A BIOGUIDED PRECLINICAL EVALUATION OF ANTINOCICEPTIVE EFFECTS OF *MONDIA WHYTEI* PLANT EXTRACTS.

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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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ABBREVIATIONS

Ach	-	Acetylcholine
5-HT	-	5-hydroxytryptamine (serotonin)
ALQ	-	Anterolateral quadrant
AMPA	-	α-amino-hydroxy-5-methyl-4-isoxazoleproprionic acid
ANOVA	-	Analysis of variance
AR	-	Analytical grade
CCK	-	Cholecystokinin
CGRP	-	Calcitonin gene-related peptice
CNS	-	Central Nervous System
GABA	-	γ-amino butyric acid
GC-MS	-	Gas chromatography-Mass spectrometer
GR	-	General Reagent
IASP	-	International Association for the Study of Pain
ICIPE	-	International Centre for Insect Physiology and Ecology
i.d	-	Intradermally
i.p	-	Intraperitoneally
L-NAME	-	L- Nitro Arginine Methyl Ester
mAChR	-	Muscarinic acetylcholine receptor
mGluR	-	Metabotropic glutamate receptor
nAChR	-	Nicotinic acetylcholine receptor
NE	-	Norepinephrine
NIST	-	National Institute of Standards and Technology
NK-A	-	Neurokinin A
NMDA	-	N-methyl-D-aspartate
NO	-	Nitric oxide
NRM	-	Nucleus raphe magnus
NS	-	Nociceptive specific
NSAID's	-	Non Steriodal Anti Inflammatory Drugs

PAG	-	Periaqueductal gray
s.e.m	-	Standard Error of the Mean
SI	-	Primary somatosensory cortex
SII	-	Secondary somatosensory cortex
SP	-	Substance P
TLC	-	Thin Layer Chromatography
WDR	-	Wide dynamic range
WHO	-	World Health Organization

CODES FOR VARIOUS PLANT EXTRACTS

AR	-	Aqueous root extract
AB	-	Aqueous bark extract
AL	-	Aqueous leaf extract
ER	-	Ethanol root extract
EB	-	Ethanol bark extract
EL	-	Ethanol leaf extract
CR	-	Chloroform root extract
CB	-	Chloroform bark extract
CL	-	Chloroform leaf extract
CR_d	-	Defatted chloroform root extract
MR_d	-	Defatted methanol root extract
HR	-	Hexane root extract
A_fMR_d	-	Aqueous portion of the defatted methanol root extract
C_fMR_d	-	Chloroform portion of the defatted methanol root extract

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ABSTRACT

Mondia whytei is a forest floor plant with aromatic rhizomatous roots. It belongs to the *Asclepiadaceous* family.

In this study, the antinociceptive activities of various parts of this plant were evaluated and investigated with the aim of isolating the active compounds in a bioguided manner. Mechanisms of action of the isolated compounds were also elucidated.

Soxhlet extraction of the leaves, barks and roots powder of *Mondia whytei* using water, ethanol and chloroform were carried out. Acetic acid induced writhing tests on Swiss albino mice were carried out on each extract. Acetic acid induced writhing test is simple to perform, reproducible and sensitive to antinociceptive effects of various drugs. It was therefore chosen as a screening test. The chloroform root (CR) extract showed the most potent antinociceptive activity with an ID_{50} of 198.8 mg/kg body weight.

The root powder was defatted using n-hexane and the marc extracted with chloroform and methanol successively. The defatted chloroform root extract (CR_d) was found to have reduced antinociceptive activity with an ID_{50} of 350 mg/kg body weight. This is a 76% reduction in antinociceptive effects compared to the CR.

Another defatted root powder was extracted with methanol only and then partitioned with chloroform. The chloroform soluble portion (C_fMR_d) showed an increased

antinociceptive activity with an ID_{50} of 50 mg/kg body weight. This is a 75% increase in antinociceptive effects compared to the CR.

C_fMR_d was fractionated with 30% methanol in dichloromethane and TLC carried out. Five fractions were obtained as follows : C_fMR_d I with R_f = 0.71, C_fMR_d II with R_f = 0.48, C_fMR_d III with R_f = 0.37, C_fMR_d IV with R_f = 0.18 and C_fMR_d V with R_f = 0.1. The five fractions were each subjected to writhing test at a fixed dose of 10 mg/kg, C_fMR_d I showed 29.7% inhibition, C_fMR_d II; 6.5%, C_fMR_d III; 5.4%, C_fMR_d IV; 1.7% and C_fMR_d V; 2.9%. A dose response curve gave an ID_{50} of 14.8 mg/kg and 28.6 mg/kg body weight for C_fMR_d I and C_fMR_d II respectively.

White crystals of C_fMR_d I were obtained at room temperature with a melting point of 168 ^{0}C whereas C_fMR_d II crystals were off-white at room temperature with a melting point of 79 ^{0}C. GC-MS analysis of C_fMR_d I showed a prominent molecular ion peak at m/z 412.4, empirical formula $C_{29}H_{48}O$, with a fragmentation pattern characteristic for sterols. It was confirmed by National Institute of Standards and Technology (NIST 05a) spectral library as a stigmasterol. C_fMR_d II showed a prominent molecular ion peak at m/z 364.0, empirical formula $C_{26}H_{52}$, and confirmed as 9-hexacosene.

The antinociceptive effects of stigmasterol and 9-hexacosene were tested using formalin test in mice. Formalin test was used since it is a superior antinociceptive test in several aspects compared to the other antinociceptive tests. The pain stimulus bears resemblance to most clinical pain and the two phases observed in the test may represent different types

of pain, acute and chronic pain respectively. Stigmasterol reduced the time spent licking, biting and/or lifting the injected paw in both the early phase (Neurogenic pain) and the late phase (Inflammatory pain) of formalin test. This reduction was found to be dose dependent and was statistically ($p<0.001$) significant at a dose of 30 mg/kg body weight and above. No motor, neurological, or other behavioral deficits were observed.

9-hexacosene produced dose-dependent and statistically ($p<0.001$) significant antinociceptive effects on the late phase only and at a dose of 7.5 mg/kg body weight and above. The dose of 100 mg/kg body weight was equipotent to indomethacine dose of 50 mg/kg body weight (Scheff'e post hoc test for multiple comparisons). No motor, neurological, or other behavioral deficits were observed.

Stigmasterol had both centrally and peripherally mediated antinociceptive effects whereas 9-hexacosene had only peripherally mediated antinociceptive effects. Stigmasterol and 9-hexacosene were isolated for the first time from *Mondia whytei*. They were shown to be the compounds strongly involved in the antinociceptive effects of *Mondia whytei* roots. This study authenticates the use of *Mondia whytei* in the management of pain since it contains stigmasterol and 9-hexacosene which have shown significant antinociceptive properties.

Key Words:

Antinociception, *Mondia whytei*, stigmasterol, 9-hexacosene

CHAPTER ONE

1.0 INTRODUCTION

1.1 GENERAL

Pain is one of the foremost causes of suffering in human beings as well as in 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 and research has produced dramatic improvements in the diagnosis and treatment of a number of painful disorders.

The Greeks and Romans were the first to advance theory of sensation, the idea that brain has a role in producing the perception of pain (Ottoson, 1983). It was not until 14th and 15th centuries that evidence began to accumulate in support of these theories. Leonardo da Vinci and his contemporaries came to believe that the brain was the central organ responsible for sensation. Da Vinci also developed the idea that the spinal cord transmits sensations to the brain.

In the 17th and 18th centuries, the study of the body and the senses continued to be a source of wonder to the world's philosophers. In 1664, the French philosopher René Descartes described what to this day is still called "pain pathway."

In the 19th century, pain came to dwell under a new domain that is science. This led to the scientists understanding causes and mechanisms of pain.

According to the International Association for the Study of Pain (IASP, 1979), pain is defined 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. 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. It may be accompanied by anxiety or emotional distress. The cause of acute pain can be diagnosed and treated. Acute pain is self-limiting and is confined to a given period of time and severity. Chronic pain on the other hand represents a disease itself. Environmental and psychological factors tend to worsen it. Chronic pain persists over a longer period of time than acute pain and is resistant to most medical treatments.

Acute pain therefore acts as a warning sign that something is not quite right and that one should take action like withdrawal from the tissue damaging stimulus, take medication or consult a doctor. On the contrary, chronic pain causes unnecessary suffering and lowers productivity. The need to treat this kind of pain cannot be over emphasized.

Analgesics and local anaesthetics were developed from compounds isolated from higher plants. Cocaine was the first local anaesthetic and was isolated from *Erythroxylon coca* in 1860 and has been used since 1884 as an ophthalmic anaesthetic.

Opiods 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.

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. The search for new analgesic compounds has been therefore a priority of pharmacologists and pharmaceutical industries (Mattison *et al.*, 1998).

The ideal analgesic drug should have pain-deadening qualities of morphine but without the side effects of morphine. Therefore to develop the future generation of pain killers one has to take full advantage of the body's pain "switching center" by formulating compounds that will prevent pain signals from being amplified or stop them altogether. Blocking or interrupting pain signals, especially when there is no injury or trauma to tissue, is a significant objective in developing pain medications.

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 should

form a logical search strategy for new analgesic drugs (Farnsworth, 1989; Mattison *et al.*, 1998). *Mondia whytei*, a wild-growing flowering plant is one of such plants.

Mondia whytei belongs to the *Asclepiadaceous* family. The family is mostly found in the tropics and subtropical regions. In Kenya, it is commonly found growing in the wild around the Kakamega forest and adjacent areas such as Kisero, Malava and Bunyala forest blocks. The species is also found on the main hill top ranges of western Kenya (Beentje, 1994).

Mondia whytei is locally known as Mukombela (Luhya), Ogomba (Luo), Olkonkola (Maasai), Mkonkora (Kamba) and Muhukura (Kikuyu) (Kokwaro, 2006). The roots are a rhizome and have been traditionally used to treat pain especially visceral pains like dysmenorrhea, gastrointestinal colic pains and post partum pains (Kokwaro, 2006). It is also claimed to have anti-inflammatory, anti-pyretic and anti-microbial activity (Jain *et al.*, 1996).

There is no scientific research information available from literature search concerning the antinociceptive activities of this plant.

Drug discovery 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 which involves fractionation of active extract and fractions until pure active ingredient are obtained was employed in this project.

1.2 JUSTIFICATION OF THE STUDY

There is need to develop analgesic drug devoid of side effects associated with opiods and NSAIDS. *Mondia whytei* roots have been reported to treat pain. However, there is no systematic research done on it. Isolation of pure compounds responsible for analgesic effects of *Mondia whytei* is hoped to yield novel analgesic compounds with qualities of an analgesic drug.

This research was therefore carried out so as to validate the use of *Mondia whytei* as a painkiller and also to isolate in a bioguided manner the active compounds responsible for the antinociceptive activities of the leaves, stem bark and the root skeels.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 PAIN: HISTORY, DEFINITION AND TERMS

The study of pain started way back in 1846. Researchers of the time found it difficult to find a suitable definition of the term pain. According to Ernest Heinrich Weber (1978), pain represented a bodily need, like hunger or nausea, with no proper stimulus (Weber, 1978). Although this was not correct, it provided a step ahead in science. Following Muller's theory of specific nerve energies (Muller, 1842), pain spots were demonstrated in the skin. Consequence 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)

Although pain is a universal experience of mankind and everybody knows what is meant by it, no satisfactory definition was given until late 20^{th} century. Earlier researchers had suggested on 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. 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 (Beecher, 1957). It is also extremely 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 "unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage." In non-human 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". Price (1999) has challenged the way pain is defined by IASP. He doubted the association between an experience of unpleasant sensation and actual or potential tissue damage. 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 as is the case with the definition given by IASP.

The IASP definition of pain indicates that pain can arise from two different stimuli i.e. the actual damage and the potential damage. 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.

Following tissue injury, a powerful and persistent signal is sent to the nervous system to activate the nociceptive elements activated from the damaged region. This will have an effect of altering the animal's behavioral and motivational states so that behavior conducive to healing and restorative processes is favored, whereas behavior that might

exacerbate the trauma is suppressed. On the other hand, pain that arises from the threat of tissue damage is associated with very different circumstances and has very different behavioral implications (Dennis and Melzack, 1983). In this case, the animal may prevent or greatly reduce the damage by initiating some actions. This depends on the time interval between the initial contact of a potentially damaging stimulus and the onset of tissue damage. Such a threat-related, damage-minimizing system causes pain sensation.

Merskey *et al.* (1986) defined some pain terminology commonly used in the field of pain research. These include analgesia, hypoalgesia, pain tolerance level, pain threshold, hyperaesthesia, hyperalgesia and allodynia. Analgesia is the absence of pain in response to stimulation, which would normally be painful, while hypoalgesia is diminished pain response to normally painful stimulus. Pain tolerance is the greatest level of pain, which a subject is prepared to tolerate. Hyperaesthesia is an increased sensitivity to stimulation, excluding the special senses. Hyperalgesia is an increased responsiveness to a painful stimulus. It is usually associated to inflammation or tissue injury. Hyperalgesia can be induced by heat, exposure to ultraviolet radiation, or injection of hyperalgesic agents such as prostaglandins, histamines, bradykinins, capsaicin, etc, into the skin (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 elicits hyperalgesia to heat and mechanical stimuli. The hyperalgesia that occurs at the site of injury is called primary hyperalgesia, while the hyperalgesia felt in the area surrounding the injury is called secondary hyperalgesia (Hardy *et al.*, 1950;

LaMotte *et al.*, 1982, Raja *et al.*, 1984). Primary hyperalgesia to heat stimuli is believed to be mediated by sensitization of peripheral C and A-delta nociceptors (Meyer and Campell, 1981; LaMotte *et al.*, 1982; Torebjork *et al.*, 1992). Secondary hyperalgesia is due to the sensitization of neurons in the central nervous system caused by discharges of nociceptors (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.

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).

Hyperalgesia is not restricted to the skin but can also occur in the viscera. Hyperalgesia involving the viscera can exist in three forms (Giamberardino, 2000). Visceral hyperalgesia is a form of primary hyperalgesia because it involves the site of injury. It is caused by inflammation or excess stimulation of the visceral structures. Referred hyperalgesia is where pain from the viscera is referred to somatic tissues. Viscero-visceral hyperalgesia is hyperalgesia of one visceral organ that manifest clinically due to

an algogenic condition of another viscus whose segmental afferent innervations partially overlaps (Giamberardino, 2000).

Allodynia is pain from normally non-painful stimuli. It is due to activity in non-nociceptive, fast conducting, thinly myelinated A-beta tactile afferents, which evoke pain in the event of inflammation or after nerve injury (Price *et al.*, 1989, 1992 and Torebjork *et al.*, 1992). Nociceptor activity originating in the area of the injury triggers and maintains a state of central sensitization that amplifies the sensory effects of A-beta tactile input, rendering it painful (Price *et al.*, 1989, 1992; Kajander *et al.*, 1992 and Torebjork *et al.*, 1995).

In man, pain threshold 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 at 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).

2.2 PAIN TRANSMISSION

2.2.1 NOCICEPTION

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

The nociceptive pathways can be described as a three-neuron chain that transmits nociceptive information from the periphery to the cerebral cortex. The *first-order neurons* have their cell bodies in the dorsal root ganglion from where two axons project, one to peripheral tissues and the other to the dorsal horn of the spinal cord. The *second-order neurons* originate from the spinal cord and ascend to the thalamus or other regions of the brainstem. From the thalamus, the *third-order neurons* project to the cerebrocortex (Cross, 1994; Millan, 1999).

2.2.2 PERIPHERAL ACTIONS

In most peripheral tissues throughout the body such as the skin, muscles, joints and viscera, the presence of nociceptors has been described. The nociceptors are free, naked nerve-endings that can be directly activated by strong mechanical, thermal or chemical stimuli; or activated after being sensitized during tissue injury, inflammation, ischemia or low pH (Cross 1994; Riedel & Neeck, 1996; Willis and Westlund, 1997). The

sensitization is mediated by second-messenger systems such as production and release of prostaglandins, bradykinin, serotonin and histamine in the injured area.

Receptors for bradykinin, serotonin and histamine are present on the surface of most primary nociceptive afferents, together with opiate, γ-aminobutyric acid (GABA) and capsaicin receptors (Willis and Westlund 1997).

The nociceptors are associated with the first-order neurons. There are two types of first-order afferent nerve fibres; Aδ- and C-fibres. The Aδ- fibres are myelinated, 2 – 6μm in diameter and conduct nerve signals with a velocity of about 30 – 100 m/s. The C-fibres are unmyelinated and thereby thinner (0.4 – 1.2 μm) than the Aδ – fibres. They are also slower in conducting nerve signals with a velocity of 12 – 30 m/s (Besson and Chaouch, 1987; Almeida *et al.*, 2004).

Stimulation of cutaneous Aδ – fibres results in pricking pain, while C-fibre activation in the skin causes a dull and burning pain sensation. Aδ – and C-fibres are also present in muscular and articular tissue. Noxious stimuli in muscles give rise to an aching and less localized pain, irrespective of fibre type (Almeida *et al.*, 2004). In joints, the occurrence of silent nociceptors is common, i.e. nociceptors that cannot be activated under normal conditions, but are sensitized during inflammation and respond to noxious stimuli. Different visceral disorders can give rise to painful sensation, a sensation that is often referred to a cutaneous zone (Willis and Westlund, 1997). Aδ – and C-fibres have been

described in viscera, but much is yet to be discovered regarding how visceral nociception arises (Cervero *et al.,* 1978; Besson and Chaouch, 1987)

2.2.3 ASCENDING NOCICEPTIVE PATHWAYS

a) Spinal Cord Dorsal Horn

The morphological structure and organization of the spinal cord has been known for long and is well described (Rexed, 1952). The superficial dorsal horn of the spinal cord grey matter includes the marginal zone and the substantia gelatinosa, also known as the Rexed's lamanae I and II (Rexed, 1952; Furst, 1999), and it is mainly here where the afferent Aδ-and C-fibres terminate and where the switch-over to second-order neurons occur (Cervero *et al.,* 1979a, 1979b)

In the superficial dorsal horn, a large variety of receptor classes and neurotransmitters are found (Rexed, 1952; Cervero *et al.,* 1976). Peripheral noxious stimuli lead to nociceptor activation followed by release of neurotransmitters in the dorsal horn. The most important neurotransmitter classes for nociceptive transmission are excitatory amino acids and neuropeptides (Furst, 1999).

The nociceptive transmission by excitatory amino acids, such as glutamate and aspartate, is mediated by ionotropic and metabotropic glutamate receptors. The ionotropic receptors can be divided into three subcategories; N-methyl-D-aspartate (NMDA), A-amino-hydroxy-5-methyl-4-isoxazole-proprionic acid (AMPA) and kainite receptors. The metabotropic glutamate receptors (mGluRs) consist of at least eight subtypes, of which at

least two are present in the spinal cord (Riedel and Neeck, 1996; Coggeshall and carlton, 1997; Furst, 1999).

Neuropeptides and their receptors are abundant in the dorsal horn and may be involved in many functions, including nociceptive transmission. Substance P (SP), can be considered the most important neuropeptide with respect to its nociceptive mechanisms, but other peptides such as neurokinin-A (NK-A), calcitonin gene-related peptide (CGRP) and cholecystokinin (CCK) are also associated with transmission of nociceptive information. SP and NK-A exert their action via neurokinin-receptors and CGRP via CGRP-receptors (Riedel and Neeck, 1996; Coggeshall and Carlton, 1997; Furst, 1999; Millan, 1999).

Nitric oxide (NO) also plays an important role in nociceptive transmission (Furst, 1999). NO acts as a non-adrenergic neurotransmitter and has been proposed to initiate presynaptic glutamate release and thereby enhance nociception (Furst 1999). This is supported by findings showing that the NO synthase inhibitor L-nitro arginine methyl ester (L-NAME) enhances the antinociceptive effect of oxotremorine (Machelska *et al.*, 1999).

b) Ascending Tracts

From the spinal cord dorsal horn, the nociceptive information is transmitted to the brain via the second-order neurons. These have their cell bodies in the dorsal horn and their axon terminations in the brain, and are mainly of two types; wide-dynamic-range (WDR) and nociceptive-specific (NS) neurons. The WDR neurons respond to and distinguish between non-noxious and noxious stimuli, while the NS neurons respond solely to

noxious stimuli (Cross, 1994). The second-order neurons reach the brain via several afferent pathways (Almeida *et al.*, 2004).

The *spinothalamic tract* ascends in the anterolateral quadrant (ALQ) of the spinal cord and terminates in the contralateral thalamus via two projections (Hodge and Apkarian, 1990). In the lateral projection, axons mainly originate in laminae I and V and terminate in the ventral posterior lateral nucleus and the ventral posterior inferior part of the lateral thalamus. In the medial projection, axons originate from deeper parts of the dorsal horn and from the ventral horn, and terminate in the central lateral locus (Cross, 1994). The lateral part of the thalamus is reported to be involved in the sensory-discriminative component of pain, while the medial part is involved in motivational-affective aspects of pain (Willis and Westlund, 1997 and Almeida *et al.*, 2004).

The *spinomesencephalic tract* includes projections to different areas in the midbrain. Most axons originate, similarly to cells of the spinothalamic tract i.e. in laminae I and IV – VI while some have their origin in lamina X or in the ventral horn (Willis and Westlund, 1997). The tract terminates in regions such as periaquaductal gray (PAG), nucleus cuneiformis, intercolliculus nucleus, deep layers of the superior colliculus, and anterior and posterior pretectal nuclei (Willis and Westlund, 1997). It has been suggested that different components of the tract have different functions. The projections to the PAG seem to contribute to aversive behavior as well as activation of descending pain modulation while the deep layers of superior colliculus are likely to be of importance in orientation (Almeida *et al.*, 2004).

The *spinoreticular tract* originates from deep layers of the dorsal horn, from laminae VI and VII of the ventral horn, ascends through ALQ and terminates in the reticular formation of the brainstem (Cross, 1994). One part of the tract terminates in several nuclei in pons and medulla, such as nucleus gigantocellularis, nuclei reticularis, pontis caudalis and oralis, nucleus paragigantocellularis and nucleus subcoeruleus (Cross, 1994). Another major termination is in the parabrachial region, including the locus coeruleus and the parabrachial nuclei (Willis & Westlund, 1997). The spinoreticular tract is important for motivational-affective aspects of pain and for descending modulatory mechanisms (Millan, 1999; Almeida *et al.*, 2004).

In addition, several other ascending nociceptive pathways have been described. The spino-limbic tracts consist 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 (Cervero *et al.*, 1977; Willis and Westlund, 1997; Millan 1999; Almeida *et al.*, 2004).

c) Cortical Structures

The nociceptive information is transmitted from the thalamus to the cerebral cortex via the 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 (Cross, 1994). Neurons from the medial nuclei are projected to the anterior cingulated gyrus, which has been suggested to be involved in perception of suffering and emotional reactions to pain. Several other areas of the cerebral cortex have also been described as important for the processing of nociceptive information and the experience of pain (Davis *et al.,* 1932). The secondary somatosensory cortex (SII), regions of the interior and anterior parietal cortex, the insular cortex and the medial prefrontal cortex have all been identified as regions activated by noxious stimuli from cutaneous and intramuscular tissue (Riedel and Neeck, 1996; Timmermann *et al.,* 2001).

d) Basal Ganglia

The basal ganglia, including the caudate nucleus, putamen, globus pallidus and substantia nigra, are important regions of the brain regarding motor functions (Barker, 1988). However, the basal ganglia have also been shown to be important for processing of nociceptive somatosensory information (Chudler and Dong, 1995). Several studies have suggested the basal ganglia to be involved in the sensory-discriminative dimension of pain, the effective dimension of pain, the cognitive dimension of pain, modulation of nociceptive information, and sensory gating of nociceptive information to higher motor areas (Chudler and Dong, 1995).

2.3 ABNORMAL PAIN CONDITIONS

The neuroanatomical system presented in figure 1 describes the mechanisms of the nociceptive system during normal conditions, i.e. when an acute painful sensation arises as a warning signal following tissue damage. This type of pain is often denominated acute nociceptive pain. However, if the pain sensation for some reason persists and no longer serves as a warning signal, chronic pain may develop. Chronic pain is a major cause of suffering and is difficult to treat. It can be defined as a continuous or intermittent pain or discomfort which has persisted for at least three months (IASP 1979; Smith *et al.*, 2001). Chronic pain may develop as a result of several changes in the nociceptive system. One significant factor for this is central sensitization at the spinal level, which has been linked to the wind-up phenomenon (Priest and Hoggart, 2002). Another important factor for the initiation of chronic pain is neuropathic pain, i.e. damage to the central or peripheral nervous system (Ossipov *et al.*, 2000; Garry *et al.*, 2004).

