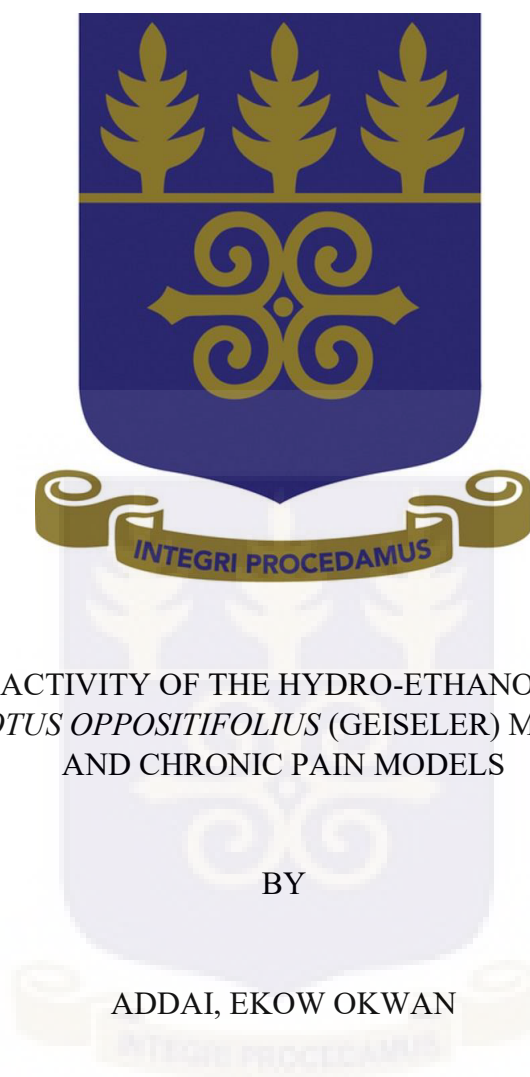


UNIVERSITY OF GHANA
COLLEGE OF HEALTH SCIENCES



ANTINOCICEPTIVE ACTIVITY OF THE HYDRO-ETHANOLIC EXTRACT OF THE
LEAVES OF *MALLOTUS OPPOSITIFOLIUS* (GEISELER) MULL. ARG. IN ACUTE
AND CHRONIC PAIN MODELS

BY

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A THESIS SUBMITTED TO THE SCHOOL OF GRADUATE STUDIES IN PARTIAL
FULFILLMENT OF THE AWARD OF MASTER OF PHILOSOPHY DEGREE IN
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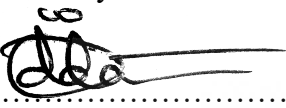
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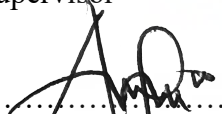
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I dedicate this work to the Almighty God.



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LIST OF ABBREVIATIONS

MOE	<i>Mallotus oppositifolius</i> extract
5-HT₃	5-Hydroxytryptamine
ATP	Adenosine Triphosphate
CNS	Central Nervous System
COX	Cyclooxygenase
DPX	Dibutyl phthalate Polystyrene Xylene
ED₅₀	Effective Dose at 50%
EDTA	Ethylenediamine Tetraacetic Acid
GABA	Gama Amino Butyric Acid
IASP	International Association for the Study of Pain
ICR	Institute of Cancer Research
NMDA	N-methyl-D-aspartate
NPC	National Pharmaceutical Council
NSAIDs	Non -steroidal Anti- inflammatory drugs
PAG	Periaqueductal Gray
SNRI	Serotonin-Norepinephrine Reuptake Inhibitor
SSRI	Selective Serotonin Reuptake Inhibitor
TCA	Tricyclic Antidepressants
TNF	Tumour necrosis factor

ABSTRACT

BACKGROUND: *Mallotus oppositifolius* is a medicinal plant commonly used in folk medicine to treat disorders involving pain and inflammation, among other ailments. However, the potential of this herb for the treatment of pain has only rudimentary scientific validation. The study sought to investigate the analgesic activity of the hydro-ethanolic extract of the leaves of *M. oppositifolius* (MOE) in order to provide detailed scientific justification for its traditional folkloric use.

METHODS: Preliminary phytochemical screening was conducted on the MOE. Antinociceptive activity of MOE (30, 100 and 300 mg/kg) in acute pain models was evaluated using the formalin test, the acetic acid-induced writhing assay and the hot plate test. The mechanism of antinociception was evaluated by employing various antagonists in the formalin test including naloxone (2 mg/kg), glibenclamide (8 mg/kg), theophylline (10 mg/kg), ondansetron (0.5 mg/kg), yohimbine (3 mg/kg), nifedipine (10 mg/kg) and atropine (5 mg/kg). For the chronic pain model, peripheral neuropathy was induced in ICR mice using paclitaxel (2 mg/kg, *i.p.*) for 5 days and the effect of the extract on paclitaxel-induced peripheral neuropathy was evaluated daily for 5 days post induction using the Randall-Selitto test for mechanical hyperalgesia, hot plate test for thermal hyperalgesia and cold water at 4°C for cold allodynia with pregabalin (10, 30 and 100 mg/kg) as the positive control. Sub-chronic toxicity study was carried out with male Sprague-Dawley rats divided into four groups; group 1 received vehicle *p.o.* while groups 2-4 were orally treated with MOE daily at 30, 100 and 300 mg/kg for 90 days consecutively. At the end of the test mice were sacrificed for their organs for histopathological analysis and their whole blood and serum for haematological and biochemical analysis respectively.

RESULTS: Phytochemical screening confirmed the presence of secondary metabolites including saponins, alkaloids, flavonoids, glycosides, sterols, tannins and reducing sugars. MOE together with morphine and diclofenac (positive controls) showed significant ($P<0.05$) and dose-dependent decrease in the nociceptive scores in the formalin test, the acetic acid induced writhing assay and an increase in antinociceptive scores in the hot plate test. Naloxone, theophylline, ondansetron, glibenclamide, atropine, yohimbine and nifedipine failed to block the antinociceptive effects of MOE (100 mg/kg) in the formalin test. The extract significantly ($P<0.05$) and dose-dependently increased the paw withdrawal latencies to mechanical and thermal hyperalgesia after the 5-day induction of neuropathy with paclitaxel in mice. In the 90-day sub-chronic toxicity study, data analysis of mortality, body weight gain, clinical observations, haematology, biochemistry, organ weights and histopathological findings did not show significant differences between control and treated groups.

CONCLUSION: The oral administration of a hydro-ethanolic leaf extract of *Mallotus oppositifolius* produces dose-dependent antinociceptive effects in formalin, acetic acid-induced and thermal acute pain tests, and also significantly inhibited mechanical and thermal hyperalgesia in paclitaxel-induced peripheral neuropathy in mice. There was no extract-treatment related toxicity in rats following a 90-day daily oral administration. The findings support the traditional uses of the plant in alleviating pain.

Chapter One

INTRODUCTION

1.1. Background

Pain is defined by the International Association for the Study of Pain (IASP) as an unpleasant sensory and emotional experience associated with actual or potential tissue damage (Merskey *et al.*, 1994). In fact, a study by Baigi and Stewart (2015) indicated that pain is a major reason for underemployment, absenteeism from work and unemployment, therefore, causing financial loss to individuals and countries. Pain is a consequence of many diseases and is among the main reasons why several people seek medical care (American Pain Society, 2000). There have been substantial advances in the management of pain with the introduction of new drugs through research in the last decade. However, many people continue to suffer from pain due to the fact that current analgesics are used inappropriately (Chen *et al.*, 2012; Moore, 2013) and they are fraught with many side effect and low therapeutic efficacy (Boakye-Gyasi *et al.*, 2017; Rajdev *et al.*, 2018).

