ASSESSMENT OF THE ANALGESIC PROPERTIES OF *MAERUA ANGOLENSIS* DC (CAPPARIDACEAE) IN ANIMAL MODELS OF PAIN

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DECLARATION

The experimental work described in this thesis was carried out at the Department of Pharmacology, Faculty of Pharmacy and Pharmaceutical Sciences, College of Health Sciences, KNUST in the period from 2012 to 2015. This work has not been submitted for any other degree.

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ABSTRACT

Maerua angolensis is a medicinal plant used traditionally to relieve pain. Although the leaves, roots and stem barks are used, their efficacy, and safety have not been proven scientifically. This study therefore assessed the antinociceptive properties of *Maerua angolensis*. The antinociceptive activity on hydroethanolic extracts of the leaf, root and stem bark (30 – 300 mg/kg, *p.o.*) in the writhing test showed significant (P<0.0002) reduction of pain induced by acetic acid with the stem bark being more potent. Subsequently, the stem bark was extracted with petroleum ether, ethyl acetate or hydroethanol to obtain three (3) extracts which at 3 - 30 mg/kg, *p.o.* significantly (P<0.0006) reduced pain in both neurogenic and inflammatory phases of the formalin test in rats with the petroleum ether extract being more potent in neurogenic while ethyl acetate was more potent in inflammatory phase. Phytochemical results of the three solvent extracts shows presence of saponins, steroids, tannins, terpenoids, alkaloids, glycosides, flavonoids, oils and fats. The two most potent extracts were combined and subsequently referred to as MAE, fractionated in column to two (2) fractions F1 and F32 and purified leading to isolation of four (4) compounds C1, C2, C3 and C5 identified and characterized by ^{1}H-NMR, GCMS and IR spectroscopy to be fatty acid and fatty acid esters namely octadecanoic acid methyl ester, bis (2ethylhexyl) phthalate, octadecanoic acid and oleic acid methyl ester, respectively. MAE and fractions (3 – 30 mg/kg, *p.o.*) produced significant (P<0.05) antinociceptive effects in writhing, formalin, prostaglandin E_2-induced mechanical hyperalgesia, bradykinin- and epinephrine-induced thermal hyperalgesia, tail-flick and paw withdrawal tests exhibiting both peripheral and central analgesic action. MAE and fractions reduced the number of jumps (intensity of withdrawal syndrome of morphine dependence) by mice but their effect was blocked by bicuculline and aminophylline. MAE and fractions suppress morphine withdrawal syndrome via stimulation of

GABAergic and adenosinergic transmission. MAE and fractions (3 and 10 mg/kg) did not compromise the motor coordination of mice in the rotarod test, suggesting lack of central depressant effect in their antinociceptive effect. MAE and fractions effects on mechanical hyperalgesia, tactile and cold allodynia measured with Von Frey filaments and cold water in a mouse model of vincristine-induced neuropathy showed inhibition of pain suggesting their analgesic effects in cancer patients with vincristine-induced neuropathy. Theophylline, L-NAME, atropine, glibenclamide and yohimbine reversed MAE and fractions antinociception in writhing test while naloxone and ondansetron additionally reversed it in the tail-flick test. MAE and fractions inhibited capsaicin- and glutamate-induced nociception implying involvement of $TRPV_1$ and glutamate receptors. Peripheral analgesic action of MAE and fractions involved ATP sensitive K^+ channels, adenosinergic, muscarinic, α_2 adrenergic and NO-cGMP paths while central action in addition involved 5-HT_3 and opioid receptors. The compounds (1 – 10 mg/kg, *p.o.*) in writhing and wiping tests in mice reduced pain suggesting their peripheral and central analgesic action. PCPA, ondansetron, capsazepine and naloxone reversed antinociception of compounds in wiping test indicating involvement of 5-HT_3, $TRPV_1$ and opioid receptors. The compounds (3 - 30 µg/ml) reduced locomotor activity of zebrafish larvae exposed to acetic acid. Acute and sub-acute toxicity tests of MAE in rats revealed LD_{50} above 3000 mg/kg orally with no significant changes in body weight, relative organ weights, haematological and serum biochemical indices but significant histological changes in livers at 1000 and 3000 mg/kg MAE, showing its relative safety at therapeutic dose. Collectively, this study provides scientific data for the use of *Maerua angolensis* in the treatment of pains and contribute to the analgesic knowledge of this species.

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ABBREVIATIONS

5-HT	5-Hydroxytryptamine
AIDS	Acquired immunodeficiency syndrome
ALP	Alkaline phosphatase
ALT	Alanine transaminase
AMPA	α-Amino-3-hydroxyl-5-methylsoxazole-4-propionic acid
ANOVA	Analysis of variance
AST	Aspartate transaminase
ATP	Adenosine triphosphate
AUC	Area under curve

B1	Bradykinin 1 receptor
B2	Bradykinin 2 receptor
C1	Compound 1
C2	Compound 2
C3	Compound 3
C5	Compound 5
cAMP	Cyclic-adenosine monophosphate
cGMP	Cyclic-guanosine monophosphate
CGRP	Calcitonin gene related peptide
COX	Cyclooxygenase
DNIC	Diffuse noxious inhibitory control
DRG	Dorsal root ganglion
ED_{50}	Effective dose-50
EDTA	Ethylene diamine tetra acetic acid
MAE	*Maerua angolensis* extract
F1	Fraction 1
F32	Fraction 32
GABA	Gama-amino butyric acid
GCMS	Gas chromatography mass spectrophotometer
HDL	High density lipoprotein
HNMR	Hydrogen nuclear magnetic resonance
i.p.	Intraperitoneal
ICR	Imprinting control region
IASP	International association for the study of pain
iGluRs	Ionotropic glutamate receptors

IL	Interleukin
IR	Infra-red
LC	Locus coeruleus
LDL	Low density lipoprotein
L-NAME	N^G-nitro-arginine methyl ester
LD_{50}	Lethal dose-50
MABEAE	Ethyl acetate stem bark extract of *Maerua angolensis*
MABHAE	Hydroalcoholic stem bark extract of *Maerua angolensis*
MABPEE	Petroleum ether stem bark extract of *Maerua angolensis*
MAEB	Aqueous ethanol stem bark extract of *Maerua angolensis*
MAEL	Aqueous ethanol leaf extract of *Maerua angolensis*
MAER	Aqueous ethanol root extract of *Maerua angolensis*
mGluRs	Metabotropic glutamate receptors
MPE	Maximum possible effect
NaCl	Sodium chloride
NGF	Nerve growth factor
NF-$_K$B	Nuclear factor-$_K$B
NMDA	N-methyl D-aspartate
NO	Nitric oxide
NOAEL	NO-observed-adverse-effect level
NRM	Nucleus raphe magnus
NRPG	Nucleus reticularis paragigantocellularis
PAG	Periaqueductal grey matter
PBS	Phosphate buffered saline
PCPA	Para-chloro phenylalanine methyl ester

PKA	Protein kinase A
PKC	Protein kinase C
p.o.	*Per os*
PG	Prostaglandin
PNS	Peripheral nervous system
PWT	Paw withdrawal threshold
RVM	Retro ventral medulla
SEM	Standard error of mean
SG	Substantia gelatinosa
SP	Substance P
TENS	Transcutaneous electrical nerve stimulation
TRPV	Transient receptor potential vanilloid
WDR	Wide dynamic range
WHO	World Health Organization

Chapter 1

INTRODUCTION

1.1 GENERAL INTRODUCTION

Pain is a disabling supplement of many medical conditions worldwide (Schim and Stang, 2004). It is associated with most pathological conditions in humans and affects thinking, sleeping, emotions, and performance of daily chores (Dib-Hajj *et al*., 2010; Wilhelm *et al*., 2009), thereby making the control of pain an important therapeutic priority. Pain is the major cause of all first visits to hospitals for consultations and the most common symptom of disease or injury (Porth, 2011). Medical attention is often sought by patients to relieve pain which may range from mild discomfort to agonized distress.

The management of pain is essential in most cases, and in some conditions like advanced cancer, the only viable therapeutic option is with analgesics; but potent and safe analgesics are limited (Schim and Stang, 2004). In many pathological conditions, particularly HIV/AIDS, diabetes, sickle cell disease and cancer, the management of pain remains a cause for concern.

1.1.1 Analgesics

Analgesics are drugs that are often used to prevent and relieve pain. The non– opioids, paracetamol and acetyl salicylic acid including other non–steroidal anti– inflammatory drugs (NSAIDs), are particularly suitable for mild to moderate pain in musculoskeletal conditions, whereas the opioids such as morphine are more suitable for moderate to severe pain, particularly of visceral origin. However, both opioids and NSAIDs have known toxic and lethal effects which limit their clinical use
(Pinheiro *et al*., 2012; Walder *et al*., 2001; Whelton, 2000).

Gastric irritation is a major side effect of NSAIDs, whereas the frequent use of opioids causes physical dependence and tolerance. Also, availability of NSAIDs have restricted approval particularly for children due to their tendency to cause bleeding, renal impairment, and worsens asthma, especially when given in large doses or inappropriately, while opioids have the risk of respiratory depression, emesis and constipation (Bozkurt, 2005). Paracetamol is similar in efficacy to acetyl salicylic acid, but has no demonstrable anti–inflammatory activity and cannot be used in inflammatory pains, though it is less irritant to the stomach but may cause hepatic damage principally in over dosage. The use of opioids and NSAIDs apart from being associated with these side effects, are not effective in neuropathic pain. This necessitates the need to search for potent and safe analgesics from medicinal plants which are known to have a long history of use in traditional medicine.

1.1.2 Medicinal plants

Medicinal plants, especially in developing countries, have been the subject of intense research due to their potential as sources of commercial drugs or as lead compounds in drug development (Zhang, 2004). It is on record that about 80% of people living in the world, notably those living in the developing world, depend on traditional medicine for their primary health care needs (Schippmann *et al*., 2006). This is due to the perceived low cost, easy access and the belief that these medicines are devoid of adverse effects as well as blending readily into the sociocultural life of the people (da Nóbrega Alves *et al*., 2008).

The World Health Organization (WHO) in an effort to promote cost effective complementary therapies has been encouraging research on natural product based drugs for the treatment of various ailments and their inclusion in the healthcare

programmes of developing countries (Somboro *et al*., 2011). It is therefore imperative that efforts be made in verifying the ethno medicinal use and safety of these medicinal plants, to develop cheaper, effective and safe drugs and even monitor their usage. Moreover, herbal medicines being in use since ancient times with good absorption are often more available and affordable and sometimes are perceived as more effective with less adverse effects than conventional drugs (Li *et al*., 2003).

Maerua angolensis DC (Family: Capparidaceae) is a medicinal plant used traditionally in the treatment of various painful conditions in Nigeria and some West African countries (Burkill, 1985; Mothana *et al*., 2009). Various parts of the plant notably the leaves, roots and stem barks are claimed to relieve pain and also used to manage psychosis, epilepsy, diabetes, diarrhoea, hepatitis, vomiting, nasal infection, insomnia, stomach ulcer, miscarriage, boils and arthritis (Adamu *et al*., 2007; Magaji *et al*., 2008; Magaji *et al*., 2009; Mohammed *et al*., 2008). Apparently, lack of scientific proof of analgesic efficacy and safety of this plant claimed by the traditional healers called for this study.

1.2 MAERUA ANGOLENSIS

Botanical name: *Maerua angolensis* DC

Family: Capparidaceae

Common/local names

Bead–bean (English); Konini-bere, Osono nantini (Twi); Pugodugo (Nabdam); Pudingo (Frafra); Chichiwaa, Gazare, Zumuwaa, Kiyafa (Hausa); Baguhi (Fulfulde); Ukon ugwak (Izere); Gyel–gyel baro, Leggael baali, that is tree of sheep (Fulani); Shegara el zeraf, that is giraffe tree (Arabic) (Burkill, 1985; Mothana *et al.*, 2009).

1.2.1 Plant description

Maerua angolensis is a tree whose size varies from medium to big and grows up to 10 – 20 metres high (Figure 1.1). The leaves are elliptic to lanceolate, up to 7 cm long, shiny green above, paler below with the mid–rib prominent and whitish, thinly textured, hairless; apex round or notched with a hair-like tip; petiole almost as long as the leaf, yellowish with a swelling and a bend below the blade. The stem is white consisting of young branches with conspicuous pale lenticels and straggling branches drooping at the ends and carrying the abundant, conspicuous white flowers. The flowers are axillary, solitary, in terminal spikes or in clusters on small lateral spurs, without petals, stamens numerous and long, white fading to yellow. Flowering time is around December. Fruit is up to 15 cm long, pod–like, often restricted between the seeds. The wood is hard and heavy, yellowish and fine grained (Burkill, 1985).

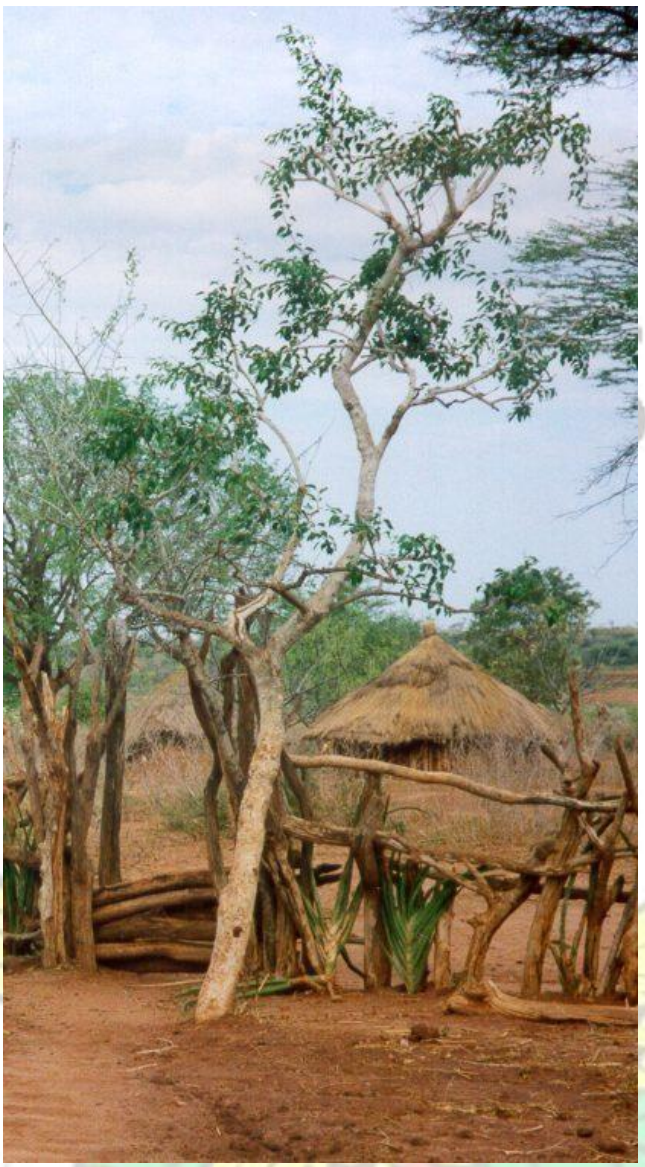


Figure 1.1 *Maerua angolensis* tree in its natural habitat (Adapted from Burkill, 1985).

1.2.2 Geographical distribution

It is commonly found growing in bushy and rocky areas but planted on graves in Nupe area of Nigeria. It is widespread in the savannah area of tropical Africa to South Africa and Swaziland. It is a native of tropical Africa found in hot and dry open woodland (Burkill, 1985; Mothana *et al*., 2009).

1.2.3 Traditional uses

The roots, leaves, stem barks, flowers, fruits and seeds of *Maerua angolensis* are used traditionally in Africa for their medicinal and/or non–medicinal uses.

1.2.3.1 Medicinal uses

The plant has the following applications in traditional medicine:

- Used in the treatment of jaundice, hepatitis and liver disease
- Used as an anticonvulsant
- The decoction of the stem bark or leaf is used locally for the management of psychosis and epilepsy
- The leaf sap is dropped into fresh wounds as an antiseptic dressing
- A decoction of the leaf or stem bark is used in the treatment of peptic ulcer disease
- The stem bark and root decoction are used as an aphrodisiac
- The leaves, roots and stem barks are used to relieve headache, toothache, arthritis, gout, oedema, swellings and rheumatism. A decoction of the leaf is laid on a painful area to relief pain
- The raw fruit is used as a laxative
- Stem bark decoction is used in the management of diabetes mellitus
- A decoction of the root and leaf is used as an antibiotic
- A decoction of the leaf is used to prevent abortion (Adamu *et al*., 2007; Burkill, 1985; Hedberg *et al*., 1982; Musa *et al*., 2011).

1.2.3.2 Non-medicinal uses

- The fruit is mixed with galena and rubbed on the eye lids by youths in Northern Nigeria as a love charm to render themselves irresistible to girls
- In Ghana the tree is said to afford good fodder for sheep and goats
- Powdered leaves are used as fish poison

- The leaves of *Maerua angolensis* is used as a source of vegetable especially by rural populace
- The leaves are used for sauces, condiments, spices and flavourings in soups
- The fruits and seeds are eaten in some areas
- The plant is used as an ornamental for garden planting
- Trees are used for smoking of milk so as to preserve milk in Central Tanzania. The wood is burnt and produces smoke that is forced into gourds used to store the milk. This smoke is believed to increase the shelf–life of milk and studies have shown that traditional smoking of milk inhibits growth and activity of mesophyllic and thermophilic lactic acid bacteria.
- Wood products are used as building materials and as fuel and lighting (Ansah and Nagbila, 2011; Burkill, 1985; Emmanuel *et al*., 2011; Komwihangilo *et al*., 2007).

1.2.4 Previous work on the stem barks of *Maerua angolensis*

- Mohammed *et al*. (2008) reported the effect of aqueous methanolic stem bark of *Maerua angolensis* extract on blood glucose levels of streptozocin– induced diabetic wistar rats where the aqueous methanolic stem bark extract of the plant was concluded to possess anti–diabetic effect in streptozocininduced diabetic rats
- A crude extract of the stem bark was also reported to dose-dependently inhibit carrageenan–induced paw oedema in rats (Adamu *et al.*, 2007)
- Preliminary gastrointestinal studies on aqueous-methanolic stem bark extract of the plant by Magaji *et al*. (2008) showed the anti-diarrhoeal activity of the aqueous methanolic stem bark of the plant against castor oil–induced diarrhoeal model in mice

- The effects of the hydroalcoholic stem bark extract of the plant in mice and chicks during some neuropharmacological studies suggests that the plant has central nervous system depressant properties (Magaji *et al*., 2009)
- Preliminary phytochemical screening of the methanolic stem bark extract has shown the presence of saponins, steroids, tannins, flavonoids, alkaloids, glycosides, terpenoids, carbohydrates and proteins (Adamu *et al*., 2007; Magaji *et al.*, 2009; Mohammed *et al.*, 2008)
- Information on the median lethal dose (LD_{50}) of the stem bark extract of the plant in mice revealed LD_{50} of 3,807.9 mg/kg orally and greater than 500 mg/kg intraperitoneally (Magaji *et al*., 2008; Mohammed *et al.*, 2008).

1.3 PAIN

The International Association for the Study of Pain (IASP) defines pain as an unpleasant sensory and emotional experience associated with actual or potential tissue damage or described in terms of such damage (Le Bars *et al.*, 2001). Pain is a perception often associated with a disease or physical trauma and serves as a natural warning and protective mechanism (Schim and Stang, 2004). It triggers reactions and induces learned avoidance behaviours which may limit the potentially damaging consequences. It is often useful in making proper diagnosis. Pain is an output of a nociceptive system generated when chemical, mechanical, thermal or electrical stimuli exceed a certain threshold value known as pain threshold which triggers the release of pain mediators.

The nociceptive system found in highly evolved animals is a system responsible for nociception and also forms a component of the overall set of controls accountable for homeostasis. Nociception is defined as the neural processes of encoding and

processing noxious stimuli (Loeser and Treede, 2008). It is a process where stimuli having potential to damage tissue produces afferent activity in the peripheral and central nervous system. Nociception triggers a variety of autonomic responses and can also result in a subjective experience of pain in highly evolved animals. The nociceptors when stimulated transmit signals along the spinal cord to the brain and also detect thermal, mechanical, chemical or electrical changes above a set threshold thereby initiating the afferent activity. Severe pain, however, can occur independently of any obvious predisposing cause as in trigeminal neuralgia, or persist long after the precipitating injury has healed as in phantom limb pain (Decosterd and Woolf, 2000; Flor *et al*., 2006; Flor, 2008; Gronseth *et al*., 2008).

Pain threshold is a point at which pain begins to be felt and is an entirely subjective phenomenon. The intensity at which a stimulus begins to evoke pain for a given noxious stimulus varies from individual to individual and for a given individual over time (Yuan *et al*., 2008), thus, it is necessary to distinguish between the individual‘s pain tolerance and pain threshold. Pain tolerance is the time that a continuous pain stimulus is tolerated or the amount of pain that a person can withstand before breaking down emotionally and/or physically. Patients usually seek for medical advice when they are beyond pain tolerance level. Pain threshold is fairly constant but pain tolerance varies enormously (DeWall and Baumeister, 2006).

1.3.1 Classification of Pain

Pain is classified according to its duration, pathogenesis, intensity, quality, time course, and location. Classification of pain is thus complicated and can be a source of confusion for many clinicians though useful as the initial strategy for treating pain.

Despite these classifications, pain syndromes commonly occur with different mixture of pain types (O'Connor *et al*., 2008).

1.3.1.1 Acute pain

Acute pain, often referred to as adaptive pain, is pain of recent onset limited to less than three to six months duration. It is a protective mechanism that alerts a person to a problem and prompts the person to take action (Bjordal *et al*., 2006; Ito *et al*., 2001). It is usually transient in nature and often related to inflammation which could be as a result of infection or injury. Acute pain being proportional to the stimulus intensity can be easily localized and declines rapidly after the stimulus is removed. In the absence of acute pain, it is doubtful whether human survival would be possible at all (Schim and Stang, 2004).

1.3.1.2 Chronic Pain

Chronic pain, often referred to as maladaptive pain, is pain that passed the usual course of the condition that originally caused the pain; lasting for at least six months or longer beyond the time of healing of an injury and has little protective significance (Sauer *et al*., 2010). It can be intermittent repeated pain, as with angina pectoris and migraine headache, or consistent as with cancer and back pain. Chronic pain, is accompanied by release of neurotransmitters by the body, which increases the body's perception of pain caused by sensitization of primary afferent and spinal cord neurons resulting in an increased sensitivity to both noxious and non-noxious stimuli. People who suffer from chronic pain have bio psychosocial effects and the intensity of the pain can cause them to become inactive leading to more physiological problems like osteoporosis, cardiovascular disease and obesity. The sleeping patterns of patients can also be

affected by chronic pain. Chronic pain can lead to emotional disturbances (fear and anger), and psychiatric disorders (anxiety and depression) (Wilhelm *et al*., 2009).

1.3.1.3 Nociceptive pain

This is a transient but constant acute localized pain in reaction to a stimulus having potential to damage tissue and occurs when the body‘s nervous system is working properly. Nociceptive pain arises from stimulation of nociceptors from somatic and visceral structures. The noxious stimulus activates nociceptors, on Aδ- and C-fibres, which transmit signals along the spinal cord to the brain triggering a variety of autonomic responses and subjective experience of pain (Loeser and Treede, 2008). Withdrawal reflexes are usually elicited thus nociceptive pain protects tissue from further damage.

1.3.1.4 Neuropathic pain

This is a persistent spontaneous burning sensation and hypersensitivity to pain as a result of the body‘s nervous system not working properly due to disease, injury, dysfunction or actual damaging stimuli of a nerve or group of nerves in the peripheral or central nervous system (Woolf, 2004). It involves sensitization of the nervous system. Peripheral sensitization is associated with increase in the stimulation of peripheral nociceptors that amplifies pain signals to the central nervous system, whereas, central sensitization is associated with hyper-stimulation of neurons that originate in the dorsal horn of the spinal cord thereby increasing pain signals to the brain and subsequently increase in pain sensation.

Neuropathic pain is commonly characterized by allodynia, hyperalgesia and dysethesia (spontaneous pain episode). It is severe, chronic and less responsive to classical

analgesics such as opioids, however it responds well to anticonvulsants and antidepressants (Benbouzid *et al.*, 2008a; Woller *et al*., 2012). The sensory abnormalities such as exaggerated responses to noxious stimuli in neuropathic pain resembles the secondary hyperalgesia which accompanies tissue damage in stroke but differs from primary hyperalgesia of nociceptive pain (Benbouzid *et al.*, 2008a; Woller *et al*., 2012). Neuropathic pain can also change the patient's quality of life by prying with emotional well–being (Benbouzid *et al*., 2008b).

1.3.1.5 Inflammatory Pain

This is a spontaneous pain and hypersensitivity to pain in response to tissue damage and inflammation subsequent to sensitization of peripheral nerve terminals (Woolf, 2004). Inflammatory pain is adaptive in that it elicits physiologic responses which protect the tissue from further damage by preventing its contact or movement until healed.

1.3.1.6 Functional pain

This is a spontaneous pain and hypersensitivity to pain in response to abnormal central processing of normal input in the brain. It is due to abnormal functioning of the CNS resulting into abnormal response but there is normal peripheral tissue and nerves.

1.3.2 Neural Mechanisms of Pain

The neurobiological mechanisms (peripheral and central) accountable for different pains give insight into how different types of pain are generated by varied etiologic factors and in which patients (Apkarian *et al*., 2005). By these mechanisms, therapeutic approach can be targeted specifically at the particular mechanisms of the type of pain an individual patient is experiencing. The nociceptive system comprising of neurons involved in nociception (sole mechanism that causes nociceptive pain) extends from

the periphery through the spinal cord, brain stem and thalamus to the cerebral cortex, which perceives the pain sensation. Multiple mechanisms capable of producing pain include nociception, peripheral sensitization, central sensitization, ectopic excitability and decreased inhibition (Apkarian *et al.*, 2005; Basbaum *et al*., 2009; Jackson *et al*., 2006; LaCroix-Fralish and Mogil, 2009). Nociception is comprised of transduction, conduction, transmission and perception processes. The descending pathways modulate nociceptive messages by exerting both inhibitory and facilitatory actions in the dorsal horn.

1.3.2.1 Peripheral mechanisms

The conversion of a noxious thermal, mechanical or chemical stimulus into electrical activity in the peripheral terminals of nociception sensory fibres constitute transduction which is mediated by specific receptor ion channels expressed only by nociceptors (Basbaum *et al*., 2009), whilst conduction constitute the passage of action potentials from the peripheral terminal along axons to the central terminal of nociceptors in the CNS. The first stage in the transmission of nociceptive pain, which is, synaptic transfer and modulation of input from neuron to neuron, therefore involves activation of specialized sensory receptors, the primary afferent nociceptors. The primary afferent nociceptors include mechanoreceptors, thermo-receptors and chemoreceptors which respond to mechanical, thermal and chemical stimuli respectively. There are two distinct types of nociceptors and peripheral nerve fibres that sub serve two distinct sensory experiences. These are the thinly myelinated Aδ–fibre and the unmyelinated C–fibre axons (Reeves *et al*., 2005). Aδ–fibres nociceptors, with a multipunctate receptive field, transduce phasic pain (fast or first pain) with pricking, stabbing or sharp quality which cause organisms to withdraw, whilst C–fibre nociceptors, which are polymodal, usually in a single receptive area, convey messages

generated by tissue damage (tonic pain, that is, slow or second pain) with burning, itching or aching quality which cause organisms to stop. The C– fibre nociceptors is morphine sensitive, but the Aδ–nociceptors is not (Le Bars *et al.*, 2001).

The specificity and threshold of the nociceptor transducers is thus the first and most important sieve in the activation of nociception and defines the diverse types of primary sensory neurons: unimodal, which react only to one type of stimulus or polymodal, which react to numerous types of stimuli. The skin, joint and visceral nerves, apart from polymodal nociceptors, contain Aδ- and C-fibres silent nociceptors/mechano–insensitive nociceptors that are activated only when sensitized to mechanical and thermal stimuli during inflammation (Basbaum *et al*., 2009).

The Aδ nociceptors are connected to the spinal cord dorsal horns via meium diameter myelinated Aδ nerve fibres which are found mainly in and just beneath the skin. They are activated by noxious stimuli such as pressure, surgery and are known as high threshold mechanoreceptors. Some also respond to heat and are known as mechano-thermal nociceptors. In muscle, there are also certain numbers of Aδ (group II and III) nerve fibres.

Nerve fibres are generally classified by size and whether they originate from skin or muscles – large diameter myelinated nerves Aβ (skin) or type I (muscle) carry touch and proprioception sensation respectively. Small diameter myelinated Aδ (skin) or types II and III (muscle) carry pain sensations, the smallest unmyelinated C (skin) and type IV (muscle) also carry pain sensations. Types II, III, IV and C also carry non painful messages (Basbaum *et al*., 2009).

The non-myelinated afferent neurons contain several neuropeptides, in particular substance P (SP), calcitonin gene related peptide (CGRP) and serotonin which are

released as mediators at both the central and the peripheral terminals, and play an important role in the pathology of pain (Rang *et al*., 2007). The action potentials initiated by a noxious stimulus applied to the peripheral nerve terminal of a nociceptor are conducted from the periphery to the CNS along the sensory neuron axon, running through peripheral nerves to the dorsal root ganglion (DRG) and into the spinal cord and further to the brain stem or cerebral cortex producing the conscious pain response. Nerve or tissue injury causes the production and release of chemical mediators (bradykinin, serotonin and prostaglandin) locally or from cells infiltrating site of injury. A mixture of these mediators add to changes in vascular permeability which result into oedema and erythema, in addition to sensitization of the peripheral nociceptors mainly C–fibres thereby initiating a cascade of events that alter ionic conductance of the peripheral nociceptor terminal.

Peripheral nociceptor sensitization as a result of nerve/tissue damage or inflammatory insults is associated with aberrations of the normal physiological pathway, giving rise to peripheral hyperalgesia (increased amount of pain due to increased sensitivity of nociceptors to both thermal and mechanical stimuli) and allodynia (pain evoked by a non-noxious stimulus due to reduction in excitation threshold of polymodal nociceptors and recruitment of silent nociceptors that add significantly to the inflammatory nociceptive input to the spinal cord). Hyperalgesia involves both sensitization of peripheral nociceptive nerve terminals and central facilitation of transmission at the level of the dorsal horn and thalamus (neuroplasticity). The peripheral hyperalgesia is due to the action of mediators such as serotonin and prostaglandin E_2 released from axon terminals, damaged skin, inflammatory cells and the microvasculature surrounding the injury site acting on the nerve terminals.

The development of neuropathic pain as result of nerve injury also sensitizes nociceptors. A damaged nerve alters gene expression within the damaged fibres which alters the neurochemistry of the damaged axons shifting the phenotype of the damaged pathways from one of transducing and transmitting sensory information to one of accomplishing regeneration and survival. The nerve begins to fire spontaneously and with increased evoked activity due to increase sensitivity to mechanical stimulation and adrenaline (Gong *et al*., 2010; Miranda *et al*., 2007).

1.3.2.2 Central mechanisms

The central mechanisms of pain transmission depend on the balance between inhibitory and facilitatory influences, integration of which occurs at the spinal cord, brain stem and multiple cortical regions. The periaqueductal grey matter (PAG), the nucleus raphe magnus (NRM) and the nociception inhibitory neurons within the dorsal horns of the spinal cord mediate central analgesia system by inhibiting nociception-transmitting neurons also located in the dorsal horns of the spinal cord. Transmission of pain via the spinal cord involves the lateral spinothalamic tract which has two pathways for nociceptive information to reach the brain. The first is the neospinothalamic tract for fast spontaneous pain which travels through Aδ-fibres to terminate on the dorsal horn of the spinal cord and synapse with the dendrites of the neospinothalamic tract. Axons of these neurons travel up the spine to the brain and end on the ventro basal complex of the thalamus synapsing with the dendrites of the somatosensory cortex. The second pathway is the paleospinothalamic tract which is involved in slow increasing pain transmission via slow C-fibres to laminae II and III of the dorsal horns (substantia gelatinosa). Impulses are transmitted from substantia gelatinosa to nerve fibres that end in lamina V, also in the dorsal horn, synapsing with neurons that join fibres from the fast pathway, crossing to the opposite site and

travelling upwards through the anterolateral pathway (Eippert *et al*., 2009; Kivell and Prisinzano, 2010).

The gatekeeper function is by neurons in the substantia gelatinosa of the dorsal horn which regulate transmission at the spinal synapse (first synapse of the nociceptive pathway, between the primary afferent fibres and the spinothalamic tract transmission neurons). The substantia gelatinosa is rich in both opioid peptides and opioid receptors thus may be an important site of action for morphine-like drugs (Bingel and Tracey, 2008). Numerous other transmitters and receptors mediate the processing of noxious information within the spinal cord. Similar ‗gate' mechanisms also operate in the thalamus.

In the medial thalamus, many cells respond specifically to noxious stimuli in the periphery, and lesions in this area cause analgesia. There are two types of neurons – nociceptive specific and wide dynamic range neurons. The nociceptive specific (high threshold) neurons are located more superficially in the dorsal horn and respond only to noxious stimuli (Aδ- and C-fibre stimulation), whist wide dynamic range (convergent) neurons are more deeply located and respond to all types of stimuli (Aβ-, Aδ- and C-fibre stimulation) (Davis and Moayedi, 2013).

Melzack and Wall (1967) developed a theory on pain mechanisms, which postulated that in each dorsal horn of the spinal cord, there is gate-like mechanisms which inhibits or facilitates the flow of afferent impulses into the spinal cord before it evoke pain perception and response. The theory states that the opening or closing of the ‗gate' is dependent on the relative activity in the large diameter (Aβ) and small diameter fibres (Aδ and C), with activity in the large diameter fibres tending to close the ‗gate' and activity in the small diameter fibres tending to open it. Many physiological studies

have helped in providing evidence that pain can be modulated depending on the balance of activity between nociceptive input and other inputs but the gate-control theory of pain surpassed all as shown in the schematic diagram of the gate control system (Figure 1.2).

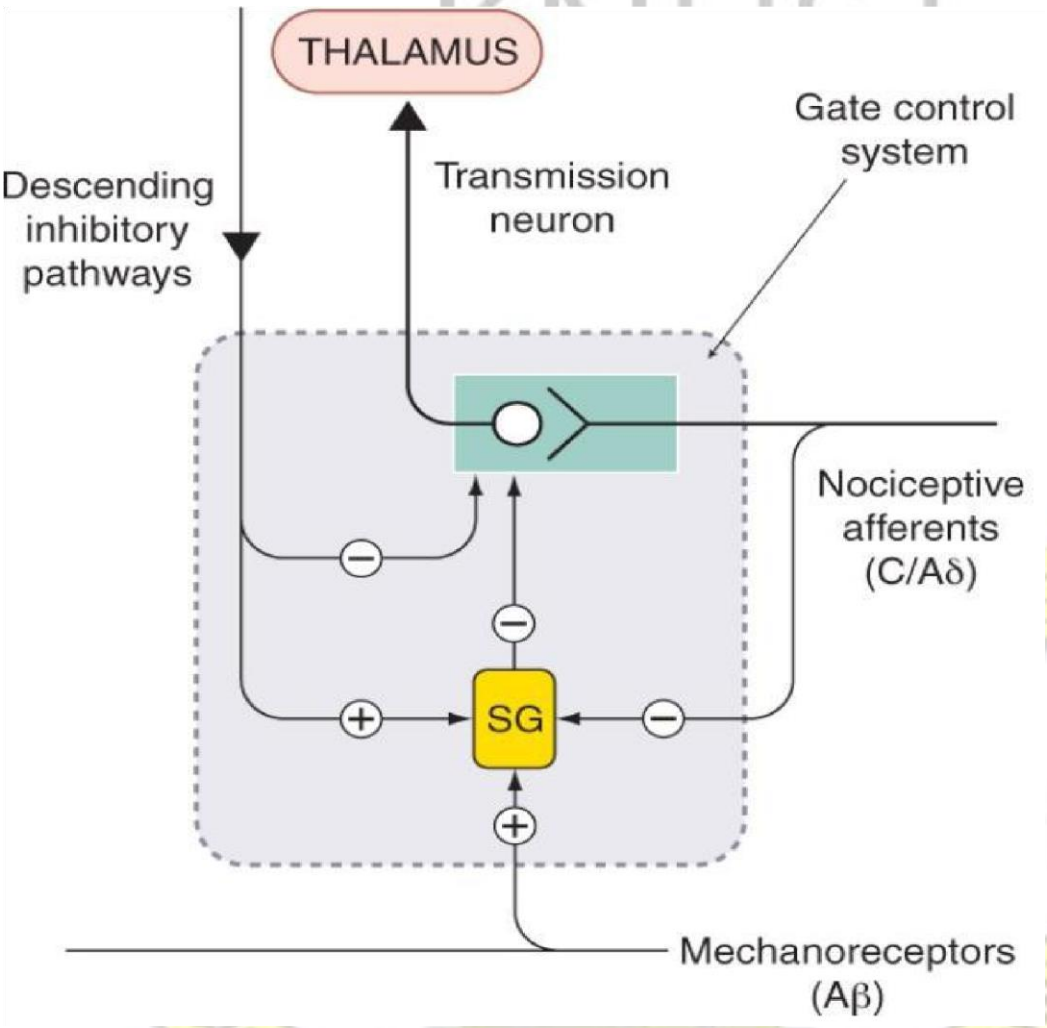


Figure 1.2 Schematic diagram of the gate control system

This system regulates the passage of impulses from the peripheral afferent fibres to the thalamus through transmission neurons originating in the dorsal horn. Neurons in the substantia gelatinosa (SG) of the dorsal horn act to inhibit the transmission pathway. Inhibitory interneurons are activated by descending inhibitory neurons or by non-nociceptive afferent input. They are inhibited by nociceptive C-fibre input, so the persistent C-fibre activity facilitates excitation of the transmission cells by nociceptive or non-nociceptive inputs. This auto-facilitation causes successive bursts of activity in the nociceptive afferents to become increasingly effective in activating transmission neurons (Adapted from Rang *et al*., 2007).

It has also been shown that the dorsal horn neurons which can potentially transmit noxious information to supraspinal levels can have their cells activities decreased during transcutaneous electrical nerve stimulation (TENS) of somatic receptive fields (Garrison and Foreman, 1994; Sluka *et al*., 2005). These authors also showed that there is a differential effect in that more cells respond to conventional high frequency low intensity TENS variable than to low frequency high intensity TENS variable.

This is consistent with the concept of the gate control theory of pain in that less noxious information will be involved in the pain perception process.

The essential site of control in the gate control theory of pain is the substantia gelatinosa (SG), which caps the grey matter of the spinal horn in the spinal cord. The control mechanism is referred to as a _gate‘ which is operated by both external and internal influences. Pain impulses can only pass through when the gate is open but not when it is closed (Dennis and Melzack, 1983; Djaldetti *et al*., 2004). Thus, if nociceptive input exceeds Aβ fibre input, then the gate is opened and the impulse ascend the spinal cord to the brain, but if Aβ fibre input exceeds nociceptive input then the gate is closed and the pain impulse is stopped or diminished to the action of the inhibitory neurotransmitter and therefore does not pass up the spinal cord.

The position of the gate is in addition influenced by the brain‘s descending inhibitory pathways (Rang *et al*., 2007). Therefore, entry into the CNS can be visualized as a gate, which is opened by pain (generated impulses) and closed by low intensity stimuli such as rubbing or mild electric stimulation. Furthermore, it can also be closed by endogenous opioid mechanism which can be activated from the brain or peripherally by acupuncture or by gentle rubbing massage, electrical stimulation and hot or cold

therapies as they activate the large diameter fibres which close the gate thus decreasing pain perception (Yuan *et al*., 2008).

Many transmitters and receptors mediate the processing of noxious information within the spinal cord. Transmitter actions could either be fast or slow kinetics. Glutamate and adenosine triphosphate (ATP) action at ionotropic receptors are fast but neuropeptides that act through G-protein coupled metabotropic receptors are slow. Fast kinetics evoke immediate and short effects on neurons (encoding input to the neuron), but slow kinetics modulate synaptic processing. Glutamate, a principal transmitter of primary afferent and dorsal horn neurons, activates ionotropic αamino-3-hydroxyl-5-methyl-4-isoxazole propionic acid (AMPA), Kainate and Nmethyl-D-aspartate (NMDA) receptors, while neuropeptides like SP activates neurokinin receptors (Bingel *et al*., 2007; Bolay and Moskowitz, 2002; Schaible, 2007). In chronic pain states, impairment of the descending pain inhibitory system gives rise to an enhanced descending pain facilitatory system mainly mediated through the glutamatergic pain system. Descending facilitatory mechanisms exert excitatory actions both on the terminals of nociponsive peripheral afferent neurons and on intrinsic dorsal horn neurons.

The ascending nociceptive transfer systems to the supraspinal targets through which the global sensation of pain is finally modulated and experienced distinguishes two components of pain – sensory discriminative and affective-cognitive/affectivemotivational. The perception and detection of noxious stimuli is by the sensory discriminative component, whereas the relationship between pain and mood, attention to and memory of pain, capacity to cope with and tolerate pain and its rationalization is by the affective-cognitive/affective motivational component (Brooks and Tracey, 2005).

The thalamo-cortical system produces the conscious pain response, while the lateral thalamo-cortical system discriminate innocuous and noxious stimuli. Oshiro *et al*. (2007), however, suggested that brain mechanisms supporting discrimination of sensory features of pain also involve medial thalamo-cortical system and frontal regions traditionally associated with affective processing. The medial thalamocortical system whose structures are part of the limbic system produces the affective motivational features (Baumgärtner *et al*., 2006). The basal ganglia, hypothalamus, amygdala and cerebellum which form the subcortical structures are postulated to function in nociception transmission and pain perception (Borsook *et al*., 2010).

Impulses from brain stem nuclei also descend onto the spinal cord influencing pain signals transmission at the dorsal horn and thus constitute one of the gating mechanisms that regulate impulse transmission in the dorsal horn. Opioids apart from acting directly on the dorsal horn as well as on the peripheral terminals of nociceptive afferent neurons also excite neurons in the PAG and in the nucleus reticularis paragigantocellularis (NRPG). The PAG of the mid brain is a key part of the descending system that receives inputs from various other brain regions, including the hypothalamus, cortex and thalamus, forming the main pathway through which cortical and other inputs act to regulate the nociceptive gate in the dorsal horn.

The PAG and NRPG project to the rostroventral medulla (RVM) which includes the NRM. From the NRM, 5-hydroxytryptamine (5-HT) and encephalin containing neurons run to the SG of the dorsal horn and exert an inhibitory influence on transmission (Bingel and Tracey, 2008; Rang *et al*., 2007). There is also a diffuse noxious inhibitory control (DNIC) from a descending inhibition of wide dynamic range (WDR) neurons due to strong noxious stimulation. Impulses from nociceptive neurons with input from the point of stimulation are propelled to the RVM triggering

DNIC of nociceptive WDR neurons in the neuraxis (Bingel and Tracey, 2008). The descending inhibitory pathways could therefore be an important site of action for opioid analgesics, since opioid antagonists like naloxone prevents electrical induced analgesia in both PAG and SG of the dorsal horn known to be rich in encephalincontaining neurons. A noradrenergic pathway from the locus coeruleus (LC) has a similar inhibitory effect on transmission on the dorsal horn, and it has been suggested that tricyclic antidepressants control pain through this pathway (Jann and Slade, 2007; Rang *et al*., 2007).

Segmental and descending inhibitory controls can suppress transmission in the somatosensory system within the dorsal horn. The inhibition normally occur presynaptically on the primary afferent terminally or post-synaptically on the dorsal horn neuron. Serotoninergic, noradrenergic and dopaminergic pathways form the key machinery of these descending mechanisms. The descending pathways in general inhibit nociception by modulating the release of neurotransmitters from nociceptive terminals. The inhibitory neurotransmitters include adenosine, glycine, serotonin, γamino butyric acid (GABA), cannabinoids and opioid peptides, of which the opioid peptides plays an important role in regulating pain transmission.

Three distinct family of opioid peptides have been identified – encephalins, endorphins and dynorphins. There are four distinctive types of opioid receptors – mu (μ), delta (δ), kappa (k) and sigma (ORL-1) which are concentrated in the superficial dorsal horn. The actions of all clinically used opioids can now be explained in terms of their acting as agonist at one of the four opiate receptors (inhibitory) found in the brain, spinal cord and peripheral nervous system. The actions of opioids important for analgesia and their side effects involve reducing transmitter release from nerve terminals so that neurons are less excited by excitatory transmitters and direct

inhibition of neuronal firing so that the nociceptive information flow from the neuron is reduced and also inhibitions of inhibitory neurons leading to disinhibition (D'Mello and Dickenson, 2008). This dual action of opioids can result in a total block of sensory inputs as they arrive in the spinal cord. Also, further activation of descending pathways directly and indirectly inhibits nociponsive peripheral nerves which are mediated through the inhibition of excitatory interneurons and the excitations of inhibitory interneurons (Vanegas *et al*., 2010).

Central sensitization or spinal hyper-excitability as a result of inflammation and nerve damage where neurons in the superficial, deep and ventral cord demonstrate increased response to input following noxious stimuli is a simple form of learning and synaptic plasticity. Pleger *et al*. (2006) reported the typical changes in spinal cord neurons to include progressive increase in neuronal activity throughout the duration of stimulus (wind up), magnification and persistence of the duration of neuron (facilitation), expansion of receptive field, reduction of action potential threshold, induction of oncogene and long term potentiation or strengthening of synaptic transmission efficacy after activity across the synapse.

The facilitation of synaptic transmission is an important component of pathological hyperalgesia such as that associated with inflammatory responses (Brooks and Tracey, 2005). The mediators responsible for central facilitation include SP, nerve growth factor (NGF) and CGRP which increase the electrical excitability, chemosensitivity and peptide content of nociceptive afferent neurons, and also promotes the formation of synaptic contact (Gao and Ji, 2009; Ji *et al*., 2003). Excitation of nociceptive sensory neurons depends on voltage-gated sodium channels, and certain sodium channel subtypes are found in these neurons but not elsewhere. Joshi and Ogunnaike (2005) showed that improved expression of these channels underlies the sensitization to

external stimuli occurring in inflammatory pain and hyperalgesia. This explains why many antiepileptic and antidysrhymic drugs which act by blocking sodium channels also find clinical application as analgesics.

1.3.3 Pain and depression

Some studies have shown that pain and depression are associated through common neurochemical mechanisms (Fishbain *et al.*, 2000; Leo, 2005; Rahman *et al*., 2006). The common physical symptom in depressed patients is pain. Chronic pain can lead to anxiety and depression (Gatchel *et al*., 2007), thus making depression common in patients with chronic pain. Antidepressants at lower dose than that used to treat depression are therefore often used to manage pain in both depressed and nondepressed patients where the onset of analgesic activity is more rapid than antidepressant activity. The mechanism of antidepressants analgesia is by reinforcement of descending inhibitory controls from the PAG and NRM by inhibiting CNS monoamine reuptake pre-synaptically (Kajdasz *et al*., 2007; Matsuzawa-Yanagida *et al*., 2008).

1.3.4 Pain and the immune system

Resident immunocompetent cells such as fibroblasts, macrophages, mast cells and a host of others residing within unhealthy nerves respond to infection, inflammation and/or trauma through production and release of pro-inflammatory mediators but in healthy nerves, the cells do not release such inflammatory mediators, rather, they provide active surveillance of the nerves (Moalem-Taylor *et al*., 2007). Activation of the immune cells leads to the release of chemokines which recruit neutrophils and macrophages from the damage myelin and disrupting the blood-nerve barrier. Degradative enzymes and acids are also released in reaction to nerve injury which exposes peripheral nerve proteins. These peripheral nerve proteins are normally hidden within the myelin sheath and not detected by immune cells. The immune derived

enzymes and acids attack myelin and interrupt further the blood-nerve barrier which under normal circumstances, the blood-borne immune cells have limited access to with the exception of circulating activated T-lymphocytes (Gao and Ji, 2010). Activation of immune cells due to partial nerve damage contributes to the exaggerated pain and axonal hyper excitability and wallerian degeneration (MoalemTaylor *et al*., 2007). The development of wallerian degeneration and neuropathic pain can therefore be delayed by delaying macrophage recruitment to the site of nerve damage.

1.3.5 Models of nociception in animals

Animals are used to study pain and to assess analgesic activity by observing either their behavioural or non-behavioural responses, however, this practice seems to be associated with ethical, technical and philosophical problems despite the fact that scientific as well as moral reasons abound for such practice. Responses such as flexion reflexes and vocalization are commonly monitored in conscious animals as the threshold for obtaining such responses to stimuli would also produce pain if applied to humans. The stimulus is stopped once the response has been obtained. At times algogenic substances are applied briefly. The characteristics of the input (stimulus applied which could be thermal, mechanical, chemical or electrical) and the output (reaction of the animal or response) must be specified when describing nociceptive tests. The behavioural parameters that are measured should also be described and this might involve defining the responses as a function of their increasing complexity. The inputs and outputs of these systems are very ultimately linked by the characteristics (temporal nature) of the stimulus which must be quantifiable, reproducible and non-invasive (Le Bars *et al*., 2001).

The behavioural models of nociception are more popular in assessing analgesic activity and have been carefully characterized for their validity and reproducibility

(Mogil, 2009) because the most reliable signs of pain are physical manifestations. Though pains in animals cannot be monitored directly, however, their responses to nociceptive stimuli can be predicted through observation despite the fact that such responses do not necessarily mean that there is a connected sensation. The behavioural models, therefore, have some limitations since animals cannot communicate verbally so measurement of pain is frequently an estimation. There is a high level of subjectivity between the responsiveness of different animals and quantification of these behaviours by the observer during measurement leading to a high level of experimental bias. Also, none of the different pain models using different types of nociceptive stimuli such as thermal, mechanical, chemical or electrical is ideal even though chemical stimuli possibly most strongly imitate acute clinical pain (Le Bars *et al*., 2001; Mogil, 2009) and the reactions being monitored are more or less at all times motor responses (spinal reflexes to multifaceted behaviours). Other physiological functions may also alter or be associated with the monitored reactions in most of the pain models, for example, if an animal is already in a state of stress and neurovegetative reactions are exacerbated, observations will not be suitable scientifically from a physiological point of view.

Other limitations of animal models of nociception include:

- Monitoring responses around a nociceptive threshold whose pain may be mild but clinical pain is more or less always severe
- Eliciting responses from healthy and inflamed tissues might be different from tissues that are diseased
- Assessing threshold responses to stimuli is a problem resulting to increase in intensity.

These limitations can be rectified through experimental designs with the appropriate controls such as use of appropriate negative and positive control groups; use of a single

observer throughout the duration of an experiment; and blinding an experimenter/observer so as not to be aware of any treatment the animals has received.

Animal models of pain can be classified into acute, inflammatory, chronic and neuropathic pain models.

1.3.5.1 Animal behavioural models of acute pain

Mogil (Mogil, 2009) broadly classified animal behavioural models of acute pain into phasic pain (using short duration stimuli) and tonic pain (using long duration stimuli).

1.3.5.1.1 Phasic pain

These models employ the use of short duration stimuli in seconds with stimulation of the somatic sites to a certain extent than the visceral sites. The models can be classified based on the nature of the stimulus into thermal, mechanical and electrical. The thermal models can further be sub classified into hot plate and tail flick tests, the mechanical into tail clip and paw pressure tests whereas the electrical into tail stimulation and dental pulp stimulation tests.

The hot plate model uses heat as the noxious stimuli. The animal paw is heated by contact with a hot plate (50 – 56 ˚C) and paw withdrawal or paw licking latency time is recorded similar to tail flick latency in tail flick or tail withdrawal thermal nociceptive test in which the distal portion of the tail is heated by a radiant heat source. The hot plate and tail flick or tail withdrawal thermal nociceptive tests are models of central pain which produces significant analgesic effects for opioid compounds,

tricyclic antidepressants, NMDA antagonists and acetylcholine agonists El Tahir and Ageel, 2002).

The tail clip and paw pressure tests (mechanical stimuli model) engage the application of pressure of increasing intensity to a punctiform area on the hind paw, or on the tail. The Randall-Selitto test allows the application of linearly increasing pressure between the third and fourth metatarsals of the hind paw through a blunt Perspex cone and the interruption of the test when the threshold is reached. The measured parameter is the threshold (weight in grams) for the appearance of a given behaviour. When the pressure increases, there is reflex withdrawal of the paw, withdrawal movement in which the animal tries to release its trapped limb, followed by a struggle and vocalization. The reflex withdrawal of the paw is a spinal reflex due to peripheral pain, while the struggle and vocalization involves supraspinal structures due to central pain (Ito *et al*., 2001).

1.3.5.1.2 Tonic pain

These models are sometimes referred to as persistent pain models and they employ the use of long duration stimuli in tens of minutes usually by single injection of algogenic agents that stimulates nociceptive fibres. In these models the visceral sites are stimulated to a certain extent than the somatic sites. The models can be classified based on the site of the injection into intradermal, example, formalin test; intraperitoneal, example, writhing test; and injection into hollow organs (Le Bars *et al*., 2001; Mogil, 2009).

The formalin test uses 2 – 5% formalin solution as a chemical noxious stimulus into the hind paw of rodents which causes persistent pain due to peripheral tissue injuries and inflammation of the cells. The animal licks, bites, flinches and elevates its paws

from the floor. The model produces biphasic nociceptive responses. The first phase lasts about 10 min and begins immediately after formalin injection. The activation of primary afferent fibres (C–fibres) is believed to be responsible for this phase. The second phase normally peaks 15 – 30 min after formalin injection. This phase is mediated by peripheral inflammation and by central sensitization (facilitatory processes in the spinal cord) due to the prolonged afferent input to the spinal cord. Clinical symptoms such as hyperalgesia associated with tissue injury are believed to be caused by peripheral inflammation and central sensitization, thereby making drugs with anti-inflammatory and/or analgesic properties to be effectively tested using this model (El Tahir and Ageel, 2002; Hunskaar and Hole, 1987).

The acetic acid-induced writhing test, a model of visceral pain uses the intraperitoneal injection of acetic acid (0.6 – 0.9 %v/v) into mice or rats and the frequency and/or duration of exaggerated abdominal distension and outstretching of hind limbs responses per 5 min segments is counted for 30 min (Ito *et al*., 2001; Woode and Abotsi, 2011).

1.3.5.2 Models of inflammatory pain in animals

These employ agents capable of inducing inflammatory responses, example intraplantar injection of carrageenan. Chronic inflammation induces hyperalgesia which is seen a few hours to days after peripheral injection of chemical irritant such as carrageenan (Arya and Kumar, 2005). The hyperalgesia is assessed by applying thermal or mechanical stimulus to the inflamed and normal paws. The second phase of formalin test is also a model of inflammatory pain.

1.3.5.3 Animal models of chronic pain

These models are often referred to as chronic inflammatory pain models and they employ the use of long duration stimuli lasting for several days to weeks usually by injecting carrageenan, kaolin, turpentine, mustard oil or other compounds into some parts of experimental animals such as the knee or ankle joint to produce prolonged allodynia/hyperalgesia (Radhakrishnan *et al*., 2003).

1.3.5.4 Animal models of neuropathic pain

Animal models of neuropathic pain according to Kerr and David (2007) evokes a unique set of anatomical and physiological changes at the level of the spinal cord, the dorsal root ganglia and the nerve to produce strong and reliable changes in nociceptive behaviours in response to both thermal and mechanical stimulation. Animal models of neuropathic pain developed to imitate the abnormal sensitivity to thermal and mechanical stimuli known to occur in humans with neuropathic pain include alloxan- or streptozotocin-induced diabetic neuropathy, inflammatory neuropathy, neuropathic herpes pain, didanosine-induced neuropathic pain, and nerve-injured neuropathy (Bhangoo *et al*., 2007; Bhangoo *et al*., 2009; Zhang *et al*., 2006).

The nerve injured neuropathy model is further sub-classified into - Seltzer or partial ligation model (half of sciatic nerve is tightly ligated), chronic constriction injury (Bennette model in which the whole sciatic nerve is loosely and constrictively ligated), and segmental spinal nerve ligation (Chung model) (Mizoguchi *et al*., 2009). Many chemotherapy-induced peripheral neuropathic pain models exist for studying the underlying mechanisms of neurogenic pain all of which has some degree of neuronal damage but differ in the time course and mechanisms associated with the hyperalgesia. Pain associated with traumatic injury (phantom limb pain after amputation) and

chemically-induced nerve damage (cancer chemotherapies-induced neuropathic pain) are classical examples of peripheral neuropathy models (Decosterd and Woolf, 2000). Antiepileptics and antidepressants but not opioids, NSAIDs and steroids have been found to be effective in vincristine-induced neuropathic pain model (Hall *et al*., 2006; Higuera and Luo, 2004; Zhang *et al*., 2006).

1.3.6 Pharmacological management of pain

Mild to moderate pain is commonly treated with non-opioids such as acetaminophen, ibuprofen or acetyl salicylic acid but when the pain persist opioids such as hydrocodone or codeine frequently administered in fixed dose combinations with non-opioids is usually added to the therapy. The WHO recommended separate dosage forms of the opioid and non-opioid when higher doses of opioid are needed (El Tahir and Ageel, 2002). Moderate to severe or persistent pains are treated with more potent opioids such as morphine and methadone.

The drug treatment of pain can be viewed under three perspectives – drugs currently in clinical use, drugs in preclinical use and future drugs. A wide range of drugs currently in clinical use in management of pain collectively termed analgesic agents include the opioids and the NSAIDs. The opioids are classified into morphine analogues (for example morphine, codeine and nalorphine) and synthetic derivatives with structures unrelated to morphine (for example pethidine and pentazocine). The NSAIDs are classified into non selective cyclooxygenase (COX) inhibitors (for example acetyl salicylic acid, indomethacin and ibuprofen) and COX-2 selective inhibitors (for example valdecoxib and etoricoxib).

Pharmacologically, these analgesic agents have long been used in the control of pain. However, availability of potent and safe analgesic agents is limited necessitating the

use of local anaesthetics (for example lidocaine), antiepileptics (for example carbamazepine), 5-HT receptor agonists (for example sumatriptan) and antidepressants (for example amitriptyline) to manage various types of pain ranging from trigeminal neuralgia, migraine to neuropathic pain by suppressing abnormal discharges in pathologically distorted neurons and reducing membrane excitability (Bultz and Carlson, 2005).

The known toxic and lethal effects of drugs currently in clinical use has made the search for safer and more potent drugs increasingly important. New approaches to control pain are therefore continually being exploited from knowledge of neurobiology of pain including the different chemical mediators and signalling pathways accountable for pain. This has led to the development of potential analgesic drugs some of which are in preclinical use.

Potential analgesic drugs includes the enkephalinase inhibitors (for example thiorphan), capsaicin-sensitive channel ($TRPV_1$) receptor antagonists, neuropeptides antagonists, glutamate antagonists acting on NMDA or AMPA receptors, antagonists at the metabotropic glutamate receptors (mGluRs), agonists at nicotinic acetylcholine receptors (for example epibatidine), agonists at cannabinoid receptors, conventional NSAIDs that have NO- donating groups attached to them by ester linkages, kinin antagonists and α_2-adrenergic agonists (Eid and Cortright, 2009; Krause *et al*., 2005; Negus *et al*., 2006; Oertel and Lötsch, 2013; Rice *et al*., 2008; Sawynok, 2003).

Similarly, future analgesic drugs should exploit useful drug targets that play vital function in nociception such as the various ion channels that play a role in nociceptive nerves in particular certain sodium channel subtypes which are specific for these nerve terminals, tyrosine kinase-linked receptors, adenosine analogues and adenosine kinase

inhibitors (Dib-Hajj *et al*., 2009; Fiorucci and Antonelli, 2006; Li and Zhang, 2012; Pertwee, 2005).

1.4 JUSTIFICATION OF THE STUDY

Pain is a principal reason of medically associated job absence because it accompanies nearly every disease condition and is the most common cause for medical actions (Schim and Stang, 2004). Pain causes millions hospital visits yearly costing billions dollars every year in health care loss due to lack of effective analgesic or wrong treatment (Kivell and Prisinzano, 2010; Schim and Stang, 2004). Some consequences of inadequately treated pain include metabolic disorders manifesting as weight loss, and increase in the use of healthcare resources (Joshi and Ogunnaike, 2005). The conventional analgesics have serious adverse effects such as gastric ulcerations, renal damage, respiratory depression, tolerance and addiction among other side effects coupled with the fact that they are not effective in neuropathic pain, making it necessary to search for more effective remedies with mild side effects but cheap and reliable. Good management of pain with costeffective analgesics is therefore needed to overcome these problems.

Some medicinal plants have a long history of use in traditional medicine to manage pain. The validation of traditional claims of these medicinal plants will provide the scientific basis for the conservation of tropical medicinal plants that are vanishing, the deployment of the beneficial ones as phytomedicines in the primary health care and the development of potential bioactive constituents. These could provide novel compounds or precursors in drug development and utilization of isolated compounds as research tools in drug development.

Maerua angolensis is one of such medicinal plants reputed for its wide ethnomedicinal use including psychosis, diabetes, stomach ulcer and various painful conditions in Nigeria and some West African countries (Adamu *et al*., 2007; Magaji *et al*., 2009; Meda *et al*., 2013; Mohammed *et al*., 2008; Mothana *et al*., 2009; Musa *et al*., 2011; Okatch *et al*., 2012). However, there is no comprehensive study on the assumed antinociceptive effects and the safety/toxicity profile of this plant. This study, therefore, investigates the traditional claim of pain relief by *Maerua angolensis* and provides scientific evidence for analgesic effects of the plant using different assays of chemical, mechanical and thermal models of pain in rodents. Further isolation and evaluation of the active chemical constituents of *Maerua angolensis* responsible for the antinociceptive effects and clarification of the structural characters of responsible components will lead to standardization and quality control of this herbal thereby improving patient acceptability of the product.

1.4.1 Aim of the study

The study aimed to assess the analgesic activity of *Maerua angolensis* and to provide scientific evidence supporting the traditional uses of the plant in pain management.

1.4.2 Specific objectives of the study

- To investigate the analgesic activity of the aqueous ethanol extract of the leaf, root and stem bark of *Maerua angolensis* in mice using acetic acid-induced writhing test and to determine the most potent plant part
- To evaluate the analgesic activity of the petroleum ether, ethyl acetate and aqueous ethanol extracts of the stem bark of *Maerua angolensis* in mice and rats using acetic acid-induced writhing and formalin-induced nociception tests and to determine the most potent solvent extract of the stem bark in the formalin test

- To investigate the analgesic activity of the petroleum ether/ethyl acetate extract (MAE) and fractions of the stem bark of *Maerua angolensis* in rodents using:
    - ✓ Acetic acid-induced writhing test
    - ✓ Formalin-induced nociception
    - ✓ Tail-flick test in Hargreaves thermal nociception model
    - ✓ Paw withdrawal test in Hargreaves thermal hyperalgesia model
- To assess the effect of MAE and fractions on the withdrawal syndrome of morphine dependence in mice
- To assess the motor function in the antinociceptive effects of MAE and fractions
- To assess the effect of MAE and fractions on vincristine-induced neuropathic pain in mice
- To investigate the mechanism(s) of action of the petroleum ether/ethyl acetate stem bark extract and fractions of *Maerua angolensis* in the acetic acid-induced writhing test and the tail-flick test in Hargreaves thermal hyperalgesia model (exploring the participation of or otherwise of different pathways such as adenosinergic, nitric oxide, muscarinic, ATP sensitive K^+ channels, adrenergic, serotoninergic and opioidergic in their antinociceptive activity). Mechanism(s) of antinociception would also be investigated in the prostaglandin E_2-induced mechanical hyperalgesia, bradykinin-, and epinephrine-induced thermal hyperalgesia, glutamate- and capsaicin-induced nociception.
- To isolate, identify and characterize the bioactive compounds from the stem bark using H-NMR, GCMS, and other spectroscopic techniques
- To investigate the analgesic activity of the compounds isolated from the stem bark of *Maerua angolensis* in animal models including the acetic acid-induced mouse writhing

assay, the hypertonic saline-induced corneal pain in mice/possible mechanisms of antinociception of the compounds in the test and the acetic acidinduced locomotor activity in zebrafish larvae

- To assess the safety and toxicity of the petroleum ether/ethyl acetate extract in rats.