2.4 PAIN MODULATION

2.4.1 ANTINOCICEPTION

Although the ability to experience pain is vital for the survival of all mammals, it is essential for the organism to be able to control and modulate the pain sensation. The pain control is termed antinociception. The transmission of nociceptive information is rigorously controlled and modulated at most levels in the central nervous system, and the modulation appears to be hierarchically organized in the descending pain modulatory system (Willis and Westlund, 1997) (Figure 1)

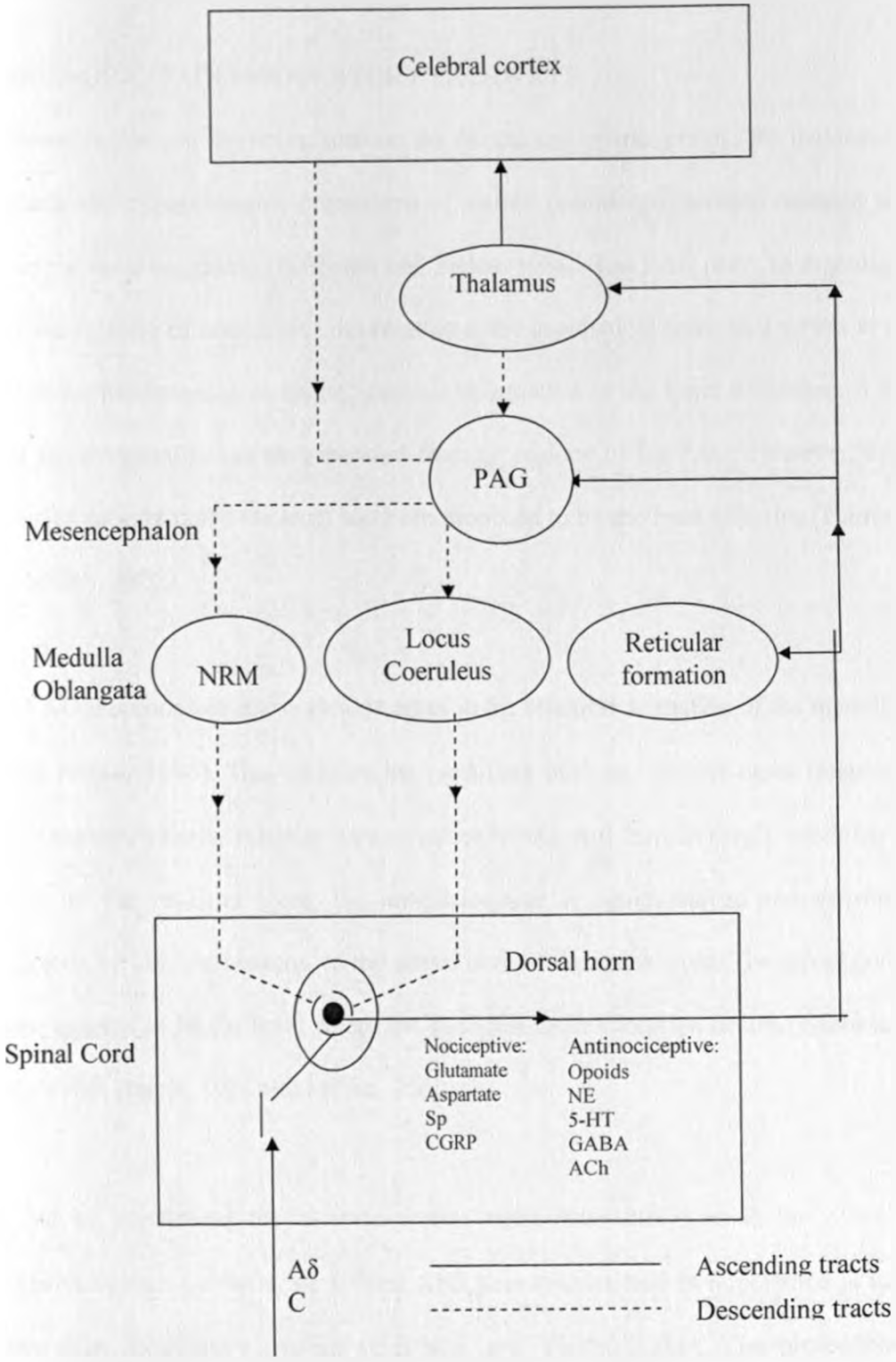


Figure 1: Simplified overview of the nociceptive and antinociceptive pathways (Abelson, 2005)

2.4.2 DESCENDING PAIN MODULATORY PATHWAYS

From different regions in the brain, such as the frontal and insular cortex, the thalamus, the amyglada and hypothalamus, projections of mainly opioidergic neurons descend to the PAG in the mesencephalon (Basbaum and Fields, 1984). 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 generated from all regions of the PAG. However, the ventral portion (dorsal raphe nucleus) has been proposed to be the most effective (Harris, 1996 and Millan, 2002).

From the PAG, neurons project to various areas in the reticular formation of the medulla (Riedel and Neeck, 1996). This includes the medullary nucleus, nucleus raphe magnus, reticularis magnocellularis, nucleus paragigantocellularis and noradrenergic medullary cell groups. In the reticular areas, the antinociception is again relayed and neurons project, directly or via interneurons, to the dorsal horn of the spinal cord. The spinal cord dorsal horn appears to be the level where the strongest antinociception occurs (Basbaum and Fields, 1984; Harris, 1996 and Millan, 2002).

Opioids can be considered the most important neurotransmitter type in the overall antinociceptive system but there are several other neurotransmitters of importance in the descending pain modulatory system (Basbaum and Fields, 1984). The projections descending from the medulla to the dorsal horn mainly consist of serotonergic and noradrenergic neurons (Harris and Westbrook, 1995; Harris, 1996; Millan, 2002).

2.4.3 ANTINOCICEPTION AT THE SPINAL CORD LEVEL

The ability to modulate pain at the spinal level has been known for a long time. In 1885, James Leonard Corning showed that cocaine administered into the perispinal space resulted in some degree of anaesthesia in humans (Corning, 1885).

a) GABA

In the spinal cord dorsal horn, nociceptive transmission can be inhibited in numerous ways. One of the major inhibitory transmitters is γ-amino butyric acid (GABA). Binding sites for $GABA_A$ and $GABA_B$-containing neurons have been localized in almost all structures in the spinal cord, including inteneurons and synaptic terminals. The highest concentration of GABA is in the dorsal horn, especially laminae I – III. There are two main GABA receptor types: the ligand-gated Cl^- channel $GABA_A$ and the GTP-binding protein coupled receptor $GABA_B$ (Malcangio and Bowery, 1996). Both types are important in antinociception at the spinal level. Activation of GABAergic interneurons reduces the release of glutamate, SP and CGRP from primary nociceptive afferents (Furst, 1999; Sehadrack and Zieglagansberger, 1998).

b) Opioids

Opioid receptors are important in supraspinal as well as spinal antinociceptive mechanisms. The three major types of opioid receptors are μ, K and δ – receptors. The μ-receptor is generally considered the most essential in antinociceptive actions, but the K and δ-receptors have also been shown to mediate antinociception. In addition, there is a

fourth type of opioid receptor; the ε-receptor, which is thought to mediate β-endophin-induced analgesia, but the existence of such a receptor, is still controversial. The endogenous ligands for opioid receptors can be divided in three different families of opioid peptides; endorphins, enkephalins, and dynorphins (Kanjhan, 1995; Furst, 1999; Tseng, 2001). Opioids can exert their antinociceptive activity through numerous mechanisms. For instance, 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 (Taddese *et al.*, 1995; Ossipov *et al.*, 2004). In addition, the opioid system has been found to interact with NMDA receptors, which might not only contribute to the antinociceptive actions of opioids, but also to 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 (Mao, 1999).

c) Monoamines

The monoamines serotonin and norepinephrine are important neurotransmitters in spinal antinociception (Yaksh, 1979). Descending serotonergic and noradrenergic neurons terminate in the spinal cord dorsal horn, where endogenous serotonin (5-HT) and norepinephrine (NE) is released to inhibit nociceptive transmission (Furst, 1999). There are several types of serotonergic and noradrenergic receptor types present in the spinal cord. Serotonergic receptors can be divided into three main classes; 5-HT_1, 5-HT_2 and 5-HT_3. In particular, 5-HT_1 and 5-HT_3 have been shown to play a role in spinal

antinociception, although some subtypes seem to also facilitate nociceptive transmission (Ali *et al.*, 1994; Hoyer *et al.*, 1994; Ali *et al.*, 1996; Furst, 1999).

The noradrenergic receptors involved in antinociception are generally $\acute{\alpha}_2$-adrenergic receptors, which consist of three subtypes: $\acute{\alpha}_{2a}$, $\acute{\alpha}_{2b}$ and $\acute{\alpha}_{2c}$. Stimulation of spinal $\acute{\alpha}_2$-adrenergic receptors results in a very potent antinociception, as seen after intrathecal administration of $\acute{\alpha}_2$ adrenergic agonists (Reddy *et al.*, 1980; Eisenach *et al.*, 1989; Saunders and Limbird, 1999).

d) Nitric Oxide

Nitric oxide (NO) is known to be involved in the transmission of nociceptive information, but several studies have also suggested a role for NO in antinociception (Xu and Tseng, 1994). It has been reported to contribute to the antinociceptive actions of morphine as well as of adrenergic and cholinergic agonists, at both spinal and supraspinal levels (Iwamoto and Marion, 1994a; Iwamoto and Marion, 1994b and Xu *et al.*, 1997).

e) Acetylcholine

Several researchers have reported the involvement of acetycholinergic receptor system in antinociception at both the supraspinal and the spinal level (Yaksh *et al.*, 1985). Systemic as well as intrathecal administration of muscarinic agonists produces potent antinociception in several species (Gower, 1987; Gillberg *et al.*, 1989; Zhuo and Gebhart, 1991; Iwamoto and Marion, 1993b; Abram and O'Connor, 1995). Different pharmacological studies have attempted to determine the spinal muscarinic subtypes

relevant for the antinociceptive effect, suggesting involvement of the M1, M2, M3 and M4 subtypes (Bartolini *et al.*, 1992; 1987; Ellis *et al.*, 1999 and Duttaroy *et al.*, 2002).

Neuronal nicotinic receptors are considered a promising target in pain treatment (Flores and Hargreaves, 1998). An involvement of nicotinic receptors in antinociception has been known for several decades. In 1932, antinociception of nicotine was reported (Davis *et al.*, 1932), an effect that has been verified by other studies (Sahley and Berntson, 1979; Iwamoto, 1991). Other nicotinic agonists that produce antinociception after supraspinal or systemic administration are epibatidine (Qian *et al.*, 1993 and Curzon *et al.*, 1998), A85380 and ABT – 594 (Bannon *et al.*, 1998; Bitner *et al.*, 1998). The antinociceptive effects of nicotinic agonists administered into the spinal cord are somewhat controversial, since both nociceptive and antinociceptive effects have been observed (Khan *et al.*, 1998).

The acetylcholinergic receptor system has been found 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 GABAergic (Baba *et al.*, 1998; Chen and Pan 2003), opioids (Harris *et al.*, 1969; Pert, 1975; Chen and Pan, 2001) and adrenergic (Detweiler *et al.*, 1993; Klimscha *et al.*, 1997; Pan *et al.*, 1999; Honda *et al.*, 2002; Honda *et al.*, 2003) receptor systems. Nicotinic receptors are also involved in modulation of nociceptive information by other receptor systems. Interactions with particularly the serotonergic and adrenergic systems have been demonstrated (Iwamoto and Marion, 1993a; Bitner *et al.*, 1998; Cordero-Erausquin and Changeux, 2001). In

addition, both muscarinic and nicotinic receptors have been suggested to play an important role in the antinociceptive mechanism of NO in the spinal cord (Xu *et al.*, 1996; Xu *et al.*, 2000; Abelson and Hoglund, 2002a).

Based on these findings, there is little doubt that the acetylcholinergic receptor system is an important component in antinociceptive mechanisms. However, the underlying mechanisms responsible for the cholinergic contribution to spinal antinociception are far from being fully understood.

One conceivable explanation for cholinergic contribution to antinociception could be that stimulation of muscarinic or nicotinic receptors results in a release of acetylcholine in the spinal cord, and that acetylcholine in turn inhibits the nociceptive transmission. In 1945, a study showed that subcutaneous injection of the acetylcholine esterase inhibitor neostigmine (prostigmine) significantly increased the antinociceptive effect of morphine in humans (Flodmark and Wramner, 1945). In more recent studies, intrathecal administration of neostigmine has revealed that part of the antinociceptive effect of this substance is mediated at the spinal cord level in both humans and animals (Bouaziz *et al.*, 1995). Since neostigmine prevents degradation of acetylcholine in the synaptic cleft, the amount of acetylcholine should be increased. This strengthens the theory that endogenous acetycholine is contributing to the inhibition of nociceptive information at the spinal cord level. A few studies have been performed to evaluate this theory (Bouaziz *et al.*, 1996; Eisenach *et al.*, 1996) but much is yet to be discovered.

Studies on the release of acetylcholine in the spinal cord of rats using microdialysis have demonstrated that both muscarinic and nicotinic receptor ligands dose-dependently affects the release of acetylcholine in the dorsal region of the rat spinal cord (Hoglund *et al.*. 2000). The microdialyis technique established in this study has made it possible to accurately perform *in vivo* studies of the pharmacological mechanisms of acetylcholine, in relation to pain modulation in the spinal cord.

Intravenously administered oxotremorine, a muscarinic receptor agonist dose-dependently increased the intraspinal release of acetylcholine whereas atropine, a muscarinic receptor antagonist decreased it (Abelson and Hoglund, 2002a).

A dose of 10 and 30 mg/kg intravenously administered lidocaine significantly increased the intraspinal release of acetylcholine. This effect was reduced by pretreatment with intraspinally administered atropine or mecamylamine. This suggests that the antinociceptive effects of systemically administered lidocaine are mediated through an action on muscarinic and nicotinic receptors (Abelson and Hoglund, 2002b).

Clonidine and rilmenidine, $\acute{\alpha}_2$-adrenergic receptor agonists increased spinal acetylcholine release while yohimbine and efaroxan, $\acute{\alpha}_2$-adrenergic receptor antagonists caused a decrease in spinal acetylcholine release. The binding of $\acute{\alpha}_2$-adrenergic receptor ligands to nicotinic receptors might affect intraspinal release of acetylcholine (Abelson and Hoglund, 2004).

It has been shown that an increased intraspinal acetylcholine release could be involved in the non-inflammatory pain suppression by aspirin but not paracetamol (Abelson *et al.*, 2004)

2.5 NOCICEPTIVE TESTS

The tests give nociceptive measures that involve different physiological and anatomical substances and which also require varying degrees of integration and co-ordination (Amit and Galina, 1986). Nociceptive tests serve as models for studying human pathological conditions as well as screening potential analgesic drugs.

Earlier nociceptive tests described for mice and rats have undergone vast modifications to cater for the pharmacological and physiological differences between narcotic and non-narcotic analgesics and to improve their sensitivity (Hunskaar, 1987a). Simplicity and reproducibility are two other criteria that have been used for rating nociceptive tests (Taber, 1974).

2.5.1 ACETIC ACID INDUCED WRITHING TEST

The acetic acid induced writhing test is a useful nociceptive test for visceral pain studies. However, it has also been used to study cutaneous pain with great success. It involves administering an irritant into the peritoneum of an animal. In rats 0.5 ml of 10% acetic acid is used to induce abdominal constriction response (Schmauss and Yaksh, 1984).

The conventional abdominal constriction test has been modified and used to study the antinociceptive effects of drugs (Bentley *et al.*, 1981). In the modified test, drugs to be tested are administered intraperitonealy 6 minutes after the acetic acid. The abdominal constriction test is commonly used as a screening method because of its simplicity and reproducibility. Acetic acid test is sensitive enough for investigating the antinociceptive

effects of drugs. The noxious agent causes a behavioral response in the experimental animal. In rats and mice, the behavioral response consists of a wave of constriction and elongation passing caudally along the abdominal wall. This sometimes is accompanied by twisting of the trunk and followed by extension of the hind limbs (Vander Wende and Margolin, 1956; Siegmund *et al.*, 1957; Collier *et al.*, 1968; Bentley *et al.*, 1981; Schmauss and Yaksh, 1984). The behavioral response has been given various terms such as "writhing" (Vander Wende and Margolin, 1956), "stretching" (Koster *et al.*, 1959), "cramping" (Murray and Miller, 1960) and "squirming" (Whittle, 1964).

In the conventional abdominal constriction response, drugs are given subcutaneously 15, 20 or 30 minutes before intraperitoneal administration of acetic acid, and the number of writhes counted for two periods of 2 min, beginning 6 min after the acetic acid injection. A writhe has been defined as a stretching of the hind limbs accompanied by a contraction of the abdominal muscles (Ward and Takemori, 1983).

Pain researchers have used various concentrations and volumes of acetic acid to induce pain in rodents. In mice, 1.0 ml/kg of 6% (Ward and Takemori, 1983) or 10 ml/kg of 0.6% (Bentley *et al.*, 1981) acetic acid has been administered. In rats 0.5 ml of 9% acetic acid has been used to induce abdominal constriction response (Schmauss and Yaksh, 1984).

Like other behavioral assays, the acetic acid test has some drawbacks. The major one is that the animal is not able to terminate the noxious input and this is considered ethically

unacceptable (Zimmerman, 1983). The test is also time-consuming, and it produces many false positives (Emele and Shanaman, 1963). The mechanisms of the action of drugs in this test is not well understood (Hunskaar, 1987). Some researchers have suggested the existence of receptors in the peripheral tissue (Bentley *et al.*, 1981) which are possibly different from those in the CNS (Bentley *et al.*, 1981).

Like other pain tests, the abdominal constriction test has been carried out in rodents and to a small extent in other small animals. In the recent past, a modified acetic acid test suitable for use in amphibians has been reported (Stevens, 1992). This method has been described as inexpensive and useful for measuring nociceptive threshold in amphibians. In this method, graded concentrations of dilute acetic acid were applied using a Pastuer pipette to hind limb of the unrestrained animal. When the concentration of dilute acetic acid reaches the nociceptive threshold, the animal responds by wiping the drop off the dorsal surface of the thigh with a rapid movement of either hind limb.

Stevens (1992) termed this nocifensive behavior as the "Wiping response". The response was described as easy to score and the nociceptive threshold measured was stable over a period of hours and days (Pezalla, 1983; Stevens, 1992). The test is comparable to tail-flick in rodents (Stevens, 1992). Evidence indicates that the amphibian model of acetic acid test activates C-fibre polymodal nociceptors (Adrian *et al.*, 1926). This allows for characterization of the analgesic effects of pharmacological agents acting on a response driven by acute activation of unmyelinated C-polymodal nociceptors (Stevens, 1992).

Unlike the conventional abdominal constriction test in rodents, the amphibians' model can be terminated by wiping the acetic acid drop (Stevens and Rothe, 1997).

2.5.2 ADJUVANT INDUCED ARTHRITIS

The test provides a chronic pain model for studying the effects of analgesics. Sodium urate crystal or mycobacterium butyricum with Freud's adjuvant (Pircio *et al.*, 1975) have been used to induce polyarthritis in rats. Eodema of the paw and vocalization following manipulation of the joint are two of the pain responses that have been used to study effects of analgesics in this test.

2.5.3 FORMALIN TEST

The formalin test assesses the way an animal responds to moderate, continuous pain generated by injured tissue. Dubuisson and Dennis (1977) developed the formalin test to rate pain in saline and morphine treated rats. Prior to 1977, Lewis and Kallgren (1939) had induced experimental pain in human subjects by injecting small volumes of hypertonic saline. Selye (1949) used formalin as a stimulus in studies of peripheral inflammation in rats while Melzack and Melinkoff (1974) also used small amounts of dilute formalin to induce pain in cats.

Dubuisson and Dennis (1977) described a modified procedure for carrying out the formalin test in cats and rats. The authors described in detail the behavior induced by formalin and developed a scheme for quantifying the assumed pain. The behavior observed included elevation, licking, biting or shaking the injected paw or reducing the

weight put on it. The different behavioral states were quantified on the basis of the total time spent in pain behaviors.

In the formalin test, two distinctive phases of nociceptive behavior in rats have been described (Dubuisson and Dennis, 1977). The first phase occurs immediately after the injection of formalin and lasts for about 5 minutes, while the second phase starts approximately 20 minutes after the injection and lasts for about 40 minutes

The formalin test, as described by Dubuisson and Dennis (1977), has been widely used in a number of animals including, rats (Dennis *et al.,* 1980; Dennis and Melzack, 1980,1983; Gamble and Milne, 1990), monkeys (Alreja *et al.,* 1984), guinea-pigs (Takahashi *et al.,* 1984), naked mole rats (Kanui *et al.,* 1993; Karim *et al.,* 1993) and mice (Hunskaar *et al.,* 1985).

Formalin is the aqueous solution of 37% (w/w) formaldehyde. A 10% formalin solution contains 3.7% formaldehyde. In the formalin test, various volumes in the range of 30 – 100 microlitres have been used (Tjolsen *et al.,* 1992). The most common concentrations that have been used are from 1 to 5% (Dubuisson and Dennis, 1977; Hunskaar *et al.,* 1985, 1986a, Tjolsen *et al.,* 1991). In the naked mole rats, 20 microlitres of 10% formalin has been reported to produce quantifiable behavioral response (Kanui *et al.,* 1993, Karim *et al.,* 1993).

In the initial studies, formalin was injected in one of the forepaws (Dubuisson and Dennis, 1977; Dennis *et al.,* 1980; Takahashi *et al.,* 1984). This site of injection has been questioned because licking of the forepaw can occur during normal grooming behavior. The hind paw has been used as the site of injection by many researchers (Coderre *et al.,* 1984; Fasmer *et al.,* 1985, 1986a; Hunskaar *et al.,* 1985, 1986a, 1987; Hunskaar and Hole, 1987; Kanui *et al.,* 1993; Karim *et al.,* 1993; Tjolsen *et al.*, 1991).

The most commonly used location for the injection of formalin is the dorsal surface of the paw. Some researchers have, however, used plantar surface of the paw (Coderre *et al.,* 1984; Gamble and Milne, 1990). In most cases the formalin is injected subcutaneously.

In the formalin test, the two phases of nociception are thought to involve two distinctly different types of pain (Dubuisson and Dennis, 1977; Hunskaar *et al.,* 1985; Hunskaar and Hole, 1987). The first phase is probably due to chemical stimulation of nociceptors (Dubuisson and Dennis, 1977; Hunskaar *et al.,* 1985). This is supported by the data showing that formalin predominantly evokes activity in C-fibres, and not in A-delta afferents (Heapy *et al.,* 1987).

The second phase starts 15 – 20 min after formalin injection and is thought to be partly due to peripheral inflammatory processes (Tjolsen *et al.,* 1992). Non-steroidal anti-inflammatory drugs (NSAIDs) such as indomethacin have been reported to reduce

nociceptive behavior during the second phase, but not in the early phase (Hunskaar and Hole, 1987; Rosland *et al.*, 1990).

Since NSAIDs are basically anti-inflammatory drugs, their effect on the late phase suggests that this phase of formalin test may be due to inflammatory process caused by noxious stimuli. There are also reports indicating that substance P and bradykinin are involved in the early phase, while histamine, serotonin and prostglandins are involved in the second phase (Shibata *et al.*, 1989).

There is evidence suggesting that spinal processes induced by the first phase, as well as the local inflammatory changes during the second phase are necessary for the full manifestation of the second phase. This evidence is based on electrophysiological recordings in the dorsal horn (Dickenson and Sullivan, 1987).