In many countries, the use of medicinal plants for the management of pain is common (Kukuia *et al.*, 2016). In Ghana, the traditional use of plants for the treatment and management of pain and other diseases such as epilepsy, malaria, fever, and depression is widespread (Agyare *et al.*, 2014; Bremer Christensen *et al.*, 2015; Kukuia *et al.*, 2016). About 25 percent of drugs currently in use are derived from plants (Wachtel-Galor & Benzie, 2011). Among the extensively used plants for medicinal purposes is *Mallotus oppositifolius*, a dioecious shrub that is extensively distributed across Africa in countries including Cameroon, Senegal, Angola, Ethiopia, Mozambique, Madagascar, Ghana and Nigeria (Farombi *et al.*, 2001; Farombi, 2003). In Ghana, it is called ‘sroti’ by the Ewes and ‘sratadua’ by the Akans (Burkill *et al.*, 1985). In West Africa, the leaves are chewed or crushed and placed on cuts and sores for antibacterial

effect and wound healing. Crushed leaves are also commonly applied to burns for analgesic effect (Adekunle & Ikumapayi, 2006; Agyare *et al.*, 2014). The leaves are used for many conditions including worm infestation, diarrhoea, malaria, epilepsy and pain among others (Farombi, 2003; Adekunle & Ikumapayi, 2006). Very little work has been done on the analgesic effect of *M. oppositifolius*. Documenting the traditional knowledge and scientifically authenticating traditional medicinal plants is a very important path (Uprety *et al.*, 2010). In the work done by Farombi *et al.* (2001), phytochemicals such as the alkaloids, anthraquinones, phenols, cardenolides and flavonoids were observed to be present through phytochemical screening. Gbedema *et al.* (2010) also reported that the leaf extract of *M. oppositifolius* exhibited significant antimicrobial activity against *Bacillus subtilis*, *Pseudomonas aeruginosa* and *Staphylococcus aureus*.

Despite the many traditional indications of *M. oppositifolius*, this research will focus on scientifically validating its folkloric use as an analgesic in traditional medicinal practice in Ghana.

1.2. Problem Statement

Pain can have very detrimental effects on the quality of life of an individual. It can disrupt normal living, normal sleep, functional capability and can degrade or deteriorate an individual's health (Elliott *et al.*, 1999). In creating more efficacious medications for pain management, the complexity of the disease condition (e.g. diabetes, cancer) from which the pain arises can be a challenge (Jarvis & Boyce-Rustay, 2009). Even though pain medication, which includes opioids and non-opioids, is widespread, pain management in patients with mild to severe pain in many countries is still ineffective (Boakye-Gyasi *et al.*, 2017). This is frequently attributed to both an improper use of analgesics and the unavailability of effective and efficacious analgesics contributing to an increase in the cost of pain management (Boakye-

Gyasi *et al.*, 2017). It has been shown that current analgesics are ineffective in treating chemotherapy-induced peripheral neuropathy which develops in about 30 to 40% of cancer patients (Deng *et al.*, 2012; Amoateng *et al.*, 2015). This often leads to the discontinuation of their cancer chemotherapy (Deng *et al.*, 2012).

Current optimized pain management strategies in individuals/patients, especially those having complicated neuropathic pain, include combining analgesics from several drug classes such as anticonvulsants (pregabalin, gabapentin and carbamazepine), tricyclic antidepressants (amitriptyline) and analgesics (fentanyl patches and tramadol) to address multiple pain mechanisms (Amoateng *et al.*, 2015; Amoateng *et al.*, 2017). This presents a number of severe side effects (emesis, gastrointestinal problems, renal damage, addiction) and limited efficacy in pain management which in the end impedes effective symptom control (van den Bent, 2005). Only about 40 to 60% of patients being given current conventional treatments, which includes opioid analgesics, tricyclic antidepressants, and anticonvulsants, achieve partial pain relief (Dworkin *et al.*, 2007; Dworkin *et al.*, 2010; Amoateng *et al.*, 2017). Therefore, analgesics that are cost-effective, efficacious, easy to access and have fewer side effects are needed. Natural medicinal plants could be very important sources of novel analgesics.

1.3. Justification

Although pharmacological management of pain remains an important therapeutic option, current pain treatment is far from perfect and is riddled with considerable side effects (Woode & Abotsi, 2011; Amoateng *et al.*, 2015). In addition, many patients battling with pain are not satisfied, prompting the need for research into finding new and promising therapeutic agents with minimum adverse effects and an improved efficacy to treat/manage pain. Plants used in traditional medicine are alternative sources of drug discovery (Kukuia *et al.*, 2018), from which we can obtain lead compounds and commercial drugs (Iliya *et al.*, 2016). Many breakthrough

drugs currently in use were discovered from plant sources. Examples are morphine, which was isolated from *Papaver somniferum* and is used clinically for the management of pain, and vincristine, which was isolated from *Vinca roseus* and is used as an anticancer agent (Staerk *et al.*, 2002; Norn *et al.*, 2005).

Also, the use of plant sources by traditional healers as drugs in disease management brandishes these sources as a strong potential for drug discovery and pharmacological management (Balunas & Kinghorn, 2005; Abotsi *et al.*, 2017). This makes the assessment of medicinal plants as alternative or complementary therapies an important strategy.

Mallotus oppositifolius (family Euphorbiaceae) is an example of such plants used traditionally in managing pain and a spectrum of other diseases including epilepsy and malaria (Woode *et al.*, 2012). The genus *Mallotus* is a widely known rich source of natural bioactive compounds (Kabran *et al.*, 2015). Previous preliminary screening of central effects of the hydroethanolic leaf extract of *Mallotus oppositifolius* revealed that it possessed significant antinociceptive effect in a tail immersion test model in mice (Woode *et al.* (2012). Literature search reveals that aside the aforementioned study, there are no extensive scientific reports that support either the short term or long-term use of the leaves of *M. oppositifolius* traditionally in the management of pain as an effective therapy. Against this backdrop, this study seeks to scientifically validate the pharmacological activity of *Mallotus oppositifolius* leaf extract in the management of pain, investigate to provide information on its long-term-usage effects and elucidate its possible mechanism(s) of action. This will go a long way to aid in our pursuit of discovering alternative drugs that are more effective and safer for the management of pain.

1.4. Hypothesis

Being traditionally used in the management of pain, the hydro-ethanolic extract of *M. oppositifolius* will demonstrate an analgesic effect in the experiments to be conducted.

1.5. Aim of Study

The study seeks to investigate an antinociceptive effect of the hydroethanolic leaf extract of *M. oppositifolius* in acute and chronic models of pain, elucidate the possible mechanism(s) of action and conduct a sub-chronic oral toxicity of the leaf extract.

1.6. Specific Objectives

1. To qualitatively assess the phytochemicals, present in *M. oppositifolius* extract.
2. To assess the antinociceptive properties of the leaf extract using animal models for nociceptive pain (acute) and a peripheral neuropathy model (chronic).
3. To elucidate the possible mechanisms through which the leaf extract exerts its analgesic effect.
4. To evaluate the 90-day sub-chronic oral toxicity of the leaf extract.

