J. and Stone, T. T. (1932). Visceral pain. *Surgical Gynecology and Obstetrics,* 55: 418.

De Sousa, D.P. (2011). Analgesic-like activity of essential oils constituents.*Molecules*. 7; 16 (3): 2233-52.

Dennis, S. G. and Melzack, R. (1983). Effects of cholinergic and dopaminergic agents on pain and morphine analgesia as measured by three pain tests. *Neurology,* 81: 167-176.

Deutsch, S. I. (1992). GABA-active steroid: endogeneous modulators of GABA- gated chloride ion conductance. *Clinical Neuropharmacol*ogy, 15: 352-364.

Dolan, S. and Nolan, A. M. (1999). N-methyl-D-aspartate induced mechanical allydonia is blocked by nitric oxide synthetase and cyclooxygenase-2 inhibitors. *Neurology Report*, 10: 449-452.

Dolan, S., Field, L. C. and Nolan, A. M. (2000). The role of nitric oxide and prostaglandin signaling pathways in spinal nociceptive processing in chronic inflammation. *Pain,* 86: 311-320.

Dubner, R. and Ren, K. (1999). Endogenous mechanisms of sensory modulation. *Pain*, 82, (1), S45–S53.

Dubuisson, D. and Dennis, S. G. (1977). The formalin test: A quantitative study of the analgesic effects of morphine, meperidine, and brain stem stimulation in rats and cats. *Pain, 4*: 161-174.

Emele, J. F. and Shanaman, J. (1963). A method for measuring analgesia. *Proceedings of the Society of Experimental Biology*, 114: 680 – 682.

Farnsworth, N. R. (1989). Screening plants for new medicines. In Wilson E.O. Editor, Biodiversity, Part II, National Academy Press, Washington, pp 83-97.

Fasmer, O. B., Berge, O. G., Treiter, L. and Hole, K. (1986). Changes in nociception after 6 – hydroxydopamine lesions of descending catecholaminergic pathways in mice. *Pharmacology Biochemistry and Behaviour*, 24: 1441 – 1444.

Ferreira, S. H. and Vane, J. R. (1974). New aspects of mode of action of non-steroid anti-inflammatory drugs. *Annual Review of Pharmacology and Toxicology*, 14: 57-73.

Ferreira, S. H. (1983). Prostaglandins, aspirin like drugs and analgesia. *Nature New Biology*, 240: 200-203.

Ferreira, S. H., Lorenzetti, B. B. and Correa, F. M. A. (1978). Central and peripheral analgesic action of aspirin like drugs. *European Journal of Pharmacology,* 53: 29-48.

Fertz, E. E. (1968). Pyramidal tract effects on interneurones in the cat lumbar dorsal horn. *Journal of Neurophysiology*, 31: 69 – 80.

Fields, H. L. and Bausbaum, A. I. (1999). Central nervous system mechanisms of pain modulation. In Wall PD, Melzack R (eds) Textbook of Pain, 4th ed. Churchill Livingstone, Edinburgh, 309-330.

Finger, S. (2001). Origins of neuroscience: a history of explorations into brain function. USA: Oxford University Press;

Franklin, K. B. and Mathies, B. K. (1992). Formalin pain is expressed in decerebrate rats but not attenuated in morphine. *Pain,* 51:199-206.

Furst, S. (1999). Transmitters involved in antinociception in the spinal cord. *Brain Research Bulletin,*48: 129-141.

Gamse, R., Holzer, P. and Lembeck, F. (1979). Indirect evidence for presynaptic location of opiate receptors on chemosensitive primary sensory neurones. *Archives of Pharmacology*, 308: 281-285.

Gendron L., Lucido, A. L., Mennicken, F., O'Donnell, D., Vincent, J. P., Stroh, T. and Beaudet, A. (2006). Morphine and Pain-Related Stimuli Enhance Cell Surface Availability of Somatic δ-Opioid Receptors in Rat Dorsal Root Ganglia. *Journal of Neuroscience,* 26:953 -962.

GenéRosa M., Segura Laura, TomàsAdzet, Esther Marin, José Iglesias(1998).*Heterothecainuloides*: Anti-inflammatory and analgesic effect. *Journal of Ethnopharmacology,* 60, 2, 157–162

Giamberardino, M. A. (2000). Visceral hyperalgesia. In proceedings of the World conference on Pain in *Progress in Pain Research and Management*, *16*, 523-550.

Giordano, J. (2005). The neurobiology of nociceptive and antinociceptive systems. An invited review. *Pain Physician Journal*, 8: 277-290

Gutstein, H. B., Rubie, E. A., Mansour, A., Akil, H. and Woodgett, J. R. (1997). Opioid effects on mitogenactivated protein kinase signaling cascades. *Anaesthesiology*, 87: 1118-1126.

Guzman, F. and Lim, R. K. S. (1968). The mechanism of action of non narcotic analgesics. *Medical Clinics of North America,* 52: 3-14.

Haley, I. E., Sullivan, A. F. and Dickenson A. H. (1990b). Evidence of spinal N-methyl-D aspartate receptor involvement in prolonged chemical nociception in the rat. *Brain Research*, 518: 218-226.

Handwerker, H. O., Neher, K. D. (1976). Characteristics of C-fibre receptors in the cat's foot responding to stepwise increase of skin temperature of noxious levels. *Pflugers Archives*, 30; 365 (2-3):221-9.

Hao, X.Y., Peng, L., Yeo, L., Huang, N. H., Shen, Y. M. (2004). A study on anti-inflammatory and analgesic effects of alkaloids from *Toddalia asiatica. Journal of Chinese Integrative Medicine*, 6:450-452[Abstract]

Hardy, J. D., Wolff, G. and Goodell, H. (1950). Experimental evidence on the nature of cutaneous hyperalgesia. *Journal of Clinical Investigations*, 29: 115-140.

Harris, J. A. (1996). Descending antinociceptive mechanisms in the brainstem: Their role in the animal's defensive system. *Journal of Physiology (Paris)*, 90:15-25.

Hole, K. and Tjolsen, A. (1993). The tail flick and formalin tests in rodents; changes in skin temperature as a confounding factor. *Pain,* 53: 247-254.

Hollander, C., Nystrom, M., Janciauskiene, S. and Westin U. (2003). Human mast cells decrease SLPI levels in type II-like alveolar cell model in vitro. *Cancer Cell International,* 3, 14–22.

Hornak, J. P. (1997). The Basics of NMR: A non-technical overview of NMR theory, equipment, and techniques. http://www.cis.rit.edu/htbooks/nmr/Retrieved, 2013-02-23.

Hudspith, M. J., Siddall, P. J. and Munglani, R. (2005). Physiology of Pain. In: Hemmings HC, Hopkins PM, (eds). Foundations of Anaesthesia. *2nd ed*. Elsevier, 2000, pp. 267–286.

Hunskaar, S. and Hole, K. (1987). The formalin test in mice: dissociation between inflammatory and non-inflammatory pain. *Pain,* 30: 103-114.

Hunskaar, S., Berge, O-G., Hole, K. (1986a). Dissociation between antinociceptive and anti-inflammatory effects of acetylsalicylic acid and indomethacin in the formalin test. *Pain,* 25(1):125-32.

Hunskaar, S., Berge, O-G. and Hole, K. (1986b). A modified hot-plate test sensitive to mild analgesics. *Behavioural Brain Research*, 21:101-108.

Hunskaar, S., Fasmer, O. B. and Hole, K. (1985). Formalin test in mice, a useful technique for evaluating mild analgesics. *Journal of Neuroscience Methods*, 14:69-76.

I.A.S.P. (1979). Pain terms: a list with definitions and notes on usage. Recommended by an I.A.S.P. subcommittee on taxonomy. *Pain,* 6: 249-252.

Iwamoto, E. T. (1991). Characterization of the antinociception induced by nicotine in the pedunculopontine tegmental nucleus and the nucleus raphe magnus. *Journal of Pharmacology and Experimental Therapeutics,* 257: 120-133.

Jeruto, P., Lukhoba, Ouma, G., Otieno, D, Mutai, C. (2008). An ethnobotanical study of medicinal plants used by the Nandi in Kenya. *Journal of Ethnopharmacology,* 116 (2): 370-376

Ji, R. R., Zhang, Q., Law, P. Y., Low, H. H., Elde, R. and Hokfelt, T. (1995). Expression of mu, delta and kappa opioid receptor- like immunoreactivities in rat dorsal root ganglia after carrageenan-induced inflammation. *Journal of Neuroscience*, 15: 815-8166.

Jones, W.P., Kinghorn, A.D., (2005). Natural product isolation: Extraction of Plant Secondary Metabolites. *Methods in Biotechnology 20:323*-351.

Junger, H., Moore, A. C. and Sorkin, L. S. (2002). Effects of full- thickness burns on receptor sensitization in anaesthetized rats. *Burns*, 8: 772-777.

Kabli, N. and Cahill, C. M. (2007). Anti-allodynic effects of peripheral delta opioid receptors in neuropathic pain. *Pain*, 127: 84-93.

Kandel, E. R. and Schwartz, J. H. (2005). Somatic Sensory system IV: Central representation of pain and analgesia. In: Principles of neuroscience. Edward Arnold (publishers) Ltd., Pg. 204 -209.

Kanui, T.I and Hole, K. (1990). Morphine induces aggression but not analgesia in the naked mole-rat (*Heterocephalus glaber*). *Comparative Biochemistry and Physiol*ogy, 96: 131–133.

Kavaliers, M. (1988). Evolutionary and comparative aspects of nociception. *Brain Research Bulletin*, 21: 923-931.

Kavimani S., Vetrichelvan, T., Ilango, R., Jaykar, B. (1996). Anti-inflammatory activity of volatile oil of *Toddalia asiatica. Indian Journal of Pharmaceutical Sciences,* 58(2):67-70

Keeler, H. (2007). Understanding NMR spectroscopy. Lectures by James Keeler, University of Cambridge. http://www-keeler.ch.cam.ac.uk/lectures/Irvine/ Retrieved 2013-02-24.

Kiefer, B. L. (1995). Recent advances in molecular recognition and signal transduction of active peptides: receptors for opioid peptides. *Cellular and Molecular Neurobiology*, 15: 615-653.

King, T. E. Joynes, R. L., Grau, J. W. (1997). Tail-flick test: II. The role of supraspinal systems and avoidance learning.*Behavioural Neuroscience*, 111(4):754-67.

Kokwaro, J. O. (2009). Medicinal Plants of East Africa: Third Edition. Nairobi: University of Nairobi Press, 478 Pp.

Kondo, I., Marvizon, J. C., Song, B., Salgado, F., Codeluppi, S. and Yaksh, T. L. (2005). Inhibition by spinal mu and delta agonists of afferent evoked substance P release. *Journal of Neuroscience*, 25: 3651-3660.

Koster, R., Anderson, M. and De Beer, E. J. (1959). Acetic acid for analgesic screening. *Federation Proceedings,* 8: 412.

LaMotte, R. H., Shain, C. N., Simone, D. A. and Tsai, E. F. (1991). Neurogenic hyperalgesia: psychophysical studies of underlying mechanisms. *Journal of Neurophysiology*, 66:190-211.

LaMotte, R. H., Thalhammer, J. G.,Torebjork, H. E. and Robinson, C.J. (1982). Peripheral neural mechanisms of cutaneous hyperalgesia following mild injury by heat. *Journal of Neuroscience*, 6: 765-781.

Le Bars, D., Gazariu, M. and Cadden, S.W. (2001). Animal models of nociception. *Pharmacology Reviews,* 53: 597-652.

Li, J. L., Ding, Y. Q., Li, Y. Q., Li, J. S., Nomura, S., Kaneko, T. and Mizuno, N. (1998). Immunocychemical localization of mu opioid receptor in primary afferent neurons containing substance P or calcitonin gene-related peptide. A light and electron microscope study in the rat. *Brain Research,* 794: 347-352.

Lu, S.Y., Qiao, Y.J., Xiao, P.G., Tan, X.H. (2005). Identification of ant-viral activity of *Toddalia asiatica* against influenza type A virus. *ZongguoZong Yao ZaZhi,* 13: 998-1001

Majewska, M. D. (1987). Action of steroids on neurons: role in personality, mood, stress and disease. *Integrative Psychiatry*, 5: 258-273.

Mansour, A., Watson, S. J. and Akil, H. (1995). Opioid receptors: past, present and future. *Trends in Neurosciences,* 18: 69-70.

Mao, J. (2004). NMDA and opioid receptors: Their interaction in antinociception, tolerance and neuroplasticity. *Brain Research Reviews,* 30: 289-304.

Mascolo, N., Pinto, A., Capasso, F., Yenesew, A. and Dagne, E. (I988). Antipyretic and analgesic studies of the ethanolic extract of *Tecleanobilis* (delile) *Phototherapy Research*, 2 (3): 154-156.

Mattison, N., Trimple, A. G. and Larsen, I. (1998). New drug development in the United States, 1963 through 1994. *Clinical Pharmacology Therapeutics Journal,* 43: 290-301.

Maundu, P. M. (1999). Traditional food plants of Kenya. National Museums of Kenya.

McCurdy, C. R. and Scully, S. S. (2005). Analgesic substances derived from natural products. Natureceuticals. *Life Science*, 78 (5): 476-484.

McGettigan, P. and Henry, D. (2006). Cardiovascular Risk and Inhibition of Cyclooxygenase A; Systematic Review of the Observational Studies of Selective and Nonselective Inhibitors of Cyclooxygenase 2. *Journal of American Medical Association,* 296 (13):1633-1644.

Meldrum, M. (2003). "A History of Pain Management." Opioids : Past, Present and Future. *Journal of American Medical Association*, 290:2470

Meller, S. T., Cummings, C. P., Traub, R. J. and Gebhart, G. F. (1994). The role of nitric oxide in the development and maintenance of the hyperalgesia produced by intraplanter injection of carrageenan in the rat. *Neuroscience*, 60: 367-374.

Melzack, R. & Casey, K.L. (1968). Sensory motivational and central control determinants of pain: A new conceptual model. In D. Kenshalo (Ed.), The Skin Senses (pp. 423-443), Springfield: Thomas.

Melzack, R. and Katz, J. (2004). The Gate Control Theory: Reaching for the Brain. In: Craig KD, Hadjistavropoulos T. Pain: psychological perspectives. Mahwah, N.J: Lawrence Erlbaum Associates,

Melzack, R. and Wall, P. D. (1965). Pain mechanisms: a new theory. *Science*, 150 (3699): 971–9.

Mense, S. (1993). Nociception from skeletal muscle in relation to clinical muscle pain. *Pain*, 4: 241-289.

Merskey, H., Mumford, J. M., Nathan, P. W., Noordenbos, W. and Sunderlard, S. (1986). Pain terms, part II. *Pain Supplement,* 3: S216.

Meyer, R. A. and Campell, J. N. (1981). Myelinated nociceptive afferents account for the hyperalgesia that follows a burn to the hand. *Science,* 231: 1527-1529.

Millan, M. J. (1999). The induction of pain: An integrative review. *Progressive Neurobiology,* 54: 1-164.

Minami, M., Maekawa, K., Yabuuchi, K. and Satoh, M. (1995). Double in situ hybridization study on coexistence of mu, kappa and delta opioid receptor mRNAs with preprotachykininA mRNA in the rat dorsal root ganglia. *Brain Research,* 30: 203-210.

Minja, M. M. J. (1999). The Maasai Wonder Plants. Paper presented at the 'People and Plants' training workshop held at the Tropical Pesticides Research Institute, Arusha, Tanzania, 15th - 18th march 1999. Registered in the data bank prelude; REF**:** HM 09.

Mousa, S. A., Machelska, H., Schafer, M. and Stein, C. (2002). Immunohistochemical localization of endomorphin-1 and endomorphin -2 in immune cells and spinal cord in a model of inflammatory pain*. Journal of Neuroimmunology*, 126: 5-15.