The involvement of N-methyl-D-aspartate (NMDA) receptors in the second phase of formalin test has also been suggested (Haley *et al.*, 1990b; Yamamoto and Yaksh, 1992). NMDA receptors play a major role in the development of central hyperalgesia that occurs following peripheral application of noxious stimulus (Haley *et al.*, 1990b; Coderre *et al.*, 1993; Dolan *et al.*, 2000). Injection of formalin sets up a cascade of events leading to the activation NMDA receptors in the spinal cord. This will lead to the release of excitatory amino acids such as glutamate, which is necessary for both phases of the formalin response (Dickenson and Sullivan, 1987). In addition to glutamate, nitric oxide is also released in the spinal cord. There is evidence indicating that the release of glutamate requires the production of nitric oxide, which is believed to play a role during prolonged

nociception such as that caused by formalin injection (Meller *et al.*, 1994). Glutamate acts on specific receptors in spinal cord to cause hyperalgesia.

The behavioral response in the formalin test can be influenced by a number of factors, including ambient temperature (Rosland, 1991). This is more so in the late phase than in the early phase. In mice, Rosland (1991) reported weaker response in a room temperature of 20 – 21 ^{0}C than 23 – 24 ^{0}C or 26 – 28 ^{0}C. The ambient temperature is thought to influence the tissue temperature, which in turn influences the inflammatory reactions taking place in the second phase. Besides the ambient temperature, other environmental factors such as sound, odors, bring light, elevated atmospheric pressure or activity by humans in the room during the test period may also influence and modify the response in the formalin test (Berge *et al.*, 1991).

Animals injected with formalin can show a wide range of behaviors, including a reduction in the weight put on the injected paw, total elevation of the paw, and licking, biting and shaking of the paw. These behaviors have been widely used to mark the end-points in the formalin test (Dennis *et al.*, 1980; Denis and Melzack, 1983; Alreja *et al.*, 1984; Coderre *et al.*, 1984; Kanui *et al.*, 1993; Karim *et al.*, 1993). A need to have a simpler way of scoring has resulted in modification of the scoring method. The time the animal spends licking and / or biting the injected paw or the total time paw is elevated from the floor has been adopted for use in most animal models (Fasmer *et al.*, 1985, 1986a; Hunskaar *et al.*, 1985, 1986a; Hunskaar and Hole, 1987; Rosland *et al.*, 1990; Kanui *et al.*, 1993; Karim *et al.*, 1993).

The formalin test is the most common behavioral nociceptive assay test and has been used in a number of studies investigating the effects and functions of exogenous and endogenous substances such as opioids and monoamines (Coderre *et al.*, 1984). The test has also been used to study mechanisms of nociception in the nervous system using lesions, local anaesthetics, or electrical stimulation (Fasmer *et al.*, 1985, 1986a and Tjolsen *et al.*, 1991).

The formalin test is sensitive to analgesic effects of weak analgesics such as acetylsalicylic acid and paracetamol (Hunskaar *et al.*, 1985; Hunskaar and Hole, 1987; Rosland *et al.*, 1990). Other nociceptive tests such as hot plate and tail flick may not show the analgesic effects of such drugs.

Another advantage of the formalin test is that the pain induced is almost similar to chronic pain in human subjects. Some of the shortcomings of the formalin test include tissue damage, paw mutilation, and prolonged subjection of the animal to pain. The use of low concentrations of formalin minimizes the occurrence of some of these disadvantages.

2.5.4 TAIL-FLICK TEST

The tail-flick test in rodents (D'Amour and Smith, 1941) has been one of the standard methods for investigating nociception and analgesia. Thermal stimulus is applied to the

tail and the latency to a flick of the tail is recorded. This test has a number of limitations despite its regular use for screening drugs and studying pain mechanisms in animals.

The test employs a transient stimulus which may not reflect clinical pain. The restraint the animal is subjected to may induce fear and discomfort which may distort the results (Hargreaves *et al.,* 1988), Tail-skin temperature has also been reported to influence tail-flick latency in this test (Tjolsen *et al.,* 1988). Despite these drawbacks, the tail-flick test is useful for studying the tail-flick reflex, a spinally intergrated nociceptive reflex, and also for evaluating new drugs, especially opiates.

2.5.5 HOT-PLATE TEST

The hot-plate test measures the response to a brief noxious heat stimulus. This test was originally proposed and used for measuring the ability of drugs to inhibit the reflex responses of mice placed in contact with a hot surface maintained at constant temperatures between 55 and 70 ^{0}C (Woolfe and MacDonald, 1944). Eddy and Leimbuch (1953) described the renowned constant temperature hot-plate test, where the temperature is maintained at 55 $\pm$ 0.5 ^{0}C.

The constant temperature hot-plate test has been widely used and with great success. It has been claimed by some researchers, however, that the method may demonstrate no or only weak analgesia of non-narcotics and narcotic antagonists (Ankier, 1974; Taber, 1974; Hunskaar *et al.,* 1986 b).

Ankier (1974) was able to demonstrate the antinociceptive activity of narcotics and narcotic antagonists, when he performed a hot plate test at 50, 55 and 59 ^{0}C in mice. Hunskaar *et al.*, (1986 b) described an increasing temperature hot-plate test in mice and rats. The antinociceptive effects of weak narcotic and non-narcotic analgesics were demonstrated when the hot-plate temperature was increased in steps of 2 ^{0}C from 42 – 52 ^{0}C, it was therefore concluded that the increasing temperature hot-plate test is useful for both mice and rats in evaluating and screening both narcotic and non-narcotic analgesics.

In the hot-plate test the animal may show one or more of the following pain-related responses: jumping, kicking and dancing, thumping of the foot, lifting the foot off the plate and licking the forepaws, the hindpaws or both (Hunskaar *et al.*, 1986 b). Licking of the forepaws, hindpaws or both have been the main criteria for determining the end-point (Woolfe and MacDonald, 1944; Ankier, 1974; Yaksh and Rudy, 1977; Berge *et al.*, 1983; Fasmer *et al.*, 1983; Hunskaar *et al.*, 1986 b). Most researchers have however used the latency to the licking or stepping of the hind paw rather than the licking of the forepaws, because the latter has a relatively shorter latency and may not necessarily be elicited by noxious stimuli (Hunskaar *et al.*, 1986 b).

To avoid tissue damage of the paws of the animals, a cut off time or temperature or both is chosen whenever a hot-plate test is conducted. Cut off temperatures of up to 70 ^{0}C, depending on the exposure time, have been used in the hot plate test.

2.6 MECHANISM OF ACTION OF ANALGESIC DRUGS

2.6.1 OPIOID DRUGS

Opioids consist of the most effective class of analgesics in clinical use today (Akil *et al.*, 1998). The endogenous opioid peptides and the exogenous opiates cause analgesia by acting on the same systems in the body of an animal. 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 receptors (DORs) and κ opioid receptors (KORs) (Wang *et al.*, 1994; Simonin *et al.*, 1995). The opioid receptors show a high degree of structural homology but 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 e*t 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 release from central and peripheral terminals of sensory neurons (Yaksh, 1988; 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 (Zöllner 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 (Mansour e*t al,* 1995; Buzas and Cox, 1997; Cheng *et al.*, 1997; Coggeshal *et al.*, 1997; Zhang *et al.*, 1998; Wang and Wessendorf, 2001; Silbert *et al.*, 2003; Rau *et al.*, 2005; Gendron e*t al.*, 2006). The peripheral antinociceptive effects of opioids are mediated by peripheral opioid receptors (Bartho *et al.*, 1990; Stein *et al.*, 1990; Stein, 1995). 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; Ständer *et al.*, 2002; Mousa *et al.*, 2007a, b;) 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 CGRP) from central and peripheral terminals (Junger *et al.*, 2002). These events lead to antinocicpetive and anti-infalmmatory effects particularly within the injured tissue.

The expression of these peripheral opioid receptors is upregulated 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.*, 2003l Troung *et al.*, 2003; Zöllner *et al.*, 2003; Püehler *et al.*, 2004; Shaqura *et al.*, 2004; Walczak *et al.*, 2005; Kabli and Cahill, 2007). The upregulation in receptor expression occurs acutely i.e. within minutes or hours after tissue damage / neural damage. The molecular mechanisms that underlie this upregulation are varied and include increased receptor trafficking to the cell membrane as well as increased receptor protein synthesis among others (Zöllner *et al.*, 2003; 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 Grey 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.

2.6.2 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 NSAIDs 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. Since the discovery that NSAIDs act by inhibiting prostaglandin biosynthesis (Vane, 1971), their mechanism of action has been linked to it (Ferreira, 1972; Ferreira and Vane, 1974; Ferreira *et al.,* 1978).

On 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 A_2 and cyclooxygenase I and II. NSAIDs are said to prevent PG biosynthesis by inhibiting cyclic endoperoxides (Fig. 2). 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).

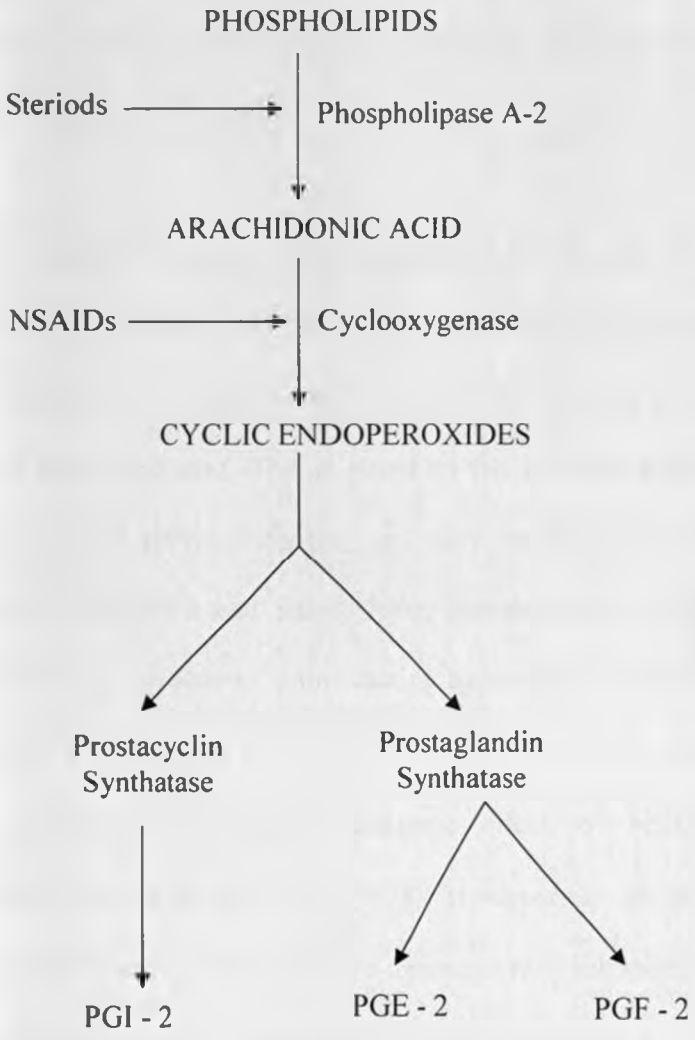


Figure 2a: The sites of action of steroidal and non-steroidal anti-inflammatory drugs (Terenius, 1981)

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 an experiment 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. A study has also shown that the serotonergic systems may play a role in the analgesic effect of paracetamol in mice (Tjolsen *et al.*, 1990). The differences in potency of NSAIDs also suggest that they have other modes of action in addition to their common effect on cyclooxygenase enzyme.

2.6.3 STEROIDAL ANTI-INFLAMMATORY DRUGS

Anti-inflammatory steroids are so far the most potent anti-inflammatory agents. They are capable of suppressing the cardinal signs of inflammation regardless of the cause. There are several ways in which anti-inflammatory steroids exert their effects on inflammation (Hunskaar and Hole, 1987). It is clearly established that this class of drugs at least exert their anti-inflammatory properties by inhibiting phospholipase A_2 activity (Fig. 2), which is necessary for the release of arachidonic acid (Terenius, 1981; Vane and Botting, 1987). Anti-inflammatory steroids inhibit phosphiolipase A_2 indirectly by causing the release of an inhibitory protein, which has been variously termed macrocortin, lipomodulin, renocortin, or lipocortin (Vane and Botting, 1987). Steroids 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 phospholipase A_2 activity.

There is a general view that steroids do not have analgesic effect. However, there are few reports suggesting that these drugs may cause potent analgesia. For instance, in the formalin test, the analgesic effect of hydrocortisone and dexamethasone has been demonstrated in the chronic 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. 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).

2.7 PRINCIPLES OF EXTRACTION OF SPECIFIC PHYTOCHEMICAL GROUPS

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 includes 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 (Figure 2b). This protocol can be modified in order to extract targeted specific phytochemical group of interest. Solvent extraction is the most scientifically preferred method of extraction. 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, chloroform and hexane (Peter and Amala, 1998). Thus in extracting a crude plant, different solvents can be mixed or they can be used in sequence in the same sample materials.

Different solvent extraction methods exist. The most simple and easy to use of these methods are maceration, hydro-distillation using steam and soxhlet extraction (Jones and Kinghorn, 2005).

In maceration, the homogenized plant sample is soaked in a solvent in a closed container and left at a room temperature. 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

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 → dichloromethane → methanol → 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.

This method does have limitations. One major problem is that because the solvent is being recycled, the extract that collects in the lower container is continuously being heated and may suffer thermal degradation reactions. Secondly, the total amount of certain substances extracted will exceed their solubility in that particular solvent. Thus they may precipitate out in the lower container and require a much greater volume of solvent for latter dissolution. Thirdly, if the operation is carried out in a large scale it may not be suitable for use with solvents with relatively high boiling points such as methanol and water, since the whole apparatus below the condenser needs to be at this temperature for effective movement of the solvent vapour. Finally, unlike the reflux method, this method is limited to extraction with pure solvents and cannot be used for extraction with any solvent mixture since the vapour would have a different composition to the liquid solvent in the lower flask.

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; volaticity, flammability and boiling point, toxicity, reactivity and the cost of solvent (Jones and Kinghorn, 2005).

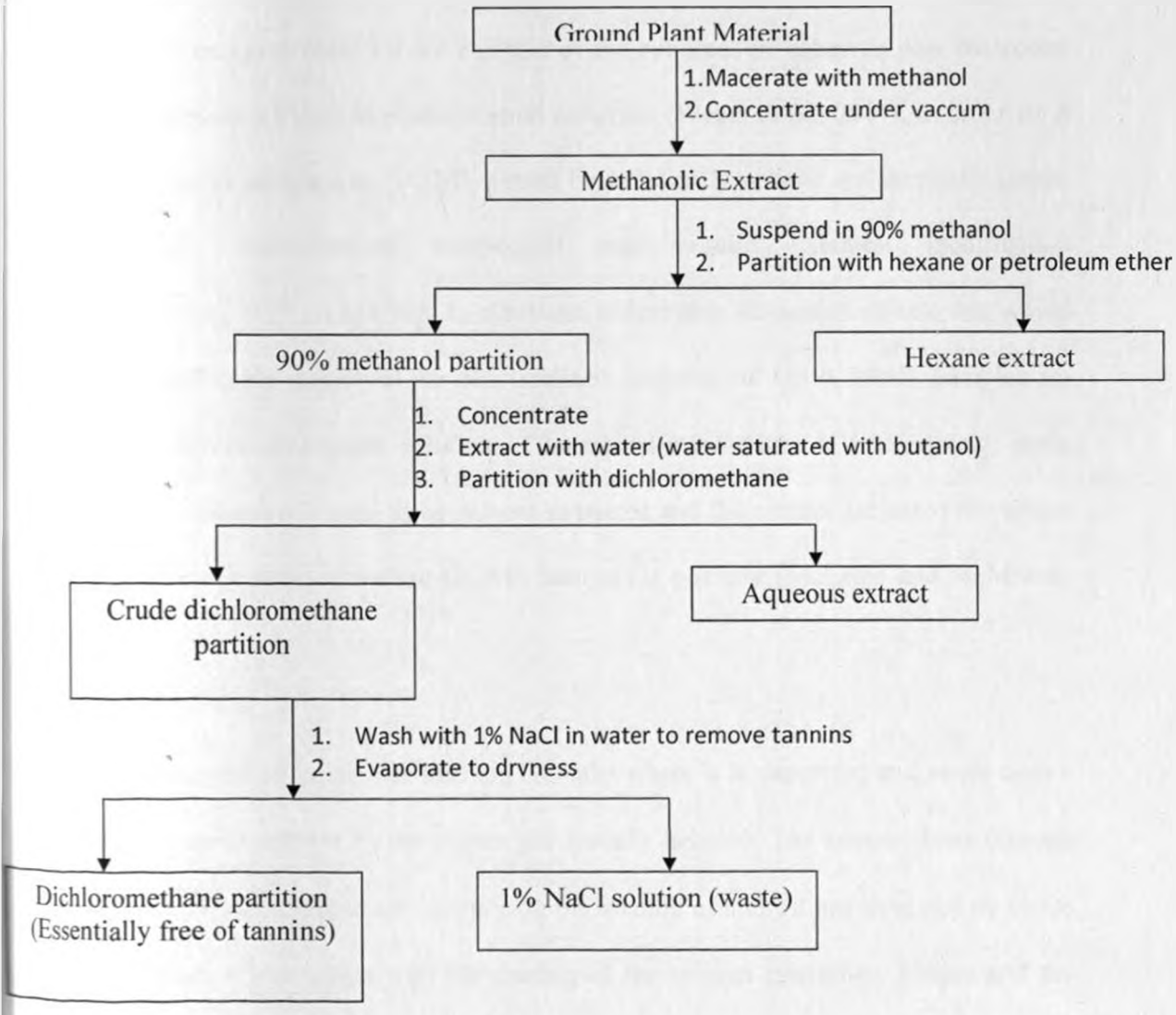


Figure 2b. General procedure for preparing extracts representing a range of polarities including a virtually tannin – free chloroform extract (Sarker *et al.*, 2007)

2.8 GAS CHROMATOGRAPHY MASS SPECTROMETRY (GC-MS)

Gas chromatography mass spectrometry (GC/MS) is an instrumental technique, comprising a gas chromatograph (GC) coupled to a mass spectrometer (MS), by which complex mixtures of chemicals may be separated, identified and quantified (Gordon, 1984). This makes it ideal for the analysis of the hundreds of relatively low molecular weight compounds found in environmental materials (Weber *et al.,* 2007). In order for a compound to be analysed by GC/MS it must be sufficiently volatile and thermally stable. In addition, functionalised compounds may require chemical modification (derivatization), prior to analysis; to eliminate undesirable adsorption effects that would otherwise affect the quality of the data obtained (Eugene and Grob, 2004). Samples are usually analyzed as organic solutions. Consequently materials of interest (e.g. soils, sediments, tissues etc.) need to be solvent extracted and the extract subjected to various 'wet chemical' techniques before GC/MS analysis is possible (McEwen and McMaster, 1998).

The sample solution is injected into the GC inlet where it is vaporized and swept onto a chromatographic column by the carrier gas (usually helium). The sample flows through the column and the compounds comprising the mixture of interest are separated by virtue of their relative interaction with the coating of the column (stationary phase) and the carrier gas (mobile phase). The latter part of the column passes through a heated transfer line and ends at the entrance to ion source where compounds eluting from the column are converted to ions (Gordon, 1984).

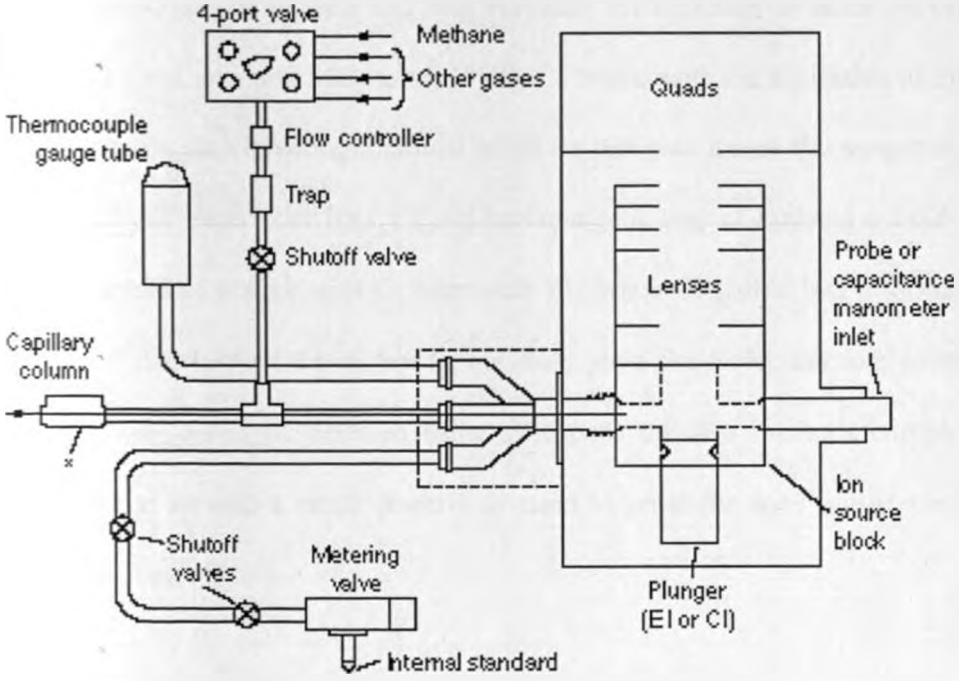


Figure 3: A typical GC-MS interface for fused silica capillary GC columns. The end of the GC column enters the ion source of the mass spectrometer (Gordon, 1984)

Two potential methods exist for ion production. The most frequently used method is electron ionisation (EI) and the occasionally used alternative is chemical ionisation (CI) (Niessen, 2001). For EI a beam of electrons ionise the sample molecules resulting in the loss of one electron. A molecule with one electron missing is called the molecular ion and is represented by $M^{+\cdot}$ (a radical cation) (Robert and Adams, 2007). When the resulting peak from this ion is seen in a mass spectrum, it gives the molecular weight of the compound. Due to the large amount of energy imparted to the molecular ion it usually

fragments producing further smaller ions with characteristic relative abundances that provide a 'fingerprint' for that molecular structure. This information may be then used to identify compounds of interest and help elucidate the structure of unknown components of mixtures (McEwen and McMaster, 1998). CI begins with the ionisation of methane (or another suitable gas), creating a radical which in turn will ionise the sample molecule to produce $[M+H]^+$ molecular ions. CI is a less energetic way of ionising a molecule hence less fragmentation occurs with CI than with EI, hence CI yields less information about the detailed structure of the molecule, but does yield the molecular ion; sometimes the molecular ion cannot be detected using EI, hence the two methods complement one another. Once ionised a small positive is used to repel the ions out of the ionisation chamber (Niessen, 2001).

The next component is a mass analyser (filter), which separates the positively charged ions according to various mass related properties depending upon the analyser used. Several types of analysers exist: quadrupoles, ion traps, magnetic sector, time-of-flight, radio frequency, cyclotron resonance and focusing to name a few. The most common are quadrupoles and ion traps. After the ions are separated they enter a detector the output from which is amplified to boost the signal. The detector sends information to a computer that records all of the data produced, converts the electrical impulses into visual displays and hard copy displays. In addition, the computer also controls the operation of the mass spectrometer (McEwen *et al.*, 1996).

2.9 *MONDIA WHYTEI*

2.9.1 BOTANICAL DESCRIPTION

Mondia whytei is a forest floor plant with aromatic rhizomatous roots and belongs to the *Asclepiadaceous* family. There are two species in the genus *Mondia;* namely *Mondia whytei* and *Mondia econata.* The two species are differentiated by their morphological characteristics. *Mondia econata* has a corona with long appendages, while *Mondia whytei* is a climber, which grows up to 3 – 6 m high. The leaves are broadly ovate with a base cordate, apex acuminate and corona of 11 – 12 mm long. The fruit follicles are ovoid with dimensions of 7 – 8 by 1.5 – 2.0 mm (Kokwaro, 2006).

Asclepiadaceous has more than 300 genera and 2000 species (Van Heerden and Steyn, 1999). However there are about 104 species in the family that have been identified in East Africa (Agnew and Agnew, 1994). *Asclepiadaceous* species are closely related to *Aporcynaceous* and *Rubiaceous* species. *Asclepiadaceous* are characterized by milky latex, leaves opposite with minute stipules, numerous flowers (usually with a corona) and anthers with pollen or sticky pollinia. Their fruits have two follicles while the seeds have a sticky corona. The family is mostly found in the tropics and subtropical regions.