Chapter Two

LITERATURE REVIEW

2.1. Pain: General Overview

Pain is a sensory experience associated with hurting and soreness, and is intrinsically unpleasant (Cimmino *et al.*, 2011). The sensation of pain is mostly evoked by a potential noxious stimulus or an actual noxious stimulus applied to the body (Schaible, 2007). Pain has also been reported in patients suffering from nerve injury or even brain injury (Rang *et al.*, 2016). Under normal circumstances, the degree of pain felt is directly linked to the noxious stimuli and this causes the sufferer to evade situations and behaviours that are known to evoke pain. Pain is essentially a sensation but is often described in terms of suffering, associated with alterations in autonomic output and avoidance motor reflexes (Woolf, 2004). This means that the perception of pain and its thresholds result from complex interactions between emotional, behavioural and sensory factors (Cole, 2002). Also, the pain experienced by a person is subjective and is modulated by genetic factors as well as other factors such as mood, beliefs and cognition (e.g., fear, distraction, anxiety and catastrophizing) making it expedient to tailor the management of pain to a particular patient (Marchand, 2008; Nix, 2017; Zortea *et al.*, 2018).

Pain that is experienced as a result of a disease is dissimilar to “normal pain”, since this usually occurs without an external noxious stimulus (Schaible, 2007). This is why it is defined by the IASP as an unpleasant sensory and emotional experience associated with actual or potential tissue damage. Their definition does not link pain exclusively to a tissue damage, noxious stimulus or a pathophysiological cause, as pain is a subjective experience that can occur without a noxious stimulus (Treede, 2018).

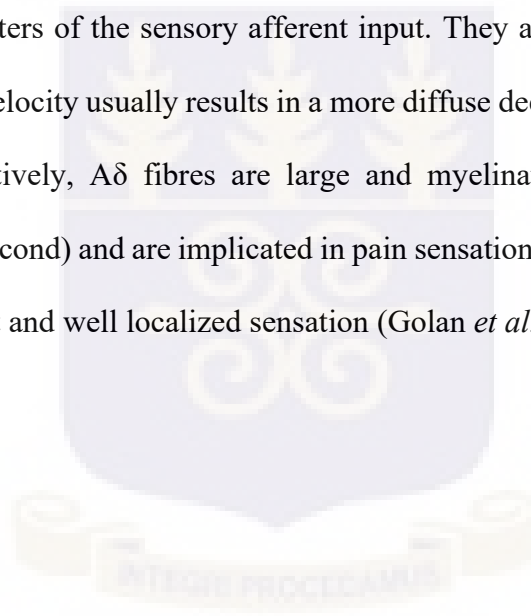
Pain is not always a bad thing. When a person experiences pain as a direct consequence of any activity they are performing, they usually halt the activity because they recognize the pain felt as a warning sign that harm is currently occurring. On the other hand, if the pain persists, the person's productivity plummets. Here, pain has ceased to provide the person with a useful signal since the pain-causing activity has long ceased. Based on these, pain can be described as either adaptive or maladaptive (Tan *et al.*, 2011). Adaptive pain, also referred to as helpful pain, contributes to the survival of an organism by producing behaviour or mechanisms that protect from damaging stimuli or promotes healing and recovery. It triggers the withdrawal reflex and heightens sensitivity after tissue damage to decrease the risk of further damage. Maladaptive pain has often been referred to as unhelpful pain as it can persist and remain a problem even without the noxious stimulus or after the site of injury is healed (Woolf, 2004; Marchand, 2008). It may develop in cases where changes in the nervous system causes it to operate abnormally and it may be accompanied by hyperalgesia or allodynia. Hyperalgesia is an increase in the response to noxious stimuli, whereas allodynia is when a person perceives pain from normally innocuous stimulus. These changes have been referred to central or peripheral sensitization, respectively (Mense & Gerwin, 2010).

Pain cannot easily be measured as it is a subjective experience (first-person perspective) that requires consciousness. This description of pain as an experience differentiates pain from nociception (Treede, 2018). Nociception can be measured with a number of methodologies including electrophysiological techniques (Fabrizi *et al.*, 2011; Johnson, 2016). It is a neural process that involves the transduction and processing of noxious stimulus to higher centres via certain pathways. Pain therefore results from an intricate interplay between certain signalling pathways, modulation of perception from the CNS, as well as the perception of the sufferer (Steeds, 2016). Nociception differs in many ways from pain (Cohen *et al.*, 2018). As pointed out earlier, pain is subjective and only exists in the individual. However, nociception is an

observable event that occurs in response to stimuli (Treede, 2018). There can be pain without nociception, and nociception without pain (NPC, 2001).

2.1.1. The Nociceptive System, Pain Pathways and Mechanisms

The skin, muscles, organs, ligaments, bones and joints are interlaced with a dense network of nerve fibres that register nociceptive events. Together, neurons that are involved in nociception collectively form the nociceptive system. These are the C-fibres and the $A\delta$ fibres, and sometimes the $A\beta$ fibres which play a dynamic inhibitory role when recruited (Marchand, 2008; Golan *et al.*, 2016). The C-fibres have a slow conduction velocity (0.5m–2m per second) and represent three-quarters of the sensory afferent input. They are unmyelinated and small. Their slow conduction velocity usually results in a more diffuse deep pain sensation when they are activated. Comparatively, $A\delta$ fibres are large and myelinated with faster conduction velocity (5m–30m per second) and are implicated in pain sensations that is described as a rapid pinprick, sharp, transient and well localized sensation (Golan *et al.*, 2016).