Mousa, S. A., Straub, R. H., Schafer, M. and Stein, C. (2007). Beta- endorphin, met-enkephalin and corresponding opioid receptors within synovium of patients with trauma, osteoarthritis and rheumatoid arthritis.*Annals of the Rheumatic Diseases*, 66: 871-879.

Muller, J. (1842). Elements of Physiology. 3rd Edition Pp142. Taylor, London.

Nabwami, J., Tabuti, J., Bekundai, M. A. (2007). Characterization of the natural habitat of *Toddalia asiatica* in the Lake Victoria basin: soil characteristics and seedling establishment.*African Crop Science Conference Proceedings,* (8) 2057-2061

Nakamura-Craig, M. and Smith, T. W. (1989). Substance P and peripheral inflammatory hyperalgesia. *Pain*, 38: 91-98.

Orwa, J. A, Ondiko, I. J. O., Minja, R. J. A. , Bekunda, M. (2008). Use of *Toddalia asiatica* (L) Lam. (Rutacae) in traditional medicine practice in East Africa. *Journal of Ethnopharmacology,* 115(2): 257-262

Ossipov, M. K., Lai, J., King, T., Vanderah, T. W., Malan, T. P. J., Hruby, V. J. and Porreca, R. (2004). Antinociceptive and nociceptive actions of opioids. *Journal of Neurobiology*, 61: 126-148.

Ottoson, D. (1983). Pain. In Physiology of the Nervous System. 2nd Edition. The McMillan Press Ltd, Pp 458-502.

Peter, J. H. and Amala, R. (1998). Laboratory Handbook for the Fractionation of Natural Extracts. Chapman and Hall, London. Pp 52.

Pieters, L. A. and Vlietinck, A. J. (2005). Bio-guided isolation of pharmacologically active plant components, still a valuable strategy for finding new lead compounds. *Journal of Ethnopharmacology*, 100: 57-60.

Price, D. D. (1999). Physiological mechanisms of pain and analgesia. In Progress in Pain Research and Management.Vol. 75, IASP Press, Seattle.

Price, D. D. and Mayer, D. J. (1974). Physiological Laminar organization of the dorsal horn of *M .mulatta. Brain Research*, 79: 321 – 325.

Puehler, W., Zollner, C., Brack, A., Shaqura, M. A., Krause, H., Schafer, M. and Stein, C. (2004). Rapid upregulation of mu opioid receptor mRNA in dorsal root ganglia in response to peripheral inflammation depends on neuronal conduction. *Neuroscience,* 129:473-479.

Raja, S. N., Campell, J. N. and Meyer, R. A. (1984). Evidence for different mechanisms of primary and secondary hyperalgesia following heat injury to the glabrous skin. *Brain,* 107: 1179-1188

Ramwell, P. W., Shaw, J. E. (1966). Spontaneous and evoked release of prostaglandins from cerebral cortex of anesthetized cats.*American Journal of Physiology*, 211(1):125-34.

Ramwell, P.W., Shaw, J. E. and Jessol, R. (1966). Spontaneous and evoked release of prostaglandins from frog spinal cord. *Journal of Physiology*, 211: 998-1004.

Rau, K. K., Caudle, R. M., Cooper, B. Y. and Johnson R. D. (2005). Diverse immunocychemical expression of opioid receptors in electrophysiologically defined cells of dorsal root ganglia. *Journal of Chemical Neuroanatomy*, 29: 255-264.

Riedel, W. and Neeck, G. (2001). Nociception, pain, and antinociception: Current concepts. *Z. Rheumatology*, 60: 404-415.

Rosland, J. H. (1991). The formalin test in mice: the influence of ambient temperature. *Pain,* 45: 211 – 216.

Rosland, J. H., Tjolsen, A., Maehle, B. and Hole, K. (1990). The formalin test in mice- effect of formalin concentration. *Pain,* 42: 235-242.

Sahley, T. L. and Berston, G. G. (1979). Antinociceptive effects of central and systemic administration of nicotine in the rat. *Psychopharmacology,* 65: 276-283.

Sarker, S. D., Latif, Z. and Gray, A. I. (2007). Natural Products Isolation. In *Kluwer international series in engineering and computer science: Power electronics & power systems*. Satyajit D. Sarker, Zahid Latif, Alexander I. Gray (Eds). Vol. 20; 2nd ed. Humana Press Inc. Tolowa N.J.

Saunders, C. and Limbird, L. E. (1999). Localization and trafficking of alpha2-adrenergic receptor subtypes in cells and tissues. *Pharmacology and Therapeutics,* 84: 193-205.

Schlage, C., Mabula, C., Mahunnah, R. L. A. and Heinrich, M. (1999). Medicinal Plants of the Washambaa (Tanzania): Documentation and Ethnopharmacological Evaluation. *Plant Biology*, 2: 83-93.

Schmidt, B.M., Ribnicky, D. M., Lipsky P. E., Raskin I. Nat. (2007).*Chemical Biology,* 3:360–366.

Scholz, J., Woolf, C. J. (2002). Can we conquer pain?*Nature Neuroscience*, 5: 1062-7.

Seltzer, Z., Dubner, R. and Shir, Y. (1990). A novel behavioral model of neuropathic pain disorders produced in rats by partial sciatic nerve injury. *Pain,* 43:205—218.

Shah, N, Sattar, A, Benanti, M, Hollander, S, Cheuck, L. (2006)."Magnetic resonance spectroscopy as an imaging tool for cancer: a review of the literature.".*The Journal of the American Osteopathic Association*106 (1): 23–27.

Shaqura, M. A., Zollner, C., Mousa, S. A., Stein, C. and Schafer, M. (2004). Characterization of mu opioid receptor binding and G-protein coupling in rat hypothalamus, spinal cord and primary afferent neurons during inflammatory pain. *Journal of Pharmacology and Experimental Therapeutics*, 308: 712-718.

Shibata, M., Ohkubo, T., Takahashi, H. and Inoki, R. (1989). Modified formalin test: characteristic biphasic response. *Pain,* 38:347-352.

Shibata, M., Ohkubo, T., Takahashi, H. and Inoki, R. (1989). Modified formalin test: characteristic biphasic pain response. *Pain,* 38, 347-352.

Siddall, P. J. and Cousins, M. J. (1998). Introduction to pain mechanisms. In: Cousins M. J and Bridenbaugh P. O., eds. Neural Blockade in Clinical Anesthesia and Management of Pain. Philadelphia: Lippincott-Raven, 675-713.

Silbert, S.C., Beacham, D. W. and McCeskey, E. W. (2003). Quantitative single cell differences in mu opioid receptor mRNA distinguish myelinated and unmyelinated nociceptors. *Journal of Neuroscience,* 23: 34-42.

Simonin, F., Gaveriaux-Ruff, C., Befort, K., Matthes, H. and Lannes, B. (1995). Kappa – opioid receptors in humans: cDNA and genomic cloning, chromosomal assignment,

functional expression, pharmacology and expression pattern in the central nervous system. *Proceedings of the National Academy of Science.* 92(15): 7006-7010.

Skilling, S. R, Smullin, D. H, Larson, A. A. (1990). Differential effects of C- and N-terminal substance P metabolites on the release of amino acid neurotransmitters from the spinal cord: potential role in nociception. *Journal of Neuroscience,* 10 (4):1309–1318.

Sorkin, L. S. and McAdoo, D. J. (1993). Amino acids and serotonin are released into the lumbar spinal cord of the anesthetized cat following intradermal capsaicin injection. *Brain Research*, 607: 89-98

Stander, S., Gunzer, M., Metze, D., Luger, T. and Steinhoff, M. (2002). Localization of mu receptors 1A on sensory nerve fibre in human skin. *Regulatory Peptides Journal,* 110: 75-83.

Stein, C., Clark J. D., Vasco, M, R., Wilcox, G. R., Overland, A. C., Vanderah, T. W. and Spencer, R. H. (2009). Peripheral mechanisms of pain and analgesia. *Brain Research Review,* 60: 90-113.

Takahashi, H., Shibata, M. and Naruse, S. (1984). A modified formalin test for measuring analgesia in mice. *Japanese Journal of Oral Biology,* 26:543-548.

Thienhaus, O. and Cole, B. E. (2002). The classification of pain". In Weiner, Richard S. *Pain management: A practical guide for clinicians*. CRC Press. p. 29.

Tjølsen, A., Berge, O.G., Hunskaar, S., Rosland, J.H. and Hole, K. (1992). The formalin test: an evaluation of the method. *Pain*, 51(1): 5-17.

Towett, P. K. and Kanui, T. I. (1993). Effects of Pethidine on thermal pain and behaviour in the mole-rat. *Pharmacology Biochemistry and Behaviour*, 45, 153-159.

Troung, W., Cheng, C., Xu, Q. G., Li, X. Q. and Zochodne, D. W. (2003). Mu opiod receptors and analgesia at the site of peripheral nerve injury. *Annals of Neurology*, 53: 366-375.

Turk, D. C.; Okifuji, A. (2001)."Pain terms and taxonomies". In Loeser, D.; Butler, S. H.; Chapman, J.J. (Eds). 3rd edition of Bonica's management of pain. Lippincott Williams & Wilkins. pp. 18–25.

Vane, J. R. and Botting, R. (1987). Inflammation and the mechanism of action of anti-inflammatory drugs. *Journal of the Federation of American Societies for Experimental Biology*, I 1: 89-96.

Walczak, P., Konopacka, M., Otlewska, A. (2005). Genetic diversity among Lactococcus sp. and Leuconostoc sp. strains using PCR-RFLP of insertion sequences ISS1-type, IS904, and IS982.*Polish Journal of Microbiology*, 54 (3):183-9.

Wall, P. D. (1967). The lamina organization of dorsal horn and effects of descending impulses. *Journal of physiology*, 188: 403 – 424.

Wang, J. and Wessendorf, M. W. (2001). Equal portions of small and large DRG neurons express opioid receptor mRNAs. *Journal of Comparative Neurology*, 429: 590-600.

Weber, E. H. (1978). The Sense of Touch. London Academic Press. 2nd edition Pp 52-58. Original work published in 1934.

Wemmer, D. (2000). Structure and Dynamics by NMR. In Bloomfield, Victor A., Crothers, Donald M., and Tinoco, Ignacio. *Nucleic acids: Structures, Properties, and Functions*. Sausalito, California: University Science Books.

Willis, W. D. and Westlund, K. N. (1997). Neuroanatomy of the pain system and of the path-ways that modulate pain. *Clinical Neurophysiology*, 14: 2-31.

Wills, W. D. and Coggeshall, R. E. (1978). Sensory mechanism of the spinal cord. Wills, W.D. and coggeshall, R.E. 9 eds. Prenun. Press, New York Pp: 53- 124.

Wolfe, G. and Macdonald, A. D. (1944). The evaluation of analgesic action of pethidine hydrochloride (Demerol). *Journal of Pharmacology and Experimental Therapeutics*, 120: 52-57.

Woolf, C. J., Chong, M. S. (1993). Preemptive analgesia--treating postoperative pain by preventing the establishment of central sensitization. *Anesthesia and Analgesia*, 77 (2): 362-79.

World Health Organization. (2002). Traditional Medicines Strategy 2002-2005.

Xu, Z., Tong, C., Pan, H. L., Cerda, S. E. and Eisenach, J. C. (1997). Intravenous morphine increases release of nitric oxide from spinal cord by an α-adrenergic and cholinergic mechanism. *Journal of Neurophysiology*, 78: 2072-2078.

Yaksh, T. L., Plant R. L. and Rudy, T. A. (1988). Studies on the antagonism by raphe lesions of the antinociceptive action of systemic morphine. *European Journal Pharmacology*, 41: 399-408.

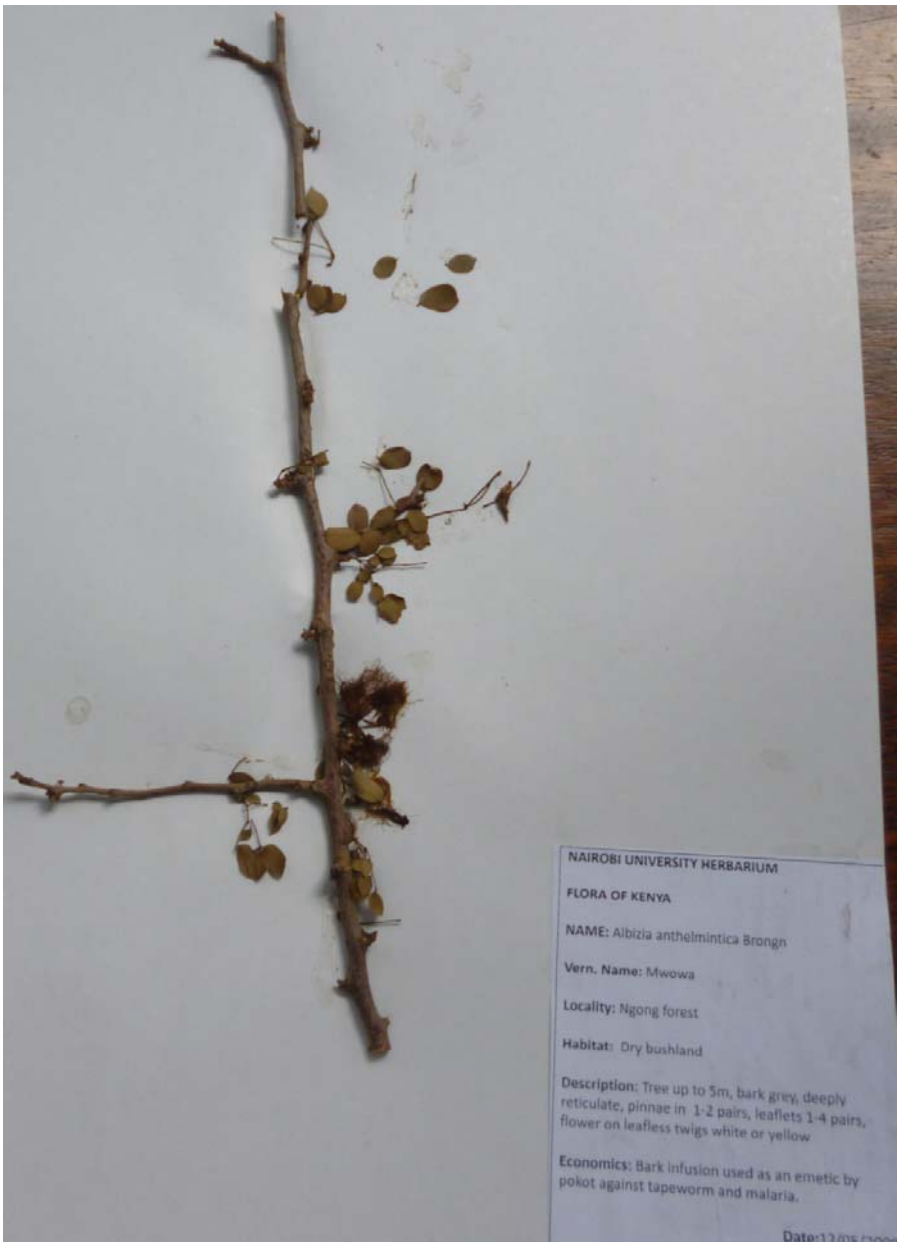
Zhang, J., Ferguson, S. S., Barak, L. S., Bodduluri, S. R., Laporte, S. A., Law, P. W. and Caron, M. G. (1998). Role for G protein-coupled receptor kinase in agonist specific regulation of mu opioid receptor responsiveness. *Proceedings of the National Academy of Science*, 95: 7157- 7162.

Zollner, C. and Stein, C. (2007). Opioids. Handbook of Exp. Pharmacol. 7th ed.: Ed. A. Herz. Pp 31-63.