Mondia whytei is locally known as Mukombela (Luhya), Ogomba (Luo), Olkonkola (Maasai), Mkonkora (Kamba) and Muhukura (Kikuyu). Outside Kenya, the plant is known as Omurondwa (Lunyore – Uganda), Ilivi (Sudan), Omondi (Zulu) and Mbombogazi (Swahili of Tanzania) (Kokwaro, 2006).

2.9.2 DISTRIBUTION

Mondia whytei is distributed widely in Africa. It is found in Guinea in West Africa through Sudan, Uganda, Kenya, Tanzania, Malawi, South Africa and westwards to Angola (Beentje, 1994). In Kenya, *Mondia whytei* is common in wet and humid areas. It is particularly common in western Kenya. The plant grows widly in undisturbed forest or fallow lands. It is a primary colonizer and does well in savannah thickets, but is mostly found on the outskirts of forests. Sometimes *Mondia whytei* is found in the interior of the forest where conditions are favourably hot and humid. The species does well in agroforestly cultivation. Currently, it is found in the Kakamega forest and outlying areas such as Kwisero, Malava and Bunyala forest blocks. The species is also found on the main hill top ranges of western Kenya (Beentje, 1994).

2.9.3 UTILIZATION

Mondia whytei roots have traditionally been used to treat various forms of pains that include dysmenorrhea, gastrointestinal colic pains and post partum pains. It is also consumed inorder to increases milk production and appetite in both human and livestock. The roots skeels are claimed to possess aphrodisiac effects upon chewing and also enhances fertility (Kokwaro, 2006). It is claimed to have anti-inflammatory, anti-pyretic and anti-microbial activity (Jain *et al.*, 1996).

2.9.4 CHEMISTRY AND PHARMACOLOGICAL PROFILE

Asclepiadaceous family has been found to contain compounds with potential flavoring and taste modifying qualities that are needed by food and pharmaceutical industries (Yoshikawa *et al.*, 1993). Both leaves and roots have shown antibacterial and antifungal activities (Mukonyi and Ndiege, 1999). The freeze dried aqueous root extract have been shown to have parasympathomimetic effects on the isolated rabbit heart and jejunum (Githinji *et al.*, 2007)

2-hydroxy-4-methoxybenzaldehyde and 3-hydroxy-4-methoxybenzaldehyde glycoside has been isolated and identified (Msonthi, 1994; Mukonyi and Ndiege, 1999).

Fig 3.a

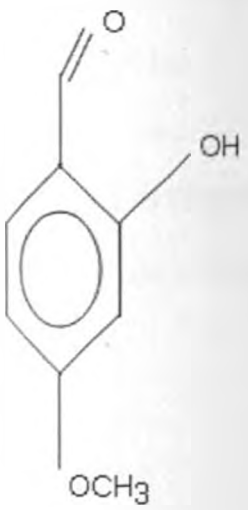


Fig 3.b

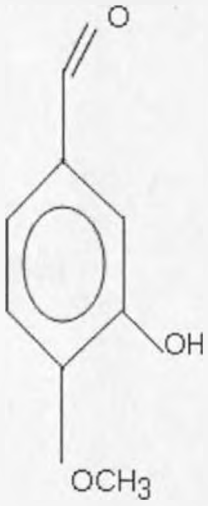


Figure 4.a: Structure of 2-hydroxy – 4 – methoxybenzaldehyde

Figure 4.b: Structure of 3-hydroxy – 4 – methoxybenzaldehyde

2.10 AIM

The aim of this study was to screen various parts of *Mondia whytei* for antinociceptive activities and to isolate in a bioguided way the active compounds.

2.11 OBJECTIVES

a) Broad Objective

To evaluate the antinociceptive effects of *Mondia whytei* using *in vivo* biological systems.

b) Specific Objectives

1. To carry out extraction, fractionation, purification and characterization of the chemical constituents of *Mondia whytei.*
2. To evaluate the antinociceptive activities of *Mondia whytei* extracts and the isolated compounds.

2.12 NULL HYPOTHESIS

Mondia whytei extracts do not possess antinociceptive effects

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.0. (A) EXTRACTION, FRACTIONATION, PURIFICATION AND IDENTIFICATION

3.1. A. MATERIALS

3.1. A.1 SAMPLING

Mondia whytei was collected from Kakamega forest and the adjacent environs in August 2006. It was identified and authenticated at the University of Nairobi, Department of Botany herbarium. It was allocated voucher specimen no. CG/MW/608 as a reference. The plant was sorted into leaves, barks and roots.

Each part was cut into small pieces to facillatate drying. The drying was carried out for three weeks under room temperature in a well aerated and dry room away from direct sunlight.

One kilogramme of each part of the plant was pulverized using a Molly grinder and the powders separately stored in an air-tight amber coloured glass containers at room temperature awaiting extraction.

3.1. A.2 REAGENTS

1. Acetic acid (glacial) (Riedel, Seelze, Germany, Analar)
2. Absolute Ethanol (Riedel de Haens AG, Seelze, Germany)

3. Ferric chloride hexahydrate (Seelze, Hannover AG, Germany)
4. Hydrochloric acid (Concentrated) (Riedel de Haens AG, Germany)
5. Iodine (Resumblimed) (May and Baker Ltd., England. Laboratory grade)
6. Lead II acetate (May and Baker Ltd., Dangenham, England. Laboratory grade)
7. Picric acid (BDH Chemicals, Poole, England, Analar)
8. Potassium hydroxide pellets (May and Baker Ltd., Dangenham, England. Laboratory grade)
9. Potassium iodide (Sigma, St. Lious, USA)
10. Ammonia solution (25% Ammonia) (Alpha Chemicals Ltd., Kenya. Laboratory grade)
11. Anisaldehyde (Aldrich Chemicals Co. Ltd., England)
12. Benzene, Crystallizable (Kobian Ltd,. Nairobi, Kenya, General Reagent)
13. Bismuth subnitrate (May and Baker Ltd., Dangenham, England. Laboratory grade)
14. 3,5 – dinitrobenzoic acid (BDH Chemicals, Poole, England, Analar)
15. Sodium carbonate, anhydrous (May and Baker Ltd., Dangenham, England. Laboratory grade)
16. Sodium hydroxide (Spectra Chemicals Ltd., Nairobi, Kenya. Laboratory grade)
17. Sulfuric acid (Concentrated) (Riedel de Haens AG, Seelze, Germany)
18. Vanillin (BDH Chemicals, Poole, England, Analar)
19. Magnesium filings (BDH Chemicals, Poole, England, Analar)
20. Mercuric chloride (May and Baker Ltd., Dangenham, England. Laboratory grade)

3.1. A.3 SOLVENTS

1. Chloroform (Loba Chemie PVT. Ltd. Mumbai. India. Laboratory Reagent)
2. Methanol (Loba Chemie PVT. Ltd. Mumbai. India. Laboratory Reagent)
3. Ethanol (Loba Chemie PVT. Ltd. Mumbai. India. Laboratory Reagent)
4. Dichloromethane (Loba Chemie PVT. Ltd. Mumbai. India. Laboratory Reagent)
5. N-Hexane (Loba Chemie PVT. Ltd. Mumbai. India. Laboratory Reagent)
6. Acetone (Loba Chemie PVT. Ltd. Mumbai. India. Laboratory Reagent)
7. Dimethyl Suphoxide (E. Merck, Darmstardt, Gemany, Analar)

3.1. A.4 CHROMATOGRAPHIC MATERIALS

1. Pre-coated TLC plates silica gel $60F_{254}$ Merck, Germany
2. Silica gel for column chromatography (0.040 – 0.65 μm diameter), Merck, Germany

3.1. A.5 INSTRUMENTS

1. Melting point determination apparatus (Stuart Melting Point Apparatus, Germany)
2. Spectrometer: Hewlett Packard 6890 series Gas chromatography interphased with mass spectrometer (GC-MS)

3.2. A. METHODS

3.2. A.1 EXTRACTION OF THE PLANT PARTS WITH CHLOROFORM, ETHANOL AND WATER

One hundrend grammes (100 g) of the leaf, bark and root powder were extracted separately with chloroform, ethanol and water using soxhlet apparatus for 48 hours at 60-80 ^{0}C. Fresh powder was used in each extraction. The extract was ultrafiltered using sintered glass connected to butchner funnel and under sunction pressure. The filtrate was then reduced to minimum volume using rotary vacuum evaporator and allowed to dry under room temperature. Water extracts were freeze dried. The extracts were then stored in amber colored sample bottles. They were well labeled and waited bioassaying.

3.2. A.2 DEFATTING OF THE ROOT POWDER PRIOR TO SUCCESSIVE EXTRACTION WITH CHLOROFORM AND METHANOL

One hundrend grammes of the root powder was extracted with n-hexane (defatting) using soxhlet apparatus for 48 hours at 60-80 ^{0}C. The marc was then successively re-extracted with chloroform and then methanol using soxhlet apparatus for 48 hours. Each of the extract was reduced to minimum volume using rotary vacuum evaporator and allowed to dry under room temperature. The extracts were then stored in well labeled amber colored sample bottles to await bioassay.

3.2. A.3 · SCREENING FOR VARIOUS PHYTOCHEMICAL GROUPS IN THE ROOTS

Spot tests for alkaloids were carried out according to the method described by Fansworth and Euler, 1962. One gramme of the root powder was extracted by warming it on a water bath with 10 ml of 10% sulphuric acid for 5 minutes. It was filtered and a portion of the extract tested for alkaloids by adding 2 drops of Mayer's reagent. Presence of alkaloids is indicated by formation of white precipitates.

Tests for saponins was carried out according to the method described by Evans, 1996. Two grammes of the root powder was boiled in 20 ml of distilled water in a water bath and then filtered. Ten mililitres (10 ml) of the filtrate was mixed with 5 ml of distilled water and shaken vigorously for a stable persistent froth. Three (3) drops of olive oil were added and the mixture shaken vigorously, then observed for the formation of emulsion.

The test for flavonoids was carried out according to the method described by Geissman, 1955. Five millilitres of dilute ammonia solution was added to a portion of the aqueous filtrate of the root powder. This was followed by addition of concerntrated sulphuric acid. Yellow colouration indicates the presence of flavonoids.

The test for total anthraquinones (bound and free) and phenolics was carried out according to the method described by Harbone, 1973. Half gramme of the root powder was boiled with 5 ml dilute sulphuric acid for 5 minutes. It was filtered while hot and upon cooling the filtrate was added an equal volume of carbon tetrachloride and vigorously mixed. A rose pink to red colour in the ammoniacal layer indicates presence of anthraquinones and phenolics. Chloroform root extracts were subjected to a test for carotenoids (Willstatter and Everest, 1913). One gramme of each extract was boiled in 20

ml water. The filtrate was then treated with concentrated sulphuric acid. Blue colour indicates presence of carotenoids.

The root powder was subjected to modified Stas Otto extraction of glycosides and the glycosides subjected to Kedde's test for unsaturated lactone ring/cardenolide and Keller-Killian's test for a 2-deoxy sugar (Sim, 1967). One gramme of the root powder was extracted with 10 ml of 70% alcohol by heating on a water bath for 2 minutes and then filtered on cooling. Ten milliliters of water and 5 drops of lead subacetate were added to the filtrate and a precipitate formed. It was filtered and 10% sulphuric acid added drop wise until no further precipitate formed. The precipitates were filtered and extracted with two successive 5 ml portions of chloroform. The two chloroform extracts were combined and washed with 1 ml distilled water. For the Kedde's test, one portion of the chloroform extract was evaporated to dryness, added one drop of 90% alcohol and 2% 3, 5-dinitrobenzoic acid in 90% alcohol and then made alkaline with 20% sodium hydroxide solution. A purple reaction colour with 3, 5-dinitrobenzoic acid indicates a positive result. Keller-Killan test was done by evaporating the chloroform portion to dryness and adding 0.4 ml glacial acetic acid containing trace of ferric chloride. It was then transferred to a test tube and 0.5 ml of concerntrated sulphuric acid added carefully down the side of the test tube. A green-blue colour in the upper acetic acid layer confirms a positive test.

Thin Layer Chromatography (TLC) evaluation for saponins, flavonoids, and alkaloids were carried out according to the methods described by Wagner and Bladt, 1996. The presence for steriodal nucleus was detected by TLC as described by Sim, 1967 and Waldi

1965. Two millilitres of acetic anhydride was added to 0.5 g ethanolic extracts of each sample. Presence of steroids is indicated by colour change from violet to blue or green.

3.2. A.4 PARTITIONING OF THE DEFATTED METHANOL ROOT EXTRACT AND ISOLATION OF THE CRYSTALS FROM THE ROOTS

(A) Defatting of the Root Powder.

Five hundrend grammes (500 g) of the powdered root was defatted with 2.5 litres n-hexane in a soxhlet apparatus for 48 hours.

The n- hexane extract was reduced to minimum volume using rotary vacuum evaporator and allowed to dry under room temperature to yield yellowish pasty mass (HR) that was 18% of the starting root powder. It was stored in an amber coloured glass container awaiting bioassay. The left over residue marc was processed further as outlined below.

(B) Extraction of Marc with Methanol.

The residue marc was transferred to a soxhlet extractor and extracted with 2.5 litre methanol for 48 hours. The extract was filtered and evaporated to dryness to afford a brown pasty mass (MR_d) that was 66.2% of the marc.

Eighteen grammes of the defatted methanol root extract (MR_d) were dissolved in 2 ml methanol and 6 ml of distilled water (1:3) to form aqueous methanol solution. Eight millilitres (8 ml) chloroform was added, vigorously shaken and left standing for two hours. Two distinct layers formed. These were separated to yield aqueous soluble portion of the defatted methanol root extract (A_fMR_d) and the chloroform soluble portion of the defatted methanol root extract (C_fMR_d). Each portion of the extract was evaporated to dryness and the yield determined.

(C) The Chloroform Soluble Portion of the Defatted Methanol Root Extract.

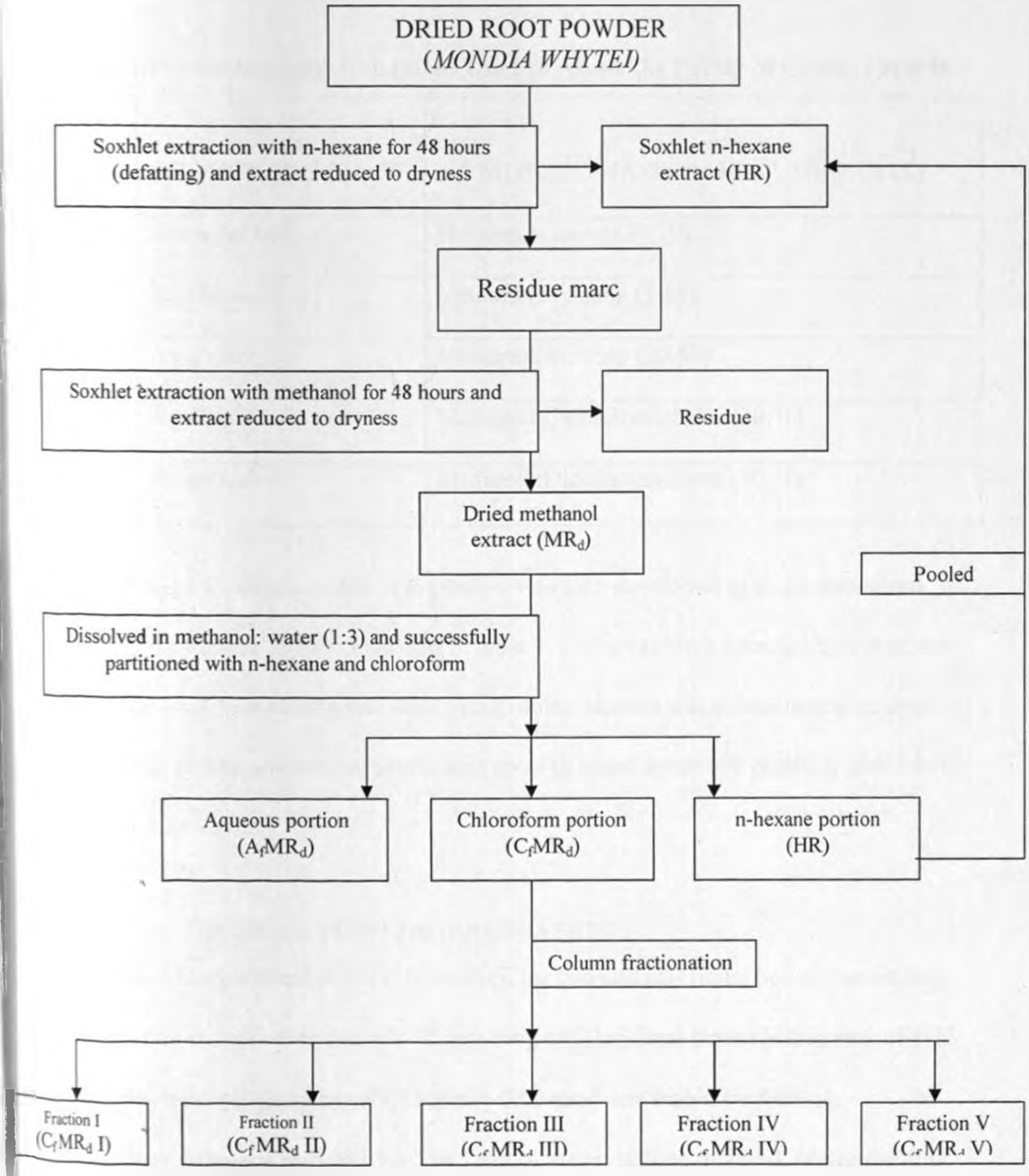
The chloroform soluble portion of the defatted methanol root extract (C_fMR_d), when evaporated to dryness gave a brown oily mass that was 6% of the total defatted methanol root extract.

Twelve grammes of C_fMR_d were subjected to column fractionation. The column consisted of deactivated silica gel (120 g, column length 57 cm, internal diameter 2.8 cm).

The column was eluted with 30% methanol in dichloromethane. The fractions were collected using a fraction collector set at 4 – 5 ml per test tubes. Two hundred and sixty fractions were collected.

Every fifth fraction collected was spotted on pre-coated silica gel analytical plates and developed with 30% methanol in dichloromethane. Fractions with similar retardation factor (R_f) value were pooled together, reduced to minimum volume and left to crystallize at room temperature. Five fractions were obtained (Appendix 1). The crystals from these five fractions were separately stored in well labelled amber colored sample bottles awaiting bioassays.

FIGURE 5: A FLOW DIAGRAM SHOWING BIO-GUIDED ISOLATION OF C_fMR_d I AND C_fMR_d II FROM THE ROOT OF *MONDIA WHYTEI*



3.2. A.5 EVALUATION OF THE ISOLATES FOR PURITY BY THIN LAYER CHROMATOGRAPHY

Alluminium pre-coated silica gel 60 F_{254} were used for analytical thin layer chromatography (TLC). The chromatographic phases used were as shown in table below.

Table 1: Chromatographic Conditions Used to Assess the Purity of C_fMR_d I and II

CODE	STATIONARY PHASE	MOBILE PHASE (IN VOLUME PARTS)
A	Silica gel $60F_{254}$	Hexane:Acetone (30:70)
B	As above	Methanol:Acetone (5:95)
C	As above	Methanol:Acetone (20:80)
D	As above	Methanol:Dichloromethane (30:70)
E	As above	Methanol:Dichloromethane (50:50)

After spotting the sample on the TLC plate, it was then developed in a chromatogram tank using the solvent system indicated in table 1. The spots were detected by use of u.v (256 to 366 nm). Visualizing was done using iodine vapours and subsequently sprayed with vanillin in concentrated sulphuric acid so as to detect spots that probably could not take the iodine vapour.

3.2. A.6 MELTING POINT DETERMINATION

C_fMR_d I and II were dried at 50 °C in an oven for two and half hours before the melting point was determined. One end of a 75 mm long capillary tube (Internal diameter of 1.15 $\pm$ 0.05 mm, external diameter of 1.55 mm $\pm$ 0.05 mm) was sealed by flaming.

The capillary tube was packed with 3 to 5 mg of the crystalline material. Melting points were determined using a Stuart Melting Point Apparatus.

3.2. A.7 GC-MS EVALUATION AND IDENTIFICATION OF C_fMR_d I AND II

Three microgrammes of each isolate were solubilized in dichloromethane and then subjected to gas chromatography-mass spectrometer analysis using a Hewlett Packard gas chromatography 6890 series II plus linked to Hewlett Packard Mass Spectrometer system equipped with a capillary columnn HP5 – 5 (30 m x 0.25 mm, 0.25 µm film thickness). The temperature was programmed from 230 ^{O}C to 300 ^{0}C at a rate of 4 ^{0}C per minute with 10 minutes hold. Injection was at 280 ^{0}C. Helium was used as a carrier gas with a constant flow of 0.8 ml per minute. The ionization voltage was 70 electron volt. The identification was accomplished using computer searches by NIST05a Wiley MS Data library (Agilent, 2005).

3.0 (B) MATERIALS AND METHODS FOR ANTINOCICEPTIVE TESTING

3.1. B MATERIALS

3.1. B.1 TEST ANIMALS

Adult male and female Swiss albino mice weighing 25 – 30 g were used. The animals were housed in colony cages with free access to food and water and allowed to acclimatize for one week. They were kept in rooms with temperature of 23 $\pm$ 0.5 ^{O}C and relative humidity of 50%. Diurnal rythms was regulated with a 12-h light: 12-h dark cycle with lights on 7.00 a.m to 7.00 p.m. The animals were handled humanely in accordance with the institutional animal welfare committee guidelines.

3.1. B.2 DRUGS AND PREPARATIONS

Drugs used during the experiments were as follows:-

Acetylsalicylic acid (Svaneapoteket, Bergen, Norway) and indomethacine (Merck, Sharp and Dohme, USA) were dissolved in Tris buffer 0.1 M, pH 7.4 – 7.6. Dexamethasone phosphate (Merck, Sharp and Dohme, USA) and the polar plant extracts were dissolved in 0.9% NaCl. The non polar plant extracts were dissolved in 5% dimethylsulphoxide.

3.2. B. METHODS

3.2. B.1 EXPERIMENTAL DESIGN

The animals were randomly picked up by touch and pick technique and assigned to an experimental unit based on the type of test article being evaluated. Each experimental unit comprised a treated group and control group.

In all experiments each animal was used only once. The test materials were prepared and appropriate dilutions made in clearly labeled sample bottles. The samples were then decoded by an independent person to ensure blinding. The coded samples were thereafter given to the researcher who administered them as per the codes. The coding was broken after data analysis.

3.2. B.2 DRUG ADMINISTRATION

All injections were given intraperitoneally (i.p) in volume of 10 ml/kg. Aspiration prior to injection was performed to ensure that the drug was not injected into the intestines. In all experiments an equal volume of vehicle was used as control. The drugs were injected 30 minutes prior to pain assessment.

3.2. B.3 NOCICEPTIVE TESTING

(A) ACETIC ACID INDUCED WRITHING TEST.

The test was performed as described in section 2.5.1. Prior to testing, the mice were adapted to the test environment for at least 18 hours.

Two hours before testing the animals were placed individually in an observation chamber made of perspex box (30 x 30 x 30 cm). The animals did not have access to water and food two hours prior to the experiment. With a minimum restraint, 0.1 ml/10 g body weight of 0.7 v/v acetic acid was administered i.p.

Immediately after the injection of the Acetic acid, each animal was isolated and placed in a box (30 x 30 x 30 cm) for observation. The numbers of writhing and stretching as described in section 2.5.1 were counted over a period of thirty minutes. The number of writhing and stretching was recorded and expressed as the percentage protection of writhing using the following ratio:

(control mean-treated mean) x 100/control mean.

(B) FORMALIN TEST

Prior to testing, the mice were adapted to the testing environment for at least 18 hours. Two hours before testing the animal were placed individually in a Perspex box (30 x 30 x 30 cm) which served as the observation chamber and did not have access to water or food during this brief period. After the adaptation period the animal was taken out of the cage, and with minimum restraint, 20 µl of formalin solution (5% in saline) was injected just under the skin of the plantar surface of the right hind paw using a microlitre syringe with

a 26 - gauge needle. The mouse was then put back in the chamber and the observation period started. The amount of time in seconds the animal spent licking the injected paw was recorded over a period of one hour in blocks of five minutes.