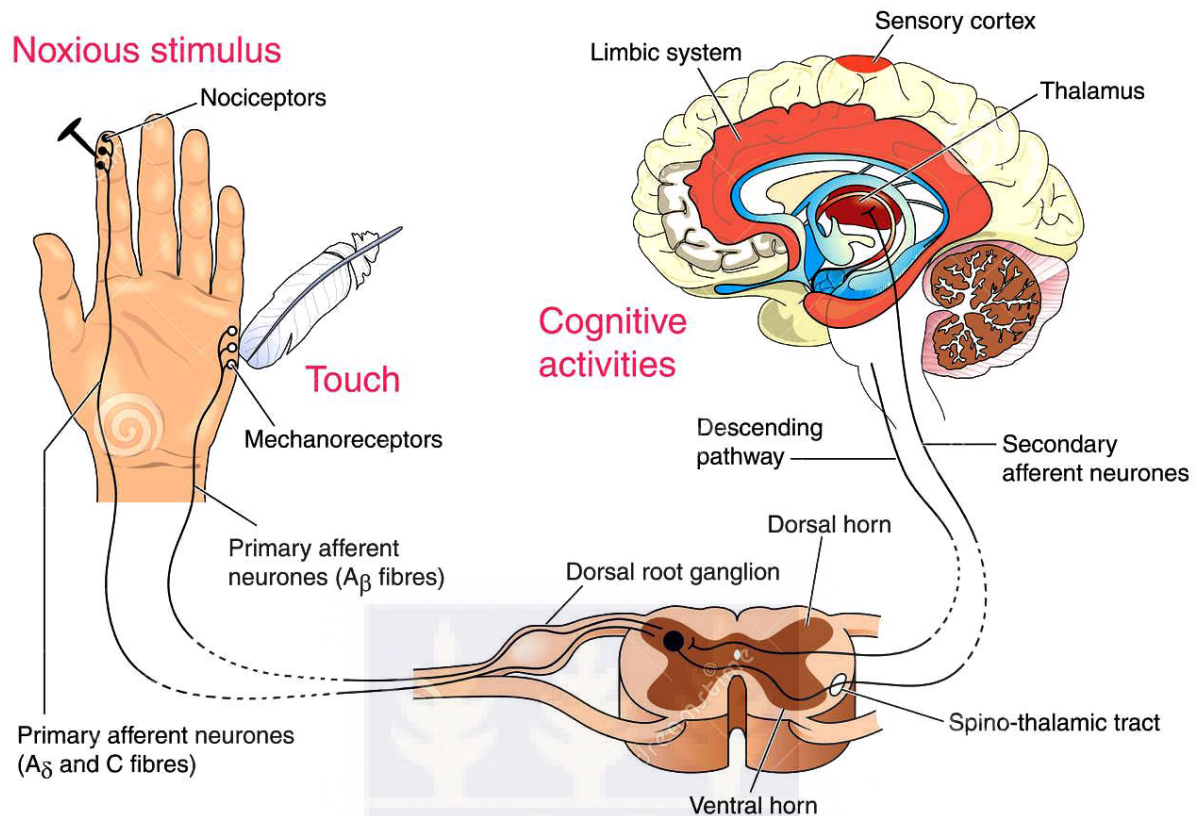


Figure 2.1. Overview of the pain pathway (dreamstime.com, 2019)

As the illustration in Figure 2.1 depicts, nociceptors or primary afferent neurons from the periphery synapse with certain 2nd order neurons and interneurons inside the dorsal horn. These second order neurons project into the thalamus, and by implication the brain, which is known to produce conscious pain responses upon stimulation by noxa. The secondary afferents travel to superior centres via two major routes: the spino-thalamic tract, that sends afferents to the thalamus, and the spino-reticular tract, which transmits stimuli to the medial thalamus, the nucleus raphe magnus and the periaqueductal grey area which are part of the descending pain modulation pathway. From the thalamus, the nociceptive signal is passed on to the somatosensory cortex among other CNS centres to produce the sensation of pain (Woolf, 2004; Marchand, 2008; Rang *et al.*, 2016).

Multiple mechanisms that produce pain exist and clinical pain syndromes could involve a combination of these mechanisms. The ideal way to treat pain would be to identify and target the precise pain mechanisms that are operative in a particular patient and devise a treatment plan aimed toward either reducing excitatory activity or enhancing inhibitory mechanisms (Woolf, 2004; Marchand, 2008). Some of these pain mechanisms include nociception, decreased inhibition, central and/or peripheral sensitization, phenotypic switches, ectopic excitability, etc. (Marchand, 2008; Rang *et al.*, 2016).

2.1.2. Phases of Nociceptive pain

The processes involved can be broken down into five different phases. These are transduction, conduction, transmission, modulation and perception (Benzon & Raj, 2008) (Figure 2.2). Transduction is defined as the conversion of noxious stimuli into electrical activity at the peripheral terminals of nociceptors via specialized proteins and voltage-gated receptors on the membranes of these primary afferents (NPC, 2001; Benzon & Raj, 2008). Chemical noxious stimuli may refer to those released by damaged cells including cholecystikinin, bradykinin and prostaglandins, which when released sensitize other nociceptors that are nearby (Schaible, 2007; Nix, 2017). This step can be inhibited by opioids, NSAIDS and local anaesthetics (Benzon & Raj, 2008).

Conduction involves the movement of an action potential from the peripheral terminals of a nociceptor down to its presynaptic terminal within the dorsal horn in the central nervous system at specific laminae (Boos & Aebi, 2008). This process depolarizes the presynaptic terminal. Transmission encompasses transfer and modulation at the synapses from one neuronal terminal to another (Boos & Aebi, 2008; Marchand, 2008; Nix, 2017). It begins when an action potential arrives at the presynaptic terminal of the nociceptor. C-fibres release glutamate, substance P and calcitonin at the terminal which activate the postsynaptic α -amino-3-hydroxy-5-methyl-4-

isoxazole propionic acid (AMPA) receptors, NK1 receptors and CGRP receptors respectively (Brooks & Tracey, 2005; Krebs *et al.*, 2017) (Figure 2.3). The result is the influx of ions which depolarize the interneurons and 2nd order neurons they synapse with. Depolarization results in the generation of an action potential which is then relayed through certain tracts to the brain stem, medulla or the hypothalamus (Brooks & Tracey, 2005). Both transmission and conduction can be modulated by the use of local anaesthetics, opioids and alpha-2-agonists.

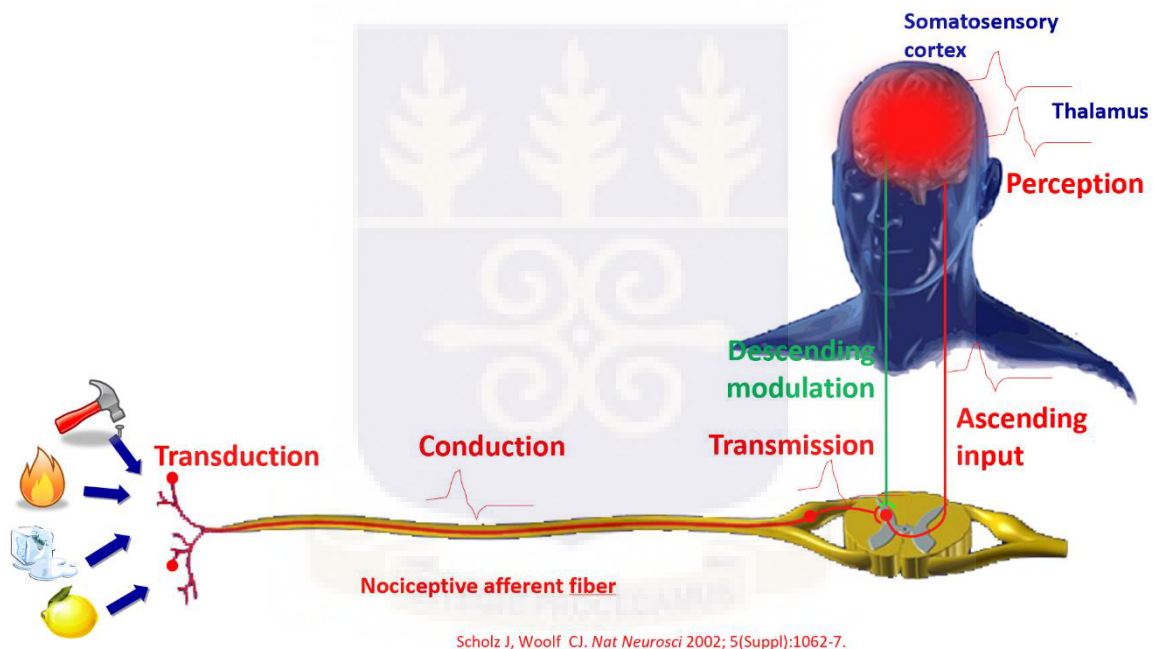


Figure 2.2. An illustration showing the various phases of the pain pathway

Modulation of nociceptive transmission has been classified as an adaptive process and involves both excitatory and inhibitory mechanisms (Schaible, 2007). When the brainstem is activated by nociceptive impulses, descending modulation occurs. In a physiologically normal state, responses of 2nd order neurons can naturally be facilitated or suppressed. This might be impaired in a diseased state. Modulation has been divided into peripheral and central based on

where it occurs along the pain pathway. Three of the ways peripheral modulation can be achieved are outlined below:

Inhibiting the terminals of nociceptors from being sensitized by using certain drugs (eg. COX inhibitors) (Rang *et al.*, 2016).