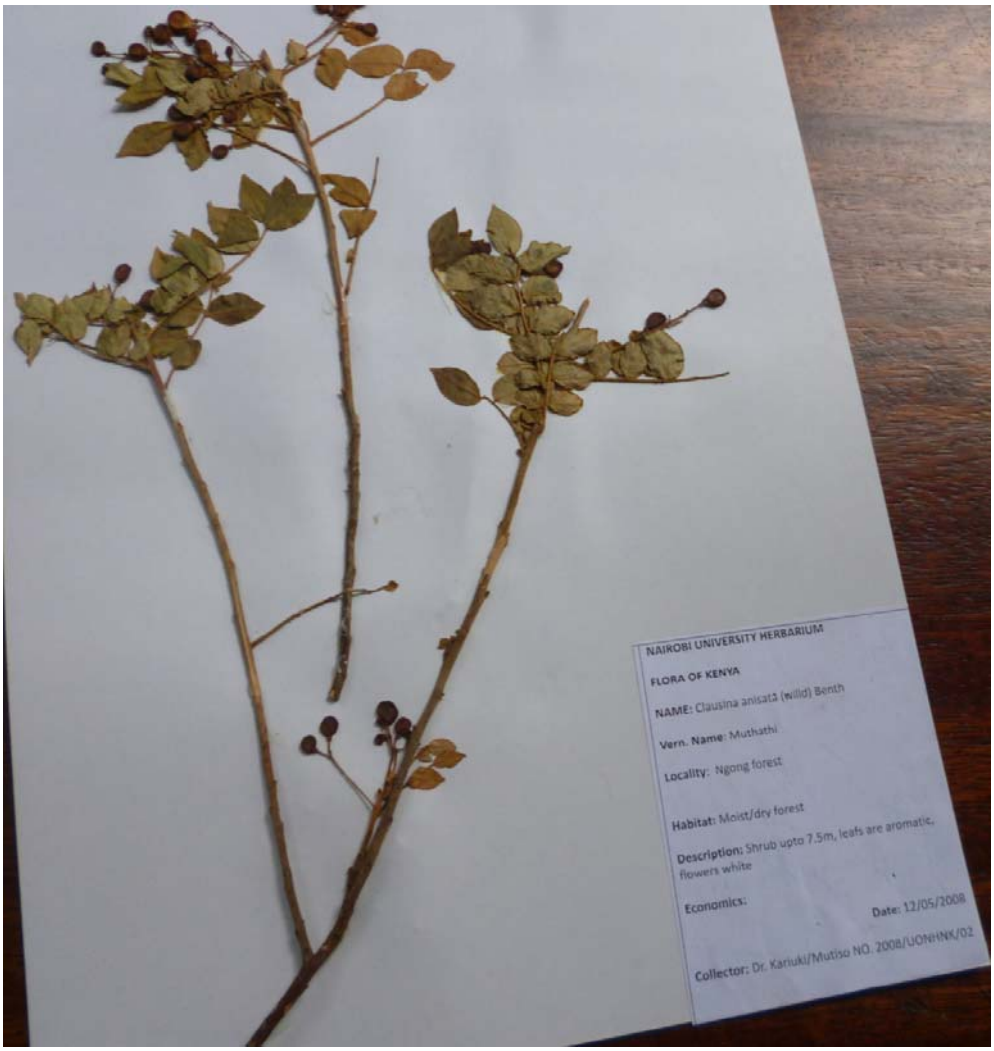
4.6 APPENDICES

Appendix 1: Herbarium Pictures of the Plants

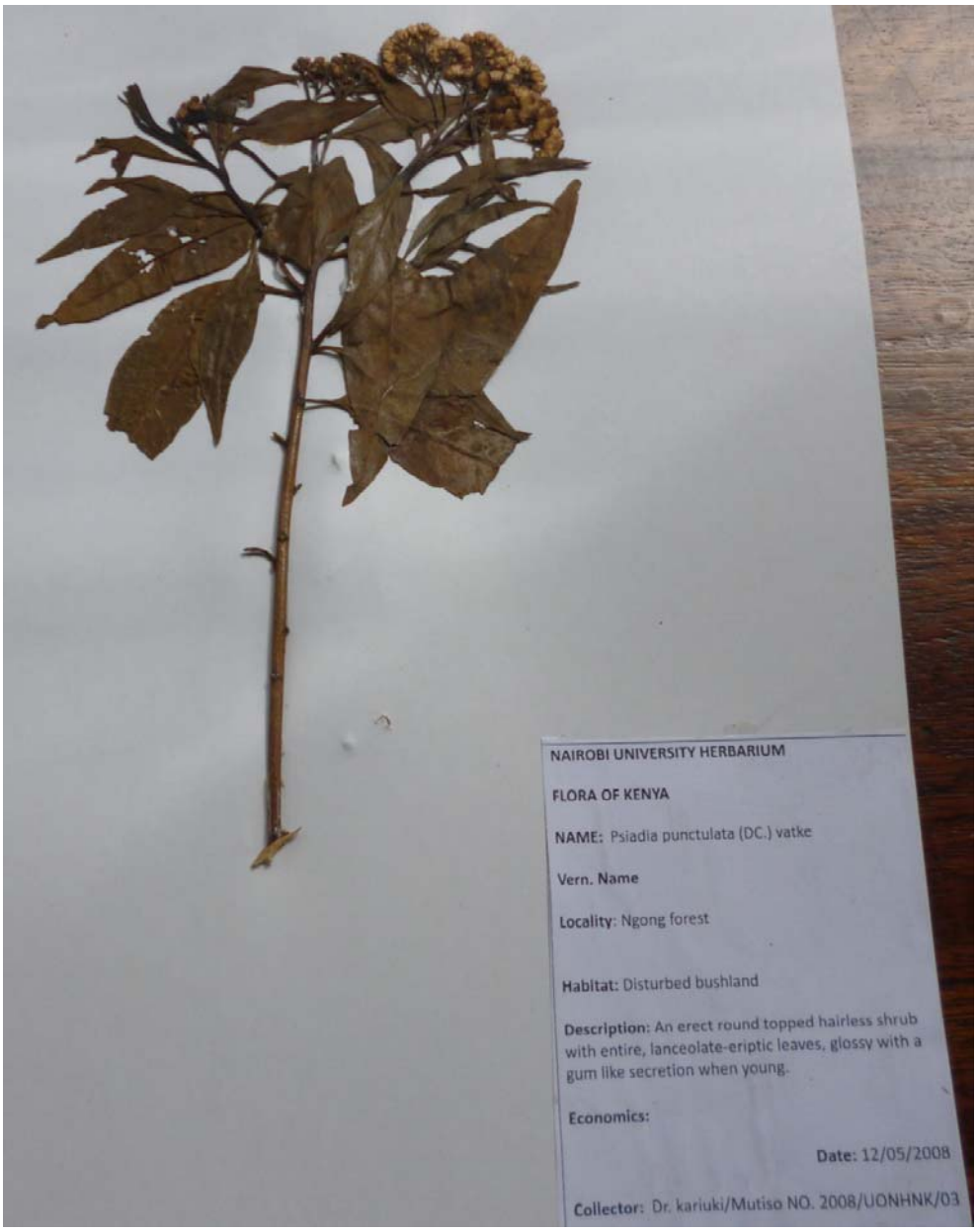
Albizia anthelmintica **HNK/MP/09/01**



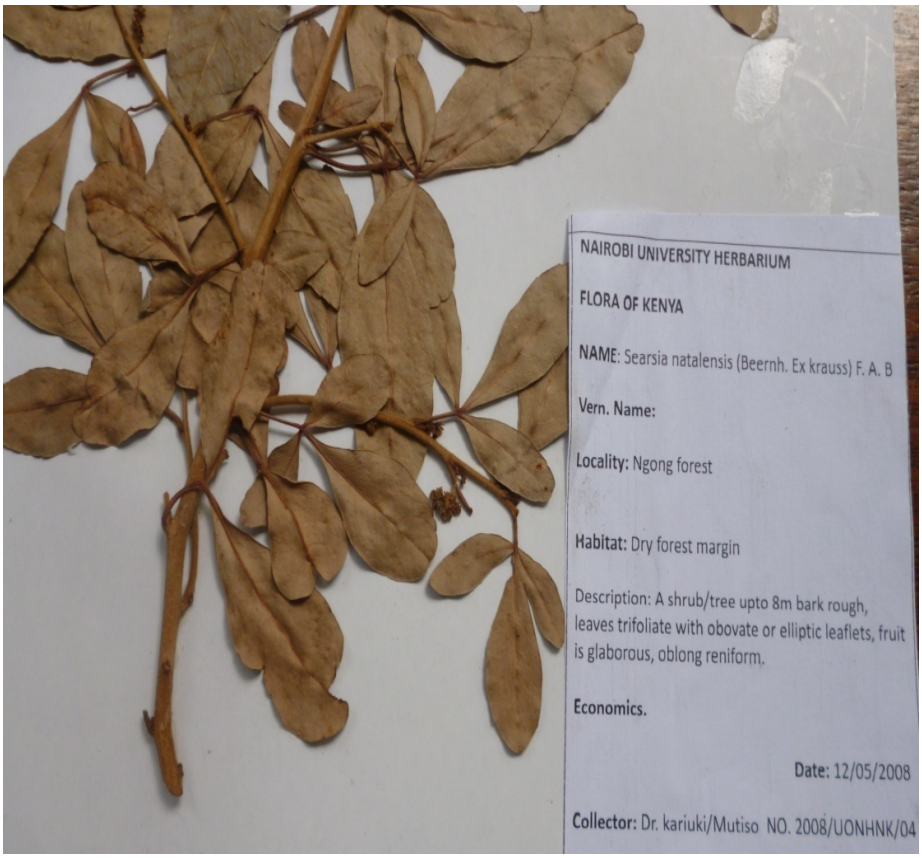
Clausena anisata **HNK/MP/09/02**



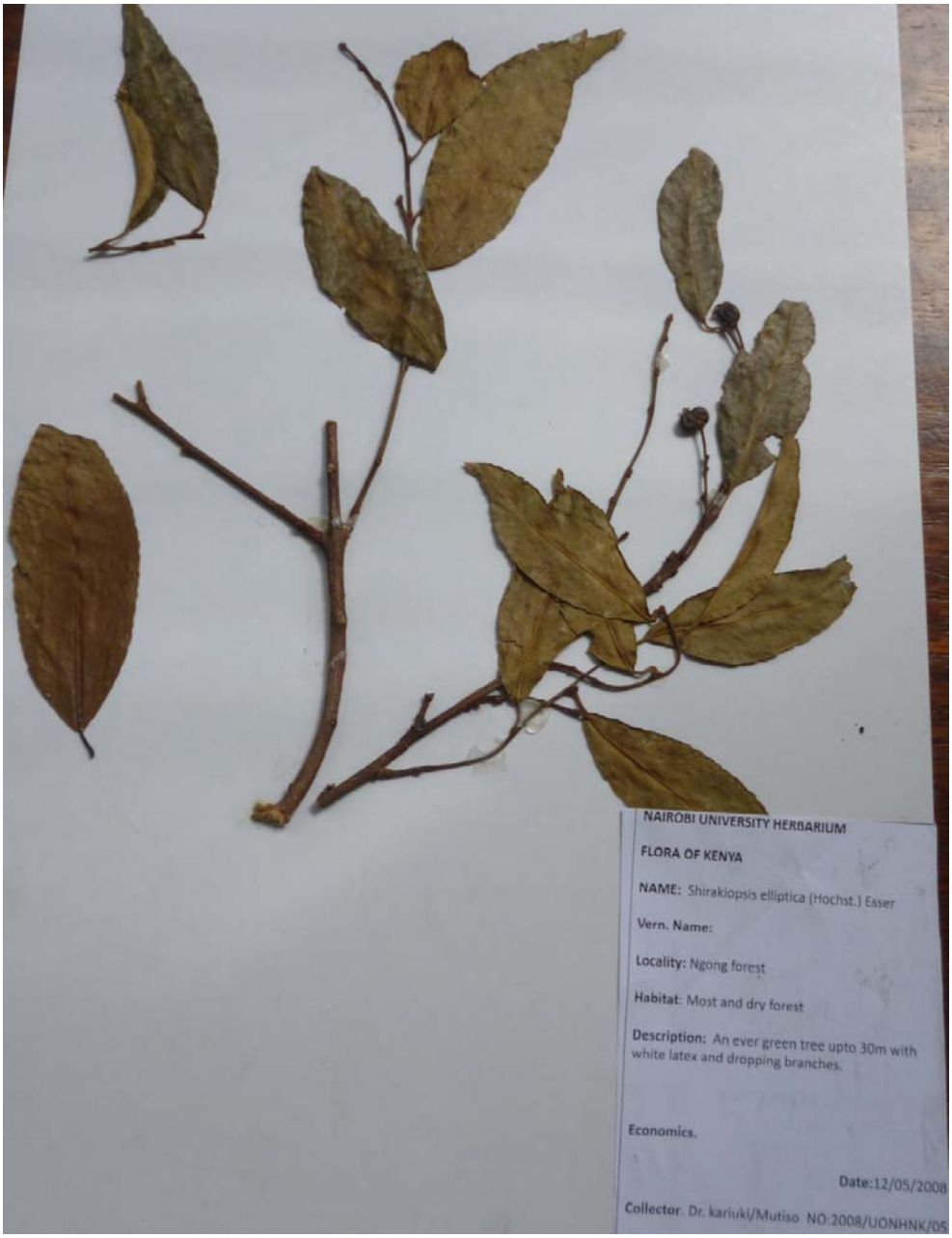
***Psadia punctulata* HNK/MP/09/03**



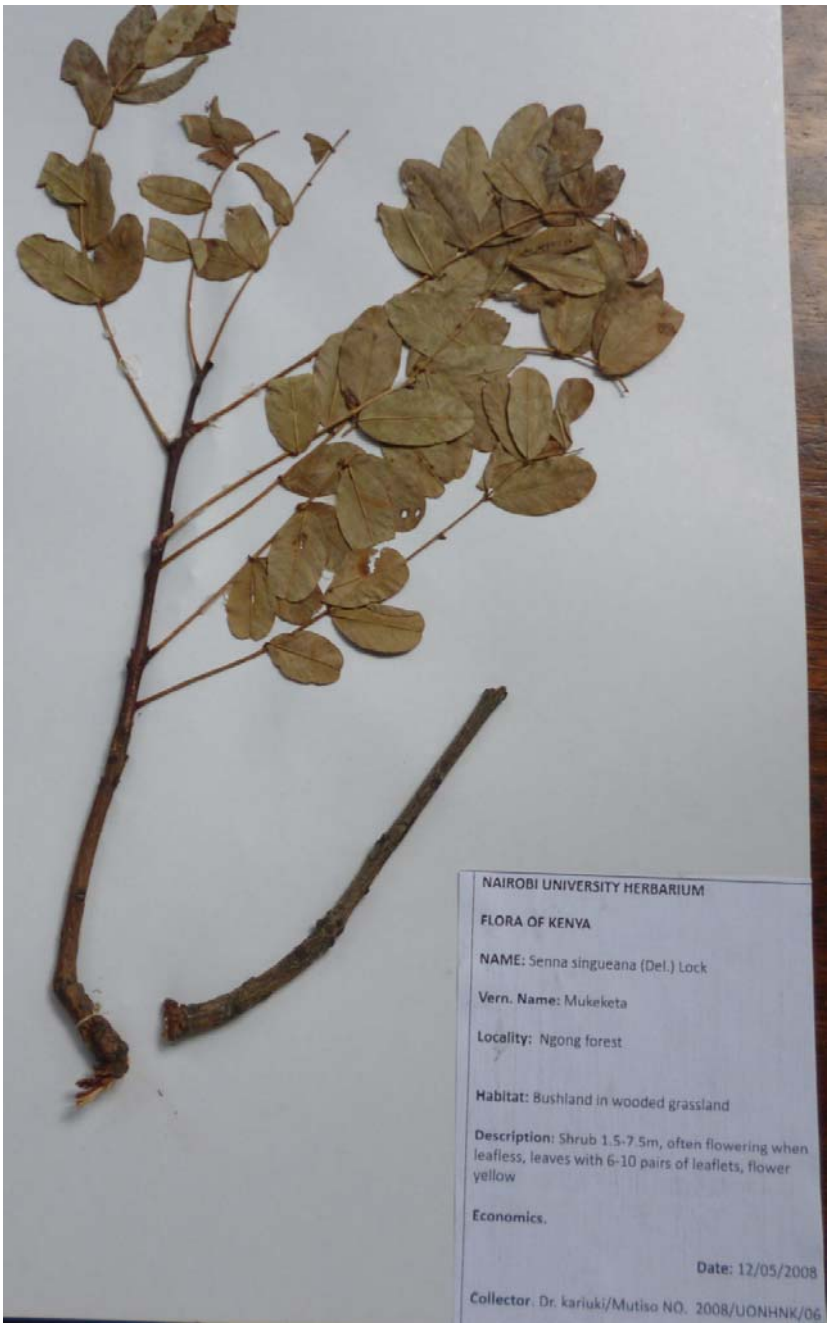