3.3 STATISTICAL ANALYSIS

Data was analyzed using analysis of variance (ANOVA). When the analysis was restricted to two means, the student's t-test (paired and one tailed) was used. Scheffe' post hoc test was done for multiple comparisons. Multivariate analysis of variance (MANOVA) was used when analyzing data with more than two dependent variables or/and independent variables. The level of significance was set at $p<0.05$. Results are presented as mean $\pm$ standard error of the mean (s.e.m). The absolute values of the number of writhes and the time spent in pain behaviors as recorded were used in statistics calculation.

Statistical Package for Social Sciences (SPSS) version 16.0 statistical package was used for data analysis.

CHAPTER FOUR

4.0 RESULTS

4.1 YIELD OF THE AQUEOUS, ETHANOL AND CHLOROFORM PLANT EXTRACTS.

The highest yield was that of ethanol root (ER) extract which was 44.3% of the extracted root powder. ER extract contained plant oils. Ethanol leaves (EL) extracts had 43.2% yield. EL was green in colour which suggested presence of chlorophyll material. Ethanol bark (EB) yielded 22.5% of the extracted bark powder. EB extracts consisted of green pasty materials suggesting presence of chlorophyll.

Chloroform root (CR), leaves (CL) and bark (CB) resulted in 28.0, 20.4 and 19.4 percent yields. These three extracts contained plant oils. Water extracts had the least yield with aqueous root (AR), aqueous leaves (AL) and aqueous bark (AB) yielding 15.0, 12.0 and 8.6 percent of the extracted powder (Table 2)

Table 2. Percentage yield from the bark, leaves and roots of *Mondia whytei* using chloroform, ethanol and water

Extract	% Yield
Aqueous Root - (AR)	15.0
Ethanol Root - (ER)	44.3
Chloroform root - (CR)	28.0
Aqueous Leaves - (AL)	12.0
Ethanol Leaves - (EL)	43.2
Chloroform Leaves - (CL)	20.4
Aqueous Bark - (AB)	8.6
Ethanol Bark - (EB)	22.5
Chloroform Bark - (CB)	19.4

4.2 EFFECTS OF AQUEOUS, ETHANOL AND CHLOROFORM EXTRACTS OF *MONDIA WHYTEI* BARK, ROOT AND LEAF IN ABDOMINAL WRITHING

Table 3 shows the effects of varied doses of *Mondia whytei* bark extracts on the number of abdominal writhes. Aqueous bark (AB), ethanol bark (EB) and Chloroform bark (CB) extracts significantly ($p<0.001$) and dose dependently reduced the number of abdominal writhes. The effects of AB and EB on abdominal writhing were not statistically significant ($p=0.124$ and $p=0.152$ respectively) at a dose of 25 mg/kg.

Aqueous root (AR), ethanol root (ER) and chloroform root (CR) extracts significantly ($p<0.001$) and dose dependently reduced the number of abdominal writhes (table 4)

Aqueous, Ethanol and chloroform leaf extracts (AL, EL and CL respectively) significantly ($p<0.001$) and dose dependently reduced the number of abdominal writhes (Table 5).

Chloroform root extracts induced higher inhibitory effects on abdominal writhes compared to the other extracts (Figure 6). The dose that lowered the number of abdominal writhes by half compared to the control (ID_{50}) for CR extract was 198.8 mg/kg (figure 7).

Table 3: Effects of increasing doses of *Mondia whytei* water, ethanol and chloroform bark extracts on abdominal writhes.

		AQUEOUS BARK (AB)		ETHANOL BARK (EB)		CHLOROFORM BARK (CB)	
Doses		Mean ± S.E.M	% Inhibition	Mean ± S.E.M	% Inhibition	Mean ± S.E.M	% Inhibition
Control Saline (10 ml/kg)		87.9±2.13	(-)	89.0±1.70	(-)	88.9±2.47	(-)
EXTRACT	25 mg/kg	83.9±3.60 n.s	4.6	85.3±3.20 n.s	4.2	82.9±2.35***	6.7
	50 mg/kg	77.5±3.72***	11.8	80.3±2.91***	9.8	71.8±2.39***	19.2
	100 mg/kg	77.6±3.72***	11.7	76.7±3.50***	13.8	64.0±3.27***	28.0
	200 mg/kg	69.8±3.34***	20.6	68.0±4.24***	23.6	61.9±2.64***	30.5
	400 mg/kg	60.7±2.21***	30.9	59.4±2.59***	33.2	50.1±3.25***	43.6

*****p< 0.001; n.s = not significant; n = 6**

Table 4: Effects of increasing doses of *Mondia whytei* water, ethanol and chloroform root extracts on abdominal writhes.

		AQUEOUS ROOT (AR)		ETHANOL ROOT (ER)		CHLOROFORM ROOT (CR)	
Doses		Mean ± S.E.M	% Inhibition	Mean ± S.E.M	% Inhibition	Mean ± S.E.M	% Inhibition
Control Saline (10 ml/kg)		90.5±2.37	(-)	88.8±5.51	(-)	87.0±2.87	(-)
EXTRACT	25 mg/kg	81.8±3.12***	9.6	80.9±3.41*	8.8	74.1±3.31***	14.8
	50 mg/kg	74.0±3.02***	18.2	80.2±4.10*	9.6	66.0±3.43***	24.1
	100 mg/kg	61.1±2.28***	32.4	76.0±3.40***	14.4	52.2±3.49***	40.0
	200 mg/kg	58.7±3.02***	35.1	62.4±4.84***	29.7	43.2±3.19***	50.3
	400 mg/kg	49.5±2.51***	45.3	57.0±7.24***	35.8	33.2±2.27***	61.8

*** $p < 0.05$ and *** $p < 0.001$; n = 6**

Table 5: Effects of increasing doses of *Mondia whytei* water, ethanol and chloroform leaf extracts on abdominal writhes.

		AQUEOUS LEAVES (AL)		ETHANOL LEAVES (EL)		CHLOROFORM LEAVES (CL)	
Doses		Mean ± S.E.M	% Inhibition	Mean ± S.E.M	% Inhibition	Mean ± S.E.M	% Inhibition
Control Saline (10 ml/kg)		88.0±2.54	(-)	88.4±2.91	(-)	88.8±2.66	(-)
EXTRACT	25 mg/kg	84.9±3.41 n.s	3.5	87.0±3.13 n.s	1.6	81.7±2.31***	8.0
	50 mg/kg	79.3±2.06**	9.9	76.9±2.96***	13.0	75.0±3.16***	15.5
	100 mg/kg	72.3±2.67***	17.8	72.9±2.42***	17.5	68.6±2.99***	20.2
	200 mg/kg	66.8±3.36***	24.1	66.2±3.58***	25.1	60.8±2.06***	31.5
	400 mg/kg	60.1±2.13***	31.7	63.0±3.09***	29.2	57.7±2.11***	35.0

****p< 0.01 and ***p< 0.001; n.s = not significant; n = 6**

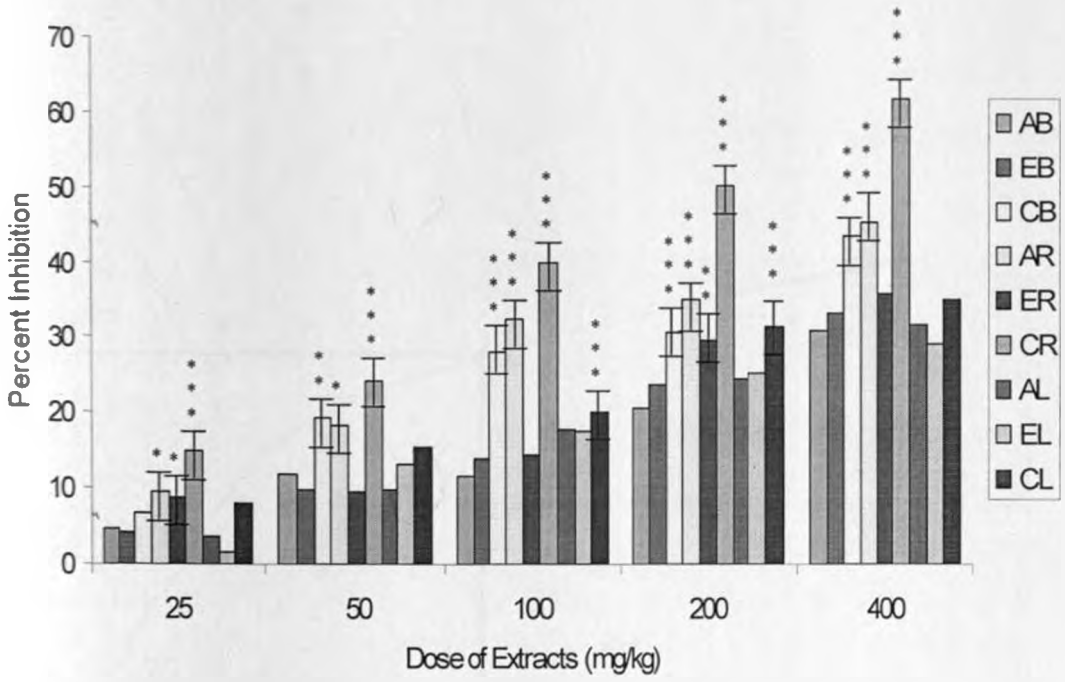


Figure 6: Effects of intraperitoneally administered extracts on the number of abdominal writhes in acetic acid induced writhing test.

Significant differences between different extract within each dose are indicated by;

*** p< 0.05, ** p< 0.01 and *** p< 0.001 (MANOVA with Sheffe' post hoc test)**

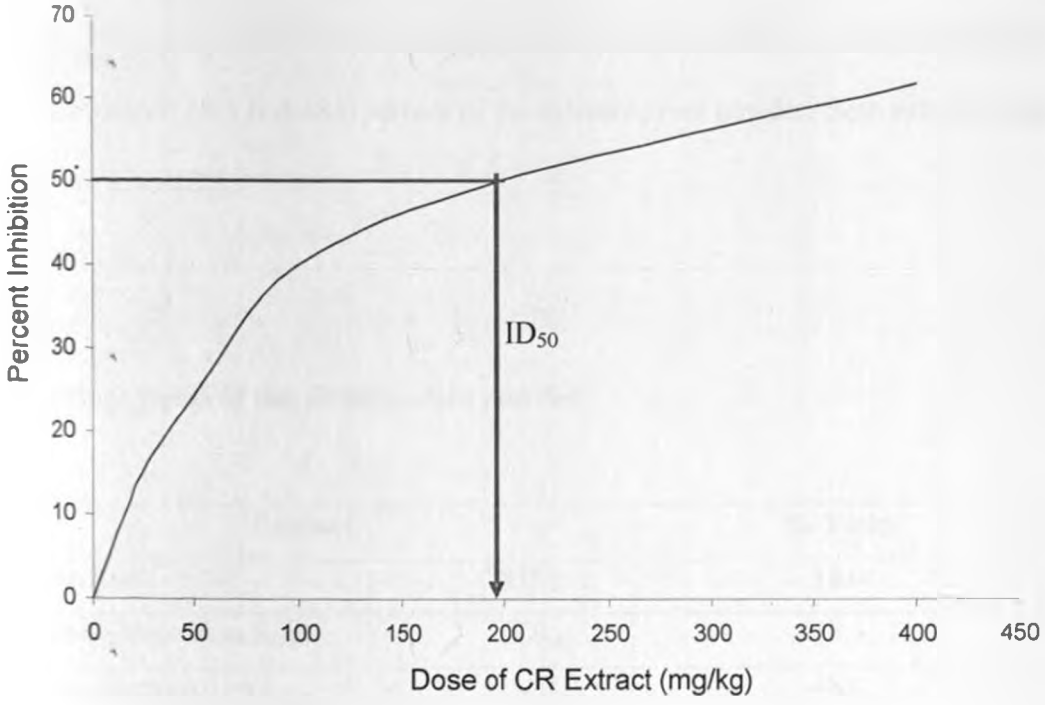


Figure 7: Dose response curve of CR extract. The ID_{50} is 198.9 mg/kg

4.3 YIELDS FROM THE DEFATTED ROOT POWDER

The root powder was first extracted exhaustively with n-hexane (defatting) and then successively with chloroform and methanol. Hexane root (HR) extract yielded 18.0% of the extracted root powder. It was an oily mass. Chloroform (CR_d) and methanol (MR_d) extracts yielded 18.3 and 48.0 percent of the extracted root powder. Both extracts were pasty in consistency.

Table 6

Percentage yields of the defatted root powder.

Extract			% Yield
Hexane Root	-	(HR)	18.0
Defatted Chloroform Root	-	(CR_d)	18.3
Defatted Methanol root	-	(MR_d)	48.0

4.4 EFFECTS OF THE DEFATTED *MONDIA WHYTEI* N-HEXANE, CHLOROFORM AND METHANOL ROOT EXTRACTS ON ABDOMINAL WRITHING

Table 7 shows the effects of various doses of the defatted *Mondia whytei* root extracts on the number of abdominal writhes. Hexane root (HR), defatted methanol root (MR_d) and defatted chloroform root (CR_d) extracts induced significan ($p<0.001$) reduction in the number of abdominal writhes at all dose levels compared to the control. This effect was dose dependent.

Figure 8 is the bar graph of the effects of various extracts at varied doses. It reveals defatted chloroform root extracts (CR_d) having higher inhibitory effects on abdominal writhes compared to the other extracts. The effects of CR_d extracts were significantly ($p< 0.01$) differenct at all dose levels tested compared to HR extracts. MR_d extracts had effects that were significantly ($p< 0.01$) different from HR extracts except at a dose of 50 mg/kg.

From the dose response curve (Figure 9) the ID_{50} for CR_d was 350 mg/kg. The effects of this dose was statistically significant ($p<0.001$) compared with the control saline. HR and MR_d could not achieve their ID_{50} at the the highest dose tested i.e 400 mg/kg (Figure 9). Defatting the extract prior to antinociceptive testing caused the ID_{50} of CR to rise from 198.8 mg/kg to 350 mg/kg. This suggested wide solubility range of the active compounds between n-hexane and chloroform. In order to concentrate the active compounds in one solvent, solvent-solvent partitioning was carried out as described in section 3.2. A.4.

Table 7: Effects of increasing doses of defatted *Mondia whytei* root extracts on abdominal writhes.

		N-HEXANE ROOT EXTRACT (HR)		DEFATED CHLOROFORM ROOT (CRd)		DEFATED METHANOL ROOT (MRd)	
Doses		Mean ± S.E.M	% Inhibition	Mean ± S.E.M	% Inhibition	Mean ± S.E.M	% Inhibition
Control Saline (10 ml/kg)		89.3±2.07	(-)	85.2±3.31	(-)	89.5±2.88	(-)
EXTRACT	25 mg/kg	84.8±1.94*	5.0	80.0±1.41*	6.1	81.2±1.94***	9.3
	50 mg/kg	76.8±2.23***	14.0	69.0±1.26***	19.0	74.7±1.97***	16.5
	100 mg/kg	72.7±2.07***	18.6	61.0±2.10***	28.4	63.2±2.14***	29.4
	200 mg/kg	65.8±1.47***	26.3	50.3±2.07***	41.0	60.0±1.41***	33.0
	400 mg/kg	60.2±1.94***	32.6	39.8±1.83***	53.3	49.0±2.37***	45.3

*** $p < 0.05$ and *** $p < 0.001$; n = 6**

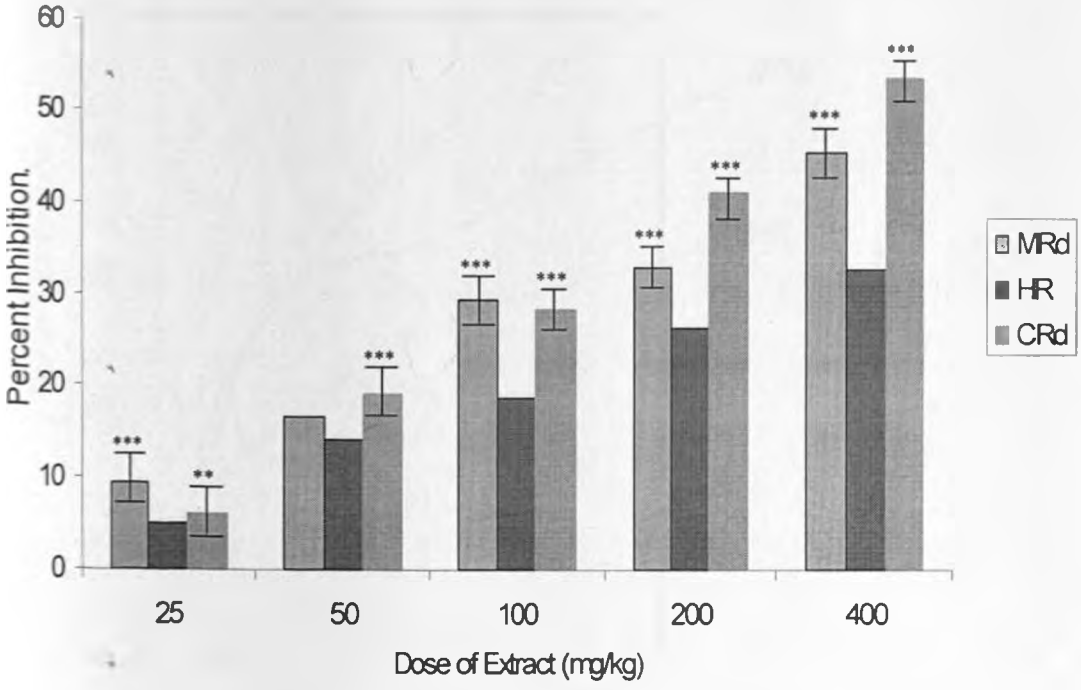


Figure 8: Effects of intraperitoneally administered MR_d, HR and CR_d extracts on the number of abdominal writhes.

Significant differences between different extracts within each dose are indicated by; ** $p< 0.01$ and *** $p< 0.001$

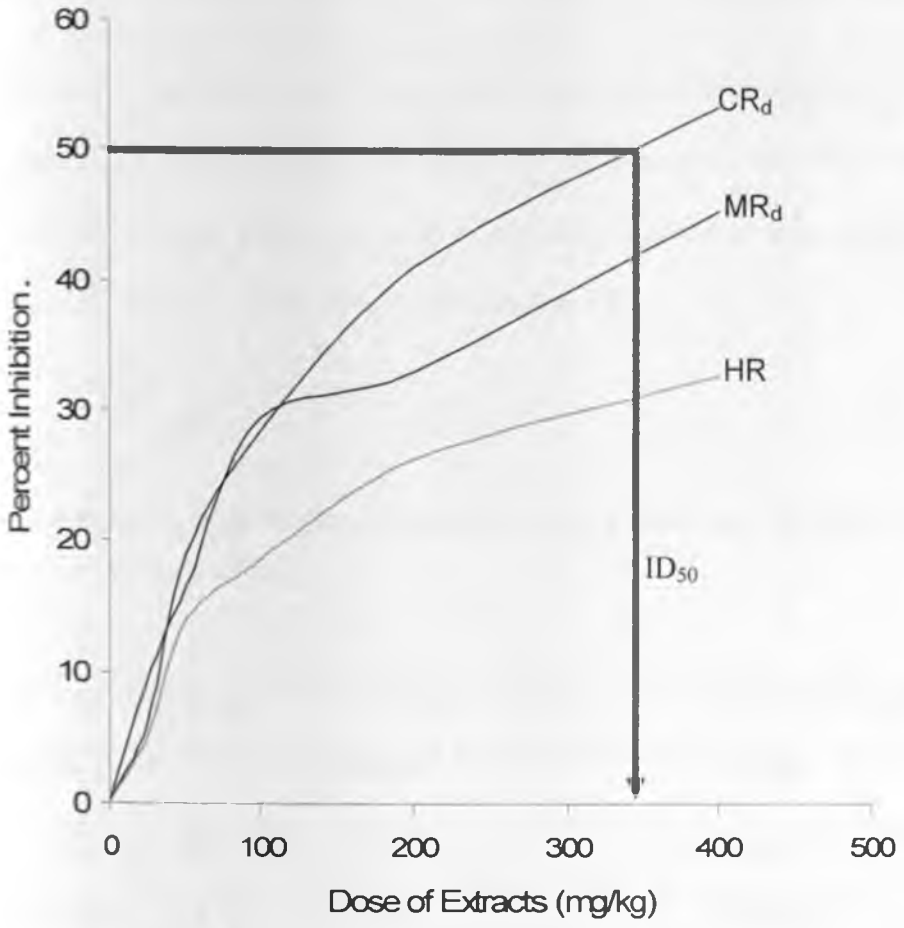


Figure 9: Dose response curves of CR_d, MR_d and HR. The ID_{50} is 350 mg/kg for CR_d.

4.5 PHYTOCHEMICAL GROUPS DETECTED IN THE CRUDE ROOT POWDER AND THE CHLOROFORM ROOT EXTRACT OF *MONDIA WHYTEI*

Preliminary investigation by TLC and spot tests revealed anthraquinones, cardenolides, carotenoids, flavonoids, steroids, tannins and 2-Deoxy sugars in the whole root powder. Chloroform extracts when subjected to phytochemical tests were shown to contain carotenoids, steroids, flavonoids and tannins (Table 8)

Table 8

Phytochemical groups detected in the crude root powder and the chloroform root extracts of *Mondia whytei.*

Phytochemical Group	Crude Root Powder	Chloroform Root Extract.
Alkaloids	Absent	Absent
Anthraquinones Glycosides	Present	Absent
Cardenolides	Present	Absent
Carotenoids	Present	Present
Flavanoids	Present	Present
Steroids	Present	Present
Saponins	Absent	Absent
Tannins	Present	Present
2-Deoxy Sugar	Present	Absent

4.6 CHLOROFORM SOLUBLE PORTION OF THE DEFATTED METHANOL ROOT EXTRACT (C_fMR_d).

The chloroform soluble portion of the defatted methanol root extract (C_fMR_d), when evaporated to dryness gave a brown oily mass, 6% of the total methanol extract.

Twelve grammes of the C_fMR_d were subjected to column fractionation using 30% methanol in dichloromethane as the eluent. Two hundrend and sixty fractions were obtained. Every fifth fraction was spotted on an analytical TLC plate and developed in a chromatographic chamber using 30% methanol in dichloromethane. Fractions with similar TLC profile as represented by their R_f values were pooled together. Five major fractions were obtained and labelled I – V (Appendix 1). The pooled fractions were reduced to minimum volume and left to crystallize at room temperature. These fractions were separately stored in amber colored sample bottles awaiting bioassay.

Fraction I yielded 342 mg of white crystalline compound and exhibited the most potent antinociceptive activity with an ID_{50} of 14.8 mg/kg body weight in mice. This compound was given a code $C_f MR_d$ I and it was recrystallized in acetone to give white crystals which were non UV active, turned yellow in iodine chamber and black when sprayed with vanillin in concentrated sulfuric acid. It had an R_f value of 0.71 in methanol: dichloromethane (3:7) and a melting point of 168 ^{0}C.

Fraction II yielded off-white crystals at room temperature. It was coded $C_f MR_d$ II and recrystallized in acetone to give an off-white crystals. These crystals were non UV active, turned yellow in iodine chamber and black when sprayed with vanillin in concentrated sulfuric acid. It had an R_f value of 0.48 in methanol: dichloromethane (3:7) and a melting point of 79 ^{0}C

4.7 EFFECTS OF THE AQUEOUS AND CHLOROFORM SOLUBLE PORTIONS OF THE DEFATTED *MONDIA WHYTEI* METHANOL ROOT EXTRACTS IN THE ABDOMINAL WRITHING

A_fMR_d and C_fMR_d significantly ($p< 0.001$) and dose dependently inhibited the abdominal writhes. The percentage inhibitions of the abdominal writhes ranged from 6.0 to 33.1 for A_fMR_d and 22.3 to 90.6 for C_fMR_d respectively. (Table 9)

At a dose of 200 mg/kg and 400 mg/kg, C_fMR_d showed no significant difference ($p=0.520$).

A_fMR_d and C_fMR_d dose dependently inhibited the abdominal writhing. C_fMR_d was more potent ($p< 0.001$) compared to A_fMR_d (Table 9). It had an ID_{50} of 50 mg/kg body weight. Based on these findings, C_fMR_d was subjected to fractionation as set out in section 3.2. A.4 c and further nociceptive testing as set out in section 4.8.

A_fMR_d at a dose of 400 mg/kg could not achieve the ID_{50} (Figure 10)

Table 9: Effects of increasing doses of aqueous and chloroform soluble portions of the defatted *Mondia whytei* methanol root extracts (A_fMR_d and C_fMR_d) on abdominal writhes.