Chapter 2

PLANT COLLECTION, EXTRACTION AND PRELIMINARY ANALGESIC STUDIES OF THE LEAF, ROOT AND STEM BARK

2.1 INTRODUCTION

The leaves, roots and stem bark of the plant *Maerua angolensis* are used to relief pain in traditional medicine (Adamu *et al*., 2007; Mothana *et al*., 2009). The medicinal plant practitioners normally used the fresh plant parts which are separately shade-dried and pulverized into powder. The powdered material is soaked in water or alcohol and taken orally daily to relieve pain. In this study, a preliminary test was performed to investigate antinociceptive effects of the hydroethanolic extracts of the leaf, root and stem bark of *Maerua angolensis* in animal models of pain. The acetic acid-induced writhing and formalin tests, animal models that predict both peripherally- and centrally-mediated pain were used. The study will help to substantiate the traditional uses of the plant as claimed by the medicinal plant practitioners and provide an alternative to current analgesics.

2.2 MATERIALS AND METHODS

2.2.1 Plant Collection and identification

Fresh leaves, roots and stem bark of *Maerua angolensis* were collected from the Samaru campus of Ahmadu Bello University, Zaria–Nigeria during the month of August, 2012. The plant materials were kept in the press to avoid distortion of the plant structure before being transported from Nigeria to Ghana. Identification of the plant materials was by Dr. Kofi Annan at the Department of Herbal Medicine,

Faculty of Pharmacy and Pharmaceutical Sciences, College of Health Sciences, Kwame Nkrumah University of Science and Technology (KNUST), Kumasi–Ghana.

A voucher specimen (KNUST/FP/12/051) was kept at the herbarium of the Faculty.

2.2.2 Extraction of the leaf, stem bark and root in aqueous ethanol

The leaves, roots and stem bark were separately shade-dried for fourteen days, and pulverized into coarse powder. The powder (100 g) were extracted by cold maceration with 1 L 70% (v/v) ethanol over a period of four days. Each was filtered using filter paper number 4 and then concentrated under reduced pressure in a rotary evaporator (Rotavapor R-215, BÜCHI Labortechnik AG, Flawil, Switzerland) at a temperature of 60 °C to give a greenish, brownish and yellowish syrupy mass respectively for the leaf, root and stem bark. These were dried in the hot air oven (Gallenhamp®, England) at 50 °C for five days. The final yields were 17.6, 7.1 and

10.4% (w/w) for the leaf, root and stem bark respectively. They were kept in a refrigerator for use. The leaf, root and stem bark extracts are subsequently referred to as MAEL, MAER and MAEB, respectively.

2.2.3 Extraction of the stem bark in petroleum ether, ethyl acetate and aqueous ethanol

Dried and powdered stem bark (4 kg) was sequentially extracted, for 4 days, with 10 L of petroleum ether, ethyl acetate and hydroethanol (in order of increasing polarity) by cold maceration. Each was filtered and treated as described in *section 2.2.2* to give yields of 2.23, 3.87 and 7.8% (w/w) respectively for the petroleum ether, ethyl acetate and hydroethanol extracts. They were kept in a refrigerator for use. The petroleum ether, ethyl acetate and hydroethanol stem bark extracts are subsequently referred to as MABPEE, MABEAE and MABHAE respectively.

2.2.4 Animals

Male and female Imprinting Control Region (ICR) mice weighing 20 – 25 g and Sprague–Dawley rats (190 – 200 g) of either sex were used in the study. All animals were housed in groups of five in stainless steel cages (34 × 47 × 18 cm) with softwood shavings as bedding in the animal facility of the Department of Pharmacology, KNUST. They were given free access to food and water and were maintained under normal laboratory conditions of temperature (25 ± 1 °C) and a 12 h/12 h day/night cycle. The investigation conformed to the Guide for the Care and

Use of Laboratory Animal published by the US National Institutes of Health (NIH No. 85 – 23, revised 1996). All protocols were also approved by the Institutional Animal Ethics Committee. In all the experimental studies, each group consisted of 5 animals.

2.2.5 Drugs and chemicals

The following drugs and chemicals were used: Acetic acid and formalin (BDH, Poole, England), diclofenac sodium (Troge Medical GmbH, Hamburg, Germany), morphine hydrochloride (Phyto–Riker, Accra, Ghana). All drugs used in the nociceptive tests were dissolved in normal saline. All extracts were freshly prepared as suspension in normal saline containing 2% Tween-40 (vehicle) before use.

2.2.6 Preliminary analgesic screening

2.2.6.1 Writhing test on MAEL, MAER and MAEB

MAEL, MAER, MAEB (30, 100, and 300 mg/kg, *p.o.*), Diclofenac sodium (10 mg/kg, i.p.); as reference analgesic agent or normal saline (10 ml/kg, i.p.); for control were administered to groups of mice. Acetic acid (0.6% v/v) was given (10 ml/kg,

i.p.) 1 h after the oral and 30 min after the intraperitoneal administration to all mice. The number of abdominal constrictions (writhing) were recorded for 15 min for analysis with a camcorder (Everio^{TM}, model GZ-MG1300, JVC, Tokyo, Japan) placed directly opposite a mirror inclined at 45° below the floor of the chamber to allow a complete view of the animals and attached to a computer. Tracking of the writhing was done with the help of the public domain software JWatcher^{TM}, Version 1.0 (University of California, LA, USA, and Macquarie University, Sidney, Australia, available at http://www.jwatcher.ucla.edu/) to obtain the frequency of writhes per 5 min segments, starting 5 min after acetic acid administration. A significant reduction in the number of acetic acid–induced abdominal constrictions by any treatment compared with control treated mice was considered as an antinociceptive response (Koster *et al*., 1959; Sikdar *et al*., 2013; Taiwe *et al*., 2011; Tang *et al*., 2007).

2.2.6.2 Writhing test on MABPEE, MABEAE and MABHAE

Various groups of mice were given MAPEE, MABEAE, MABHAE (30, 100 and 300 mg/kg, *p.o*.), diclofenac (10, 30 and 100 mg/kg, i.p.) or normal saline (10 ml/kg, i.p.). Acetic acid (0.6% v/v) was given (10 ml/kg, i.p.) 1 h after the oral and 30 min after the intraperitoneal administration to all mice. The number of writhing were recorded for 30 min for analysis with a camcorder and tracking of the behaviour was done with the help of the public domain software JWatcher^{TM} as described in *section 2.2.6.1*. A significant reduction in the number of writhing by any treatment compared with control treated mice was considered as an antinociceptive response (*section 2.2.6.1*).

2.2.6.3 Formalin test on MABPEE, MABEAE and MABHAE

The formalin test was carried out as described by Fischer *et al*. (2013) and Tjølsen *et al*. (1992). Rats were given MABPEE, MABEAE, MABHAE (3, 10 and 30 mg/kg, *p.o*.), morphine (1, 3 and 10 mg/kg, i.p.) or normal saline (10 ml/kg, i.p.). Formalin (5%) was given by intraplantar injection (10 µl) into the dorsal surface of the right hind paw 1 h after the oral and 30 min after the intraperitoneal administration to all rats to induce pain (J Cobos and Portillo-Salido, 2013; Le Bars *et al*., 2001). A mirror inclined at 45° below the floor of the chamber allowed a complete view of the paws.

The behaviour of the rats were then captured (1 h) for analysis by a camcorder placed directly opposite to the mirror and attached to a computer. Pain response was scored for 1 h, starting immediately after formalin injection. A nociceptive score was determined for each 5-min time block by measuring the amount of time spent biting/licking of the injected paw (Woode *et al.*, 2009). Tracking of the biting/licking of the injected paws was done with the help of the public domain software JWatcher™. Average nociceptive score for each time block was calculated by multiplying the frequency and time spent in biting/licking. The first phase of the nociceptive response normally peaks 0 – 5 min and the second phase 15 – 30 min after formalin injection corresponding to the neurogenic and inflammatory pain responses respectively (Godínez-Chaparro *et al*., 2013; Hunskaar and Hole, 1987).

Data were expressed as the mean ± SEM of scores between 0 – 10 (first phase) and 10 – 60 min (second phase) after formalin injection.

2.2.7 Thin-layer chromatography (TLC) analyses of MABPEE, MABEAE and

MABHAE

MABPEE, MABEAE and MABHAE were separately subjected to TLC analyses carried out on aluminium sheet pre-coated with normal phase silica gel 60 F_{254} (Merck, 0.20 mm thickness) and eluted with petroleum ether: chloroform (90:10).

2.2.8 Phytochemical analyses of MABPEE, MABEAE and MABHAE

Phytochemical tests were conducted on MABPEE, MABEAE and MABHAE to verify the presence of saponins, tannins, terpenoids, steroids, flavonoids, alkaloids, glycosides as well as oils and fats.

2.2.8.1 Saponins

The frothing test was used to detect presence of saponins. MABPEE, MABEAE or MABHAE (200 mg) was mixed with 5 ml of water in a test tube by shaking and the mixture observed for the presence of a froth which does not break easily upon standing (Usman *et al.*, 2009).

2.2.8.2 Tannins

About 500 mg of MABPEE, MABEAE and MABHAE were separately boiled with 25 ml of water for 5 minutes, cooled and filtered. The volumes of the filtrates were adjusted to 25 ml with water. To 1 ml of the filtrates were added 10 ml of water and 5 drops of 1%, ferric chloride and observed for a blue-black or green precipitate formation (Ferric chloride test). The method was repeated using 5 drops of 1% lead acetate (Lead acetate test) and observed for formation of precipitate or any change in colour (Evans, 2009).

2.2.8.3 Terpenoids

The Salkowski test was utilized to detect the presence of terpenoids. MABPEE, MABEAE and MABHAE (500 mg) were separately extracted with 2 ml chloroform in a test tube followed by addition of 1 ml concentrated sulphuric acid. The presence of terpenoids is confirmed by the presence of reddish-brown colouration at interface (Jana and Shekhawat, 2010).

2.2.8.4 Flavonoids

In the ammonia test, dilute ammonia solution (5 ml) was added to aqueous filtrates of MABPEE, MABEAE or MABHAE followed by addition of concentrated sulphuric acid and observed for yellow colouration (Ayoola *et al*., 2008).

2.2.8.5 Alkaloids

The Dragendorff‘s test was used to detect the presence of alkaloids in the extracts. MABPEE, MABEAE and MABHAE (500 mg) were separately boiled with 10 ml of dilute hydrochloric acid in a test tube for 5 minutes. The supernatant liquid were filtered into different test tubes and 1 ml of the filtrates were taken into which three drops of Dragendorff‘s reagent (potassium bismuth iodide solution) were added, shaken and observed for the appearance of an orange-red spot and precipitate formation (Abotsi *et al.*, 2012; Saxena *et al*., 2012; Sofowora, 1993).

2.2.8.6 Glycosides

In the Fehling‘s test, 200 mg of MABPEE, MABEAE and MABHAE were separately boiled in 5 ml dilute sulphuric acid on a water bath for 2 minutes. The mixtures were cooled, filtered and made clearly alkaline with 2 to 5 drops of 20%

NaOH. 1 ml each of Fehling's A and B solutions were added to the filtrates, heated on a water bath for 2 minutes and observed for a red-brown precipitate (Evans, 2009).

2.2.8.7 Oils and fats

In the spot test to detect presence of oils and fats, a small quantity of MABPEE, MABEAE and MABHAE were separately pressed in between two filter papers. Oil stain on the filter papers indicates the presence of oils and fats (Saxena *et al*., 2012).

2.2.8.8 Steroids

The Lieberman-Burchard's test was conducted to detect steroids where 500 mg each of MABPEE, MABEAE and MABHAE were separately extracted with 2 ml of chloroform in test tubes. 2 ml of acetic anhydride was added to each extract. Concentrated sulphuric acid was then carefully added at the side of the test tubes. A blue colour that emerged at the interface indicated the presence of steroids (Jana and Shekhawat, 2010; Sofowora, 1993).

2.2.9 Fractionation of petroleum ether/ethyl acetate stem bark extract of *Maerua angolensis* (MAE)

The MAE (10 g) was fractionated using a chromatography column (60 cm length × 3 cm width) dry packed with silica gel 60 F_{254}; Merck Damstadt, Germany and by eluting sequentially and exhaustively with 100% petroleum ether, petroleum ether and ethyl acetate (90:10) and petroleum ether and ethyl acetate (50:50) in this order (in order of increasing polarity) to get the fractions responsible for the analgesic activity. A piece of cotton wool was used to plug the bottom portion of the column above which the silica gel (column adsorbent) was gradually added and tapping the column to level off (dry packing technique). The extract dispensed in a crucible bowl was mixed well with

small amount of the silica gel with the aid of a pestle. The extract/silica gel mixture was then gradually added into the column on top the column adsorbent and tapping the column to also level off. Another cotton wool was packed on top near the open end of the column before the solvent systems were separately poured into the column to fractionate the extract sequentially. Precautions were taken to ensure there was no entrapment of air bubbles or crack in the stationary phase. Serially labelled collecting bottles were used to collect 100 ml of each fraction when running the column.

A total of 64 fractions were collected in 100 ml aliquots. Continuous elution with 100% petroleum ether yielded 31 fractions (fractions 1 – 31). Further elution with petroleum ether and ethyl acetate (90:10) yielded 24 fractions (fractions 32 – 55) while 9 fractions (fractions 56 – 64) resulted from continuous elution with petroleum ether and ethyl acetate (50:50). Fractions with similar TLC profiles indicating similar phytocomponents were combined and concentrated. Fractions 1 - 2 were combined giving a yield of 5.5% (w/w) and coded F1 while fractions 32 - 34 were combined giving a yield of 6.7% (w/w) and coded F32 and were kept in a refrigerator for later use. Fractions 3 – 31 and 35 – 64 did not show any spot on TLC and were discarded.

2.2.10 Data analysis

Data were expressed as mean ± standard error of the mean (SEM) per group. Statistical differences between control and treated groups were tested by two–way (treatment × time) repeated measures analysis of variance (ANOVA) with Bonferroni's *post hoc* test. Differences between other means were by one-way ANOVA with Newman–Keuls *post hoc* test. The ED_{50} (dose responsible for 50% of the maximal effect of the extract or drug) and 95% confidence intervals values were determined by using nonlinear regression (three-parameter logistic). GraphPad® Prism 5.01 for

Windows (GraphPad® Prism Software, San Diego, CA, USA) was used for all statistical analyses. Differences were considered significant at $P<0.05$.

2. 3 RESULTS

2.3.1 Effect of MAEL, MAEB and MAER on the writhing test

Injection of acetic acid intraperitoneally to the control mice pre-treated with normal saline produced writhing (exaggerated distension of the abdomen combined with the outstretching of the hind limbs). MAEL, MAEB and MAER dose-dependently and significantly suppressed the time-course curve of acetic acid-induced writhes similar to diclofenac (Figure 2.1 a, c and e). Two-way ANOVA (treatment × time) revealed a significant (MAEL: $F_{4, 80} = 212.4$, $P<0.0001$; MAEB: $F_{4, 80} = 148.2$, $P<0.0001$ and MAER: $F_{4, 80} = 20.52$, $P<0.0001$) effect of drug treatments on the acetic acidinduced abdominal constrictions.

MAEL; MAEB and MAER (30 – 300 mg/kg, *p.o.*) 1 h before acetic acid injection significantly ($F_{4, 20} = 99.58$, $P<0.0001$; $F_{4, 20} = 91.29$, $P<0.0001$ and $F_{4, 20} = 9.19$, $P = 0.0002$) and dose-relatedly reduced the number of the acetic acid-induced abdominal constrictions in mice over 15 min with AUC of 72.6 ± 3.48, 63.7 ± 4.14 and 51 ± 15.96% respectively (Figure 2.1 b, d, f) at the highest doses (300 mg/kg) used. Similar effect was observed in mice pre-treated with diclofenac (10 mg/kg, i.p.) 30 min before acetic acid injection.

The ED_{50} values calculated from the dose-response curves, for MAEL, MAER and MAEB in the writhing test obtained by *F*-test (Table 2.1) showed the MAEB was most potent (49.45 ± 0.08 mg/kg) in preventing the nociception caused by acetic acid. Thus,

the stem bark extraction with various solvents was chosen for further studies in writhing and formalin tests.

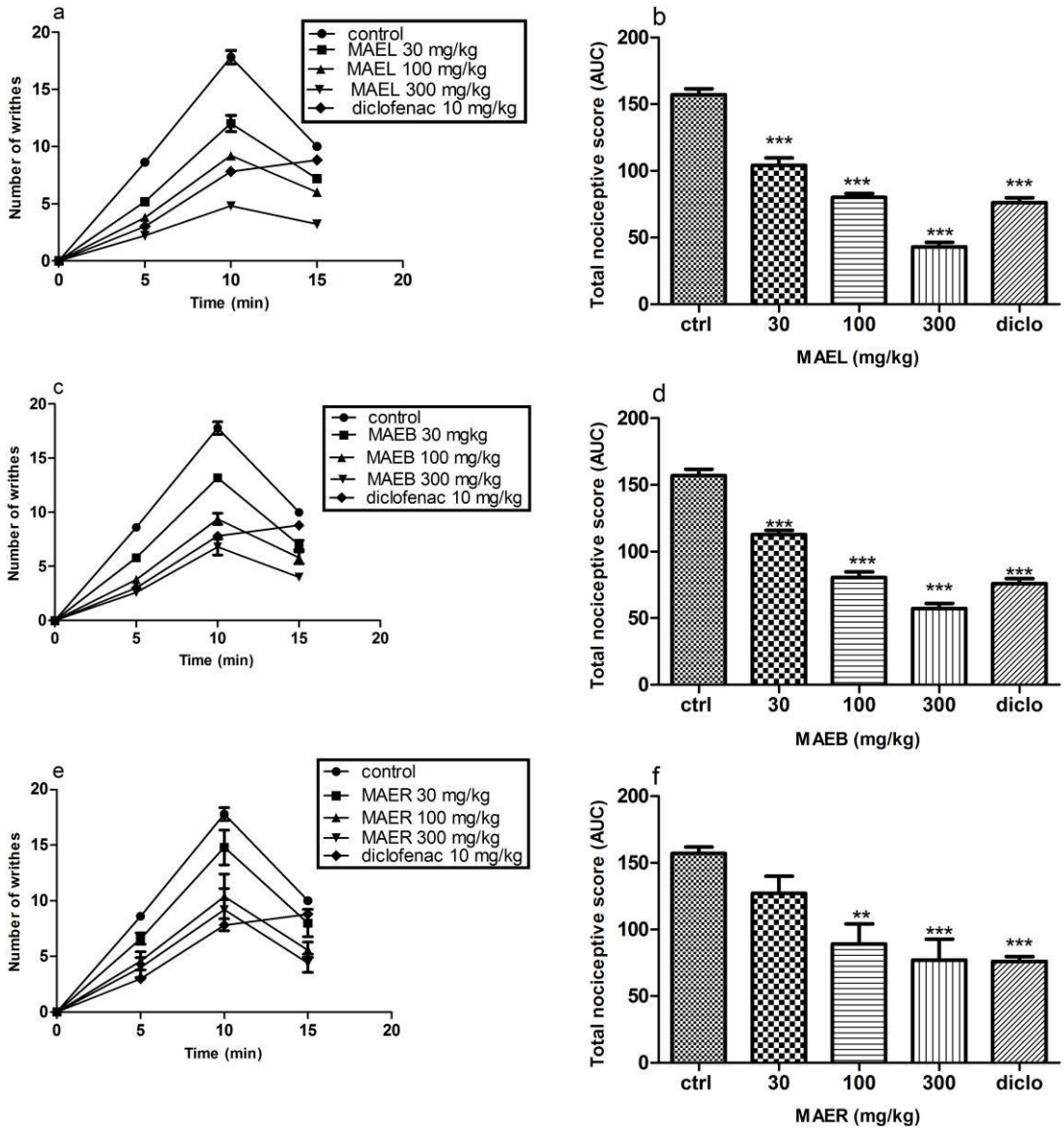


Figure 2.1 Effect of MAEL, MAEB, MAER (30 – 300 mg/kg, *p.o.*) and diclofenac (10 mg/kg, i.p.) on the time course curve of acetic acid-induced abdominal writhes (a, c and e) and the total nociceptive score (calculated as AUC) (b, d and f) in ICR mice. Data are expressed as mean ± SEM (n = 5). **P<0.01; ***P<0.001 compared to vehicle-treated group (one-way ANOVA followed by Newman-Keuls *post hoc* test).

Table 2.1: ED_{50} values for MAEL, MAER and MAEB (30 – 300 mg/kg, *p.o.*) in the acetic acidinduced writhing test

Treatment	ED_{50} (mg/kg)
MAEL	52.31 ± 0.09
MAER	56.25 ± 0.36
MAEB	49.45 ± 0.08

Values are expressed as mean ± SEM, (n = 5)

2.3.2 Effect of MABPEE, MABEAE and MABHAE on the writhing test

Injection of acetic acid i.p. produced writhing, exhibited as an exaggerated distension of the abdomen combined with the outstretching of the hind limbs seen more in control mice pre-treated with normal saline. MABPEE, MABEAE, MABHAE and diclofenac dose-dependently reduced the time-course curve of acetic acid-induced abdominal constrictions (Figure 2.2 a, c, e and g). Two-way ANOVA (treatment × time) revealed a significant (MABPEE: $F_{3,\ 112}$ = 34.07, P<0.0001; MABEAE: $F_{3,\ 112}$ = 15.68, P<0.0001; MABHAE: $F_{3,\ 112}$ = 28.16, P<0.0001 and diclofenac: $F_{3,\ 112}$ = 41.10, P<0.0001) effect of drug treatments on the acetic acid-induced abdominal constrictions. MABPEE, MABEAE and MABHAE (300 mg/kg, *p.o.*) dose-dependently and significantly ($F_{3,\ 16}$ = 9.87, P = 0.0006; $F_{3,\ 16}$ = 3.89, P = 0.0290 and $F_{3,\ 16}$ = 6.96, P = 0.0033) suppressed the number of abdominal writhes over 30 min with maximal inhibition of 70.4 ± 19.79, 72.3 ± 12.26 and 76.7 ± 13.45% respectively (Figure 2.2 b, d and f). Similarly, diclofenac (10 – 100 mg/kg, i.p.) 30 min before acetic acid injection dose-dependently and significantly ($F_{3,\ 16}$ = 12.09, P= 0.0002) attenuated the acetic acid-induced writhes by a maximum of 83.7 ± 13.02% (Figure 2.2 h). Table 2.2 shows the ED_{50} values obtained by F-test for

MABPEE, MABEAE, MABHAE and diclofenac in the writhing test in mice.
MABHAE was the most potent extract (25.37 ± 0.31 mg/kg) though less potent than diclofenac (5.06 ± 0.22 mg/kg).

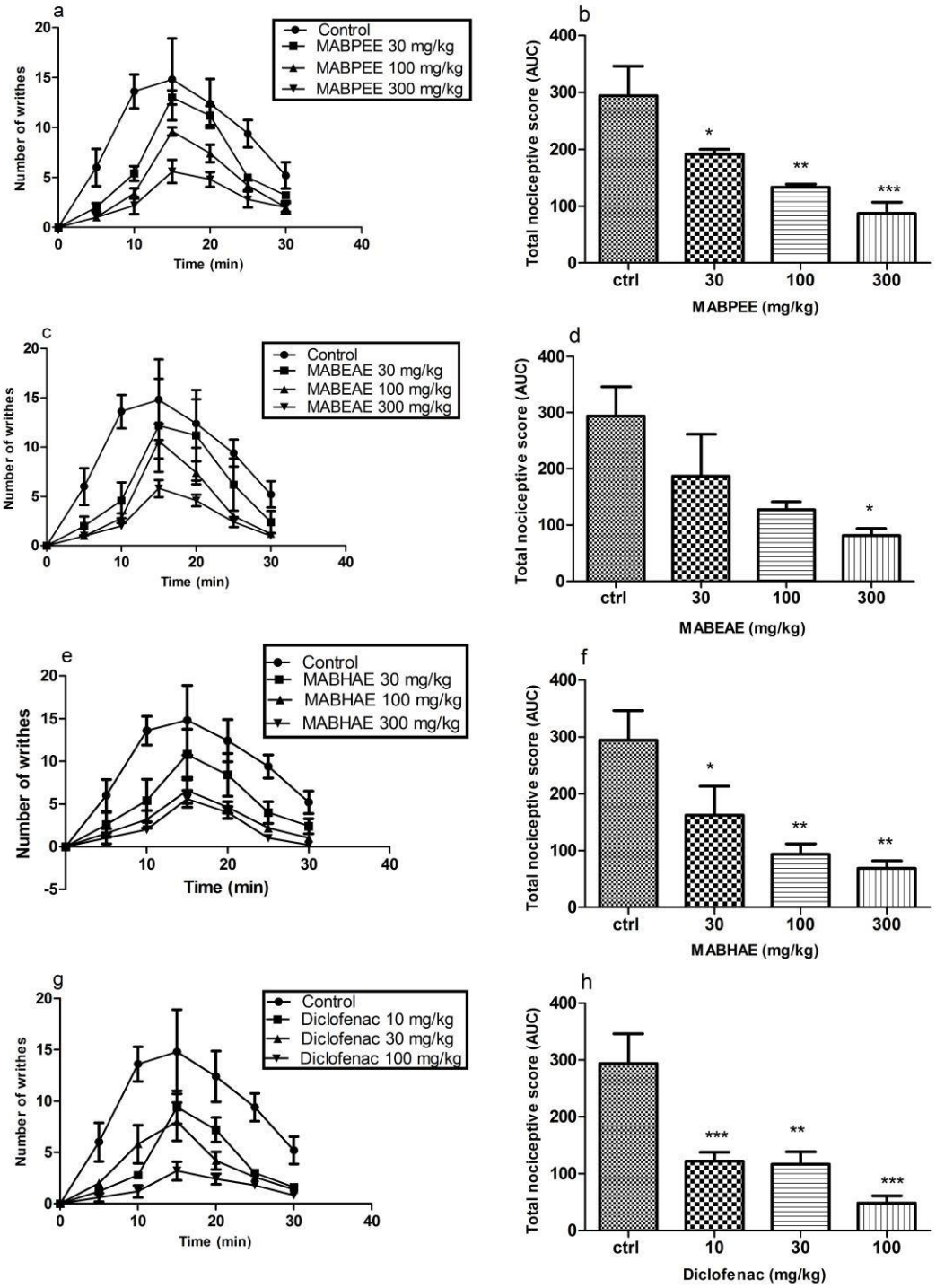


Figure 2.2 Effect of MABPEE, MABEAE, MABHAE (30 – 300 mg/kg, *p.o.*) and diclofenac (10 – 100 mg/kg, i.p.) on the time course curve of acetic acid-induced abdominal writhes (a, c, e and g)

and the total nociceptive score (calculated as AUC) (b, d, f and h) in ICR mice. Data are expressed as mean ± SEM (n = 5). *P<0.05; **P<0.01; ***P<0.001 compared to vehicle-treated group (one-way ANOVA followed by Newman-Keuls *post hoc* test).

Table 2.2: ED_{50} values for MABPEE, MABEAE, MABHAE and diclofenac in the writhing test

Treatment	ED_{50} (mg/kg)
MABPEE	39.42 ± 0.13
MABEAE	38.14 ± 0.43
MABHAE	25.37 ± 0.31
Diclofenac	5.06 ± 0.22

Values are expressed as mean ± SEM, (n = 5)

2.3.3 Effect of MABPEE, MABEAE and MABHAE on the formalin test

Intraplantar injection of 5% formalin (10 µl) into the dorsal surface of the right hind paw produced a biphasic nociceptive response, exhibited as biting, licking and flinching of the injected paw. This was seen more in control rats pre-treated with normal saline. This characteristic biphasic licking response consisted of an initial intense response to pain which starts immediately after formalin injection and lasted for 10 min (first or neurogenic phase) with maximum effect at approximately 5 min followed by a slowly rising but longer-lasting response (second or inflammatory phase) from 10 min and lasted until 1 h with maximum effect at approximately 30 min after formalin injection.

Administration of MABPEE, MABEAE, MABHAE (3 – 30 mg/kg, *p.o.*) or morphine (1 – 10 mg/kg, i.p.) to the rats dose-dependently and significantly attenuated the time-

course curve of formalin-induced nociception when compared with the vehicle treated-group (Figure 2.3 a, c, e and g). Two-way ANOVA (treatment × time) revealed a significant (MABPEE: $F_{3, 208} = 92.76$, $P<0.0001$; MABEAE: $F_{3, 208} = 75.11$, $P<0.0001$; MABHAE: $F_{3, 208} = 40.86$, $P<0.0001$ and morphine: $F_{3, 208} = 116.1$, $P<0.0001$) effect of drug treatments on the formalininduced nociception.

MABPEE, MABEAE and MABHAE (3 – 10 mg/kg, *p.o.*) 1 h before formalin injection dose-dependently and significantly suppressed paw licking time in the neurogenic ($F_{3, 16} = 19.44$, $P<0.0001$; $F_{3, 16} = 13.90$, $P = 0.0001$ and $F_{3, 16} = 9.86$, $P = 0.0006$) and the inflammatory phase ($F_{3, 16} = 30.72$, $P<0.0001$; $F_{3, 16} = 24.00$, $P<0.0001$ and $F_{3, 16} = 16.94$, $P<0.0001$) over 1 h. The maximal inhibition of the neurogenic phase was 81.7 ± 2.78, 74.1 ± 6.31 and 85.3 ± 4.1% and the inflammatory phase was 94.1 ± 6.28, 94 ± 1.53 and 84.3 ± 4.14% respectively (Figure 2.3 b, d and f) at doses of 30 mg/kg when compared with vehicle treated control rats. In a similar manner, morphine pre-treatment (1 – 10 mg/kg, i.p.) 30 min before formalin injection resulted in a distinct dose–dependent and significant reduction of response time in the early ($F_{3, 16} = 36.82$, $P<0.0001$) and late ($F_{3, 16} = 34.80$, $P<0.0001$) phases of formalin-induced licking with maximal inhibition of the neurogenic as 96.7 ± 0.67% and the inflammatory phase as 95 ± 1.37% (Figure 2.3 h).

The calculated mean ED_{50} values obtained by *F*-test for the antinociceptive effects of MABPEE, MABEAE, MABHAE and morphine in the formalin test (Table 2.3) showed MABPEE was more potent in inhibiting the neurogenic pain and MABEAE more potent in blocking pain emanating from inflammation. Morphine was, however, most potent in both the neurogenic and the inflammatory phase of the formalininduced paw licking.

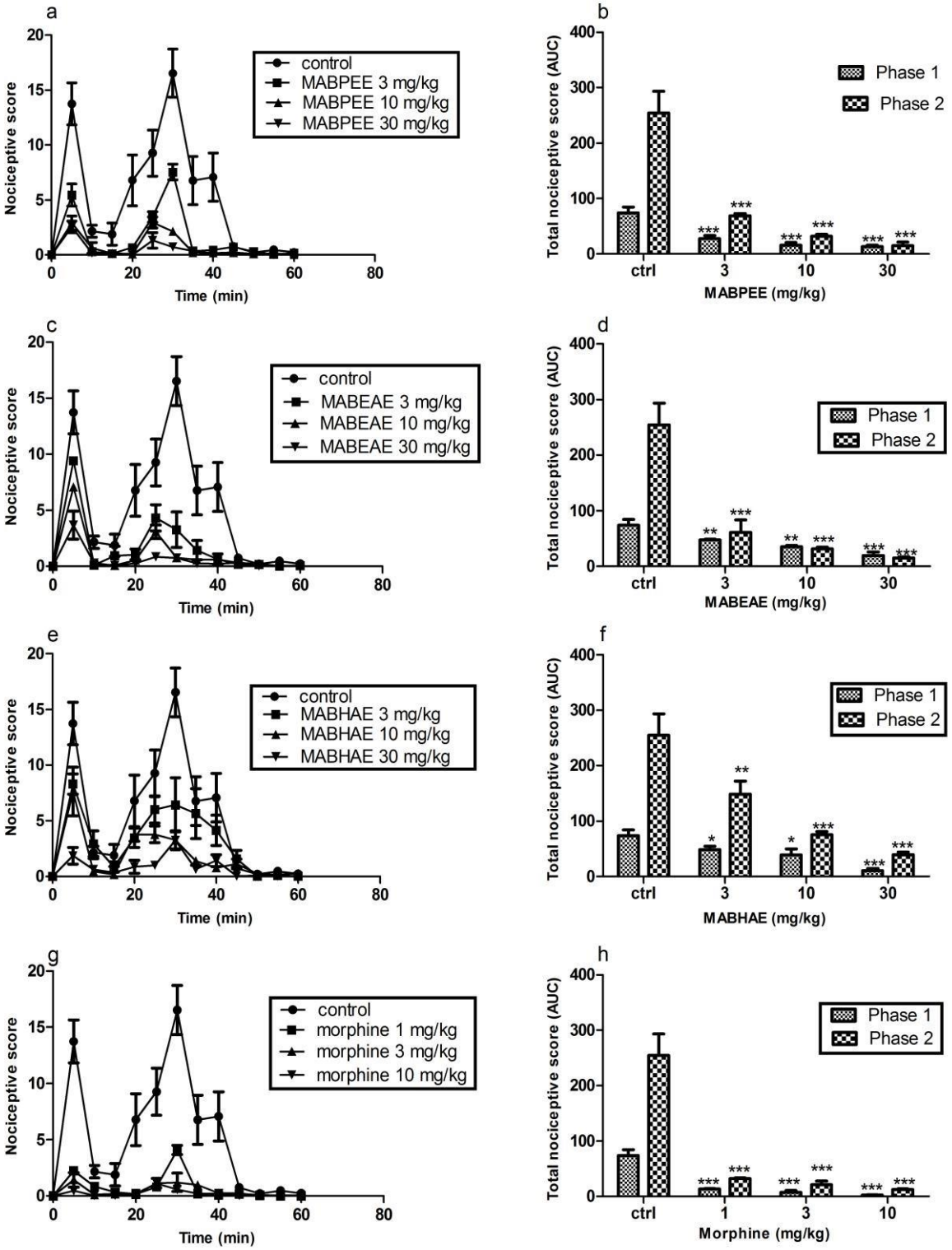


Figure 2.3 Effect of MABPEE, MABEAE, MABHAE (3 – 30 mg/kg, *p.o.*) and morphine (1 – 10 mg/kg, i.p.) on the time course curve of formalin-induced nociception (a, c, e and g) and the total nociceptive score (calculated as AUC) (b, d, f and h) in Sprague-Dawley rats. Data are expressed as mean ± SEM (n = 5). **P*<0.05; ***P*<0.01; ****P*<0.001 compared to vehicle-treated group (one-way ANOVA followed by Newman-Keuls *post hoc* test).

Table 2.3: ED_{50} values for MABPEE, MABEAE, MABHAE and morphine in the formalin test

Treatment	ED_{50} (mg/kg) in early phase	ED_{50} (mg/kg) in late phase
MABPEE	1.13 ± 0.20	1.00 ± 0.06
MABEAE	4.62 ± 0.16	0.79 ± 0.20
MABHAE	9.89 ± 0.28	3.81 ± 0.13
Morphine	0.20 ± 0.13	0.10 ± 0.03

Values are expressed as mean ± SEM, (n = 5)

2.3.4 TLC analyses of MABPEE, MABEAE and MABHAE

The TLC profile of MABPEE and MABEAE (Plate 2.1) were found to be similar indicating possibility of collective elution of components with similar Rf values. The two extracts were then combined and subsequently referred to as petroleum ether/ethyl acetate stem bark extract of *Maerua angolensis* (MAE).

Plate 2.1 TLC profile of MABPEE (P.E), MABEAE (E.A) and MABHAE (H.A) in petroleum ether: chloroform (90:10) solvent revealing MABPEE and MABEAE to be similar indicating possibility of collective elution of components with similar Rf values.

2.3.5 Phytochemical analyses of MABPEE, MABEAE and MABHAE

The phytochemical tests showed the presence of saponins, tannins, steroids, terpenoids, flavonoids, alkaloids, glycosides as well as oils and fats (Table 2.4).

Table 2.4: Phytochemical constituents of MABPEE, MABEAE and MABHAE

CONTITUENT	MABPEE	MABEAE	MABHAE
Saponins	-	-	+
Tannins	+	+	+
Terpenoids	+	+	+
Flavonoids	+	+	+
Alkaloids	-	-	+
Glycosides	+	+	+
Oils and fats	+	+	-
Steroids	+	+	+

+ present; - not present; MABPEE petroleum ether extract of the stem bark of *M. angolensis*; MABEAE ethyl acetate extract of the stem bark of *M. angolensis*; MABHAE hydroalcohol extract of the stem bark of *M. angolensis*.

2.4 DISCUSSION

Various parts of *Maerua angolensis* are traditionally used in the treatment of pain without any scientific evidence. The outcome of this study demonstrates that the extract of the hydroethanolic leaf, stem bark and root and the petroleum ether, ethyl

acetate stem bark extracts of *Maerua angolensis* have significant analgesic activity in chemical-induced pain models in mice and rats. In acetic acid-induced writhing test, the plant leaves, roots and stem bark extracts as well as the petroleum ether, ethyl acetate stem bark extracts suppressed the pain sensation in a dose-dependent manner however; stem bark extract was the most potent whereas petroleum ether and ethyl acetate extracts were the most potent in first and second phase of formalin test respectively. Similar effects were observed in mice and rats pre-treated with diclofenac and morphine used as reference analgesic agents in writhing and formalin tests respectively. These then may be a confirmation to the usefulness of *Maerua angolensis* for the management of pain. It is also an indication that all the extracts are readily absorbed following oral administration.

Acetic acid-induced abdominal writhing test is a model of acute persistent nociception and a typical model for inflammatory pain in which acetic acid is used as the algogenic agent (Shamsi Meymandi and Keyhanfar, 2013). Acetic acid when injected intraperitoneally induces visceral pain in the animals via stimulation of primary afferent sensory Aδ and C nerve fibres (Sawynok, 2003). Generally, the test is popular in detecting peripheral analgesic agents (Aliyu *et al*., 2005; SanchezMateo *et al*., 2006). Abdominal constriction test has good sensitivity and is capable of detecting antinociceptive compounds at doses that may be inactive with other antinociceptive tests (Sawynok, 2003).

Related studies have established that acetic acid indirectly induces the release of proinflammatory prostanoids (prostaglandins) which in turn cause the production of prostanoid-dependent pain causing molecule bradykinin, important in the mechanism of pain transduction in primary afferent nociceptors (Chen *et al.*, 2013b; Roome *et al*.,

2011). Additionally, prostaglandins sensitize peripheral pain through activation of prostanoid (EP) receptors present on the peripheral terminals of sensory neurons (Austin and Moalem-Taylor, 2013; Lin *et al*., 2006). Acetic acid also liberates sympathetic nervous system mediators that stimulate the nociceptive neurons, all of which are sensitive to NSAIDs and opioid analgesics (Danjuma *et al.*, 2011; Jothimanivannan *et al*., 2010; Sanchez-Mateo *et al*., 2006).

Diclofenac, a non-opioid analgesic inhibits the production and release of prostaglandins accompanied by reduction in the abdominal writhes. The results indicated that the leaf, root and stem bark hydroethanolic extracts as well as the petroleum ether and ethyl acetate stem bark extracts of *Maerua angolensis* could reduce the number of writhing in the animal behavioural model of pain similar to diclofenac, implying that extracts had antinociceptive effects which might be due to inhibition of synthesis and/or release of pro-inflammatory prostanoids peripherally. Antinociception may have also occurred spinally through inhibition of proinflammatory mediators-mediated central sensitization. This activity probably is due to the presence of flavonoids, alkaloids, saponins, glycosides, steroids, terpenoids, tannins as well as oils and fats seen in the present results of qualitative phytochemical screening of the stem bark extracts.

These phytochemical constituents are secondary metabolites which in difference to primary metabolites, are not directly involved in growth, development or reproduction of organisms but have formed the basis of medicine (Gomes *et al*., 2009; Jenke-Kodama *et al*., 2008; Maganha *et al*., 2010). Some flavonoids are known to possess potent analgesic properties (Ching and Faloduna, 2011; Manthey *et al*., 2001; Meotti *et al*., 2006). More so, flavonoids are known to potently inhibit prostaglandins, which are pro–inflammatory signalling molecules. Some flavonoids inhibit phosphodiesterases involved in cell activation (Kumar *et al*., 2013; Manthey *et al.*,

2001) the effect of which is on the biosynthesis of protein cytokines that mediate adhesion of circulating leucocytes to sites of injury. The presence of alkaloids, glycosides, steroids, saponins, tannins, terpenoids and flavonoids in the aqueous methanol stem bark extract of *M. angolensis* has earlier been reported in literature (Adamu *et al*., 2007; Magaji *et al*., 2009).

The drawback of writhing test is that it has poor specificity - drugs such as muscle relaxants used as ‗adjuvant' in pain management exhibit antinociceptive activity in this test, leaving gap for the misinterpretation of results (Pietrovski *et al*., 2006). Furthermore, the results of this writhing test alone cannot establish whether the antinociception was central or peripheral. In view of the disadvantage of writhing test, formalin test was employed to assess the antinociceptive properties of various solvent extracts of the stem bark and to determine the most potent solvent extract in the test. The results indicated that the petroleum ether, ethyl acetate and aqueous ethanol stem bark extracts of *Maerua angolensis* were effective in formalin test, also a chemical-induced pain model, suggesting peripheral and central antinociceptive activity. This too may be another confirmation to the traditional use of *Maerua angolensis* for the management of pain.

Formalin test is the most predictive model of acute tonic pain and unlike writhing test has an advantage of discriminating pain into central and/or peripheral components (Higgs *et al*., 2013; Trongaskul *et al*., 2003). It has been reported that formalin–induced persistent nociception in rodents paws produced a marked biphasic licking response (Hunskaar and Hole, 1987). The first phase (early or neurogenic phase) of the nociceptive behaviour (paw licking/biting response) after formalin injection which starts immediately after injection might be due to direct stimulation of nociceptors such as transient receptor potential ankyrin1 ($TRPA_1$) and transient receptor potential

vanilloid1 ($TRPV_1$) receptors by formalin or involvement of substance P (SP) and bradykinin in nociceptors sensitization, while the second phase (late or inflammatory phase) which appears a little later is taken to be due to a combination of an inflammatory reaction in the peripheral tissue and changes in central processing (Tjølsen *et al*., 1992). Hunskaar and Hole (1987) have established that central analgesics, such as opioids (morphine) inhibit both phases, while peripherally acting agents, such as steroids (hydrocortisone) and NSAIDs (diclofenac) curb mainly the late phase.

A significant and dose–dependent antinociceptive effect was obvious for the tested MABPEE, MABEAE and MABHAE against both neurogenic and inflammatory pain behaviour caused by formalin injection in rats similar to morphine. Analgesic effect in the second phase of the formalin test is predictive of anti-hyperalgesic activity of the extracts in neuropathic pain models (Fishbain *et al.*, 2000; Le Bars *et al*., 2001; Taneja *et al*., 2013) but this needs further investigation. It is possible that the mechanism of action of the solvent extracts could be peripherally by blocking SP or bradykinin known to be involved in nociceptor sensitization or by inhibiting $TRPA_1$ or $TRPV_1$ receptors at the spinal site in phase 1. It could also be centrally by blocking pro-inflammatory pain mediators known to be involved in phase 2 or by inhibiting nociceptive effects of transmitters like glutamate which act as descending pain facilitators.

In inflammatory pain, arachidonic acid is converted into a variety of intermediate substances with the help of endogenous enzymes, cyclooxygenase-1 and -2 which are elevated resulting in an increase in prostaglandin E_2 production. Melgaard *et al*. (2013) suggested that prostaglandin E_2, may mediate an increase in nitric oxide (NO)

production resulting in increased vasodilatation and capillary permeability leading to oedema and sensitization of pain fibres. It is likely the solvent extracts are acting by inhibiting the enzyme cyclooxygenase, thereby decreasing prostaglandin production which otherwise would cause pain.

The TLC analyses of the various solvent extracts of the stem bark showed several constituents in the extracts which were confirmed by phytochemical analysis to be the various secondary metabolites. Additionally, the TLC profile of MABPEE and MABEAE were similar indicating possibility of collective elution of components with similar Rf values which could be responsible for their similar antinociceptive activity. MABPEE was more potent in inhibiting the neurogenic pain and MABEAE more potent in blocking pain emanating from inflammation. In contrast, the TLC profile of MABHAE was different. Petroleum ether is a non-polar solvent and therefore it might have eluted the non-polar constituents of the stem bark. On the other hand aqueous ethanol being a polar solvent might have eluted the polar constituents of the stem bark. Ethyl acetate then might have eluted other constituents of the stem bark which could be polar or non-polar. MABPEE and MABEAE in equal amount were thus combined and subsequently referred to as MAE which was fractionated to examine which fraction has the antinociceptive activity and to aid isolate the compound(s) responsible for the activity.

2.5 CONCLUSION

The present study shows that the petroleum ether, ethyl acetate and aqueous ethanol extracts of the stem bark of *Maerua angolensis* have antinociceptive effects. The petroleum ether extract was potent in the neurogenic pain and the ethyl acetate extract most potent in the inflammatory pain. The TLC profile of MABPEE and

MABEAE were similar indicating possibility of collective elution of components with similar Rf values thus the two were combined and referred to as MAE. Phytochemical analysis confirmed the constituents in the extracts to be saponins, tannins, flavonoids, terpenoids, steroids, alkaloids, glycosides as well as oils and fats. The results support the traditional uses of this plant in neurogenic and inflammatory pain.

2.6 RECOMMENDATIONS

The following are being recommended for further work:

- Investigate antinociceptive effects of MAE and fractions (F1 and F32) in various experimental animal models of pain including thermal and chemical models of nociception to further substantiate the traditional claim of pain relief by the plant
- Assess effect of MAE and fractions on morphine dependence withdrawal symptoms because treatment of acute morphine dependence and withdrawal up to date is limited to opioid replacement therapy and symptomatic treatment of withdrawal signs
- Rotarod test to determine whether the antinociceptive effects of MAE and fractions are due to sensory blockade or impairment of motor function.

Chapter 3

ANALGESIC ACTIVITY OF MAE AND FRACTIONS

3.1 INTRODUCTION

Some medicinal plants including *Maerua angolensis* have a long history of use in traditional medicine to manage pain. In the previous chapter, it was shown that *Maerua angolensis* hydroethanolic leaf, root and stem bark extracts possessed antinociceptive

effect in writhing test with the stem bark extract being most potent. Since the petroleum ether and ethyl acetate stem bark extracts were most potent in the neurogenic and inflammatory pain, MAE and its fractions were selected for further study in various experimental animal models of pain including chemical and thermal models of nociception to further substantiate this traditional claim of pain relief. The effect of MAE and fractions (F1 and F32) on withdrawal syndrome of morphine dependence in mice was also assessed because treatment of acute morphine dependence and withdrawal up to date is limited to opioid replacement therapy and symptomatic treatment of withdrawal signs. The antinociceptive effects of MAE and fractions were finally assessed for motor performance in mice using the rotating rod method.

3.2 MATERIALS AND METHODS

3.2.1 Animals

Sprague–Dawley rats (190 – 200 g) and ICR mice (20 – 25 g) (n = 5) of either sex were used in the study. All animals were housed, fed and cared for as described previously (*section 2.2.4*).

3.2.2 Drugs and chemicals

The following drugs and chemicals were used: Acetic acid, aminophylline, carrageenan sulphate and formalin (BDH, Poole, England), diclofenac sodium
(Troge Medical GmbH, Hamburg, Germany), morphine hydrochloride (Phyto–Riker, Accra, Ghana), naloxone, muscimol, baclofen and bicuculline (Sigma-Aldrich Inc., St. Louis, MO, USA). All drugs, extract and fractions used in the nociceptive tests were prepared as described earlier (*section 2.2.5*).

3.2.3 Analgesic activity of MAE and fractions in the writhing test

The antinociceptive effect of the extract and fractions was assessed in writhing test, a model that is very sensitive and popular in detecting peripherally acting analgesic agents. MAE, F1, F32 (3, 10 and 30 mg/kg, *p.o.*), diclofenac (3, 10 and 30 mg/kg, i.p.) or normal saline (10 ml/kg, i.p.) were administered to thirteen groups of male mice. Acetic acid (0.6% v/v) was given (10 ml/kg, i.p.) 1 h after the *p.o*. and 30 min after the i.p. administration to all mice. Mice were then placed individually in a testing chamber (Perspex chamber, 15×15×15 cm). The number of writhing for 30 min were recorded for analysis with a camcorder and tracking of the behaviour was done with the help of the public domain software JWatcher™ as described (*section 2.2.6.1*). A significant reduction in the number of writhing by any treatment compared with control treated mice was considered as an antinociceptive response (*section 2.2.6.1*).

3.2.4 Analgesic activity of F1 and F32 in the formalin test

The formalin test was carried out as described (*section 2.2.6.3*). Each male rat was assigned and acclimatized to one of 25 formalin test chambers (Perspex chamber 15×15×15 cm) for 30 min before the test. The rats were then given F1, F32 (3 - 30 mg/kg, *p.o.*), morphine (0.3, 1 and 3 mg/kg, i.p.) or normal saline (10 ml/kg, i.p.). Formalin (5%) was given by intraplantar injection (10 µl) in the right hind paw 1 h after the *p.o*. and 30 min after the i.p. administration to all rats to induce pain as described in *section 2.2.6.3*. The animals were immediately returned individually into the testing chamber and their nociceptive behaviours were then captured (1 h) for analysis with a camcorder and tracking of the behaviour was done with the help of the public domain software JWatcher™ as described (*section 2.2.6.3*). The average nociceptive score for each time block was calculated by multiplying the frequency and time spent in biting/licking of the injected paws. The first phase of the nociceptive response normally peaks 0

– 5 min and the second phase 15 – 30 min after formalin injection corresponding to the neurogenic and inflammatory pain responses respectively (*section 2.2.6.3*). Data were expressed as the mean ± SEM of scores between 0 – 10 min (first phase) and 10 – 60 min (second phase) after formalin injection.

3.2.5 Analgesic activity of MAE and fractions in the tail-flick test

Tail-flick latencies in Hargreaves thermal nociception model were determined in mice. Thermal nociceptive latencies were measured in the mouse tail by means of radiant heat source using the IITC Model 336 Paw/Tail stimulator Analgesia Meter (Woodland Hills, CA., USA) (Fecho *et al*., 2005; Galbraith *et al*., 1993; Meotti *et al*., 2006; Moriyama *et al*., 2005; Negus *et al*., 2006). Mice were individually placed in a transparent plexi-glass observation chamber on a clear glass platform for acclimatization period of 15 min in the testing environment. The test head of the paw/tail stimulator was used to present a focused beam of radiant light to the distal portion of the tails. The idle intensity of the light (intensity of light innocuous to the animals) was set at 10% of the maximum intensity and it was used to accurately direct the beam of light to the appropriate region of the tail, while the active intensity of the light was set at 50% maximum. The thermal nociceptive stimulus was manually directed to the tail resulting into a focused beam of radiant light being delivered to the tail until the mouse flicked the tail. Basal reaction times of mice were taken before the administration of MAE, F1, F32 (3 - 30 mg/kg, *p.o.*), morphine (0.3 - 3 mg/kg, i.p.) or normal saline (10 ml/kg, i.p.). A timer was set to automatically turn off the light source when the mouse withdrew the tail, and the tail withdrawal latency (TWL) recorded was defined as the time required for the tail to show an abrupt withdrawal. TWLs were measured again at 1, 2, 3 and 4 h post–drug administration. A cut–off time of 25 s was used in order not to cause any tissue injury to the tail. Mice received two training

sessions before the day of testing. Antinociceptive effects exerted by drugs were calculated from the TWLs as a percentage of the maximum possible effect (% MPE) using the following formula:

$\frac{T2-T1}{T0-T1} \times 100$ where T1 and T2 are the pre- and post-drug latencies respectively, and T0 is the cut-off time.

3.2.6 Analgesic activity of MAE and fractions in the paw withdrawal test Paw withdrawal latencies (PWLs) in Hargreaves thermal hyperalgesia model were determined in the rats‘ paws similar to the thermal nociceptive latencies measured in the mouse tail (*section 3.2.5*). However, the test head of the paw/tail stimulator was used to present a focused beam of radiant light on to the mid plantar region of the right hind paws. The thermal nociceptive stimulus was manually directed under the foot pad before and after the intraplantar injection of carrageenan into the right hind paw. Baseline measurements were taken followed by administration of carrageenan (100 µl of a 2% solution) into the right hind paw 1 h for the *p.o*. or 30 min for the i.p. route post treatment with MAE, F1, F32 (3 - 30 mg/kg, *p.o*.), morphine (0.3 - 3 mg/kg, i.p.) or normal saline (10 ml/kg, i.p.). A timer was set to automatically turn off the light source when the rat withdrew the paw, and the PWLs (time required for the paw to show an abrupt withdrawal) recorded. PWLs were measured again hourly for 4 h post carrageenan administration. Rats received two training sessions before the day of testing. A cut–off time of 25 s was chosen as the maximum time the rat‘s paw will be stimulated with the light in order to prevent any tissue damage. Antihyperalgesic effects exerted by drugs were calculated from the PWLs as % MPE using the formula described previously (*section 3.2.5*).

3.2.7 Assessment of the effect of MAE and fractions on the withdrawal syndrome of morphine dependence

To induce morphine dependence, morphine was injected subcutaneously (s.c.) to mice at doses of 50, 50 and 75 mg/kg three times daily at 10.00, 13.00 and 16.00 h, respectively for 3 days. On day 4, a single final dose of morphine (50 mg/kg) was injected (Hosseinzadeh and Nourbakhsh, 2003; Roome *et al*., 2011). To precipitate morphine withdrawal, naloxone was injected (5 mg/kg, s.c.) 2 h after the last administration of morphine. After the naloxone challenge, mice were immediately placed in a transparent glass cylinder (30 cm high, 20 cm in diameter). The number of jumping episodes (withdrawal symptoms) was recorded for 30 min. Different groups of morphine-dependent mice (as described above) were pre-treated either with MAE, F1, F32 (3 – 30 mg/kg, *p.o*.), muscimol (0.5, 1 and 2 mg/kg, i.p.), baclofen (0.5, 2 and 3 mg/kg, i.p.) or normal saline (10 ml/kg, i.p.). An hour after the *p.o*. and 30 min after the i.p administration, the final dose of morphine was administered 2 h before naloxone and the number of jumps for 30 min recorded.

To investigate the possible mechanisms of MAE and F1 inhibition of withdrawal syndrome of morphine dependence, different groups of morphine-dependent mice were pre-treated with MAE or F1 (10 mg/kg, *p.o*.) and bicuculline (3 mg/kg, i.p.) or aminophylline (20 mg/kg, i.p.) 1 h before the final dose of morphine. Naloxone (5 mg/kg, s.c.) was administered 2 h after the last dose of morphine and the number of jumps for 30 min recorded. The results are stated as a change in the number of jumps as compared to the control or to MAE or F1.

3.2.8 Measurement of motor performance in the analgesic effect of MAE and fractions

The motor function test using the rotarod apparatus was performed in order to determine whether any of the observed antinociceptive effects of the extract and fractions resulted from sensory blockade or from an impairment of motor function (Gareri *et al.*, 2005; Kondo *et al.*, 2008). This is more so as models such as acetic acid-induced writhing test is non-specific for evaluating antinociceptive effects of drugs since drugs such as muscle relaxants used as ‗adjuvant' in pain management show antinociceptive effect in this model (Pietrovski *et al*., 2006). Naïve mice were trained for three successive days on the rotarod (Ugo Basile, model 7600, Comerio, Varese, Italy) at the speed of 25 rev per min. A preliminary selection of mice was made on the previous day of experiment excluding those that did not remain on the rotarod bar during a 2 min session each. On the test day, selected mice (ten groups) were tested 1 h for the *p.o*. or 30 min for the i.p. route after receiving MAE, F1, F32 (3 - 30 mg/kg, *p.o*.) or normal saline (10 ml/kg, i.p.). The mice were repeatedly tested for their motor coordination performance on the rotarod (cut off time 120 s) hourly for 4 h after drug administration. Impairment of coordinated motor movements was defined as the inability of the mice to remain on the rotarod for a test period of 120 s.

3.2.9 Data analysis

Data were expressed as mean ± SEM per group. Raw data for the paw withdrawal and tail-flick tests were calculated as the % MPE. The time-course curves were subjected to two-way ANOVA (treatment × time) with Bonferroni's *post hoc* test.

Total nociceptive score for each treatment was calculated in arbitrary unit as the AUC. To determine the percentage inhibition for each treatment, the following formula was used:

$$\%\ inhibition = \frac{AUC_{control} - AUC_{treatment}}{AUC_{control}} \times 100$$

Differences in AUCs were analyzed using one-way ANOVA with drug treatment as a between subjects factor. Further comparisons between vehicle and drug-treated groups were performed using the Newman-Keuls' *post hoc* test. ED_{50} for each drug and 95% confidence interval values were determined by using an iterative computer least squares method, with the following nonlinear regression (three-parameter logistic) using the formula:

$$Y = \frac{a + (b - c)}{(1 + 10^{(Log\ ED_{50} - X)})}$$

Where X is the logarithm of dose and Y is the response. Y starts at a (the bottom) and goes to b (the top) with a sigmoid shape.

The fitted mid-points (ED_{50}s) of the curves were compared statistically using *F* test (Ramón-Azcón *et al*., 2008). GraphPad® Prism for Windows version 5.01 (GraphPad® Software, San Diego, CA, USA) was used for all statistical analyses and ED_{50} determinations. $P<0.05$ was taken to be statistically significant.

3.3 RESULTS

3.3.1 Analgesic activity of MAE and fractions in the writhing test

Injection of acetic acid i.p. produced writhing, exhibited as an exaggerated distension of the abdomen combined with the outstretching of the hind limbs during the 30 min observation period in control mice pre-treated with normal saline. MAE, F1, F32 and diclofenac dose-dependently and significantly reduced the time-course curve of acetic acid-induced abdominal constrictions (Figure 3.1 a, c, e and g). Two-way

ANOVA (treatment × time) revealed a significant (MAE: $F_{3, 112}$ = 25.29; P<0.0001, F1: $F_{3, 112}$ = 13.55; P<0.0001, F32: $F_{3, 112}$ = 33.08; P<0.0001 and diclofenac: $F_{3, 112}$ = 47.43; P<0.0001) effect of drug treatments on the acetic acid-induced abdominal constrictions.

MAE, F1 and F32 (3 – 30 mg/kg, *p.o.* 1 h before acetic acid injection) dosedependently and significantly ($F_{3, 16}$ = 6.346, P=0.0049; $F_{3, 16}$ = 4.354, P=0.0201 and $F_{3, 16}$ = 11.27, P=0.0003) reduced the number of abdominal writhes over 30 min with maximal inhibition of 89.3 ± 9.3; 67.4 ± 19.2 and 78.6 ± 15.5% respectively

(Figure 3.1 b, d and f) at doses of 30 mg/kg. Similarly, diclofenac (3 – 30 mg/kg, i.p. 30 min before acetic acid injection) dose-dependently and significantly ($F_{3, 16}$ = 14.50, P<0.0001) suppressed the acetic acid-induced writhes by a maximum of 87.9 ± 10.7% (Figure 3.1 h) at the dose of 30 mg/kg.

Table 3.1 shows the ED_{50} values for MAE, F1, F32 and diclofenac in the writhing test in mice. F32 was more potent (3.673 ± 0.19 mg/kg) than F1 and MAE though less potent than diclofenac (2.250 ± 0.17 mg/kg).

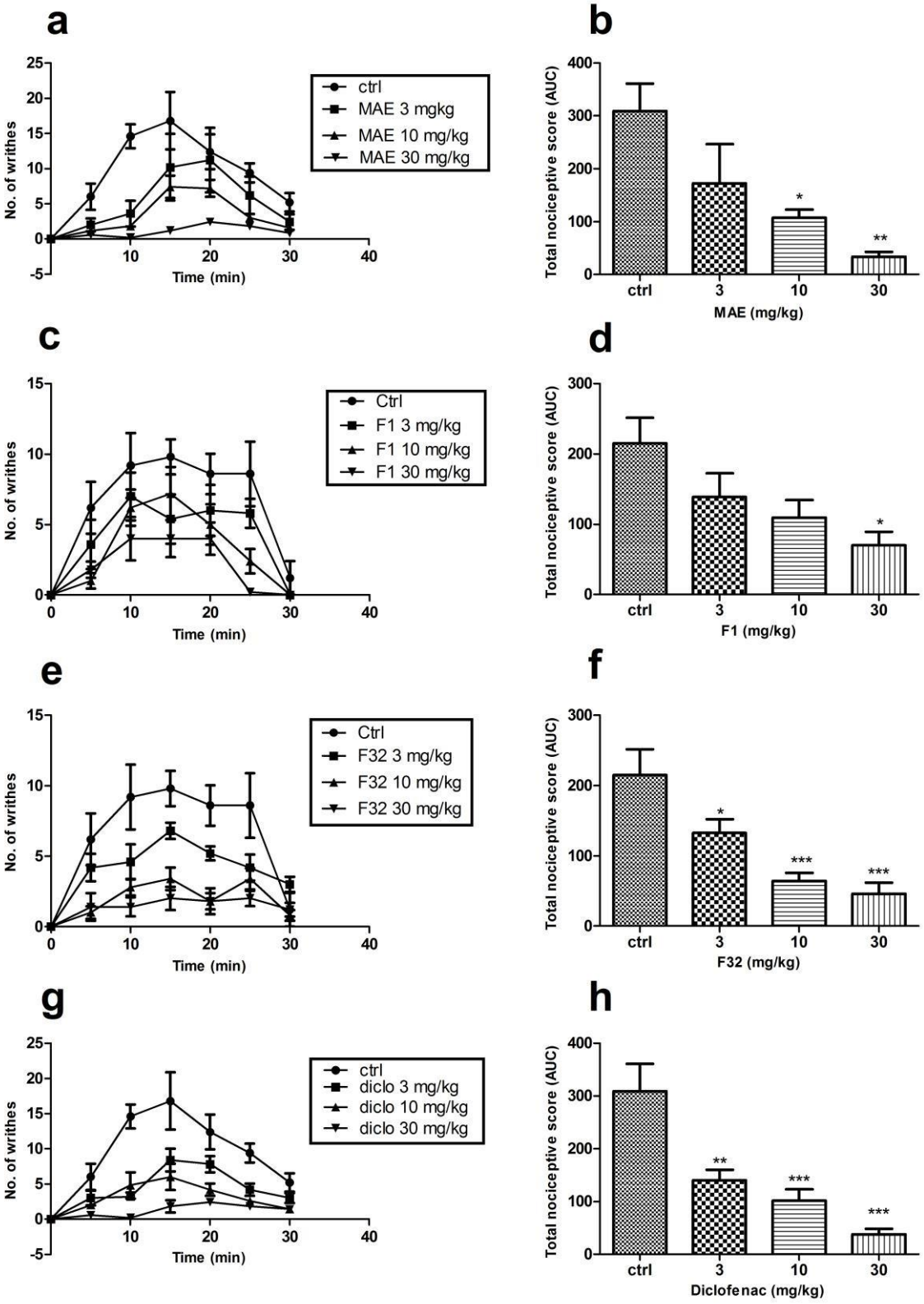


Figure 3.1 Effect of MAE, F1, F32 (3 – 30 mg/kg, *p.o.*) and diclofenac (3 – 30 mg/kg, i.p.) on the time course curve of acetic acid-induced abdominal writhes (a, c, e and g) and the total nociceptive score (calculated as AUC) (b, d, f and h) in ICR mice. Data are expressed as mean ± SEM (n = 5). *P<0.05; **P<0.01; ***P<0.001 compared to vehicle-treated group (one-way ANOVA followed by Newman-Keuls *post hoc* test).