***Rhus Natalensis* HNK/MP/09/04**



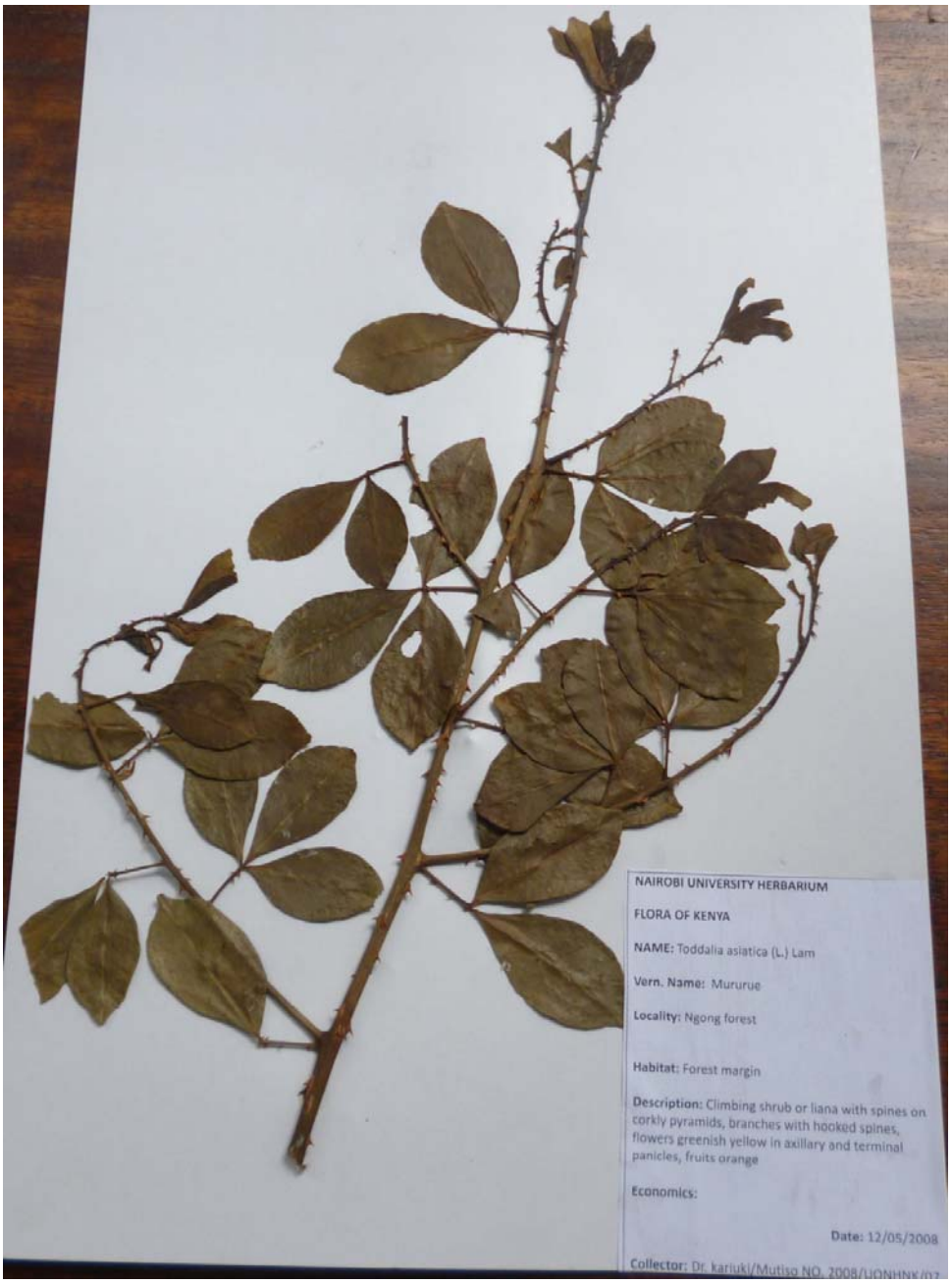
***Sapinum ellipticum* HNK/MP/09/05**



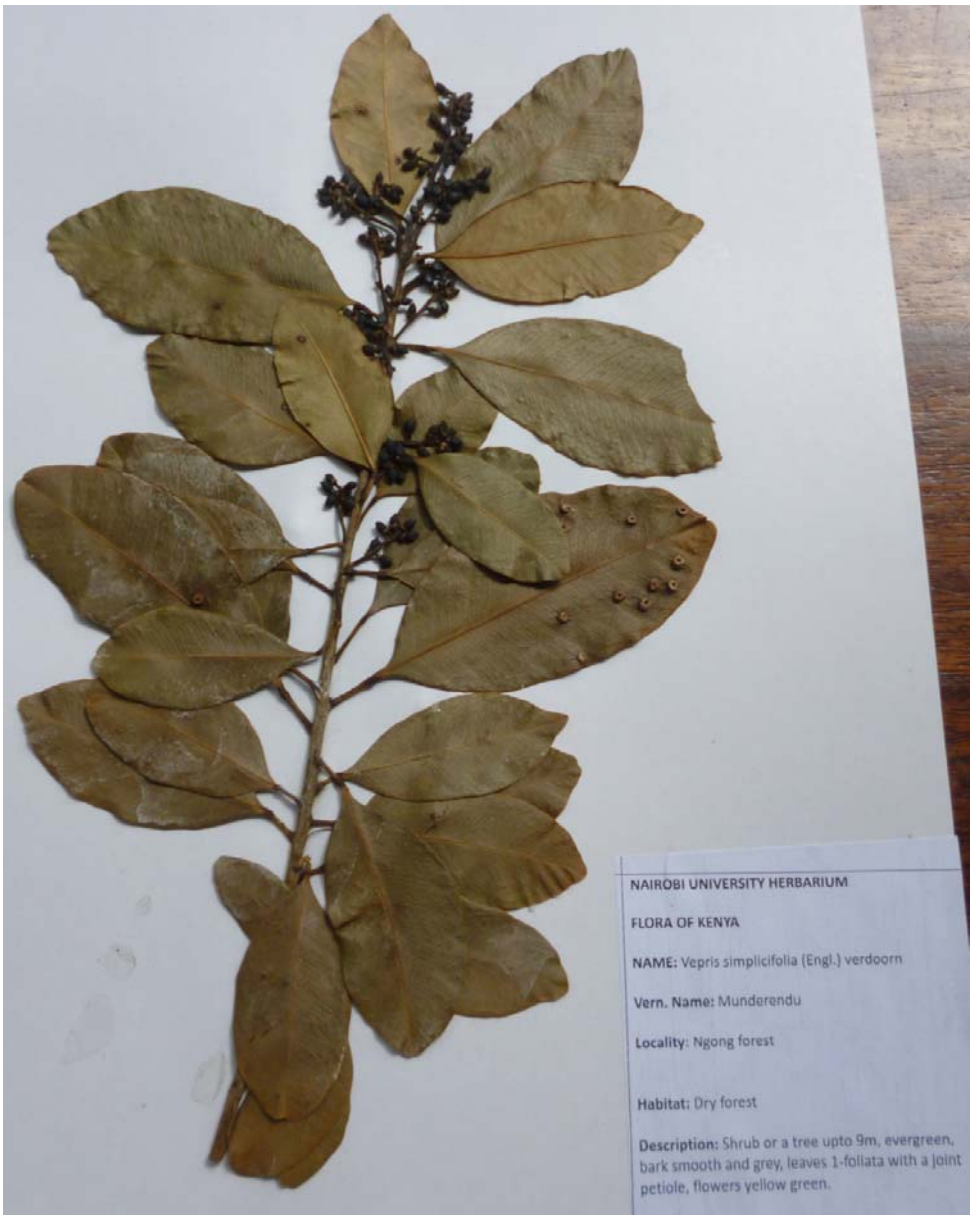
***Senna singueana* HNK/MP/09/06**



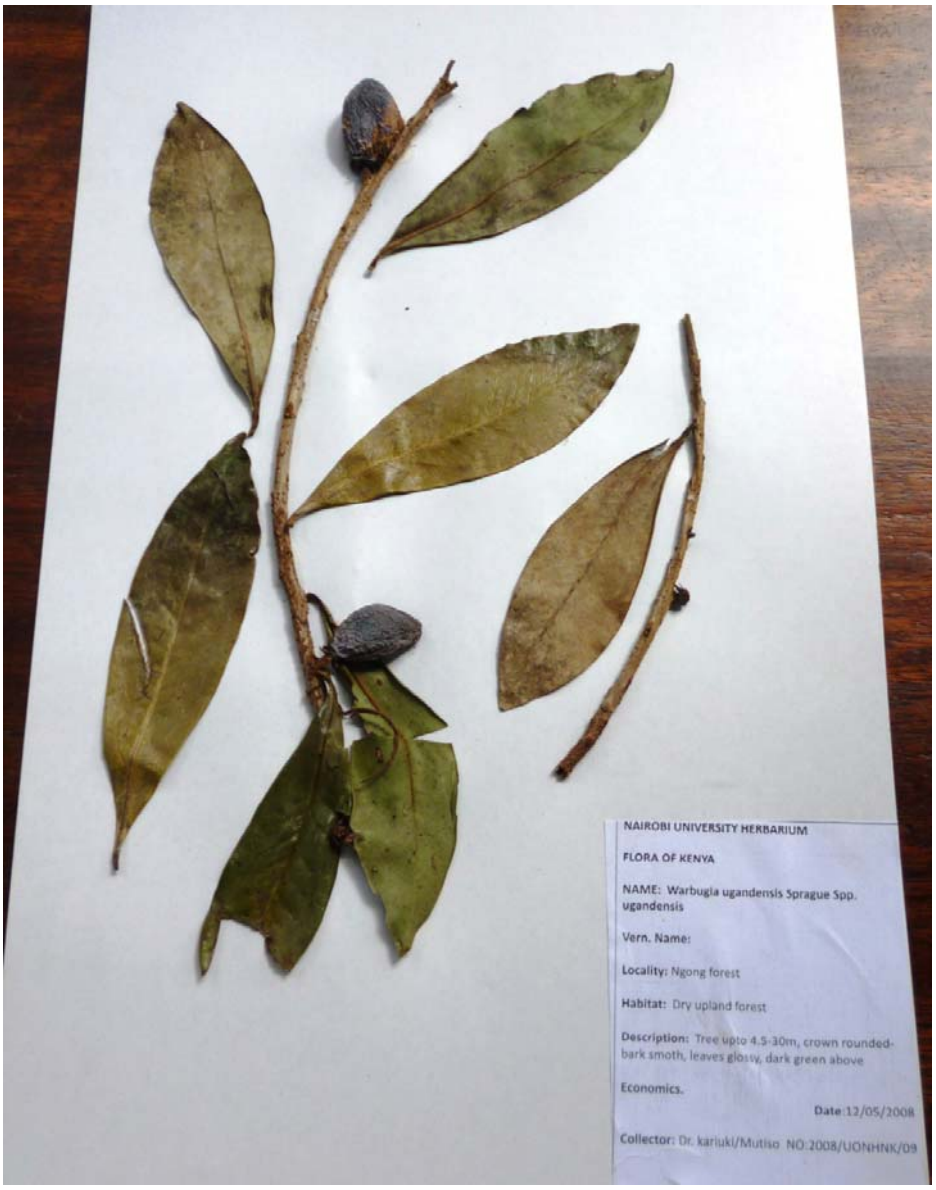
***Toddalia asiatica* HNK/MP/09/07**



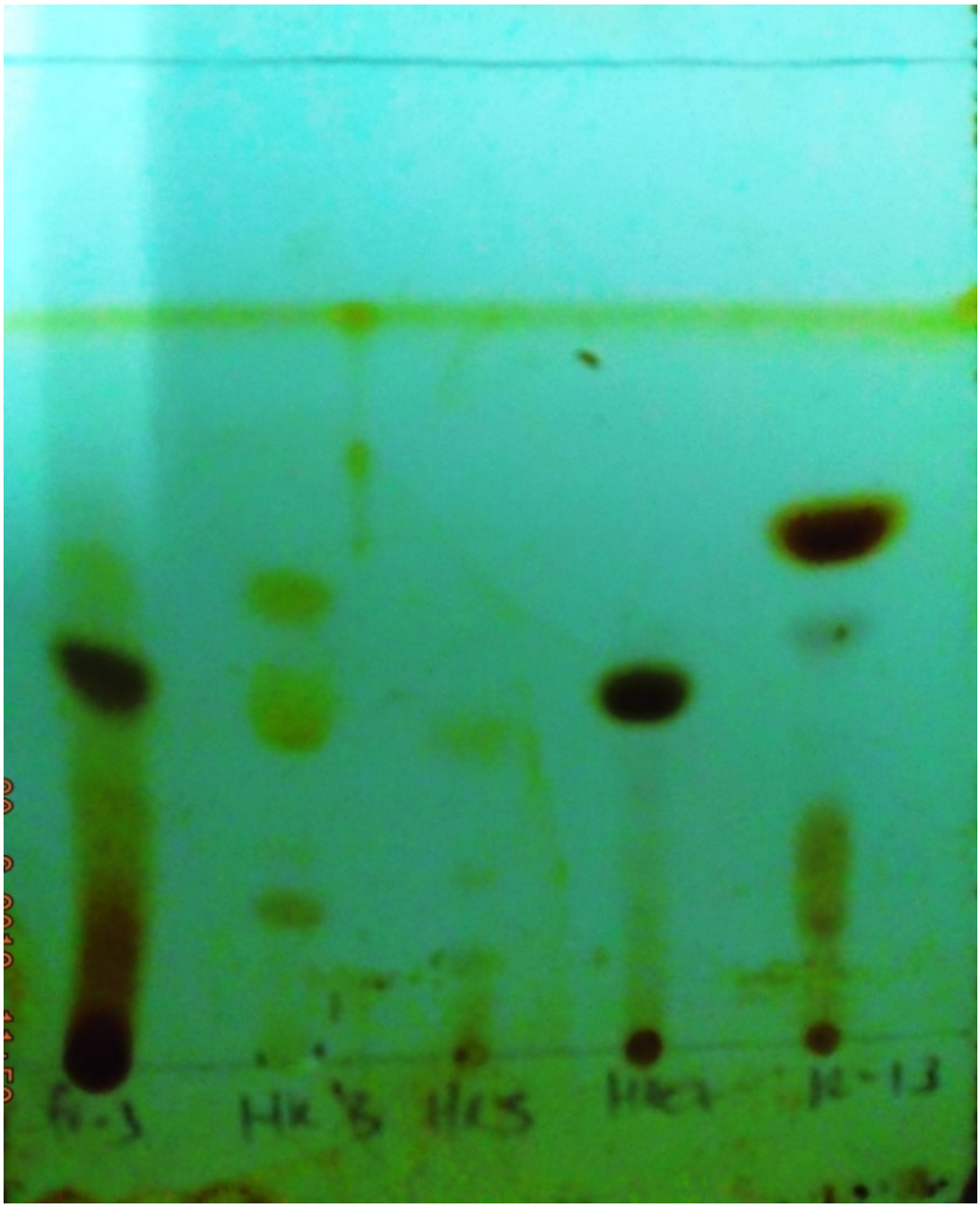
***Teclea simlicifolia* HNK/MP/09/08**



***Warburgia ugandensis* HNK/MP/09/09**



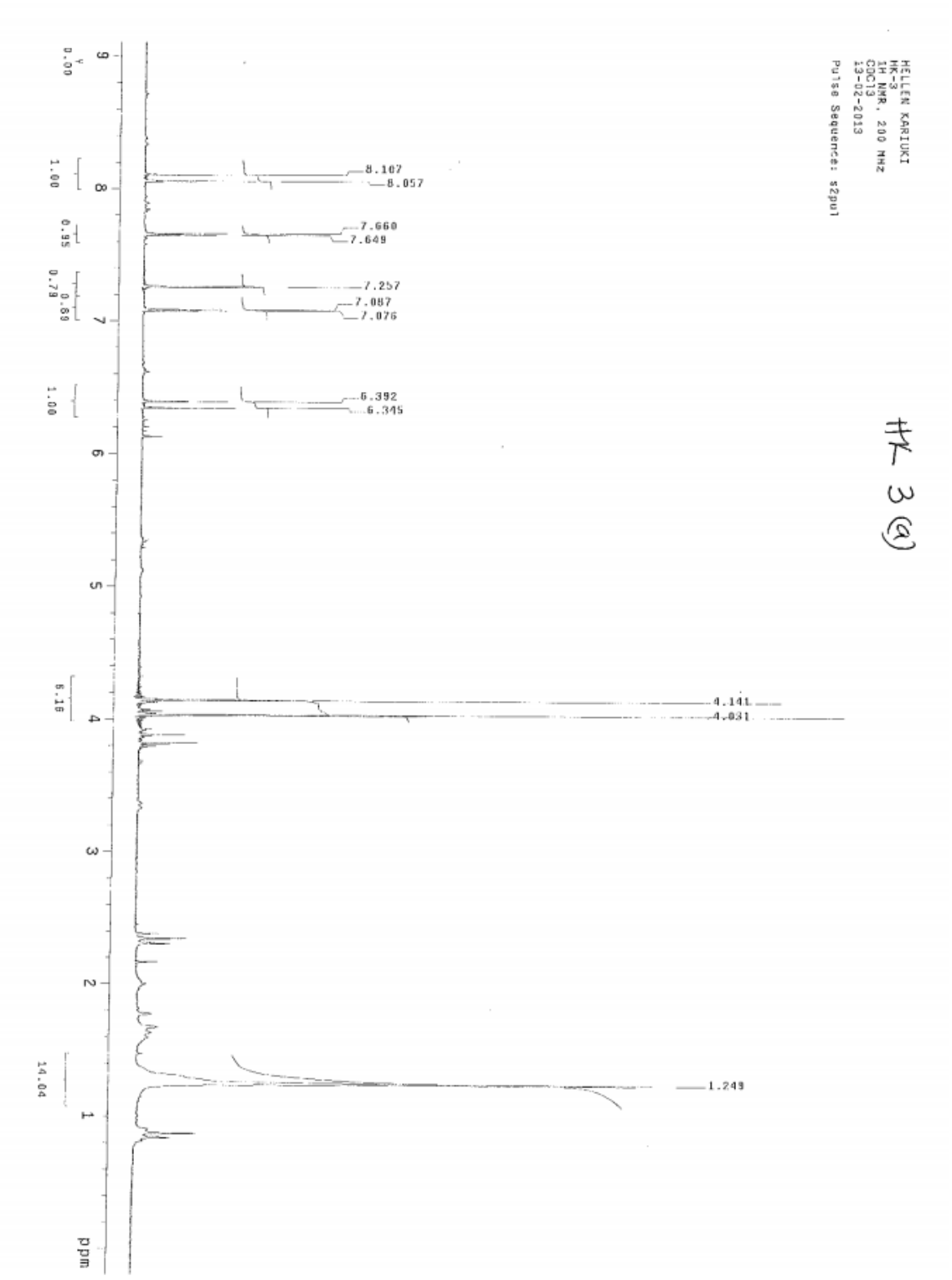