		AQUEOUS PORTION A_fMR_d		CHLOROFORM PORTION C_fMR_d	
Doses		Mean ± S.E.M	% Inhibition	Mean ± S.E.M	% Inhibition
Control Saline (10 ml/kg)		88.7±1.63	(-)	88.2±1.17	(-)
EXTRACT	25 mg/kg	83.8±1.60**	6.0	68.5±1.05***	22.3
	50 mg/kg	78.2±2.04***	11.8	45.3±0.82***	48.6
	100 mg/kg	72.2±1.72***	18.6	15.3±1.37***	82.6
	200 mg/kg	64.8±2.14***	30.0	9.8±1.94***	88.9
	400 mg/kg	59.3±1.63***	33.1	8.3±0.82***	90.6

$^{**}p< 0.01$ and $^{***}p< 0.001$; n = 6

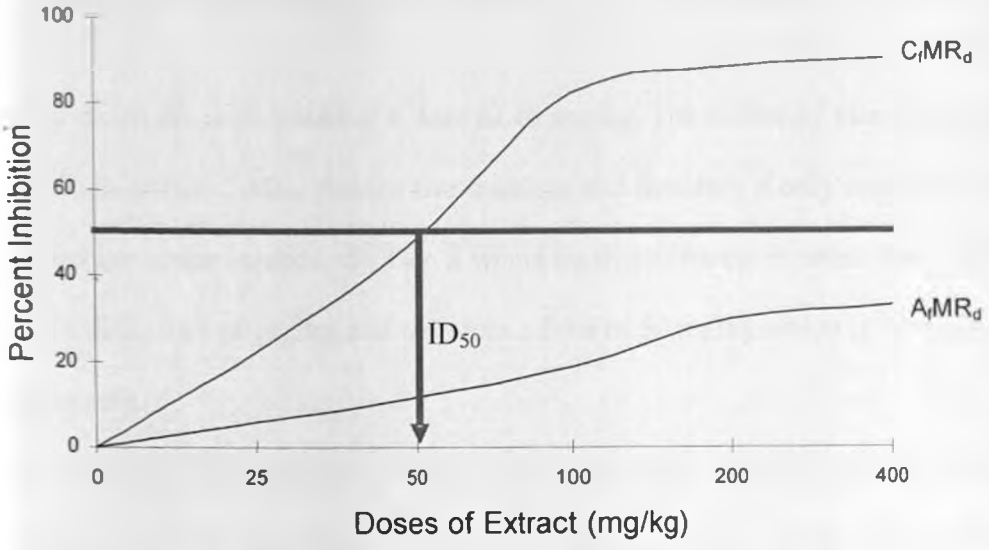


Figure 10: Dose response curves of A_fMR_d and C_fMR_d. The ID_{50} is 50 mg/kg for C_fMR_d. .

4.8 EFFECTS OF C_fMR_d FRACTIONS (I – V) ON THE ABDOMINAL WRITHING

The five fractions were tested at a dose of 10 mg/kg. The choice of this dose was based on the findings that C_fMR_d yielded five fractions and therefore if only one of the fractions contained the active compounds, then it would be five times more potent than C_fMR_d. The ID_{50} of C_fMR_d was 50 mg/kg and therefore a fifth of 50 mg/kg which is 10 mg/kg would be equipotent.

Fraction I caused the highest inhibition of the abdominal writhes at 29.7% followed by fraction II at 6.5%. The effects of these two fractions were significantly different ($p< 0.001$) compared to the control.

Fraction III caused 5.4% inhibition of the abdominal writhes and this was significantly different ($p< 0.01$) compared to the control. Fraction IV and V caused 1.7% and 2.9% inhibition of the abdominal writhes respectively. This inhibition was not significantly different compared to the control. (Table 10, Figure 11).

The effects of fraction II were not significantly different from that of fraction III and IV ($p= 0.908$ and 0.069 respectively).

The effects of fraction III were not significantly different from that of fraction IV and V ($p= 0.069$ and 0.35 respectively).

The effects of fraction I were found to be significantly different ($p<0.001$) from that of the other four fractions. Fraction I was therefore the most potent fraction.

Table 10: Effects of intraperitoneal adminstration of 10 mg/kg body weight of C_fMR_d fraction I to V on the number of abdominal writhes.

	Fraction Number	Mean ± s.e.m.	Inhibition in Abdominal Writhing (%)
Control Saline	10 ml/kg	86.3 ± 1.03	Nil
C_fMR_d 10 mg/kg	Fraction I	60.7 ± 1.63***	29.7
	Fraction II	80.7 ± 1.23***	6.5
	Fraction III	81.7 ± 1.86**	5.4
	Fraction IV	84.8 ± 2.04 n.s	1.7
	Fraction V	83.8 ± 1.83 n.s	2.9

****P< 0.01 and ***P< 0.001; n.s = not significant; n = 6**

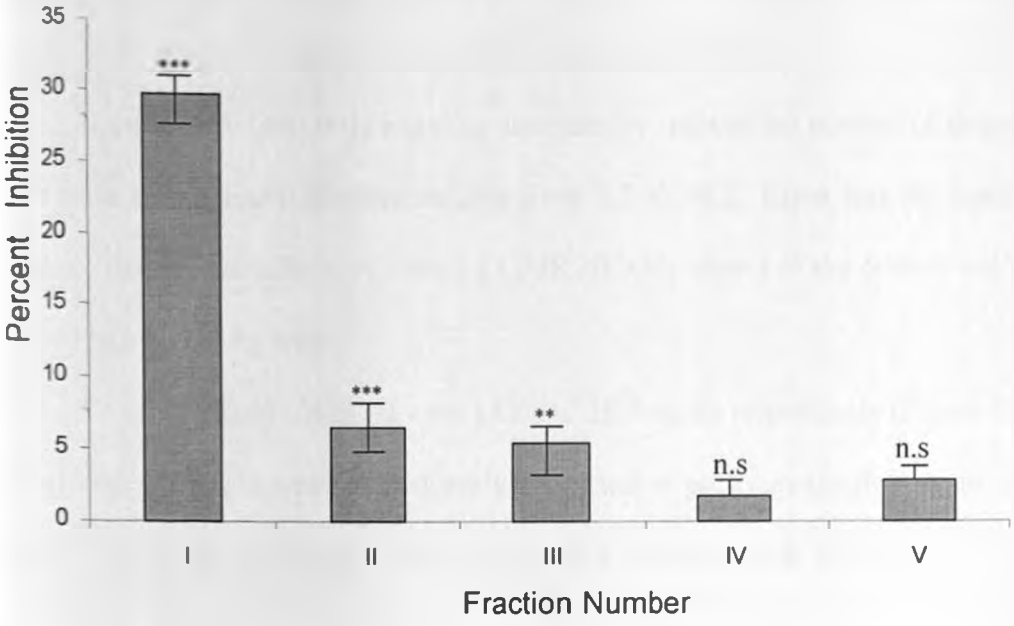


Figure 11: Effects of intraperitoneal adminstration of 10 mg/kg body weight of C_fMR_d fraction I to V on the number of abdominal writhes.

****P< 0.01 and ***P< 0.001; n.s = not significant**

4.9 EFFECTS OF C_fMR_d I AND C_fMR_d II IN ABDOMINAL WRITHING

C_fMR_d I significantly ($p<0.001$) and dose dependently reduced the number of abdominal writhes with percentage inhibitions ranging from 10.7 to 84.3 (Table 11, Figure 12). The lower doses of 25 and 30 mg/kg did not induce significant effects.

C_fMR_d II significantly ($p<0.001$) and dose dependently reduced the number of abdominal writhes with percentage inhibition ranging from 5.7 to 54.2. There was no significant difference between the effects of 5 mg/kg C_fMR_d II with respect to the control and when compared to a 10 mg/kg dose.

The ID_{50} for C_fMR_d I and C_fMR_d II were 14.8 and 28.9 mg/kg respectively (Figure 12).

C_fMR_d I and C_fMR_d II were subsequently subjected to purity evaluation as set out in section 3.2.A.5 and spectroscopic identification as set out in section 3.2. A.7.

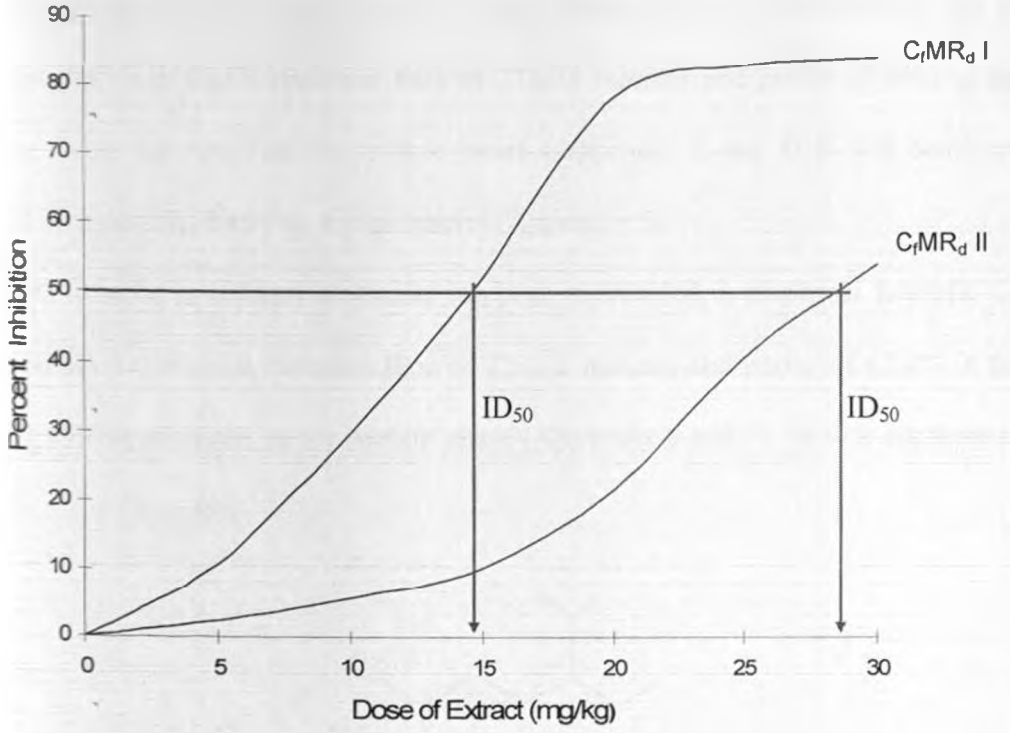


Figure 12: Dose response curve of C_fMR_d I and C_fMR_d II.

The ID_{50} is 14.8 and 28.9 mg/kg respectively.

4.10 SPECTROSCOPIC IDENTIFICATION OF COMPOUND C_fMR_d I AND II USING GC-MS

GC-MS analysis of C_fMR_d I showed a prominent molecular ion peak at m/z 412.4, empirical formula, C_{29} H_{48} O, with a fragmentation pattern characteristic for sterols (Appendix 4). It had a retention time of 27.624 minutes and purity of 95% in the GC using dichloromethane as the mobile phase (Appendix 2 and 3) It was confirmed by NIST05a spectral library as a stigmasterol (Appendix 5).

C_fMR_d II had a prominent molecular ion peak at m/z 364.0, empirical formula, C_{26} H_{52} (Appendix 8). It had a retention time of 22.412 minutes and purity of 62.4% in the GC using dichloromethane as the mobile phase (Appendix 6 and 7). It was confirmed as 9-hexacosene (Appendix 9)

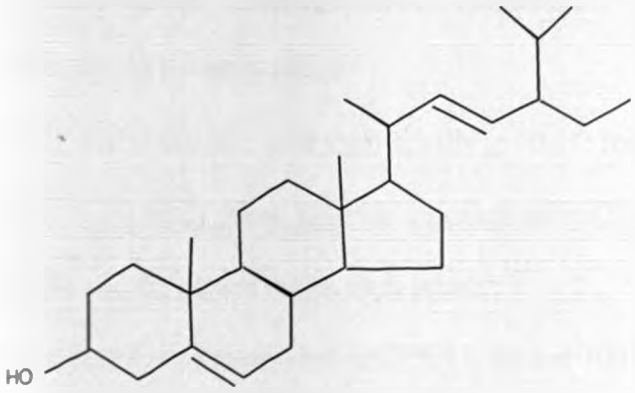


Figure 13: Structure of stigmasterol

Figure 14: Structure of 9-hexacosene

4.11 EFFECTS OF STIGMASTEROL AND 9-HEXACOSENE COMPARED WITH INDOMETHACINE, ACETYLSALICYLIC ACID AND DEXAMETHASONE IN THE EARLY AND LATE PHASE OF THE FORMALIN TEST

(a) The Formalin Test

Injection of 20 µl of 5% formalin subcutaneously into the plantar surface of the mouse produced distinct behavioural responses, licking, biting and lifting the injected paw. Two distinct periods of high pain behaviour were identified: the early phase lasting for the first 5 minutes and a second, the late phase, starting 20 to 60 minutes after the injection of formalin.

(b) Stigmasterol

Intraperitoneal administration of stigmasterol significantly reduced the time spent in pain behaviour at a dose of 30 mg/kg ($p<0.05$) and 100 mg/kg ($p<0.001$) during the early phase. 7.5 mg/kg and 15 mg/kg showed no significant effect on the time spent in pain behaviour also in the early phase.

400 mg/kg acetylsalicylic acid significantly ($p<0.01$) reduced the time spent in pain behaviour in the early phase whereas indomethacine (50 mg/kg) and dexamethasone (30 mg/kg) showed no effects in the early phase.

In the late phase, stigmasterol dose (7.5, 15, 30 and 100 mg/kg) dependently reduced the time spent in pain behaviour. This reduction was statistically significant ($p<0.05$).

Indomethacine (50 mg/kg), acetylsalicylic acid (400 mg/kg) and dexamethasone (30 mg/kg) significantly ($p<0.01$) reduced the time spent in pain behaviour in the late phase.

There was no significant difference ($p=0.72$) between the effects of acetylsalicylic acid (400 mg/kg) and stigmasterol (100 mg/kg) in the late phase. No overt motor, neurological or behavioural deficits were observed at all the doses tested

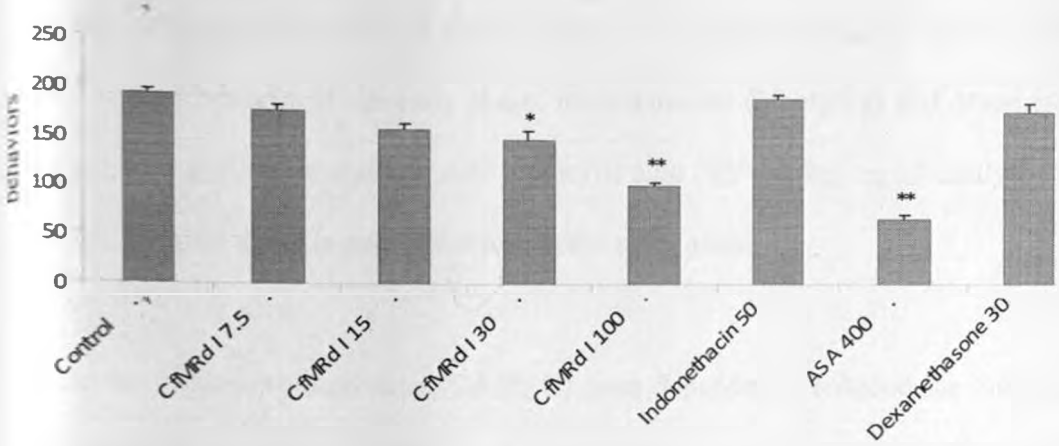
Table 12

Effects of stigmasterol, indomethacine, acetylsalicylic acid and dexamethasone following intraperitoneal injection.

DRUG	DOSE	Time (Seconds) spent in pain behavior
EARLY PHASE		
Vehicle	10 ml/kg	190.76 ± 5.18
Stigmasterol (C_fMR_d I)	7.5 mg/kg	174.39 ± 5.57 n.s
	15 mg/kg	155.81 ± 5.99 n.s
	30 mg/kg	144.78 ± 9.80 *
	100 mg/kg	99.36 ± 2.51 **
Indomethacine	50 mg/kg	186.21 ± 4.52 n.s
Acetylsalicylic Acid	400 mg/kg	65.45 ± 4.65 **
Dexamethasone	30 mg/kg	172.37 ± 9.09 n.s
LATE PHASE		
Vehicle	10 ml/kg	263.22 ± 2.89
Stigmasterol (C_fMR_d I)	7.5 mg/kg	171.16 ± 5.53 *
	15 mg/kg	136.8 ± 7.85 **
	30 mg/kg	103.72 ± 4.4 **
	100 mg/kg	80.62 ± 4.82 **
Indomethacine	50 mg/kg	67.76 ± 8.43 **
Acetylsalicylic Acid	400 mg/kg	85.09 ± 6.8 **
Dexamethasone	30 mg/kg	91.63 ± 6.07 **

*** $p< 0.05$ and ** $p< 0.01$; n.s = not significant; n = 6**

Early Phase



Late Phase

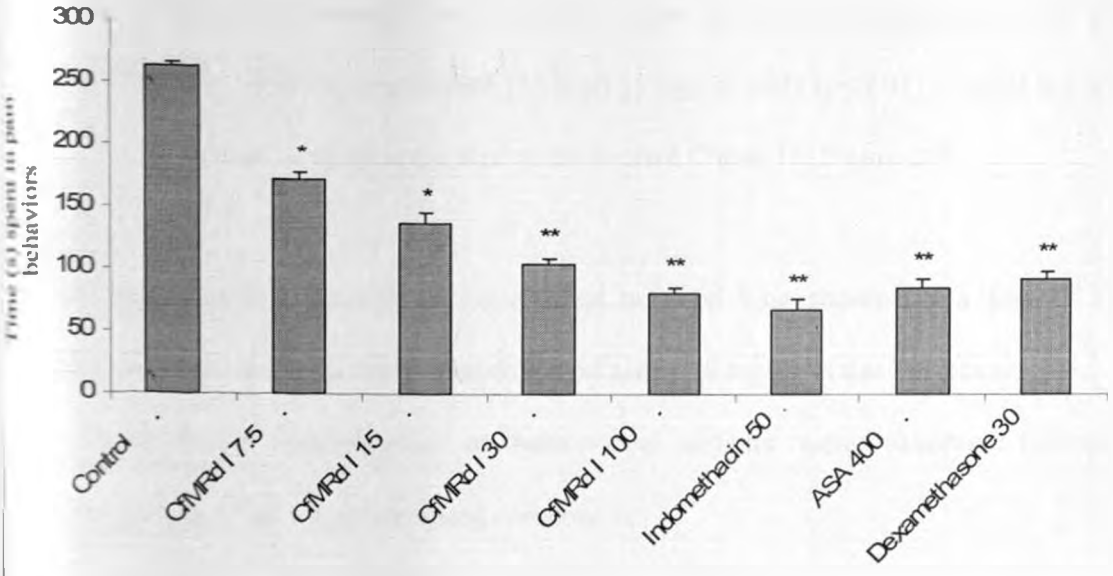


Figure 15

Effects of intraperitoneally administered stigmasterol, indomethacin, acetylsalicylic acid and dexamethasone on time (s) spent in pain behaviors in formalin test.

*** $p < 0.05$ and ** $p < 0.01$**

(c) 9-hexacosene

Intraperitoneal administration of varying doses of 9-hexacosene had no effect on the time spent in pain behavior in the early phase. Indomethacine (50 mg/kg) and dexamethasone (30 mg/kg) had no effects either. Acetylsalicylic acid (400 mg/kg) significantly ($p<0.001$) reduced the time spent in pain behaviour in the early phase.

In the late phase, 9-hexacosene (C_fMR_d II) dose dependently reduced the time spent in pain behavior (Table 13, Figure 16). The effects of all the doses were statistically significant at $p<0.01$ compared to the control. Indomethacine (50 mg/kg), acetylsalicylic acid (400 mg/kg) and dexamethasone (30 mg/kg) significantly ($p<0.01$) reduced the time spent in pain behavior when compared to the control (Table 13, Figure 16).

There was comparable antinociceptive effect between 9-hexacosene at a dose of 100 mg/kg, indomethacine 50 mg/kg and dexamethasone 30 mg/kg in the late phase.

No overt motor, neurological or behavioural deficits were observed following administration of all the above tested compounds.

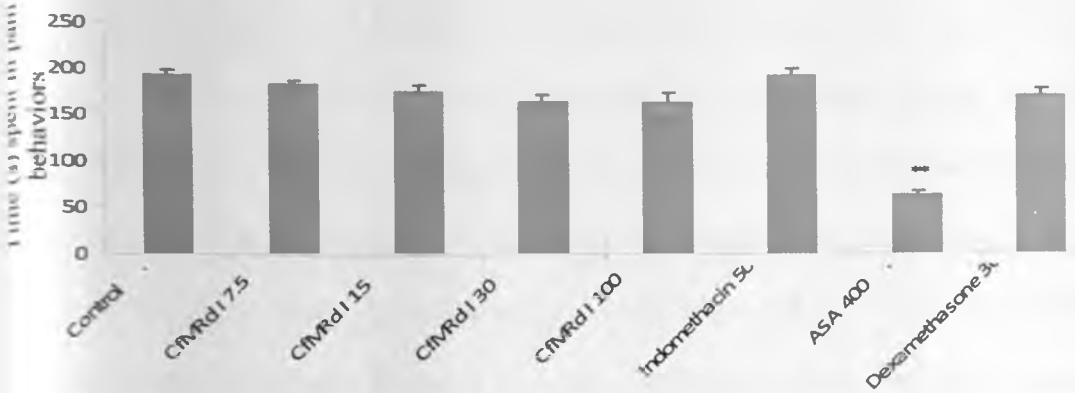
Table 13

Effects of 9-hexacosene, indomethacine, acetylsalicylic acid and dexamethasone following intraperitoneal Injection.

DRUG	DOSE	Time (seconds) spent in pain behaviour
EARLY PHASE		
Vehicle	10 ml/kg	193.35 ± 5.50
9-hexacosene (C_fMR_d II)	7.5 mg/kg	184.74 ± 2.69 n.s
	15 mg/kg	176.40 ± 5.80 n.s
	30 mg/kg	165.22 ± 5.86 n.s
	100 mg/kg	164.12 ± 9.76 n.s
Indomethacine	50 mg/kg	191.89 ± 6.19 n.s
Acetylsalicylic Acid	400 mg/kg	62.86 ± 3.52 **
Dexamethasone	30 mg/kg	169.53 ± 6.53 n.s
LATE PHASE		
Vehicle	10 ml/kg	258.67 ± 6.06
9-hexacosene (C_fMR_d II)	7.5 mg/kg	476.93 ± 6.67 *
	15 mg/kg	126.50 ± 6.81 **
	30 mg/kg	109.69 ± 2.91 **
	100 mg/kg	71.31 ± 3.00 **
Indomethacine	50 mg/kg	78.42 ± 2.04 **
Acetylsalicylic Acid	400 mg/kg	132.65 ± 2.70 **
Dexamethasone	30 mg/kg	118.46 ± 2.42 **

*** P< 0.05 and **P< 0.01; n.s = not significant; n = 6**

Early Phase



Late Phase

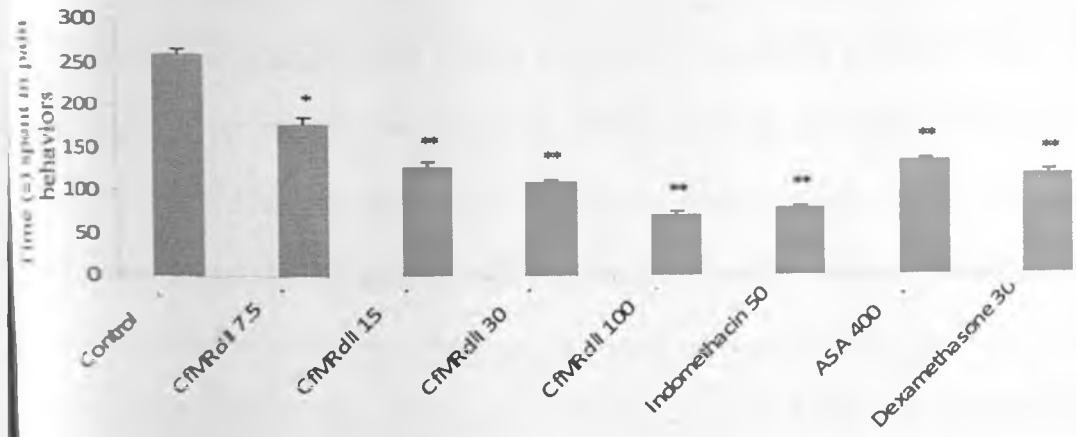


Figure 16:

Effects of intraperitoneally administered 9-hexacosene (C_fMR_d II), indomethacin, acetylsalicylic acid and dexamethasone on time (s) spent in pain behaviors in formalin test.