Table 3.1: ED_{50} values for MAE, F1, F32 and diclofenac in the writhing test

Treatment	ED_{50} (mg/kg)
MAE	4.176 ± 0.34
F1	3.687 ± 0.39
F32	3.673 ± 0.19
Diclofenac	2.250 ± 0.17

Values are expressed as mean ± SEM, (n = 5)

3.3.2 Analgesic activity of F1 and F32 in the formalin test

Intraplantar injection of 5% formalin (10 µl) into the dorsal surface of the rat right hind paw produced a biphasic nociception, exhibited as biting, licking and flinching of the injected paw seen more in control rats pre-treated with normal saline. This characteristic biphasic licking response consisted of an initial intense response to pain which start immediately after formalin injection and lasted for 10 min (first or neurogenic phase) with maximum effect at approximately 5 min followed by a slowly rising but longer-lasting response (second or inflammatory phase) from 10 min and lasted until 1 h with maximum effect at approximately 30 min after formalin injection.

Administration of F1, F32 (3 – 30 mg/kg, *p.o.*) and morphine (0.1 – 3 mg/kg, i.p.) to the rats dose-dependently and significantly attenuated the maximal algesic effect of formalin-induced nociception when compared with the vehicle-treated group (Figure 3.2 a, c and e). Two-way ANOVA (treatment × time) revealed a significant (F1: $F_{3, 208} = 103.3$, $P<0.0001$; F32: $F_{3, 208} = 245.7$, $P<0.0001$ and morphine: $F_{3, 208} = 313.6$, $P<0.0001$) effect of drug treatments on the formalin-induced nociception.

F1 and F32 (3 – 30 mg/kg, *p.o.*) 1 h before formalin injection dose-dependently and significantly suppressed total nociception on the neurogenic ($F_{3, 16}$ = 34.47, P<0.0001 and $F_{3,16}$ = 119.5, P<0.0001) and the inflammatory ($F_{3, 16}$ = 152.6, P<0.0001 and $F_{3, 16}$ = 1105, P<0.0001) phases over 1 h with maximal inhibition of the neurogenic as 92 ± 7 and 98 ± 2% and the inflammatory phase as 93 ± 4 and 93 ± 1% respectively (Figure 3.2 b and d) at doses of 30 mg/kg when compared with control rats. In a similar manner, morphine pre-treatment (0.3 – 3 mg/kg, i.p.) 30 min before formalin injection resulted into a distinct dose-dependent and significant reduction of response time in the early ($F_{3, 16}$ = 145.1, P<0.0001) and the late ($F_{3, 16}$ = 1682, P<0.0001) phases of formalin-induced licking with maximal inhibition of the neurogenic as 96 ± 1% and the inflammatory phase as 97 ± 1% (Figure 3.2 f).

The calculated mean ED_{50} values for the antinociceptive effects of F1, F32 and morphine in the formalin test (Table 3.2) showed F1 was more potent in inhibiting the neurogenic pain and F32 more potent in blocking pain emanating from inflammation. Morphine was, however, most potent in both the neurogenic and the inflammatory phase of the formalin-induced licking.

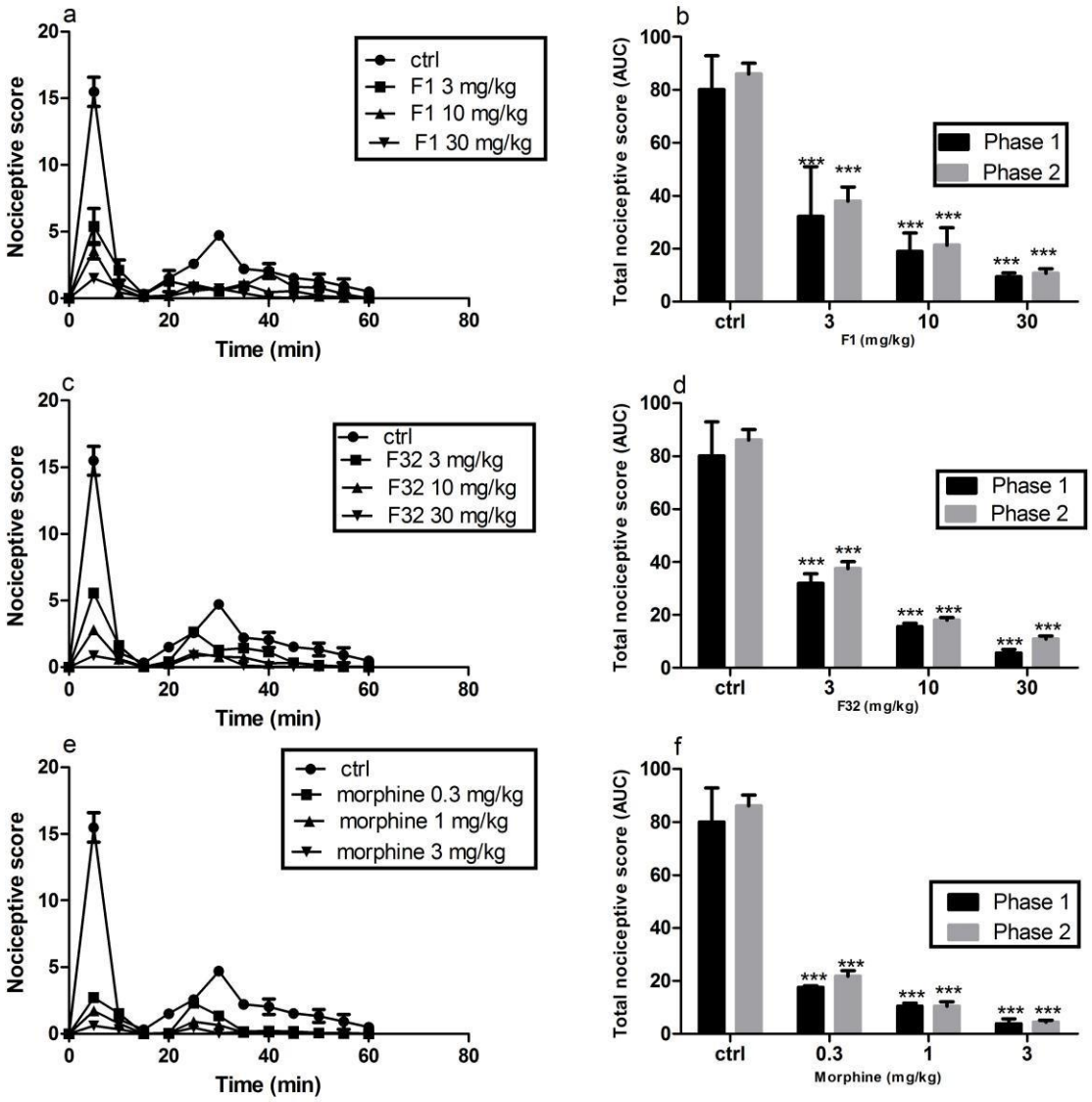


Figure 3.2 Effect of F1, F32 (3 – 30 mg/kg, *p.o.*) and morphine (0.3 – 3 mg/kg, i.p.) on the time course curve of formalin-induced nociception (a, c and e) and the total nociceptive score (calculated as AUC) (b, d and f) in Sprague-Dawley rats. Data are expressed as mean ± SEM (n = 5). ******P*<0.001 compared to vehicle-treated group (one way ANOVA followed by NewmanKeuls *post hoc* test).

Table 3.2: ED values for F1, F32 and morphine in the formalin test

Treatment	ED_{50} (mg/kg) in early phase	ED_{50} (mg/kg) in late phase
F1	1.66 ± 0.18	2.46 ± 0.09
F32	1.94 ± 0.04	2.18 ± 0.03
Morphine	0.07 ± 0.04	0.11 ± 0.03

Values are expressed as mean ± SEM, (n = 5)

3.3.3 Analgesic activity of MAE and fractions in the tail-flick test

Significant increase in tail-flick latency calculated as % MPE was observed in all mice pre-treated with test drugs [(MAE: $F_{3, 80}$ = 57.71, P<0.0001; F1: $F_{3, 80}$ = 119.9, P<0.0001; F32: $F_{3, 80}$ = 135.4, P<0.0001; morphine: $F_{3, 80}$ = 136.8, P<0.0001) twoway ANOVA (treatment × time) (Figure 3.3 a, c, e and g)]. MAE (3 – 30 mg/kg, *p.o.*) produced a significant ($F_{3, 16}$ = 22.38, P<0.0001), dose-related reduction of thermal nociception with maximum effect at the highest dose used (Figure 3.3 b). F1 and F32 (3 – 30 mg/kg, *p.o.*) respectively also dose-dependently and significantly ($F_{3, 16}$ = 63.36, P<0.0001 and $F_{3, 16}$ = 59.73, P<0.0001) attenuated thermal nociception in the mice (Figure 3.3 d and f). Morphine, the reference analgesic (0.3 – 3 mg/kg, i.p.) exhibited significant ($F_{3, 16}$ = 75.56, P<0.0001), dose-related antinociception with a maximum effect at 3 mg/kg (Figure 3.3 h).

The ED_{50} values (Table 3.3) in the tail-flick test showed antinociception of morphine (0.2092 ± 0.1063 mg/kg) was more potent than F1 (1.656 ± 0.1224 mg/kg) and F32.

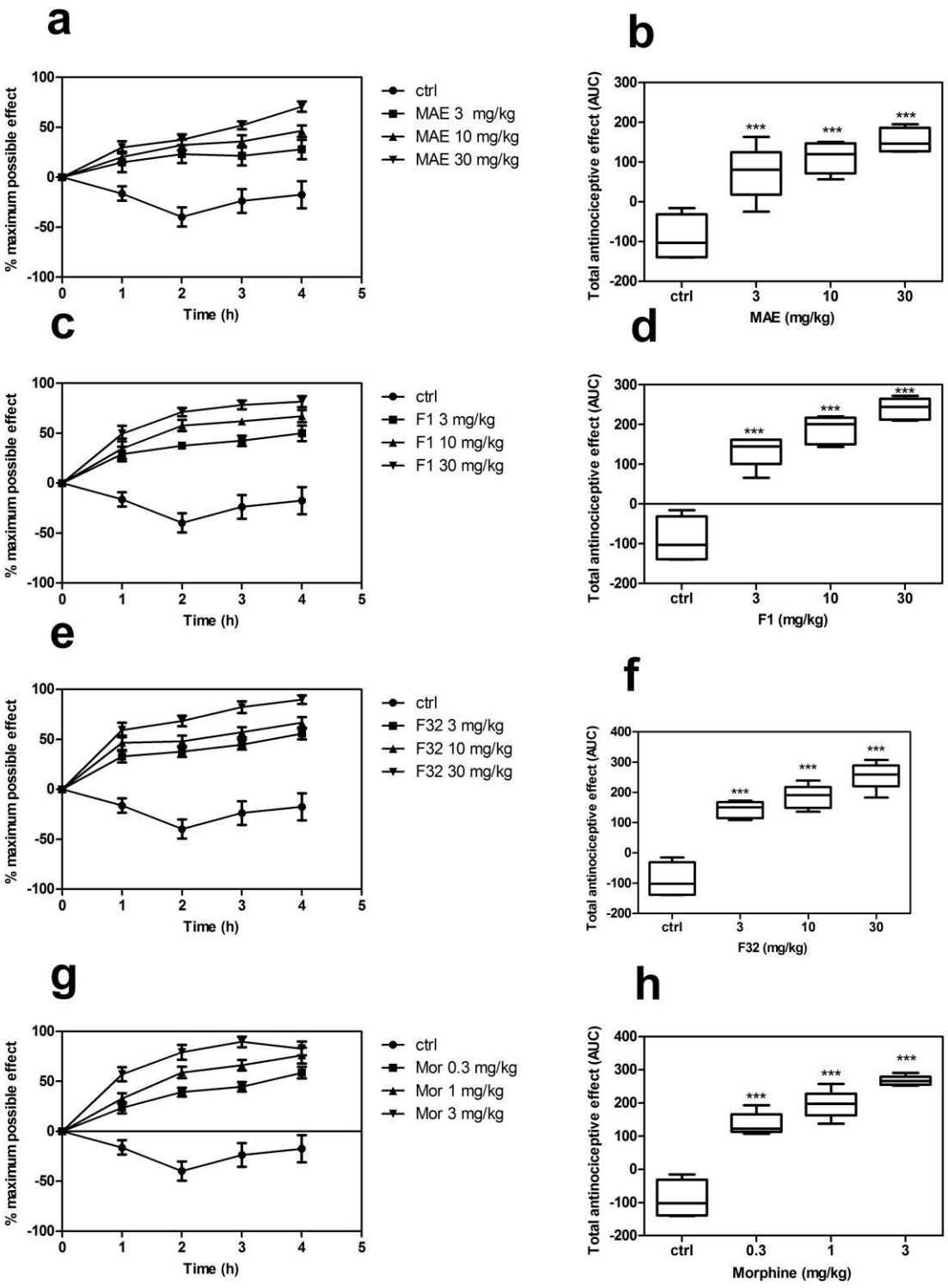


Figure 3.3 Effect of MAE, F1, F32 (3 – 30 mg/kg, *p.o.*) and morphine (0.3 – 3 mg/kg, i.p.) on the time course curve of tail-flick in Hargreaves thermal hyperalgesia model (a, c, e and g) and the total antinociceptive score (calculated as AUC) (b, d, f and h) in ICR mice. Data are expressed as mean ± SEM (n = 5). The lower and upper margins of the boxes (b, d, f and h) represent the 25th and 75th percentiles, with the extended arms representing the 10th and 90th percentiles, respectively. The median is shown as the horizontal line within the box. ***P<0.001 compared to vehicle-treated group (one-way ANOVA followed by Newman-Keuls *post hoc* test).

Table 3.3: ED values for MAE, F1, F32 and morphine in the Hargreaves tail-flick test

Treatment	ED_{50} (mg/kg)
MAE	1.760 ± 0.2235
F1	1.656 ± 0.1224
F32	1.680 ± 0.1403
Morphine	0.209 ± 0.1063

Values are expressed as mean ± SEM, (n = 5)

3.3.4 Analgesic activity of MAE and fractions in the paw withdrawal test

Paw withdrawal latency was higher for the tested drugs compared to the control group. Two-way ANOVA (treatment × time) showed dose-related reduction of time course curve which was significant [(MAE: $F_{3, 80}$ = 323.8, P<0.0001; F1: $F_{3, 80}$ = 335.8, P<0.0001; F32: $F_{3, 80}$ = 296.6, P<0.0001; morphine: $F_{3, 80}$ = 320.3, P<0.0001) (Figure 3.4 a, c, e and g)]. MAE (3 – 30 mg/kg, *p.o.*) caused a significant ($F_{3, 16}$ = 81.06, P<0.0001), dose-related reduction of AUC with maximum effect at the highest dose used (Figure 3.4 b). F1 and F32 (3 – 30 mg/kg, *p.o.*) also dosedependently and significantly ($F_{3, 16}$ = 84.61, P<0.0001 and $F_{3, 16}$ = 72.21, P<0.0001) inhibited carrageenan-induced thermal hyperalgesia in the rats respectively (Figure 3.4 d and f). The reference analgesic agent, morphine (0.3 – 3 mg/kg, i.p.) showed significant ($F_{3, 16}$ = 77.34, P<0.0001) and dose-dependent anti-hyperalgesia effect with a maximum effect at 3 mg/kg (Figure 3.4 h).

Table 3.4 shows the ED_{50} values in the paw withdrawal test where the antinociception of morphine was more potent (0.1172 ± 0.0683 mg/kg) than F32

(0.6913 ± 0.0777 mg/kg) and F1.

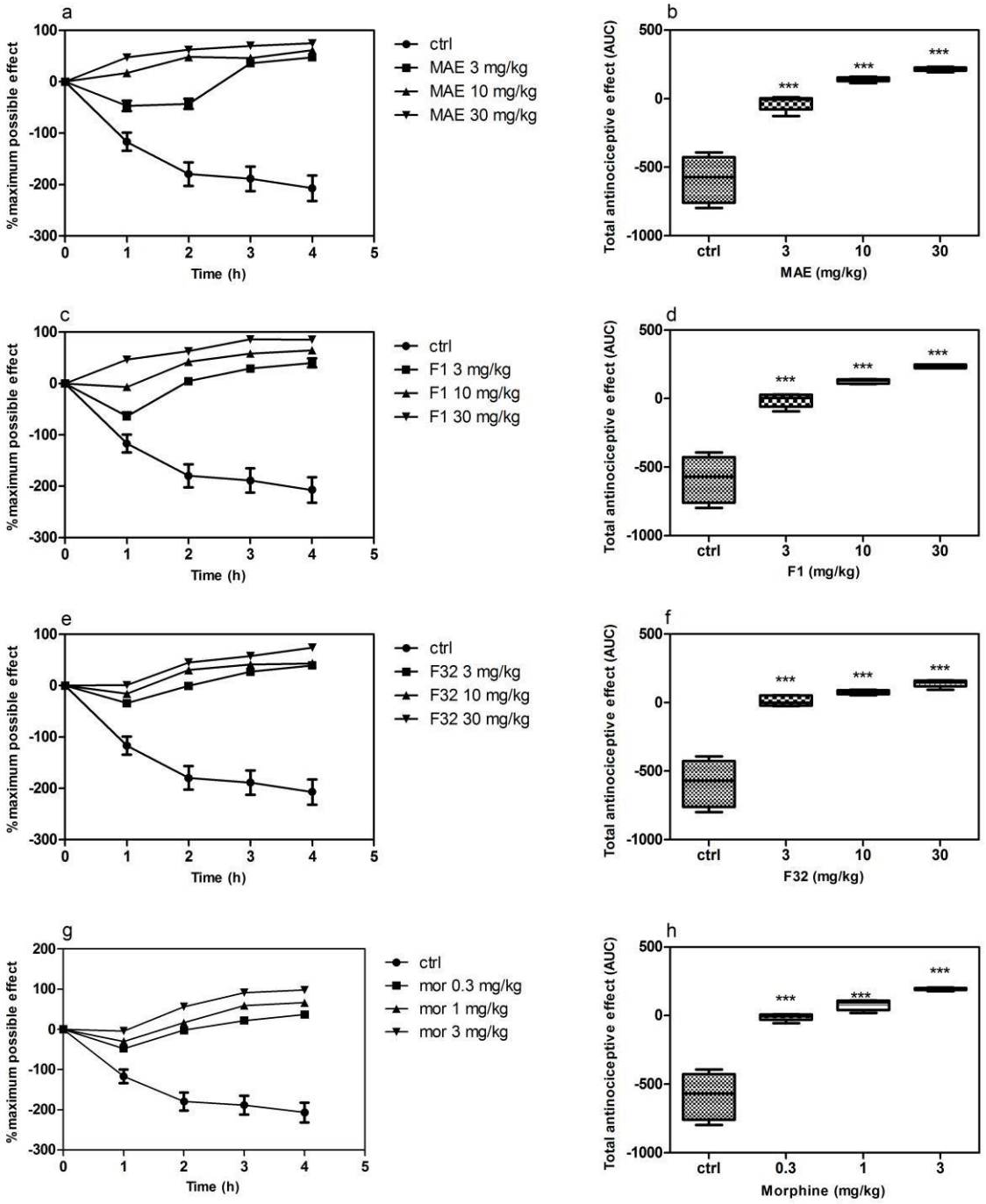


Figure 3.4 Effect of MAE, F1, F32 (3 – 30 mg/kg, *p.o.*) and morphine (0.3 – 3 mg/kg, i.p.) on the time course curve of paw withdrawal in Hargreaves thermal hyperalgesia model (a, c, e and g) and the total antinociceptive score (calculated as AUC) (b, d, f and h) in Sprague-Dawley rats. Data are expressed as mean ± SEM (n = 5). The lower and upper margins of the boxes (b, d, f and h) represent the 25th and 75th percentiles, with the extended arms representing the 10th and 90th percentiles, respectively. The median is shown as the horizontal line within the box. ***P<0.001 compared to vehicle-treated group (one-way ANOVA followed by Newman-Keuls *post hoc* test).

Table 3.4: ED values for MAE, F1, F32 and morphine in the paw withdrawal test

Treatment	ED_{50} (mg/kg)
MAE	1.548 ± 0.0485
F1	1.484 ± 0.0521
F32	0.6913 ± 0.0777
Morphine	0.1172 ± 0.0683

Values are expressed as mean ± SEM, (n = 5)

3.3.5 Assessment of the effect of MAE and fractions on the withdrawal syndrome of morphine dependence

Administration of MAE, F1 and F32 1 h before the last dose of morphine in the presence of naloxone significantly ($F_{3, 16}$ = 6.980, P=0.0032; $F_{3, 16}$ = 4.598, P=0.0167 and $F_{3, 16}$ = 3.152, P=0.0539 respectively) and dose-dependently suppressed the jumping behaviour in mice. MAE, F1 and F32 at the highest doses used blocked the morphine-dependent withdrawal effect by 80.7 ± 7.4, 71.0 ± 12.9 and 67.7 ± 10.6%, respectively (Figure 3.5 a, b and c). The intraperitoneal administration of the $GABA_A$ and $GABA_B$ receptor agonists, muscimol and baclofen (30 min before the final dose of morphine), also significantly ($F_{3, 16}$ = 4.519, P=0.0177 and $F_{3, 16}$ = 14.38, P<0.0001 respectively) and dose-dependently reduced the jumping reaction (Figure 3.5 d and e).

The mechanism involved against morphine withdrawal symptoms was tackled using antagonist of $GABA_A$ receptors (bicuculline) and the non-selective adenosine receptors antagonist (aminophylline). The inhibitory effect of MAE was reversed in the presence of bicuculline and aminophylline (Figure 3.6 a and b, respectively), whereas the effect of F1 was found to be suppressed in the presence of

aminophylline but not bicuculline (Figure 3.6 c and d).

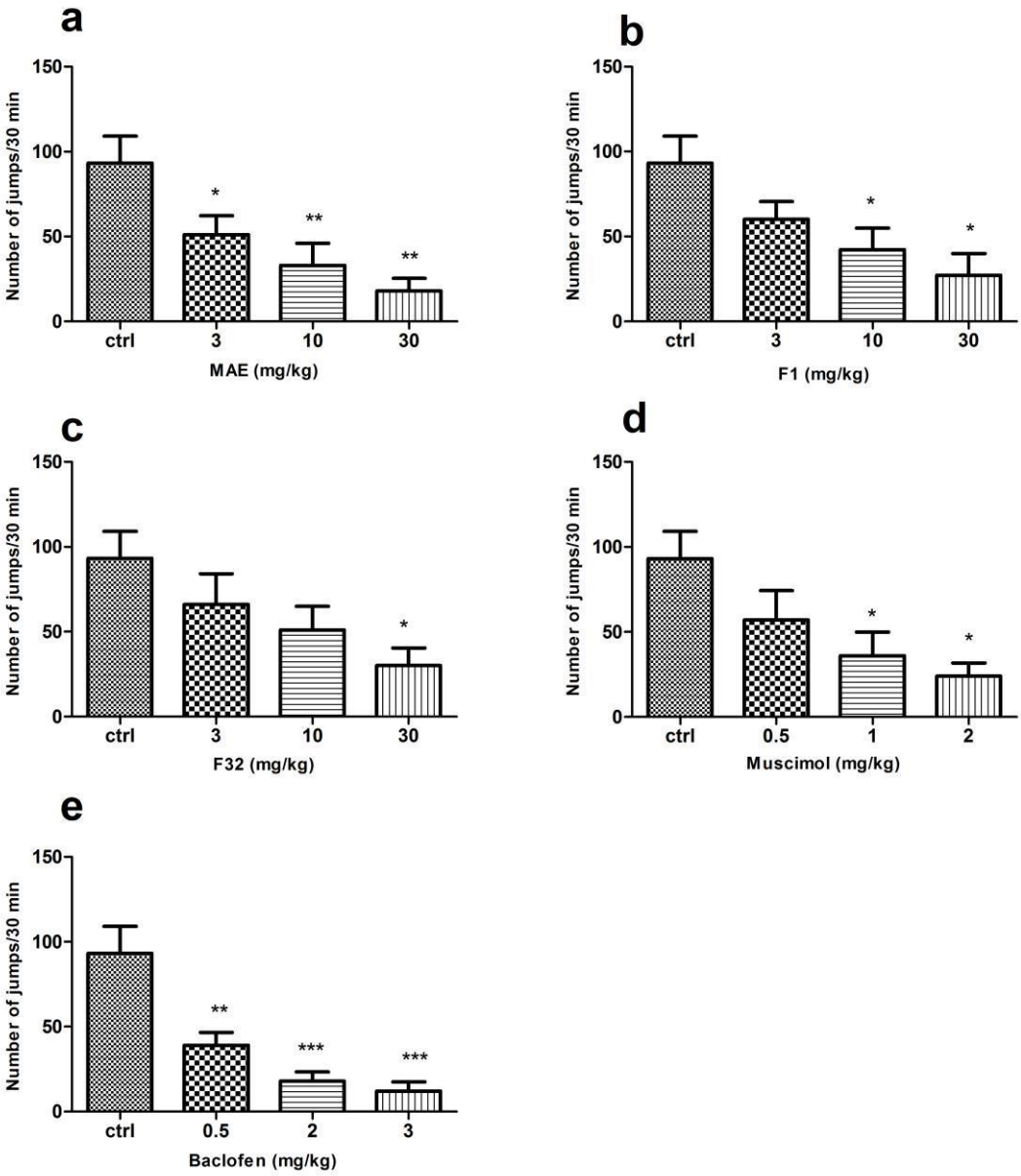


Figure 3.5 Effect of (a) MAE, (b) F1, (c) F32 (3 – 30 mg/kg, *p.o.*), (d) muscimol (0.5 – 2 mg/kg, i.p.) and (e) baclofen (0.5 – 3 mg/kg, i.p.) on the withdrawal syndrome of morphine dependence in ICR mice. Each column represents the mean of 5 mice, and the error bar indicates the SEM. Asterisks denote the significance levels compared with control groups (one -way ANOVA followed by Newman Keuls *post hoc* test): **P*<0.05, ***P*<0.01 and ****P*<0.001

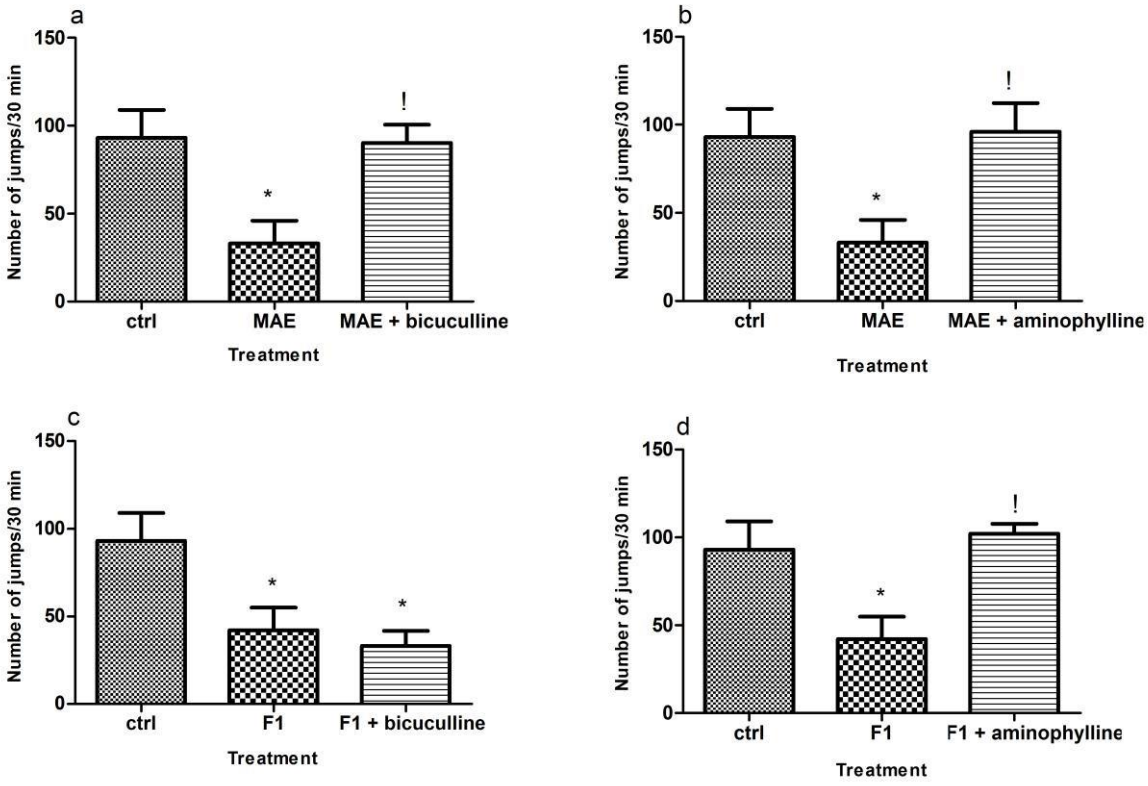


Figure 3.6 Effect of bicuculline (3 mg/kg, i.p.) and aminophylline (20 mg/kg, i.p) on the inhibitory effect of MAE (a and b) and F1 (c and d) against morphine dependence withdrawal symptoms. Each column represents the mean of 5 mice, and the error bar indicates the SEM. *P<0.05 compared to control, !P<0.05 compared to MAE or F1 (one-way ANOVA followed by Newman-Keuls *post hoc* test).

3.3.6 Measurement of motor performance in the analgesic effect of MAE and fractions

Table 3.5 shows the time of mice stay on the rotating bar in the rotarod test. The petroleum ether/ethyl acetate extract and the fractions of *Maerua angolensis* at 1, 2, 3, and 4 h after oral administration did not significantly alter the motor response of the mice at the dose of 3 – 10 mg/kg; however, significantly affect the motor response of the animals at the highest dose (30 mg/kg) when compared with animals that received normal saline. The control response in the rotarod test was 95.24 ± 0.14 verse 94.36 s

in the presence of MAE ($F_{3,\ 16}$ = 4.503, P = 0.0179); F1 ($F_{3,\ 16}$ = 5.046, P = 0.0119) and F32 ($F_{3,\ 16}$ = 5.705, P = 0.0075).

Table 3.5: Effect of the oral treatment of the petroleum ether/ethyl acetate extract and the fractions prepared from the stem bark of *Maerua angolensis* on motor performance of ICR mice in the rotarod test

Treatment	Dose (mg/kg)	Time on the rotating bar (s)
Normal saline	-	95.24 ± 0.14
MAE	3	94.60 ± 0.25
MAE	10	94.52 ± 0.31
MAE	30	94.12 ± 0.10**
F1	3	94.66 ± 0.25
F1	10	94.52 ± 0.31
F1	30	94.12 ± 0.10**
F32	3	94.38 ± 0.18
F32	10	94.42 ± 0.38
F32	30	93.86 ± 0.19**

Data are expressed as mean ± SEM of the total time spent on the rotating bar during a 2 min test, n = 5. Data were analysis by one–way ANOVA, followed by Newman–Keuls *post hoc* test. **P<0.01, significantly different compared to the vehicle-treated group.

3.4 DISCUSSION

Different chemical and thermal models of nociception were used to investigate the antinociceptive activities of MAE, F1 and F32. Results of the study demonstrate that oral administration of the petroleum ether/ethyl acetate extract and fractions prepared from stem bark of *Maerua angolensis* produced significant dose–dependent and distinct antinociceptive effect when evaluated in various models of chemical and thermal nociception in rodents. The antinociceptive effect observed in the chemical model in which acetic acid was used as the algogenic agent is similar to previous studies (Aliyu *et al.*, 2005; Lin *et al*., 2006; Sanchez-Mateo *et al.*, 2006; Sawynok, 2003) which involves the stimulation of local peritoneal receptors and primary afferent sensory Aδ and C nerve fibres leading to generation of visceral pain in the animals. The intraperitoneal injection of acetic acid also induces an increase in the concentration of glutamate and aspartate in the cerebrospinal fluid (Feng *et al*., 2003). Inhibition of writhing by MAE and the fractions is therefore suggestive of peripheral analgesic action probably through inhibition of synthesis and/or release of pro-inflammatory pain mediators peripherally. Antinociception of MAE and fractions may have also occurred spinally through inhibition of pro-inflammatory mediators-mediated central sensitization.

Similarly, the antinociceptive effect observed in the chemical model in which formalin was used as the algogenic agent was similar to previous studies (Fischer *et al.*, 2013; Fishbain *et al*., 2000; Godinez-Chaparro *et al*., 2013; Hunskaar and Hole, 1987; J Cobos and Portillo-Salido, 2013; Taneja *et al*., 2013) involving the bradykinin, SP and transient receptor potential family receptors as well as inflammatory reaction in the peripheral tissue and changes in central processing leading to generation of neurogenic and inflammatory pain in the animals. A significant and dose–dependent

antinociceptive effect was evident for all the tested fractions of *Maerua angolensis* against both neurogenic and inflammatory pain behaviour caused by formalin injection in rats suggesting that they may be effective in neuropathic pain.

It is possible that the mechanism of action of the fractions could be peripheral by blocking substance P or bradykinin known to be involved in nociceptor sensitization or by inhibiting $TRPA_1$ or $TRPV_1$ receptors at the spinal site in phase 1. Since formalin test discriminates pain into central and/or peripheral components, mechanism could also be centrally by blocking pro-inflammatory pain mediators known to be involved in phase 2 or by inhibiting nociceptive effects of transmitters like glutamate which act as descending pain facilitators. It is likely the fractions are acting by inhibiting Cox, thereby decreasing prostaglandin production which otherwise would cause pain. Notwithstanding, the exact mechanism of

antinociception of these fractions needs to be established.

To support that the petroleum ether/ethyl acetate extract and the fractions from the stem bark of *Maerua angolensis* have antinociceptive activity, tail–flick and paw withdrawal tests in Hargreaves thermal hyperalgesia model were conducted. These tests are specific and popular in detecting centrally acting analgesic drugs and have selectivity for opioid–derived analgesics (Gupta *et al*., 2013; Spetea, 2013; Taïwe *et al*., 2011). The primary heat hyperalgesia generated by a strong thermal stimulation in the stimulated skin area is mainly caused by sensitization of primary afferent nociceptors (Raja *et al*., 1984).

It has been demonstrated that the excitation of $TRPV_1$ receptor-bearing primary afferents is essential for the induction states of central sensitization in humans (Jürgens *et al*., 2014; Magerl *et al*., 2001). Specific agonists such as capsaicin and noxious heat selectively excites these afferents. Moreover, it has since been demonstrated that

activation of the mu–opioid receptors mediates antinociceptive effects (Jaremko *et al*., 2014). The extract and fractions then probably exerts central antinociception via activation of opioid receptors similar to morphine (reference analgesic) but this needs further investigation. Activation of mu-opioid receptor inhibits the activity of $TRPV_1$ via $G_{o/i}$ proteins and the cAMP pathway (EndresBecker *et al*., 2007). This result give further credence to the traditional use of the plant in the treatment of pain.

The present results furthermore show that MAE and fractions reduced the morphine withdrawal signs. Withdrawal from acute morphine dependence is accompanied by centrally mediated side effects, such as physical dependence (Roome *et al*., 2011; Zhang and Schulteis, 2008). Physical dependence is distinguished by excessively definite behavioural abstinence signs such as hyperirritability, anxiety and restlessness after withdrawal of morphine or administration of opioids antagonists (Karami *et al*., 2013; Zhang and Schulteis, 2008). In morphine-dependent mice abstinence sign such as jumping is produced on administration of naloxone. In this study, the extract and fractions showed inhibitory outcome against withdrawal syndrome of morphine dependence which was comparable in extent to drugs acting on GABAergic systems, such as muscimol and baclofen. Some neurotransmitters, including GABA, dopamine, noradrenaline, serotonin, adenosine and glutamate have been associated with the expression of opioid withdrawal (Roome *et al*., 2011).

It has earlier been reported that sensitization to opioids seems to be linked with increased dopaminergic transmission in nucleus accumbens which was established to be related to accelerated locomotor, and behavioural response (Bartoletti *et al*., 2007). It is well known that morphine also produces an increase in whole brain GABA concentration (Tabatabai *et al*., 2014). GABA is an inhibitory neurotransmitter which

acts on $GABA_A$ and $GABA_B$ receptors, localized on dopaminergic and glutamatergic neurons, and regulate the release of dopamine and its afferent inputs in nucleus accumbens and ventral tegmental area (Bartoletti *et al.*, 2007). However in intermittence or withdrawal of morphine GABA discharge is consequently decreased which result in up-regulation of dopaminergic system leading to abstinence behaviour (Tabatabai *et al*., 2014). Thus, GABA-related compounds modify the behavioural sensitization to opiates. The result in this study show that MAE and fractions similar to muscimol and baclofen ($GABA_A$ and $GABA_B$ receptor agonists, respectively), blocked naloxone-induced jumping behaviour in morphine-dependent mice in a dose-related way, suggesting involvement of GABAergic system in their antinociception.

The inhibitory effect of MAE against abstinence behaviour was significantly antagonized in the presence of bicuculline ($GABA_A$ receptor antagonist), implying that effect of MAE is mediated through $GABA_A$ receptors. This is consistent with some studies (Bartoletti *et al*., 2007; Ghannadi *et al*., 2012; Hajhashemi *et al*., 2010; Hosseinzadeh and Nourbakhsh, 2003; Karami *et al.*, 2013; Roome *et al*., 2011) that have shown other plants extracts are able to modulate morphine withdrawal syndrome. Therefore, MAE may modulate morphine withdrawal syndrome via potentiating the GABA system.

Similarly, non-selective adenosine receptor antagonist aminophylline suppressed the inhibitory effect produced by MAE and F1 on naloxone-induced withdrawal syndrome in mice signifying their mechanism, at least in part, through adenosinergic system. The role of cAMP and adenosine in acute opioid withdrawal has since been proposed at behavioural level. Chronic morphine treatment up-regulates adenylyl cyclase that leads to an increase in extracellular cAMP and adenosine modulating

GABA release. Though in withdrawal of morphine, there is an adenosine-dependent inhibition of GABA release so, adenosine analogues or an increase in endogenous adenosine neutralizes sign of morphine withdrawal. It is on record that inhibition of A_1 adenosine receptor or adenosine tone inhibits GABA release by inhibition of phosphodiesterase activity (Roome *et al*., 2011). Hence, MAE and F1 effect on GABA release through adenosine system is confirmatory to their inhibitory responses against morphine withdrawal.

Treatment of morphine dependence and withdrawal syndrome is limited to opiate replacement therapy and symptomatic treatment of withdrawal signs (Tabatabai *et al.*, 2014), but from this study and several similar studies (Doosti *et al*., 2013; Tabatabai *et al*., 2014), herbal treatment may be a rational option for the treatment of morphine dependence and withdrawal. In summary, results imply that stimulation of GABAergic system may be a possible way for the antinociception of MAE and F1. This outlook may be valuable to minimize the adverse effects associated with opioid analgesics known to activate NMDA receptors. Additionally MAE and F1 with possibility of being GABA receptor agonists can be effective in neuralgia and chronic pain associated with spasticity.

The results of the study further demonstrates that the antinociceptive activities of orally administered *Maerua angolensis* at doses of 3 and 10 mg/kg did not impair motor function of the mice in the rotarod test, however, there was statistically significant interference in motor performance at 30 mg/kg. The rotarod test is used to assess impairment of motor function in the antinociception of drugs and is useful in complementing models such as writhing test which is non-specific for evaluating antinociceptive effects of drugs since drugs like muscle relaxants used as adjuvant in pain management show analgesic effect in this model (Pietrovski *et al.*, 2006).

3.5 CONCLUSION

The extract and fractions of *Maerua angolensis* stem bark possesses both peripheral and central antinociceptive effects in the murine models of chemical and thermal nociception. Extract and fractions also suppressed morphine withdrawal symptoms via stimulation of GABAergic and adenosinergic transmission. All these contribute to the analgesic knowledge of this species and support the traditional use of the plant in the treatment of pain, so can be exploited for development in therapy.

3.6 RECOMMENDATION

- Assess effect of MAE and fractions on neuropathic pain since MAE and fractions showed antinociceptive effects in the formalin test.

Chapter 4

EFFECT OF MAE AND FRACTIONS ON NEUROPATHIC PAIN 4.1

INTRODUCTION

Neuropathic pain is a chronic pain that arises from a disease or injury to the CNS or the peripheral nervous system (PNS) leading to its damage or abnormal function (Quintans *et al.*, 2014). It has a poor response towards therapy (Quintans *et* al., 2014). Common symptoms of neuropathic pain include sensory abnormalities such as hyperalgesia and allodynia (Schim, 2009). Millions of people worldwide are suffering from neuropathic pain (Hall *et al*., 2006). The transient receptor potential (TRP) family of ion channels have been shown to play a role in neuropathic pain (Alessandri-Haber *et al*., 2004) and because formalin has a direct effect on these receptors in the neurogenic phase, the formalin test is utilized to predict agents that

may be active in neuropathic pain (Vissers *et al*., 2006). Since MAE and fractions showed antinociception in the formalin test, they were also envisaged to be effective in neuropathic pain but this needs further investigation. In this chapter, the analgesic effect of MAE and fractions were therefore assessed in the mouse model of vincristine-induced neuropathic pain.

Vincristine and other chemotherapeutic agents have been reported to produce peripheral neurotoxicity with patients reporting sensory abnormalities and neuropathic pain during and after therapy (Flatters and Bennett, 2004; Lynch *et al*., 2005; Thibault *et al*., 2008). Pain from the malignancy itself and pain from the treatment of the cancer are the two main causes of cancer related pain (Wolf *et al*., 2008). The current analgesics are unable to treat cancer chemotherapy-induced neuropathic pain which is severe enough for patients to discontinue their treatment and thus worsen the quality of their life (Park *et al*., 2012).

4.2 MATERIALS AND METHODS

4.2.1 Animals

ICR mice (20 – 25 g) (n = 5) were utilized in this experiment. The mice were bestowed with the needed conditions as described previously (*section 2.2.4*).

4.2.2 Drugs and chemicals

The following drugs were used: Pregabalin (Lyrica®) from Pfizer Pharmaceuticals, Arzneimittelwerk Godecke, Freiburg, Germany; vincristine sulphate purchased from Celon Laboratory Ltd, Gajularamaram, India.

4.2.3 Vincristine-induced neuropathic pain

Vincristine sulphate was dissolved in normal saline and stored as a stock concentration of 1 mg/10 ml at 4 °C. The mice received vincristine (0.1 mg/kg/day, i.p.) in two cycles of five consecutive days with two days off between the cycles (days 1 – 5 and days 8 – 12) to induce neuropathic pain with no significant motor deficit (Weng *et al*., 2003). On day 15, baseline nociception was measured using the Von Frey (4, 8 and 15 g), Randall-Selitto and cold allodynia (cold water at 4.5 °C) tests. The mice subsequently were treated with MAE, F1, F32 (3 – 20 mg/kg, *p.o.*), pregabalin (10 – 100 mg/kg, *p.o*.) or normal saline (10 ml/kg, i.p.).

Three sets of experiments namely Von Frey (tactile allodynia, intermediate and mechanical hyperalgesia), Randall-Selitto mechanical hyperalgesia and cold allodynia were later conducted in order to investigate the effects of MAE, F1, F32 and pregabalin on vincristine-induced neuropathic pain.

4.2.3.1 Evaluation of tactile allodynia, intermediate and mechanical hyperalgesia

To assess the effect of MAE, F1, F32 and pregabalin on mechanical allodynia/hyperalgesia, mice were put in restrainers and restricted. Tactile allodynia was evaluated by means of Von Frey filaments (IITC Life Science Inc. Model 2888, Woodland Hills, CA, USA) with bending forces of 4 g. Chemotherapy-induced responses to 4 g are best explained as tactile allodynia (pain from a normally innocuous stimulus) as normal mice never withdraw from this stimulus (Flatters and Bennett, 2004; Park *et al*., 2012; Siau *et al*., 2006). Intermediate and mechanical hyperalgesia were evaluated with Von Frey filaments of bending forces of 8 and 15 g respectively. Responses to 15 g are best explained as hyperalgesia (exaggerated pain response from

a normally noxious stimulus) as normal mice withdraw from this stimulus 5 – 10% of the time; while the responses to 8 g are intermediate.

In ascending order of force, each filament was applied to the mid-plantar area (avoiding the base of the tori) of each hind paw five times, with each application held for 5 s. Withdrawal responses to the Von Frey filaments from both hind paws were counted and expressed as an overall percentage (Flatters and Bennett, 2004; Park *et al.*, 2012; Siau *et al*., 2006).

4.2.3.2 Evaluation of mechanical hyperalgesia in the Randall-Selitto test

Mechanical nociception was determined with the IITC Life Science Model 2888 (Woodland Hills, CA, USA). The mouse‘s hind paw was placed into the pressure applicator, and a constantly increasing pressure stimulus (maximum cut-off of 250 g) was applied to the dorsal surface of the paw until withdrawal or vocalization happened, at which weight the nociceptive threshold value was documented. For each mouse, two readings were documented for each hind paw, and the results reported as the mean value of readings from both hind paws (Woode *et al.*, 2013).

4.2.3.3 Evaluation of cold allodynia

Cold allodynia was evaluated by immersion of the animal‘s hind paw into a water bath containing cold water (4.5 °C) and latency to paw withdrawal was determined using a digital timer (Lynch *et al*., 2005). Only one hind paw was assessed in each immersion at a time, with the maximum cut-off time of 20 s. For each mouse two readings were documented for each hind paw, and the data reported as the mean of both hind paw values (Park *et al*., 2012).

4.2.4 Data analysis

All data were analysed as described previously (*section 3.2.9*).

4.3 RESULTS

4.3.1 Evaluation of vincristine-induced cytotoxicity

Vincristine treated mice exhibited signs of neuropathy which showed as decrease in body weight (Figure 4.1) and mortality of some mice. The initial mean weight of the mice was 20.44 ± 0.33 g which gradually decreased to a mean weight of 18.17 ± 0.32 g. The percentage reduction in body weight was 11.11%. Five vincristine treated mice died; one died on day six and two died each on day ten and twelve.

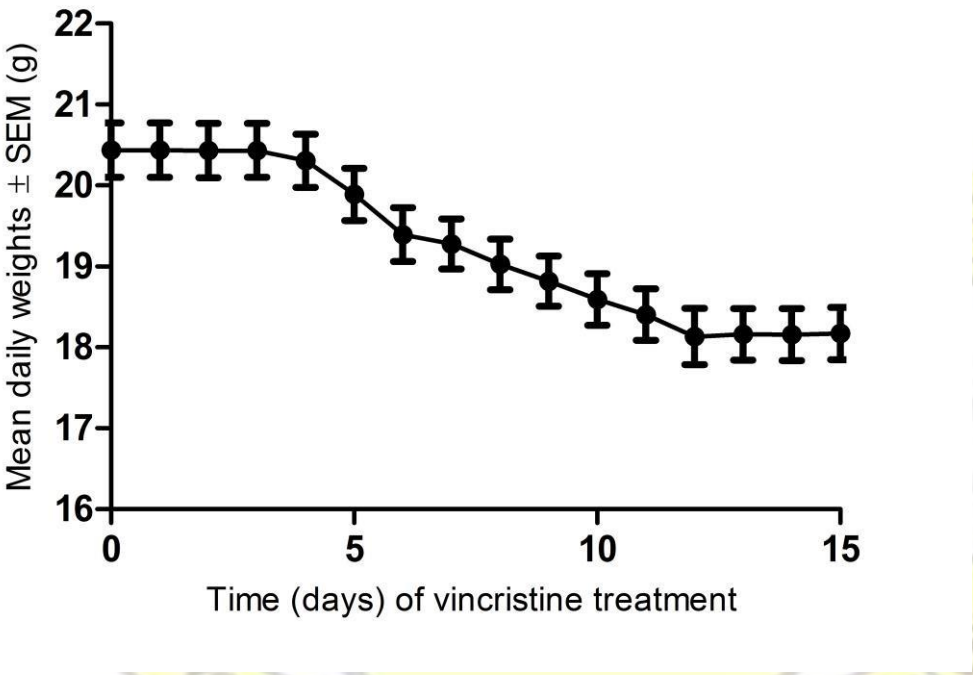


Figure 4.1 Daily weights for groups of mice (n = 80) receiving vincristine (0.1 mg/kg, i.p.).

4.3.2 Assessment of tactile allodynia using Von Frey filament of 4 g

Administration of vincristine intraperitoneally in two cycles of five consecutive days with two days off between the cycles generated a distinct and protracted active tactile allodynia in mice. An hour after the drug treatments, tactile allodynia was determined

using Von Frey filament of 4 g and the normal saline treated mice (control) exhibited increase response to tactile allodynia compared to the drug treated mice. MAE, F1 and F32 (3 – 20 mg/kg, *p.o.*) dose-relatedly reduced the response to paw withdrawal ($F_{3,16}$ = 11.56, P=0.0003; $F_{3,16}$ = 46.66, P<0.0001 and $F_{3,16}$ = 17.51, P<0.0001 respectively, Figure 4.2 a, c and e) producing tactile anti-allodynia of 51.7 ± 11.3, 66.7 ± 9.4 and 60.7 ± 11.6% respectively at the highest doses used (Figure 4.2 b, d and f).

The anticonvulsant, pregabalin (10 – 100 mg/kg, *p.o.*) dose-dependently and significantly ($F_{3, 16}$ = 14.20, P<0.0001, Figure 4.2 g) reduced tactile allodynia with the highest dose (100 mg/kg) producing an anti-allodynic effect of 54.7 ± 16.6% (Figure 4.2 h). F1 was more potent (3.426 ± 0.1467 mg/kg) than pregabalin (17.23 ± 0.2431 mg/kg) (Figure 4.3), MAE and F32.

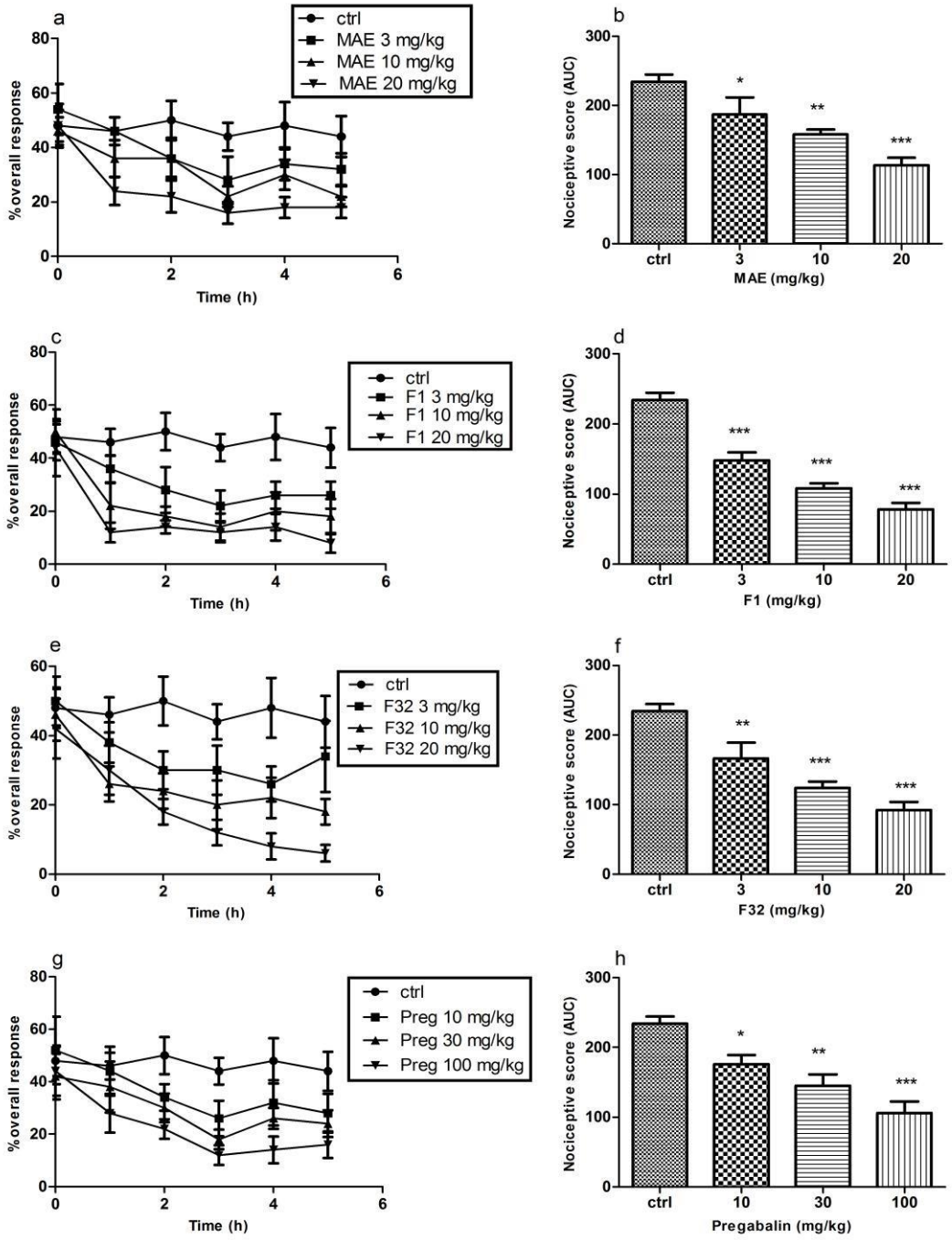


Figure 4.2 Effect of (a) MAE (3 – 20 mg/kg *p.o.*), (c) F1 (3 – 20 mg/kg *p.o.*), (e) F32 (3 – 20 mg/kg *p.o.*) and (g) pregabalin (10 – 100 mg/kg *p.o.*) on the time course curve of vincristine-induced neuropathic pain (tactile allodynia, 4 g Von Frey filament) and the total nociceptive score (calculated as AUC) (b, d, f and h respectively) in ICR mice. Data are expressed as mean ± SEM (n = 5). *P<0.05, **P<0.01, ***P<0.001 compared to vehicle-treated group (one-way ANOVA followed by Newman-Keuls *post hoc* test).

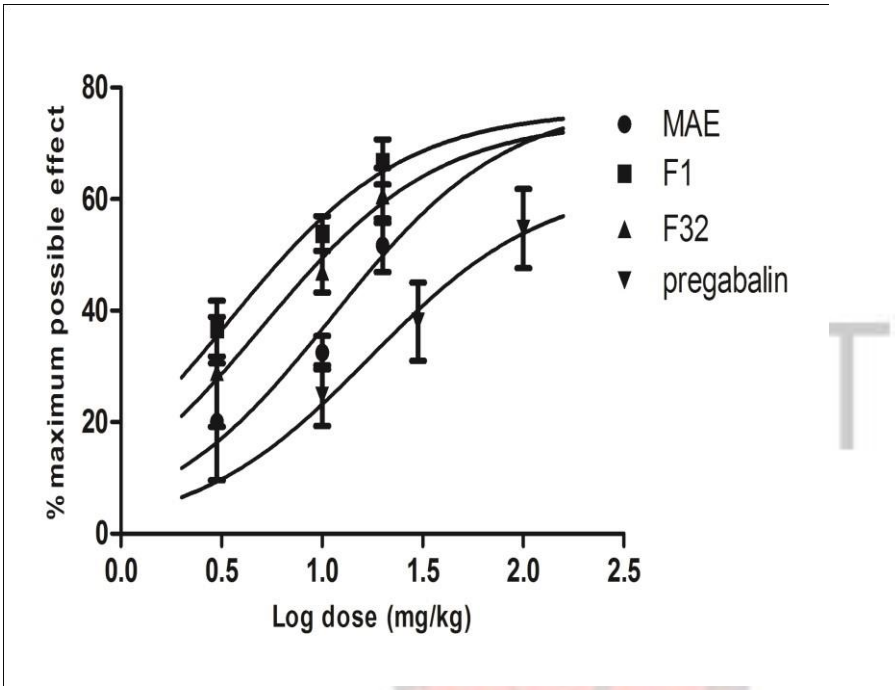


Figure 4.3 Dose-response curves for the tactile anti-allodynic effect of MAE, F1, F32 (3 – 20 mg/kg *p.o.*) and pregabalin (10 – 100 mg/kg *p.o.*) in vincristine-induced neuropathic pain. Each point is the mean ± SEM (n = 5).

4.3.3 Assessment of intermediate hyperalgesia using Von Frey filament of 8 g

Von Frey filament of 8 g was used to measure the effect of MAE, F1, F32 (3 – 20 mg/kg *p.o.*) and pregabalin (10 – 100 mg/kg *p.o.*) on intermediate hyperalgesia (intermediate to tactile allodynia and mechanical hyperalgesia). MAE, F1, F32 and pregabalin produced dose-dependent and significant anti-hyperalgesia (MAE: $F_{3, 16}$ = 12.79, P=0.0002; F1: $F_{3, 16}$ = 35.79, P<0.0001; F32: $F_{3, 16}$ = 14.22, P<0.0001; pregabalin: $F_{3, 16}$ = 28.99, P<0.0001; Figure 4.4 a, c, e and g). The highest doses of MAE, F1, F32 and pregabalin produced maximum anti-hyperalgesic effects of 45.8 ± 12.3, 62.1 ± 13.2, 53.5 ± 15.5 and 61.2 ± 9.8% respectively (Figure 4.4 b, d, f and h). MAE was more potent (12.04 ± 0.339 mg/kg) than pregabalin (47.79 ± 0.1781 mg/kg) (Figure 4.5), F32 and F1.

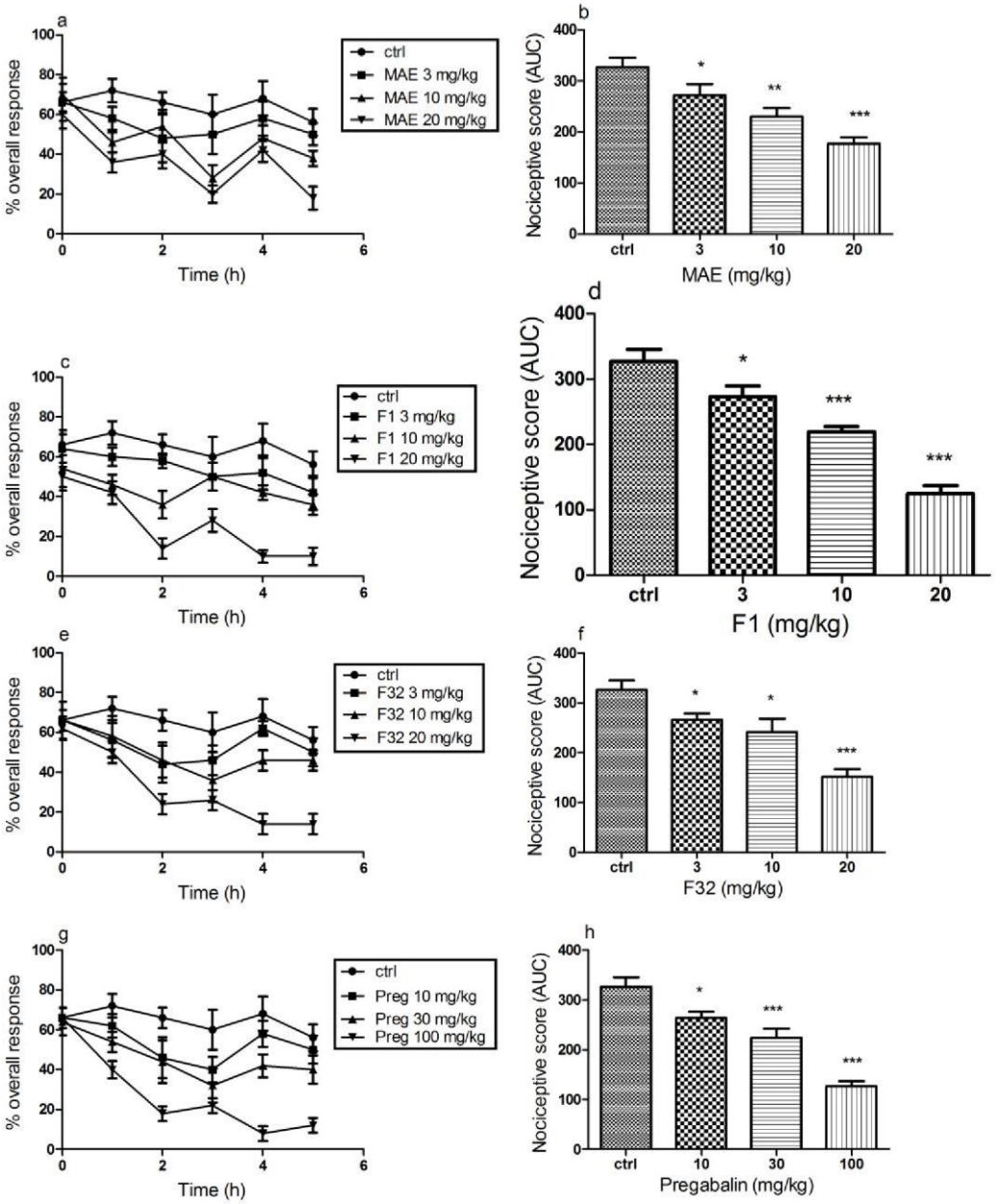


Figure 4.4 Effect of (a) MAE (3 – 20 mg/kg *p.o.*), (c) F1 (3 – 20 mg/kg *p.o.*), (e) F32 (3 – 20 mg/kg *p.o.*) and (g) pregabalin (10 – 100 mg/kg *p.o.*) on the time course curve of vincristine-induced neuropathic pain (8 g Von Frey filament) and the total nociceptive score (calculated as AUC) (b, d, f and h respectively) in ICR mice. Data are expressed as mean ± SEM (n = 5). **P*<0.05, ***P*<0.01, ****P*<0.001 compared to vehicle-treated group (one-way ANOVA followed by Newman-Keuls *post hoc* test).