Appendix 2: TLC profile of compounds of *T. asiatica*(UV light)



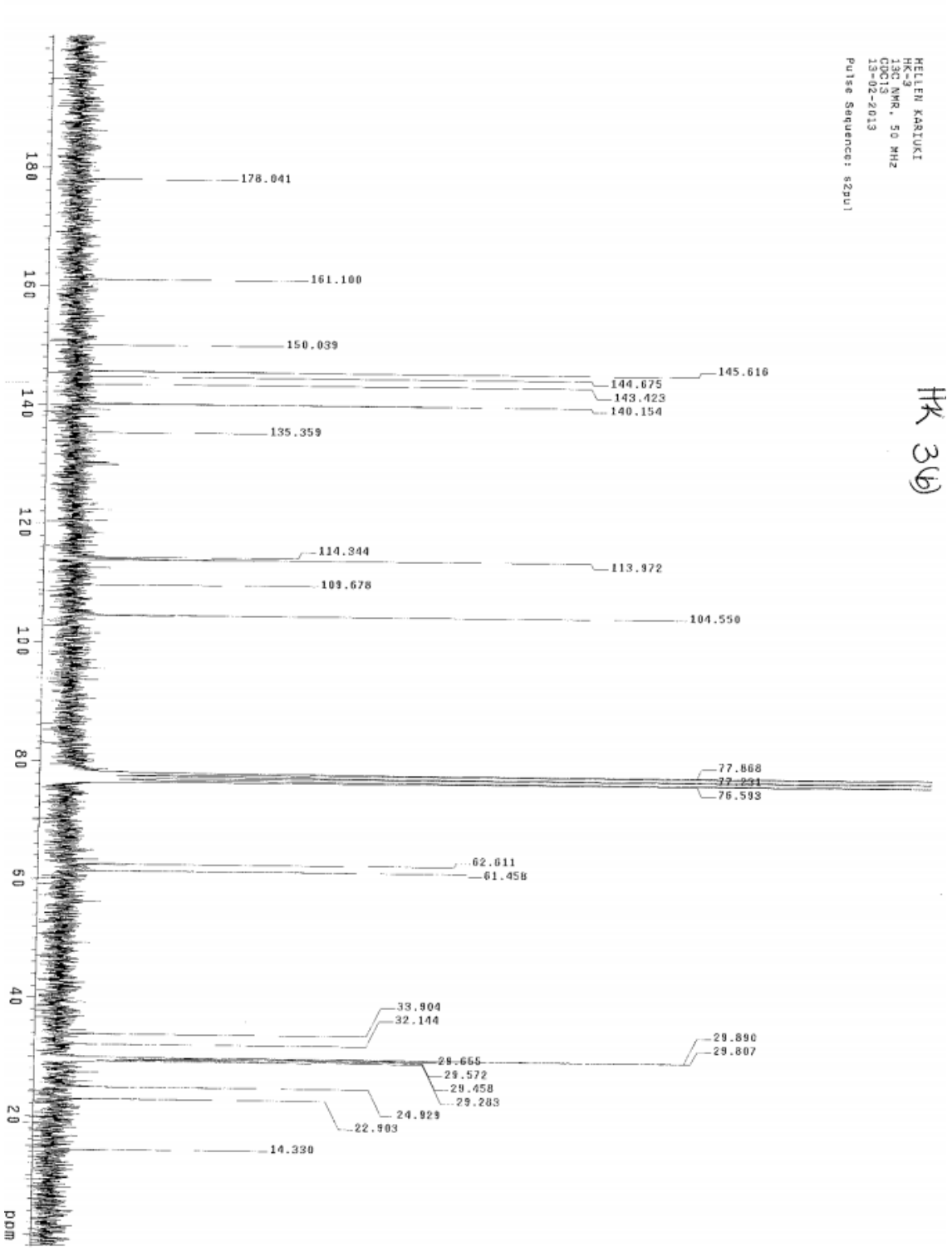
Crude extract	HK3	HK18	HK7	HK13

Mobile phase was Ethyl acetate: Hexane (3:7 ratios)

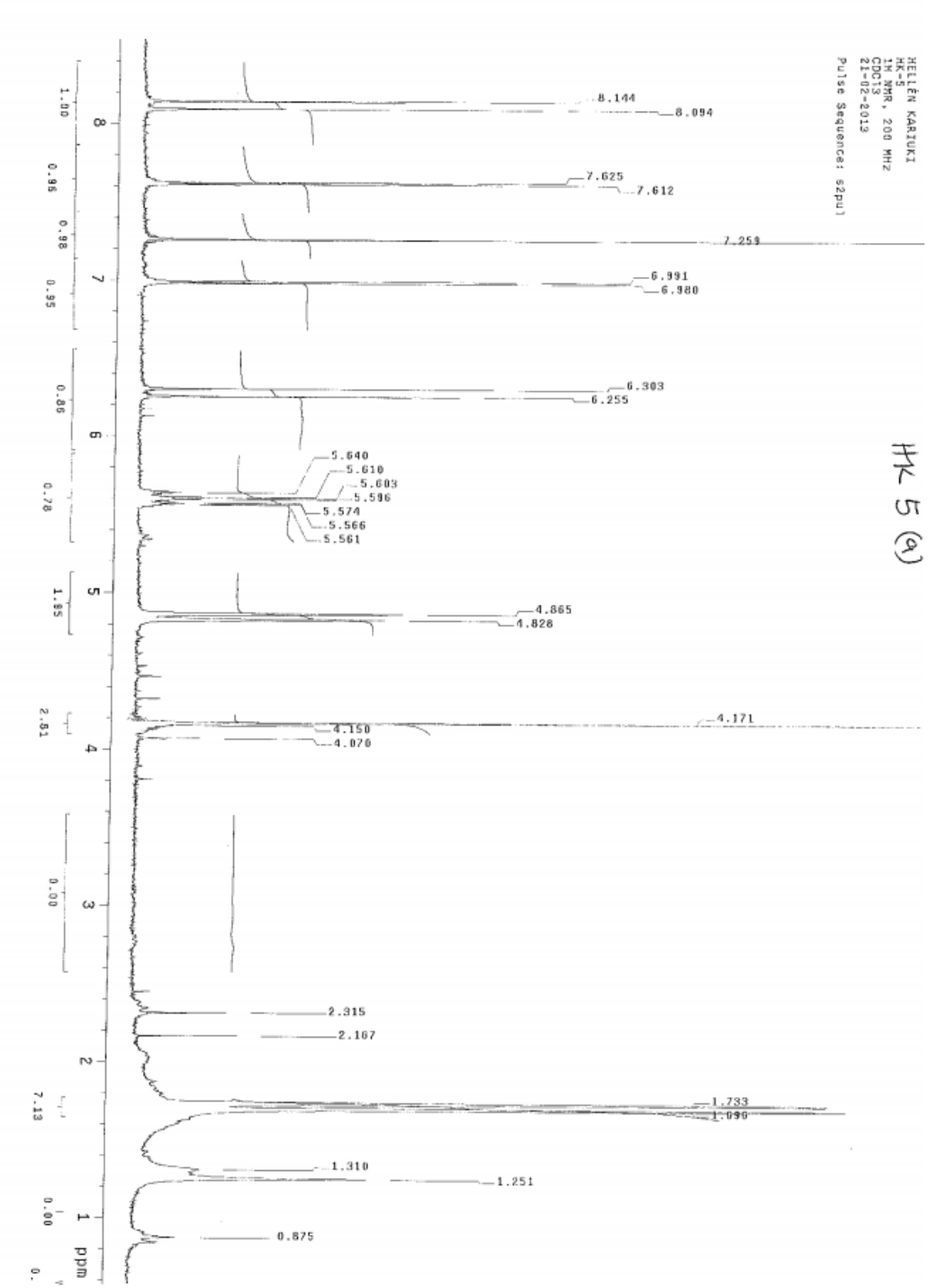
Appendix 3: ^{1}H NMR Spectrum of HK3 at 200 MHz



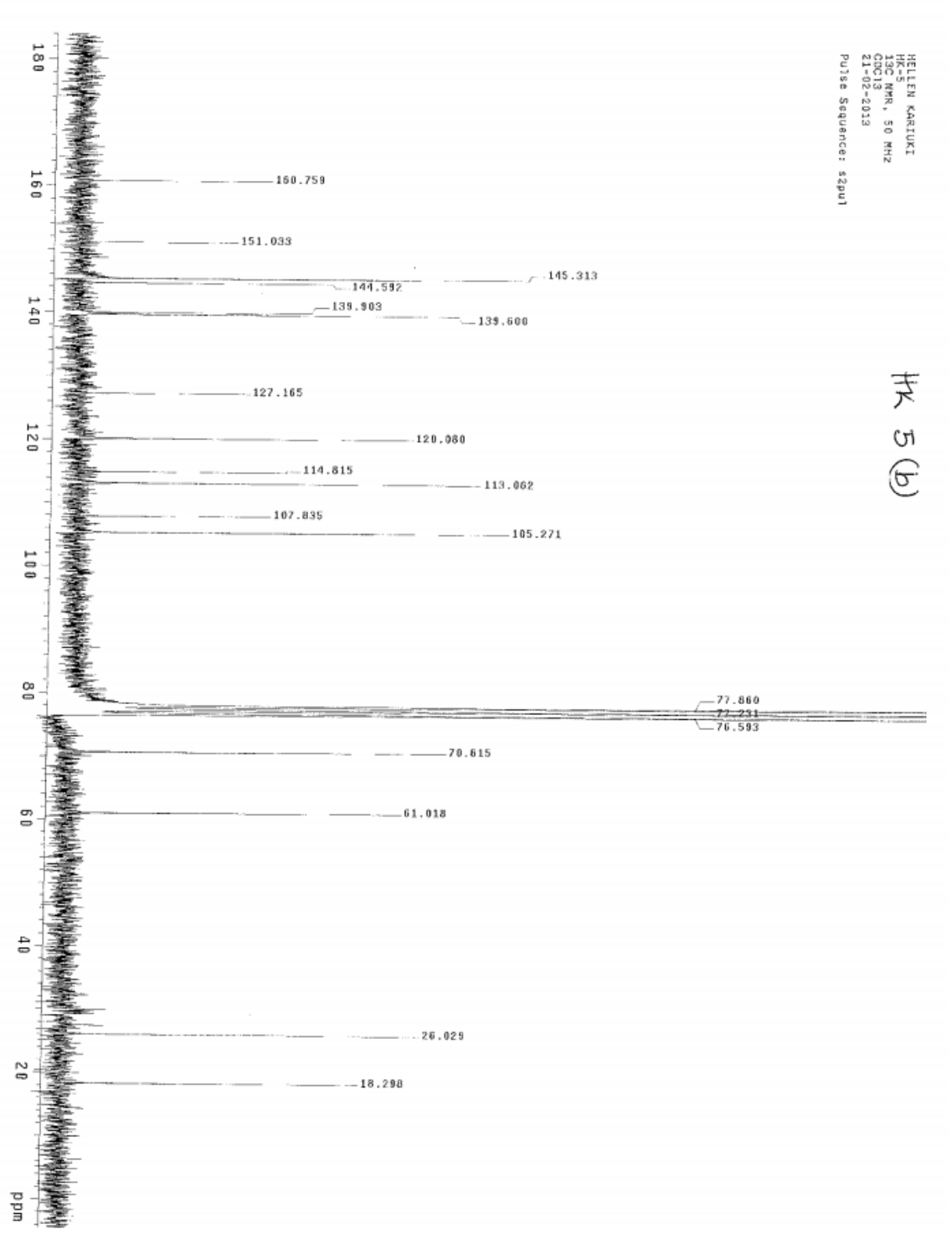
Appendix 4: ^{13}C NMR spectrum of HK3 at 50 MHz