*** $p < 0.05$ and ** $p < 0.01$**

CHAPTER FIVE

5.0 DISCUSSION

Acetic acid induced writhing test in mice was used to screen *Mondia whytei* for antinociceptive activity. This test is commonly used as a screening method because it is easy to perform and is sensitive (Taber, 1974). Significant linear correlations have been found between the mouse ED_{50} values for several drugs and the various recommended human analgesics dosages (Pong *et al.*, 1985). It therefore means that ED_{50} values for stigmasterol and 9-hexacosene obtained in this study may be extrapolated in order to determine the projected human daily dose.

The main disadvantage of this method is its lack of specificity as many drugs without analgesic effects in man can effectively inhibit writhing responses (Handershot and Fursaith, 1959; Chernorv *et al.*, 1967). In addition, there is a large variation in response between mouse strains (Brown and Hughes, 1962). The entire research used two thousands one hundrend and twelve mice. Adult male and female swiss albino mice weighing 25 – 30 mg were used. Equal number of male and female was assigned to each experimental unit. This countered variation of result attributed to gender differences. Acclimatization of the animals to the experimental environment was done for two weeks before subjecting them to the tests. Also adaptation of the animals to the testing chamber was done two hours prior to testing. This had an overall effect of reducing stress to the animal which would otherwise lead to variations in the results obtained since stress can result in stress induced analgesia.

Formalin test was used to evaluate the antinociceptive mechanisms of stigmasterol and 9-hexacosene. Formalin test has several advantages over the other tests (Dubuisson and Dennis, 1977). There is no restraint during the observation period. The animals are not stressed as stress can alter pain sensitivity of the animal. The pain stimulus bears resemblance to most clinical pain. The stimulus elicits a continous response that enables a temporal nociceptive profile to be measured. Two phases of pain behavior were identified representing different types of pain. The pain of the early phase (0 – 5 minutes) is evoked by direct stimulation of nociceptors while the late phase (20 – 60 minutes) is due to inflammation (Shibata *et al.*, 1986).

However, room temperature has been shown to influence the licking response in the late phase of the formalin test (Rosland, 1991). An increase in room temperature would cause an increase in the intensity and duration of licking in the late phase. It is therefore recommended that the room temperature in the testing chamber should be carefully controlled to obtain reliable results especially in the late phase of the formalin test (Rosland, 1991). To avoid this source of error, all the tests were conducted in a controlled testing chamber temperature of $24 \pm 0.5\ ^{0}C$.

Chloroform root (CR) extracts caused the highest inhibition of the abdominal writhes compared to the ethanol and water extracts (Table 3, 4 and 5). These findings suggested that the active compounds responsible for antinociceptive activities of *Mondia whytei* are non polar in nature. Phytochemical tests done on the chloroform root extract confirmed the presence of carotenoids, flavonoids, steroids and tannins. CR extracts also contained

plenty of plant oils. However, plant oils are known to have low pharmacological activity (Peter and Amala, 1998). CR had an ID_{50} of 198.8 mg/kg, the dose that lowered the number of abdominal writhes by half compared to the control. This was statistically significan ($p<0.01$) whereas ethanol and water root extracts could not achieve ID_{50} at the highest dose tested (400mg/kg).

Defatting of the root powder with n-hexane prior to extracting with chloroform was meant to remove plant oils and therefore lower the ID_{50} of the defatted chloroform root extract (CR_d). On the contrary, the ID_{50} of CR_d was raised to 350 mg/kg (figure 9). This meant that n-hexane dissolved some of the active compounds prior to extracting with chloroform. This suggested a wide solubility range of the active compound between n-hexane and chloroform.

Solvent-solvent partitioning of the defatted methanol root extract with chloroform as set out in section 3.2.A.4 resulted in two extracts; the chloroform soluble portion of the defatted methanol root extract (C_fMR_d) and the aqueous soluble portion of the defatted methanol root extract (A_fMR_d). At the highest dose tested (400 mg/kg), C_fMR_d and A_fMR_d caused 90.6 and 33.1 percent inhibition of the abdominal writhes respectively (Table 9). C_fMR_d had an ID_{50} of 50 mg/kg body weight whereas A_fMR_d could not achieve its ID_{50} at 400 mg/kg (Figure 10). This meant that the active compounds had concentrated in the chloroform portion.

There was a probable chance that the chloroform residue in C_fMR_d was contributing to the antinociceptive behaviour by causing motor impairment. C_fMR_d could also have

muscle relaxant properties that would manifest as antinociceptive behaviour. Rotarod test was therefore carried out and no motor or neurological deficits were found in C_fMR_d treated animals. Likewise, no overt behavioural changes were noted.

Among several solvent systems tested (Table 1) for carrying out fractionation of C_fMR_d. 30% methanol in dichloromethane gave the highest number of well separated spots on TLC. Five fractions were obtained upon fractionation of C_fMR_d. These fractions were labeled I to V. TLC separation of fractions I to V revealed spots with R_f values of 0.71, 0.48. 0.37, 0.18 and 0.1 respectively (Appendix 1). The fractions were tested for antinociceptive effects at the same dose level of 10 mg/kg. This dose was chosen on assumption that all the active compounds in C_fMR_d were taken up in one out of the five fractions. Since the ID_{50} of C_fMR_d was 50 mg/kg, it therefore implied that if all the active compounds end up in one of the five fractions then that fraction would have five times the potency thereby lowering its ID_{50} to 10 mg/kg. At a dose of 10 mg/kg, fraction I to V caused in 29.7, 6.5, 5.4, 1.7 and 2.9 percent inhibition of the abdominal writhes respectively (Table 10 and figure 11). These results indicated that the antinociceptive compounds were distributed in all the five fractions but not equally. Fraction I and II exhibited statistically significant ($p<0.001$) antinociceptive effects at a dose of 10 mg/kg (Table 11). The ID_{50} was 14.8 mg/kg and 28.8 mg/kg for fraction I and II respectively (Figure 12).

White crystals were obtained from fraction I (C_fMR_d I) at room temperature with a melting point of 168 ^{0}C whereas fraction II (C_fMR_d II) yielded off-white crystals at room

temperature with a melting point of 79 ^{0}C. GC-MS analysis of C_fMR_d I showed a prominent molecular ion peak at m/z 412.4, empirical formula $C_{29}H_{48}O$, with a fragmentation pattern characteristic for sterols. It was confirmed by National Institute of Standards and Technology (NIST 05a) spectral library as a stigmasterol (Appendix 5). C_fMR_d II showed a fragmentation pattern with a prominent molecular ion peak at m/z 364.0, empirical formula $C_{26}H_{52}$, and confirmed as 9-hexacosene (Appendix 9). Stigmasterol and 9-hexacosene (Figure 13 and 14) were isolated from the roots of *Mondia whytei* for the first time.

When tested at a dose of 30 mg/kg and above, stigmasterol caused significant ($p<0.05$) dose dependent in the amount of time the animal spent in pain behavior in both the early phase and the late phase of the formalin (Table 12 and figure 15). These results suggested that the antinociceptive effects of stigmasterol is both non-genomic characterized by its fast action in exerting antinociceptive effect in the early phase and also genomic as characterized by delayed action in exerting antinociceptive effects in the late phase of formalin test. Stigmasterol like dexamethasone is a steroid and was expected to exert antinociceptive effects on the late phase of the formalin test. This is because steroids are known to inhibit phospholipase A_2 (Vane and Botting, 1987) thereby blocking generation of pro-inflammatory mediators.

The finding that stigmasterol exerted antinociceptive effects in the early phase of formalin test was unpredicted and therefore generated interest in understanding the mechanisms underlying the basis of steroidal activity. It was thought to act like a

neurosteroid. Neurosteroids are those neuroactive steroids which are synthesized *de novo* in the brain in contrast to the exogenous steroids. Pregnane steroids directly activate $GABA_A$ receptor. Both the potentiation and activation appear to be mediated through a site(s) distinct from the well known barbiturate and benzodiazepine allosteric sites of the $GABA_A$ receptor. This potentiation is stereoselective and mediated by a steroid-induced prolongation of the burst duration of the $GABA_A$ –activated channel. The possession of a hydroxyl group in α – configuration at C-3 of the steroid ring A is an important determinant of potency (Harrison. *et al.*, 1987). Stigmasterol unlike dexamethasone possesses the hydroxyl group in α – configuration at C-3 of the ring A.

γ-aminobutyric acid (GABA), the principal inhibitory neurotransimitter in the CNS, activates two types of receptor, the ionotropic $GABA_A$ receptor and the metabotropic (G protein-coupled) $GABA_B$ receptor. The $GABA_A$ receptor is a pentameric protein (Schofield *et al.*, 1987) whose activation by agonists opens an associated chloride ion channel, leading to an increase in chloride ion influx that results in membrane hyperpolarization (Majewska, 1987). Steroids like stigmasterol therefore likely exerts their antinociceptive effects in the early phase of formalin test by altering the neuronal excitability. Stigmasterol at a dose of 100 mg/kg was equipotent to acetylsalicylic acid at a dose of 400 mg/kg. Acetylsalicylic acid at a dose of 400 mg/kg was found to exert antinociceptive effects equipotent to morphine 5 mg/kg body weight in mice early phase of the formalin test (Hunskaar, 1987).

On the other hand, 9-hexacosene exhibited antinociceptive effects only in the late phase of the formalin test. It produced graded, dose-dependent and statistically significant ($p<0.05$) antinociceptive effects (Table 13). 30 mg/kg of 9-hexacosene was equipotent to 400 mg/kg body weight acetylsalicylic acid (Figure 16). The same dose of 9-hexacosene had no statistically significant antinociceptive effects during the early phase whereas 400 mg/kg acetylsalicylic acid significantly reduced the amount of time the animal spent in pain behavior in the early phase ($p<0.05$).

9-hexacosene and indomethacine were found to be equipotent at doses of 100 mg/kg and 50 mg/kg body weight respectively in the late phase.

9-hexacosene is an unsaturated hydrocarbon with a double bond at C-9 with structural resemblances to arachidonic acid. Structure activity relationship exist between 9-hexacosene and arachidonic acid and may therefore explain the antinociceptive effects of 9-hexacosene. Considering the spatial arrangement of arachidonic acid inside the cyclo-oxygenase enzyme (a 534 amino acid molecule), it is possible that 9-hexacosene enters into the cyclo-oxygenase enzyme tunnel and because of the slight differences with the normal substrate (arachidonic acid), 9-hexacosene is unable to form linkages at appropriate sites within the cyclo-oxygenase enzyme and therefore cannot be hydrolyzed to form pro-inflammatory mediators. 9-hexacosene therefore may act as a false substrate. This is the first time such a mechanism of action is being postulated and it is expected that it will open a new way of thinking in as far as developing new safe, effective and efficacious analgesics is concerned.

5.1 CONCLUSION

Stigmasterol and 9-hexacosene were isolated from *Mondia whytei* for the first time. The two compounds were shown to strongly contribute to the antinociceptive effects of *Mondia whytei* roots.

Stigmasterol dose dependently caused graded and statistically significant ($p<0.05$) antinociceptive effects in the early and late phase of the formalin test. Stigmasterol at a dose of 100 mg/kg was equipotent to acetylsalicylic acid at a dose of 400 mg/kg in the late phase of formalin test.

9-hexacosene dose dependently caused graded and statistically significant ($p<0.05$) antinociceptive effects in the late phase of the formalin test. 9-hexacosene and indomethacine were found to be equipotent at doses of 100 mg/kg and 50 mg/kg body weight respectively.

There were no motor or neurological deficits observed in the treated animals using rotarod test. No behavioural deficits were observed following intraperitoneal injection of stigmasterol or 9-hexacosene.

5.2 RECOMMENDATION

1. *Mondia whytei* is claimed to have aphrodisiac, appetite stimulating and antimicrobial properties. Further research work should be carried out in order to verify these claims.
2. Though the data obtained in this study confirms the analgesic properties of the isolated compounds (stigmasterol and 9-hexacosene) in mice, its safety need to be evaluated in animal toxicity tests and the conventional clinical trials.

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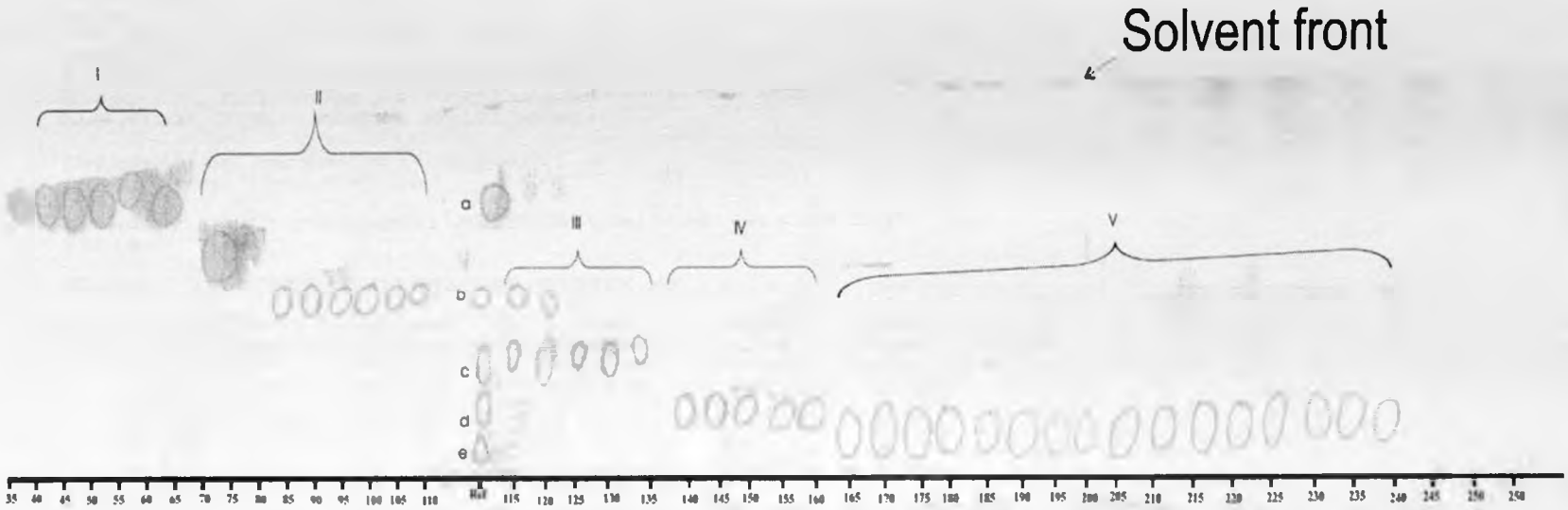
5.4 APPENDICES



SPOT OF EVERY FIFTH TEST TUBE COLLECTED
(done on a pre-coated silica gel TLC analytical plate)

Appendix 2: ***Purity Data of C_fMR_d I as Analyzed using Gas Chromatography.***

Mass Spec Lab @ ICIPE- BCED Area Percent Report

Data Path : D:\DATA\15062009B\
Data File : OK15062009B.D
Acq On : 15 Jun 2009 11:44
Operator : OK
Sample : Cf-MRd-1-Solid in DCM
Misc : 0.0003g Cf-MRd-1-Solid in 1.5ml DCM
ALS Vial : 2 Sample Multiplier: 1

Integration Parameters: autoint1.e
Integrator: ChemStation

Method : C:\msdchem\1\METHODS\training version 1.M
Title :

Signal : TIC: OK15062009B.D\data.ms

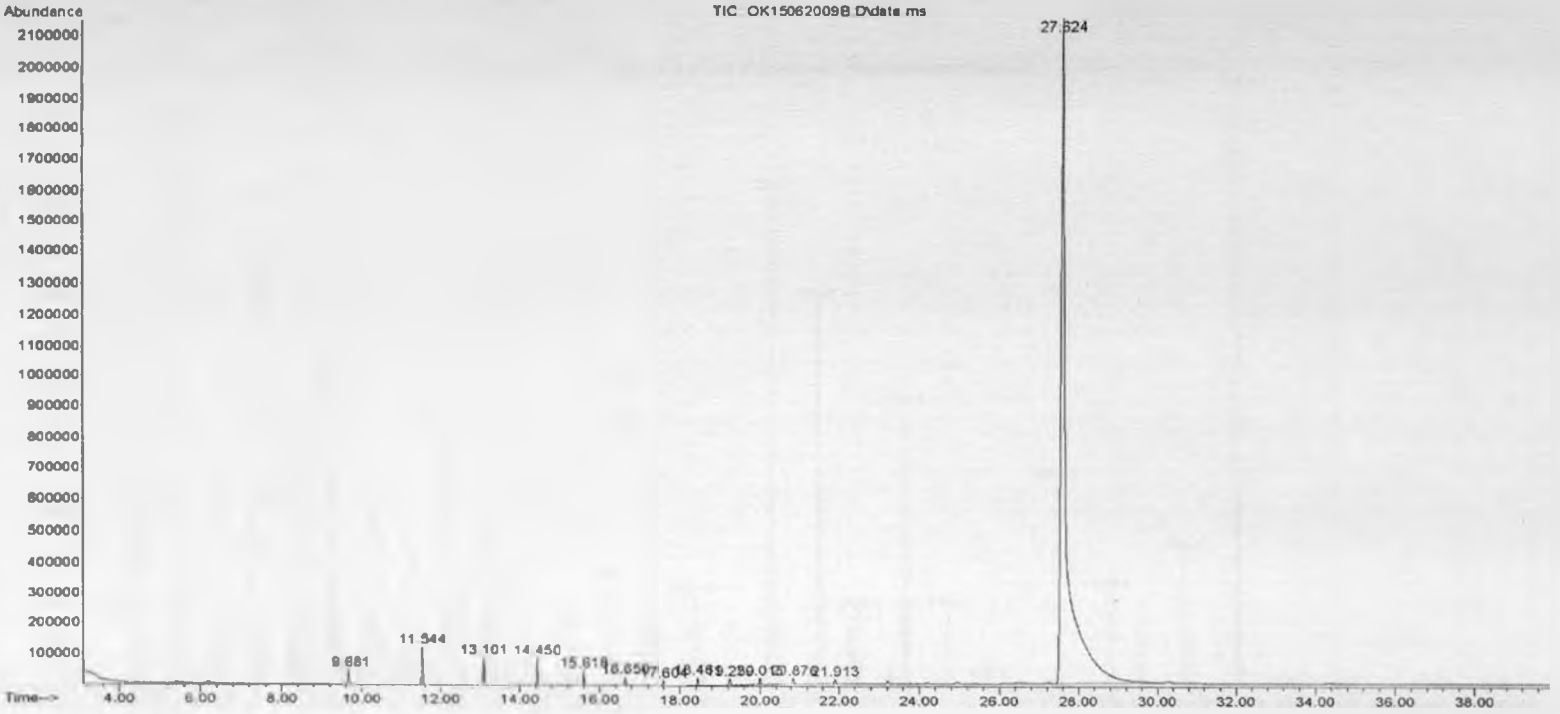
peak #	R.T. min	first scan	max scan	last scan	PK TY	peak height	corr. area	corr. % max.	% of total
1	9.681	289	294	301	BB	36995	965519	0.44%	0.420%
2	11.544	372	378	384	BB 2	109138	2571165	1.17%	1.119%
3	13.101	442	447	453	BB 2	82722	2008141	0.92%	0.874%
4	14.450	501	507	512	BB	71740	1301654	0.59%	0.566%
5	15.618	554	559	564	BB	37046	781768	0.36%	0.340%
6	16.650	600	606	610	BB	24694	531591	0.24%	0.231%
7	17.604	641	648	651	BB 2	15627	378521	0.17%	0.165%
8	18.461	680	686	691	BB	22127	448090	0.20%	0.195%
9	19.259	710	722	725	BB 2	18920	470986	0.22%	0.205%
10	20.015	747	756	761	BB 2	20175	555304	0.25%	0.242%
11	20.876	788	794	798	BB	17493	495496	0.23%	0.216%
12	21.913	831	841	846	BV	13788	458088	0.21%	0.199%
13	27.624	1086	1096	1209	BV 2	2064051	218893438	100.00%	95.229%

Sum of corrected areas: 229859761

training version 1.M Thu Jun 18 17:15:23 2009

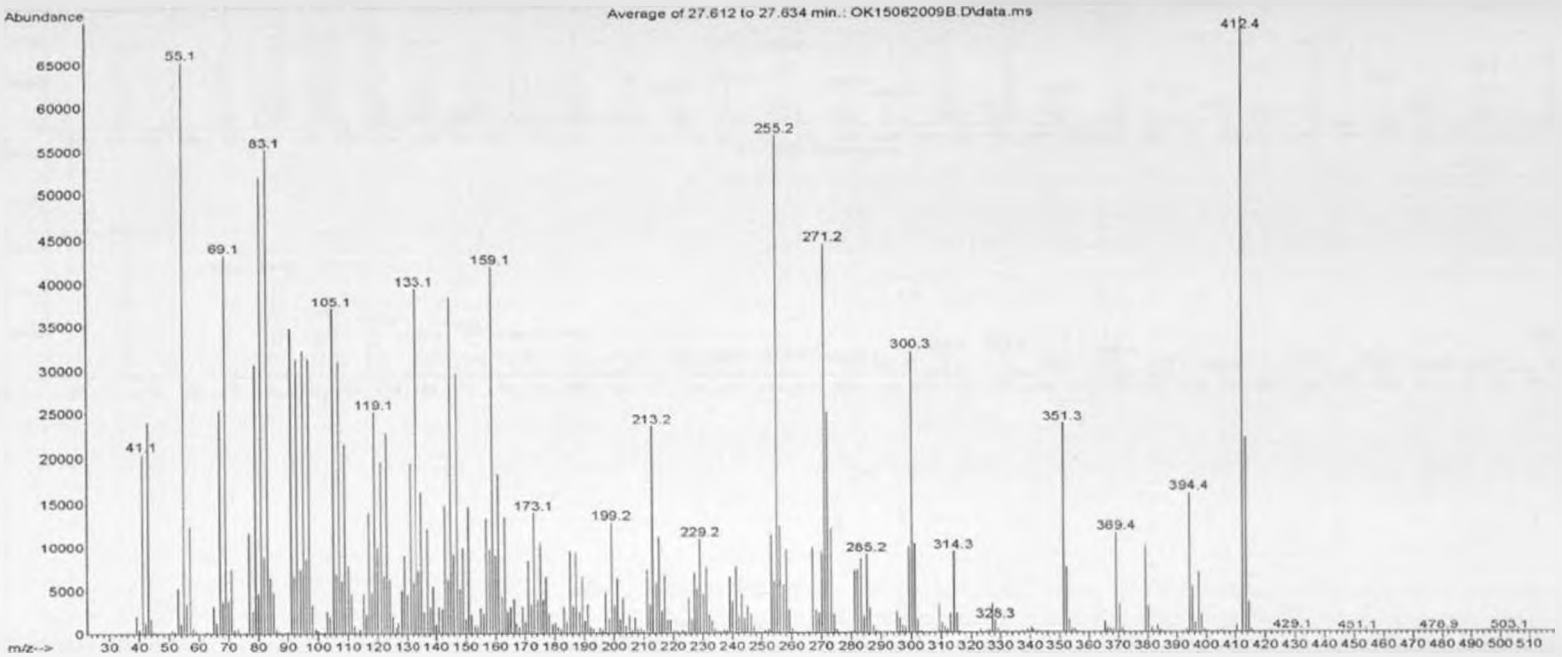
Appendix 3. Retention Time for C_fMR_d I in Gas Chromatography.

```
File       :D:\DATA\15062009B\OK15062009B.D
Operator   : OK
Acquired   : 15 Jun 2009  11:44     using AcqMethod VOLATILES 60-280 FAST RISE 70MIN RUN.M
Instrument :   ICIPE MSD
Sample Name: Cf-MRd-1-Solid in DCM
Misc Info  : 0.0003g Cf-MRd-1-Solid in 1.5ml DCM
Vial Number: 2
```