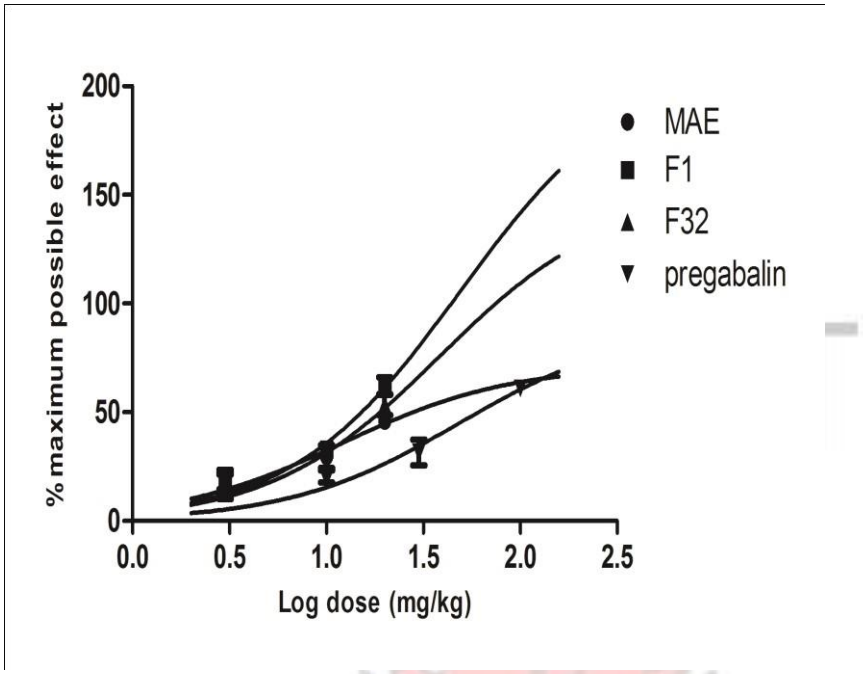


Figure 4.5 Dose-response curves for the anti-hyperalgesic effect of MAE, F1, F32 (3 – 20 mg/kg *p.o.*) and pregabalin (10 – 100 mg/kg *p.o.*) in vincristine-induced neuropathic pain (8 g Von Frey filament). Each point is the mean ± SEM (n = 5).

4.3.4 Assessment of mechanical hyperalgesia using Von Frey filament of 15 g

An hour after the different drug treatment, mechanical hyperalgesia was determined using Von Frey filament of 15 g and the normal saline treated mice (control) exhibited increase response to mechanical hyperalgesia compared to the drug treated mice. Von Frey filament of 15 g was used to measure the effect of MAE, F1, F32 (3 – 20 mg/kg *p.o.*) and pregabalin (10 – 100 mg/kg *p.o.*) on mechanical hyperalgesia. MAE, F1, F32 and pregabalin produced dose-dependent and significant antihyperalgesia (MAE: $F_{3, 16}$ = 14.30, P<0.0001; F1: $F_{3, 16}$ = 36.00, P<0.0001; F32: $F_{3, 16}$ = 24.69, P<0.0001; pregabalin: $F_{3, 16}$ = 13.04, P=0.0001; Figure 4.6 a, c, e and g). The highest doses of MAE, F1, F32 and pregabalin produced maximum antihyperalgesic effects of 43.9 ± 15.7, 59.8 ± 7.0, 54.6 ± 12.5 and 45.1 ± 15.4%

respectively (Figure 4.6 b, d, f and h). F32 was more potent (10.27 ± 0.1823 mg/kg) than pregabalin (31.66 ± 0.2112 mg/kg) (Figure 4.7).

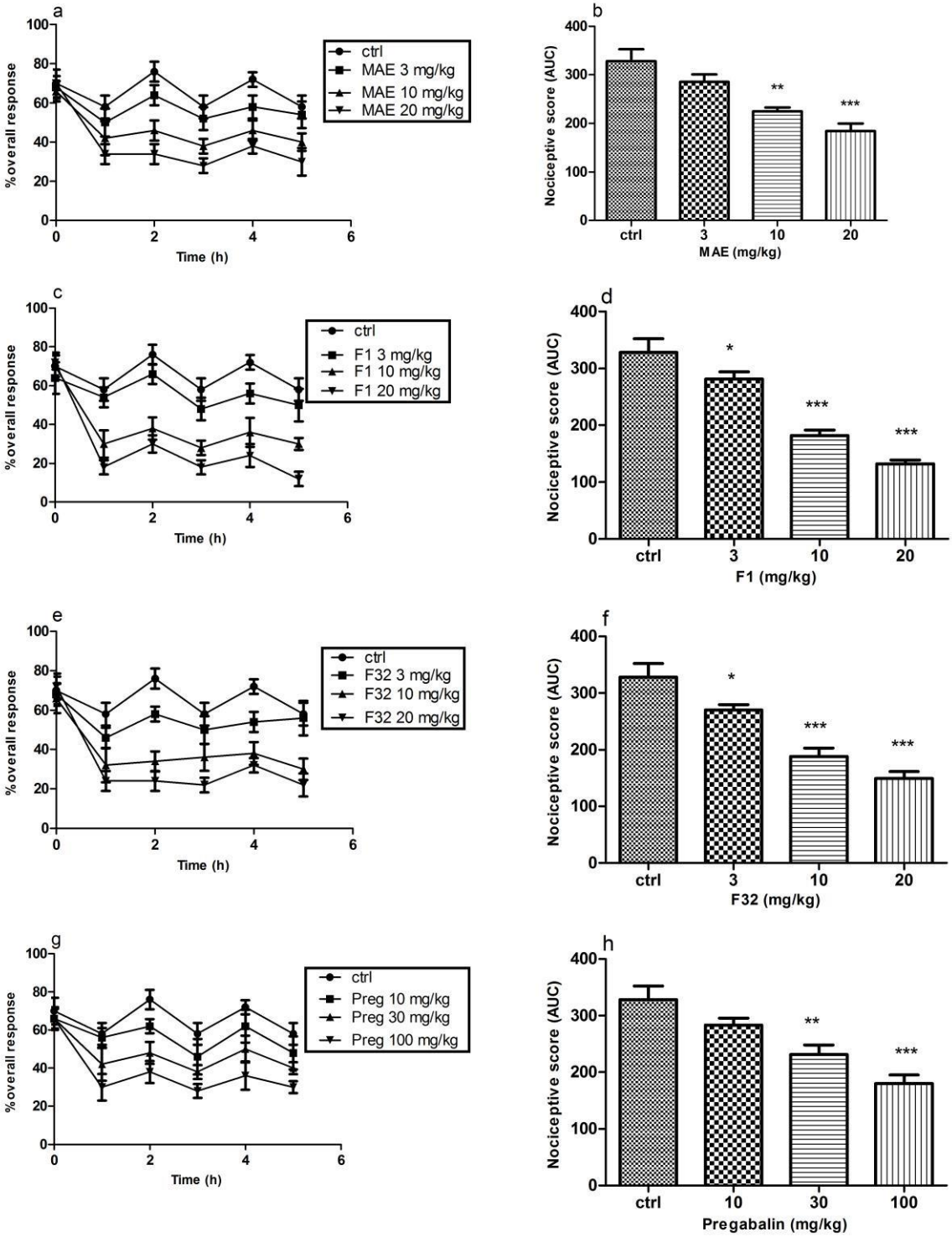


Figure 4.6 Effect of (a) MAE (3 – 20 mg/kg *p.o.*), (c) F1 (3 – 20 mg/kg *p.o.*), (e) F32 (3 – 20 mg/kg *p.o.*) and (g) pregabalin (10 – 100 mg/kg *p.o.*) on the time course curve of vincristine-induced neuropathic pain (15 g Von Frey filament) and the total nociceptive score (calculated as AUC) (b,

d, f and h respectively) in ICR mice. Data are expressed as mean ± SEM (n = 5). *P<0.05, **P<0.01, ***P<0.001 compared to vehicle-treated group (one-way ANOVA followed by Newman-Keuls *post hoc* test).

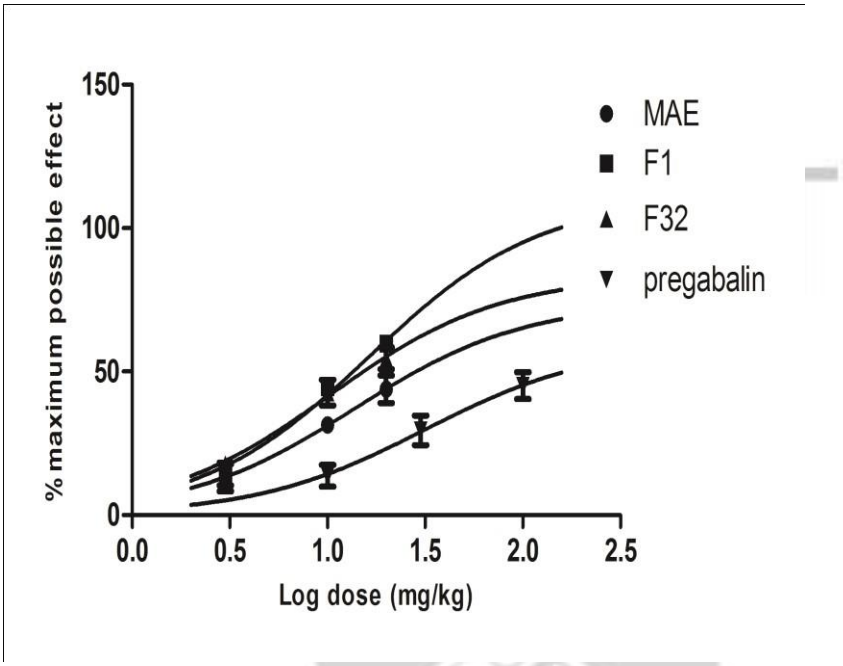


Figure 4.7 Dose-response curves for the anti-hyperalgesic effect of MAE, F1, F32 (3 – 20 mg/kg *p.o.*) and pregabalin (10 – 100 mg/kg *p.o.*) in vincristine-induced neuropathic pain (15 g Von Frey filament). Each point is the mean ± SEM (n = 5).

4.3.5 Assessment of mechanical hyperalgesia in the Randall-Selitto test

In the Randall-Selitto test, baseline mechanical hyperalgesia measured on day 15 showed that both hind paws demonstrated distinct mechanical hyperalgesia. A change in hyperalgesia was calculated as % MPE. MAE, F1, F32 (3 – 20 mg/kg, *p.o.*) and pregabalin (10 – 100 mg/kg, *p.o.*) generated dose-dependent and significant inhibition of mechanical hyperalgesia (MAE: $F_{3,16}$ = 9.162, P=0.0009; F1: $F_{3,16}$ = 39.57, P<0.0001; F32: $F_{3,16}$ = 27.94, P<0.0001 and pregabalin: $F_{3,16}$ = 20.89, P<0.0001; Figure 4.8 a, c, e and g, respectively). The highest doses of MAE, F1, F32 and pregabalin produced maximum anti-hyperalgesic effects of 72 ± 10.8, 74.1± 5.9, 62.4 ± 7.7 and 52.8 ± 14.5% respectively (Figure 4.8 b, d, f and h). F1 was more potent (9.29 ± 0.2002 mg/kg) than pregabalin (56.14 ± 0.2699 mg/kg) (Figure 4.9), F32 and MAE.

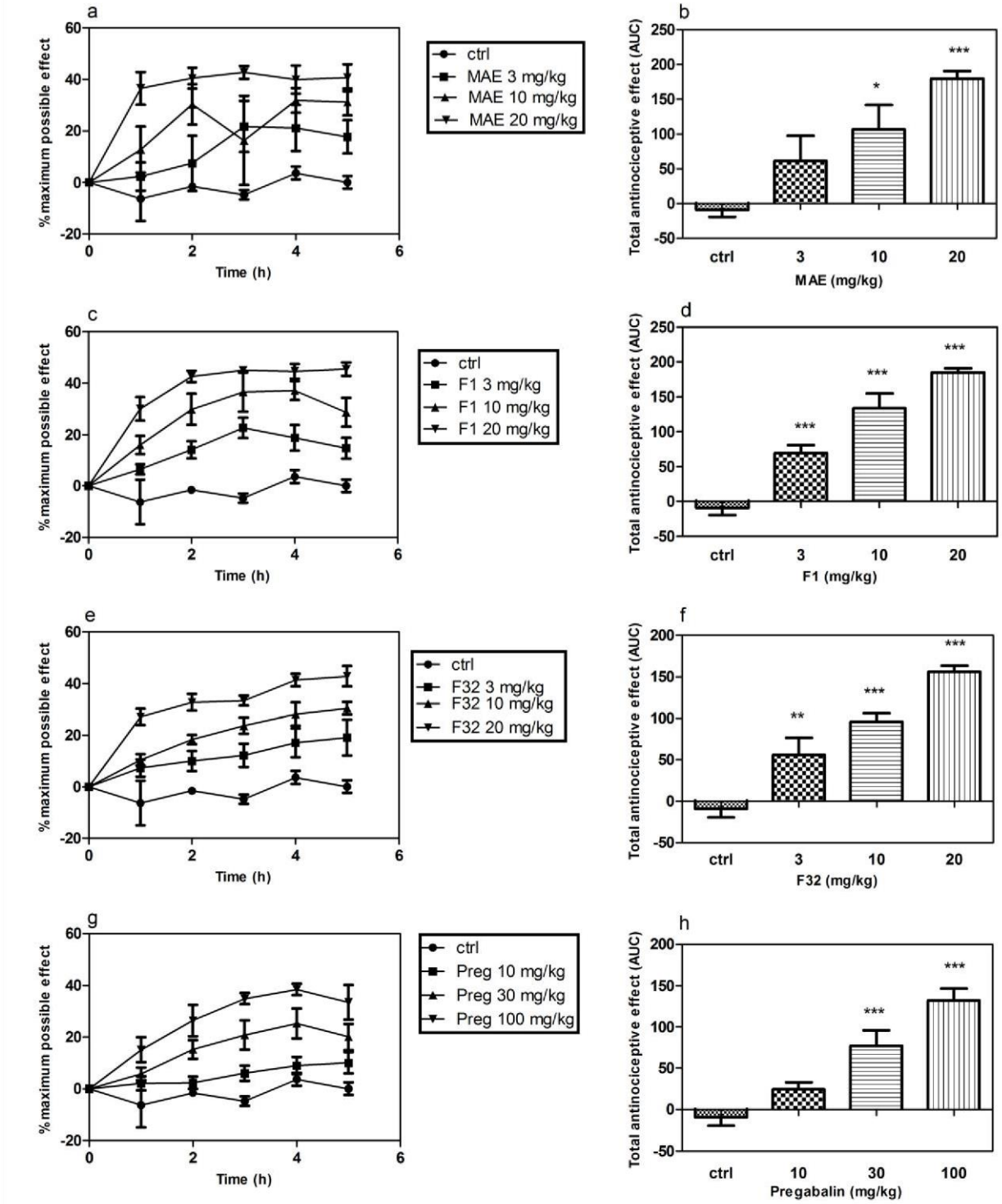


Figure 4.8 Effect of (a) MAE (3 – 20 mg/kg *p.o.*), (c) F1 (3 – 20 mg/kg *p.o.*), (e) F32 (3 – 20 mg/kg *p.o.*) and (g) pregabalin (10 – 100 mg/kg *p.o.*) on the time course curve of vincristine-induced neuropathic pain (Randall-Selitto) and the total antinociceptive score (AUC) (b, d, f and h respectively) in ICR mice. Data are expressed as mean ± SEM (n = 5). **P*<0.05, ***P*<0.01, ****P*<0.001 compared to vehicle-treated group (one-way ANOVA followed by Newman-Keuls *post hoc* test).

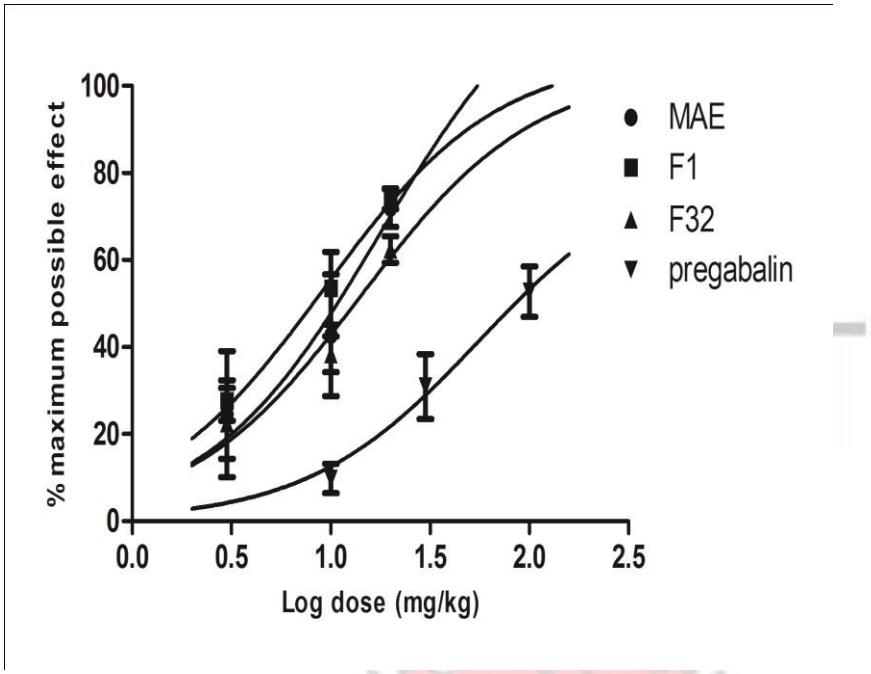


Figure 4.9 Dose-response curves for the anti-hyperalgesic effect of MAE, F1, F32 (3 – 20 mg/kg *p.o.*) and pregabalin (10 – 100 mg/kg *p.o.*) in vincristine-induced neuropathic pain (RandallSelitto). Each point is the mean ± SEM (n = 5).

4.3.6 Assessment of cold allodynia

Baseline cold allodynia was determined from both hind paws on day 15 in cold water set at a temperature of 4.5 °C. MAE, F1, F32 (3 – 20 mg/kg, *p.o.*) and pregabalin (10 – 100 mg/kg, *p.o.*) produced significant (MAE: $F_{4,20} = 42.58$, $P<0.0001$; F1: $F_{4,20} = 45.49$, $P<0.0001$; F32: $F_{4,20} = 51.39$, $P<0.0001$ and pregabalin: $F_{4,20} = 44.89$, $P<0.0001$) and dose-dependent inhibition of cold allodynia (Figure 4.10 a, c, e and g respectively) exhibited as increased latency to paw withdrawal. The highest doses of MAE, F1, F32 and pregabalin used increased the time to paw withdrawal to cold allodynia by 125.5 ± 3.07, 121.5 ± 2.77, 127.5 ± 2.70 and 116.7 ± 2.68% respectively (Figure 4.10 b, d, f and h). The potency is in the order: MAE greater than F1 greater than F32 greater than pregabalin (Figure 4.11).

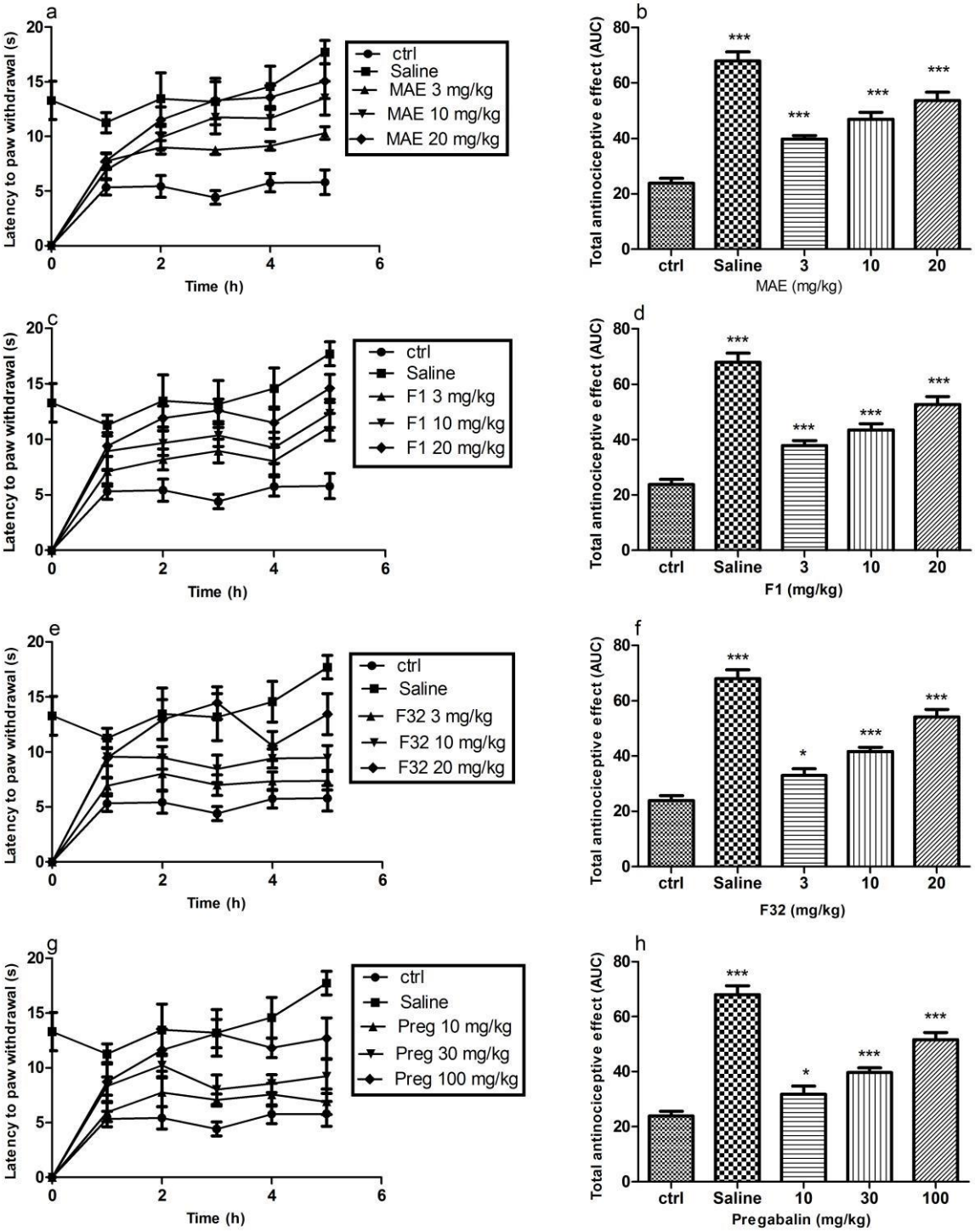


Figure 4.10 Effect of (a) MAE (3 – 20 mg/kg *p.o.*), (c) F1 (3 – 20 mg/kg *p.o.*), (e) F32 (3 – 20 mg/kg *p.o.*) and (g) pregabalin (10 – 100 mg/kg *p.o.*) on the time course curve of vincristineinduced cold allodynia and the total antinociceptive score (AUC) (b, d, f and h respectively) in ICR mice. Data are expressed as mean ± SEM (n = 5). *P<0.05, ***P<0.001 compared to vehicle-treated group (one-way ANOVA followed by Newman-Keuls *post hoc* test).

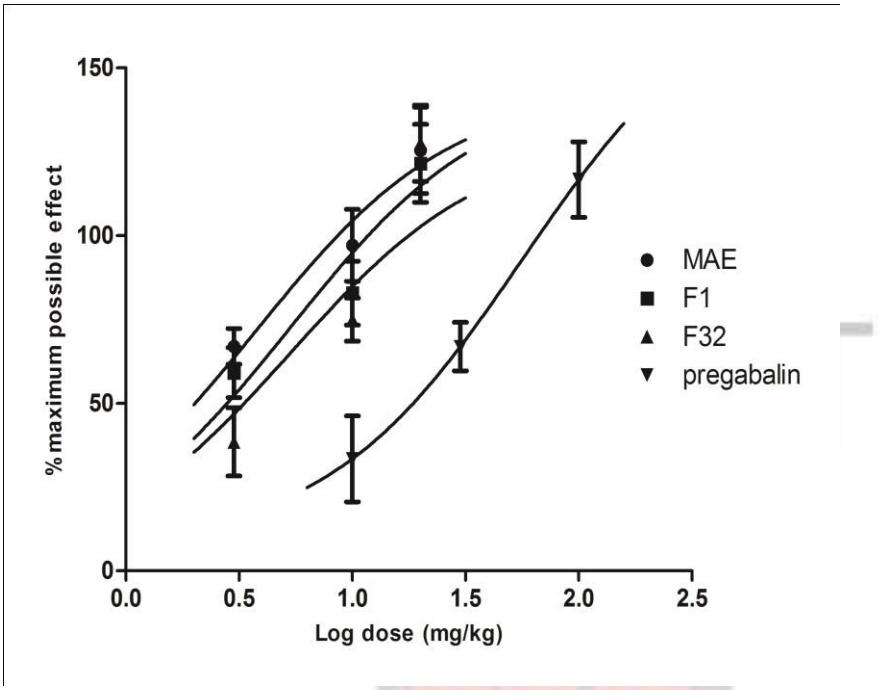


Figure 4.11 Dose-response curves for the cold anti-allodynic effect of MAE, F1, F32 (3 – 20 mg/kg *p.o.*) and pregabalin (10 – 100 mg/kg *p.o.*) in vincristine-induced neuropathic pain (cold water at 4.5 °C). Each point is the mean ± SEM (n = 5).

4.4 DISCUSSION

This study shows that orally administered petroleum ether/ethyl acetate stem bark extract and fractions of *Maerua angolensis* dose-dependently improves vincristineinduced mechanical hyperalgesia, tactile and cold allodynia which comprise the main and frequent symptoms suffered by cancer patients with chemotherapy-induced peripheral neuropathy. Though, the exact mechanism of chemotherapy-induced neuropathic pain is not known it is generally considered that each chemotherapeutic agent has separate mechanism thus, the therapy would as well require being specific for each chemotherapeutic agent (Kaley and DeAngelis, 2009). Considering the aforementioned, the antinociceptive effect of MAE and fractions can be said to be significant in the vincristine-induced neuropathic pain model. Furthermore, administration of the extract and the fractions after the occurrence of

neuropathic pain (post-treatment) suggested their curative treatment effect for chemotherapyinduced neuropathic pain.

Though the mechanism by which vincristine produces peripheral neuropathy is not clear, numerous hypotheses are rising including increase in tissue thiobarbituric acid reactive species, superoxide anion and oxidative stress (Kaur *et al*., 2010; Muthuraman *et al*., 2011). The extract and fractions may have anti-oxidative effects which might have influenced the anti-inflammatory and/or analgesic activity on vincristine-induced neuropathic pain. For now, vincristine therapy for human adenocarcinoma cells triggers nuclear factor-$_K$B (NF-$_K$B) in a dose-dependent way (Park *et al*., 2012). In addition, systemic therapy with vincristine injures Schwann cells and dorsal root ganglion (DRG) neurons of the PNS resulting in degeneration of myelinated and unmyelinated fibres (Jaggi and Singh, 2012).

It has also been reported that vincristine therapy inhibits the axonal transport of the sciatic nerve in rats (Park *et al*., 2012), thus the direct effect on peripheral nerve probably is a further mechanism of vincristine-induced neuropathic pain. Besides, the changes in the PNS and CNS, vincristine therapy result in spontaneous action of C- and Aβ-fibres which leads to spontaneous pain and abnormal sensations both peripherally and centrally hence both central and peripheral mechanisms are implicated in vincristine-induced peripheral neuropathy. Peripherally, the interleukin-6-janus-kinase-transcription-3 pathway; and centrally, the tumour necrosis factor-α (TNF-α)-p38 mitogen-activated protein kinase (MAPK) pathway have been implicated (Aley and Levine, 2002; Kiguchi *et al*., 2008; Siau *et al*., 2006). MAE and fractions therefore may have exercised analgesic effects in this study by blocking the phosphorylation of extracellular signal-related kinases and p38

MAPK action, or by decreasing NF-$_K$B.

Furthermore, the extract and fractions probably curbed the generation of NO, interleukins and TNF-α but this needs further investigation. Increase in TNF-α has been reported in the sciatic nerve of rats with vincristine-induced neuropathic pain (Kiguchi *et al*., 2008; Muthuraman *et al*., 2011). The NO second-messenger pathway has been shown to play a role in hyperalgesia in the vincristine-induced neuropathic pain model in rats (Aley and Levine, 2002). Additionally, inhibitors of NO synthase that demonstrate anti-hyperalgesia in vincristine-induced neuropathic pain imply that NO synthase pathways are linked with vincristine-induced hyperalgesia (Bujalska and Makulska-Nowak, 2009). Consequently, the stem bark of *Maerua angolensis* possibly inhibit NO, TNF-α and NF-KB in this study through its anti-inflammatory effects (Adamu *et al*., 2007). The functional improvement of the Schwann cells, myelinated axons, nerve conduction velocity and axonal transport (neuroprotective effects) could perhaps be another mechanism by which MAE and the fractions exert their action in this study.

Enhanced expression and action of α_2-δ_1 Ca^{2+} channels, a sub-unit form of N-type voltage-dependent Ca^{2+} channel is also reported in vincristine-induced neuropathic pain conditions (Yajima *et al*., 2005; Zamponi *et al*., 2009). Inhibition of calcium channels stop neuronal excitability and other cellular enzymatic cascade reactions that guide to pain sensations and pain stimuli transmission in the affected myelinated and unmyelinated C-, Aδ- and Aβ-fibres (Kumar *et al*., 2010; Schim, 2009; Zamponi *et al*., 2009). The small diameter C- and Aδ-sensory fibres (high threshold fibres) are mostly engaged in the response to cold and strong mechanical stimuli but large Aβsensory fibres (low threshold fibres) response to tactile stimuli. Pregabalin, antagonist of α_2-δ_1 sub-unit of voltage dependent calcium channels is known to be useful both clinically and experimentally in neuropathic pain (Kumar *et al*., 2010; Schim, 2009). The extract

and the fractions may have also produced analgesic effects in this study by blocking calcium channels on sensory nerves similar to pregabalin.

4.5 CONCLUSION

Orally administered MAE and fractions dose-dependently inhibited tactile and cold allodynia in addition to mechanical hyperalgesia exhibited by vincristine-induced neuropathic mice. This indicates that MAE and fractions may exercise an analgesic effect in cancer patients with vincristine-induced neuropathic pain.

4.6 RECOMMENDATION

- Assess the mechanism of antinociceptive effects of MAE and fractions so as to provide more evidence about the analgesic activity of the plant extract.

Chapter 5

MECHANISM OF ANTINOCICEPTION OF MAE AND FRACTIONS

5.1 INTRODUCTION

In the chemical and thermal behavioural models of pain, the petroleum ether/ethyl acetate stem bark extract and the fractions of *Maerua angolensis* were shown to have antinociceptive effect. Several pain mediators including bradykinin, serotonin, nitric oxide, acetylcholine, noradrenaline and prostaglandins can be implicated in the nociception (Bannwarth and Kostine, 2014; Spagnoli and Kaye, 2014).

Substances acting as descending pain facilitators or descending pain inhibitors modulates nociception in the spinal cord to the brain (Umana *et al*., 2013; Yang *et al*., 2013). The neurons from the brain accountable for the regulation of pain perception have numerous transmitters including NO, serotonin (5-HT), noradrenaline, acetylcholine, glutamate and dopamine among others (Borisovska *et al*., 2013; Dicken *et al*., 2012).

This study investigates the possible mechanism(s) of antinociceptive activity of MAE and its fractions in the acetic acid-induced writhing test and the tail-flick test in Hargreaves thermal hyperalgesia model. The participation of adenosinergic, nitric oxide, muscarinic, ATP sensitive K^+ channels, adrenergic, serotoninergic and opioidergic pathways in the antinociceptive activity of MAE and fractions were exploited. The mechanism(s) of antinociception of MAE, F1 and F32 was also investigated in the prostaglandin E_2-induced mechanical hyperalgesia, bradykinin-, and epinephrine-induced thermal hyperalgesia, glutamate- and capsaicin-induced nociception. The doses of antagonists, agonists and other drugs were chosen on the

basis of earlier literature information and in experiments in the laboratory (Woode *et al*., 2009; Woode *et al*., 2013; Woode and Abotsi, 2011).

5.2 MATERIALS AND METHODS

5.2.1 Animals

Sprague-Dawley rats (190 – 200 g) and ICR mice (20 – 25 g) of either sex were used in the study. In all the experimental studies each group consisted of 5 animals. All animals were bestowed with the needed conditions as described previously (*section 2.2.4*).

5.2.2 Drugs and chemicals

The following drugs and chemicals were used: Acetic acid, theophylline (BDH, Poole, England), diclofenac sodium (Troge Medical GmbH, Hamburg, Germany), epinephrine hydrochloride (Wuhan Grand Pharm, China), atenolol (Ernest Chemist Ltd, Accra, Ghana), captopril (Teva UK Ltd, Eastbourne, UK), morphine hydrochloride (Phyto–Riker, Accra, Ghana), Prostaglandin E_2, bradykinin acetate, Lglutamic acid, capsaicin, naloxone and N^G-Nitro-l-arginine methyl ester (L-NAME) (Sigma-Aldrich Inc., St. Louis, MO, USA), glibenclamide (Sanofi-Aventis, Guildford, UK), ondansetron (GlaxoSmithKline, Uxbridge, UK), atropine sulphate (E. Merck AG-Damstadt, Germany), yohimbine (Walter Ritter GmbH & Co. KG, Germany) and ketamine hydrochloride (Brotex Medica, Trittau, Germany). All drugs, extract and fractions used in the nociceptive tests were prepared as described earlier (*section 2.2.5*).

5.2.3 Investigation of the mechanism of antinociception of MAE and fractions in the writhing test

The mechanism of antinociceptive activity of MAE and fractions was examined using different antagonists in the writhing test. The receptor/pathways investigated were the adenosinergic, nitric oxide, muscarinic, ATP sensitive K^+ channels, serotoninergic, adrenergic and opioidergic receptor/pathways.

5.2.3.1 Involvement of the adenosinergic system

The procedure used was similar to that described previously (Katyal and Gupta, 2011; Zakaria *et al*., 2014). To examine the part played by the adenosinergic systems in the antinociception caused by the extract and fractions, mice (n = 5) were pretreated with theophylline, a non-selective adenosine receptor antagonist (5 mg/kg, i.p.). After 15 min mice were given the extract, the fractions F1/F32 (10 mg/kg, *p.o*.), diclofenac (10 mg/kg, i.p.) or normal saline as vehicle (10 ml/kg, i.p.). Another group of mice was pre-treated with vehicle and after 15 min, received MAE, F1, F32, diclofenac or vehicle. Acetic acid (0.6% v/v) was administered (10 ml/kg, i.p.) to all the mice 1 h after *p.o.* administration of the extract or the fractions and 30 min after i.p. diclofenac or vehicle administration. The nociceptive responses were then evaluated as described previously (*section 2.2.6.1*).

5.2.3.2 Participation of the nitric oxide system

To study the involvement of nitric oxide/cyclic GMP pathway in the antinociceptive activity of the extract or fractions, mice (n = 5) were pre-treated with L-NAME, a NO synthase inhibitor (10 mg/kg, i.p.). After 30 min mice were given the extract, and the fractions F1/F32 (10 mg/kg, *p.o*.), diclofenac (10 mg/kg, i.p.) or vehicle (10 ml/kg, i.p.). Another group of mice was pre-treated with vehicle and after 30 min, treated the

same way as L-NAME pre-treated mice. Acetic acid (0.6% v/v) was administered (10 ml/kg, i.p.) to all the mice 1 h after the *p.o*. and 30 min after the i.p. administration and the nociceptive responses were recorded as described earlier (*section 2.2.6.1*).

5.2.3.3 Involvement of the muscarinic system

To assess the possible participation of muscarinic receptor/system in the antinociception caused by the extract or fractions, mice (n = 5) were pre-treated with atropine, non-selective muscarinic receptor antagonist (5 mg/kg, i.p.). After 30 min mice were given the extract, F1, F32 (10 mg/kg, *p.o*.), diclofenac (10 mg/kg, i.p.) or vehicle (10 ml/kg, i.p.). Another group of mice was pre-treated with vehicle and after 30 min, treated the same way as atropine pre-treated mice. Acetic acid (0.6% v/v) was administered (10 ml/kg, i.p.) to all the mice 1 h after the *p.o*. and 30 min after the i.p. administration and the nociceptive responses were recorded as described earlier (*section 2.2.6.1*).

5.2.3.4 Participation of the ATP sensitive K^+ channels

To examine the role played by the ATP sensitive K^+ channels in the antinociception produced by the extract and fractions, mice (n = 5) were pre-treated with glibenclamide, an ATP-sensitive K^+ channel inhibitor (8 mg/kg, *p.o*.). After 30 min mice were given the extract, F1, F32 (10 mg/kg, *p.o*.), diclofenac (10 mg/kg, i.p.) or vehicle (10 ml/kg, i.p.). Another group of mice was pre-treated with vehicle and after 30 min, treated the same way as glibenclamide pre-treated mice. Acetic acid (0.6% v/v) was administered (10 ml/kg, i.p.) to all the mice 1 h after the *p.o*. and 30 min after the i.p. administration and the nociceptive responses were recorded as described previously (*section 2.2.6.1*).

5.2.3.5 Participation of serotoninergic system

To examine the input of 5-HT_3 receptors/pathway in the antinociceptive activity of the extract and fractions, mice (n = 5) were pre-treated with ondansetron, 5-HT_3 receptor inhibitor (0.5 mg/kg, i.p.). After 30 min mice were given the extract, F1, F32 (10 mg/kg, *p.o.*), diclofenac (10 mg/kg, i.p.) or vehicle (10 ml/kg, i.p.). Another group of mice was pre-treated with vehicle and after 30 min, treated the same way as ondansetron pre-treated mice. Acetic acid (0.6% v/v) was administered (10 ml/kg, i.p.) to all the mice 1 h after the *p.o.* and 30 min after the i.p. administration and the nociceptive responses were recorded as described earlier (*section 2.2.6.1*).

5.2.3.6 Participation of the adrenergic system

To determine the contribution of α_2 adrenoceptor in the antinociceptive effects of the extract and fractions, mice (n = 5) were pre-treated with yohimbine, α_2 adrenoceptor antagonist (3 mg/kg, i.p.). After 30 min mice were given the extract, F1, F32 (10 mg/kg, *p.o.*), diclofenac (10 mg/kg, i.p.) or vehicle (10 ml/kg, i.p.). Another group of mice was pre-treated with vehicle and after 30 min, treated the same way as yohimbine pre-treated mice. Acetic acid (0.6% v/v) was administered (10 ml/kg, i.p.) to all the mice 1 h after the *p.o.* and 30 min after the i.p. administration and the nociceptive responses were recorded as described previously (*section 2.2.6.1*).

5.2.3.7 Investigation of the opioid pathway

The procedure used was similar to that described previously (Otuki *et al.*, 2005) with modification (Woode *et al.*, 2013). To investigate the possible involvement of the opioid receptors or pathway in the antinociceptive activity of the extract and fractions, mice (n = 5) were pre-treated with naloxone, a non-selective opioid receptor antagonist (2 mg/kg, i.p.). After 15 min mice were given the extract, F1, F32 (10 mg/kg, *p.o.*),

diclofenac (10 mg/kg, i.p.) or vehicle (10 ml/kg, i.p.). Another group of mice was pre-treated with vehicle and after 15 min, treated the same way as naloxone pre-treated mice. Acetic acid (0.6% v/v) was administered (10 ml/kg, i.p.) to all the mice 1 h after the *p.o*. and 30 min after the i.p. administration and the nociceptive responses were recorded as described previously (*section 2.2.6.1*).

5.2.4 Investigation of the mechanism of antinociception of MAE and fractions in the tail-flick test

The mechanism of antinociceptive activity of the extract and fractions was examined using various antagonists in the tail-flick test in Hargreaves thermal hyperalgesia model. The receptor/pathways investigated were the adenosinergic, nitric oxide, muscarinic, ATP sensitive K^+ channels, serotoninergic, adrenergic and opioid receptor / pathways.

5.2.4.1 Involvement of the adenosinergic system

To examine the part played by the adenosinergic system in the antinociception caused by the extract and fractions, mice (n = 5) were pre-treated with theophylline, a non-selective adenosine receptor antagonist (5 mg/kg, i.p.). After 15 min mice were given the extract, F1, F32 (10 mg/kg, *p.o.*), morphine (3 mg/kg, i.p.) or normal saline as vehicle (10 ml/kg, i.p.). Another group of mice was pre-treated with vehicle and after 15 min, treated the same way as theophylline pre-treated mice. TWLs were determined with the IITC Analgesia Meter by delivering a focused beam of radiant light to the distal portion of the tail until the mouse flicked the tail. Basal reaction times of mice were taken before the administration of the extract, fractions, morphine or vehicle. The reaction times were taken again at 1, 2, 3 and 4 h post drug administration.

Antinociceptive effects exerted by drugs were calculated from TWLs as % MPE as described earlier (*section 3.2.5*).

5.2.4.2 Participation of the nitric oxide system

To study the involvement of nitric oxide/cyclic GMP pathway in the antinociceptive activity produced by the extract or fractions, mice (n = 5) were pre-treated with LNAME, a NO synthase inhibitor (10 mg/kg, i.p.). After 30 min mice were given the extract, F1, F32 (10 mg/kg, *p.o.*), morphine (3 mg/kg, i.p.) or vehicle (10 ml/kg, i.p.). Another group of mice was pre-treated with vehicle and after 30 min, treated the same way as L-NAME pre-treated mice. TWLs were determined with the IITC Analgesia Meter by delivering a focused beam of radiant light to the distal portion of the tail until the mouse flicked the tail. Basal reaction times of mice were taken before the administration of the extract, fractions, morphine or vehicle. The reaction times were taken again at 1, 2, 3 and 4 h post drug administration. Antinociceptive effects exerted by drugs were calculated from TWLs as % MPE as described in *section 3.2.5*

5.2.4.3 Involvement of the muscarinic system

To assess the possible participation of muscarinic receptor/system in the antinociception caused by the extract or fractions, mice (n = 5) were pre-treated with atropine, non-selective muscarinic receptor antagonist (5 mg/kg, i.p.). After 30 min mice were given the extract, F1, F32 (10 mg/kg, *p.o.*), morphine (3 mg/kg) or vehicle (10 ml/kg, i.p.). Another group of mice was pre-treated with vehicle and after 30 min, treated the same way as atropine pre-treated mice. TWLs were determined with the IITC Analgesia Meter by delivering a focused beam of radiant light to the distal portion of the tail until the mouse flicked the tail. Basal reaction times of mice were taken before the administration of the extract, fractions, morphine or vehicle. The reaction

times were taken again at 1, 2, 3 and 4 h post drug administration. Antinociceptive effects exerted by drugs were calculated from TWLs as % MPE as described in *section 3.2.5*

5.2.4.4 Participation of the ATP sensitive K^+ channels

To examine the role played by the ATP sensitive K^+ channels in the antinociception produced by the extract and fractions, mice (n = 5) were pre-treated with glibenclamide, an ATP-sensitive K^+ channel inhibitor (8 mg/kg, *p.o.*). After 30 min mice were given the extract, F1, F32 (10 mg/kg, *p.o.*), morphine (3 mg/kg, i.p.) or vehicle (10 ml/kg, i.p.). Another group of mice was pre-treated with vehicle and after 30 min, treated the same way as glibenclamide pre-treated mice. TWLs were determined with the IITC Analgesia Meter by delivering a focused beam of radiant light to the distal portion of the tail until the mouse flicked the tail. Basal reaction times of mice were taken before the administration of the extract, fractions, morphine or vehicle. The reaction times were taken again at 1, 2, 3 and 4 h post drug administration. Antinociceptive effects exerted by drugs were calculated from TWLs as % MPE as described earlier (*section 3.2.5*).

5.2.4.5 Participation of the serotoninergic system

To examine the input of 5-HT_3-receptors/pathway in the antinociceptive activity of the extract and fractions, mice (n = 5) were pre-treated with ondansetron, 5-HT_3 receptor inhibitor (0.5 mg/kg, i.p.). After 30 min mice were given the extract, F1, F32 (10 mg/kg, *p.o.*), morphine (3 mg/kg, i.p.) or vehicle (10 ml/kg, i.p.). Another group of mice was pre-treated with vehicle and after 30 min, treated the same way as ondansetron pre-treated mice. TWLs were determined with the IITC Analgesia Meter by delivering a focused beam of radiant light to the distal portion of the tail until the

mouse flicked the tail. Basal reaction times of mice were taken before the administration of the extract, fractions, morphine or vehicle. The reaction times were taken again at 1, 2, 3 and 4 h post drug administration. Antinociceptive effects exerted by drugs were calculated from TWLs as % MPE as described previously (*section 3.2.5*).

5.2.4.6 Participation of the adrenergic system

To determine the contribution of α_2 adrenoceptor in the antinociceptive effects of the extract and fractions, mice (n = 5) were pre-treated with yohimbine, α_2 adrenoceptor antagonist (3 mg/kg, i.p.). After 30 min mice were given the extract, F1, F32 (10 mg/kg, *p.o.*), morphine (3 mg/kg, i.p.) or vehicle (10 ml/kg, i.p.). Another group of mice was pre-treated with vehicle and after 30 min, treated the same way as yohimbine pre-treated mice. TWLs were determined with the IITC Analgesia Meter by delivering a focused beam of radiant light to the distal portion of the tail until the mouse flicked the tail. Basal reaction times of mice were taken before the administration of the extract, fractions, morphine or vehicle. The reaction times were taken again at 1, 2, 3 and 4 h post drug administration. Antinociceptive effects exerted by drugs were calculated from TWLs as % MPE as described earlier (*section 3.2.5).*

5.2.4.7 Investigation of the opioid pathway

The procedure used was similar to that described previously (Otuki *et al*., 2005) with modification (Woode *et al*., 2013). To investigate the possible involvement of the opioid receptors or pathway in the antinociceptive activity of the extract and fractions, mice (n = 5) were pre-treated with naloxone, a non-selective opioid receptor antagonist (2 mg/kg, i.p.). After 15 min mice were given the extract, F1, F32 (10 mg/kg, *p.o.*),

morphine (3 mg/kg, i.p.) or vehicle (10 ml/kg, i.p.). Another group of mice was pre-treated with vehicle and after 15 min, treated the same way as naloxone pre-treated mice. TWLs were determined with the IITC Analgesia Meter by delivering a focused beam of radiant light to the distal portion of the tail until the mouse flicked the tail. Basal reaction times of mice were taken before the administration of the extract, fractions, morphine or vehicle. The reaction times were taken again at 1, 2, 3 and 4 h post drug administration. Antinociceptive effects exerted by drugs were calculated from TWLs as % MPE as described previously (*section 3.2.5*).

5.2.5 Investigation of the mechanism of antinociception of MAE and fractions in the capsaicin test

The capsaicin-induced nociception was done as described previously (Sakurada *et al*., 1992) with slide modifications (Woode *et al*., 2013). The mice (n = 5) were placed individually in 1 of 20 transparent perspex testing chambers (15 × 15 × 15 cm) for 1 h adaptation in the chamber before the test. Following the adaptation period, ten groups of male mice were pre-treated with MAE, F1, F32 (3 – 30 mg/kg, *p.o*.) or vehicle (normal saline, 10 ml/kg, i.p.) 30 min for the intraperitoneal route and 1 h for the oral route before intraplantar injection of capsaicin (1.6 µg/paw, 20 µl dissolved in 0.5% ethanol) in the right hind paw. The ethanol did not cause any detectable analgesic effect on its own. The nociceptive behaviour (biting/licking of the injected paw) of the mice was captured (5 min) for analysis by a camcorder (Everio^{TM}, model GZ-MG 1300, JVC, Tokyo, Japan) placed directly opposite the mirror and attached to a computer. Tracking of the behaviour was done with help of the public domain software JWatcher^{TM}, Version 1.0 (University of California, LA, USA and Macquarie University, Sidney, Australia, available at

http://www.jwatcher.ucla.edu/) to obtain the frequency and duration of biting/licking for 5 min, starting immediately after capsaicin injection. A nociceptive score for the time block was calculated by multiplying the frequency and time spent in biting/licking the injected paw. Data were expressed as the mean ± SEM of scores between 0 and 5 min after capsaicin injection.

5.2.6 Investigation of the mechanism of antinociception of MAE and fractions in the glutamate test

The glutamate-induced nociception was performed as described earlier (Meotti *et al*., 2006; Woode *et al*., 2013). The mice (n = 5) were placed individually in 1 of 20 transparent perspex testing chambers (15 × 15 × 15 cm) for 1 h adaptation in the chamber before the test. Following the acclimatised period, thirteen groups of male mice were pre-treated with MAE, F1, F32 (3 – 30 mg/kg, *p.o*.), ketamine (1 – 10 mg/kg, i.p.) or vehicle. Mice were returned individually into the testing chambers immediately after intraplantar injection of glutamate (10 µmol/paw, 20 µl dissolved in normal saline) into the right hind paw. The amount of time spent licking the injected paw (considered as a nociceptive behaviour) was determined for 15 min immediately following glutamate administration in the same way as that described previously in the formalin test (*section 2.2.6.3*).

5.2.7 Analgesic activity of MAE and fractions in the prostaglandin E_2–induced mechanical hyperalgesia

The method is a modification of that described previously (Wilhelm *et al*., 2009; Woode *et al*., 2013). After baseline pain threshold measurements on the test day, thirteen groups of male rats received MAE, F1, F32 (3 - 30 mg/kg, *p.o*.), morphine (0.3 - 3 mg/kg, i.p.) or normal saline (10 ml/kg, i.p.) 1 h for the *p.o*. or 30 min for the

i.p. route before the intraplantar injection of prostaglandin E_2 (20 µl: 1 nmol/paw) into the right hind paw. Mechanical nociceptive thresholds were determined in the rat paw pressure test using an analgesimeter (Model No. 15776, Ugo Basile, Comerio, Varese, Italy) based on the Randall and Selitto method (Randall and Selitto, 1957). The analgesimeter was used to apply a linearly–increasing pressure, by means of a blunt perspex cone, to the dorsal region of the right hind paw of the rat until the rat squealed, struggled or withdrew the paw. Rats received two training sessions before the day of testing. An hour after prostaglandin E_2 administration, pressure was gradually applied to the right hind paw at a constant rate and paw withdrawal thresholds (PWTs) were assessed as the pressure (grams) that elicits paw withdrawal. PWTs were measured again at 30 min intervals for 2 h. A cut–off point of 250 g was chosen as the maximum weight to apply to prevent any tissue injury to the paw of the rats. A change in hyperalgesic state was calculated as a percentage of the maximum possible effect (% MPE). The % MPE was calculated using the formula

$$\% MPE = \frac{(PWT - CT)}{(250\ g - CT)} \times 100$$

Where, PWT is paw withdrawal threshold and CT is control threshold.

Anti-hyperalgesic effects exerted by drugs were calculated from the PWTs as a % MPE.

5.2.8 Analgesic activity of MAE and fractions in the bradykinin-induced thermal hyperalgesia

After baseline PWLs measurements on the test day, ten groups of male rats (n = 5) were pre-treated with MAE, F1, F32 (3 - 30 mg/kg, *p.o.*), or normal saline (10 ml/kg, i.p.) 1 h for the *p.o.* or 30 min for the i.p. route before the intraplantar injection of bradykinin (20 µl: 10 nmol/paw). Rats were pre-treated with captopril (5 mg/kg, s.c.),

an angiotensin converting enzyme inhibitor 1 h before experiments to prevent the degradation of bradykinin. Thermal nociceptive latencies were measured in the rat paw as described in *section 3.2.5*, however, the thermal nociceptive stimulus was manually directed under the foot pad before and after the intraplantar injection of bradykinin into the right hind paw. A timer was set to automatically turn off the light source when the rat withdrew the paw, and the PWLs (time required for the paw to show an abrupt withdrawal) recorded. PWLs were taken again at 1, 1.5, 2, 2.5, and 3 h post bradykinin administration. Rats received two training sessions before the day of testing. A cut–off time of 25 s was chosen as the maximum time the rat‘s paw will be stimulated with the light in order to prevent any tissue damage. Anti-hyperalgesic effects exerted by drugs were calculated from the PWLs as % MPE using the formula described previously (*section 3.2.5*).

5.2.9 Analgesic activity of MAE and fractions in the epinephrine–induced thermal hyperalgesia

After baseline PWLs measurements, rats (n = 5) were pre-treated with MAE, F1, F32 (3 - 30 mg/kg, *p.o*.), atenolol (1, 3 and 10 µg/paw) or normal saline (10 ml/kg, i.p.) 1 h for the *p.o*. or 30 min for the i.p. route before the intraplantar injection of epinephrine (20 µl: 450 nmol/paw). Thermal nociceptive latencies were measured in the rat paw as described in *section 3.2.5*, however, the thermal nociceptive stimulus was manually directed under the foot pad before epinephrine administration, and at 1, 2, 3 and 4 h post epinephrine administration into the right hind paw. Antihyperalgesic effects exerted by drugs were calculated from the PWLs as % MPE using the formula described previously (*section 3.2.5*).

5.2.10 Data analysis

Raw data for the prostaglandin E_2-induced mechanical, bradykinin- and epinephrineinduced thermal hyperalgesia tests were calculated as the % MPE. Data were expressed as mean ± SEM per group and analysed as described in *section 3.2.9*

5.3 RESULTS

5.3.1 Investigation of the mechanism of antinociception of MAE and fractions in the writhing test

5.3.1.1 Involvement of the adenosinergic system

Pre-treatment of mice with 5 mg/kg theophylline reversed antinociception of MAE (10 mg/kg, *p.o.*, Figure 5.1 a).

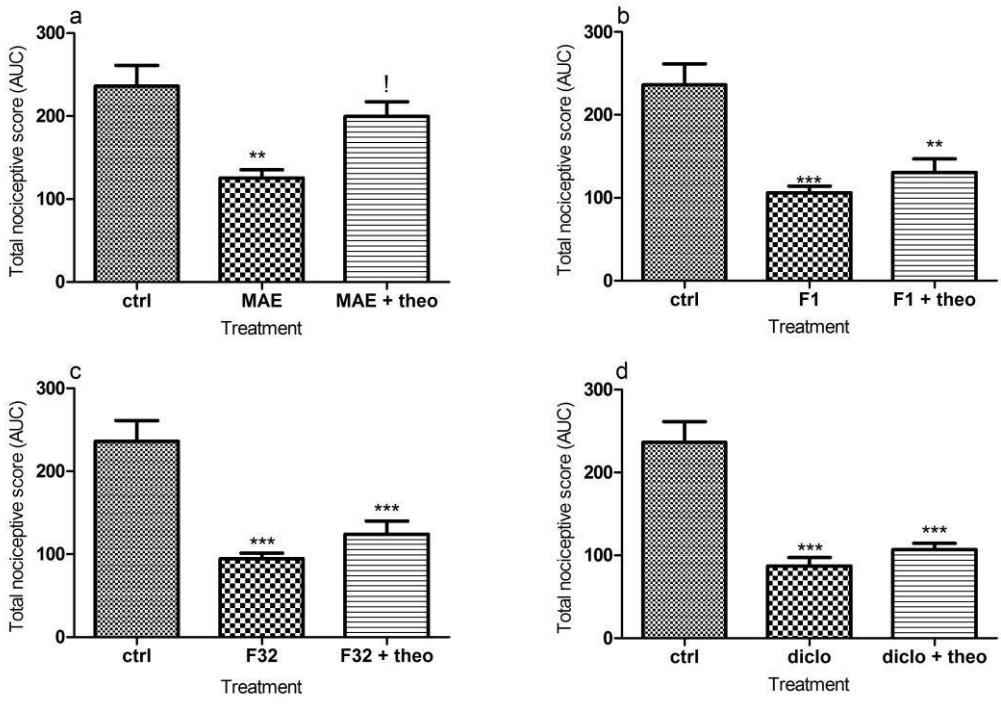


Figure 5.1 Effect of theophylline (5 mg/kg, i.p.) on the antinociceptive effects of MAE, F1, F32 (10 mg/kg, *p.o.*) and diclofenac (10 mg/kg, i.p.) in acetic acid-induced writhing test (a, b, c and d). Each column represents the mean ± SEM (n = 5). *****P*<0.01, ******P*<0.001, compared to controls, !*P*<0.05, compared to MAE (one-way ANOVA followed by Newman-Keuls *post hoc* test).

5.3.1.

2 Participation of the nitric oxide system

The antinociception of F32 (10 mg/kg, *p.o.*, Figure 5.2 c) and diclofenac (10 mg/kg, i.p., Figure 5.2 d) in the acetic acid-induced abdominal writhing test was blocked by L-NAME (10 mg/kg).

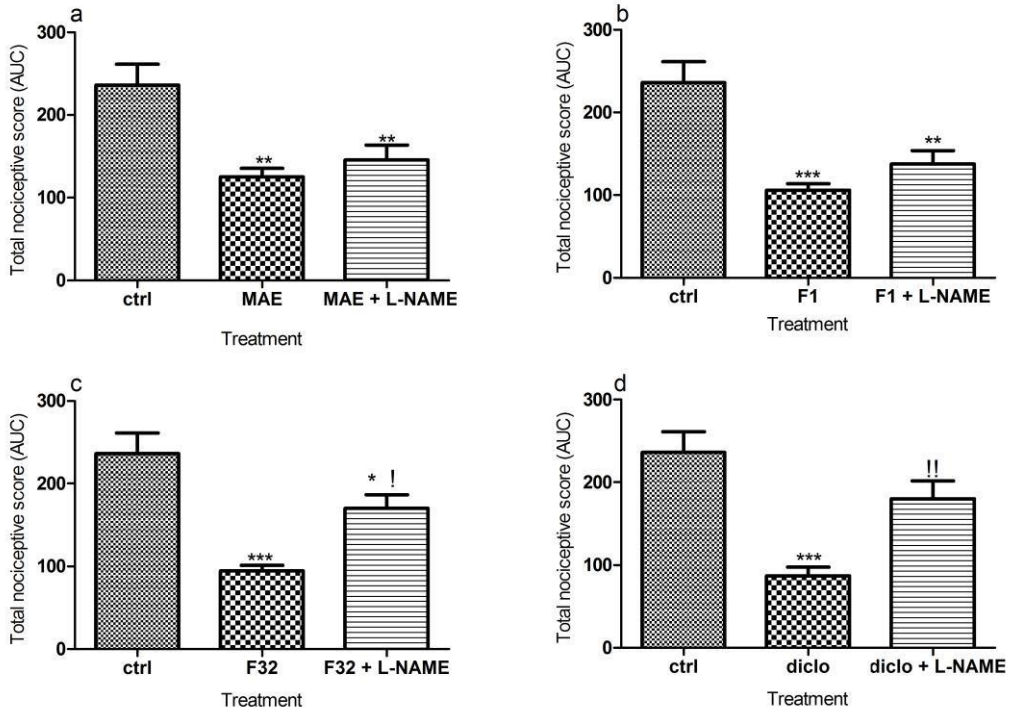


Figure 5.2 Effect of L-NAME (10 mg/kg, i.p.) on the antinociceptive effects of MAE, F1, F32 (10 mg/kg, *p.o.*) and diclofenac (10 mg/kg, i.p.) in acetic acid-induced writhing test (a, b, c and d). Each column represents the mean ± SEM (n = 5). **P*<0.05, ***P*<0.01, ****P*<0.001, compared to controls, !*P*<0.05, !!*P*<0.01, compared to F32 or diclofenac (one-way ANOVA followed by Newman-Keuls *post hoc* test).

5.3.1. 3 Involvement of the muscarinic system

Pre-treatment of mice with atropine (5 mg/kg) reversed antinociception of F1, F32 (10 mg/kg, *p.o*., Figure 5.3 b and c) and diclofenac (10 mg/kg, i.p., Figure 5.3 d).

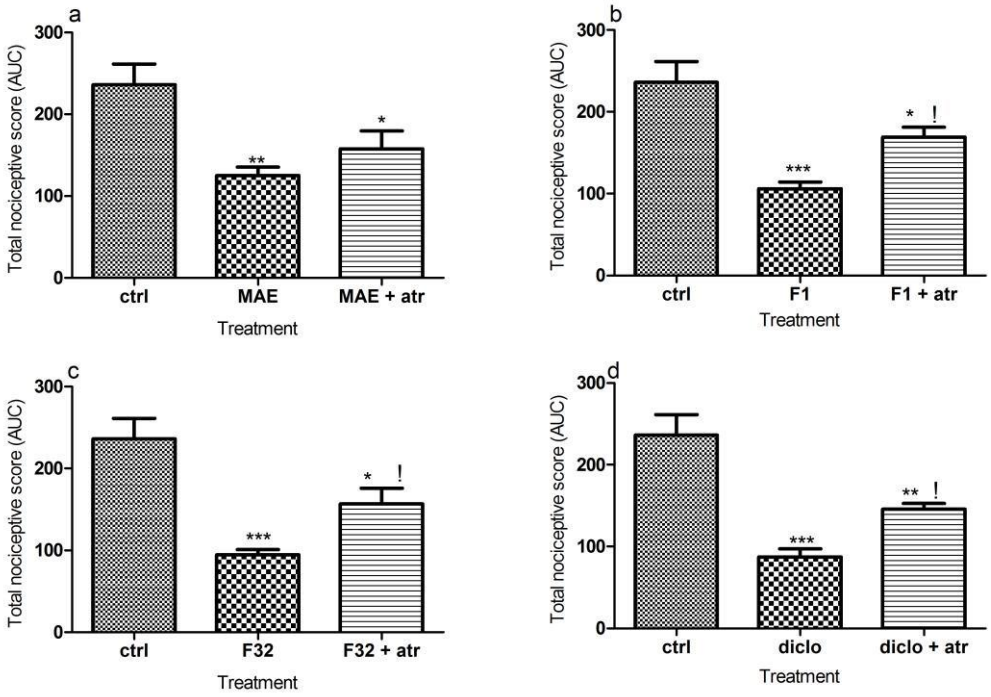


Figure 5.3 Effect of atropine (5 mg/kg, i.p.) on the antinociceptive effects of MAE, F1, F32 (10 mg/kg, *p.o*.) and diclofenac (10 mg/kg, i.p.) in acetic acid-induced writhing test (a, b, c and d). Each column represents the mean ± SEM (n = 5). **P*<0.05, ***P*<0.01, ****P*<0.001, compared to controls, !*P*<0.05, compared to F1, F32 or diclofenac (one-way ANOVA followed by NewmanKeuls *post hoc* test).

5.3.1. 4 Participation of the ATP sensitive K^+ channels

Pretreatment of mice with 8 mg/kg glibenclamide reversed antinociception of MAE, F1, F32 (10 mg/kg, *p.o*., Figure 5.4 a, b and c) and diclofenac (10 mg/kg, i.p., Figure 5.4 d).

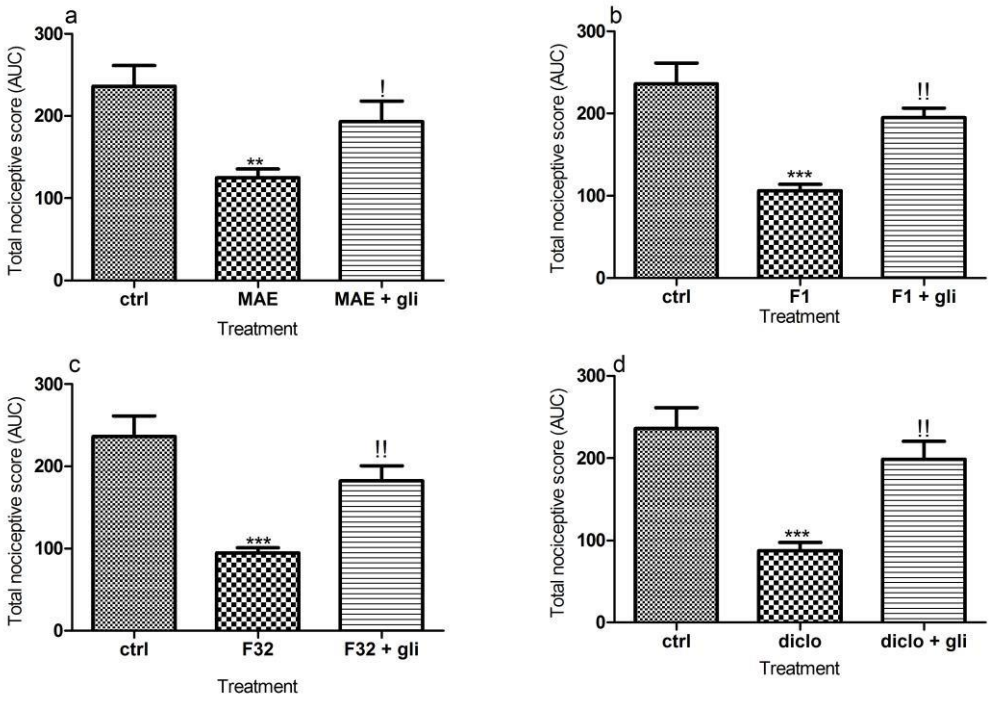


Figure 5.4 Effect of glibenclamide (8 mg/kg, *p.o*.) on the antinociceptive effects of MAE, F1, F32 (10 mg/kg, *p.o*.) and diclofenac (10 mg/kg, i.p.) in acetic acid-induced writhing test (a, b, c and d). Each column represents the mean ± SEM (n = 5). ***P*<0.01, ****P*<0.001, compared to controls, !*P*<0.05, !!*P*<0.01, compared to MAE, F1, F32 or diclofenac (one-way ANOVA followed by Newman-Keuls *post hoc* test).