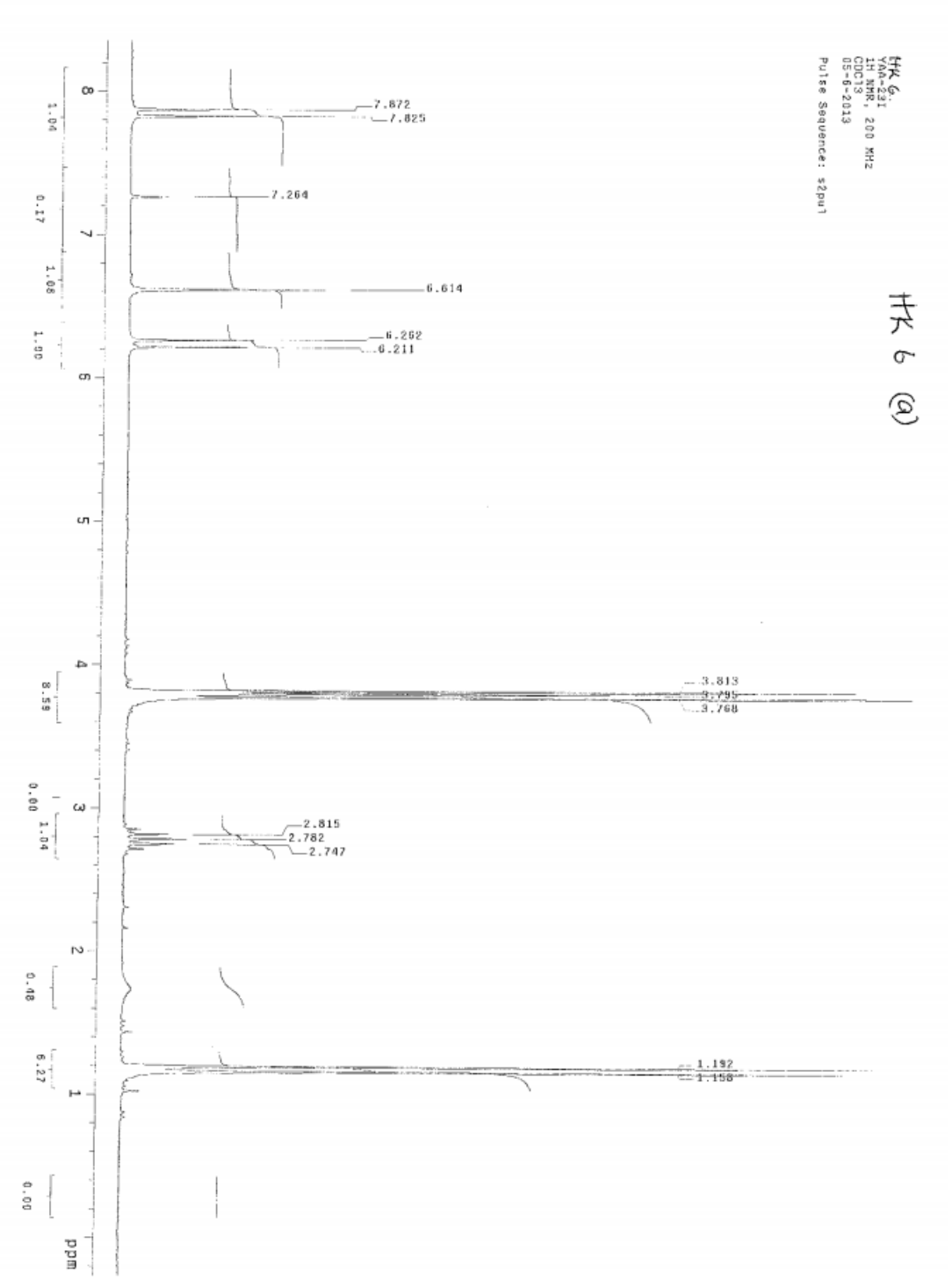
Appendix 5: ^{1}H NMR spectrum of HK5 at 200 MHz



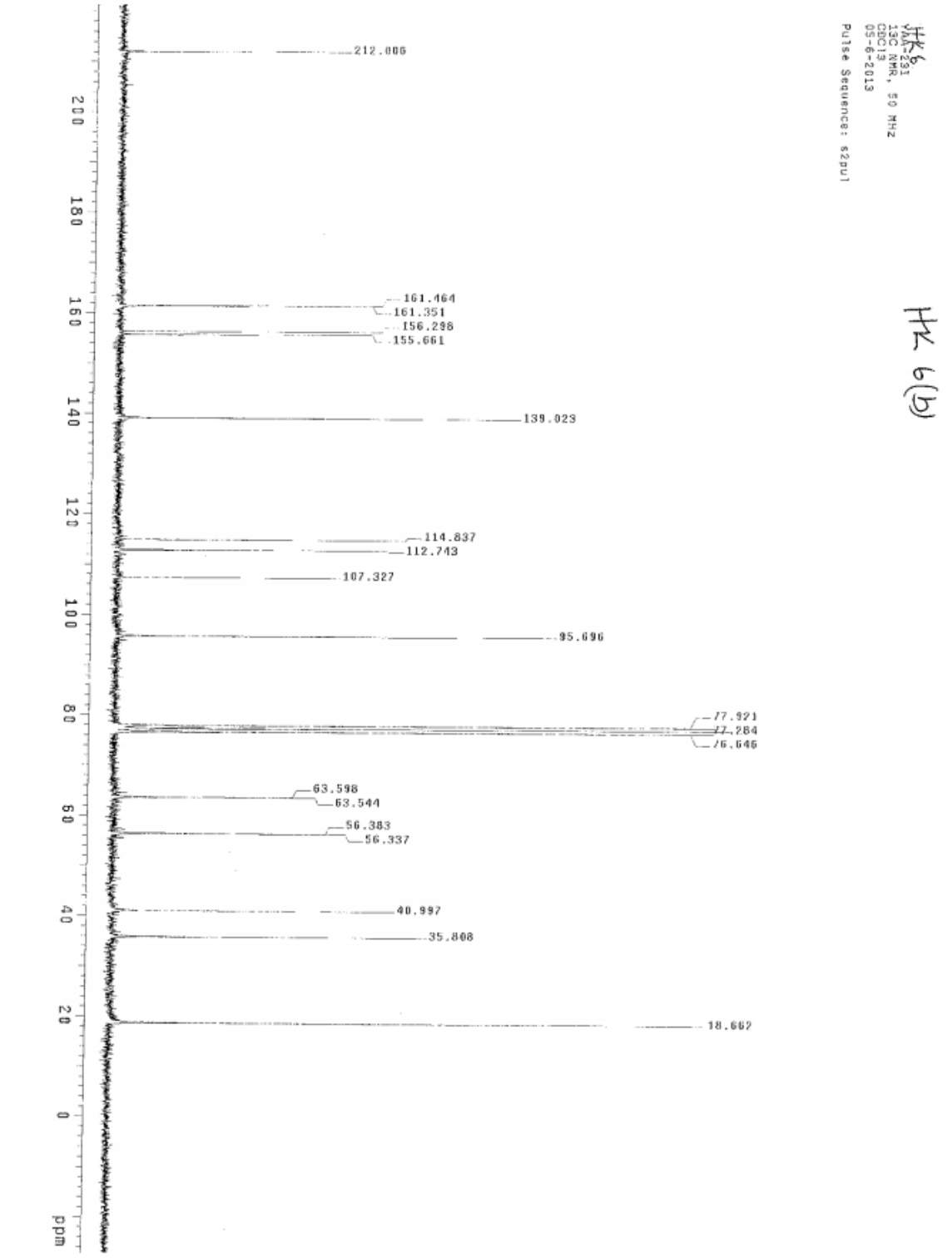
Appendix 6: ^{13}C NMR spectrum of HK5 at 50 MHz



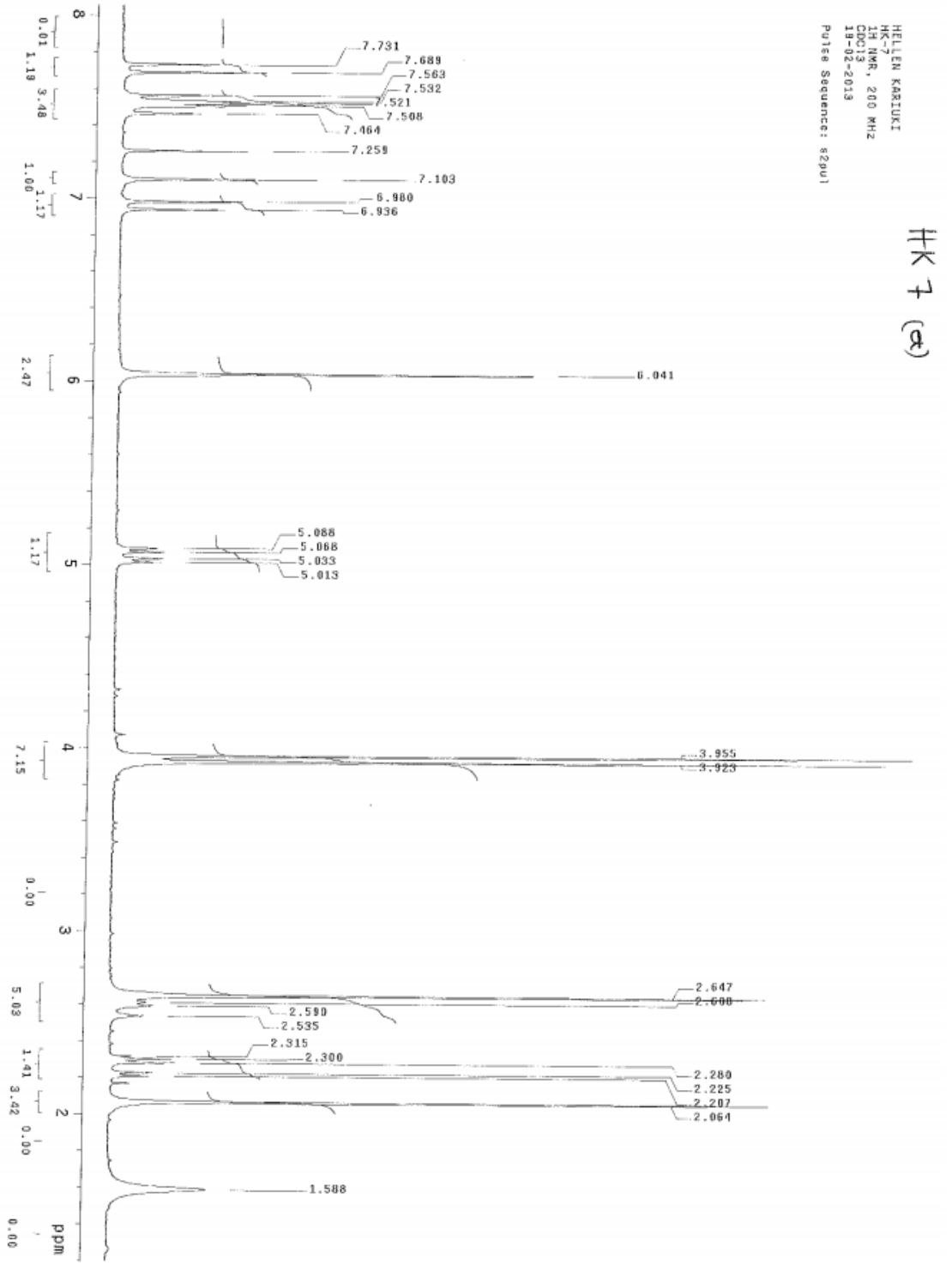
Appendix 7: ^{1}H NMR spectrum of HK6 at 200 MHz



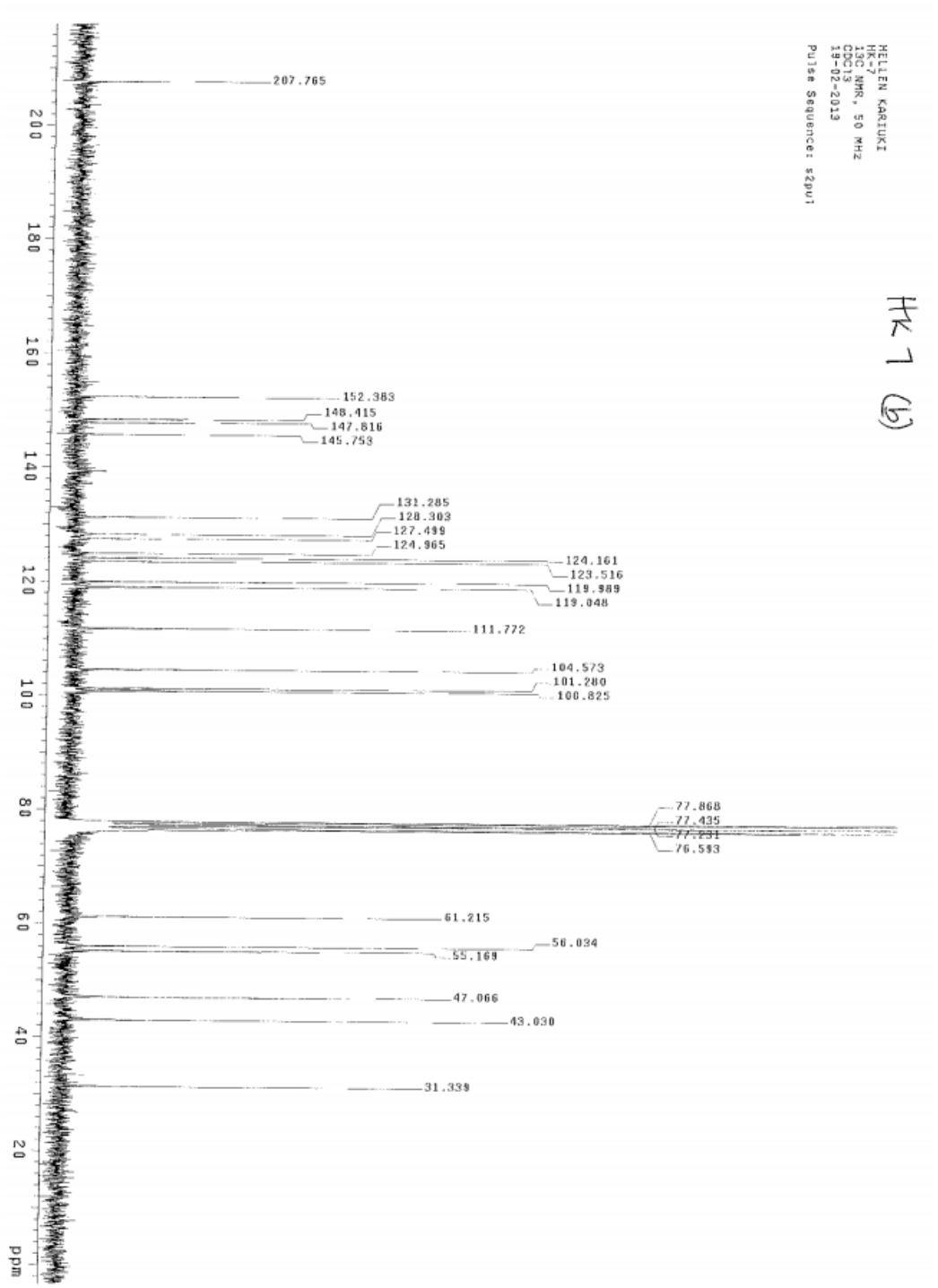
Appendix 8: : ^{13}C NMR spectrum of HK6 at 50 MHz