Inhibiting the production and transfer of action potentials in the axons. Local anaesthetics like lidocaine are commonly used (Benzon & Raj, 2008; Mense & Gerwin, 2010; Nix, 2017).

Stimulating the A β fibres can recruit inhibitory interneurons in substantia gelatinosa (Figure 2.3) to release glycine and GABA which prevent glutamate release from the afferent terminals. This prevents the depolarization of the 2nd order neuron. This phenomenon is known by many as the gate control theory (Marchand, 2008).

Two of the ways by which central modulation can be achieved are outlined below:

The use of opioids like morphine produce their analgesic effect by mimicking endogenous opioids and can activate the endorphin receptors: μ , κ and δ . The analgesia produced can be blocked by naloxone (Kieffer, 1999; Crain & Shen, 2007; Nix, 2017).

Antidepressants enhance the pain relief effect of the descending pain pathway when they increase the availability of the synaptic monoamines (Scholz & Woolf, 2002; Costigan *et al.*, 2009; Nix, 2017).

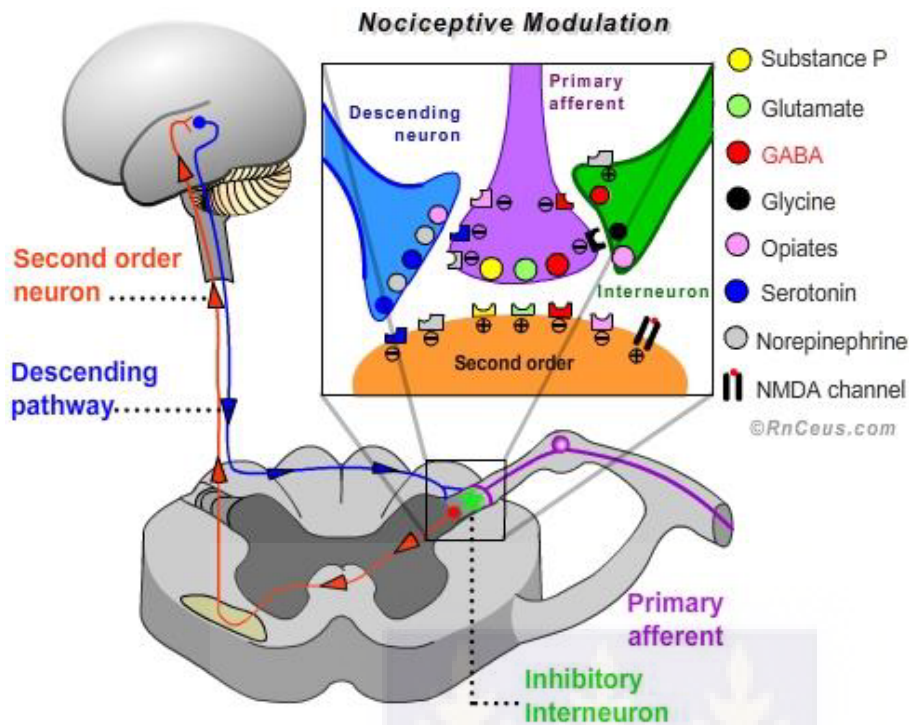


Figure 2.3. Illustration showing the substantia gelatinosa and the chemicals involved in transmission and modulation.

The perception phase is the stage where the event is recognised as pain by an individual (NPC, 2001). Both the cortical and limbic system structures are involved. The somatosensory cortex is somatotopically mapped to enable us identify the location and intensity of the noxious event occurring (NPC, 2001; Brooks & Tracey, 2005; Krebs *et al.*, 2017). Again, perception can be modulated with the use of opioids, alpha-2-agonists, TCAs, SNRIs and SSRIs.

2.1.3. Classification of Pain

Multiple classification systems for pain have been used. This includes intensity (severe, moderate and mild), duration (acute and chronic), physiology (nociceptive, neuropathic, inflammatory, (dys)functional), syndromes (cancer, migraine) and also the specific tissue involved (heart, tendons etc.), (Schaible, 2007; Marchand, 2008; Costigan *et al.*, 2009; Amoateng *et al.*, 2015; Treede *et al.*, 2015; Rang *et al.*, 2016; Nix, 2017). However, the most common classifications are based on duration and pain physiology.

2.1.3.1. Classification by Duration

Acute pain is known to arise from trauma to soft tissue or even inflammation. It has a sudden onset and a foreseeable end lasting less than 3 to 6 months. It persists as long as the triggering noxa persists. When the noxa is removed, acute pain disappears and the event ends on its own (Nix, 2017). It facilitates tissue repair/healing (Macintyre *et al.*, 2010) which is achieved through hypersensitizing the injury area (called primary hyperalgesia), and the tissues in the area of injury (secondary hyperalgesia) to all stimuli (both damaging/noxious and non-damaging) in a way that causes us to avoid contact with any external stimulus thereby allowing reparative processes to proceed unperturbed (Macintyre *et al.*, 2010). Acute pain does not always have a short duration as is always classified. Some sources of acute pain are surgery, labour, acute disease states, medical procedures etc. (NPC, 2001; Costigan *et al.*, 2009).

Chronic pain persists beyond the expected time frame (longer than 6 months) for an injury or disease process or expected period of healing (Russo & Brose, 1998; NPC, 2001). It disrupts normal living and sleep, and, unlike acute pain, serves no protective or adaptive function. Instead, it degrades the health and functional capability of the sufferer and contribute to patient morbidity. Here, the nervous system becomes the primary focus of the ongoing pathology (Flor, 2000; NPC, 2001). Chronic pain can arise from any sustained noxious stimulus like an on-going inflammation and injury, or may exist *de novo*, with no temporal relation to an inciting cause (Flor, 2000). Environmental factors, as well as other affective factors can also make chronic pain worse, and lead to disability (Golan *et al.*, 2016). Chronic pain may be either neuropathic or nociceptive or both (Golan *et al.*, 2016).

2.1.3.2. Classification by Pain Physiology

Pain can be classified based on the underlying pathophysiology. There are four main classes in this category distinguishable by their underlying pain mechanisms or pathways (Figure 2.4).

These include nociceptive, inflammatory, neuropathic and dysfunctional pain (Russo & Brose, 1998; Woolf, 2004; Costigan *et al.*, 2009; Golan *et al.*, 2016; Nix, 2017).