5.3.1.
5 Participation of serotoninergic system

The antinociception of MAE, F1, F32 (10 mg/kg, *p.o*., Figure 5.5 a, b and c) and diclofenac (10 mg/kg, i.p., Figure 5.5 d) in the acetic acid-induced abdominal writhing test was not blocked by ondansetron.

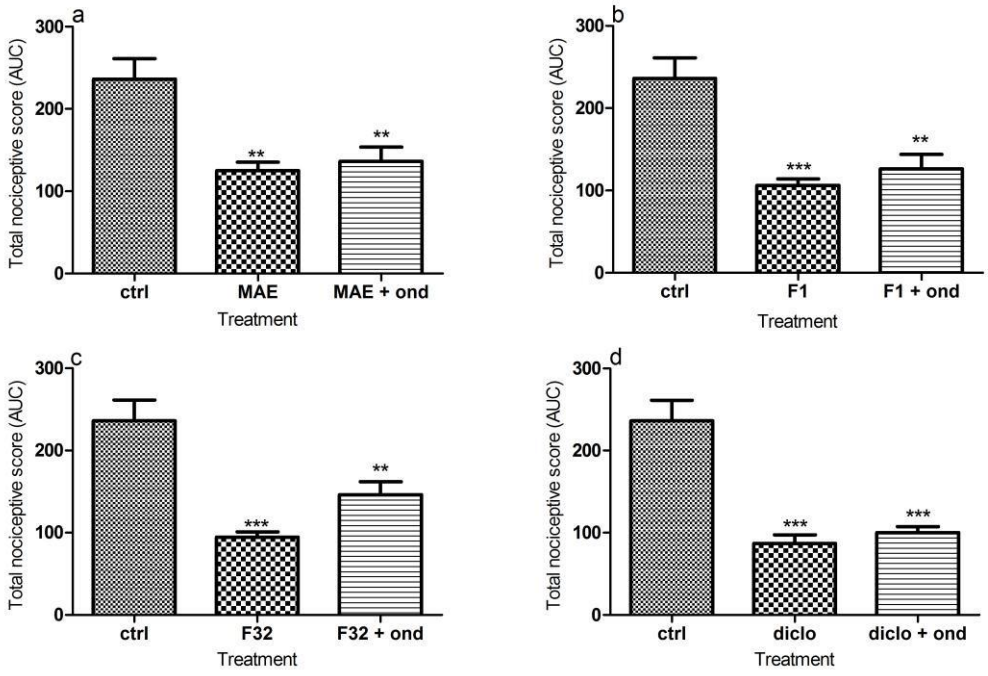


Figure 5.5 Effect of ondansetron (0.5 mg/kg, i.p.) on the antinociceptive effects of MAE, F1, F32 (10 mg/kg, *p.o*.) and diclofenac (10 mg/kg, i.p.) in acetic acid-induced writhing test (a, b, c and d). Each column represents the mean ± SEM (n = 5). *****P*<0.01, ******P*<0.001, compared to controls (one-way ANOVA followed by Newman-Keuls *post hoc* test).

5.3.1. 6 Participation of the adrenergic system

Pre-treatment of mice with 3 mg/kg yohimbine reversed antinociception of F1, F32 (10 mg/kg, *p.o*., Figure 5.6 b and c) and diclofenac (10 mg/kg, i.p., Figure 5.6 d).

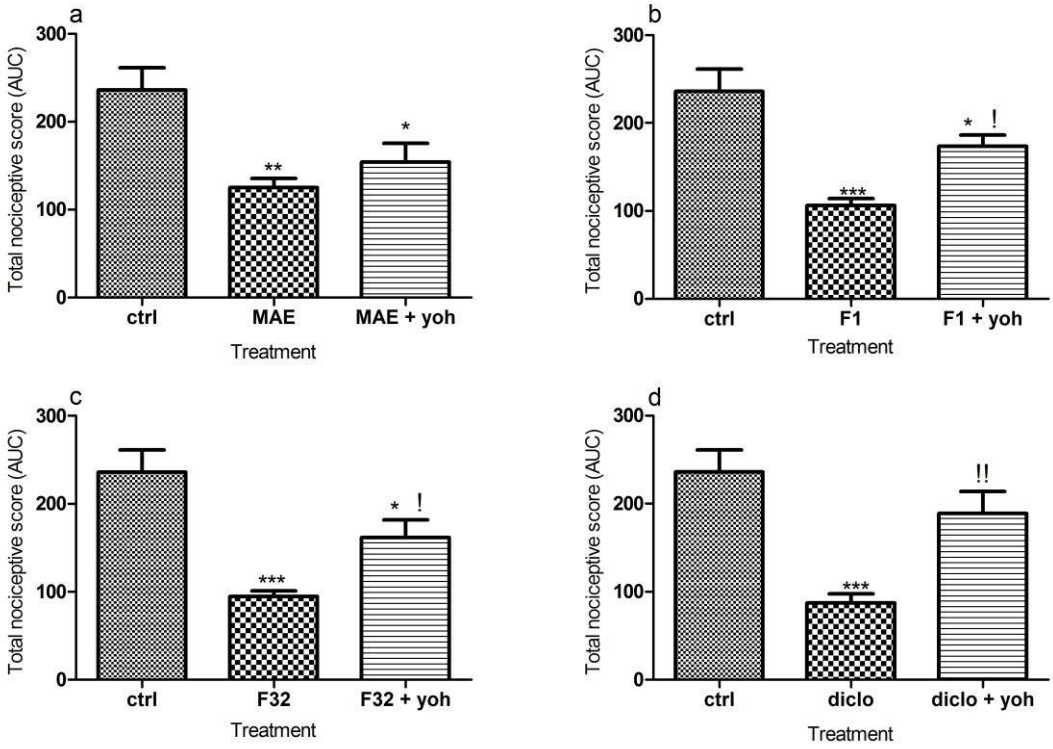


Figure 5.6 Effect of yohimbine (3 mg/kg, i.p.) on the antinociceptive effects of MAE, F1, F32 (10 mg/kg, *p.o*.) and diclofenac (10 mg/kg, i.p.) in acetic acid-induced writhing test (a, b, c and d). Each column represents the mean ± SEM (n = 5). **P*<0.05, ***P*<0.01, ****P*<0.001, compared to controls, !*P*<0.05, !!*P*<0.01, compared to F1, F32 or diclofenac (one-way ANOVA followed by Newman-Keuls *post hoc* test).

5.3.1. 7 Investigation of the opioid pathway

The antinociception of MAE, F1, F32 (10 mg/kg, *p.o*., Figure 5.7 a, b and c) and diclofenac (10 mg/kg, i.p., Figure 5.7 d) in the acetic acid-induced abdominal writhing test was not blocked by naloxone.

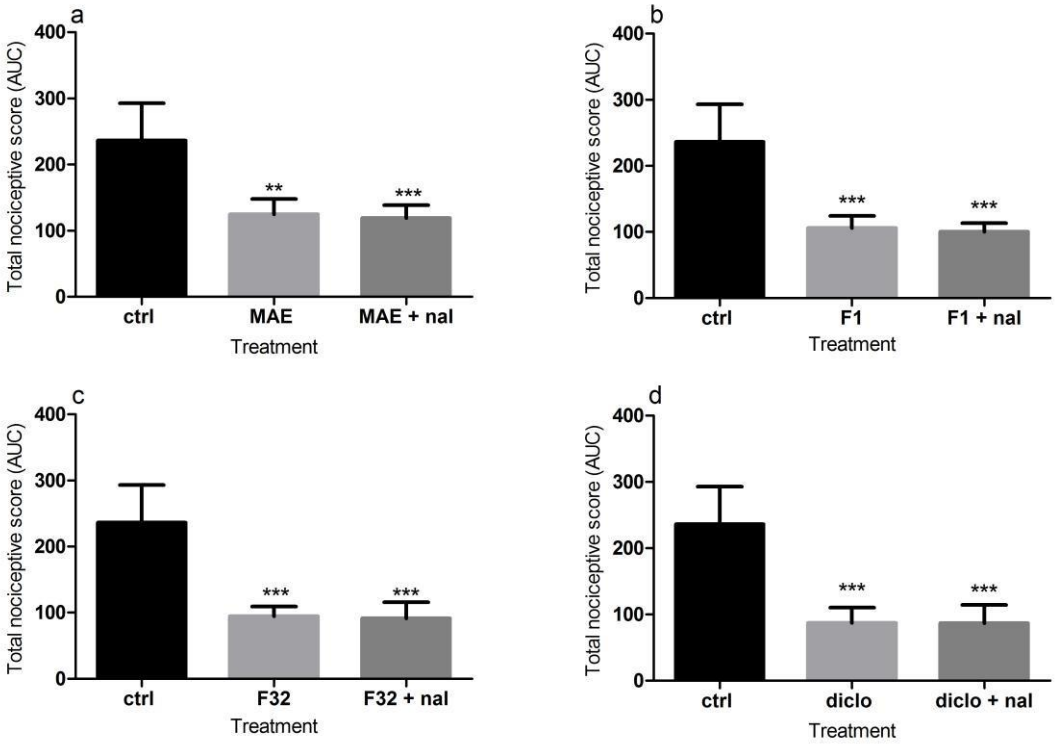


Figure 5.7 Effect of naloxone (2 mg/kg, i.p.) on the antinociceptive effects of MAE, F1, F32 (10 mg/kg, *p.o*.) and diclofenac (10 mg/kg, i.p.) in acetic acid-induced writhing test (a, b, c and d). Each column represents the mean ± SEM (n = 5). ***P*<0.01, ****P*<0.001, compared to controls (one-way ANOVA followed by Newman-Keuls *post hoc* test).

5.3.1.

5.3.2 Investigation of the mechanism of antinociception of MAE and fractions in the tail-flick test

5.3.2.1 Involvement of the adenosinergic system

Pre-treatment of mice with 5 mg/kg theophylline reversed antinociception of MAE, F1, F32 (10 mg/kg, *p.o*., Figure 5.8 a, b and c) and morphine (3 mg/kg, i.p., Figure 5.8 d).

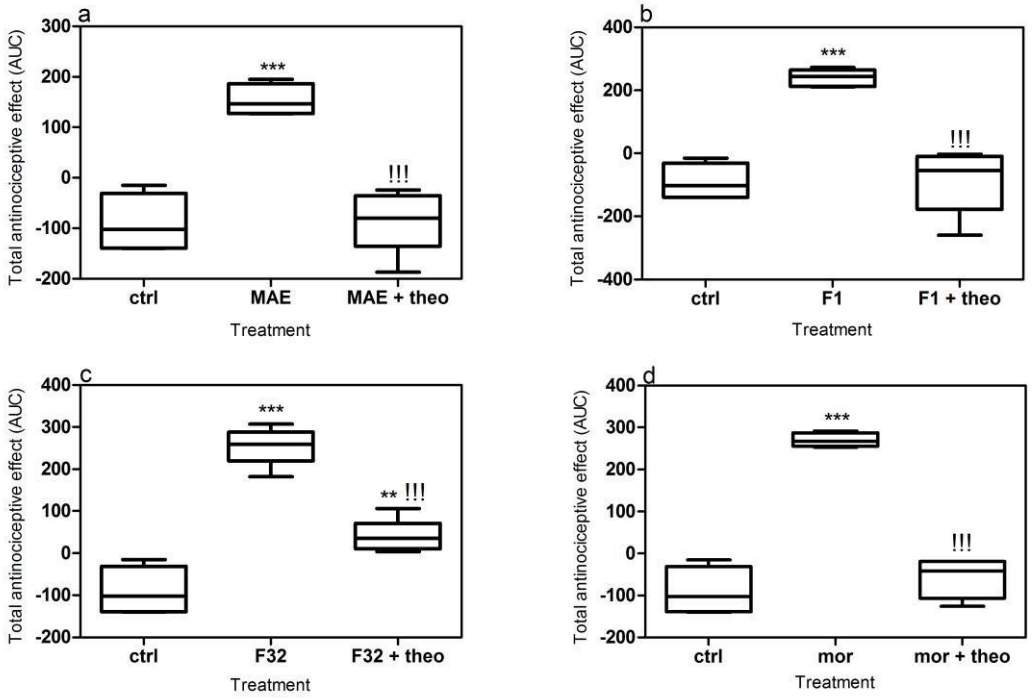


Figure 5.8 Effect of theophylline (5 mg/kg i.p.) on the antinociceptive effects of MAE, F1, F32 (10 mg/kg *p.o*.) and morphine (3 mg/kg, i.p.) in Hargreaves thermal tail-flick test. Data are expressed as mean ± SEM (n = 5). The lower and upper margins of the boxes represent the 25th and 75th percentiles, with the extended arms representing the 10th and 90th percentiles, respectively. The median is shown as the horizontal line within the box. *****P*<0.01, ******P*<0.001, compared to controls, !!!*P*<0.001, compared to MAE, F1, F32 or morphine (one-way ANOVA followed by Newman-Keuls *post hoc* test).

5.3.2.2 Participation of the nitric oxide system

The antinociception of F1, F32 (10 mg/kg, *p.o*., Figure5.9 b and c) and morphine (3 mg/kg, i.p., Figure 5.9 d) in the Hargreaves thermal tail-flick test was blocked by LNAME (10 mg/kg).

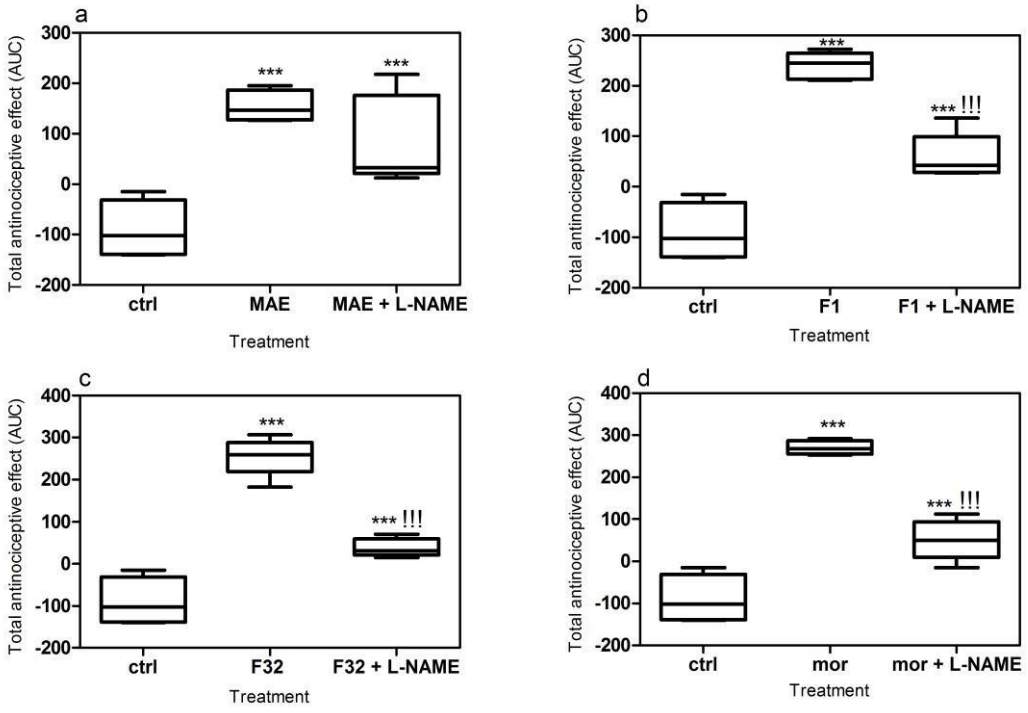


Figure 5.9 Effect of L-NAME (10 mg/kg i.p.) on the antinociceptive effects of MAE, F1, F32 (10 mg/kg *p.o.*) and morphine (3 mg/kg, i.p.) in Hargreaves thermal tail-flick test. Data are expressed as mean ± SEM (n = 5). The lower and upper margins of the boxes represent the 25th and 75th percentiles, with the extended arms representing the 10th and 90th percentiles, respectively. The median is shown as the horizontal line within the box. ******P*<0.001, compared to controls, !!!*P*<0.001, compared to F1, F32 or morphine (one-way ANOVA followed by Newman-Keuls *post hoc* test).

5.3.2.3 Involvement of the muscarinic system

Pre-treatment of mice with 5 mg/kg atropine reversed antinociception of MAE, F1, F32 (10 mg/kg, *p.o.*, Figure 5.10 a, b and c) and morphine (3 mg/kg, i.p., Figure 5.10 d).

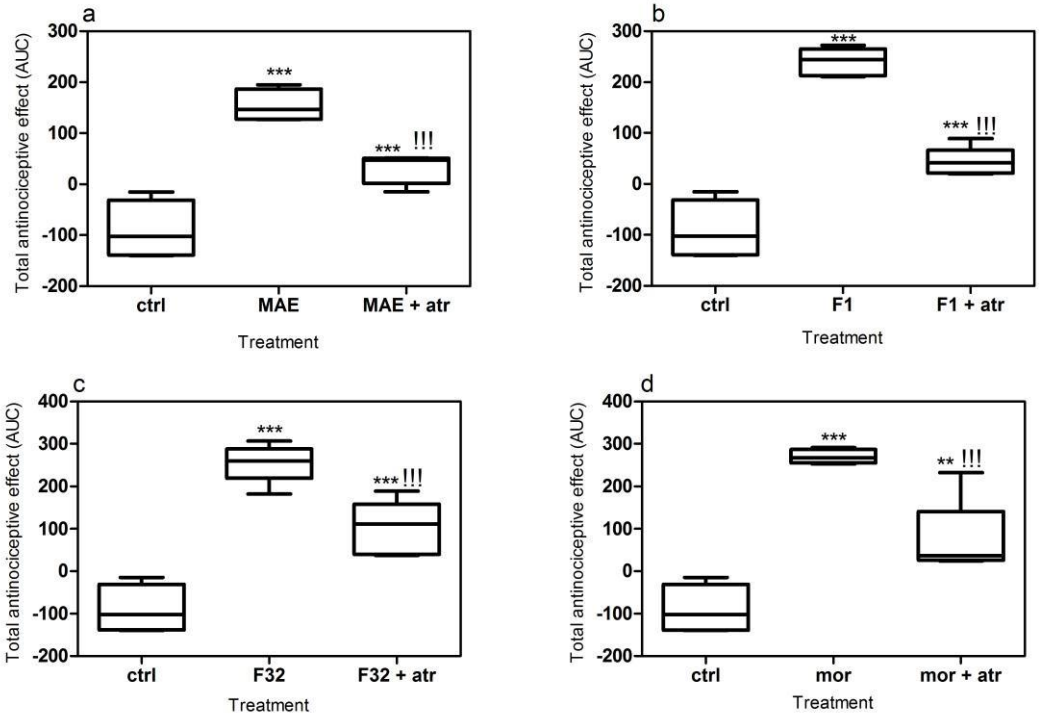


Figure 5.10 Effect of atropine (5 mg/kg i.p.) on the antinociceptive effects of MAE, F1, F32 (10 mg/kg *p.o.*) and morphine (3 mg/kg, i.p.) in Hargreaves thermal tail-flick test. Data are expressed as mean ± SEM (n = 5). The lower and upper margins of the boxes represent the 25th and 75th percentiles, with the extended arms representing the 10th and 90th percentiles, respectively. The median is shown as the horizontal line within the box. *****P*<0.01, ******P*<0.001, compared to controls, !!!*P*<0.001, compared to MAE, F1, F32 or morphine (one-way ANOVA followed by Newman-Keuls *post hoc* test).

5.3.2.4 Participation of the ATP sensitive K^+ channels

Pre-treatment of mice with 8 mg/kg glibenclamide reversed antinociception of MAE, F1, F32 (10 mg/kg, *p.o.*, Figure 5.11 a, b and c) and morphine (3 mg/kg, i.p., Figure 5.11 d).

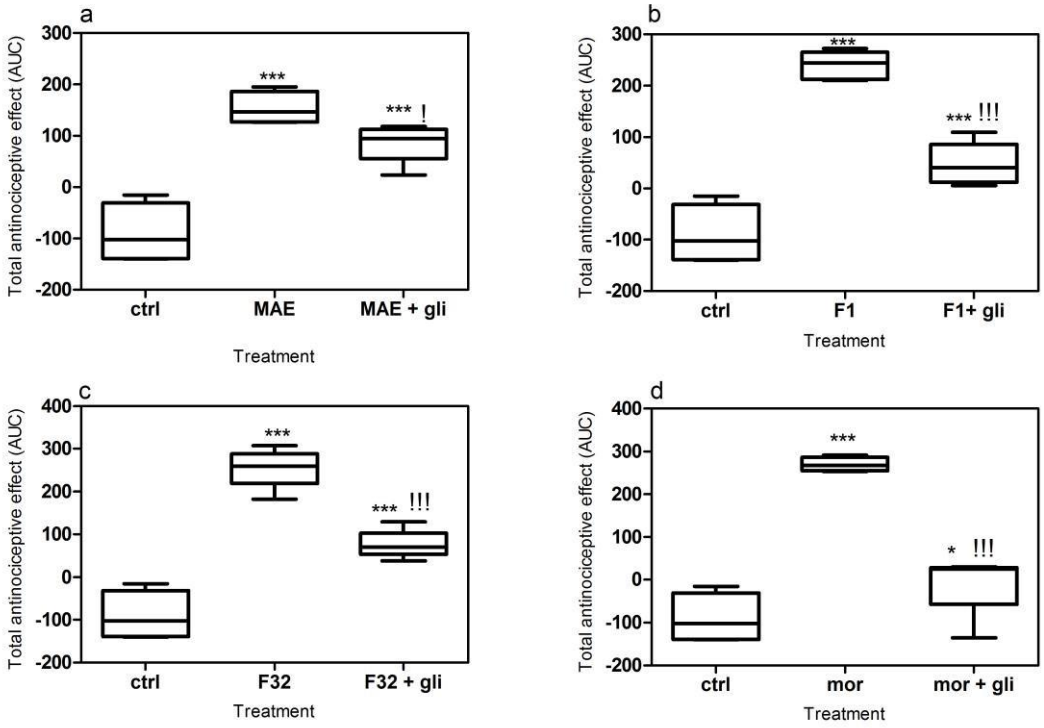


Figure 5.11 Effect of glibenclamide (8 mg/kg *p.o.*) on the antinociceptive effects of MAE, F1, F32 (10 mg/kg *p.o.*) and morphine (3 mg/kg, i.p.) in Hargreaves thermal tail-flick test. Data are expressed as mean ± SEM (n = 5). The lower and upper margins of the boxes represent the 25th and 75th percentiles, with the extended arms representing the 10th and 90th percentiles, respectively. The median is shown as the horizontal line within the box. **P*<0.05, ****P*<0.001, compared to controls, !*P*<0.05, !!!*P*<0.001, compared to MAE, F1, F32 or morphine (one-way ANOVA followed by Newman-Keuls *post hoc* test).

5.3.2.5 Participation of the serotoninergic system

The antinociception of F1, F32 (10 mg/kg, *p.o.*, Figure 5.12 b and c) and morphine (3 mg/kg, i.p., Figure 5.12 d) in the Hargreaves thermal tail-flick test was blocked by ondansetron (0.5 mg/kg, i.p.)

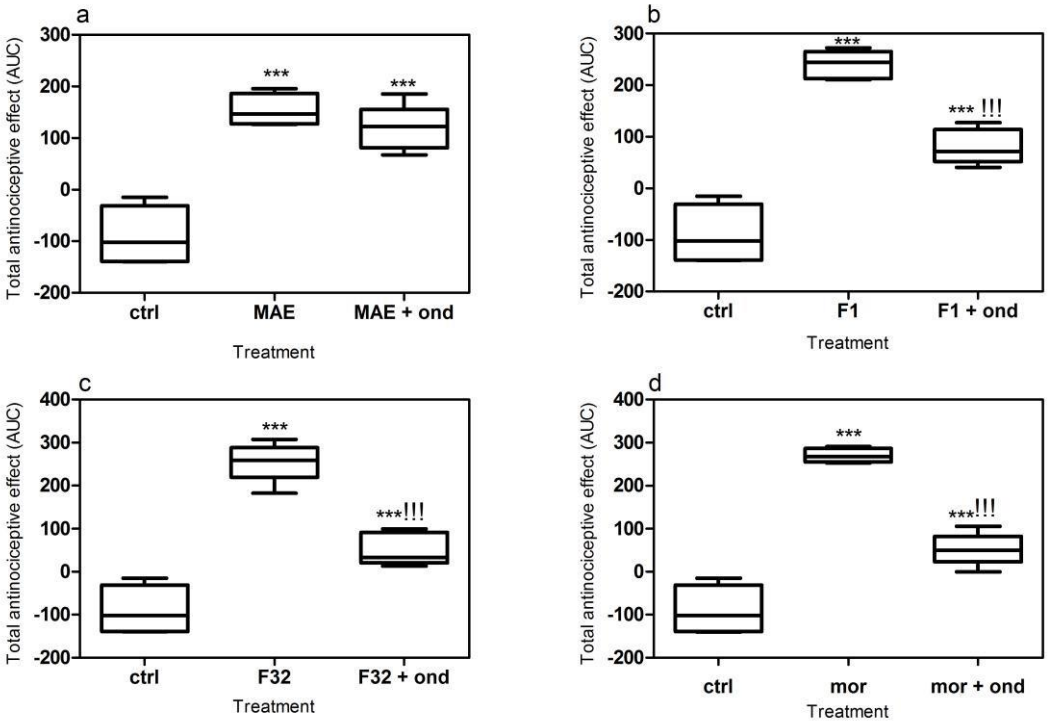


Figure 5.12 Effect of ondansetron (0.5 mg/kg i.p.) on the antinociceptive effects of MAE, F1, F32 (10 mg/kg *p.o.*) and morphine (3 mg/kg, i.p.) in Hargreaves thermal tail-flick test. Data are expressed as mean ± SEM (n = 5). The lower and upper margins of the boxes represent the 25th and 75th percentiles, with the extended arms representing the 10th and 90th percentiles, respectively. The median is shown as the horizontal line within the box. ***P<0.001, compared to controls, !!!P<0.001, compared to F1, F32 or morphine (one-way ANOVA followed by Newman-Keuls *post hoc* test).

5.3.2.6 Participation of the adrenergic system

The antinociception of F1 and F32 (10 mg/kg, *p.o.*, Figure 5.13 b and c) in the Hargreaves thermal tail-flicked test was blocked by yohimbine (3 mg/kg).

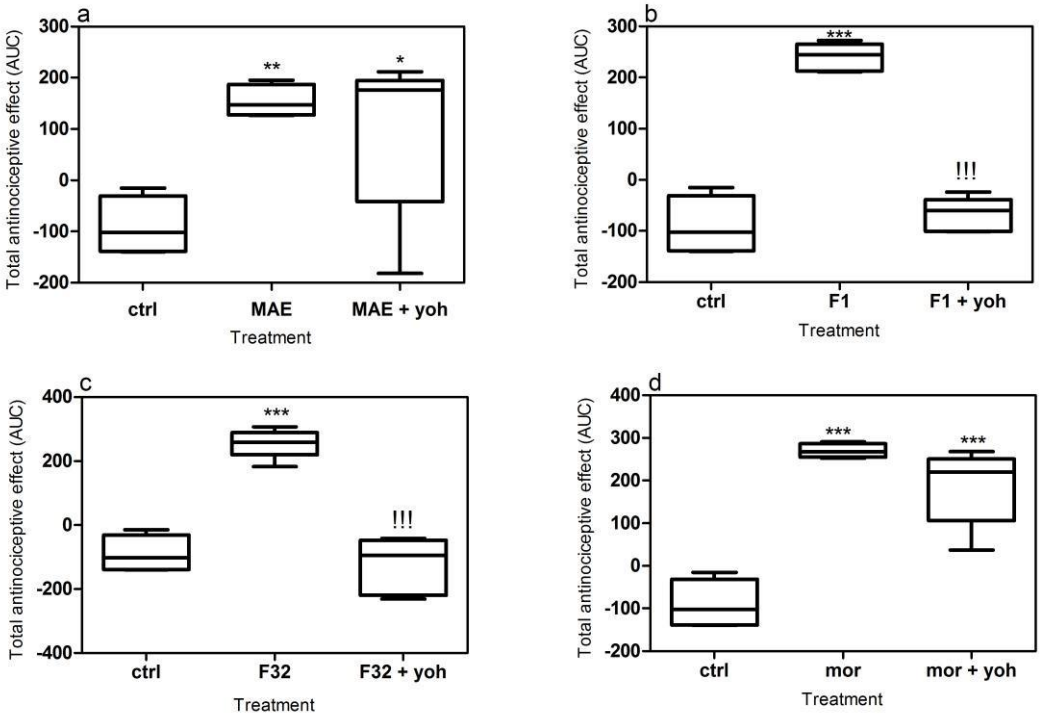


Figure 5.13 Effect of yohimbine (3 mg/kg i.p.) on the antinociceptive effects of MAE, F1, F32 (10 mg/kg *p.o.*) and morphine (3 mg/kg, i.p.) in Hargreaves thermal tail-flick test. Data are expressed as mean ± SEM (n = 5). The lower and upper margins of the boxes represent the 25th and 75th percentiles, with the extended arms representing the 10th and 90th percentiles, respectively. The median is shown as the horizontal line within the box. **P*<0.05, ***P*<0.01, ****P*<0.001, compared to controls, !!!*P*<0.001, compared to F1 or F32 (one-way ANOVA followed by Newman-Keuls *post hoc* test).

5.3.2.7 Investigation of the opioid pathway

The antinociception of MAE, F1, F32 (10 mg/kg, *p.o.*, Figure 5.14 a, b and c) and morphine (3 mg/kg, i.p, Figure 5.14 d) in the Hargreaves thermal tail-flicked test was blocked by naloxone (2 mg/kg).

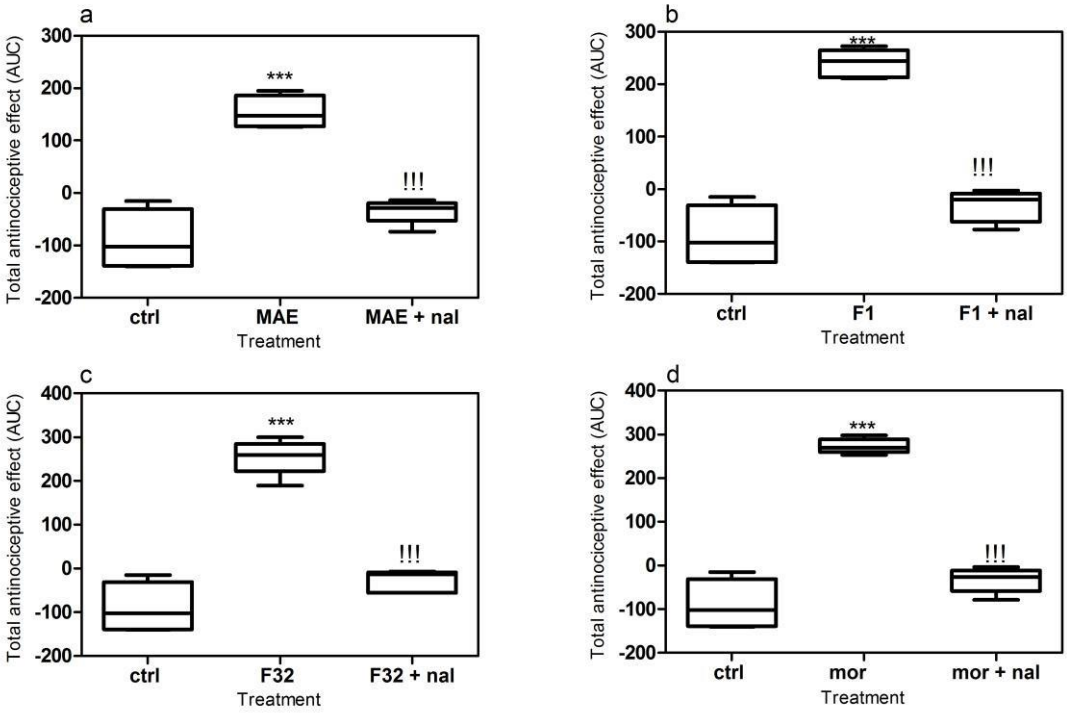


Figure 5.14 Effect of naloxone (2 mg/kg i.p.) on the antinociceptive effects of MAE, F1, F32 (10 mg/kg *p.o.*) and morphine (3 mg/kg, i.p.) in Hargreaves thermal tail-flick test. Data are expressed as mean ± SEM (n = 5). The lower and upper margins of the boxes represent the 25th and 75th percentiles, with the extended arms representing the 10th and 90th percentiles, respectively. The median is shown as the horizontal line within the box. ******P*<0.001, compared to controls, !!!*P*<0.001, compared to MAE, F1, F32 or morphine (one-way ANOVA followed by Newman-Keuls *post hoc* test).

5.3.3 Investigation of the mechanism of antinociception of MAE and fractions in the capsaicin test

Intraplantar administration of capsaicin induced a nociceptive response characterized by biting, licking and flinching of the injected paw. MAE, F1 and F32 significantly inhibited the capsaicin-induced licking. The antinociception produced by MAE (3 – 30 mg/kg, *p.o.*) was significant ($F_{3, 16}$ = 8.054, P = 0.0017) and dose-dependent with the highest dose causing a maximal antinociception of 93.5 ± 0.35% (Figure 5.15 a). F1 and F32 (3 – 30 mg/kg, *p.o.*) also produced significant ($F_{3, 16}$ = 9.385, P = 0.0008 and $F_{3, 16}$ = 6.392, P = 0.0047) and dose-dependent inhibition of capsaicin-induced nociception with the highest doses causing maximal antinociception of 97.5 ± 0.27 and

99.1 ± 0.07% respectively (Figure 5.15 b and c). The dose-response curves for the inhibition of capsaicin-induced neurogenic pain in mice shows F1 was the most potent (1.654 ± 0.1575 mg/kg, Figure 5.16).

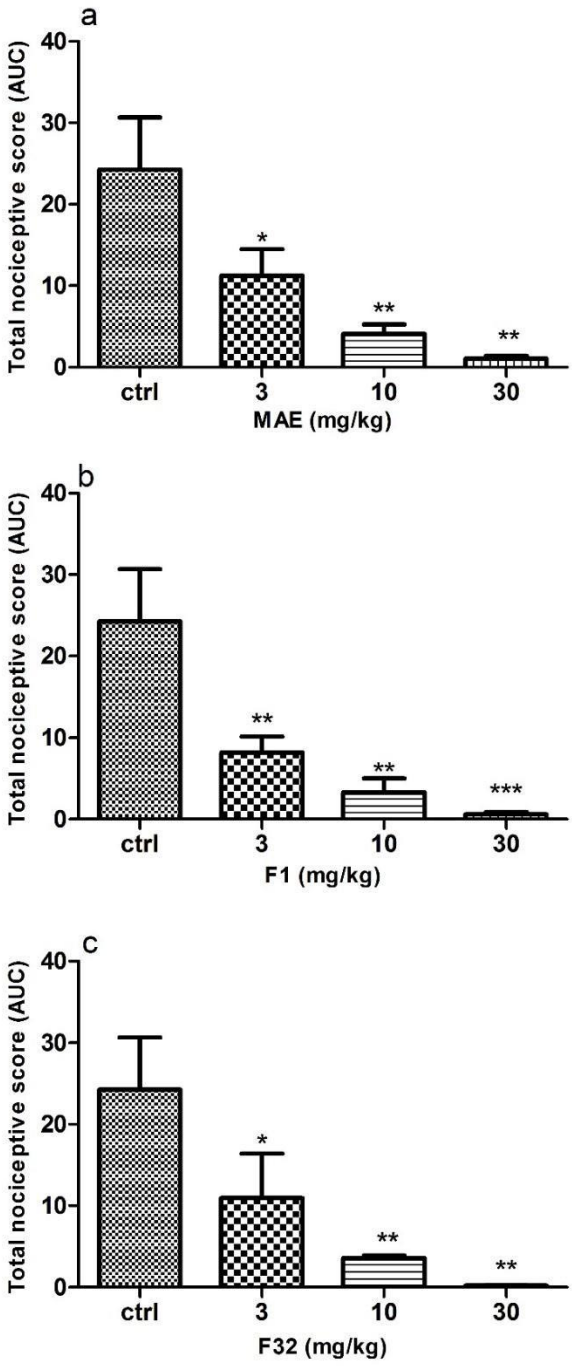


Figure 5.15 Effect of MAE, F1 and F32 (3 – 30 mg/kg, *p.o.*) on the total nociceptive score of capsaicin-induced nociception (calculated as AUC) (a, b and c) in ICR mice. Data is presented as mean ± SEM (n = 5). **P*<0.05, ***P*<0.01, ****P*<0.001, compared to vehicle-treated group (Oneway ANOVA followed by Newman-Keuls *post hoc* test).

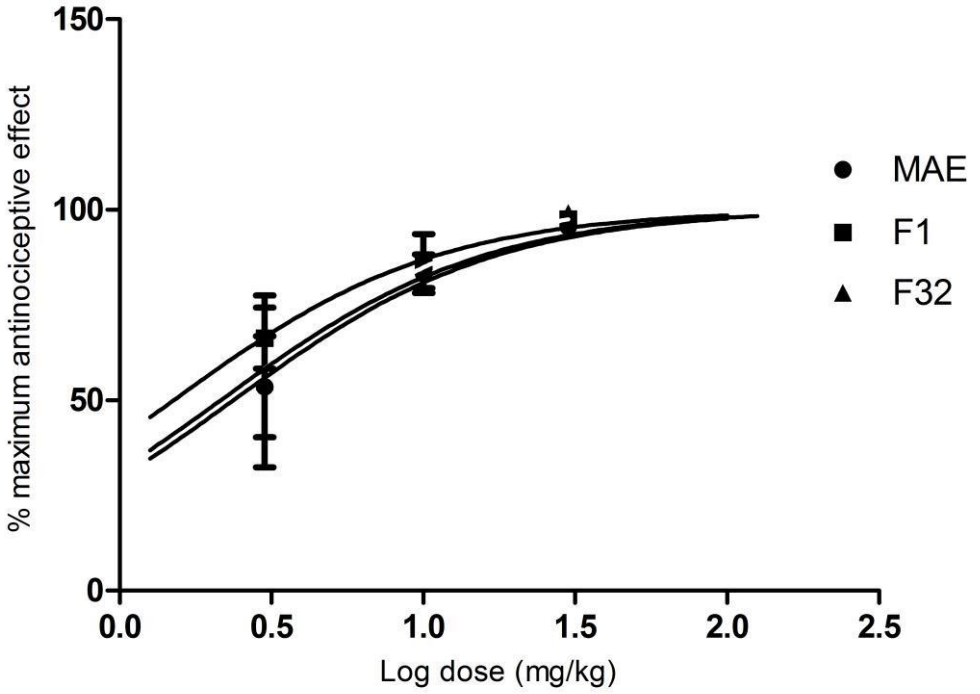


Figure 5.16 Dose-response curves for the antinociceptive activity of MAE, F1 and F32 (3 – 30 mg/kg) in the capsaicin-induced neurogenic pain test in ICR mice. Each point represent the mean ± SEM (n = 5).

5.3.4 Investigation of the mechanism of antinociception of MAE and fractions in the glutamate test

The extract, F1, F32 and ketamine significantly suppressed the time-course curve of glutamate-induced neurogenic pain during the 15 min observational period [$F_{3, 64}$ = 31.77, P<0.0001; $F_{3, 64}$ = 48.22, P<0.0001; $F_{3, 64}$ = 33.94, P<0.0001 and $F_{3, 64}$ = 43.39, P<0.0001; Two-way ANOVA (treatment x time); Figure 5.17 a, c, e and g respectively]. MAE (3 – 30 mg/kg, *p.o*. 1 h before glutamate injection) produced significant ($F_{3, 16}$ = 41.76, P<0.0001; Figure 5.17 b) and dose-dependent inhibition of glutamate-induced licking with a maximal inhibition of 74.1 ± 1.33% at the dose of 30 mg/kg. F1 and F32 (3 – 30 mg/kg, *p.o*.) 1 h before glutamate injection also produced significant ($F_{3, 16}$ = 58.97, P<0.0001 and $F_{3, 16}$ = 30.15, P<0.0001; Figure

5.17 d and f) and dose-related attenuation of glutamate-induced pain with maximal inhibitions of 84.4 ± 1.06 and 86.9 ± 1.69% respectively at the highest doses used. Similarly, ketamine (1 – 10 mg/kg, i.p.) 30 min before glutamate injection significantly ($F_{3, 16}$ = 75.43, P<0.0001; Figure 5.17 h) and dose-dependently inhibited the glutamate evoked neurogenic pain with a maximal inhibition of 84.4 ± 1.72% at the dose of 10 mg/kg. The dose-response curves for the inhibition of glutamate induced pain shows the potency of ketamine being greater than F1 greater than MAE greater than F32 (Figure 5.18).

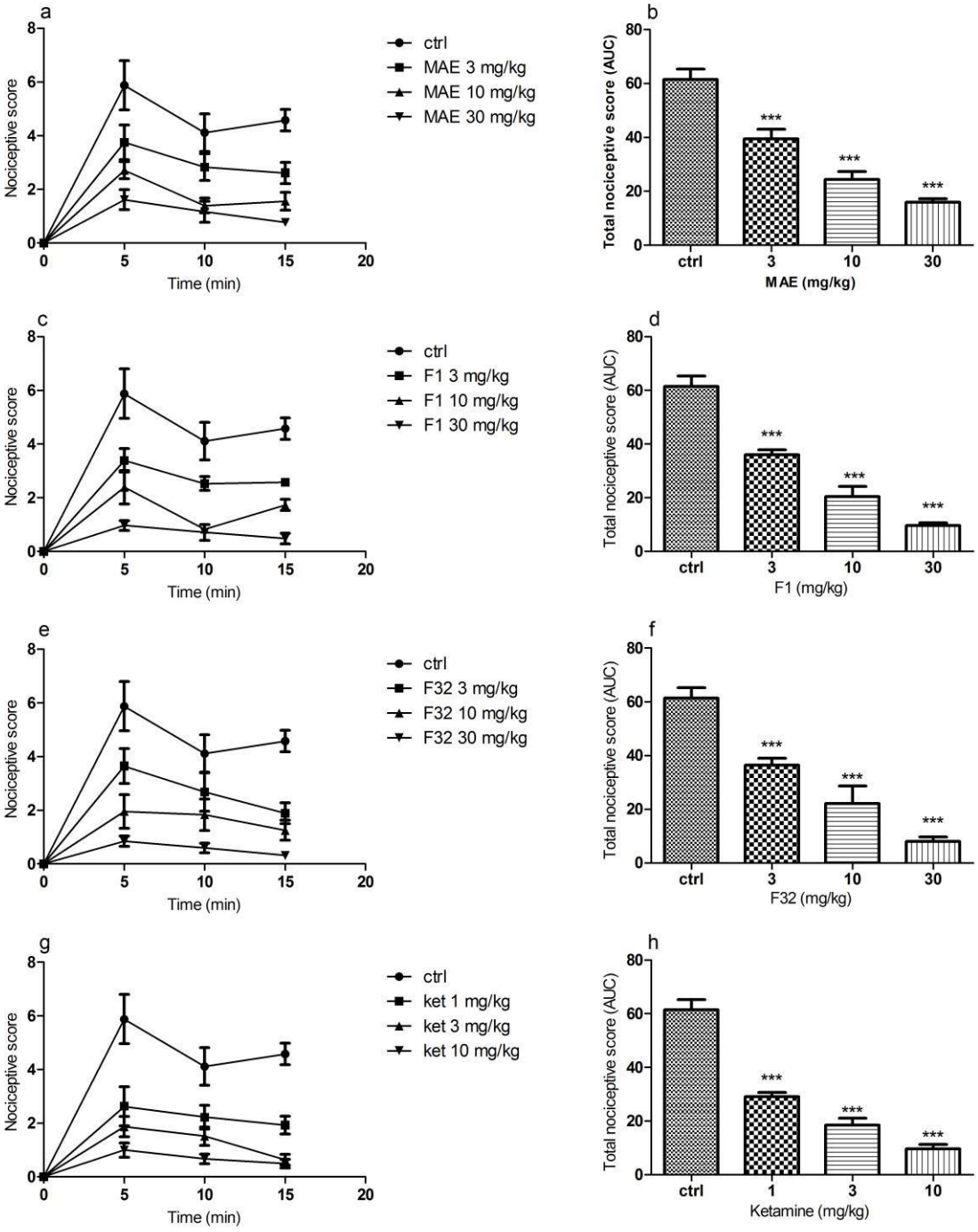


Figure 5.17 Effect of MAE, F1, F32 (3 – 30 mg/kg, *p.o.*) and ketamine (1 – 10 mg/kg, i.p.) on the time course curve of glutamate-induced nociception (a, c, e and g) and the total nociceptive score (calculated as AUC) (b, d, f and h) in ICR mice. Data are expressed as mean ± SEM (n = 5). ***P<0.001 compared to vehicle-treated group (one-way ANOVA followed by Newman-Keuls *post hoc* test).

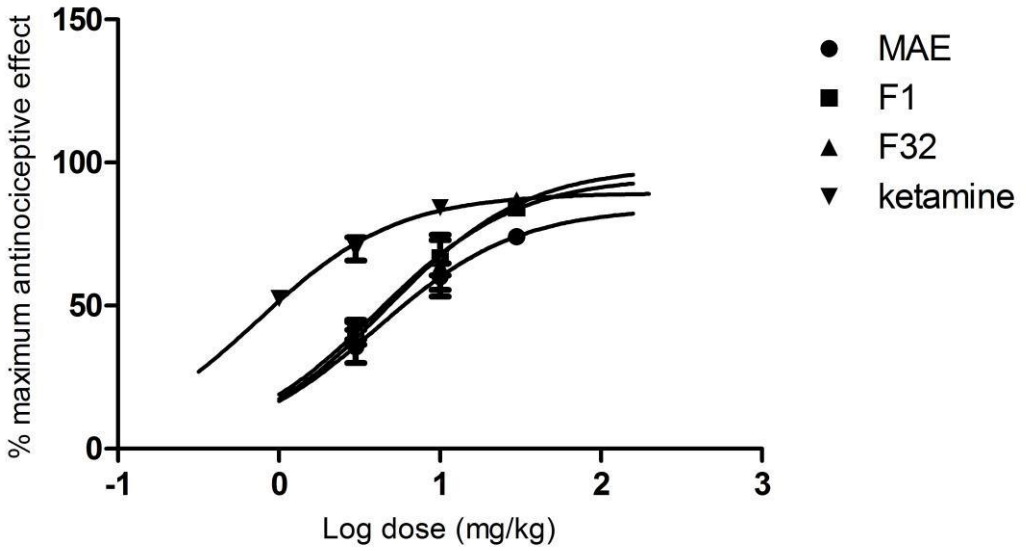


Figure 5.18 Dose-response curves for the antinociceptive activity of MAE, F1, F32 (3 – 30 mg/kg) and ketamine (1 – 10 mg/kg) in the glutamate-induced neurogenic pain test in ICR mice. Each point represent the mean ± SEM (n = 5).

5.3.5 Analgesic activity of MAE and fractions in the prostaglandin E_2–induced mechanical hyperalgesia

Intraplantar injection of the algogenic agent, prostaglandin E_2 produced a painful state which was measured using a mechanical source of stimulus. Mechanical hyperalgesia was induced in all the rats treated with prostaglandin E_2 though the control group exhibited higher hyperalgesia compared to the drug treated groups. The hyperalgesia caused was decreased by MAE, F1, F32 and morphine treatments when compared to control. MAE (3 – 30 mg/kg, *p.o.*) significantly ($F_{3,\ 16}$ = 34.39, P<0.0001) (Figure 5.19 a) and dose-dependently inhibited prostaglandin E_2-induced mechanical hyperalgesia in rats when compared to control with the maximum possible effect at the highest dose used (Figure 5.19 b). Oral administration of F1 and F32 (3 - 30 mg/kg) also significantly ($F_{3,\ 16}$ = 46.81, P<0.0001 and $F_{3,\ 16}$ = 13.06,

P<0.0005) (Figure 5.19 c and e) attenuated prostaglandin E_2-induced mechanical hyperalgesia in a dose–related manner with the maximum possible effect at 30 mg/kg (Figure 5.19 d and f). Morphine (0.3 – 3mg/kg, i.p.) used as reference analgesic produced significant ($F_{3,\ 16}$ = 25.88, *P*<0.0001) (Figure 5.19 g) and dose-dependent reduction in hyperalgesia with the maximum possible effect at the highest dose used (Figure 5.19 h). Morphine was the most potent (0.5656 ±0.1622 mg/kg) in this model followed by F1 (3.688 ±0.1208 mg/kg) (Figure 5.20).

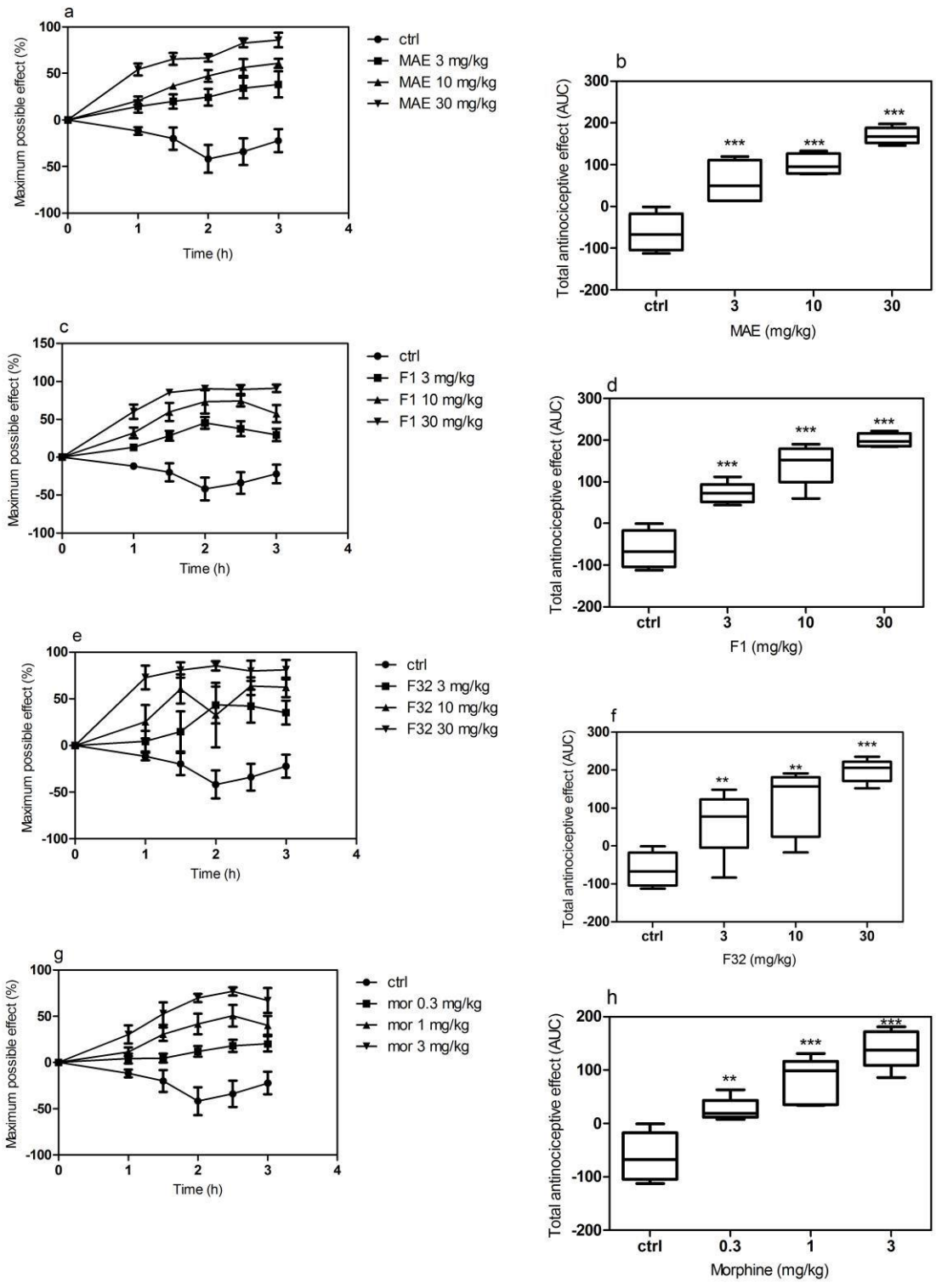


Figure 5.19 Effect of MAE, F1, F32 (3 – 30 mg/kg, *p.o.*) and morphine (0.3 – 3 mg/kg, i.p.) on the time course curve of Prostaglandin E2-induced mechanical hyperalgesia (a, c, e and g) and the total antinociceptive score (calculated as AUC) (b, d, f and h) in Sprague-Dawley rats. Data are expressed as mean ± SEM (n = 5). The lower and upper margins of the boxes (b, d, f and h) represent the 25th and 75th percentiles, with the extended arms representing the 10th and 90th

percentiles, respectively. The median is shown as the horizontal line within the box. *****P*<0.01; ******P*<0.001 compared to vehicle-treated group (one-way ANOVA followed by Newman-Keuls *post hoc* test).

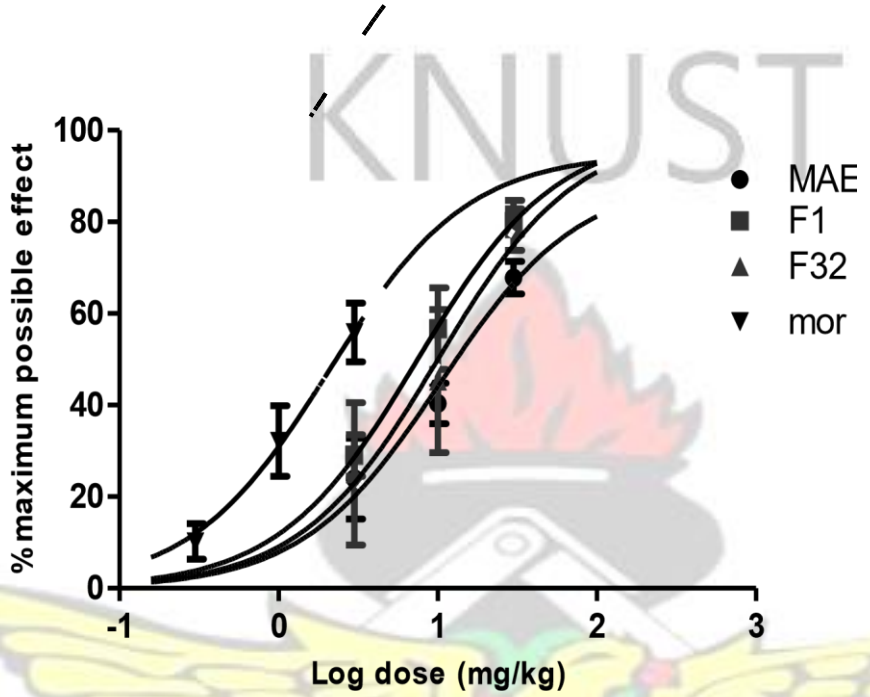


Figure 5.20 Dose-response curves for the antinociceptive activity of MAE, F1, F32 (3 – 30 mg/kg) and morphine (0.3 – 3 mg/kg) in the prostaglandin E2-induced hyperalgesia in SpragueDawley rats. Each point is the mean ± SEM (n = 5).

5.3.6 Analgesic activity of MAE and fractions in the bradykinin-induced thermal hyperalgesia

Intraplantar injection of the algogenic agent, bradykinin produced a painful state which was assessed using a thermal source of stimulus. Thermal hyperalgesia was induced in all the rats treated with bradykinin though the control group exhibited higher hyperalgesia compared to the drug treated groups. The hyperalgesia produced was lowered by MAE, F1 and F32 treatments when compared to the control. MAE (3 – 30 mg/kg, *p.o.*) significantly ($F_{3,\ 16}$ = 12.73, P = 0.0002) (Figure 5.21 a) and

dosedependently inhibited bradykinin-induced thermal hyperalgesia in rats when compared to control with the maximum possible effect at the highest dose used (Figure 5.21 b). Oral administration of F1 and F32 (3 - 30 mg/kg) also significantly ($F_{3,\ 16}$ = 26.38, P<0.0001 and $F_{3,\ 16}$ = 20.12, P<0.0001) (Figure 5.21 c and e) attenuated bradykinin-induced thermal hyperalgesia in a dose–related manner with the maximum possible effect at 30 mg/kg (Figure 5.21 d and f). F32 was more potent (4.053 ±0.2098 mg/kg) in this model followed by F1 (Figure 5.22).

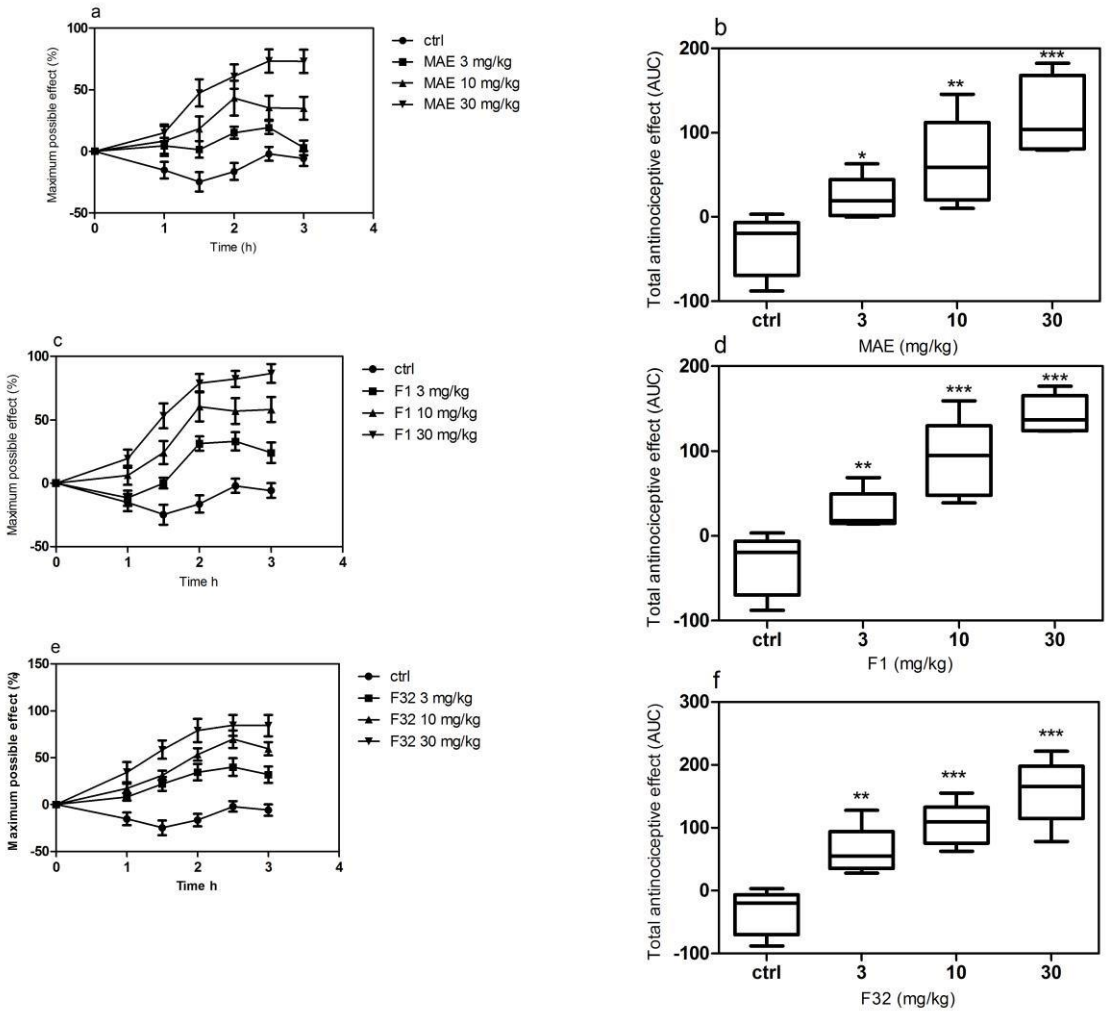


Figure 5.21 Effect of MAE, F1 and F32 (3 – 30 mg/kg, *p.o.*) on the time course curve of bradykinin-induced thermal hyperalgesia (a, c and e) and the total antinociceptive score (calculated as AUC) (b, d and f) in Sprague-Dawley rats. Data are expressed as mean ± SEM (n = 5). The lower and upper margins of the boxes (b, d and f) represent the 25th and 75th percentiles, with the extended arms representing the 10th and 90th percentiles respectively. The median is shown as the

horizontal line within the box. **P*<0.05; ***P*<0.01; ****P*<0.001 compared to vehicle-treated group (one-way ANOVA followed by Newman-Keuls *post hoc* test).

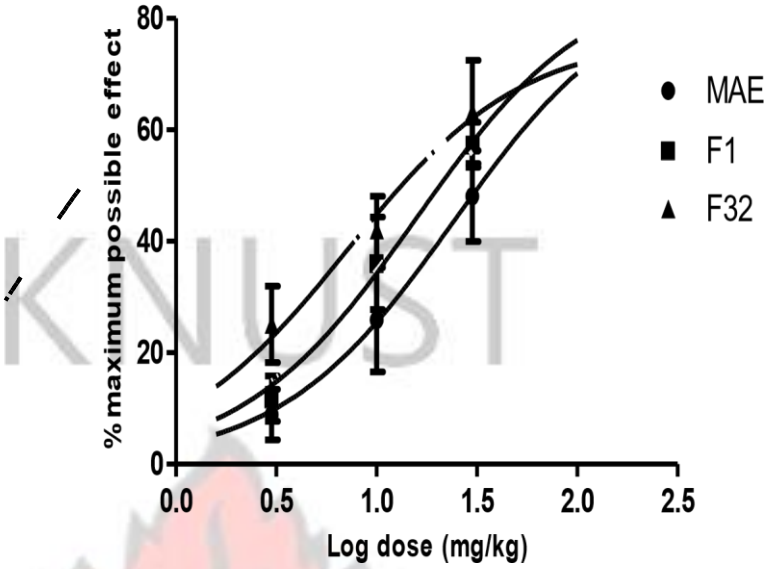


Figure 5.22 Dose-response curves for the antinociceptive activity of MAE, F1 and F32 (3 – 30 mg/kg) in the bradykinin-induced hyperalgesia in Sprague-Dawley rats. Each point is the mean ± SEM (n = 5).