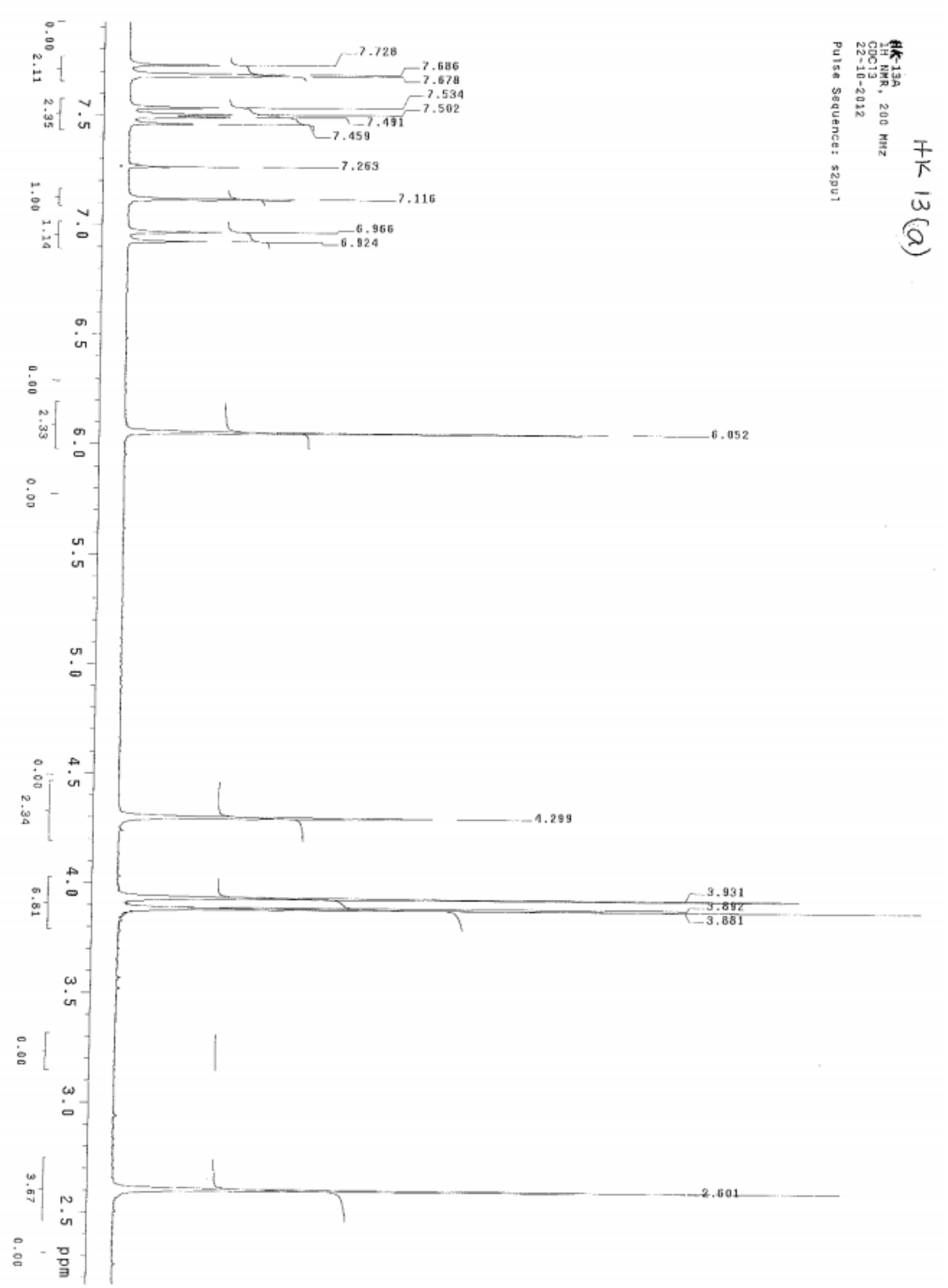
Appendix 9: ^{1}H NMR spectrum of HK7 at 200 MHz



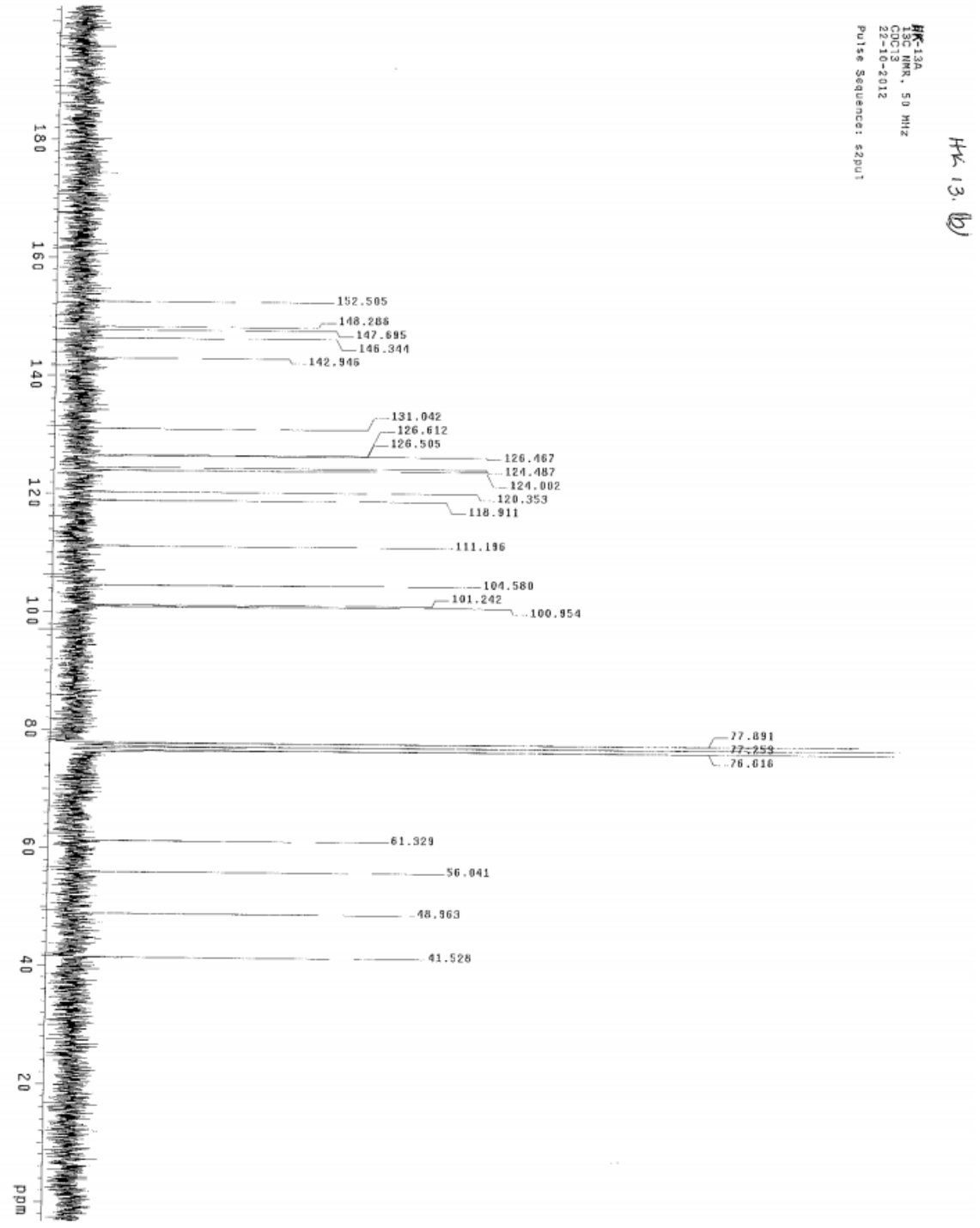
Appendix 10: ^{13}C NMR spectrum of HK7 at 50 MHz



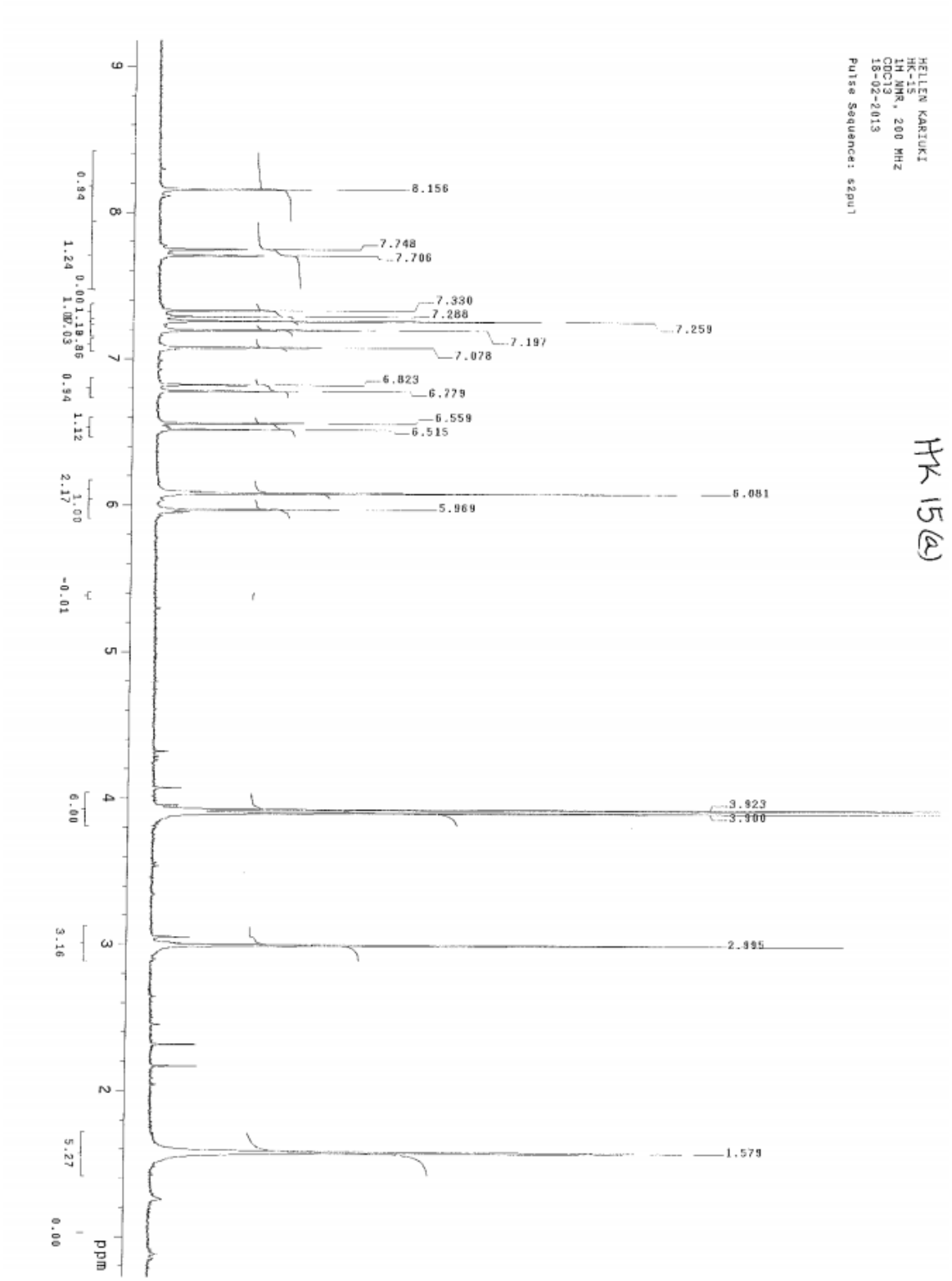
Appendix 11: ^{13}C NMR spectrum of HK13 at 200 MHZ



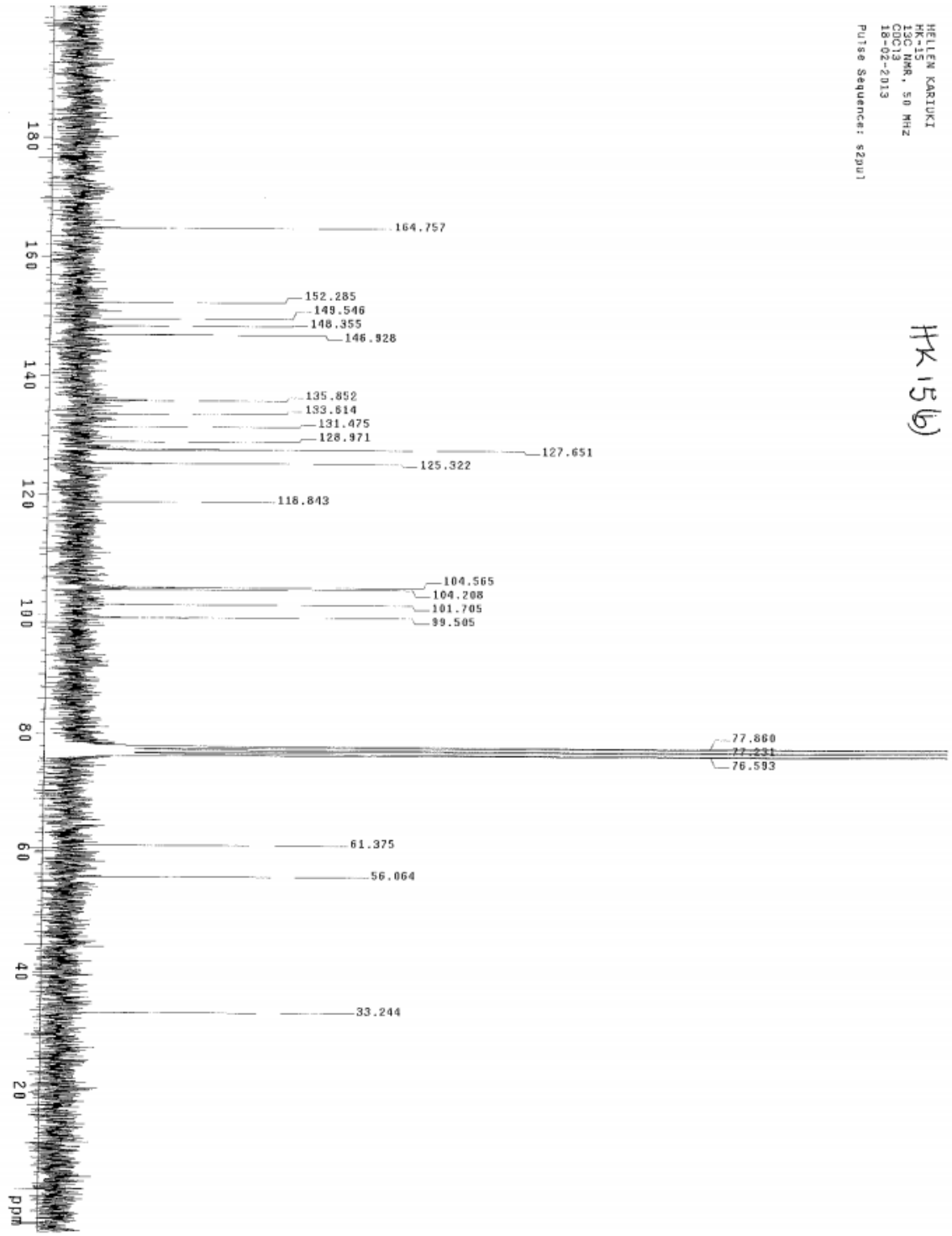
Appendix 12: ^{13}C NMR spectrum of HK13 at 50 MHz