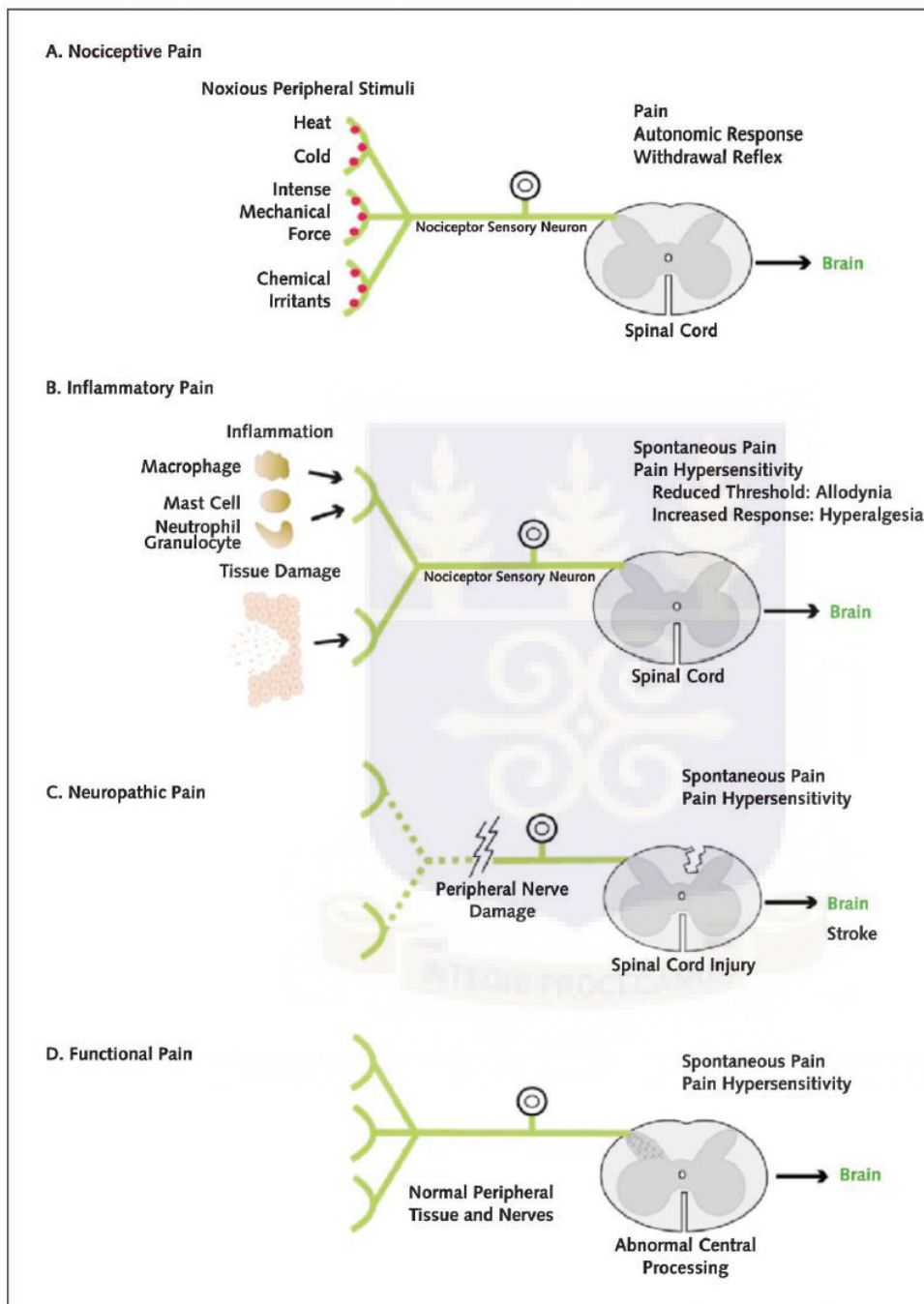


Figure 2.4. The four primary types of pain (Woolf, 2004)

2.1.3.3. Nociceptive Pain

This type is mediated by primary afferents that project into certain nociceptive pathways of the CNS. They are sensitive to intense mechanical and thermal stimuli and certain chemical

mediators (Marchand, 2008). The specific pathway is depicted in Figure 2.4. Nociceptive pain is basically an acute or chronic pain event in which both the central and peripheral neuronal structures are intact (Nix, 2017). However, when inflammation, autoimmune, or metabolic aetiology affects these structures causing lesions in the somatosensory system, the pain becomes neuropathic. Nociceptive pain is very important to survival. It serves as a protective measure against self-mutilation/hurt and additionally sparks/encourages the healing process after an encounter with noxa. It is generally transitory and is known to only occur in the presence of the noxa and persists as long as that stimulus remains (Costigan *et al.*, 2009). However, there are some cases where the pain persists even after the injury/stimulus is removed hence advancing to chronic pain. Certain diseases (like osteoarthritis) may generate recurring or persistent noxious stimuli that can result in the production of chronic nociceptive pain (Schaible, 2007; Costigan *et al.*, 2009). Clinicians are not always able to predict which case will devolve into chronic pain following acute nociceptive pain, hence the need to treat this type of pain as early as possible. When this system is chronically disabled, its protective function is inevitably lost leading to tissue damage including self-induced mutilation of the lips and tongue, loss of the tips of fingers, destruction of joints and pressure ulcers (Woolf, 2004).

2.1.3.4. Inflammatory Pain

Inflammatory pain is often grouped under nociceptive pain but according to Benzon and Raj (2008), it qualifies to stand alone as a pain category as there is evidence of peripheral and central reorganization in such pain states. This pain type occurs because of tissue injury and the linked inflammatory response that follows (Figure 2.4). This pain stimulus is aimed at addressing the consequences of tissue damage as opposed to protecting the organism against potentially damaging noxious stimulus (Costigan *et al.*, 2009). In an effort to aid/accelerate healing and repair, the CNS is forced to undergo certain changes that can result in hyperalgesia and allodynia (Juhl *et al.*, 2008).

2.1.3.5. Neuropathic Pain

It occurs when signals are aberrantly processed in the nervous system and is an indicator of damage to the nervous system (Figure 2.4). It is usually generated by a disease-inducing activity within the nociceptive system without an adequate stimulation of the peripheral sensory endings of nociceptors (Costigan *et al.*, 2009; Nix, 2017). This can continue beyond the disease-inducing activity and the pain thereof can become an independent disease. Even though central sensitization/stimulation does not last when the noxa is removed, the resulting injury to the nerve that was left causes long lasting changes in the CNS (Costigan *et al.*, 2009). From this, many believe that central sensitization can be used to explain why neuropathic pain is not proportional to the applied stimuli or why it persists even when no identifiable stimuli exist (NPC, 2001; Benzon & Raj, 2008; Nix, 2017). Neuropathic pain is broadly categorized as either central or peripheral in origin. Lesions in the nervous system, as found in peripheral neuropathic pain, is usually caused by neurotoxic chemicals, trauma arising from mechanical sources, infection and tumours. Several of these pathophysiological changes in the nervous system are implicated (Woolf, 2004; Nix, 2017). Central neuropathic pain is commonly associated with stroke, spinal cord injury etc. (Ducieux *et al.*, 2006).

According to Benzon and Raj (2008), the pharmacological management of neuropathic pain in patients is quite disappointing as no single therapy exists that can properly alleviate the pain in such individuals. Some of the drugs prescribed to patients for the management of this type of pain are NSAIDs, acetaminophen, tricyclic antidepressants (which include amitriptyline and doxepin), anticonvulsants (which include pregabalin and carbamazepine), some local anaesthetics (which include lidocaine) and opioids (such as morphine and methadone) (Rang *et al.*, 2016; Nix, 2017).

Non-pharmacological treatment options given by some psychologists include hypnosis, physical therapy, occupational therapy, individual and family therapy (Benzon & Raj, 2008).

2.2. Types of Analgesics for the pharmacological management of pain

An analgesic can be defined as any compound that selectively relieves pain without the loss of consciousness (Medical Subject Heading, 2018) and may block nerve impulse conduction which is known to alter sensory perception (Moore, 2013). This distinguishes analgesics from anaesthetics in that, anaesthetics induce a general or a local loss of sensation or sensory perception. The human body intrinsically produces certain endogenous compounds that influence pain impulse generation and perception and how the brain responds to these impulses (Lesniak & Lipkowski, 2011; Corder *et al.*, 2018). Some of these are endogenous peptides like Substance P and chemical messengers such as 5HT₃ (serotonin) and GABA (Koneru *et al.*, 2009). Medications are therefore mostly used to ameliorate the body's intrinsic pain management mechanisms.