5.3.7 Analgesic activity of MAE and fractions in the epinephrine–induced thermal hyperalgesia

Intraplantar injection of the algogenic agent, epinephrine produced a painful state which was assessed using a thermal source of stimulus. Thermal hyperalgesia was induced in all the rats treated with epinephrine though the control group exhibited higher hyperalgesia compared to the drug treated groups. The hyperalgesia produced was lowered by MAE, F1, F32 and atenolol treatments when compared to the control. MAE (3 – 30 mg/kg, *p.o.*) significantly ($F_{3,\ 16}$ = 44.02, P<0.0001) (Figure 5.23 a) and dose-dependently inhibited epinephrine-induced thermal hyperalgesia in rats when compared to control with the maximum possible effect at the highest dose used (Figure 5.23 b). Oral administration of F1 and F32 (3 - 30 mg/kg) also significantly ($F_{3,\ 16}$ = 27.96, P<0.0001 and $F_{3,\ 16}$ = 22.24, P<0.0001) (Figure 5.23 c and e) attenuated

epinephrine-induced thermal hyperalgesia in a dose–related manner with the maximum possible effect at 30 mg/kg (Figure 5.23 d and f). Atenolol (1 –

10 µg/paw) used as reference drug produced significant ($F_{3,\ 16}$ = 28.21, P<0.0001) (Figure 5.23 g) and dose-dependent reduction in hyperalgesia with the maximum possible effect at the highest dose used (Figure 5.23 h). Atenolol was the most potent in this model followed by MAE (Figure 5.24).

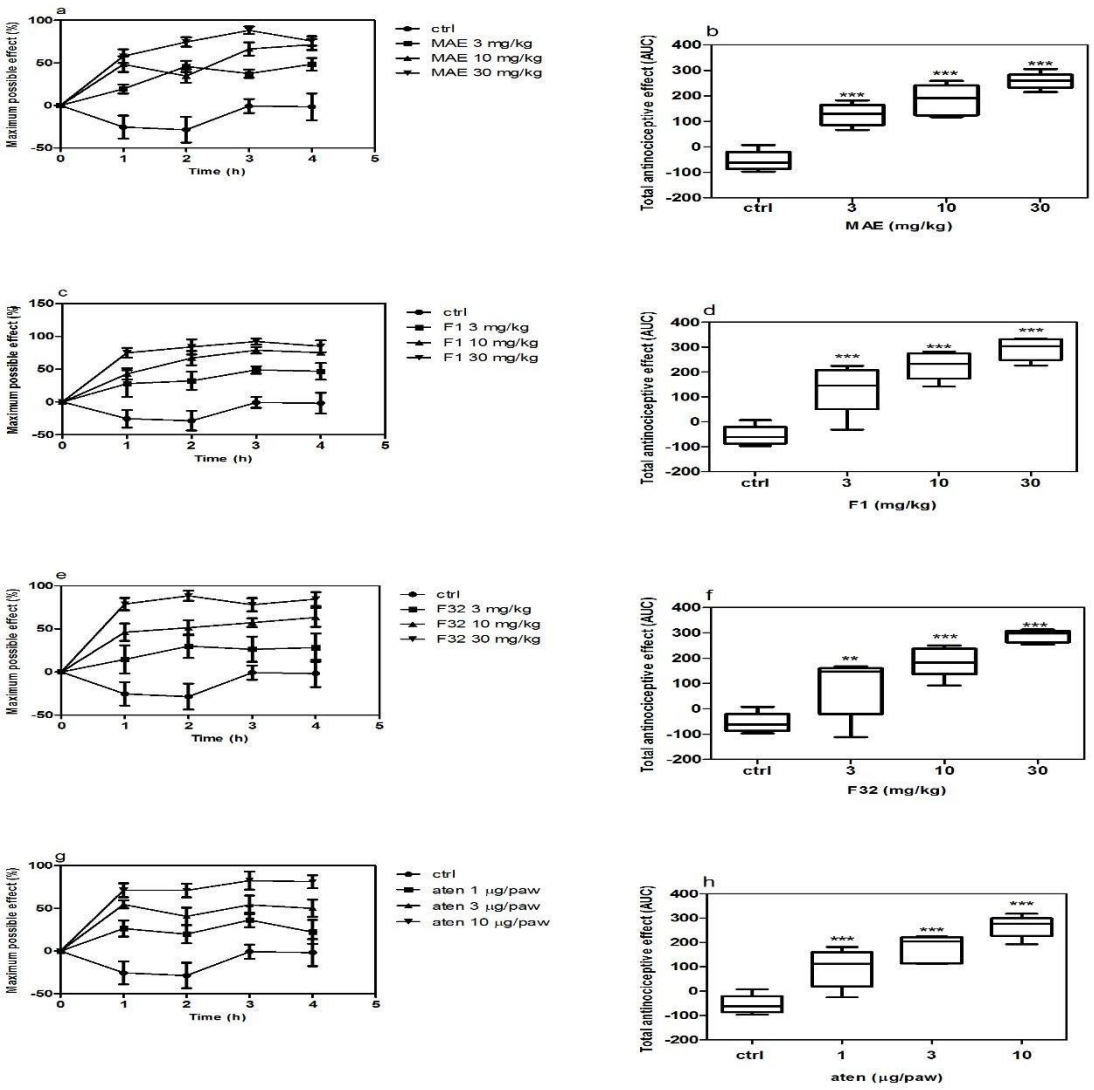


Figure 5.23 Effect of MAE, F1, F32 (3 – 30 mg/kg, *p.o.*) and atenolol (1 – 10 µg/paw) on the time course curve of epinephrine-induced thermal hyperalgesia (a, c, e and g) and the total antinociceptive score (calculated as AUC) (b, d, f and h) in Sprague-Dawley rats. Data are expressed as mean ± SEM (n = 5) . The lower and upper margins of the boxes (b, d, f and h) represent the 25th and 75th percentiles, with the extended arms representing the 10th and 90th percentiles, respectively. The median is shown as the horizontal line within the box. **P<0.01;

***P<0.001 compared to vehicle-treated group (one-way ANOVA followed by Newman-Keuls *post hoc* test).

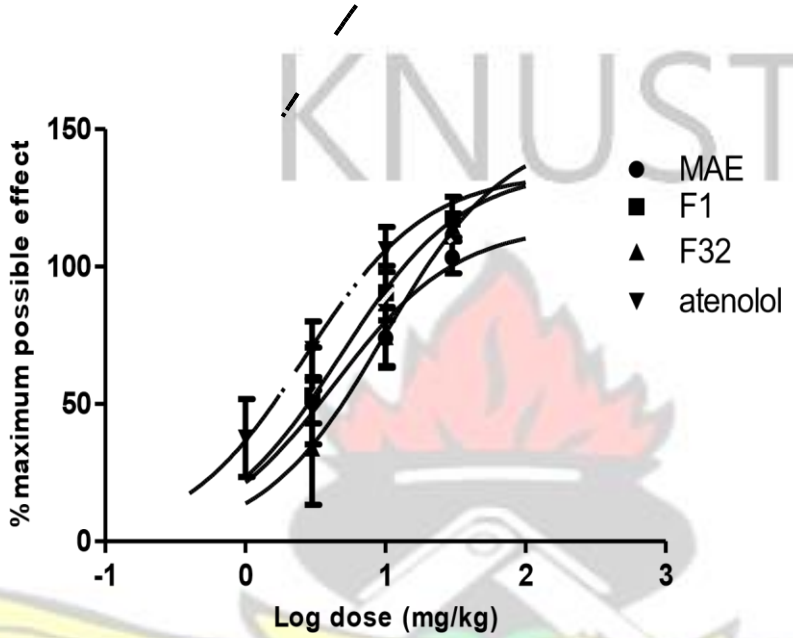


Figure 5.24 Dose-response curves for the antinociceptive activity of MAE, F1, F32 (3 – 30 mg/kg) and atenolol (1 – 10 µg/paw) in the epinephrine-induced hyperalgesia in Sprague-Dawley rats. Each point is the mean ± SEM (n = 5).

5.4 DISCUSSION

Antagonism analysis was conducted in the acetic acid-induced writhing and Hargreaves thermal tail-flick tests to detect possible mediators of pain both peripherally and centrally that are engaged in the antinociception of MAE, F1 and F32. The writhing test was chosen for this purpose because of its sensitivity and popularity in detecting peripheral analgesics (Shamsi Meymandi and Keyhanfar, 2013), whereas tail-flick test was chosen because of its popularity and specificity in detecting central analgesic activity of drugs with selectivity for opioid-derived analgesics (Gupta *et al*., 2013; Taïwe *et al*., 2011). The antinociceptive activity of MAE and fractions was

evaluated in the presence of different antagonists like theophylline, L-NAME, atropine, glibenclamide, ondansetron, yohimbine and naloxone.

Theophylline blocked antinociception of MAE in writhing test implying participation of adenosinergic system in the peripheral action of MAE. Pre-treatment of mice with theophylline, a non-selective adenosine receptor antagonist inhibited the antinociception of MAE, F1, F32 and morphine in the Hargreaves thermal tail-flick test. This suggests the involvement of adenosinergic pathway in the central antinociception of MAE, F1, F32 and morphine in the Hargreaves thermal tail-flick test.

Adenosine is an inhibitory neuromodulator that can increase nociceptive thresholds in response to noxious stimuli. Adenosine acts at numerous P1 receptors (A_1, A_{2A}, A_{2B} and A_3) all of which are coupled to G protein receptors (Katritch *et al*., 2012; Lebon *et al*., 2011). Activation of A_1 receptor peripherally produces pain suppression but A_2 receptor activation produces pain enhancement (Goldman *et al.*, 2010; Zhang *et al*., 2005). A_3 receptor activation produces pro-nociception centrally and peripherally secondary to mast cell degranulation and 5-HT and histamine release that exert nociception at sensory nerve terminal (Chen *et al*., 2013a; Fredholm, 2010).

Theophylline blocks A_1 and A_2 receptors, therefore the antinociception of the extract and fractions might be due to activation of A_1 and/or increase in endogenous adenosine centrally. Both opioid and adenosine receptor agonists share similar antinociceptive mechanisms (Maione *et al*., 2011; Ramos-Zepeda and Herrero, 2013). A_1 receptor exist as part of multireceptor complex, in union with μ-opioid and α_2-adrenoceptors on the basis of established cross antagonism, cross tolerance and cross withdrawal between

these systems (Boye *et al*., 2013) and activation of any of these receptors may affect the rest (Binder *et al.,* 2004; Chan *et al*., 2010). Adenosine

kinase (adenosine-metabolizing enzyme) decreases adenosine concentrations extracellular at sites of tissue injury; therefore inhibiting this enzyme increases adenosine concentrations. Adenosine kinase inhibitors may thus be therapeutic potential analgesic agents. The extract and fractions then could be more selective to adenosine kinase than other neurotransmitters and peptide receptors but this needs further investigation.

Pre-treatment of mice with L-NAME, a NO synthase inhibitor blocked the antinociception of F32 and diclofenac in the acetic acid-induced writhing test indicating the participation of the NO/cGMP system in the peripheral analgesic action of F32 and diclofenac. In the Hargreaves thermal tail-flick test, L-NAME inhibited the antinociception of F1, F32 and morphine indicating the participation of the NO/cGMP system in the central antinociception of the fractions. It has since been shown that NO participates in the modulation of nociceptive transmission both peripherally and centrally (Cury *et al*., 2011; Rodella *et al*., 2010). NO exercises a dual effect on nociception which may be due to the presence of different subsets of nociceptive primary sensory neurons in which NO plays opposite roles (Cury *et al.*, 2011; Hancock and Riegger-Krugh, 2008).

Atropine, a non-specific muscarinic receptor antagonist, blocked antinociception of F1, F32 and diclofenac in the acetic acid-induced writhing test. It also blocked antinociception of MAE, F1, F32 and morphine in the Hargreaves thermal tail-flick test. MAE, F1, F32, diclofenac and morphine antinociception may therefore involve the muscarinic cholinergic system. Activation of muscarinic receptors induces antinociception in various pain models (Deng and Guindon, 2013; Fiorino and Garcia-Guzman, 2012). Muscarinic M_1, M_2, M_3, M_4 and M_5 receptors mediate the antinociceptive effects of muscarinic agonists at the spinal and supra-spinal level (Jones and Dunlop, 2007; Mendes

et al., 2013; Wess *et al*., 2007). Peripheral activation of muscarinic M_2 receptors possibly adds to antinociception via decreased calcitonin gene related peptide (CGRP) release (Ebersberger *et al*., 2006). It has been established that antinociception of morphine is mediated by a descending cholinergic pathway and spinal endogenous acetylcholine acting through muscarinic receptors (Chen *et al*., 2005; Silva *et al*., 2011) therefore the inhibition of antinociception of morphine by atropine in this test is not surprising. The extract and fractions could probably be muscarinic agonists for all the 5 muscarinic receptors (M_1, M_2, M_3, M_4 and M_5). Their antinociceptive activities might be mediated through activation of muscarinic receptors which has been shown to inhibit Forskolin-induced increase in cAMP levels (Sullivan *et al*., 2007).

The ATP sensitive K^+ channel blocker, glibenclamide, administered orally inhibited the antinociceptive activity of MAE, F1, F32 and diclofenac in the acetic acidinduced writhing test and also blocked the antinociception of the extract, fractions and morphine in the Hargreaves thermal tail-flick test in the mice. This suggests that the ATP sensitive K^+ channel pathway may be contributing to both the peripheral and central antinociceptive mechanism of the extract and fractions. Pre-treatment of mice with ondansetron (5-HT_3 receptor inhibitor) did not block the antinociception of MAE, F1, F32 or diclofenac in the acetic acid-induced abdominal writhing test implying that 5-HT_3-serotoninergic pathway may not be involved in the peripheral antinociceptive mechanism of the extract and fractions. However, ondansetron pretreatment inhibited the antinociception of F1, F32 and morphine in the Hargreaves thermal tail-flick test in mice indicating that 5-HT_3-serotoninergic pathway may be contributing to the central antinociceptive mechanism of the fractions.

F1 and F32 might be increasing extra synaptic serotonin levels in the prefrontal cortex probably by inhibiting transporters (monoamine neurotransmitter uptake) (Basile *et al*., 2007) in addition to activation of serotoninergic pathways contributing to their

antinociceptive effects. Serotoninergic neurons play a fundamental role in the control of pain (Lin and Chen, 2008) and the range of subtype receptors for serotonin makes this system to exercise either facilitatory or inhibitory action (Shields and Goadsby, 2006). Spinal 5-HT_3 receptors mediate antinociception, probably through GABA release (Inocêncio Leite *et al*., 2014).

Yohimbine, α_2-adrenoceptor antagonist, might have blocked the antinociception of F1, F32 and diclofenac in the acetic acid-induced writhing test implying the participation of α_2-adrenergic mechanisms in the peripheral antinociception of F1,
F32 and diclofenac. Yohimbine might also blocked the antinociception of F1 and F32 in the Hargreaves thermal tail-flick test indicating the involvement of $\alpha_2$adrenergic mechanisms in the central antinociception of F1 and F32. $\alpha_2$adrenoceptors are significant in peripheral, spinal and supra-spinal pain modulation. Noradrenaline released from descending pathways attenuates pain by inhibitory action on α_{2A}-adrenoceptors on central terminals of primary afferent nociceptors or by direct α_2-adrenergic action on pain relay neurons (Pertovaara, 2006; Woode *et al*., 2013).

The non-selective opioid receptor antagonist, naloxone, administered intraperitoneally did not block the antinociceptive activities of the extract, fractions nor diclofenac in the acetic acid-induced writhing test indicating that opioid system is unlikely to be involved in the peripheral analgesic action of MAE, F1, F32 and diclofenac. However, pre-treatment of animals with naloxone blocked the antinociceptive effects of MAE, fractions and morphine in the Hargreaves thermal tail-flick test indicating that the opioidergic pathway may be contributing to their central antinociceptive actions. Activation of mu-opioid receptor has been shown to inhibit the activity of $TRPV_1$ via $G_{o/i}$ proteins and the cAMP pathway (EndresBecker *et al*.,

2007). Therefore, MAE and fractions may have produced their analgesic action by interacting with mu-opioid receptors.

In the capsaicin test, MAE and fractions dose-dependently blocked the pain response produced by capsaicin. Capsaicin is an agonist of the capsaicin receptor ($TRPV_1$), an excitatory ligand-gated non-selective cation channel co-expressed with transient receptor potential ankyrin 1 ($TRPA_1$) (Nilius *et al*., 2007) by a sub-population of primary afferent neurons containing SP and CGRP, that mediate pain and neurogenic inflammation (Materazzi *et al.,* 2008). $TRPV_1$ is also sensitized by prostanoids to cause hyperalgesia, where prostanoids decrease the threshold temperature for channel activation (Moriyama *et al.*, 2005).

Activation of central terminal $TRPV_1$ by capsaicin has been shown to result in an increase in synaptic release of both glutamate, excitatory amino acids, NO, neuropeptides, and pro-inflammatory mediators from the periphery, transmitting nociceptive information to the spinal cord or causing spinal sensitization through Protein kinase A (PKA) and Protein kinase C (PKC) activation (Calixto *et al*., 2005; Ferrini *et al.*, 2007; Kosugi *et al.*, 2007; Meotti *et al*., 2006). Transient receptor potential (TRP) channels are also important players in inflammatory pain. The $TRPV_1$ activation also sends an efferent signal at peripheral terminal via secretion of inflammatory agents, causing local neurogenic inflammation with vasodilation and oedema owing to increased capillary permeability (Rodrigues *et al.*, 2012). The reduction of nociceptive response by MAE and fractions then could be by regulating the $TRPV_1$ receptor activation or the inhibition of production or action of some of these mediators, which in turn reduces the neurogenic inflammation and the glutamate release, contributing to the modulation of nociceptive transmission at spinal levels.

Additionally, it has been demonstrated that μ-opioid receptor activation can block the action of $TRPV_1$ through $G_{i/o}$ proteins and the cAMP pathway (EndresBecker *et al*., 2007). Thus, MAE and fractions furthermore may have blocked capsaicin-induced pain via the opioid path.

In the glutamate test, MAE, fractions and ketamine inhibited the pain behaviour induced by glutamate. Glutamate plays a significant role in pain processing in both the central and peripheral nervous system (Freitas *et al*., 2009). The pain response generated by intraplantar glutamate in the mouse paw is mainly mediated by release of neuropeptides (neurokinins and kinins) from sensory fibres (Jesse *et al*., 2009). The mechanism responsible for the antinociceptive effect of MAE and fractions then appeared to be partly associated with an interaction with the glutamatergic system and subsequent inhibition of glutamatergic transmission.

It has also been demonstrated that the pain response induced by glutamate is largely mediated by both NMDA and non-NMDA receptors by a mechanism which greatly depends on the activation of L-arginine-nitric oxide pathway (Beirith *et al*., 2002). The analgesic effect of the extract and fractions in this test, possibly includes prevention of the generation/action of NO. This discovery is important since glutamate and its receptors (ionotropic and metabotropic) are vital for pain processing. Alteration in function of glutamatergic neurotransmission has been shown to be associated with pain-related plastic changes in the CNS and PNS (Neugebauer, 2007). In fact, drugs capable of blocking either iGluRs or mGluRs show analgesic effects in several mammalian species including human beings

(Wiech *et al*., 2004). The biting behaviour is induced by activation of iGluRs such as NMDA and AMPA because NMDA-receptor antagonists have been demonstrated to effectively improve pain-like behaviour, lessen opioid-induced hyperalgesia and

hinder opioid tolerance development in animal models and clinically (DuPen *et al.*, 2007). This result then might suggest the participation of ionotropic glutamatergic receptors (NMDA and probably AMPA) in the antinociceptive effect of MAE and fractions.

The major mechanisms by which glutamate agonists produce pain or neurotoxicity include a discharge of intracellular calcium, activation of cell mediators and opening of ion channels (Freitas *et al*., 2009). Consequently, stimulation by glutamate would result in a chain reaction with other mediators, for instance, pro-inflammatory cytokines which act synergistically in the stimulation of neurons (Kleinschnitz *et al.*, 2004). Considering these mechanisms, it would not be surprising then if the effect of MAE and fractions was associated with the interruption of any of these mediators. Moreover, inhibition of cytokine production has been shown to produce changes in glutamatergic pathways (Freitas *et al*., 2009). This then could also imply that MAE and fractions could inhibit the production of pro-inflammatory cytokines, interfering with the glutamate-induced nociception. Whatever it is, the exact mechanism of antinociception of MAE and fractions in the glutamate test needs to be further investigated.

Relevant additional outcomes of the study were that oral administration of MAE, F1 and F32 caused significant and dose-related attenuation of bradykinin– and epinephrine–induced thermal hyperalgesia and dose related suppression of the mechanical hyperalgesia induced by Prostaglandin E_2. The models are selective pain tests which could be more inferential to the mechanism of antinociception of the extract and the fractions.

Bradykinin directly stimulate the Aδ- and C–fibre nociceptors and may also release some pro–inflammatory mediators such as cytokines and substance P (Wang *et al*.,

2008). Moreover, thermal hyperalgesia induced by bradykinin may involve binding to bradykinin 1/bradykinin 2 ($B1/B_2$) receptors causing a direct activation of PKC and the indirect activation of PKA pathways eliciting hyperalgesia which in this study was measured using a thermal source of stimulus similar to the mechanical hyperalgesia induced by bradykinin (Ferreira *et al*., 2004). The extract and fractions then probably produced their antinociception by blocking the activation of B1/B2 receptors.

Epinephrine on the other hand binds to β adrenergic receptor to activate cyclic adenosine monophosphate (cAMP)/PKA independent of PKC second messenger pathways leading to hyperalgesia (Meotti *et al*., 2006). The extract and fractions then possibly inhibit β adrenergic receptors to produce their anti-hyperalgesic effects.

Prostaglandin E_2 mediates an increase in NO production which is responsible for increase in vasodilatation and capillary permeability resulting to oedema and sensitization of pain fibres (Melgaard *et al*., 2013). Prostaglandin E_2 also binds to EP receptors present on peripheral terminals of sensory neurons causing sensitization of peripheral nerves to pain stimuli and hyperalgesia (measured using a mechanical source of stimulus) through activation of PKA pathway (Austin and Moalem-Taylor, 2013; Lin *et al*., 2006; Peng *et al*., 2013; St-Jacques and Ma, 2013). The attenuation of mechanical hyperalgesia induced by prostaglandin E_2 therefore suggests involvement of EP receptors or PKA pathway in the anti-hyperalgesic effect of the extract and fractions.

Suppression of thermal hyperalgesia induced by bradykinin and epinephrine, and the attenuation of mechanical hyperalgesia induced by prostaglandin E_2 in rats then suggests involvement of B1/B2/β/EP receptors or PKA and/or PKC pathways directly or indirectly in the anti-hyperalgesic effects of MAE, F1 and F32.

5.5 CONCLUSION

The present results lead to the conclusion that the peripheral analgesic action of MAE was mediated by stimulation of ATP-sensitive K^+ channels and adenosinergic pathway while the central analgesic effect was due to activation of muscarinic and opioid receptors. The central analgesic effects of F1 and F32 were mediated by stimulation of adenosine, 5-HT_3, muscarinic, opioid, NO-cyclic GMP, ATP sensitive K^+ channels and α-$_2$ adrenergic receptor/pathway while the peripheral analgesic effects were due to activation of muscarinic, ATP-sensitive K^+ channels and α-$_2$ adrenergic pathways. F32 peripheral analgesic effect in addition was mediated by activation of NO-cyclic GMP pathway. MAE and fractions furthermore probably produce antinociception in this study by inhibiting the $TRPV_1/B_1/B_2$/β-adrenergic/EP receptors and glutamate pathways.

5.6 RECOMMENDATION

- Isolate, identify and characterize the bioactive constituents of MAE.

Chapter 6

ISOLATION, IDENTIFICATION AND CHARACTERIZATION OF BIOACTIVE COMPOUNDS FROM MAE

6.1 INTRODUCTION

Most often desired biological response from medicinal plants is due to a mixture of bioactive constituents (Musa *et al*., 2009). Isolating and purifying the active principles and testing of individual desired activity (antinociception) would enable us to know whether the activity of the extract is the synergistic effect of its various components or not. The isolation and identification of the active principles of an extract is of

paramount importance because each type of chemical compound may have a different activity or mechanism by which it exerts the same activity which may modify the action of the major active component. Additionally, it may lead to development of potential bioactive constituents which could provide novel compounds or precursors in drug development and utilization of some as research tools in drug development (Musa *et al*., 2009).

In the present study, an effort was made to isolate, identify and characterize the pharmacologically active components from MAE. The antinociceptive activity of the isolated compounds were studied in the mouse writhing assay and the hypertonic saline-induced corneal pain in mice. Since fish is known to possess the physiological and neuroanatomical structures required for nociceptive responses similar to mammals (Stevens, 2008) and zebrafish larvae have been proposed as a novel system in nociception and pain related research (Steenbergen and Bardine, 2014), the antinociceptive effects of the compounds were also studied in the acetic acid-induced locomotor activity.

6.2 MATERIALS AND METHODS

6.2.1 Isolation of bioactive constituents of MAE

Figure 6.1 shows the isolation scheme for isolation of active constituents of *Maerua angolensis*. 10 g of the petroleum ether/ethyl acetate extract of *Maerua angolensis* stem bark was fractionated to F1 and F32 as described in *section 2.2.9* and investigated for antinociceptive activity (*section 3.2.3 – 3.2.8*).

The bioactive fractions (F1 and F32) were then separately subjected to purification in a column (30 cm length × 2 cm width) wet packed with silica gel (Merck, 230 – 400 mesh generally in the ratio of 1:10). F1 (0.55 g) was eluted sequentially with 100%

petroleum ether and petroleum ether and ethyl acetate (95:5) in order of increasing polarity. Similarly, 0.67 g of the F32 was eluted continuously with petroleum ether and ethyl acetate (90:10). In the wet packing method the silica gel was mixed with petroleum ether and poured into the column. The stationary phase settled uniformly in the column and precautions were taken to ensure there was no entrapment of air bubbles or crack in the column adsorbent. F1 and F32 separately dispensed in crucible bowls were mixed well with small amount of the silica gel with the aid of a pestle. The F1 or F32/silica mixture was then gradually packed into the column that was about two-third wet packed with silica gel. The solvent systems were separately poured into the column sequentially in order of increasing polarity. Serially labelled collecting bottles were used to collect 60 ml of each sub-fraction when running the column.

A total of 13 sub-fractions were collected from F1 while 6 sub-fractions were collected from F32. Continuous elution of F1 with 100 % petroleum ether yielded 9 sub-fractions (sub-fractions 1 – 9). Further elution of F1 with petroleum ether and ethyl acetate (95:5) yielded 4 sub-fractions (sub-fractions 10 – 13) while 6 subfractions (sub-fractions 1 – 6) resulted from continuous elution of F32 with petroleum ether and ethyl acetate (90:10). Sub-fractions with similar TLC profile indicating similar phytocomponents were combined and concentrated. Sub-fractions 4, 10 and 11 from F1 with yields of 65, 120 and 90 mg were coded as C1, C2 and C3 respectively. Sub-fractions 12 and 13 also from F1 were combined and concentrated (coded as C4) gave a yield of 100 mg but later through identification and characterization, C4 and C3 were found to be alike as their spectra were identical and it was assumed they were the same compound and the two were combined but still coded C3. Sub-fractions 5 and 6 from F32 were combined and concentrated (coded as C5) giving a yield of 85 mg. Sub-

fractions 1 – 3 and 5 – 9 from F1 and subfractions 1 – 4 from F32 did not show any spot on TLC and were discarded.

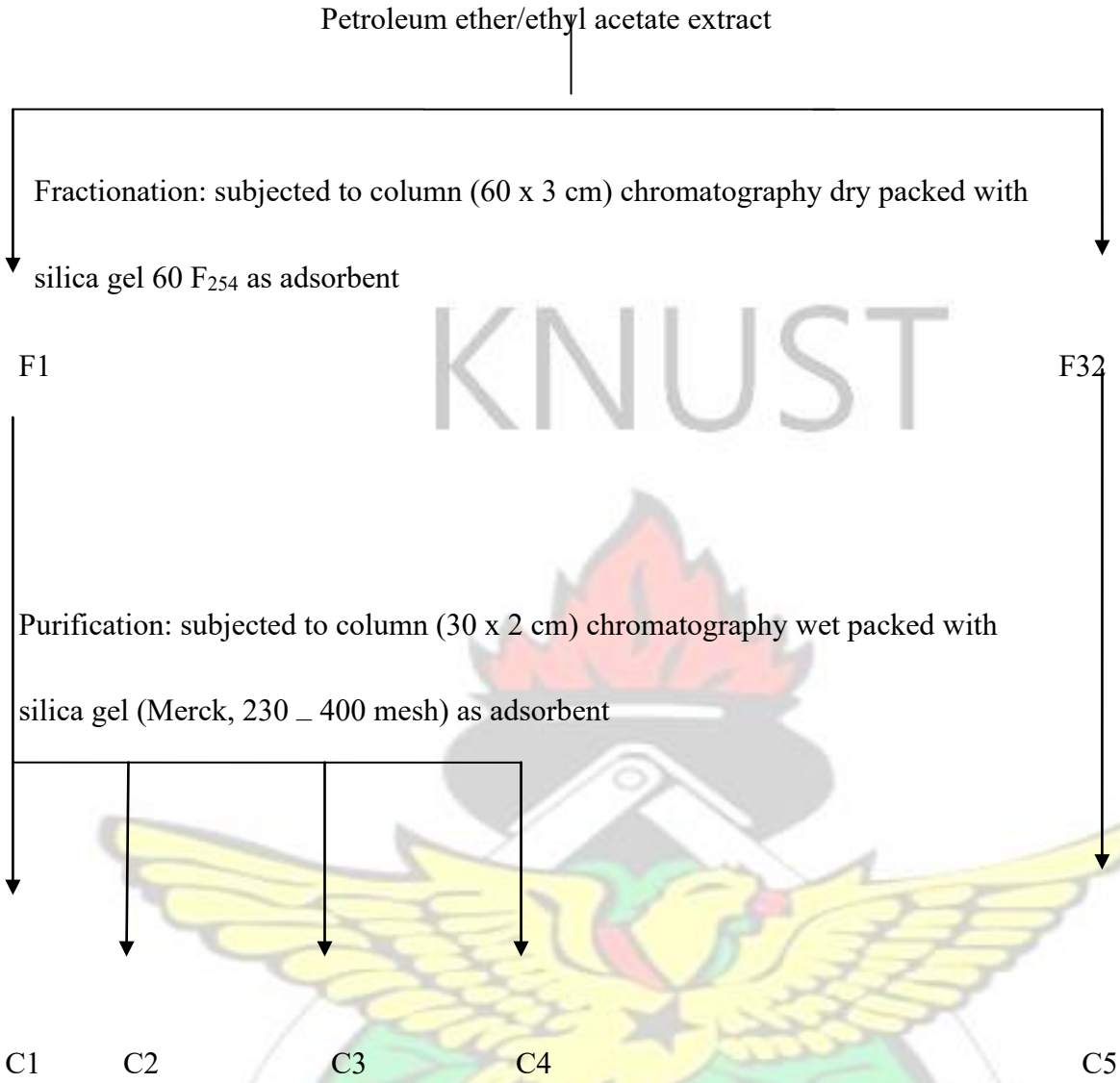


Figure 6.1 Isolation scheme for isolation of active constituents of *Maerua angolensis*

6.2.2 Identification and characterization of bioactive compounds from MAE

The samples obtained following isolation were subjected to spectroscopic analysis in an effort to determine their chemical structures. Analytical data obtained from these include ^{1}H-NMR, GCMS and Infrared (IR) spectroscopy. The ^{1}H-NMR spectra were recorded on Varian Mercury (300 MHZ) instrument. IR spectra were recorded on SHIMADZU Fourier Transform instrument. GCMS spectra were also recorded on SHIMADZU QP2010 PLUS instrument. Despite the presence of numerous peaks in the GCMS, which may be due to impurities, attention was focused on the highest peak

in the TIC peak report of the GCMS in an attempt to determine chemical structures of the compounds.

6.2.3 Animals

ICR mice (n = 5) of either sex weighing 20 – 25 g were used in the study. All mice were housed, fed and cared for as described previously (*section 2.2.4*).

Zebrafish (*Danio rerio*) larvae of 5 days post fertilization (dpf) were also selected for the study. Animal husbandry consisted of adult male and female wild type zebrafish purchased from C. S. L. Thean Yeang Aquarium, Malaysia. They were housed in semi-static recirculation system made of glass tanks with a holding capacity of approximately 20 litres with air and water temperature range of 23 – 25 ˚C. There was a constant 14 h light: 10 h dark cycle. Fish were purchased at the juvenile stage and were allowed to adapt to this facility for at least 2 months before being used as adult breeders. Fish were fed with crushed dry food (Ranaan) and brine shrimp in an alternate manner every 12 hours. They were maintained under sound management of laboratory breeding stocks including addressing key elements of husbandry, most notably water quality, nutrition, and behavioural management.

The embryos (fertilized eggs) were obtained by random mating between sexually mature individuals and collected at 7 am over a spawning tank, designed in-house, and set up the previous day. The eggs were then washed to remove debris and physically selected based on several criteria (McGrath, 2012) with the aid of a light microscope. The age of the embryos was set as day(s) post fertilization (dpf). Approximately 50 viable eggs were transferred into petri dishes containing egg media and stored at a temperature of 26 ˚C under a 14 h light and 10 h dark cycle.

The media was replaced immediately after hatching and maintained under similar conditions until 5 dpf. All treatment of 5 dpf larvae was carried out in a sterile 24 round well plate (Nunc™, Thermo Fischer Scientific, Waltham, MA USA) with a well diameter of approximately 1.7 cm and a culture area of 1.8 cm^2. Larvae were transferred gently into designated wells using a modified Pasteur pipette. A Canon® 1100d camera with 16.1 megapixel was used for imaging after the well were illuminated from the base using mini-tablet set at 100% illumination in a dark imaging box. All treatments were terminated after 3 min of exposure to acetic acid. In all the experimental studies each group consisted of six larvae (5 dpf). The investigation conforms to the standards for using zebrafish to assess compounds set out by the Organization for Economic Cooperation and Development in 1986.

6.2.4 Drugs and chemicals

The following drugs and chemicals were used: Acetic acid (BDH, Poole, England), diclofenac sodium (Troge Medical GmbH, Hamburg, Germany), morphine hydrochloride (Phyto–Riker, Accra, Ghana), naloxone, P-chlorophenylalanine methyl ester, capsazepine, piroxicam, ethyl acetate, petroleum ether (Sigma-Aldrich Inc., St. Louis, MO, USA), ondansetron (GlaxoSmithKline, Uxbridge, UK) and 5 M sodium chloride solution prepared locally. All drugs and compounds used in the nociceptive tests were prepared as described earlier (*section 2.2.5*).

6.2.5 Antinociceptive effects of C1, C2, C3 and C5 in the mouse writhing assay

The compounds (C1, C2, C3 or C5) (1, 3 and 10 mg/kg, *p.o.*), diclofenac sodium (3, 10 and 30 mg/kg, i.p.) or normal saline (10 ml/kg, i.p.) were administered to groups of mice. Acetic acid (0.6% v/v) was given (10 ml/kg, i.p.) 1 h after the *p.o.* and 30 min after the i.p. administration to all mice. The number of writhing for 30 min were

recorded for analysis with a camcorder and tracking of the behaviour was done with the help of the public domain software JWatcher™ as described (*section 2.2.6.1*). A significant reduction in the number of writhing by any treatment compared with control treated mice was considered as an antinociceptive response (*section 2.2.6.1*).

6.2.6 Antinociceptive effects of the compounds in hypertonic saline-induced corneal pain in mice and possible mechanisms of antinociception

Corneal pain was produced by a local application of 5 M NaCl to the corneal surface (Tamaddonfard *et al*., 2008). 40 µL of hypertonic saline was applied locally on the corneal surface of mice using a fine dropper (Ingale and Kasture, 2012). The number of eye wipes carried out with the ipsilateral forepaw was counted for a period of 30 s. C1, C2, C3 or C5 (1, 3 and 10 mg/kg, *p.o*.), morphine (1, 3 and 10 mg/kg, i.p.) or normal saline (10 ml/kg, i.p.) were given an hour (for the *p.o.* route) and 30 min (for the i.p. route) before the noxious agent.

To prove the possible participation of opioid, 5-HT3 and TRPV1 receptors in the outcome of C1, C2, C3 and C5, the mice were pre-treated with the respective antagonists naloxone (2 mg/kg), ondansetron (0.5 mg/kg) and capsazepine (5 mg/kg) i.p., 30 min before the *p.o.* administration of C1, C2, C3 or C5 (3 mg/kg) (Inocêncio Leite *et al*., 2014). The doses of antagonists were chosen on the basis of earlier literature information.

The possible contribution of endogenous serotonin was examined by pre-treating mice with an inhibitor of serotonin synthesis, para-chlorophenylalanine methyl ester (PCPA, 100 mg/kg, i.p.) or with normal saline (10 ml/kg, i.p.) once daily for 4 consecutive days. Mice then received C1, C2, C3 or C5 (3 mg/kg, *p.o*.) 24 h after the final dose of

PCPA or normal saline and 1 h later they were tested in the eye wiping test as described above.

6.2.7 Antinociceptive effects of the compounds in acetic acid-induced locomotor activity in zebrafish larvae

6.2.7.1 Measuring nociceptive responses to dilute acetic acid

The following concentrations of acetic acid were prepared by diluting with distilled water: 0.0003, 0.001, 0.003, and 0.01% v/v. Distilled water (0% acetic acid) was used as the medium for the control group. The acidic pH of the solutions was measured. Zebrafish larvae (5 dpf, n = 6) were then exposed to these different concentrations of dilute acetic acid. The nociceptive-specific behaviours (increase in general locomotor activity) were imaged for 3 min as described (*section 6.2.3*). The video output was analysed using Ethovision® XT version 10 (Trial). The parameters assessed included the mean velocity (mm/s), total distance travelled (mm), mean angular velocity (deg/s), mean turn angle (deg), mean meander (deg/mm), frequency of complete clockwise (CW) and frequency of counter clockwise (CCW). All treatments were terminated after 3 min of exposure to acetic acid by transferring the larvae back to egg water to reduce discomfort.

6.2.7.2 Antinociceptive effects of the compounds in 0.0003% (v/v) acetic acidinduced locomotor activity (mean velocity and total distance travelled)

Egg water in the 24 round well plates containing the 5 dpf larvae were replaced by 0.3 and 1 µg/ml concentrations of either morphine or diclofenac diluted in egg water. The egg water containing the larvae were also replaced by different concentrations of the compounds (C1, C2, C3 and C5 separately) or piroxicam ranging from 3 to 30 µg/ml. Larvae were treated for 30 min, after which the different concentrations were

replaced by egg water. The egg water in the 24 round well plates were later replaced with 0.0003% (v/v) acetic acid. A Canon® camera was used for imaging the swimming pattern of all individual larvae and Ethovision® XT version 10 (Trial) software was used to measure velocity and total distance travelled as described in *section 6.2.7.1*

6.2.8 Data analysis

Data were expressed as mean ± SEM per group and analysed as described earlier (*section 3.2.9*).

6.3 RESULTS

6.3.1 Identification and characterization of bioactive compounds from the stem bark

Following minimal spectra information obtained for the isolated compounds, the structures were identified as fatty acid and fatty acid esters. The fatty acid and fatty acid esters were isolated as whitish and yellowish waxy solids. They were positive for oils and fats test using filter papers. Full structural characterization possibly from synthetic derivatives and extensive and detailed spectroscopic experimentation may be necessary to re-affirm these observations, however, based on the information available, the compounds are speculated to be the following:

C1 is speculated to be the ester, octadecanoic acid methyl ester (Figure 6.2 C1). C1, according to ^{1}H-NMR spectra, could possibly contain a methyl ester group. This is evidenced by a signal at 3.59ppm, without taking into effect full integrations. It is difficult to predict whether the signal at 4.02 is compound related or impurity related, however, signal at 5.29ppm could possibly represent a solvent (CH_2Cl_2) peak. The upfield signals are very general and typically represent the aliphatic nature of C1.

GCMS reveals mass ion of 298, in accordance with the proposed structure. A fragment of mass 74 is probably methyl acetate whiles a fragment mass 87 is probably methyl propionate. FTIR revealed the normal C-H stretches at 2930.93 cm^{-1} and the carbonyl stretch at 1724.42 cm^{-1}.

C2 is believed to be bis (2-ethylhexyl) phthalate (Figure 6.2 C2). ^{1}H-NMR peaks shown at 7.63ppm and 7.46ppm are consistent with a doubly substituted phenyl ring. The substitution is believed to be of a 1, 2- pattern, with the peak at 7.63ppm representing the two protons adjacent to the substituents and the peak at 7.46ppm, representing two protons furthers from the substituents. A 1, 4- disubstitution pattern would probably give one signal for all four aromatic protons as they would all be in a similar chemical environment, that is why the substitution is believed to be of a 1, 2- arrangement, placing two protons in a different chemical environment to the other two protons. Peaks at 4.16ppm and 3.61ppm represent the two methylene protons closest to the oxygen atom. A proton each from the methylene group makes up 2 protons at peak 4.16ppm and the two remaining protons (one each from the other methylene group) are represented by peak 3.61ppm. Peak splitting of this manner may be due to the germinal effect. The multiplet peak at 2.40 – 2.10ppm represents the two tertiary alky protons. Multiplet at 1.70 – 1.48ppm may be attributed to the 4 methylene protons closest to the end of the long chain. Multiplet signal at 1.46 – 1.42ppm show peaks for twelve methylene protons, whiles multiplet signal at 0.96 – 0.70ppm could be for the twelve methyl protons. GCMS reveals mass ion of 390, which is consistent with the mass of the proposed structure. Fragment peaks at 279 and 167 reveal loss of first and second alkyl groups respectively, to give phthalic acid, while fragment at 149 show mass of phthalic anhydride. Infra-red data shows 3433 cm^{-1}, 2903 cm^{-1} the distinctive carbonyl peak at 1724 cm^{-1}, 1456 cm^{-1} and 1276 cm^{-1}.

C3 is proposed to be the long chain fatty acid, octadecanoic acid (Figure 6.2 C3). There are no downfield signals which could be associated with protons next to heteroatoms. That indicates the presence of an ester or an ether is unlikely. Two protons showing a possible triplet at 2.36ppm can be attributed to the methylene protons alpha to the carbonyl carbon. Two protons showing multiplet at 2.10 – 1.95ppm can be attributed to the beta protons in relation to the carbonyl group. Two protons showing multiplet at 1.80 – 1.60ppm could be attributed to the methylene protons next to the methyl group. Twenty six protons are shown as multiplet at 1.45 – 1.20ppm. The three methylene protons are also showing what appears to be multiplet at 0.90 – 0.60ppm. GCMS results reveal mass ion at 284 which is in accordance with the proposed structure. A fragmented mass at 241 may be due to C_{17} fragment. FTIR also shows the distinctive carbonyl peak at 1703 cm^{-1}. Other peaks are at 3420 cm^{-1}, and 2922 cm^{-1}.

A very weak ^{1}H-NMR means proposing a structure for C5 proved very challenging. However, it can be speculated that C5 is aliphatic in nature, with most of the signals appearing between 2.28 and 0.67ppm if related solvent peaks are disregarded. A broad like signal at 3.52ppm could represent a methyl ester, however, this is purely based on speculation. GCMS also reveals a mass ion of 296 with fragmentations at 264 (probably due to loss of methoxy group), 222 (may be due to loss of methyl acetate) and 180. FTIR also shows distinctive carbonyl peak at 1732 cm^{-1}. Based on the minimum information at hand, it can be speculated that C5 probably has a carbonyl group in the form of a methyl ester. It may be a C_{19} compound and possesses an alkene moiety as part of the structure. There are no clear ^{1}H-NMR signals to back the alkene assertion except for very weak noise like signals downfield. The GCMS however, provides some evidence with the mass of 296 as indication of loss of two protons in

the C_{19} chain which also contains possible ester functionality. C5 is speculated to be oleic acid methyl ester (Figure 6.2 C5).

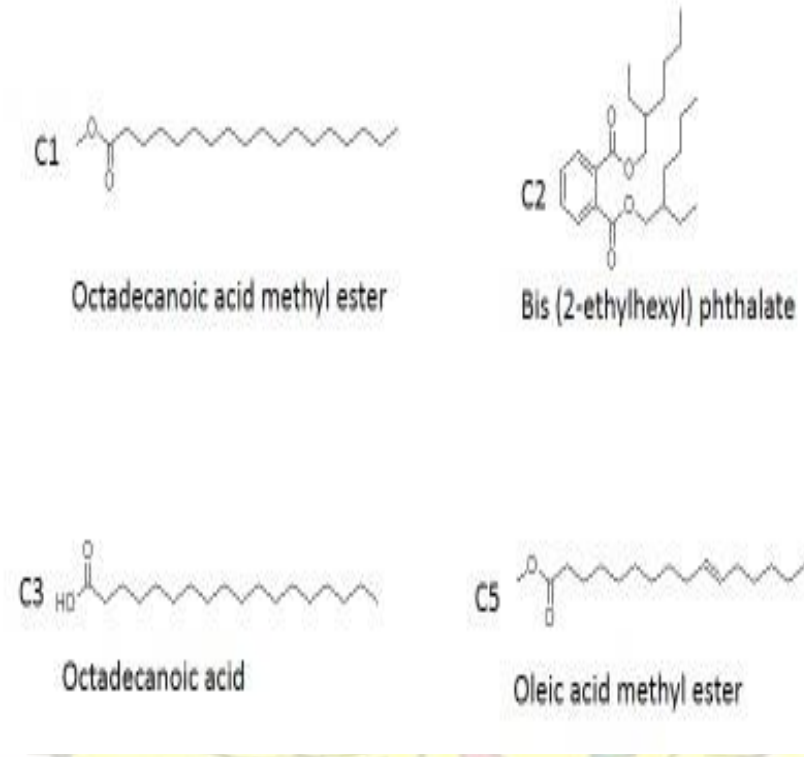


Figure 6.2 Chemical structures and IUPAC names of C1, C2, C3 and C5

6.3.2 Antinociceptive effects of C1, C2, C3 and C5 in the mouse writhing assay

Acetic acid intraperitoneally produced 75.4 ± 7.74 writhes, exhibited as an exaggerated distension of the abdomen combined with the outstretching of the hind limbs during the 30 min observation period in control mice pre-treated with normal saline (Figure 6.3). C1, C2, C3, C5 and diclofenac dose-dependently and significantly reduced the time-course curves of acetic acid-induced abdominal constrictions (Figure 6.3 a, c, e, g and i respectively). Two-way ANOVA (treatment

× time) revealed a significant (C1: $F_{3, 112}$ = 62.28; P<0.0001, C2: $F_{3, 112}$ = 46.79; P<0.0001, C3: $F_{3, 112}$ = 46.26; P<0.0001, C5: $F_{3, 112}$ = 20.65; P<0.0001 and diclofenac:

$F_{3,\ 112}$ = 33.41; P<0.0001) effect of drug treatments on the acetic acidinduced abdominal constrictions. C1, C2, C3 and C5 (1 – 10 mg/kg, *p.o.* 1 h before acetic acid injection) dose-dependently and significantly ($F_{3,\ 16}$ = 33.37, P<0.0001; $F_{3,\ 16}$ = 20.67, P<0.0001; $F_{3,\ 16}$ = 21.74, P<0.0001 and $F_{3,\ 16}$ = 10.42, P = 0.0005 respectively) reduced the number of abdominal writhes over 30 min with maximal inhibition of 78.36 ± 7.93; 69.45 ± 14.22; 72.28 ± 10.11 and 61.81 ± 9.35% respectively (Figure 6.3 b, d, f and h) at doses of 10 mg/kg. The reduction of the number of writhes by the compounds was similar to that of the NSAID diclofenac (3 – 30 mg/kg, i.p. 30 min before acetic acid injection) which dose-dependently and significantly ($F_{3,\ 16}$ = 17.73, P<0.0001) suppressed the acetic acid-induced writhes by a maximum of 72.84 ± 9.7% (Figure 6.3 j) at the dose of 30 mg/kg. C1 was more potent (0.9392 mg/kg) than diclofenac (1.353 mg/kg) (Figure 6.4).

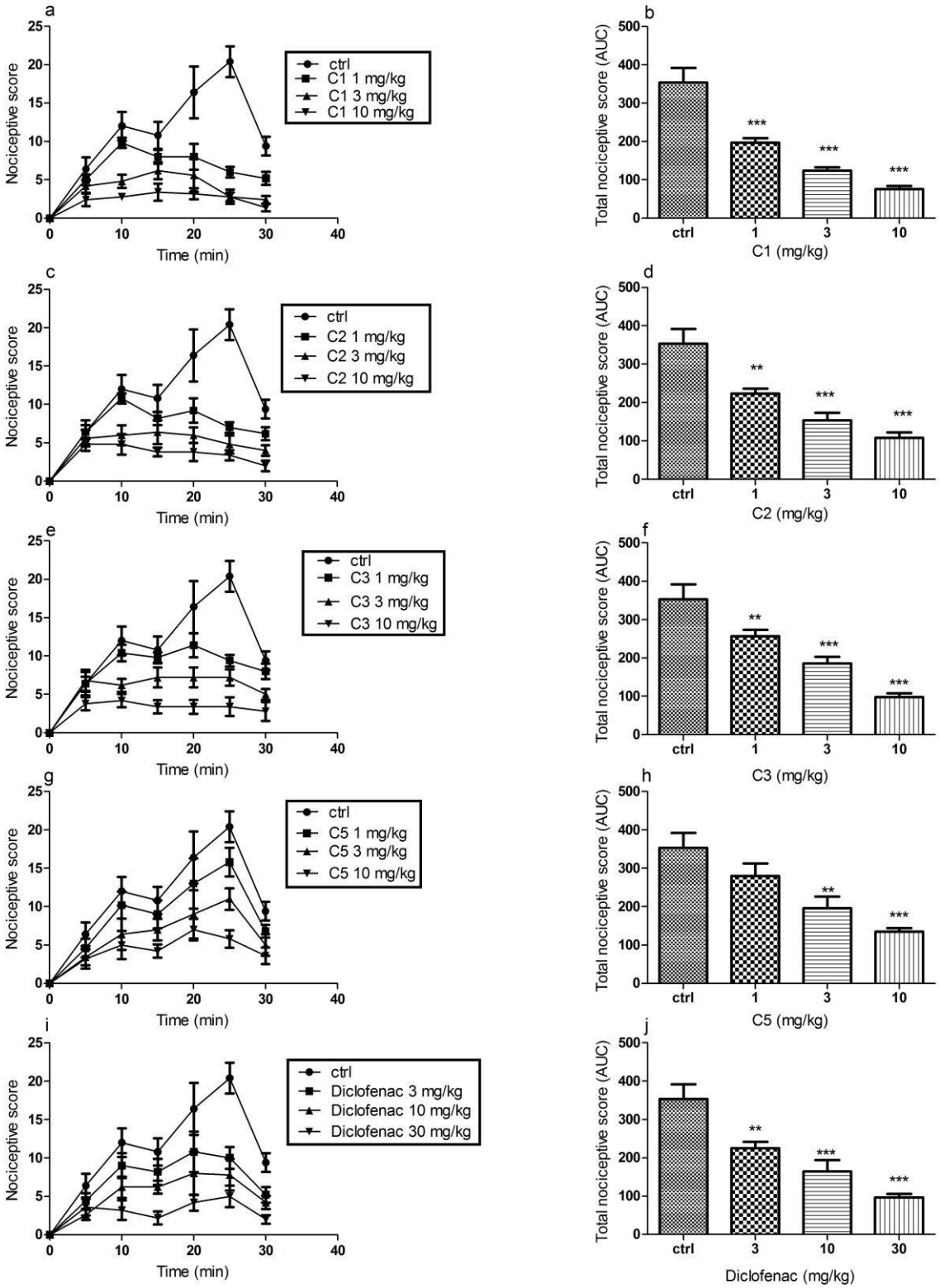


Figure 6.3 Effect of C1, C2, C3, C5 (1 – 10 mg/kg, *p.o.*) and diclofenac (3 – 30 mg/kg, i.p.) on the time course curve of acetic acid-induced abdominal writhes (a, c, e, g and i) and the total nociceptive response (calculated as AUC) (b, d, f, h and j) in ICR mice. Each column represents

the mean of 5 mice, and the error bar indicates the SEM. Asterisks denote the significance levels compared with control groups (one-way ANOVA followed by Newman Keuls *post hoc* test): *****P*<0.01 and ******P*<0.001

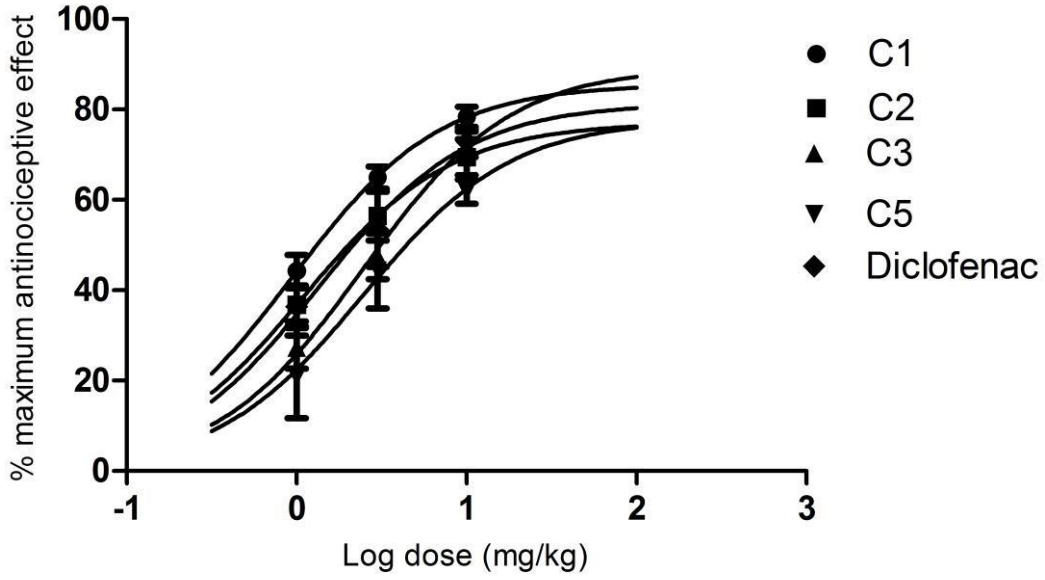


Figure 6.4 Dose-response curves for the antinociceptive effect of C1, C2, C3, C5 (1 – 10 mg/kg, *p.o.*) and diclofenac (3 – 30 mg/kg, i.p.) in acetic acid-induced abdominal pain. Each point is the mean ± SEM (n = 5).

6.3.3 Antinociceptive effects of the compounds in hypertonic saline-induced corneal pain in mice and possible mechanisms of antinociception

Orally administered C1, C2, C3 and C5 (1 - 10 mg/kg) significantly ($F_{3, 16}$ = 3.925; P = 0.0282, $F_{3, 16}$ = 6.914; P = 0.0034, $F_{3, 16}$ = 8.852; P = 0.0011 and $F_{3, 16}$ = 4.628; P = 0.0163 respectively) and dose-dependently decreased the number of eye wipes induced by the local application of hypertonic saline on the corneal surface producing antinociception of 60.98 ± 19.33, 75.61 ± 21.21, 71.95 ± 16.16 and 70.73 ± 27.82% respectively at the highest doses used (Figure 6.5 a, b, c and d). The reference analgesic, morphine (1 - 10 mg/kg, i.p.) significantly ($F_{3, 16}$ = 5.075; P = 0.0117) and dose dependently inhibited eye wiping with the highest dose (10 mg/kg) producing

antinociceptive effect of 59.76 ± 24.46% (Figure 6.5 e). C2 was more potent (0.6812 mg/kg) than morphine (0.9549 mg/kg) (Figure 6.6).

Pre-treatment of mice with 100 mg/kg PCPA, ondansetron (0.5 mg/kg) and 5 mg/kg capsazepine reversed antinociception of C1 (3 mg/kg, *p.o.*, Figure 6.7 a, b and d), while pre-treatment of mice with 5 mg/kg capsazepine reversed antinociception of C2 (3 mg/kg, *p.o.*, Figure 6.7 h). The antinociception of C3 and C5 (3 mg/kg, *p.o.*) in the hypertonic saline-induced corneal pain was blocked by PCPA (100 mg/kg, Figure 6.8 a and e), ondansetron (0.5 mg/kg, Figure 6.8 b and f), naloxone (2 mg/kg, Figure 6.8 c and g) and capsazepine (5 mg/kg, Figure 6.8 d and h).

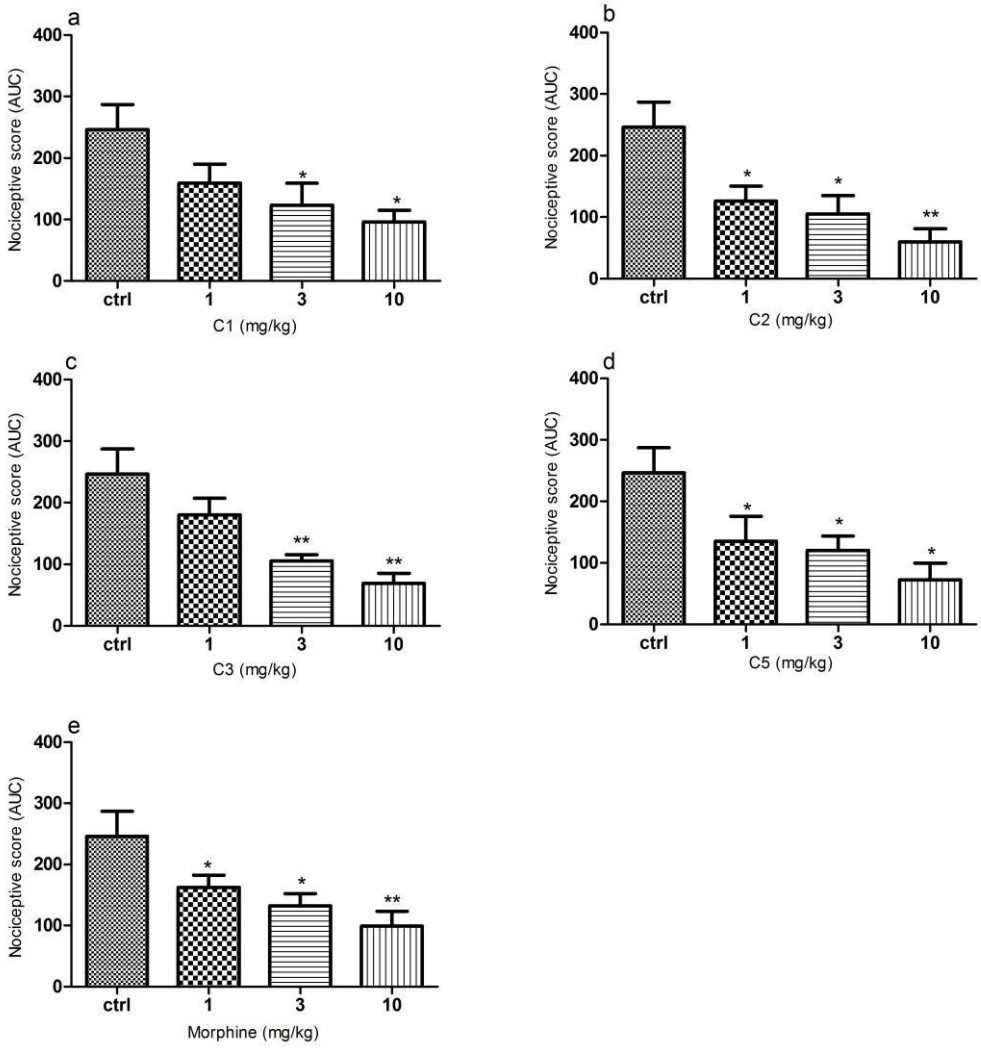


Figure 6.5 Effect of (a) C1, (b) C2, (c) C3, (d) C5 (1 – 10 mg/kg, *p.o.*) and (e) morphine (1 – 10 mg/kg, i.p.) on total nociceptive response (AUC) of hypertonic saline-induced corneal pain in ICR mice. Each column represents the mean of 5 mice, and the error bar indicates the SEM. Asterisks denote the significance levels compared with control groups (one-way ANOVA followed by Newman Keuls *post hoc* test): **P*<0.05 and ***P*<0.01

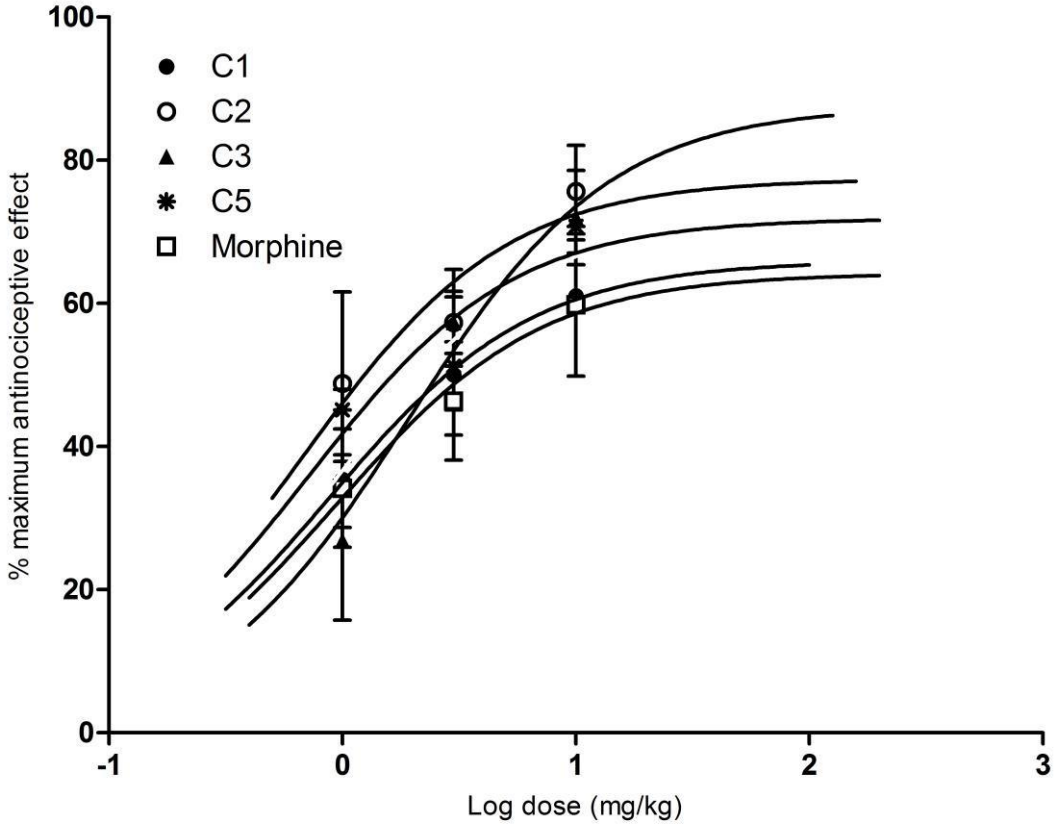


Figure 6.6 Dose-response curves for the antinociceptive effect of C1, C2, C3, C5 (1 – 10 mg/kg, *p.o.*) and morphine (1 – 10 mg/kg, i.p.) in hypertonic saline-induced corneal pain. Each point is the mean ± SEM (n = 5).