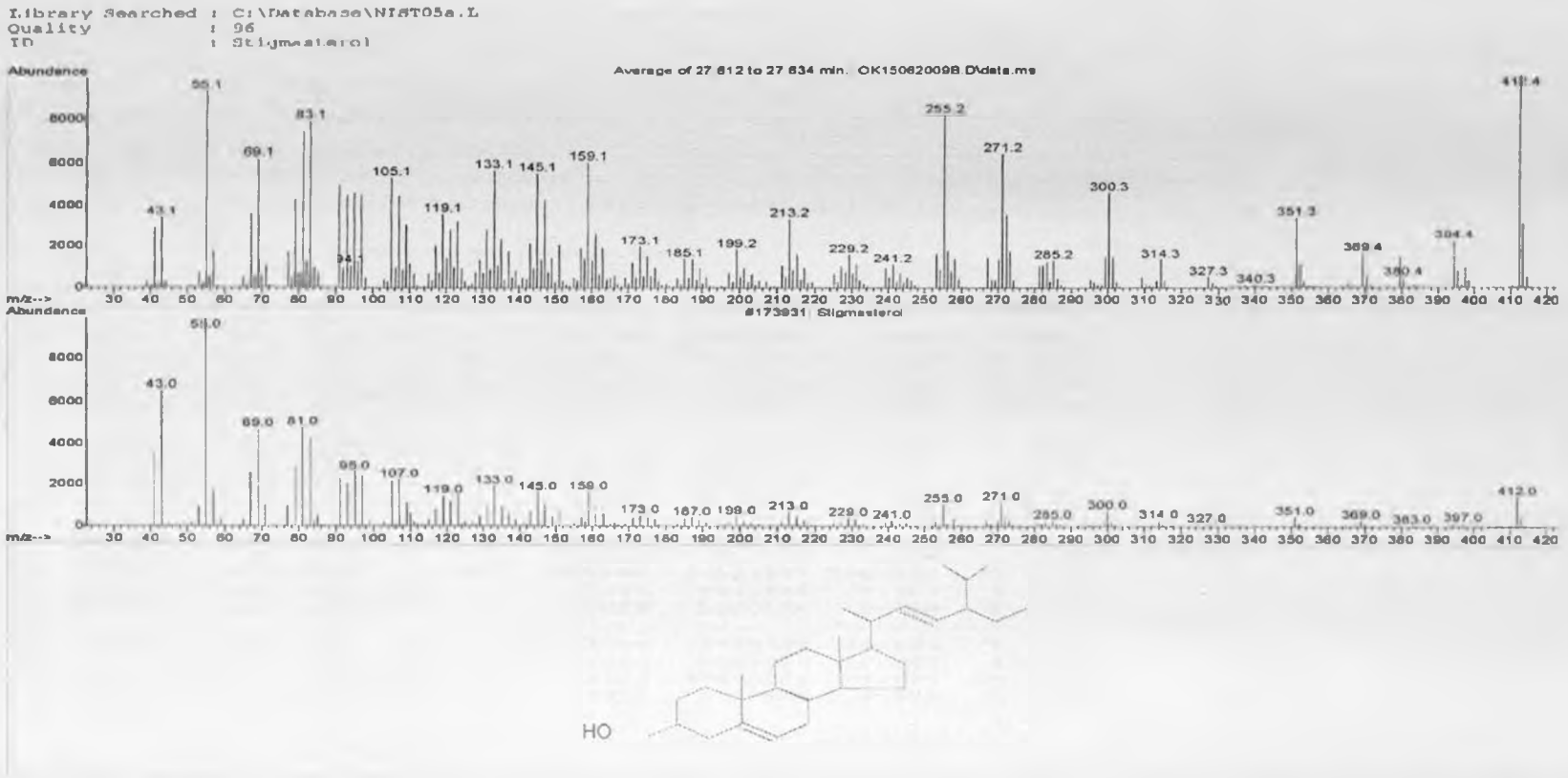


Appendix 4. Mass Spectrum of C_fMR_d I

```
File          :D:\DATA\15062009B\OK15062009B.D
Operator      : OK
Acquired      : 15 Jun 2009  11:44      using AcqMethod VOLATILES 60-280 FAST RISE 70M1N RUN.M
Instrument :    ICIPE MSD
Sample Name: Cf-MRd-1-Solid in DCM
Misc Info  : 0.0003g Cf-MRd-1-Solid in 1.5ml DCM
Vial Number: 2
```



Appendix 5. **Mass Spectrum of C_fMR_d I Compared with the Mass Spectrum of Authentic Stigmasterol Based on NIST05a Library Data.**



Appendix 6. Purity Data of C_fMR_d II

Mass Spec Lab @ ICIPE- BCED Area Percent Report

Data Path : D:\DATA\15062009B\
Data File : OK15062009C.D
Acq On : 15 Jun 2009 12:44
Operator : OK
Sample : Cf-MRd-II-Solid in DCM
Misc : 0.0003g Cf-MRd-II-Solid in 1.5ml DCM
ALS Vial : 3 Sample Multiplier: 1

Integration Parameters: autoint1.e
Integrator: ChemStation

Method : C:\msdchem\1\METHODS\training version 1.M
Title :

Signal : TIC: OK15062009C.D\data.ms

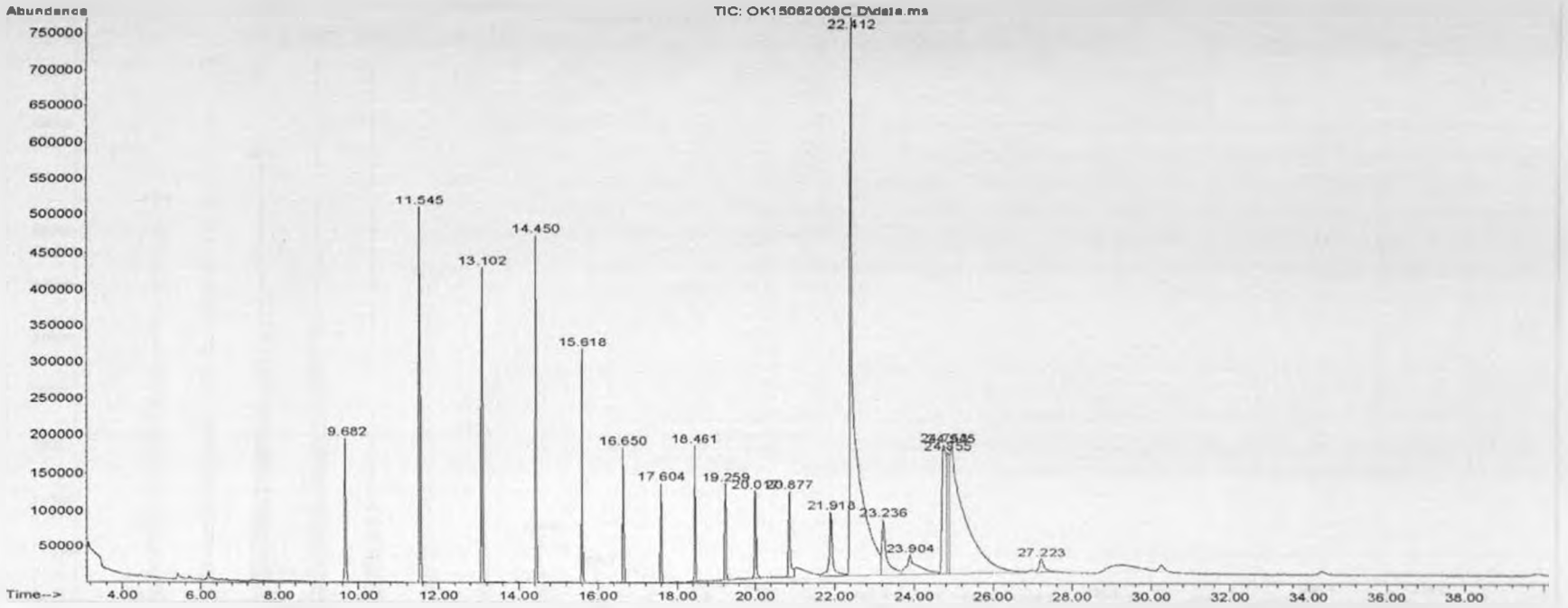
peak #	R.T. min	first scan	max scan	last scan	PK TY	peak height	corr. area	corr. % max.	% of total
1	9.682	288	294	301	BB	152770	3918380	6.17%	1.925%
2	11.545	372	378	387	BB 2	465123	11113438	17.49%	5.459%
3	13.102	444	447	451	BB 2	396492	9215019	14.51%	4.527%
4	14.450	501	507	513	BB	390205	6999359	11.02%	3.438%
5	15.618	553	559	563	BB	237572	4825580	7.60%	2.371%
6	16.650	586	606	611	BB 2	159084	3323813	5.23%	1.633%
7	17.604	636	648	653	BV 2	108661	2657625	4.18%	1.306%
8	18.461	673	686	690	BB	154155	2990158	4.71%	1.469%
9	19.259	703	722	730	BV 2	122840	3180107	5.01%	1.562%
10	20.017	738	756	767	VB 2	121497	3719321	5.85%	1.827%
11	20.877	787	794	799	BV	106073	3482104	5.48%	1.711%
12	21.918	828	841	859	VV	85406	4954729	7.80%	2.434%
13	22.412	859	863	896	VV	695288	63523987	100.00%	31.206%
14	23.236	896	900	919	VV	75261	6545245	10.30%	3.215%
15	23.904	919	929	962	VV	25959	5200564	8.19%	2.555%
16	24.753	962	967	970	VV 3	173295	11749309	18.50%	5.772%
17	24.855	970	972	974	VV 3	163089	6936028	10.92%	3.407%
18	24.945	974	976	1055	VV 4	173143	47613872	74.95%	23.390%
19	27.223	1071	1078	1094	VB	17374	1617427	2.55%	0.795%

Sum of corrected areas: 203566065

training version 1.M Thu Jun 18 18:10:11 2009

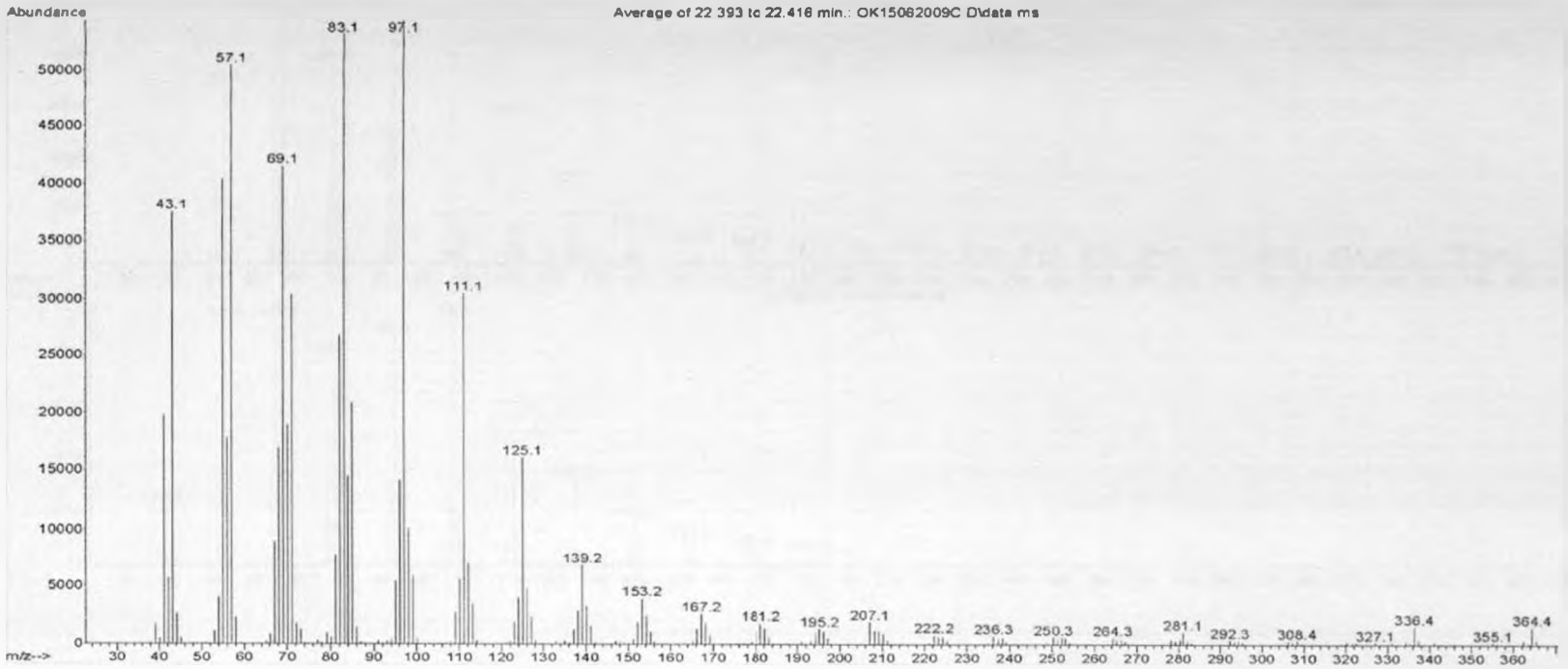
Appendix 7: Retention Time for C_fMR_d II

```
File         :D:\DATA\15062009B\OK15062009C.D
Operator     : OK
Acquired     : 15 Jun 2009  12:44      using AcqMethod VOLATILES 60-280 FAST RISE 70MIN RUN.M
Instrument   :   ICIPE MSD
Sample Name: Cf-MRd-II-Solid in DCM
Misc Info    : 0.0003g Cf-MRd-II-Solid in 1.5ml DCM
Vial Number: 3
```

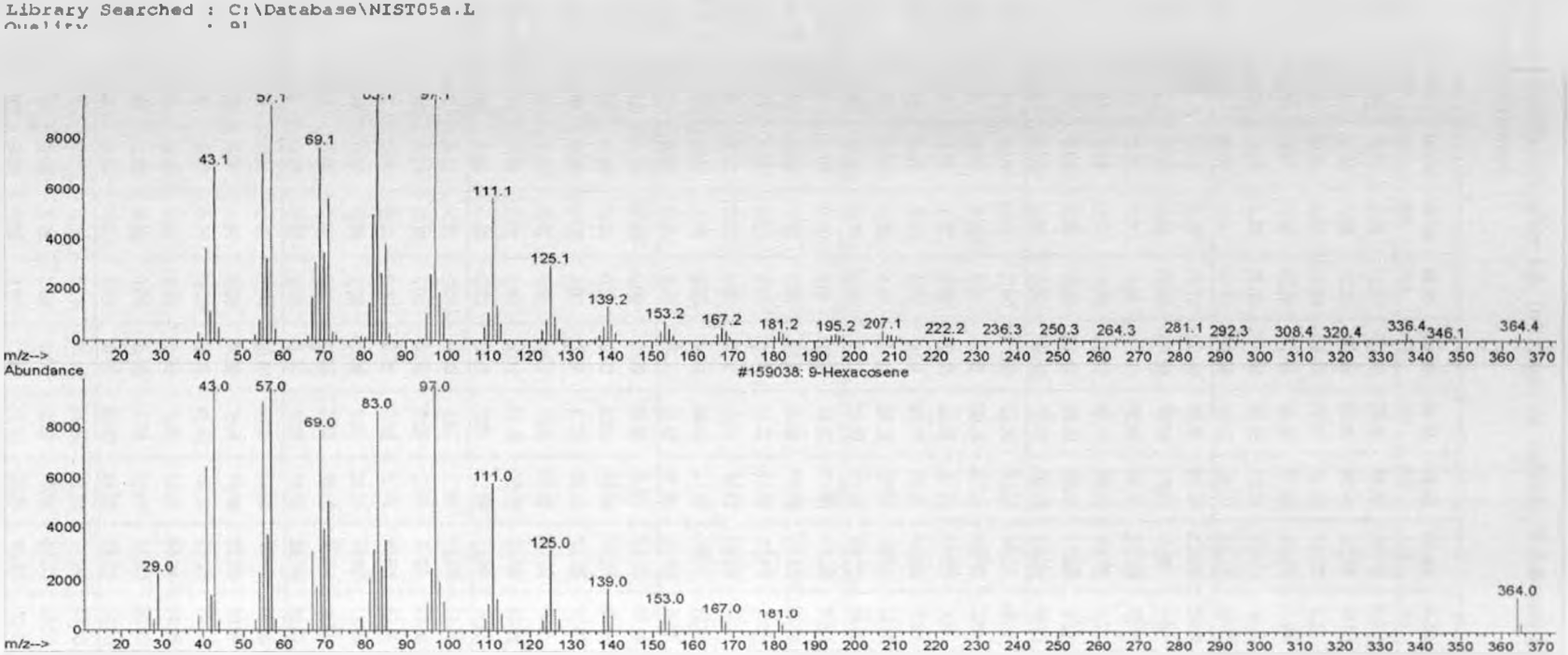


Appendix 8. Mass Spectrum of C_fMR_d II

```
File       :D:\DATA\15062009B\OK15062009C.D
Operator   : OK
Acquired   : 15 Jun 2009  12:44     using AcqMethod VOLATILES 60-280 FAST RISE 70MIN RUN.M
Instrument :    ICIPE MSD
Sample Name: Cf-MRd-II-Solid in DCM
Misc Info  : 0.0003g Cf-MRd-II-Solid in 1.5ml DCM
Vial Number: 3
```



Appendix 9. Mass Spectrum of C_fMR_d II Compared with the Mass Spectrum of Authentic 9-Hexacosene Based on NIST05a Library Data.



APPENDIX 11

Time (seconds) spent in pain behaviors after i.p. injection of vehicle, 9-hexacosene, indomethacine, acetylsalicylic acid and dexamethasone.

Treatment	Animal Number	0 - 5 min	5 -10 min	10 -15 min	15 -20 min	20 -25 min	25 -30 min	30 -35 min	35 -40 min	40 -45 min	45 -50 min	50 -55 min	55 -60 min
Vehicle	1	204.10	14.24	0.00	3.30	12.72	80.36	50.34	46.82	36.73	20.22	18.68	18.40
	2	212.34	8.36	2.26	1.26	20.36	50.94	56.49	40.68	30.26	18.47	32.38	12.32
	3	198.38	24.56	8.31	0.00	48.94	86.45	42.87	32.43	14.32	9.22	5.36	3.28
	4	182.49	26.39	0.00	0.00	46.38	62.45	48.34	36.56	25.22	21.68	8.83	11.32
	5	180.42	18.23	3.28	8.36	78.36	54.34	36.45	18.24	23.42	14.73	8.28	11.49
	6	182.34	9.42	0.00	0.00	40.43	80.38	52.53	28.23	23.23	12.43	8.23	11.42
9-hexacosene (CMR_d II) 7.5 mg/kg	1	182.40	82.60	6.30	0.00	10.72	56.30	48.20	22.34	20.62	13.56	14.30	19.22
	2	182.92	0.00	0.00	0.00	16.36	86.52	16.54	12.83	6.62	5.63	18.52	12.24
	3	193.45	42.24	0.00	0.00	0.00	43.83	36.48	34.28	28.56	16.24	12.68	7.36
	4	192.37	17.22	3.78	0.00	13.78	22.43	49.78	36.22	19.78	4.23	3.42	6.2
	5	178.96	0.00	0.00	0.00	18.23	57.47	43.20	18.54	14.66	8.23	12.47	4.80
	6	178.36	0.00	0.00	0.00	0.00	32.82	46.98	36.23	20.38	18.23	8.24	5.28
9-hexacosene (CMR_d II) 15 mg/kg	1	198.12	20.30	0.00	52.10	20.40	41.30	22.30	20.60	16.81	18.52	8.14	11.31
	2	162.34	8.53	0.00	0.00	0.00	0.00	60.20	23.26	16.85	3.50	12.10	5.25
	3	172.93	8.36	0.00	3.37	0.00	22.45	22.54	32.54	18.36	9.84	12.36	4.36
	4	182.33	13.48	3.48	0.00	0.00	0.00	49.71	18.21	16.98	7.29	14.81	6.33
	5	160.34	42.32	0.00	0.00	0.00	30.32	24.22	18.97	16.96	14.24	8.22	5.47
	6	182.34	18.32	0.00	0.00	0.00	0.00	32.03	28.24	22.36	19.42	13.48	8.42
9-hexacosene (CMR_d II) 30 mg/kg	1	183.12	10.51	2.30	0.00	0.00	18.20	13.52	28.60	10.30	16.81	9.24	6.31
	2	160.23	16.54	3.24	0.00	0.00	0.00	0.00	40.28	15.56	36.92	6.25	12.36
	3	142.83	48.56	0.00	0.00	0.00	0.00	15.67	55.68	22.45	7.38	9.48	3.97
	4	173.92	8.92	6.23	0.00	0.00	0.00	0.00	34.23	28.34	18.31	16.21	3.43
	5	158.30	16.24	0.00	0.00	0.00	0.00	40.46	32.92	18.56	10.05	10.72	6.83
	6	172.92	22.53	0.00	0.00	0.00	18.23	27.47	16.93	24.48	9.26	8.31	4.43
9-hexacosene (CMR_d II) 100 mg/kg	1	152.30	18.42	2.83	60.40	0.00	0.00	14.40	0.00	11.12	17.40	20.12	2.64
	2	142.56	16.68	3.82	0.00	0.00	0.00	0.00	18.66	14.52	9.20	4.93	13.2
	3	160.36	32.45	0.00	0.00	0.00	2.37	8.32	18.45	13.25	15.47	8.57	8.34
	4	210.93	8.32	0.00	0.00	0.00	0.00	0.00	38.49	18.92	8.22	9.63	3.49
	5	158.20	16.24	0.00	0.00	0.00	0.00	40.46	32.92	18.56	10.05	10.72	6.83
	6	160.34	22.48	0.00	0.00	0.00	0.00	0.00	32.98	12.46	8.23	18.29	6.49
Indomethacine 50 mg/kg	1	215.20	4.42	4.62	3.10	0.00	0.00	3.62	0.00	10.31	11.40	34.80	22.22
	2	184.36	12.23	8.36	0.00	0.00	0.00	0.00	0.00	8.45	28.36	18.25	16.34
	3	169.42	52.1	0.00	0.00	0.00	8.25	0.00	9.71	28.51	12.31	14.3	5.82
	4	193.43	16.94	0.00	0.00	0.00	0.00	0.00	32.34	18.43	13.96	11.97	5.23
	5	198.23	18.24	0.00	0.00	0.00	0.00	0.00	38.28	26.24	8.64	3.28	6.38
	6	190.71	8.2	3.21	0.00	0.00	0.00	0.00	18.23	24.96	16.42	8.53	4.96
Acetysalicylic Acid 400 mg/kg	1	78.40	0.00	5.26	2.43	17.78	16.61	2.52	29.60	18.62	19.90	23.42	14.60
	2	58.36	12.68	0.00	0.00	0.00	0.00	0.00	18.56	52.46	33.84	12.92	14.35
	3	62.23	0.00	0.00	0.00	18.29	0.00	42.52	16.34	13.22	18.34	19.32	5.22
	4	52.36	0.00	0.00	0.00	0.00	0.00	0.00	42.36	48.34	14.94	14.1	5.22
	5	62.83	12.42	0.00	0.00	0.00	0.00	8.24	33.46	44.83	28.43	8.04	3.42
	6	62.98	2.92	0.00	0.00	0.00	0.00	48.23	44.96	18.23	12.96	3.46	8.22
Dexamethasone 30 mg/kg	1	193.02	0.00	12.42	0.00	0.00	21.60	24.80	22.96	11.24	13.30	8.40	12.22
	2	162.33	11.26	0.00	3.26	0.00	0.00	2.28	18.56	42.33	18.57	22.34	7.48
	3	160.34	12.96	0.00	0.00	0.00	13.21	32.36	23.24	16.91	12.2	14.31	9.22
	4	172.34	3.32	0.00	0.00	0.00	0.00	0.00	24.22	36.34	32.42	15.68	10.46
	5	148.20	12.92	3.42	0.00	0.00	0.00	0.00	28.32	48.96	28.49	14.22	8.30
	6	180.92	6.93	0.00	0.00	0.00	14.42	36.96	16.36	18.24	12.43	8.49	8.93