2.2.1. Non-Steroidal Anti-Inflammatory Drugs (NSAIDs)

NSAIDs exert their therapeutic effect by inhibiting a group of endogenous enzymes known as cyclooxygenases (Zarghi & Arfaei, 2011; Nix, 2017). These enzymes mediate the production of prostaglandins from arachidonic acid (Zarghi & Arfaei, 2011; Rang *et al.*, 2016). They are usually prescribed for mild to moderate pain. The therapeutic activity of NSAIDs stems from the inhibition of the COX-2 enzyme. Inhibiting the activity of the COX-1 enzyme generates a slew of undesirable effects which include gastric bleeding, stomach ulcers, nausea, etc (Benzon & Raj, 2008; Dinarello, 2010; Nix, 2017). Selective COX-2 inhibitors are known to have a better gastric safety profile than NSAIDs that inhibit both COX-1 and COX-2. However, their usage also comes with certain side effects and may increase the risk of stroke or heart attack in certain patients (Benzon & Raj, 2008; Zarghi & Arfaei, 2011). All the side effects limit the

usage of NSAIDs over an extended period. Examples of selective COX-2 inhibitors include celecoxib, lumiracoxib etc. Other examples of commonly used NSAIDs include ibuprofen, aspirin and indomethacin (Zarghi & Arfaei, 2011).

2.2.2. Opioids

Opioids are effective analgesics and are widely used in clinical management of pain (Koneru *et al.*, 2009; Corder *et al.*, 2018). The opioid system is one of the main systems engaged in pain perception and modulation, reward, addiction and fear behaviours (Lesniak & Lipkowski, 2011). Some of the opioid peptides synthesised by the human body for these processes include endorphins, enkephalins, dynorphins and endomorphins (Przewlocki & Przewlocka, 2005). Opioids exert their effect by acting on the pre-synaptic terminal of primary nociceptive afferents thereby decreasing the release of excitatory neurotransmitters from the nociceptive neurons. They are very effective in treating acute nociceptive pain and to some extent acute neuropathic pain. However, their usage in the management of persistent pain is discouraged due to concerns about ineffectiveness, the high potential for the development of tolerance, the risk of addiction, and a slew of other side effects (Przewlocki & Przewlocka, 2005; Corder *et al.*, 2018). Some of these side effects include nausea, vomiting, constipation, sedation, confusion and myoclonus. Some commonly used opioid analgesics in clinical practice include morphine, codeine, fentanyl, oxycodone, hydromorphone, methadone and buprenorphine (Brain & Cox, 2006).

2.2.3. Adjuvant analgesics

These are drugs that are prescribed for conditions other than pain but display significant analgesic effect in some painful conditions (Lui & Ng, 2011; Mitra & Jones, 2011). They are usually co-administered with analgesics such as the NSAIDs, opioids and acetaminophen. Major classes include antidepressants (tricyclic antidepressants, selective serotonin reuptake

inhibitors), anticonvulsants (carbamazepine, pregabalin), neuroleptics (olanzapine), NMDA receptor antagonists (amantadine, ketamine), corticosteroids (dexamethasone, prednisone), local anaesthetics (lidocaine), muscle relaxants (cyclobenzaprine) etc. Some are described as multipurpose adjuvant analgesics (antidepressants, corticosteroids etc) as they are prescribed for many painful conditions (Lussier *et al.*, 2004; Nix, 2017). Others are prescribed specifically for neuropathic pain (anticonvulsants, NMDA receptor antagonists), bone pain (calcitonin, bisphosphonates), musculoskeletal pain (muscle relaxants) or pain from bowel obstruction (anticholinergics) (Lui & Ng, 2011; Mitra & Jones, 2011; Nix, 2017). These too are riddled with side effects such as orthostatic hypotension, erectile dysfunction, nausea, vomiting etc (Lussier *et al.*, 2004).

2.3. Traditional Medicine

Traditional medicine has been practiced for centuries and still remains common in the developing world (Chukwujekwu *et al.*, 2005). In Ghana, it is common to see herbal products advertised to treat a plethora of diseases. This is scientifically possible because a plant contains a slew of different compounds that can act synergistically on targeted elements of certain complex cellular pathways to produce varying effects (Farombi, 2003; Rajdev *et al.*, 2018). The use of herbal preparations was popularized by traditionalists as the best alternative to allopathic drugs which are riddled with toxicity and mild to severe side effects. It has been estimated that about 80 percent of the world's population rely primarily on medicinal plants for the management of various medical conditions (Mahomoodally, 2013; Ekor, 2014). Most of these drugs are prepared as extracts of different parts of the medicinal plants or their active constituents. Thus, natural products is seen by researchers as a good source of lead compounds based on its traditional use in treating and managing ailments (Hasan *et al.*, 2014; Kukuia *et al.*, 2016). Purified natural products from plant sources can be used as lead compounds and

structurally improved upon to produce new generations of therapeutic compounds that have a higher therapeutic value and low toxicity.

2.4. Mallotus oppositifolius

Mallotus oppositifolius is a dioecious shrub that is widely distributed in Africa across many parts of Ghana, Nigeria, Angola, Madagascar, Cameroon, Senegal, Ethiopia etc (Burkill *et al.*, 1985). It bears many local names in Ghana including “sroti” (Ewe), “sratadua” (Fanti) and “osratadua” (Twi). In Ivory Coast, it is referred to as Tomida (Abron) in the Zanzan district where it is also abundant and widely used (Farombi, 2003; Okpekon *et al.*, 2004). It has been ascribed several names in different parts of Nigeria and these include “Kafar Mutuwaa” by Hausas (Northern Nigeria), “Ija” by Yorubas (Western Nigeria) and “Nne Okpo Kirinya” by Igbos (Eastern, Nigeria) (Nwachujor *et al.*, 2014).



Figure 2.5. Photograph showing the leaves and inflorescence of *M. oppositifolius*

Mallotus oppositifolius is part of the plant family Euphorbiaceae under the tropical genus *Mallotus* which constitutes about 150 species of shrubs and trees (Woode *et al.*, 2012; Harinantenaina *et al.*, 2013) distributed in tropical and subtropical areas which also include Australia, Asia and the Pacific (Kulju *et al.*, 2007). Euphorbiaceae is a plant family that has afforded several very promising bioactive compounds and is the subject of numerous biological investigations (Beutler *et al.*, 1998; Yoder *et al.*, 2007; Harinantenaina *et al.*, 2013). From research work done by Burkill *et al.* (1985), *Mallotus oppositifolius* sometimes grows to a total height of 13 metres. It harbours a raceme inflorescence which may grow to different lengths for either the male (10-15) or female (10-18) inflorescence. Its pale yellow-green flowers are unisexual with slight differences in the length and shape of the sepals. Its fruits are deeply 3-lobed and seeded. *M. oppositifolius* is shown in Figure 2.3.1.