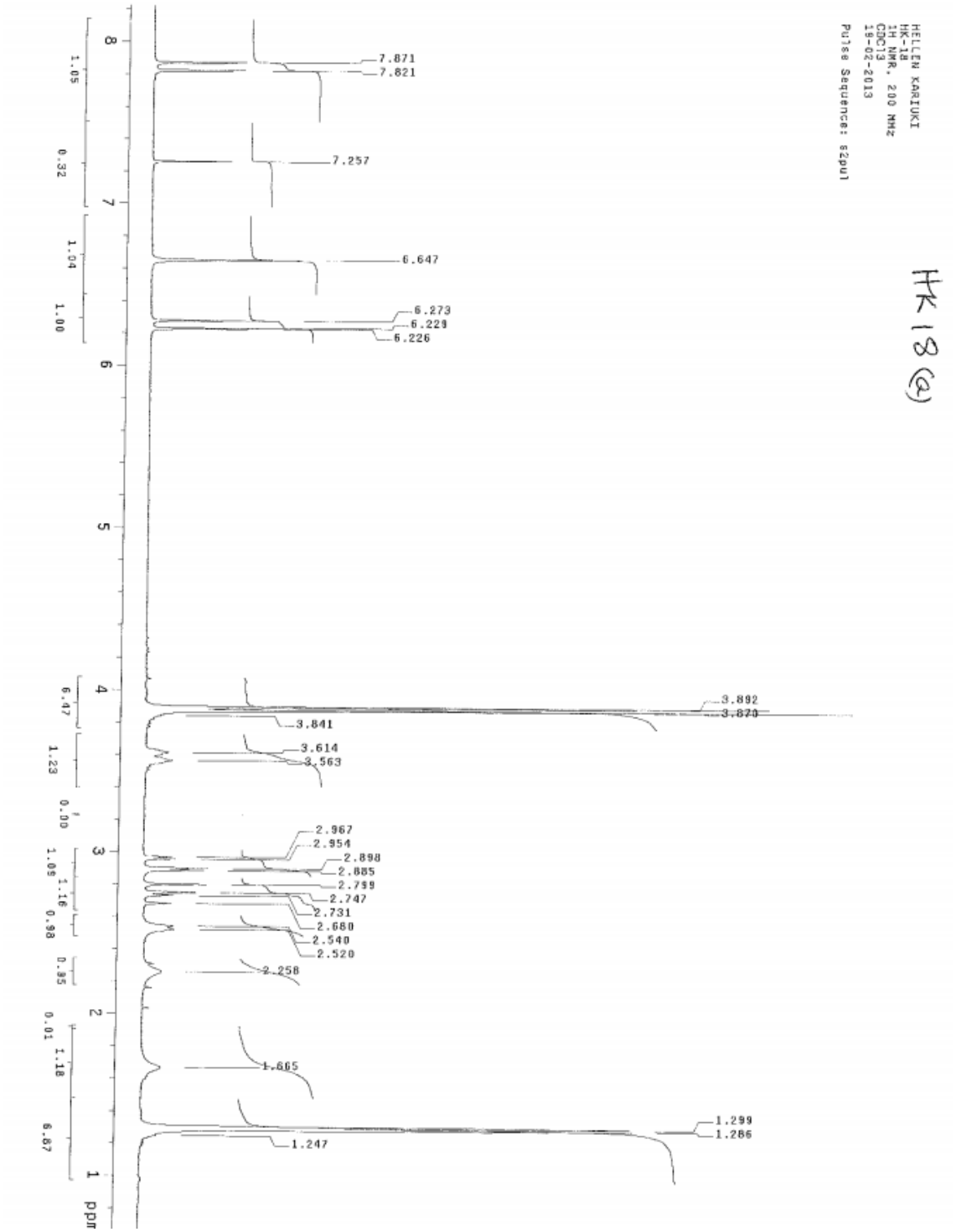
Appendix 13: ^{1}H NMR spectrum of HK15 at 200 MHz



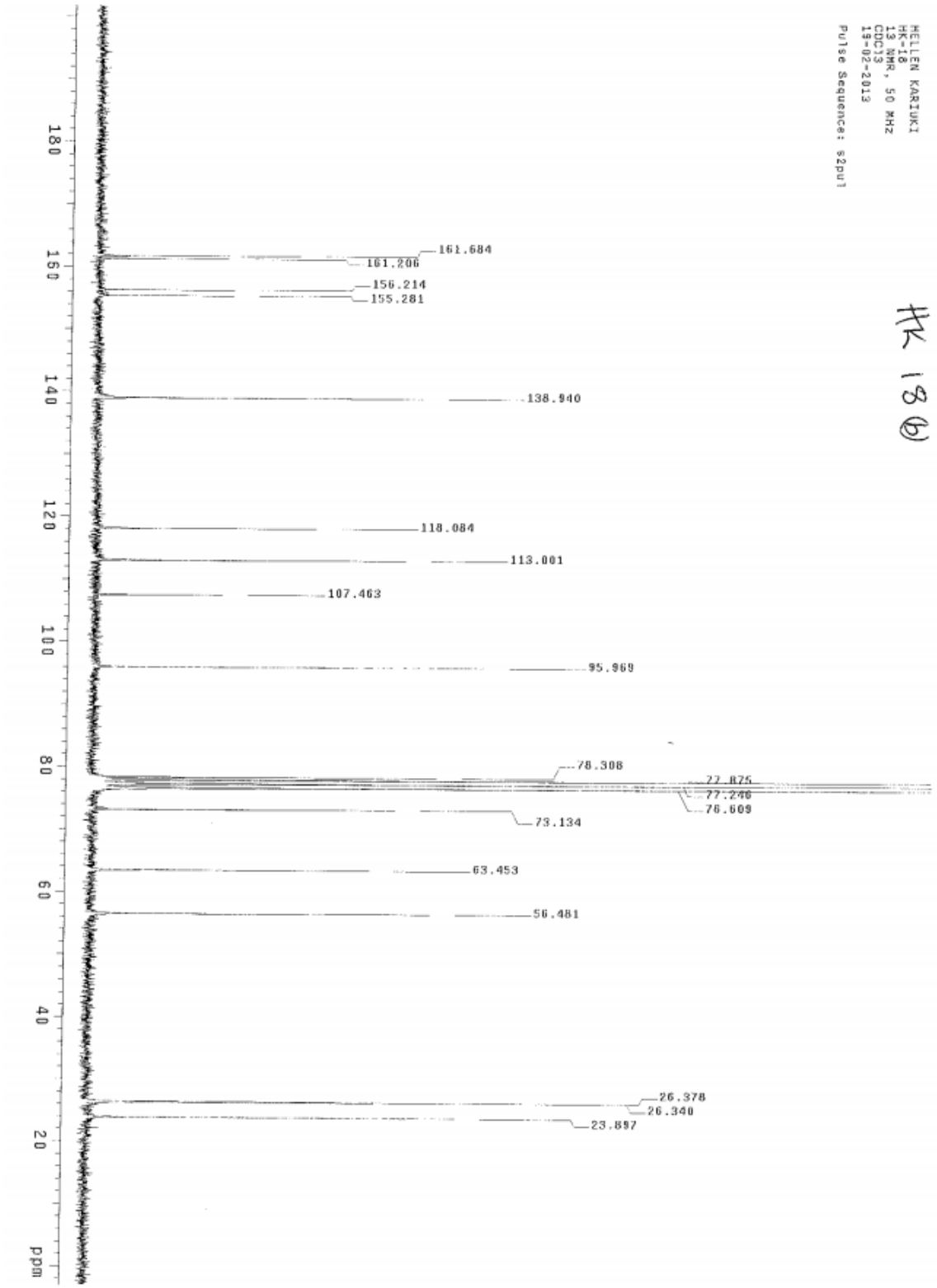
Appendix 14: ^{13}C NMR spectrum of HK15 at 50 MHz



Appendix 15: ^{1}H NMR spectrum of HK18 at 200 MHz



Appendix 16: ^{13}C NMR spectrum of HK18 at 50 MH_Z



Appendix 17: Effects(licking latency sec) of the plants extracts in the Tail Flick Test

Teclea simlicifolia **(leaves)**

NEGATIVE CONTROL	200mg / kg	100mg / kg	ASA 100mg / kg	MORPHINE 5mg / kg
6	9	9	6	7.3
7	6	6	5	8.8
6	8	7	6	8.3
6	6	7	6	8.9
7	9	8	5	8.1
6	6	7	5	7.8
7	7	7	7	6.7
7	7	6	5	8.2

Rhus natalensis **(Root)**

NEGATIVE CONTROL	200mg / kg	100mg / kg	ASA 100mg / kg	MORPHINE 5mg / kg
6.1	7.4	6.8	8	7.3
5.7	7	7.4	8.1	8.8
4.9	7.6	8	8.7	8.3
5.8	8	7.3	8.8	8.9
5.8	9.1	6.3	9.4	8.1
5.7	6.4	5.6	9.6	7.8
5.2	7.1	7.7	7.4	6.7
5.8	6.8	7.6	8.6	8.2

Toddalia asiatica **(Root)**

NEGATIVE CONTROL	200mg / kg	100mg / kg	ASA 100mg / kg	MORPHINE 5mg / kg
5	6	7	6	8
4	8	6	5	8
5	7	5	6	7
4	9	7	6	9
5	7	6	5	7
5	9	7	5	7
6	9	8	7	10
5	8	7	5	7

***Senna singuana* (Root)**

NEGATIVE CONTROL	200mg / kg	100mg / kg	ASA 100mg / kg	MORPHINE 5mg / kg
7.3	6.4	7	8	7.3
5.2	7.9	7	8.1	8.8
5.2	7	6.9	8.7	8.3
5	6.8	7.2	8.8	8.9
5.8	7.6	6.2	9.4	8.1
6.7	7.9	6.1	9.6	7.8
6	7.7	6.9	7.4	6.7
6.2	6.7	6.7	8.6	8.2

***Clausena anisata* (Root)**

NEGATIVE CONTROL	200mg / kg	100mg / kg	ASA 100mg / kg	MORPHINE 5mg / kg
5	8	13	9	9
6	8	8	11	14
10	11	7	9	12
7	7	6	10	11
10	13	6	12	9
7	8	7	9	10
10	10	7	14	10
10	10	8	12	11

***Warburgia ugandensis* (Bark)**

NEGATIVE CONTROL	200mg / kg	100mg / kg	ASA 100mg / kg	MORPHINE 5mg / kg
5	7	10	9	9
6	10	6	11	14
10	9	8	9	12
7	7	7	10	11
10	9	7	12	9
7	8	7	9	10
10	9	8	14	10
10	8	6	12	11

***Sapinum ellipticum* (Root)**

NEGATIVE CONTROL	200mg / kg	100mg / kg	ASA 100mg / kg	MORPHINE 5mg / kg
5	11	8	9	9
6	10	9	11	14
10	7	16	9	12
7	10	6	10	11
10	8	9	12	9
7	12	7	9	10
10	11	10	14	10
10	11	8	12	11

Appendix 18: Effects (licking latency sec) of *Rhus natalensis,Senna singuaenae and Toddalia asiatica* Root Extract in the Hot Plate Test

***Rhus natalensis* (Root)**

NEGATIVE CONTROL	100mg / kg	200mg / kg	ASA 100mg / kg	MORPHINE 5mg / kg
3.4	5.3	3.8	3.7	6.1
3.5	5.8	3.6	3.4	5.6
3.2	4.5	2.5	3.2	6.3
3.3	5.6	2.9	2.6	5.2
4	3.3	4	3.6	4.2
3.7	4.9	5.1	2.8	6.4
4.6	5.6	4.8	3.9	6.7
3.6	5.8	5	4.2	6.6

***Senna singuaenae* (Root)**

NEGATIVE CONTROL	100mg / kg	200mg / kg	ASA 100mg / kg	MORPHINE 5mg / kg
3.4	3.9	4.4	3.7	6.1
3.5	5	5.6	3.4	5.6
3.2	3.6	4.8	3.2	6.3
3.3	4.3	6.4	2.6	5.2
3.2	5.8	5.2	3.6	4.2
3.3	4.1	5.5	2.8	6.4
3.6	5.4	6.2	3.9	6.7
3.6	5.3	6.1	4.2	6.6

***Toddalia asiatica* (Root)**

NEGATIVE CONTROL	100mg / kg	200mg / kg	ASA 100mg / kg	MORPHINE 5mg / kg
4	4	4	8	9
4	4	6	9	7
5	5	7	8	7
3	6	8	7	7
4	5	7	8	8
5	5	5	7	7
3	4	6	5	10
5	5	7	8	8

Appendix 19: Effects (number of writhes) of *Toddalia asiatica* Root and leaf Extract in the Acetic Acid Induced Writhing Test

***Toddalia asiatica* leaf extract**

NEGATIVE CONTROL	200mg / kg	100mg / kg	50mg / kg
101	87	63	103
97	55	83	64
104	15	56	66
56	40	47	84
91	45	58	109
79	42	65	98

***Toddalia asiatica* root extract**

NEGATIVE CONTROL	200mg / kg	100mg / kg	50mg / kg
91	41	14	70
53	51	19	74
101	47	32	53
109	5	35	48
66	35	67	58
82	31	52	68

Appendix 20: Effects (time spent in pain behavior s) of *Toddalia asiatica* Root Extract in the Formalin test

Early phase

NEGATIVE CONTROL	100mg / kg	200mg / kg	50mg / kg	ASA 100mg / kg	Indomethacin 50mg / kg
192.93	193.46	182.96	194.09	167.9	228.38
247.34	193.99	179.02	194.66	172.32	218.32
180.59	202.06	190.27	162.15	159.34	257.93
265.24	258.63	169.37	249.37	162.47	197.43
242.75	212.03	161.21	200.68	202.08	220.21
197.89	197.42	196.96	218.47	144.83	218.74
232.75	220.42	181.7	213.62	186.53	230.21
215.21	218.47	191.97	227.28	138.16	199.72

Late phase

NEGATIVE CONTROL	100mg / kg	200mg / kg	50mg / kg	ASA 100mg / kg	Indomethacin 50mg / kg
173.86	66.6	123.16	350.41	190.37	11.9
217.31	104.59	239.72	234.02	182.31	15.67
175.47	145.54	168.93	185.72	88.29	49.76
191.26	93	181.8	310.7	153.2	25.92
207.04	118.72	196.02	235.39	154.78	21.5
155.11	111.03	190.86	247.48	153.96	28.43
199.19	114.04	186.91	265.27	163.53	21.33
207.92	131.14	180.9	267.82	140.04	28.16

Appendix 21: Effects(tail flick latency in secs) of the Column Chromatography Fractions from *Toddalia asiatica* Root extract in the Tail Flick Test

100 mg / kg

Negative control	Morphine 5mg / kg	ASA 100mg / kg	Hex: DCM	DCM	9:1 DCM: Meth	8:2 DCM: Meth	1:1 DCM: Meth
3.2	8.5	6.5	6.3	4	4.6	4.8	4.3
3.5	8.4	7	5.3	4.1	5.2	5.7	5.2
3	8.1	6	5	4.1	5.3	4.3	4.8
3.1	8.2	7.4	6.2	4.2	5.2	6.8	5.5
3.8	8.6	5.8	5.2	5.2	6.3	4.5	4.6
3.6	8.6	6.6	5.5	4.5	5.8	5.2	5.3
3.7	8.7	6.7	6.9	4.2	5.2	5.9	5.1
3.5	8.2	7.4	6.8	4.4	6.2	6	4.9

50 mg / kg DOSE

Negative control	Morphine 5mg / kg	ASA 100mg / kg	Hex: DCM	DCM	9:1 DCM: Meth	8:2 DCM: Meth	1:1 DCM: Meth
3.2	8.5	6.5	5	4.8	5.7	4.8	4.3
3.5	8.4	7	4.8	3.1	4	4.7	5.2
3	8.1	6	5.5	3.9	4.7	4.9	4.8
3.1	8.2	7.4	6	4.2	4	4.8	6.2
3.8	8.6	5.8	4.6	4.6	4.3	4.8	4.7
3.6	8.6	6.6	4.9	4	4.7	4.6	5.2
3.7	8.7	6.7	5.7	4.5	5.2	4.9	5.6
3.5	8.2	7.4	6.1	4.7	4.6	4.8	4.9

Appendix 22: Effects (tail flick latency in seconds) of Compounds from*Toddalia asiatica* in the Tail Flick Test

Compounds from *T. asiatica*

NEGATIVE CONTROL	HK 3	HK 6	HK 5	HK 7	HK 13	HK 15	HK 18
3.51	3.35	4.15	3.31	3.71	4.49	4.6	3.54
2.77	3.71	4.02	3.53	3.64	4.09	3.4	3.84
3.03	3.61	3.13	3.18	3.79	4.47	3.3	3.51
2.93	3.03	4.04	3.79	3.98	3.75	4.62	3.61
2.94	3.05	3.86	3.5	3.88	3.75	3.4	3.84
3.21	3.92	3.82	3.05	3.47	4.43	4.48	4.02
3.22	3.03	3.8	3.61	3.85	5.12	4.21	3.92
2.85	3.79	3.92	3.41	3.67	4.89	4.52	3.44