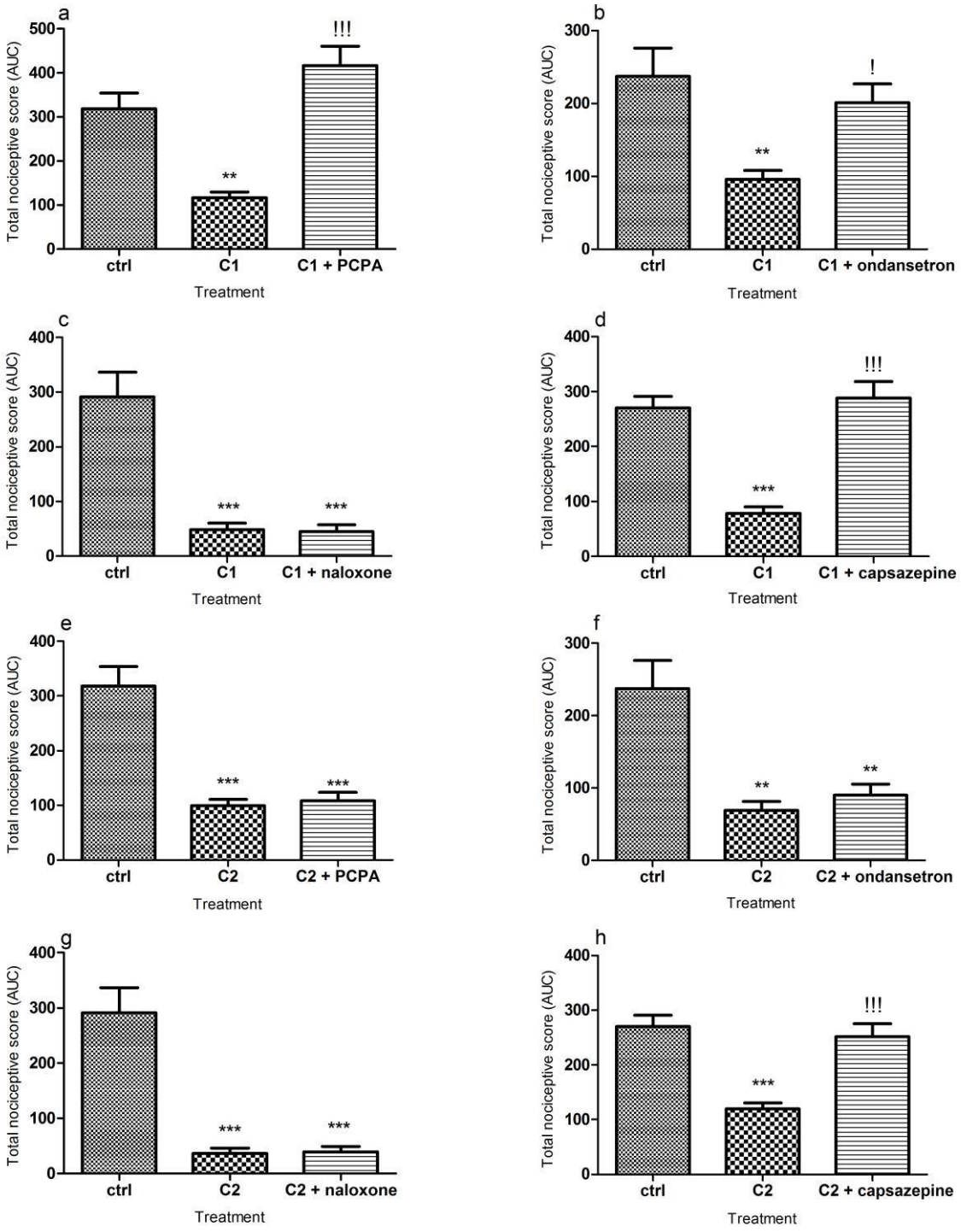


Figure 6.7 Effect of PCPA (100 mg/kg i.p.), ondansetron (0.5 mg/kg i.p.), naloxone (2 mg/kg i.p.) and capsazepine (5 mg/kg i.p.) on the antinociceptive effect of C1 or C2 (3 mg/kg *p.o.*) in hypertonic saline-induced wiping test. Each column represents the mean ± SEM (n = 5). *****P*<0.01, ******P*<0.001, compared to controls, !*P*<0.05, !!!*P*<0.001, compared to C1 or C2 (oneway ANOVA followed by Newman-Keuls *post hoc* test).

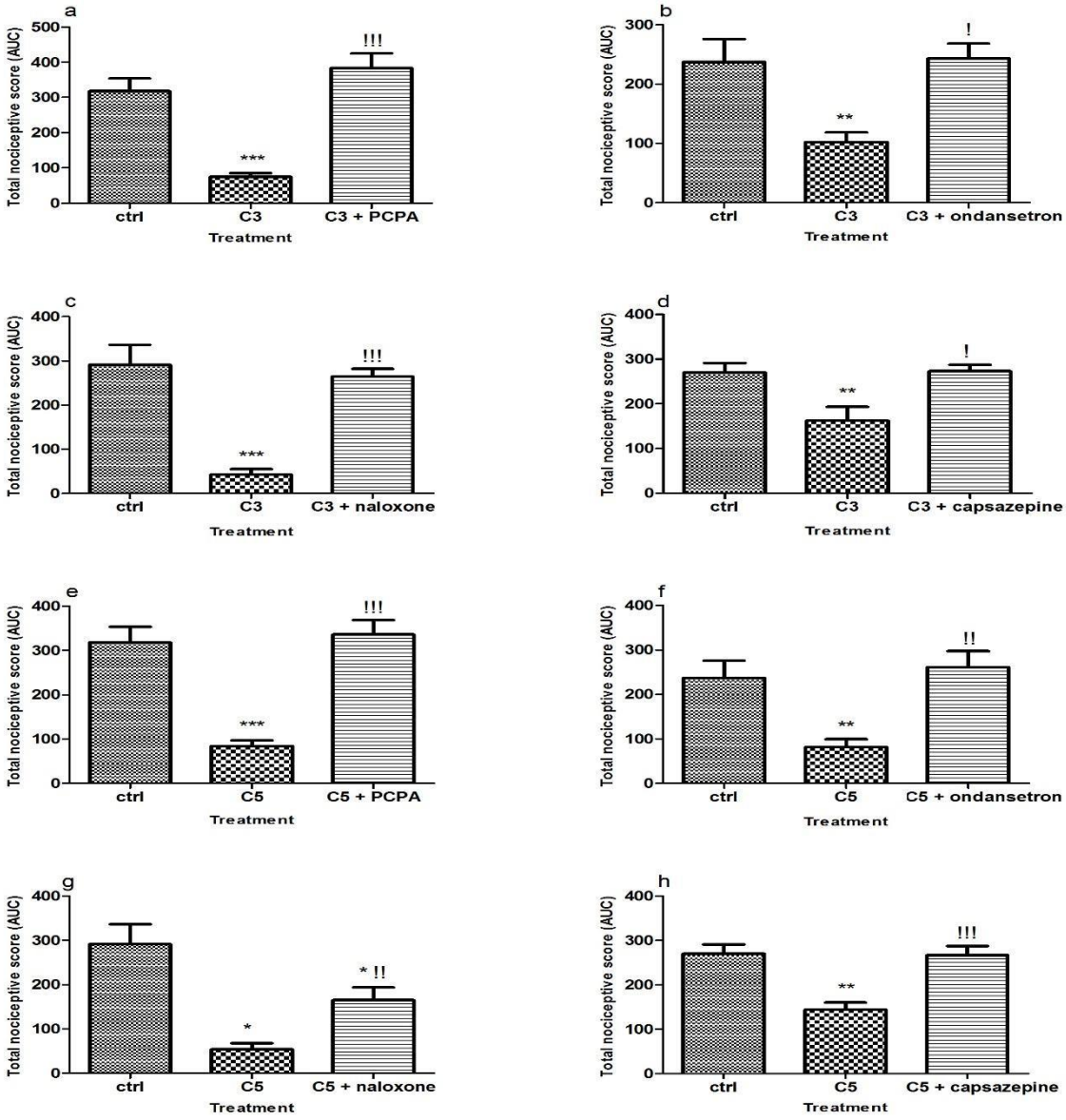


Figure 6.8 Effect of PCPA (100 mg/kg i.p.), ondansetron (0.5 mg/kg i.p.), naloxone (2 mg/kg i.p.) and capsazepine (5 mg/kg i.p.) on the antinociceptive effect of C3 or C5 (3 mg/kg *p.o.*) in hypertonic saline-induced wiping test. Each column represents the mean ± SEM (n = 5). **P*<0.05, ***P*<0.01, ****P*<0.001, compared to controls, !*P*<0.05, !!*P*<0.01, !!!*P*<0.001, compared to C3 or C5 (one-way ANOVA followed by Newman-Keuls *post hoc* test).

6.3.4 Antinociceptive effects of the compounds in acetic acid-induced locomotor activity in zebrafish larvae

6.3.4.1 Measuring nociceptive responses to dilute acetic acid

Exposure of the larvae to various concentrations of dilute acetic acid, the chemical noxious stimulus reveals the distinctive nociceptive responses (increase mean velocity and total

distance travelled) observed during 3 min exposure (Figure 6.9 and 6.10). On contact with a chemical noxious stimulus, zebrafish larvae demonstrate an initial decrease in activity, followed by an increase in activity which were higher than in control larvae during the 180 s of exposure. There was a statistically significant difference in locomotor activity between groups as determined by oneway ANOVA ($F_{4, 42} = 22.42$, $P<0.0001$ for mean velocity) and ($F_{4, 42} = 22.67$, $P<0.0001$ for total distance travelled). The swimming activity during the last minute of exposure was used as a measure for the intensity of the noxious stimulus applied and the lowest effective concentration (0.0003%) was thus chosen for subsequent experiments to minimize the discomfort of the larvae.

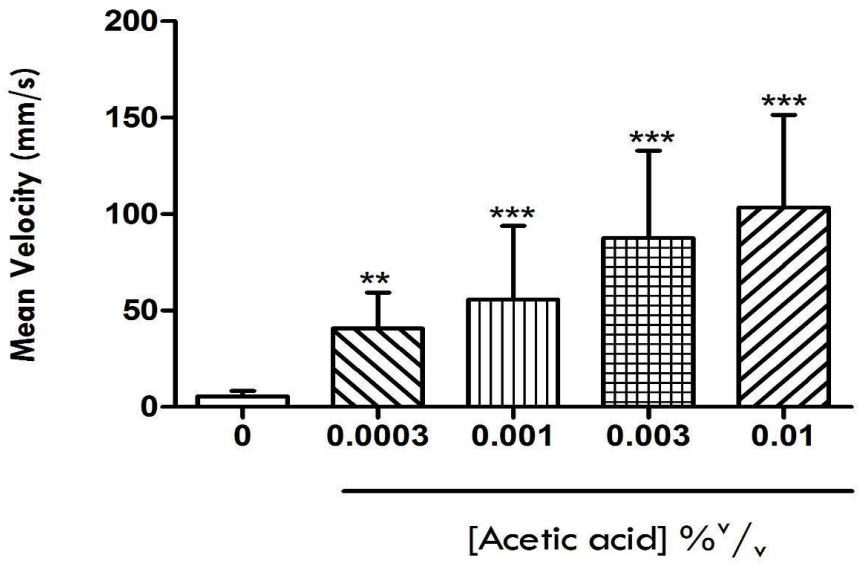


Figure 6.9 Mean velocity of zebrafish larvae against various concentrations of acetic acid over 3 min. **P<0.01, ***P<0.001 compared to 0% acetic acid (control) (one-way ANOVA followed by Newman-Keuls *post hoc* test).

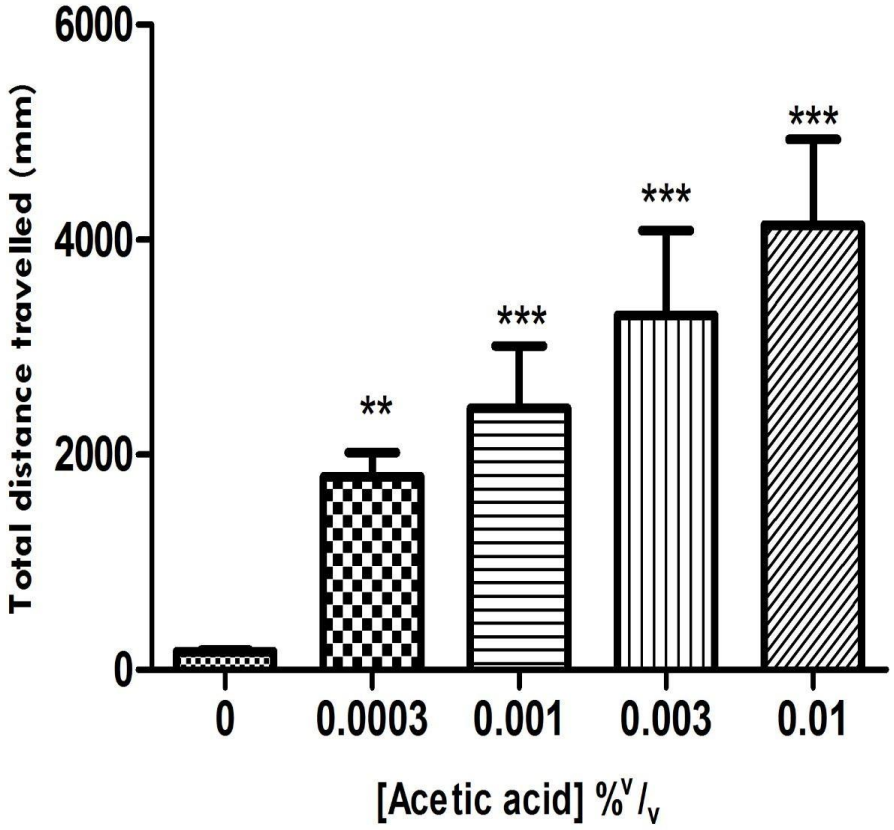


Figure 6.10 Total distance travelled by zebrafish larvae against various concentrations of acetic acid over 3 min. *****P*<0.01, ******P*<0.001 compared to 0% acetic acid (control) (one-way ANOVA followed by Newman-Keuls *post hoc* test).

6.3.4.2 Antinociceptive effects of the compounds in 0.0003% (v/v) acetic acidinduced locomotor activity (mean velocity and total distance travelled)

6.3.4.2.1 Antinociceptive effects of morphine and diclofenac in 0.0003% (v/v) acetic acid-induced locomotor activity (mean velocity and total distance travelled)

Pre-treatment with 0.3 (0.6484 ± 0.3147 mm/s, *P*<0.001) and 1 (0.7093 ± 0.2834 mm/s, *P*<0.001) µg/ml morphine (Figure 6.11 a) prevented the increase in activity (mean velocity) due to acid exposure. The increase in activity (total distance travelled) was also prevented by pre-treatment with 0.3 (0.6772 ± 0.3330 mm,

P<0.001) and 1 (39.73 ± 38.98 mm, *P*<0.001) µg/ml morphine (Figure 6.11 c). Similarly, pre-treatment with 0.3 (0.4301 ± 0.2047 mm/s, *P*<0.001) and 1 (0.1751 ± 0.2955 mm/s, *P*<0.001) µg/ml diclofenac (Figure 6.11 b) prevented the increase in activity (mean velocity) due to acid exposure. The increase in activity (total distance travelled) was similarly prevented by pre-treatment with 0.3 (0.1720 ± 0.1456 mm, *P*<0.001) and 1 (6.608 ± 6.603 mm, *P*<0.001) µg/ml diclofenac (Figure 6.11 d).

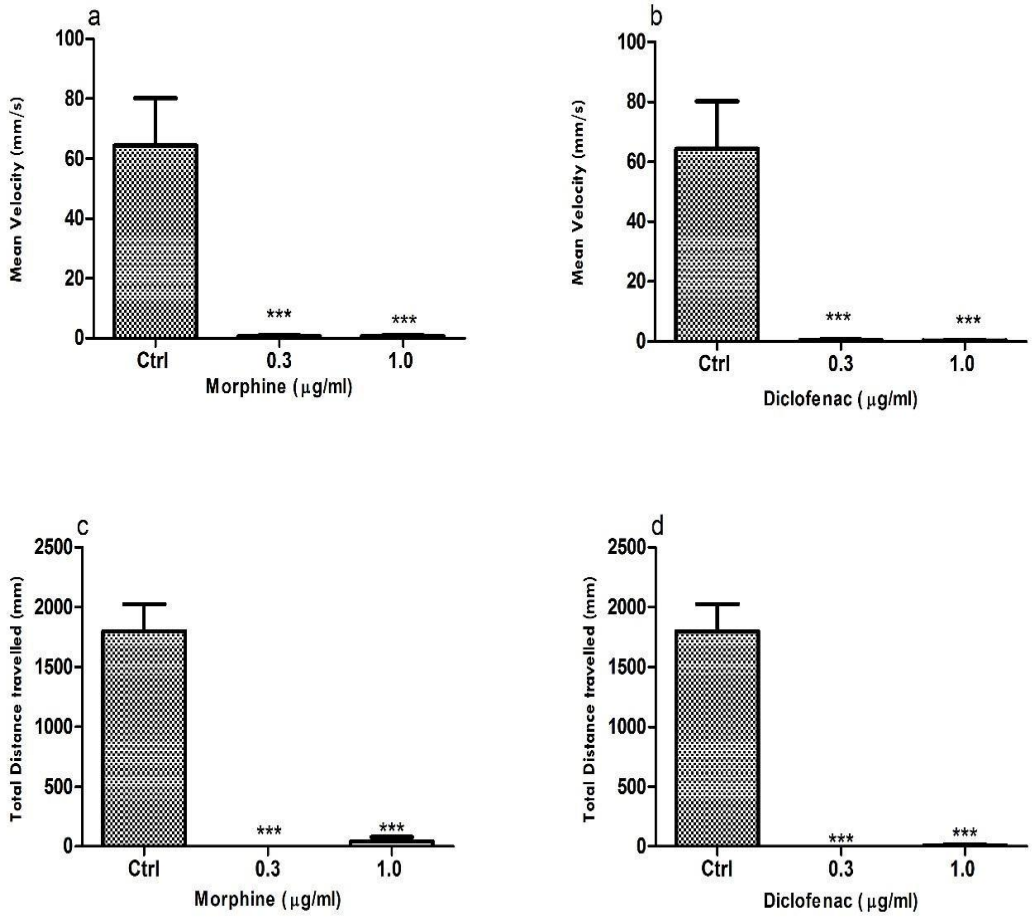


Figure 6.11 Effects of morphine and diclofenac in zebrafish larvae exposed to 0.0003% (v/v) acetic acid. (a) Mean velocity in presence of 0.3 and 1 µg/ml morphine. (b) Mean velocity in presence of 0.3 and 1 µg/ml diclofenac. (c) Total distance travelled in presence of 0.3 and 1 µg/ml morphine. (d) Total distance travelled in presence of 0.3 and 1 µg/ml diclofenac. Values represent mean ± SEM, n = 6. ******P*<0.001 compared to acetic acid exposed control (one-way ANOVA followed by Newman-Keuls *post hoc* test).

6.3.4.2.2 Antinociceptive effects of the compounds in 0.0003% (v/v) acetic acidinduced locomotor activity (mean velocity and total distance travelled)

Exposure to acetic acid (0.0003% v/v) produced locomotor activity, exhibited as increased mean velocity and total distance travelled, in control larvae treated with egg water. Pre-treatment of larvae with C1, C2, C3, C5 and piroxicam (3 – 30 µg/ml, 30 min before exposure to acetic acid) significantly (C1: $F_{3, 10} = 7.946$, P=0.0053; C2: $F_{3, 19} = 18.53$, P<0.0001; C3: $F_{3, 19} = 19.15$, P<0.0001; C5: $F_{3, 19} = 19.01$, P<0.0001 and piroxicam: $F_{3, 19} = 19.03$, P<0.0001) reduced the mean velocity over 3 min (Figure 6.12). Zebrafish larvae pre-treated with C1, C2, C3, C5 and piroxicam (3 – 30 µg/ml, 30 min before exposure to acetic acid) significantly (C1: $F_{3, 11} = 6.759$, P=0.0075; C2: $F_{3, 20} = 19.15$, P<0.0001, C3: $F_{3, 20} = 29.82$, P<0.0001; C5: $F_{3, 18} = 25.12$, P<0.0001 and piroxicam: $F_{3, 20} = 31.81$, P<0.0001) inhibited the total distance travelled over 3 min (Figure 6.13).

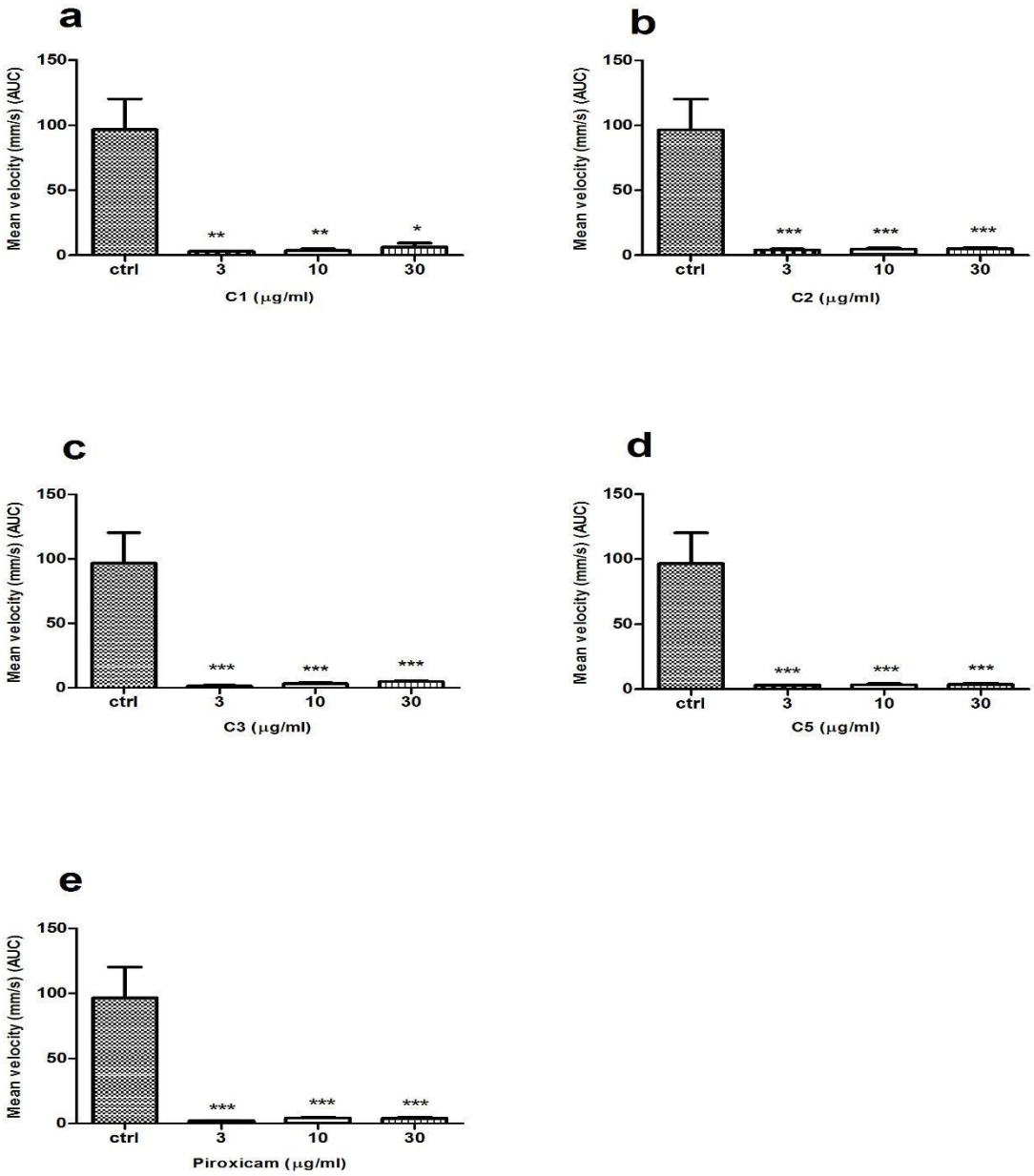


Figure 6.12 Effect of C1, C2, C3, C5 and piroxicam (3 – 30 µg/ml) on the total nociceptive score of acetic acid-induced locomotor activity (mean velocity, calculated as AUC) (a, b, c, d and e respectively) in zebrafish larvae. Data are expressed as mean ± SEM (n = 6). *P<0.05, **P<0.01, ***P<0.001 compared to acetic acid exposed control group (one-way ANOVA followed by Newman-Keuls *post hoc* test).

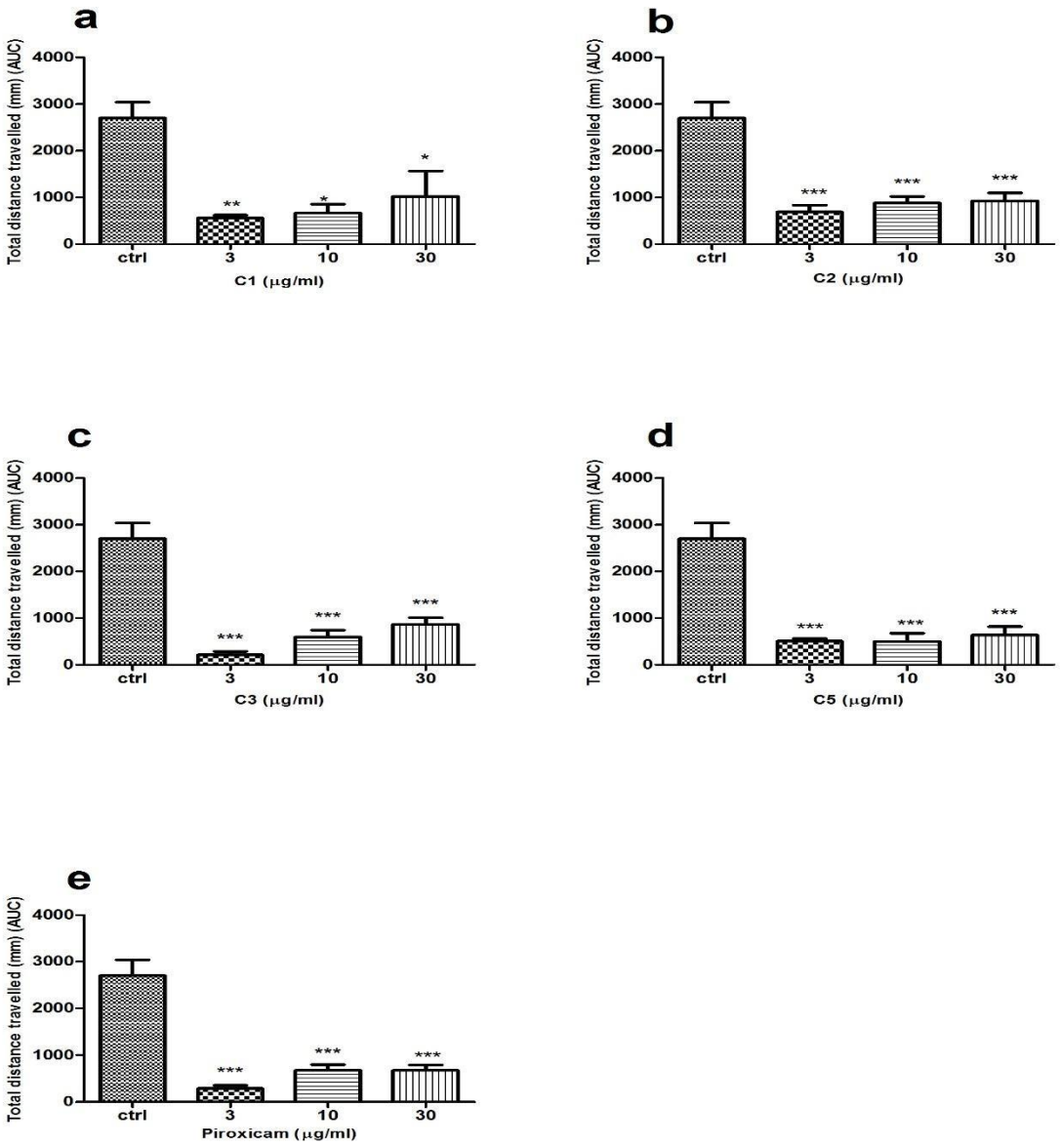


Figure 6.13 Effect of C1, C2, C3, C5 and piroxicam (3 – 30 µg/ml) on the total nociceptive score of acetic acid-induced locomotor activity (total distance travelled, calculated as AUC) (a, b, c, d and e respectively) in zebrafish larvae. Data are expressed as mean ± SEM (n = 6). *P<0.05, **P<0.01, ***P<0.001 compared to acetic acid exposed control group (one-way ANOVA followed by Newman-Keuls *post hoc* test).

6.4 DISCUSSION

The present study upon isolation, identification and characterization of the compounds from MAE also assessed the analgesic activity of C1, C2, C3 and C5

(fatty acid and fatty acid esters) in mice with the experimental models of chemogenic nociception in mice and zebrafish larvae. The fatty acid and fatty acid esters were effective in relaxing abdominal pain induced by acetic acid. The compounds, given orally, produced a dose-related analgesic activity on the abdominal constriction response, suggesting the peripheral analgesic effects of fatty acid and fatty acid esters isolated from *M. angolensis* for the first time. These types of fatty acid and fatty acid esters have earlier been isolated from some plants such as *Celtis australis*, *Alstonia scholaris* and *Mangifera indica* (Arulmozhi *et al*., 2012; Garrido *et al*., 2004; Semwal and Semwal, 2012) and were reported to possess analgesic, antiinflammatory, antipyretic, antiulcerogenic, anticancer, antihypertensive, antibacterial, antiviral activities and also associated with lowered LDL cholesterol and increased HDL cholesterol (Hui *et al*., 2009; Hunter *et al*., 2010; Khalil *et al*., 2000; Martin-Moreno *et al*., 1994; Terés et al., 2008) indicating their numerous pharmacological properties.

In related studies using seed oil from *H. Sabdariffa* and *Thespesia populnea* also containing fatty acids, it was shown that the fatty acids from these plants have analgesic activities in animal models of nociception including acetic acid-induced abdominal constriction test (Ali *et al*., 2014; Shah and Alagawadi, 2011), suggesting that fatty acids and fatty acid esters from some medicinal plants can be used as analgesics. The results presented here showed that the compounds could inhibit writhing. This was similar to diclofenac, meaning their antinociception may be due to prevention of synthesis and/or liberation of pro-inflammatory pain mediators peripherally. It may also be due to prevention of pro-inflammatory mediatorsmediated central sensitization. These outcomes are in concurrence with previous studies (Fischer *et al*., 2013; Iliya *et al*., 2014).

In wiping test, the local application of hypertonic saline to the corneal surface generated corneal pain. Related studies have established that application of 5 M NaCl

solution to the cornea and tongue briefly triggers nociceptive neurons with wide dynamic range effect in the trigeminal sub nucleus caudalis (Ro *et al*., 2007). Wiping test is a chemical model of nociception reported previously (Farazifard *et al*., 2005; Tamaddonfard *et al*., 2008) and is in agreement with findings presented here.

The management of trigeminal acute pains for example headache, dental problems, muscle spasms, corneal ulcers or post-surgery pain with the current analgesics remains a cause for concern. Safe, long-lasting pain relief with current analgesics following trigeminal acute pains is lacking necessitating the need to search for new chemical entities. Natural products being the basis of most early medicines are the most promising sources of these new chemical entities (Butler, 2008), more so some plants have a long history of use in traditional medicine to manage various types of pain.

Corneal nociceptive receptors have a significant representation in the trigeminal ganglion through the ophthalmic branch of trigeminal nerve making the cornea useful for nociception studies in trigeminal system (Inocêncio Leite *et al.*, 2014). Thin myelinated and unmyelinated fibres in cornea react to mechanical, thermal and chemical noxious stimuli (Inocêncio Leite *et al*., 2014). From the aforementioned, it can be deduced that wiping the eye with ipsilateral forepaw by mice in this study is an obvious withdrawal response to corneal chemical stimuli. Eye wiping test is a phasic analgesic test sensitive to centrally acting analgesics making the choice of morphine as the reference analgesic suitable in this study. A significant and dose– dependent antinociceptive effect was evident for the tested fatty acid and fatty acid esters from *M. angolensis* in the wiping test suggesting their analgesic effect in patients with trigeminal acute pains.

Hypertonic saline-induced corneal nociception has been used as a model of acute pain for the study of mechanisms of pain in the trigeminal system in rodents (Farazifard *et al*., 2005; Inocêncio Leite *et al*., 2014). The present study therefore attempted to characterize further possible mechanisms through which the compounds from *M. angolensis* stem bark exercise their analgesic action in chemical model of corneal nociception in mice. The analgesic effect produced by C1 was blocked by PCPA, ondansetron and capsazepine implying participation of serotoninergic system (through 5-HT3 receptors) and $TRPV_1$ receptors in its analgesic action. C1 might be increasing extra synaptic serotonin levels in the prefrontal cortex probably by inhibiting transporters (monoamine neurotransmitter uptake) (Basile *et al*., 2007) in addition to activation of serotoninergic pathway (through 5-HT3 receptors) thereby contributing to its antinociceptive effect.

Serotoninergic neurons play a fundamental role in the control of pain (Lin and Chen, 2008) and the range of subtype receptors for serotonin makes this system to exercise either facilitatory or inhibitory action (Shields and Goadsby, 2006). Spinal 5-HT3 receptors mediate antinociception, probably through GABA release (Inocêncio Leite *et al*., 2014). Furthermore, pre-treatment of mice with PCPA (tryptophan hydroxylase inhibitor) at a dose known to decrease the cortical content of serotonin and to change morphine antinociception (Inocêncio Leite *et al*., 2014) inhibited the analgesic action of C1.

Inhibition of antinociception by capsazepine, a competitive $TRPV_1$ channel antagonist suggests that C1 interaction directly with $TRPV_1$ receptors probably contributes to its antinociceptive action. $TRPV_1$ receptor is activated by capsaicin resulting in the release of neuropeptides, excitatory amino acids, nitric oxide and proinflammatory mediators from the periphery,

transmitting nociceptive information to the spinal cord or causing spinal sensitization through protein kinase A and C activation (Calixto *et al*., 2005; Meotti *et al*., 2006; Woode *et al*., 2013). The analgesic effect of C1 may therefore also involve the inhibition of production or action of some of these mediators in addition to the direct interaction with the $TRPV_1$. $TRPV_1$ antagonism additionally has been suggested to suppress pain evoked prostaglandin metabolites without the adverse effects of inhibiting cyclooxygenases (Materazzi *et al.*, 2008). Pre-treatment of mice with capsazepine also reversed antinociception of C2 indicating possible involvement of $TRPV_1$ receptors in its antinociception.

The antinociception of C3 and C5 in the hypertonic saline-induced corneal pain was also inhibited by PCPA, ondansetron and capsazepine in addition to naloxone indicating that in addition to involvement of serotoninergic system (through 5-HT3 receptors) and $TRPV_1$ receptors, opioidergic pathway may be contributing to their antinociceptive mechanism. Inhibition of C3 and C5 analgesic action by naloxone, a non-specific opioid receptor antagonist suggests that C3 and C5 interacts with opioid receptors, activation of which has been shown to also inhibit the activity of $TRPV_1$ via Go/i proteins and the cAMP pathway (Endres-Becker *et al*., 2007). Therefore, C3 and C5 may have produced their antinociception additionally through the opioid pathway.

The current rodent behavioural assays of nociception is labour intensive and only small groups of animals can be used making zebrafish larvae an alternative model system (Steenbergen and Bardine, 2014). This study therefore additionally explore the possibilities of the use of zebrafish larvae as a model system to investigate the antinociceptive effects of the isolated fatty acid and fatty acid esters. The results of nociceptive responses to dilute acetic acid revealed that upon exposure to dilute acetic acid, there was a stimulus dependent increase in zebrafish larvae locomotor activity (mean velocity and total distance travelled) which is in support of a similar study

conducted by Steenbergen and Bardine (Steenbergen and Bardine, 2014). The use of acetic acid as a noxious stimulus in zebrafish has been shown (Maximino, 2011) but the route of administration and the concentration used differ with the current study.

The submersion of zebrafish larvae in dilute acetic acid has been shown to activate nociceptive pathways where expression of COX-2, a gene known to be involved in nociception was increased (Grosser *et al*., 2002; Steenbergen and Bardine, 2014). COX-2 protein levels has also been demonstrated to be up regulated after skin injury in rats (Chen *et al*., 2012). The suppression of locomotor activity by morphine and diclofenac in this study therefore suggests their antinociceptive effects in zebrafish larvae exposed to acetic acid. Morphine has been used to study antinociceptive effects in fish with the opioid system being most studied (Mettam *et al*., 2011; Roques *et al*., 2012). The opioid system in fish and mammals are similar with both expressing opioid receptors early during growth (Gonzalez-Nunez and Rodríguez, 2009). Adult zebrafish submerged in water containing morphine have been shown to display behavioural changes and measurable morphine levels in the brain (Lau *et al*., 2006). The compounds (C1, C2, C3 and C5) could inhibit locomotor activity induced by acetic acid similar to morphine, diclofenac and piroxicam (reference analgesics) implying that the compounds have antinociceptive effects. This result gives further credence to the traditional use of the plant in the treatment of pain and the use of zebrafish larvae in pain and nociception research.

6.5 CONCLUSION

Fatty acid and fatty acid esters isolated from *Maerua angolensis* stem bark have significant antinociceptive activity in models of acetic acid-induced abdominal constriction and hypertonic saline-induced corneal pain in mice suggesting peripheral

and central analgesic action of the compounds. In the eye wiping model, these actions could be due to involvement of 5-HT_3, $TRPV_1$ and opioid receptors. The fatty acid and fatty acid esters additionally showed antinociceptive activity in zebrafish larvae exposed to acetic acid thereby giving further credence to the traditional use of *Maerua angolensis* in the treatment of pain and the use of zebrafish larvae in pain and nociception research.

6.6 RECOMMENDATION

- Toxicity studies in rodents to assess the safety and toxicity of MAE.

Chapter 7

ASSESSMENT OF THE SAFETY AND TOXICITY OF MAE IN RATS 7.1 INTRODUCTION

Plant extracts and herbal remedies have bioactive compounds which like drugs and other chemicals need to be assessed for their effect on human health, particularly during early development. It is therefore recommended that all natural products used in therapeutics must be subjected to safety tests by the same methods for new scientific

drugs (Khalil *et al*., 2006; Witaicenis *et al*., 2007). Toxicological studies of drugs and similar products for human use evaluates the safety and toxicity of such products in different animal models which also assist in the selection of a dose that can be safe in human beings (Oduola *et al*., 2007).

Acute and chronic administration of drug or plant extract in animals enables assessment of the adverse effects of such products which is critical in defining their safety and toxicity especially to the central nervous, cardiovascular, gastrointestinal, and haematological systems. Toxicity observed in animals can be correlated with adverse effects in humans but some adverse effects such as headache, hypersensitivity and idiosyncratic reactions are poorly correlated. The translation of some adverse effects from animals to humans can therefore be very challenging due to interspecies differences in the pharmacokinetics (Rhiouani *et al*., 2008). In acute toxicity studies, the safety profile of a drug is recognized (Veerappan *et al*., 2007) as the toxic effects produced by a single large dose of a drug is assessed. Data for predicting the maximum tolerated levels for the species during potential life time exposure is obtained from sub-acute, sub-chronic and chronic toxicity studies in which repeated dose of a drug is assessed. The developmental effects of thalidomide recognized in 1966 make it mandatory to assess drug effects on reproduction and development prior to approval for human use (McGrath, 2012).

Information regarding safety and toxicity of *Maerua angolensis* from traditional use is contradictory. This chapter, therefore, investigates the safety and toxicity of the petroleum ether/ethyl acetate extract of the stem bark of *Maerua angolensis* after acute and sub-acute oral administration in rats.

7.2 MATERIALS AND METHODS

7.2.1 Animals

Male Sprague-Dawley rats (168.3 – 233 g) were used in the study. The rats (n = 5) were housed, fed and cared for as described previously (*section 2.2.4*).

7.2.2 Drugs and chemicals

The following chemicals were used: 10% neutral buffered formalin, xylene, paraffin, haematoxylin-eosin, and ethanol solutions. The extract was administered as described earlier (*section 2.2.5*).

7.2.3 Acute toxicity

Male Sprague-Dawley rats were randomly divided into six groups (n = 5) and 1 day allowed for them to acclimatized in the experimental environment. The rats were fasted overnight but allowed access to water *ad libitum* before being orally treated the following day with MAE (30, 100, 300, 1000 and 3000 mg/kg) or normal saline (10 ml/kg). The rats were placed in observation cages and observed for general changes in behaviour and physiological function as well as for mortality at 0, 15, 30, 60, 120, 180 min and 24 h after treatment similar to the primary observation procedure by Irwin (Roux *et al*., 2004). Behaviours specifically related to neurotoxicity (convulsions and tremor), CNS stimulation (jumping, excitation, hypersensitivity to external stimuli, straub tail, stereotypies and aggressive behaviour), CNS depression (rolling gait, loss of traction, sedation, hypothermia, akinesia, hyposensitivity to external stimuli, loss of balance, motor incoordination, decreased muscle tone and catalepsy) and autonomic functions (body temperature, urination, respiration, defaecation, lacrimation and salivation) were observed and noted.

7.2.4 Sub-acute toxicity

Male Sprague-Dawley rats were randomized into four groups (n = 5) and treated orally with normal saline (10 ml/kg) or MAE (300, 1000 and 3000 mg/kg) respectively daily for 14 consecutive days. The extract was prepared such that not more than 2 ml was administered orally. During the experimental period, rats were monitored closely daily for general appearance, behaviour pattern, abnormalities in food and water intake and signs of toxicity.

7.2.4.1 Preparation of serum and isolation of organs

Animals were fasted overnight and sacrificed on the fifteenth day by cervical dislocation, the jugular vein was cut and blood flowed freely. About 1.5 ml of blood was collected into vacuum tubes containing 2.5 µg of ethylene diamine tetra acetic acid (EDTA) as an anticoagulant for haematological assay and 3.5 ml of the blood was collected into sample tubes without anticoagulant for biochemical assay. The blood for the biochemical assay was allowed to clot before it was centrifuged (4000 rpm at 4 °C for 10 min) to obtain serum and stored at -20 ºC until assayed for biochemical parameters the next day. The animals were then quickly dissected and the organs (liver, kidneys, brain, stomach, heart and spleen) harvested and weighed.

7.2.4.2 Effect of MAE on haematological parameters

Haematological analyses was performed using the automatic analyzer (Sysmex XT2000 L Cell-DYN 1700, Abbot Laboratories Ltd., IL, USA). The parameters examined included red blood cells (RBC), haematocrit (HCT), haemoglobin (Hb), mean cell volume (MCV), mean cell haemoglobin (MCH), mean cell haemoglobin concentration (MCHC), white blood cells (WBC), platelets, lymphocytes, neutrophils, eosinophils, basophils, and monocytes.

7.2.4.3 Effect of MAE on serum biochemical parameters

Biochemical analyses were carried out using Cobas integra 400 (Hoffmann-La Roche Ltd., Basel, Switzerland). The biochemical parameters assessed included alkaline phosphatase (ALP), alanine aminotransaminase (ALT), aspartate aminotransaminase (AST), total protein, albumin, fasting blood glucose, total bilirubin (T-BIL), direct bilirubin (D-BIL), urea, creatinine, triglyceride, total cholesterol, high density lipoprotein (HDL) and calcium.

7.2.4.4 Effect of MAE on body and organ weights

Body weights of the rats were taken on days 0 and 15. The brain, liver, kidneys, stomach, heart, and spleen were isolated and weighed. Relative organ weight (ROW) of each organ was then calculated as:

$$ROW = \frac{absolute\ organ\ weight\ (g)}{body\ weight\ on\ day\ 15\ (g)} \times 100$$

7.2.4.5 Histopathological examination of organs

Sections of the tissue from liver, kidneys, spleen, brain, heart and stomach were used for histopathological examination. Tissues were fixed in 10% neutral buffered formalin (pH 7.2) and dehydrated through a series of ethanol solutions. They were cleared with xylene, embedded in paraffin and regularly processed for histological analysis. Sections of 2 μm thickness were cut and stained with haematoxylin-eosin for examination. The stained tissues were observed through an Olympus microscope (BX-51) and photographed by INFINITY 4 USB Scientific Camera (Lumenera Corporation, Otawa, Canada).

7.2.5 Data analysis

Data were expressed as mean ± SEM per group. Statistical differences between control and treated groups were tested by one-way ANOVA with Newman–Keuls *post hoc* test. GraphPad® Prism 5.01 for Windows (GraphPad® Prism Software, San Diego, CA, USA) was used for all statistical analyses. Differences were considered significant at $P<0.05$.

7.3 RESULS

7.3.1 Acute toxicity

Rats in all the six groups (n = 5) survived during the 24 hours study period. The group administered MAE (30 - 300 mg/kg) did not display toxic signs (no observed adverse effects level) during observation period when compared to the group administered normal saline (control group). Nevertheless, the groups administered MAE (1000 - 3000 mg/kg) exhibited some toxic signs such as sedation, asthenia and increased urination and defaecation when compared to the control group (Table 7.1).

Table 7.1: Observations in the acute toxicity test after oral administration of MAE in SpragueDawley rats

Dose (mg/kg)	Mortality	Toxicity signs
0	None	None
30	None	None
100	None	None
300	None	None

1000	None	Sedation, asthenia, defaecation, and urination
3000	None	Sedation, asthenia, defaecation, and urination

The petroleum ether/ethyl acetate extract of *Maerua angolensis* in normal saline was administered orally to groups of rats (n = 5). Observations for toxicity signs were done at 15, 30, 60, 120, 180 min and 24 hours after administration. Symptoms that did not necessitate handling were also observed at 0 to 15 min immediately following administration.

7.3.2 Sub-acute toxicity

Rats in all the four groups (n = 5) survived during the 14 days study period. No MAE-related changes were seen in general appearance, behaviour pattern, abnormalities in food and water intake or signs of toxicity in rats that were administered 300 mg/kg of MAE when compared to rats that were administered normal saline. However, rats administered MAE (1000 and 3000 mg/kg) exhibited signs of sedation, increased defaecation and urination on days 1 to 2 which marginally diminished from day 3.

7.3.2.1 Effect of MAE on haematological parameters

There were no significant differences seen between control and extract treated groups for the haematological parameters measured (Table 7.2).

Table 7.2: Haematological values of control and rats treated with *Maerua angolensis* for 14 days

Parameters	MAE					
	0	300 (mg/kg)	1000 (mg/kg)	(3000 mg/kg)	$F_{3,16}$	*P* Value
Hb (g/dl)	15.82±0.77	16.41±0.94	15.33±1.37	16.03±0.96	0.1866	0.9040
PCV (%)	50.40±1.43	50.80±1.62	52.40±4.76	51.60±2.37	0.0953	0.9615
WBC (x 10^9/L)	10.06±1.36	16.05±2.54	13.00±3.01	10.08±2.29	1.439	0.2684

RBC (x 10^{12}/L)	5.79±0.32	5.35±0.14	5.10±0.28	5.85±0.39	1.384	0.2837
Platelets (x 10^{9}/L)	272.00±17.38	294.00±18.07	327.80±32.40	280.40±14.19	1.284	0.3137
MCV (fL)	88.25±6.12	95.09±4.13	102.00±6.21	90.55±9.19	0.8323	0.4955
MCH (pg)	27.54±1.71	30.71±1.99	29.91±2.05	28.24±3.26	0.3942	0.7589
MCHC (g/dL)	31.41±1.36	32.40±2.09	29.28±0.56	31.00±0.80	0.9443	0.4425
Lymphocytes (%)	56.98±4.42	53.95±1.48	55.44±4.15	58.59±6.95	0.1817	0.9072
Eosinophils (%)	2.67±0.39	3.01±0.62	1.27±0.21	2.72±0.53	2.768	0.0757
Basophils (%)	1.16±0.41	1.50±0.39	0.78±0.27	0.69±0.33	1.094	0.3802
Monocytes (%)	3.95±0.67	3.72±0.77	2.32±0.48	3.76±0.81	1.157	0.3568
Neutrophils (%)	19.18±1.36	22.56±0.72	17.79±1.25	19.33±1.94	2.103	0.1401

Values are mean±SEM (n = 5). MAE = *Maerua angolensis* extract, 0 = control. MAE treated groups were compared to control using one-way ANOVA followed by Newman-Keuls *post hoc* test.

7.3.2.2 Effect of MAE on serum biochemical parameters

There were no statistically significant differences between control and extract treated groups for the biochemical indices measured (Table 7.3).

Table 7.3: Clinical biochemistry parameters of control and *M. angolensis* treated rats for 14 days

Parameters	MAE				$F_{3,12}$	*P* Value
	0	300 (mg/kg)	1000 (mg/kg)	3000 (mg/kg)		
Glucose (mmol/L)	5.92±0.31	5.78±0.42	5.36±0.18	6.09±0.73	0.4532	0.7198
Urea (mmol/L)	5.03±0.32	5.59±0.31	6.48±0.54	6.01±0.28	2.6610	0.0955
AST (U/L)	187.90±18.19	178.90±19.03	164.50±14.22	170.50±15.77	0.3615	0.7820
ALT (U/L)	93.83±7.59	81.79±3.30	79.38±6.50	75.63±5.55	1.7470	0.2107
ALP (U/L)	323.80±47.11	278.00±29.81	276.40±38.88	264.7±31.77	0.4834	0.7000
Creatinine (mmol/L)	37.53±2.64	34.73±1.48	34.10±2.39	40.33±1.91	1.7540	0.2094

Albumin (g/L)	33.72±3.17	32.70±1.55	31.64±2.70	22.93±3.29	3.199	0.0623
Triglyceride (mmol/L)	0.82±0.08	0.79±0.08	0.74±0.03	0.70±0.08	0.5171	0.6784
T-choles (mmol/L)	1.81±0.22	1.67±0.12	1.77±0.19	1.65±0.07	0.2111	0.8867
HDL choles (mmol/L)	0.89±0.03	1.12±0.06	1.11±0.10	1.14±0.09	2.3330	0.1257
T-protein (g/L)	60.75±3.54	61.13±5.41	56.20±3.10	50.60±5.49	1.1850	0.3567
Calcium (mmol/L)	2.28±0.12	2.10±0.24	1.92±0.20	1.87±0.22	0.8400	0.4977
T-bilirub (mg/dL)	0.50±0.01	0.49±0.01	0.49±0.01	0.49±0.01	0.1027	0.9569
D-bilirub (mg/dL)	0.25±0.01	0.24±0.01	0.24±0.01	0.24±0.00	0.1818	0.9067

Values are mean±SEM (n = 4). MAE = *Maerua angolensis* extract, 0 = control. MAE treated groups were compared to control using one-way ANOVA followed by Newman-Keuls *post hoc* test.

7.3.2.3 Effect of MAE on body and some targeted organ weights

Rats in all groups increased weight over the 14 days study period with the percentage changes in body weights being more for the groups treated with MAE except the 1000 mg/kg group but there were no significant change in the body weight of rats that survived at the end of the experiment (Table 7.4) or percentage change in body weight (Figure 7.1) nor were the relative weights of some targeted organs affected (Table 7.5) when compared to controls.

Table 7.4: Effect of oral administration of MAE on body weight change of rats in the sub-acute toxicity test

Dose (mg/kg)	Initial body weight (g)	Day 15 body weight (g)
0	196.6 ± 10.40	203.3 ± 10.73
300	200.0 ± 5.45	206.7 ± 7.12^{ns}
1000	199.0 ± 7.48	202.7 ± 7.65^{ns}

3000	198.6 ± 11.40	217.7 ± 10.94^{ns}

Data are expressed as mean ± SEM (n = 5), 0 = control, ns = not significant as compared to the control using one-way ANOVA followed by Newman-Keuls *post hoc* test.

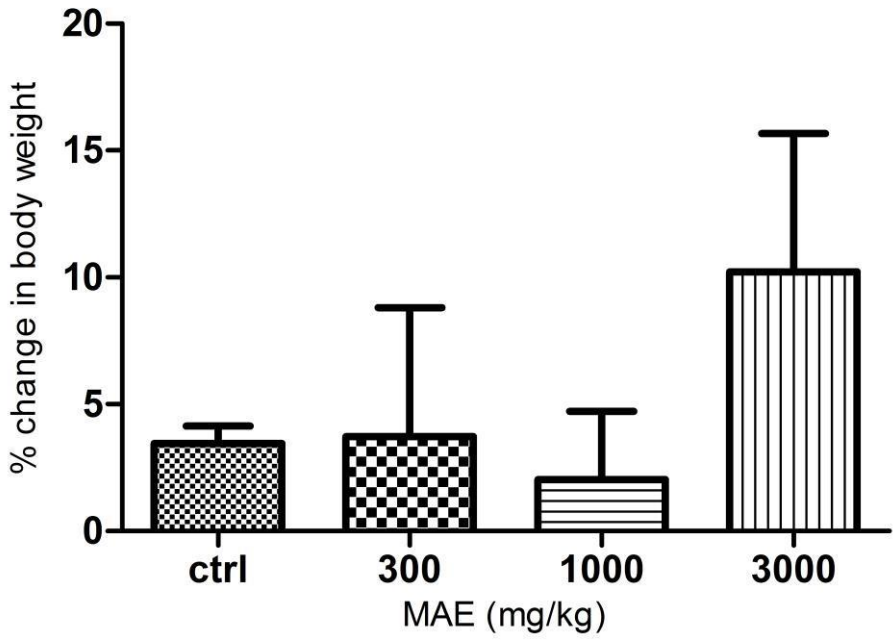


Figure 7.1 Effect of oral administration of *Maerua angolensis* extract on the % change in body weights of rats in the sub-acute toxicity test. Data are expressed as mean ± SEM, (n = 5). Extract treated groups were compared to control group using one-way ANOVA followed by NewmanKeuls *post hoc* test.

Table 7.5: Effect of oral administration of MAE on the relative organ weights (ROW) of rats in the sub-acute toxicity test

Organ	ROW			
	Control	300 mg/kg MAE	1000 mg/kg	3000 mg/kg
Liver	3.148±0.02377	3.125±0.03159^{ns}	3.226±0.0221^{ns}	3.096±0.021^{ns}
Kidneys	0.836±0.01215	0.881±0.01626^{ns}	0.937±0.00924^{ns}	0.891±0.01312^{ns}
Spleen	0.285±0.00349	0.271±0.00716^{ns}	0.296±0.01013^{ns}	0.34±0.00366^{ns}

Stomach	0.676±0.00454	0.659±0.00603^{ns}	0.695±0.00533^{ns}	0.639±0.00506^{ns}
Heart	0.344±0.00659	0.242±0.00993^{ns}	0.444±0.00924^{ns}	0.367±0.00646^{ns}
Brain	1.194±0.00542	1.18±0.00899^{ns}	1.238±0.00757^{ns}	1.203±0.00492^{ns}

Data are expressed as mean ± SEM (n = 5), ns = not significant as compared to the control group using one-way ANOVA followed by Newman-Keuls *post hoc* test.

7.3.2.4 Histopathological examination of organs

Plates 7.1 – 7.6 show the photomicrographs of sections of the isolated organs (livers, kidneys, stomachs, hearts, spleens and brains) of control group and rats treated orally with MAE (300 – 3000 mg/kg) for 14 days in the sub-acute toxicity study. Livers of control and rats treated with 300 mg/kg MAE showed normal appearance and histology of the liver with sinusoids and well-arranged hepatocytes but significant MAE-related changes at 1000 and 3000 mg/kg. The histology of the livers was not consistent with the normal ALT, AST and bilirubin levels in the serum of rats treated with 1000 and 3000 mg/kg MAE.

Generally, there were no observable changes in architecture of kidneys, stomachs, spleens, hearts and brains of MAE-treated rats at all doses compared to the control.

The morphological structure of control and MAE-treated group livers (Plate 7.1) reveals normal radial arrangement of the hepatocytes and normal nuclei within the hepatocytes with no remarkable abnormalities in both the control and the rats administered 300 mg/kg MAE. However, livers of rats treated with 1000 mg/kg MAE showed hepatocytes with nucleolus and binucleated hepatocytes. The livers of rats treated with 3000 mg/kg MAE showed disoriented sinusoids, necrotic nucleus and lymphocytic infiltrations.

The histology of the kidneys showed normal renal corpuscles with well-defined glomeruli and distal convoluted tubules in control and rats treated with 300 - 3000 mg/kg MAE (Plate 7.2). The stomach and spleen samples from both control and MAE-treated rats (Plate 7.3 and 7.4) revealed normal gastric glands and normal white pulp respectively. Similarly, the heart samples from both control and MAEtreated rats (Plate 7.5) showed normal histology of the cardiac muscles. There was no MAE-induced changes (normal brain histology with normal neuron and neuroglia) in the brains of treated rats at all doses (Plate 7.6).

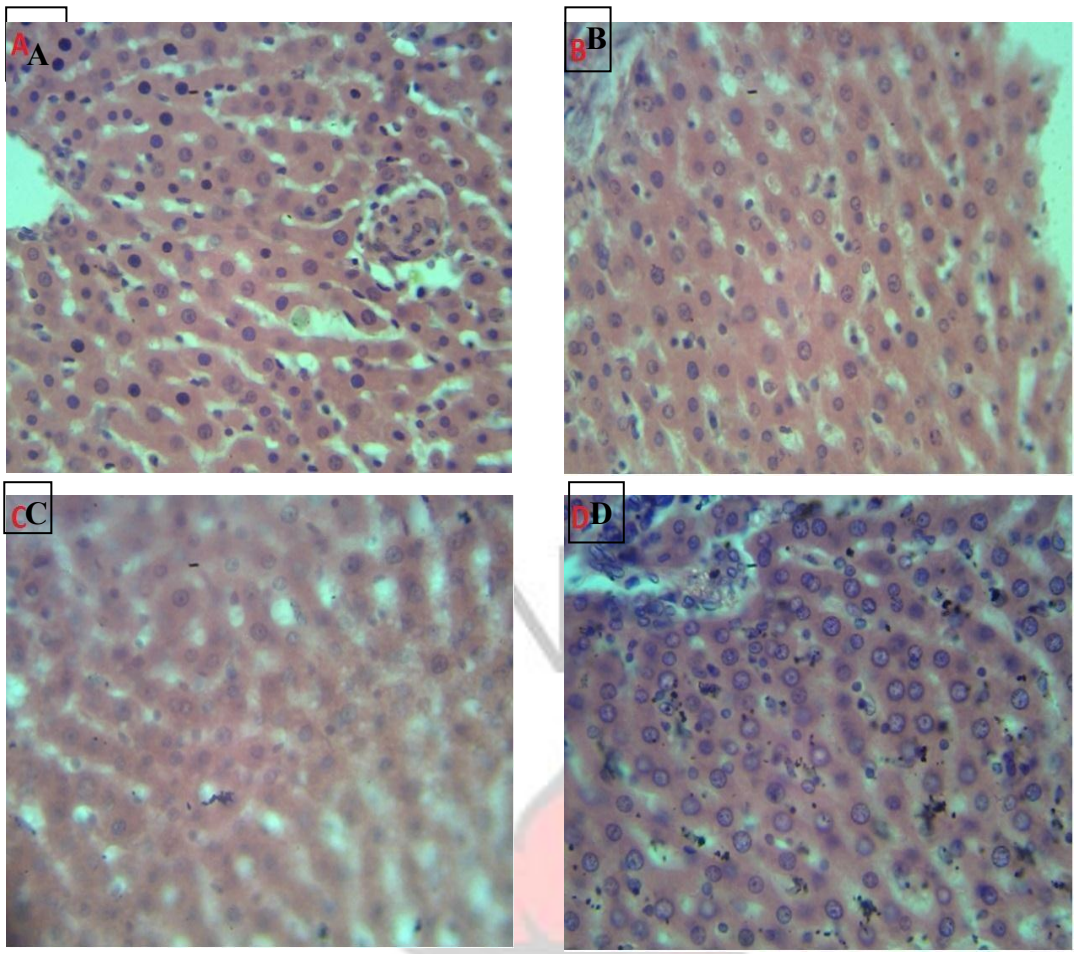


Plate 7.1 Photomicrographs (H & E stain, × 400) of liver histology of control (A) and MAEtreated rats (B = 300 mg/kg, C = 1000 mg/kg and D = 3000 mg/kg, *p.o*. for 14 days) in the subacute toxicity study.

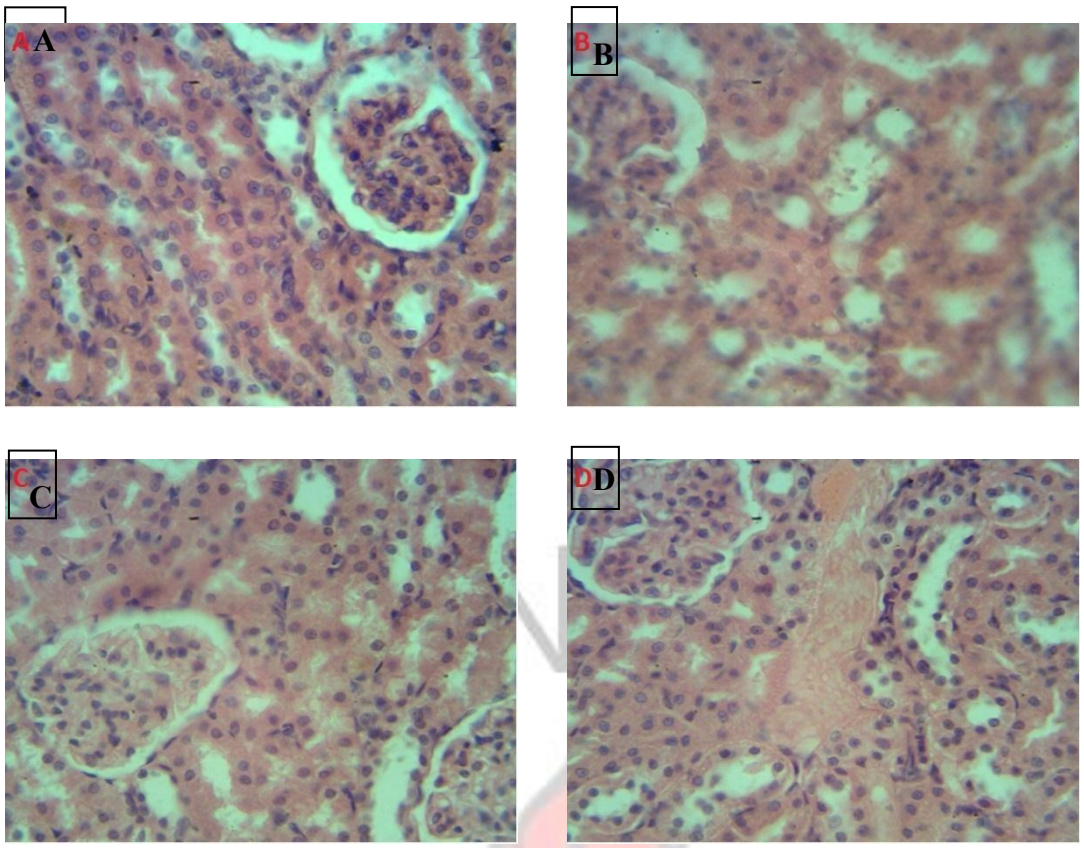


Plate 7.2 Photomicrographs (H & E stain, × 400) of kidney histology of control (A) and MAEtreated rats (B = 300 mg/kg, C = 1000 mg/kg and D = 3000 mg/kg, *p.o.* for 14 days) in the subacute toxicity study.

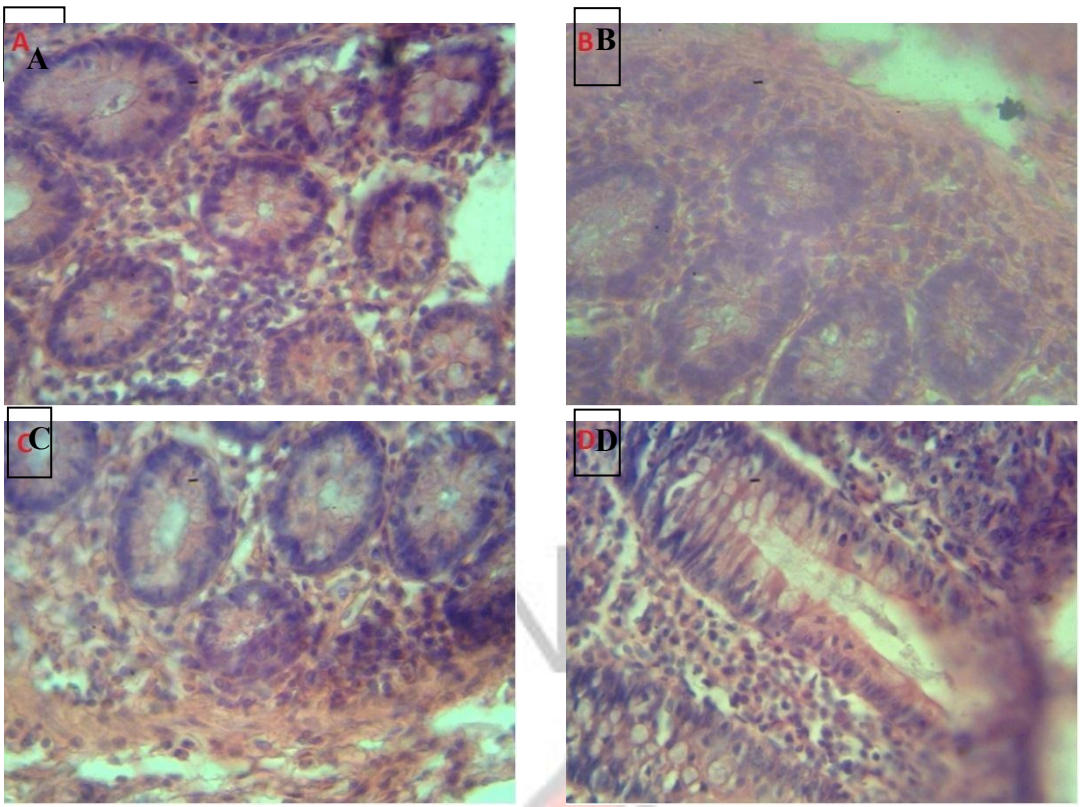


Plate 7.3 Photomicrographs (H & E stain, × 400) of stomach histology of control (A) and MAEtreated rats (B = 300 mg/kg, C = 1000 mg/kg and D = 3000 mg/kg, *p.o.* for 14 days) in the subacute toxicity study.

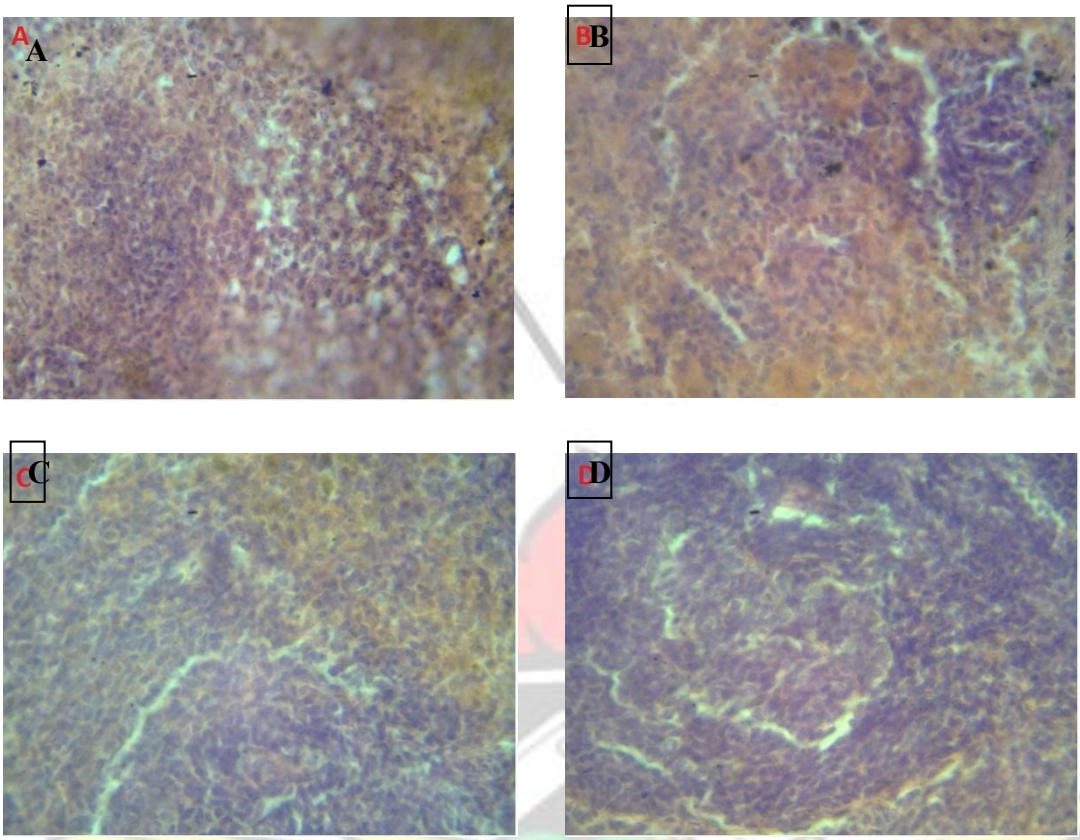


Plate 7.4 Photomicrographs (H & E stain, × 400) of spleen histology of control (A) and MAEtreated rats (B = 300 mg/kg, C = 1000 mg/kg and D = 3000 mg/kg, *p.o.* for 14 days) in the subacute toxicity study.

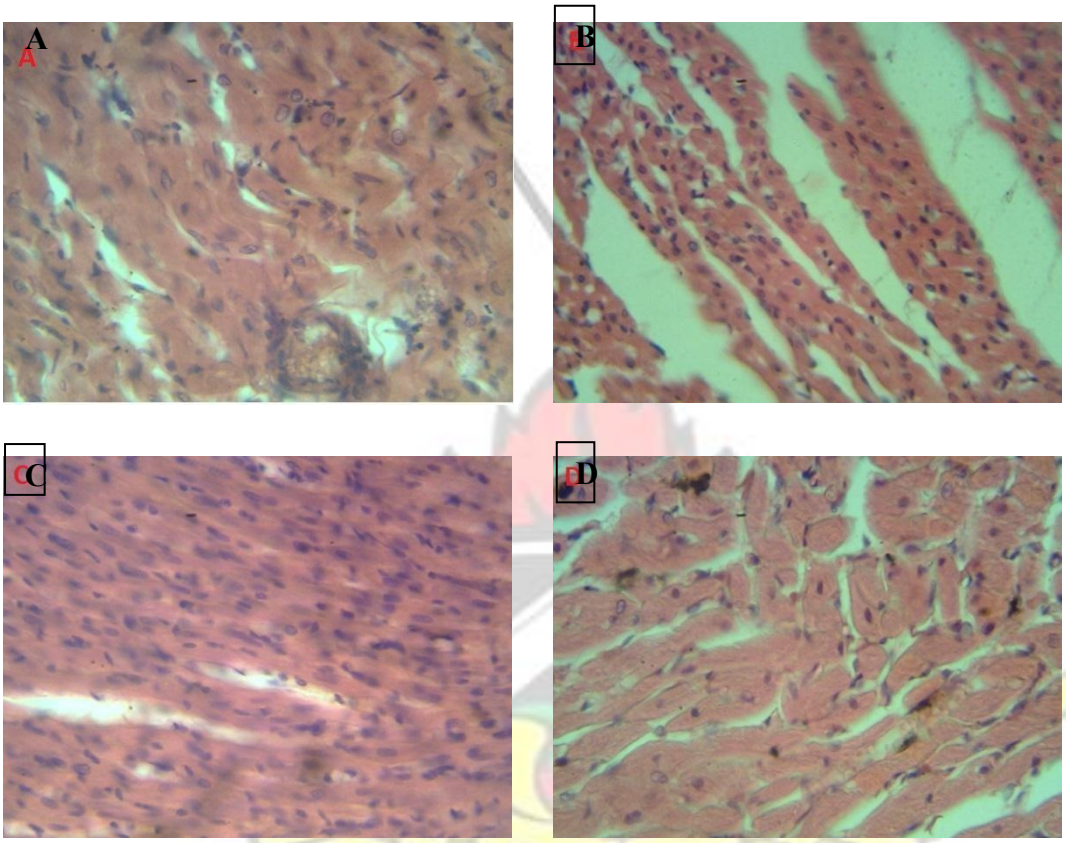
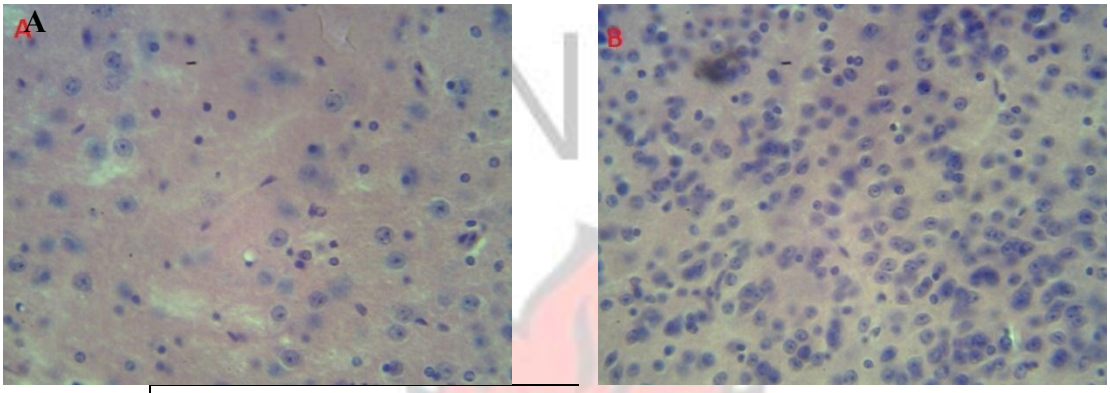
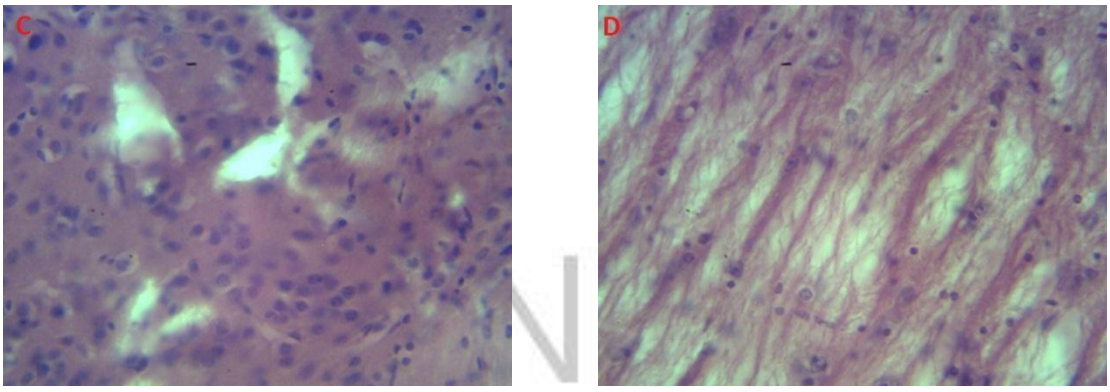


Plate 7.5 Photomicrographs (H & E stain, × 400) of heart histology of control (A) and MAEtreated rats (B = 300 mg/kg, C = 1000 mg/kg and D = 3000 mg/kg, *p.o.* for 14 days) in the subacute toxicity study.





C **D**

Plate 7.6 Photomicrographs (H & E stain, × 400) of brain histology of control (A) and MAEtreated rats (B = 300 mg/kg, C = 1000 mg/kg and D = 3000 mg/kg, *p.o.* for 14 days) in the subacute toxicity study.

7.4 DISCUSSION

Herbal products contain bioactive constituents with the potential to produce adverse effects (Bent, 2008) but they are often erroneously regarded as safe due to poor pharmacovigilance (Saidu *et al*., 2007) and because they are natural. This is a major drawback to the use of traditional herbal preparations. Revealing the toxicity profile of plant extracts intended to be used as medicines will assist in the determination of the extent of their safety and toxicity if in use as drugs. Moreover, it is not enough for drugs to be efficacious, cheap and available, but also safe for short and long term uses.

Evaluation of safety profile of phytotherapeutic products therefore is paramount in the development of drugs and in their subsequent clinical uses. The present study

conducted toxicity study in rats. However, at least two animal species are required in toxicity study because of the possible differences between species in drug metabolism and susceptibility to toxic effects.

The acute toxicity study was used to establish the median lethal dose (LD_{50}), defined as the dose of the extract that will kill 50% of the rats treated orally. This was estimated to be above 3000 mg/kg because all the rats treated with various doses of MAE in the acute toxicity survived. Rats treated with single oral dose of MAE up to 300 mg/kg and observed over a period of 24 h did not show any signs of adverse effects or altered behavioural pattern. Though, attention should be given to the increased urination, defaecation, asthenia and sedation observed at higher doses of 1000 and 3000 mg/kg few minutes post treatment, however, were all reversible in a maximum period of 24 h after the administration of the extract.

The LD_{50} of over 3000 mg/kg of MAE orally in rats suggests that MAE is relatively safe. This is because any substance with an LD_{50} of 1000 mg/kg or more orally is taken as being safe (Obici *et al*., 2008). LD_{50} is not an absolute value but inherently variable biological parameter that cannot be compared to constants such as molecular weight or melting point (Nwinyi *et al*., 2009). Accuracy should therefore not be used to describe LD_{50} but precision which is being only relevant to the experiment for which the LD_{50} was derived and does not increase the probability that in subsequent experiments, the LD_{50} will be same or even similar (Nwinyi *et al*., 2009). Acute toxicity study therefore has its own limitations as a tool for assessing toxicity (Aniagu *et al*., 2005; Orisakwe *et al*., 2002) as it does not necessarily guarantee the safety of the tested agent not withstanding its value. Acute toxicity data has limited clinical application as very low doses of a substance administered for a long period can result

in cumulative toxic effects. Therefore, sub-acute and chronic toxicity studies are almost always important in assessing the safety profile of phytomedicines (Aniagu *et al*., 2005). Nevertheless, acute toxicity studies provides some useful information that assists in the selection of dose ranges that could be used for subsequent studies. Also, the possible clinical signs induced by the substance of investigation could manifest at this level of study. It is also applied in the establishment of therapeutic index of drugs and xenobiotics (Rang *et al*., 2007).

The present study also carried out sub-acute toxicity study to assess long term, low dose effect of MAE. The study discovered that the extract at 300 mg/kg showed no adverse clinical sign or toxicity sign or death throughout the treatment duration of 14 days. Signs of sedation, urination and defaecation were however observed on the first two days which somewhat diminished from day 3 after administration of MAE at 1000 and 3000 mg/kg doses but no mortality throughout the 14 days. This is in line with the acute toxicity studies where rats treated orally with the extract doses (30 – 300 mg/kg) showed neither toxicity sign or death. This may be an indication that long term oral administration of the extract within these low dose ranges is safe. There was also no significant changes observed in water and food intake in rats treated with various doses of MAE.

Proper intake of food and water are essential to the physiological status of the animals (Feres *et al*., 2006). Since the water and food intake of MAE treated rats was not affected, it can be concluded that MAE possibly did not interfere with the nutritional benefits (weight gain, stability of appetite) expected of the rats. This was confirmed by the general but non-significant increases in the body weight observed in all the MAE treated groups throughout the study period. Change in body weight is an

indicator of adverse effect (Obici *et al*., 2008). Change in organ weight has also been shown to be a sensitive indicator of organ toxicity by some known toxicants (Nwinyi *et al.*, 2009) but there is need to examine both organ weights and their histopathology. There was no significant difference in organ to body weight ratio with MAE treated rats when compared with control rats. These could be closely related to body weight gain. The non-signicant differences in the body weight could also indicates the relative safety of MAE in rats.

When animals lose appetite (anorexia), weight loss is bound to follow owing to disturbances in carbohydrate, protein or fat metabolism (Nwinyi *et al*., 2009). The general but non-significant increases in the body weight observed indicates that MAE probably did not induce anorexia, an effect that could have spearhead in loss of body weight. The fact that MAE caused no significant changes in the relative weight of the liver, brain, heart, spleen, stomach and kidney could mean that the integrity of all the organs were not tampered with by the extract. Though, this inference can only be probably correct if the results of the effects of MAE on relative organ weight, serum biochemical indices and histopathology of these organs are taken together. Gross pathological examination of the organs showed no gross abnormalities in the morphologies/features, consistencies and appearances of the kidney, spleen, brain, stomach and heart of the rats treated for 14 days with the extract. Histopathological examinations however revealed that there were abnormalities in the liver at 1000 – 3000 mg/kg of the extract.

There are many reports of liver and kidney toxicity related to the use of herbal products (Obici *et al*., 2008; Rhiouani *et al*., 2008). The liver is the major site for the metabolism of most chemicals and has the capacity to metabolize a large number of drugs including

herbal products thus predisposing it to toxicity because metabolism does not always result in detoxification. The kidneys eliminate many drugs and their metabolites and because of its high blood flow which exposes renal parenchyma to high peak concentrations of chemicals, toxicity occur even if the toxic chemical is present briefly in the circulation. The ability of the kidneys to concentrate toxic solutes in parenchymal cells and in tubular luminal fluid is a further risk factor (Greaves, 2011).

Serum biochemical parameters (AST, ALT, ALP, total bilirubin, direct bilirubin, albumin, total protein, urea, creatinine, and calcium) as specific markers for some target organs (liver and kidney function) showed no significant differences between treated groups and control. Damage to or effect on liver often results in increase in clinical chemistry parameters such as serum enzymes like ALP, AST, ALT and analytes like total and conjugated bilirubin but decrease in total protein and albumin (Aniagu *et al*., 2005; Asiedu-Gyekye *et al*., 2014; Nawaz *et al*., 2014). The liver produces most of the plasma proteins in the blood including albumin and globulin. Total proteins and albumin were decreased but not significant in MAE treated rats especially at 3000 mg/kg. Low plasma proteins could be due to either MAE-induced reduction in synthesis or MAE-induced intestinal protein malabsorption due to rapid gastrointestinal transit. Low serum proteins have some implications pharmacologically since plasma proteins bind many molecules including drugs and carry them through circulation. Thus chronic administration of high dose of MAE alongside with another drug that is normally highly bound to plasma proteins may result in exaggerated response or even toxicity of the drug due to increased plasma concentration.

Hepatocellular damage is typified by a joint rise in serum levels of AST and ALT. Approximately 80% of AST is located in the mitochondria whereas ALT is purely cytosolic (Andy and Keeffe, 2003). AST therefore appears in higher concentrations in

liver, kidneys, heart, and pancreas and is released slowly in comparison to ALT. However, since ALT is localized primarily in the cytosol of hepatocytes, this enzyme is reflected a more sensitive marker of hepatocellular damage than AST and within limits can provide a quantitative assessment of the degree of damage sustained by the liver (Aniagu *et al*., 2004). Decrease in serum albumin is an obvious sign of hepatic disorder (Saidu *et al*., 2007). Increase ALP is associated with hepatobiliary condition such as primary biliary cirrhosis. With the exception of growing animals or animals with bone disease (where an increase in ALP is due to the osteoblast production), elevated serum ALP activity is mostly attributed to hepatobiliary origin (Ramaiah, 2007). High levels of ALP exist in cells that are rapidly dividing or are otherwise metabolically active. However, ALP levels reach remarkable levels in primary biliary cirrhosis, in conditions of disorganized hepatic architecture, and in diseases characterized by inflammation, regeneration, and obstruction of intrahepatic bile ductules (Witthawaskul *et al*., 2003). The fact that the whole blood analysis of parameters like the platelets and WBC counts were normal coupled with normal ALP ruled out all these conditions. Additionally, when bilirubin level was considered, it revealed that the bile ducts of the rats could not have been obstructed since the bilirubin is a better indicator.

Increase in ALP activity is also associated with the administration of drugs such as corticosteroids and anticonvulsants (Boone *et al*., 2005). Serum ALP activity in rats has been reported to increase rapidly following a meal and thus cannot be reliable alone to detect cholestasis (Barton *et al*., 2000). It is known that on a per gram basis, intestinal mucosa in the rat has higher ALP activity than the liver (Amacher, 2002). Thus, food intake should be taken into consideration when interpreting ALP values in

rats, especially as decreased food intake and body weight routinely noted in toxicology studies results in decrease ALP.

Bilirubin is the main pigment that is formed from the breakdown of haem in RBCs. It is conjugated in the liver and then secreted into the bile (Nazir *et al*., 2011). Therefore increased levels of bilirubin in the plasma may result from an increase in its production, a decrease in its conjugation, a decrease in its secretion by the liver, or a blockade of the bile ducts (Limdi and Hyde, 2003). In cases of increased production, or decreased conjugation, the unconjugated (indirect) form of bilirubin is increased. An increase in serum levels of unconjugated bilirubin suggests pre-hepatic or hepatic jaundice whereas an increase in conjugated bilirubin suggests post-hepatic jaundice (Nazir *et al.*, 2011). When the bile ducts are obstructed, there is a build-up of direct bilirubin. This escapes from the liver and ends up in the blood increasing plasma levels. Serum bilirubin is thus considered a true test of liver function, since it reflects the liver‘s ability to take up, process, and secret bilirubin into the bile (Limdi and Hyde, 2003). Since there were no elevations in direct, and total bilirubin fractions after treatment with MAE, it can be implied that MAE did not have any harmful effects on hepatic metabolism or biliary excretion.

The decreasing trend of serum AST, ALT and bilirubin levels in MAE treated rats even though not significant, cannot be overlooked. Clinically, decreased AST and ALT levels have no documented relevance if not in a patient already having a hepatic dysfunction; whereas an increase suggest a hepatic leakage or acute liver damage (Chand *et al*., 2011). The absence of significant changes in the relative weight of the liver, the absence of abnormalities in the morphologies/features, consistencies and appearances of the liver observed

grossly (except at 1000 – 3000 mg/kg MAE) and the non-significant difference observed in the hepatic function indices suggest absence of hepatotoxicity.

The criteria for assessing hitopathological changes of organs include necrosis, cloudy swelling, distortion of radial arrangement of hepatocytes, diffusion/enlargement of nuclei, fatty infiltration of cells, inflammatory infiltrations, fibrosis, vacuolation and neurofibrillation among other signs (Greaves, 2011). In rats treated with 1000 and 3000 mg/kg MAE, the livers showed mild distortion of radial arrangement of hepatocytes and diffusion/enlargement of nuclei within the hepatocytes. These may not be considered clinically significant since serum aminotransferases and bilirubin levels, which are markers of hepatic function, were decreased (though insignificant) even at these higher doses. Caution should nevertheless be taken in using MAE outside 3000 mg/kg.

Further toxicity studies may be required in other animal species and with more chronic toxicity studies to establish the safety profile of MAE, specifically with regards to hepatic toxicity. The non-significant decrease in serum aminotransferases and bilirubin at all doses, though clinically insignificant, suggests possible protective ability of MAE on the liver which could be the reason of its traditional use in jaundice (Mothana *et al.*, 2009) but this needs further investigation using different models and animal species.

Urea and creatinine are compounds derived from proteins, which are eliminated by the kidneys but when the kidneys are damaged, their levels increase (Akdogan *et al*., 2003). The non-significant effect of MAE on renal function indices suggest that the renal integrity of the extract treated rats is preserved. The non-significant elevation of serum creatinine up to 3000 mg/kg MAE suggests that glomerular function is intact, more so that urea was not affected. Urea is

less reliable than creatinine as a marker of glomerulus filtration rate (GFR) because it diffuses back into the renal tubular cells (Adebayo *et al*., 2003; Eteng *et al*., 2009). Absence of abnormalities in the gross and histopathological examination of the kidneys, in addition to the observed effects on renal function indices at all doses given further indicate that the excretory capability of the kidneys was not impaired.

Effect of the extract tested on serum lipid profile showed that the extract had no significant effect on total cholesterol, triglyceride and HDL cholesterol concentrations. The extract had non-significant reduction ($P > 0.05$) on total cholesterol and triglyceride but non-significant increase on HDL cholesterol at all doses administered when compared with controls. High blood cholesterol concentration is a risk factor for cardiovascular disease (Adebayo *et al*., 2005). Thus the extract may be beneficial in reducing the risk of cardiovascular disease.

The haematological system has a higher predictive value for toxicity in humans (Van Meer *et al*., 2012). Blood is the main medium of transport for many drugs and xenobiotics in the body thus initially exposing components of the blood such as RBCs, WBCs, Hb and platelets to significant concentrations of toxic compounds. There were no significant effect of MAE on the haematological parameters measured suggesting that the integrity of the blood of the rats was not negatively affected by the extract unlike some herbal extracts and conventional drugs. Some flavonoids isolated from herbs have been shown to cause haemolytic anaemia and thrombocytopenia (Nyarko *et al*., 2005). MCV, MCH and MCHC relate to individual red blood cells and Hb, PCV and RBC relate to the total population of red blood cells in the blood suggesting that the extract may neither affect the incorporation of haemoglobin into red blood cells

nor the morphology and osmotic fragility of red blood cells produced. Since HB, PCV, RBC, MCV, MCH and MCHC were not

affected, this indicate that the extract may not affect the population of red blood cells produced from the bone marrow and the oxygen-carrying capacity of each red blood cell/whole blood. The possibility of anaemia which may result from impaired red blood cell production is therefore non-existent with the extract administration.

To determine whether the defence system of the rats had been compromised, WBC count as well as differential WBC percentages was measured. The extract nonsignificantly ($P > 0.05$) increased WBC at all doses administered when compared with control implying that the extract may not contain some bioactive constituents that could cause destruction or impaired production of white blood cells. Thus administration of the extract may not predispose to infection. The non-significant effect of the extract on WBC, RBC, and platelet count at all doses administered when compared with control, imply that the extract may not possess the potential of causing a gradual and selective bone marrow depression because the bone marrow is responsible for the production of red blood cells, white blood cells and platelets (Adebayo *et al*., 2005). Platelets play a crucial role in reducing blood loss and repairing vascular injury (Adedapo *et al*., 2007; Dahlbäck, 2008). The nonsignificant effect of the extract on WBC and differential leucocyte counts further suggest that the extract may not have consequential effects on the immune system and phagocytic activity of the blood cells of the animals.

7.5 CONCLUSION

The acute and sub-acute toxicity effect of MAE administered orally in rats have shown that the extract is safe and relatively non-toxic at 300 mg/kg dose. However, further toxicity studies including sub-chronic, chronic, reproductive, developmental and

genetic as well as mutagenicity and carcinogenicity tests, effects on drug metabolizing enzymes and toxicokinetic profiling using different animal species still need to be conducted for the complete elucidation of the safety and toxicity profile of MAE. Additionally, caution should be used when using the extract especially at high doses because this result cannot be directly extrapolated to humans.

Chapter 8

GENERAL DISCUSSION, CONCLUSION AND RECOMMENDATIONS 8.1

GENERAL DISCUSSION

Pain is associated with most pathological conditions in humans that affects thinking, sleeping, emotion and performance of daily chores (Dib-Hajj *et al*., 2010; Schim and Stang, 2004), thereby making it an important therapeutic priority for control of pains. In many pathological conditions, particularly HIV/AIDS, diabetes and cancer, the management of pain remains a cause for concern. New agents with improved efficacy are required to help manage challenging pain including neuropathic pain. Medicinal plants are potential sources of commercial drugs and lead compounds in drug development (Zhang, 2004) forming important sources of new chemical substances with potential therapeutic effects (Ebadi, 2006). With the huge reserve of medicinal plants, research into those with claims of relieving pain in traditional medicine is a good strategy in the search for new analgesic agents.

The present study assessed the analgesic properties of *Maerua angolensis*, a medicinal plant used traditionally in the treatment of various diseases including pain in Nigeria and some West African countries (Meda *et al*., 2013; Mothana *et al*., 2009). The safety of this plant that could be an advantage or a limitation to the sought analgesic effects of the plant was also investigated. Almost all the parts of this plant including the fruits, seeds, flowers but notably the leaves, roots and stem barks are being used in traditional medicine. The leaves, roots and stem bark were therefore chosen for this study and extracted with the common and popular solvent, aqueous ethanol, to assess their antinociceptive activity in acetic acid-induced abdominal writhing test (a test that has good sensitivity and is capable of detecting antinociceptive compounds at doses that may be inactive with other antinociceptive

tests).

The aqueous ethanolic leaves, stem bark and roots extracts of *Maerua angolensis* exhibited significant antinociceptive activity with the stem bark extract being the most potent. The petroleum ether, ethyl acetate and aqueous ethanol stem bark extracts of *Maerua angolensis* were also effective in inhibiting pain caused by both acetic acid and formalin, chemical models of nociception, suggesting their peripheral and central analgesic properties. The petroleum ether extract was most active in neurogenic while ethyl acetate was most active in inflammatory pain, thus the two extracts were combined and subsequently referred to as petroleum ether/ethyl acetate stem bark extract of *Maerua angolensis* (MAE). The MAE was fractionated to 2 fractions (F1 and F32), purification of which lead to isolation of 4 compounds (C1, C2, C3 and C5) identified and characterized by ^{1}H-NMR, GCMS and IR spectroscopy to be fatty acid and fatty acid esters namely octadecanoic acid methyl ester, bis (2-ethylhexyl) phthalate, octadecanoic acid and oleic acid methyl ester respectively.

The extract (MAE) and fractions exhibited both central and peripheral analgesic properties in the various models of chemical and thermal nociception in rodents. In neurogenic phase of formalin test, formalin has a direct effect on transient receptor potential family of ion channels which have been found to play a role in neuropathic pain (Alessandri-Haber *et al*., 2004). The formalin test is therefore used to predict agents that may be active in neuropathic pain (Vissers *et al*., 2006). Since MAE and fractions showed antinociception in the second phase of formalin test which is predictive of anti-hyperalgesic activity of drugs in neuropathic pain models, their effectiveness in neuropathic pain is consequently not a surprise. Various painful

conditions including neurogenic and inflammatory origin are being manage in traditional medicine with the stem bark of *M. angolensis* (Adamu *et al*., 2007). MAE and fractions blocked both neurogenic and inflammatory pain produced by formalin. The inflammatory phase of formalin test involves the release of pro-inflammatory pain mediators such as prostaglandins, serotonin, histamine and TNF-α which have also been implicated in the development of neuropathic pain.

It was demonstrated in the present study that MAE and fractions improved vincristine-induced neuropathic pain. The pain induced by vincristine has been associated with increase in tissue thiobarbituric acid reactive species, superoxide anion and oxidative stress (Kaur *et al.*, 2010; Muthuraman *et al.*, 2011). Systemic therapy with vincristine also injures Schwann cells and DRG neurons of the PNS resulting in degeneration of myelinated and unmyelinated fibres (Jaggi and Singh, 2012). The cancer pain type of neuropathic pain is not sensitive to opioid due to down regulation of mu-opioid receptors in dorsal spinal cord, mediated by the activation of NMDA receptors and protein kinase A (Mizoguchi *et al*., 2009). MAE and fractions then could not have acted on opioidergic receptors to inhibit pain in this model. Enhanced expression and action of α_2-δ_1 Ca^{2+} channels, a sub-unit form of Ntype voltage-dependent Ca^{2+} channel, and voltage-dependent Na^{+} channel as well as increased in expression of α_2 adrenergic receptors on neuronal terminals is also reported in neuropathic pain conditions (Yajima *et al*., 2005; Zamponi *et al*., 2009). From the foregoing, MAE and fractions could have inhibited NMDA receptors and protein kinase A thereby suppressing the development of hyperalgesia and allodynia in neuropathic pain conditions.

The present study has also demonstrated that MAE and fractions are effective in suppression of withdrawal syndrome of morphine dependence. Withdrawal from acute

morphine dependence is accompanied by centrally mediated side effects, such as physical dependence. Some neurotransmitters, including adenosine and glutamate have been associated in the expression of opioid withdrawal (Roome *et al*., 2011). Treatment of morphine dependence and withdrawal syndrome is limited to opiate replacement therapy and symptomatic treatment of withdrawal signs, but from this study and several similar studies (Doosti *et al*., 2013; Tabatabai *et al*., 2014), herbal treatment such as MAE may be a rational option for the treatment of morphine dependence and withdrawal.

The analgesic action of MAE was mediated by stimulation of the ATP-sensitive K^+ channels, adenosinergic, muscarinic and opioid pathways/receptors. The analgesic effects of fractions involved the adenosine, 5 HT_3, NO-cGMP, ATP-sensitive K^+ channels, muscarinic, opioid and α_2 adrenergic receptors/pathways. The extract and fractions furthermore produced antinociception by inhibiting EP, B1/B2, βadrenergic receptors-cAMP, $TRPV_1$ and glutamate receptors/pathways. The involvement of protein kinase A and C pathways are very likely in the antinociception of MAE and fractions since opioidergic, muscarinic, adrenergic and adenosinergic receptors are coupled to these pathways.

The antinociceptive activity of MAE and fractions could be as a result of flavonoids, oils and fats including fatty acid and fatty acid esters among other constituents as shown in this study. Flavonoids have been demonstrated to possess potent analgesic activities (Ching and Faloduna, 2011; Meotti *et al*., 2006). Besides, flavonoids potently inhibit prostaglandins, which are pro-inflammatory signalling molecules.

Flavonoids also inhibits phosphodiesterase known to be involved in cell activation (Kumar *et al*., 2013). Fatty acid and fatty acid esters have also been isolated in some

plants such as *Celtis australis*, *Alstonia scholaris* and *Mangifera indica* (Arulmozhi *et al*., 2012; Garrido *et al*., 2004; Semwal and Semwal, 2012) and were reported to possess analgesic, anti-inflammatory, antipyretic, antiulcerogenic, anticancer, antihypertensive, antibacterial, antiviral activities and also associated with lowered LDL cholesterol and increased HDL cholesterol (Hui *et al*., 2009; Hunter *et al*., 2010; Khalil *et al*., 2000; Martin-Moreno *et al*., 1994; Terés *et al*., 2008) indicating their numerous pharmacological properties. All these suggest that fatty acids and fatty acid esters from some medicinal plants including *Maerua angolensis* can be used as analgesic agents.

The present study was able to establish that the analgesic action of fatty acid and fatty acid esters obtained from MAE involve 5-HT_3, $TRPV_1$ and opioid receptors.

Spinal 5-HT_3 receptors mediate antinociception, probably through GABA release (Inocêncio Leite *et al*., 2014). $TRPV_1$ receptor also known as capsaicin receptor is a ligand-gated non-selective cation channel present in primary sensory neurons which is activated by capsaicin resulting in increased synaptic release of glutamate, neuropeptides, excitatory amino acids, nitric oxide and pro-inflammatory mediators from the periphery, transmitting nociceptive information to the spinal cord or causing spinal sensitization through protein kinase A and C activation (Calixto *et al*., 2005; Meotti *et al*., 2006; Woode *et al*., 2013). The analgesic effect of the fatty acid and fatty acid esters may therefore also involve the inhibition of production or action of some of these mediators in addition to the direct interaction with the $TRPV_1$. $TRPV_1$ antagonism additionally has been suggested to suppress pain evoked prostaglandin metabolites without the adverse effects of inhibiting cyclooxygenases (Materazzi *et al*., 2008). Additionally, activation of opioid receptors have been shown to also inhibit

the activity of $TRPV_1$ via $G_{0/i}$ proteins and the cAMP pathway (EndresBecker *et al*., 2007).

It is on record that fatty acids and fatty acid esters isolated from some plants have analgesic and anticancer activities (Khalil *et al*., 2006; Martin_Moreno *et al.*, 1994). Since cancer patients receiving various treatments experience pain from the malignancy itself and pain due to the treatments of the cancer and the current analgesics are unable to treat cancer chemotherapy-induced neuropathic pain which is severe enough for patients to discontinue their cancer chemotherapy treatment and worsens the quality of life (Lynch *et al*., 2005; Park *et al*., 2012; Wolf *et al*., 2008) but MAE and fractions were effective in this type of pain. It is likely that MAE and fractions may possess anticancer properties. MAE and fractions could therefore be another source of anticancer agent as well as being useful in neuropathic pain linked with anticancer therapy. Furthermore, the effectiveness of fatty acid and fatty acid esters isolated from MAE in both writhing and wiping tests indicates their peripheral and central analgesic action as well as their usefulness in visceral and trigeminal acute pains. The management of trigeminal acute pains for example headache, dental problems, muscle spasms, corneal ulcers or post-surgery pain with the current analgesics remains a cause for concern. Safe, long lasting pain relief with current analgesics following trigeminal acute pains is lacking but from the present study, the fatty acid and fatty acid esters from MAE seems to provide a solution to this problem.

The present study also established that MAE administered orally is relatively nontoxic in rats at 300 mg/kg dose. An important aspect of research into new drugs including analgesics is to develop analgesics with less toxic and lethal effects so as not to limit their clinical use.

Gastric irritation is a major side effect of NSAIDs, whereas the frequent use of opioids causes physical dependence and tolerance

(Walder *et al*., 2001). Others are renal and related cardiovascular effects due to both NSAIDs and non-NSAIDs analgesics (Whelton, 2000). In this study, the histology of the livers after sub-acute toxicity studies did not reveal significant difference between control and MAE-treated rats up to 300 mg/kg but there were overt effects (distortion of radial arrangement of hepatocytes and massive enlargement of nuclei within the hepatocytes) on the livers at 1000 - 3000 mg/kg. However, no significant changes in serum biochemical parameters such as AST, ALT, ALP, total bilirubin, direct bilirubin, albumin and total protein which are specific markers for liver function were noted in rats treated at extract doses up to 3000 mg/kg. It can therefore be inferred that MAE is relatively non-toxic at 300 mg/kg dose but has the potential to cause toxicity at high dose levels so should be used with caution. Additionally, because these effects cannot be directly extrapolated to human beings, it could well occur in them even at lower doses. Further studies to show the toxicity profile of MAE on the liver is therefore recommended including chronic toxicity studies in different animal species so as to be able to ascertain whether MAE has some advantages over current analgesics especially as regards to hepatic toxicity.

8.2 CONCLUSION

It can be concluded that the present study bestowed Pharmacological data to buttress the use of the leaf, root and/or stem bark of *Maerua angolensis* as an analgesic. In addition it demonstrated that the analgesic outcome of the stem bark, the most potent plant part was to some extent owing to the existence of fatty acid and fatty acid esters. MAE and fractions inhibited withdrawal syndrome of morphine dependence mediated by stimulation of GABAergic and adenosinergic systems. Extract and fractions also

blocked vincristine-induced neuropathic pain by improving mechanical hyperalgesia as well as tactile and cold allodynia. The extract, fractions, fatty acid and the fatty acid esters exhibited peripheral and central antinociceptive activity in various animal models of nociception. Furthermore, MAE is relatively non-toxic in rats at therapeutic dose. The analgesic effect of MAE and fractions was mediated by:

- Exciting adenosinergic, muscarinic, α_2 adrenergic, 5-HT_3, opiodergic receptors, nitric oxide-cyclic GMP, ATP sensitive K^+ channels pathways
- Preventing EP, B1/B2, $TRPV_1$, glutamate, β-adrenergic receptors-cAMPprotein kinase A and C pathways.

8.3 RECOMMENDATIONS

The following are being recommended so as to improve on the analgesic knowledge of this species:

- Chronic toxicity studies in rodents and other animal species to further assess the long term safety and toxicity of MAE
- Hepatoprotective effects of MAE
- Binding studies to elucidate receptor subtypes the compounds act upon
- Western blotting or ELISA to reveal the direct inhibition of PKA and PKC in addition to possible inhibition of COX-1 and 2
- Isobolographic analysis of the isolated compounds and other clinically used analgesics to increase efficacy
- Further antinociceptive study of MAE and the compounds should be conducted in primates so as to obtain relevant scientific data before being used in human beings Neurotoxicity assays.

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APPENDICES

Appendix 1: Some Pharmacological Methods

Drug preparation and administration

Suspension of the extracts, fractions and compounds were separately prepared by weighing the quantity needed followed by the addition of 2% Tween 40 and triturated. Normal saline was then gradually added while triturating to produce the required volume. Other drugs were prepared by diluting the stock with normal saline. All drug concentrations were prepared such that the required dose was always administered in equivalent volumes not exceeding a total volume of 1 ml for oral and
0.5 ml for intraperitoneal route except in the toxicity studies.

Preparation of phosphate buffer saline

One tablet of phosphate buffer saline was dissolved in 400 ml of distilled water to obtain 1 M sodium phosphate buffer, pH 7.4.

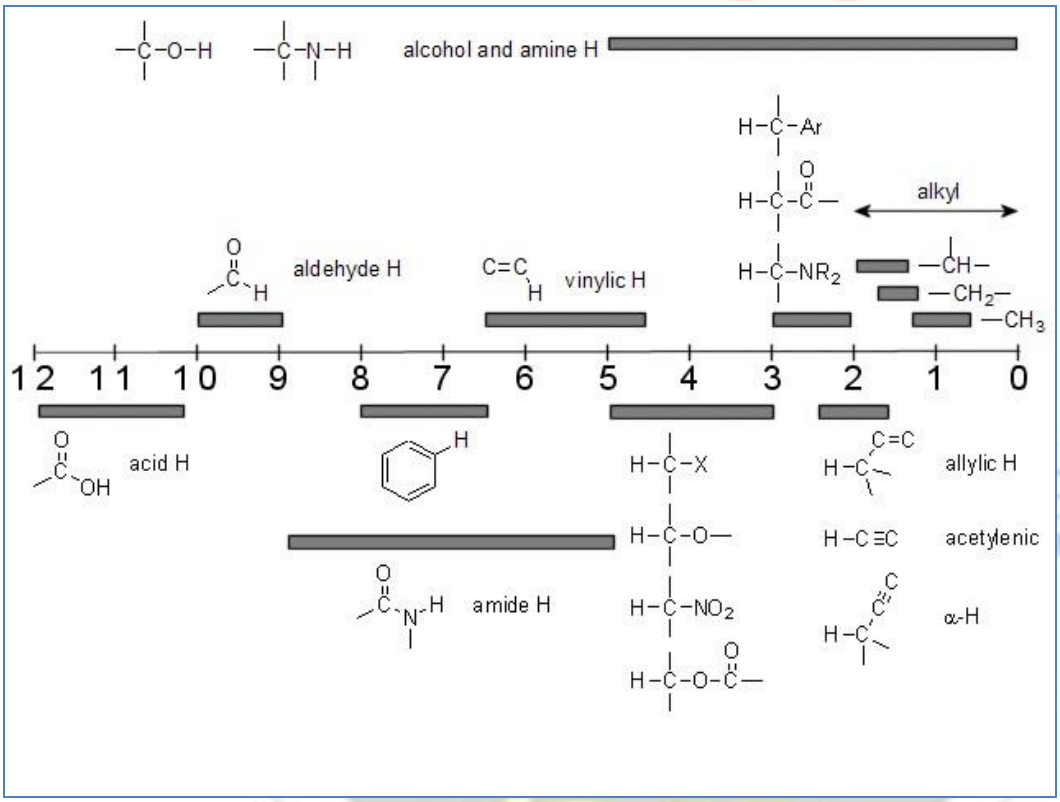
Preparation of stock solution of bradykinin

A stock solution of 1 M bradykinin was prepared by dissolving 0.5 mg of bradykinin in 5 ml of PBS and stored at -20 °C. Aliquot of 0.5ml of this solution was taken and diluted to 23 ml to obtain 1 nM/0.02 ml solution.

Preparation of hypertonic saline

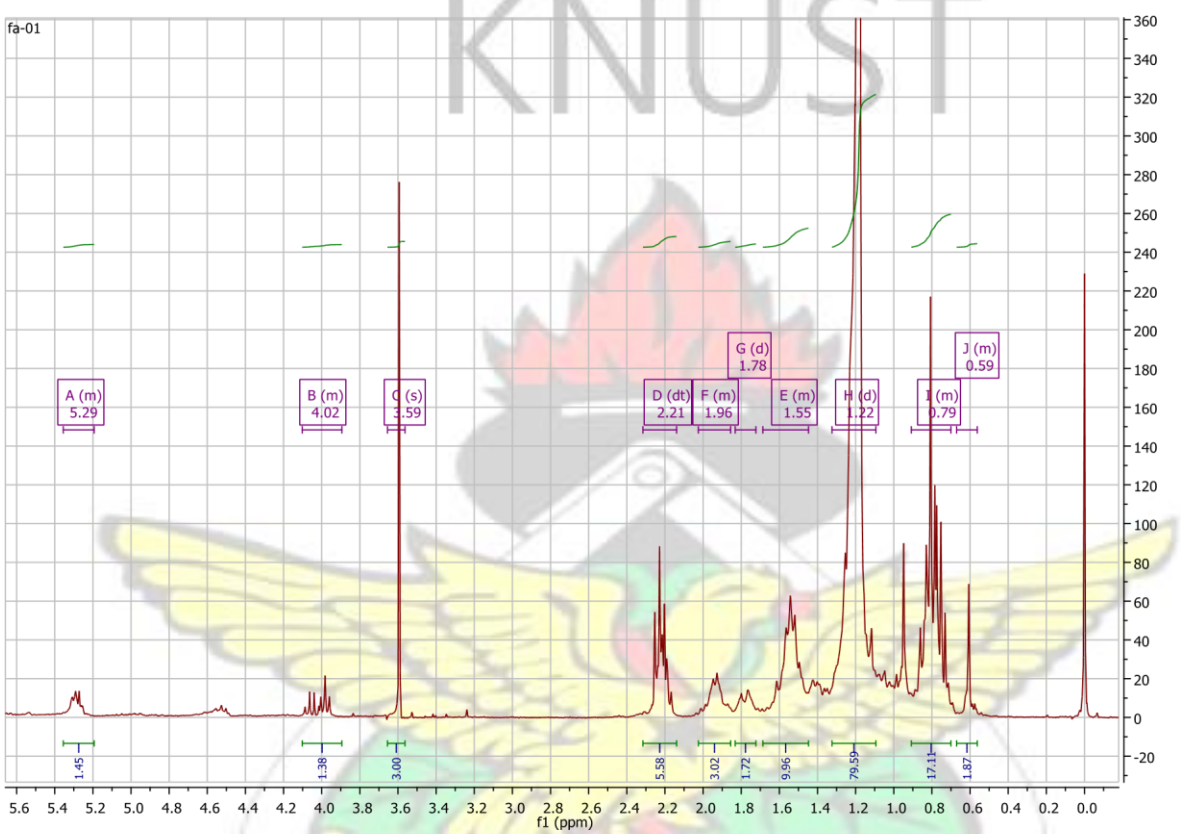
Hypertonic saline (5 M NaCl solution) was prepared by dissolving 2.92 g of NaCl in 10 ml distilled water and stored in a refrigerator.

Appendix 2: Hydrogen NMR of the Unknown Compounds Hydrogen chemical shift template



Procedure: Each (5-10 mg) sample was dissolved in 500 ul deuterated chloroform ($CDCl_3$) (7.2 ppm signal) and placed in a clean 3mm NMR tube. Compounds were analyzed using NMR spectra obtained on a Varian Mercury 300 (300 MHz). Chemical shifts (□) are given in ppm relative to the signal for the deuterated solvent and are reported consecutively as position (dH), relative integral, multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, dd = doublet of doublets, m = multiplet and where br = broad), coupling constant (J/Hz) and assignment.

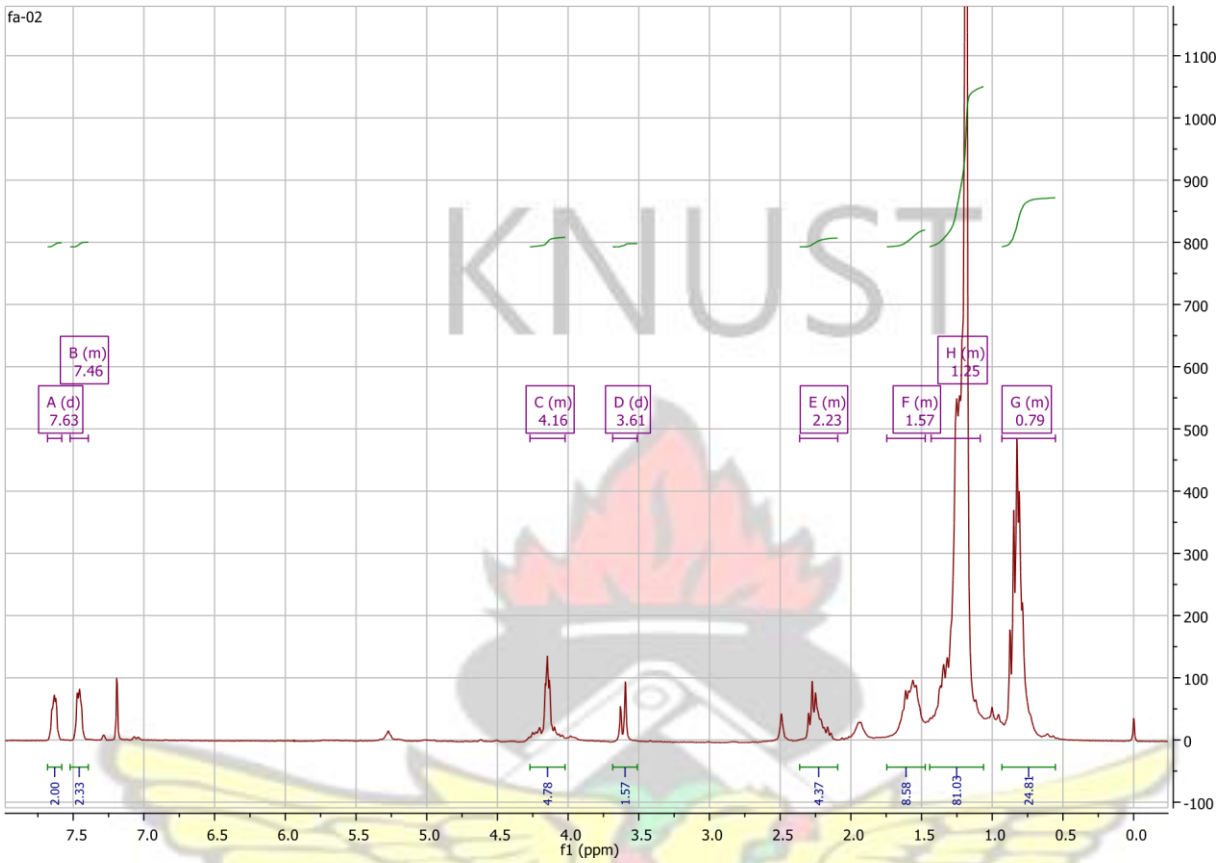
Sample 1 spectrum



δ_H (300 MHz, $cdcl_3$) 5.36 – 5.19 (1 H, m), 4.10 – 3.89 (1 H, m), 3.59 (3 H, s), 2.21 (6 H, dt, *J* 10.8, 7.3), 2.02 – 1.86 (3 H, m), 1.78 (2 H, d, *J* 9.8), 1.69 – 1.45 (10 H, m), 1.22 (80 H, d, *J* 21.6), 0.91 – 0.70 (17 H, m), 0.67 – 0.56 (2 H, m).

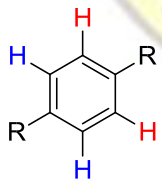
Notes: Peak C (at 3.6 ppm) is a common ester peak. Since it is a singlet, it is speculate to be a methyl ester ($-OCH_3$). This peak was used to integrate the remaining signals.

Sample 2 spectrum



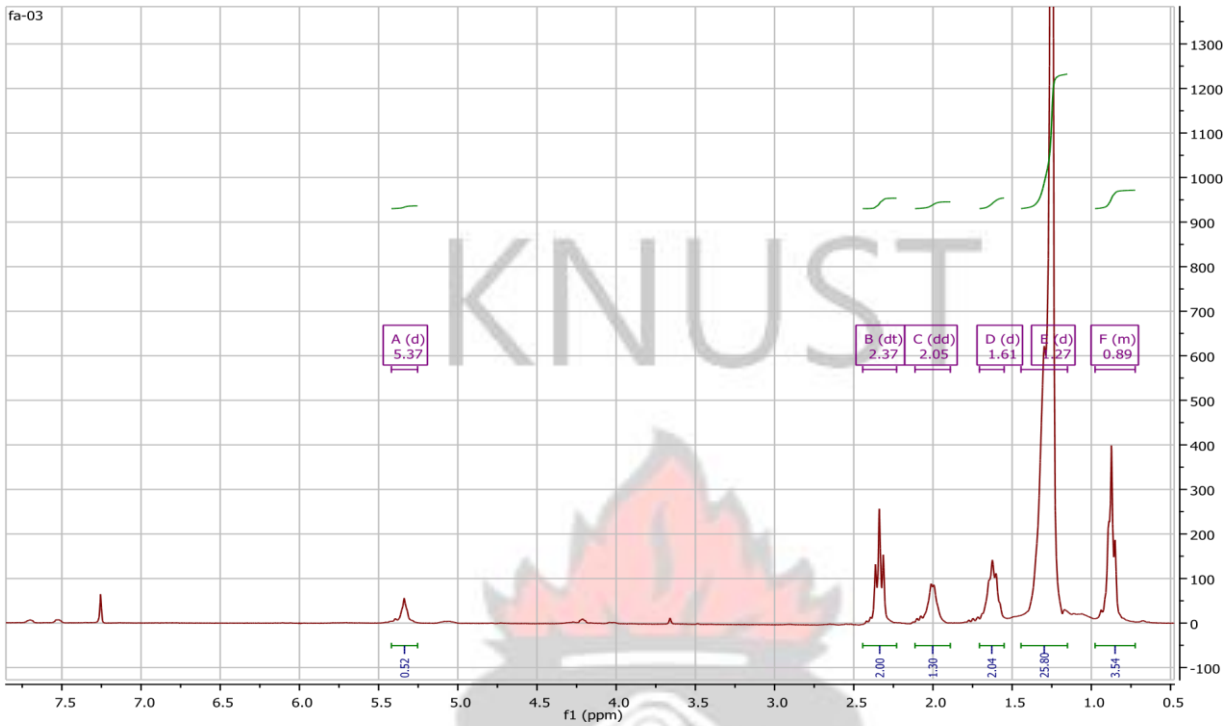
^{1}H NMR (300 MHz, cdcl$_3$) δ = 7.63 (d, *J*=3.5, 2H), 7.52 – 7.39 (m, 2H), 4.27 – 4.02 (m, 5H), 3.61 (d, *J*=10.7, 2H), 2.36 – 2.09 (m, 4H), 1.75 – 1.47 (m, 9H), 1.43 – 1.08 (m, 40H), 0.93 – 0.55 (m, 25H).

Notes: Compound 2 has 2 peaks at the 7-8 range, which is a solid indicator of aromatic hydrogen. So, integrated spectra based on the 1, 4- di substituted benzene



ring, resulting in two different hydrogens.

Sample 3 spectrum

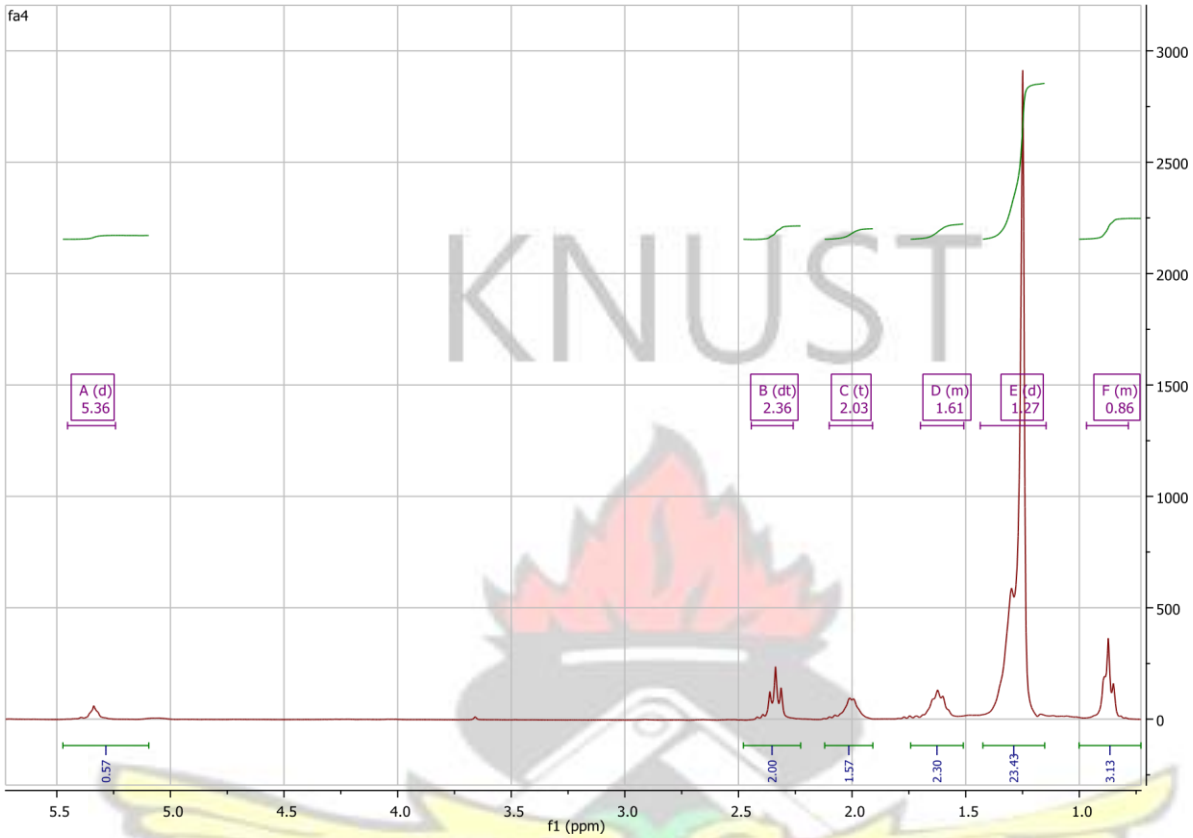


δ_H (300 MHz, $cdcl_3$) 5.37 (2 H, d, *J* 16.8), 2.37 (8 H, dt, *J* 14.9, 7.5), 2.05 (5 H, dd, *J* 25.5, 6.0), 1.61 (8 H, d, *J* 6.7), 1.27 (102 H, d, *J* 14.4), 0.98 – 0.72 (14 H, m).

Note: While simple, this was difficult to speculate which peak to use for normalization. Signal B was chosen and assumed that it represented 2 hydrogen.

This could be analyzed differently.

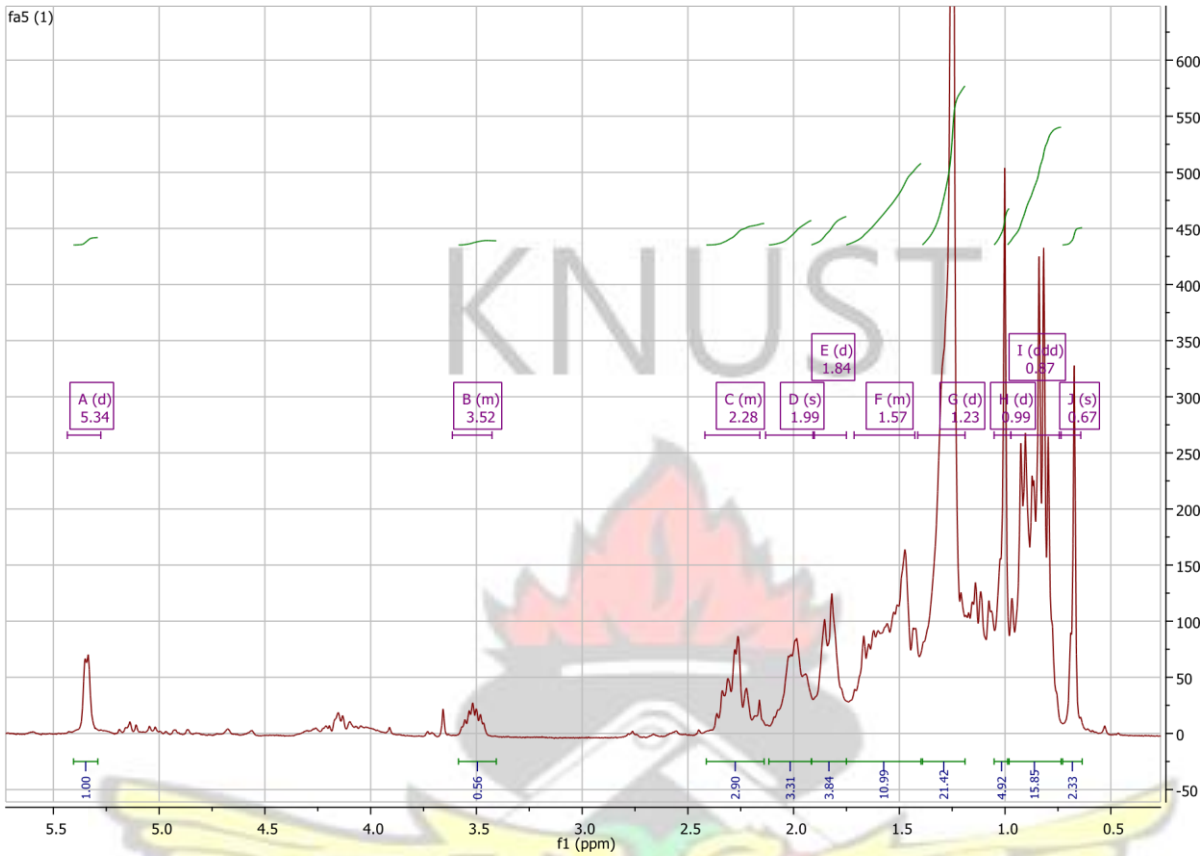
Sample 4 spectrum



δ_H (300 MHz, cdcl$_3$) 5.36 (2 H, d, *J* 16.6), 2.36 (7 H, dt, *J* 14.9, 7.5), 2.03 (5 H, t, *J* 12.1), 1.70 – 1.51 (8 H, m), 1.27 (81 H, d, *J* 14.8), 0.97 – 0.78 (11 H, m).

Note: Compounds 3 and 4 are alike. It was assumed that they are the same compounds (their spectra are identical). This spectra was normalized the same way as done above.

Sample 5 spectrum



δ_H (300 MHz, cdcl$_3$) 5.34 (1 H, d, *J* 4.3), 3.61 – 3.43 (1 H, m), 2.42 – 2.16 (3 H, m), 1.99 (3 H, s), 1.84 (4 H, d, *J* 10.2), 1.71 – 1.43 (9 H, m), 1.23 (21 H, d, *J* 11.7), 0.99 (5 H, d, *J* 8.7), 0.87 (15 H, ddd, *J* 20.5, 16.0, 9.6), 0.67 (2 H, s).