2.4.1. Ethnopharmacological Uses of *M. oppositifolius*

In folklore traditional use, the leaves, stem bark, fruits and roots of *M. oppositifolius* are prepared as either a tincture or a decoction to treat several conditions (Chukwujekwu *et al.*, 2005). Leaf and/or stem bark infusions are taken as an anthelmintic to treat intestinal helminthiasis and as treatment for diarrhoea (Okpekon *et al.*, 2004). The leaves are chewed by some traditional healers and placed on sores, skin eruptions, burns and rashes as an antibacterial, haemostatic or analgesic for fast healing and relief (Adekunle & Ikumapayi, 2006). It is also used in folklore for the treatment of epilepsy, headache or mental illness as a steam bath. For inflamed eyes and gums in the mouth, the leaves are crushed and applied. The leaves are ground with salt and applied to snakebites to nullify poisons (Farombi *et al.*, 2001). The root and leaf decoction is taken by some to treat malaria, anaemia, vomiting, pneumonia, urinary infections, leprosy, venereal diseases and chickenpox (Noumi & Yomi, 2001; Farombi, 2003; Okpekon *et al.*, 2004). A paste is also made from the roots and leaves and is applied to treat chest pains, convulsions and stomach-ache. In certain parts of East Africa, the root

decoction is drunk as an aphrodisiac (Burkill, 1994). Certain traditional medicine practitioners in the south-eastern part of Nigeria hold the claim that *M. oppositifolius* possess antidiabetic activity (Nwaehujor *et al.*, 2013). The stick is also chewed by some for teeth cleaning and oral hygiene (Burkill *et al.*, 1985; Nwaehujor *et al.*, 2014).

2.4.2. Non-Medicinal Uses of *M. oppositifolius*

M. oppositifolius leaves are commonly pastured on by cattle (Aschfalk *et al.*, 2000). The dried wood is commonly used as firewood and as supplies in making tool handles. The stem is sometimes used as support for climbing vines like yam. Farmers use the thin stems from some *M. oppositifolius* plants as rope for binding and the twigs as chewing sticks (Burkill *et al.*, 1985).

2.4.3. Previous studies on *M. oppositifolius*

Phytochemical screening of *M. oppositifolius* by Farombi *et al.* (2001), Adekunle and Ikumapayi (2006) and Woode *et al.* (2012) revealed that it constituted certain secondary metabolites such as anthocyanins, butacyanins, flavonoids, saponins, steroids, tannins, alkaloids, phenols, cardenolides and anthraquinones in both the ethanolic and aqueous leaf extracts. Heavy metal analysis by Adekunle and Ikumapayi (2006) revealed the presence of insignificant amounts (below the recommended limit of 0.95% in plants) of zinc, copper, iron and manganese. Their study also confirmed that the plant extract (aqueous and ethanolic) possessed antifungal properties that were very likely attributable to the phytochemical compounds present in the extract and not due to the presence of any heavy metals. Significant antifungal activity was exhibited against *Aspergillus flavus*, *Microsporum audouinii*, *Candida albicans*, *Penicillium sp.*, *Trichoderma mentagrophytes* and another *Trichoderma spp.*

In a study conducted by Gbedema *et al.* (2010), the leaf extract of *M. oppositifolius* displayed a significant antimicrobial activity against *Pseudomonas aeruginosa* and *Staphylococcus aureus* and *Bacillus subtilis*. It also enhanced the antibacterial activity of amoxicillin against

Staph. aureus and *B. subtilis*. Other studies on the antimicrobial activity of *M. oppositifolius* extract in-vivo and in-vitro have yielded significantly positive results (Chukwujekwu *et al.*, 2005; Gangoue-Pieboji *et al.*, 2009).

Extensive work has been done to provide scientific evidence on the anticonvulsant, antidepressant and anxiolytic activity of *M. oppositifolius* (Kukuia, 2012; Woode *et al.*, 2012; Kukuia *et al.*, 2014; Kukuia *et al.*, 2016, 2016) as a potent alternative to mainstream drugs for the management of these conditions. Mallotoxin (Rottlerin, pictured in figure 2.7) is a compound isolated from the *M. oppositifolius* plant and has been cited to display a spectrum of pharmacology (Harinantenaina *et al.*, 2013; Kukuia *et al.*, 2016; Manville & Abbott, 2018).

In past studies conducted, researchers demonstrated that the leaf extract possesses significant antioxidant effect (Nwaehujor *et al.*, 2013; Nwaehujor *et al.*, 2014), anti-inflammatory activity (Chukwujekwu *et al.*, 2005), antimalarial effect and anti-parasitic activity (Okpekon *et al.*, 2004; Harinantenaina *et al.*, 2013; Kabran *et al.*, 2015; Eaton *et al.*, 2016) and significant anti-blastocystic activity (Bremer Christensen *et al.*, 2015).

In a study by Harinantenaina *et al.* (2013), three bioactive compounds, mallotojaponin B and C (methylated form of mallotojaponin C, tetramethylmallotojaponin C, was also obtained) and mallotophenone were isolated from the ethanol extract of the leaves and inflorescence of *M. oppositifolius* by bioassay-guided fractionation (Figure 2.6). Mallotojaponins B and C showed potent antimalarial activity against chloroquine-resistant *Plasmodium falciparum*. Again, in the same study, all three isolated compounds displayed strong antiproliferative activity against the A2780 human ovarian cancer cell line. Similarly, Okpekon *et al.* (2004) tested *M. oppositifolius* methanolic extract against chloroquine-resistant FCB1 strain of *Plasmodium falciparum* with promising results. Following this, several other bioactive compounds have been isolated from *M. oppositifolius* (Kabran *et al.*, 2015).

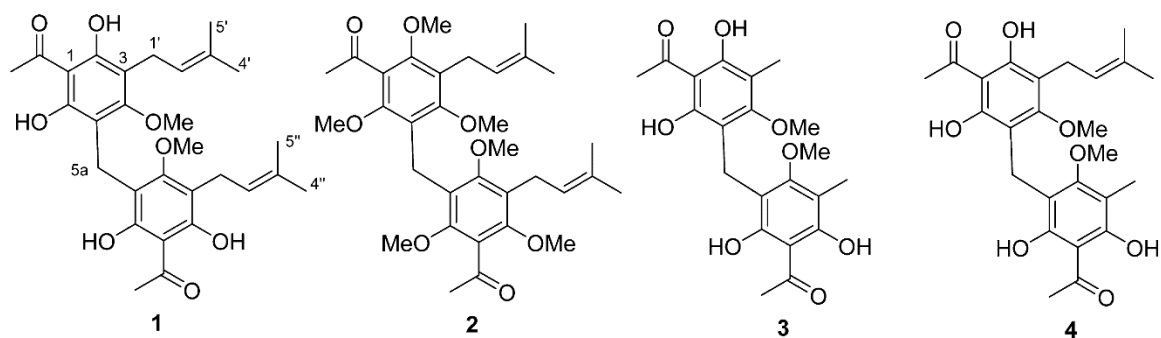


Figure 2.6. Chemical structures of mallotojapinin C (1), tetramethylmallotojapinin C (2), mallotophenone (3) and mallotojaponin B (4).

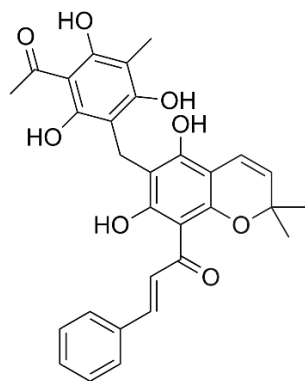


Figure 2.7. Chemical structure of Mallotoxin (Rottlerin)

Chapter Three

MATERIALS AND METHODS

3.1. Plant Material Collection

Mallotus oppositifolius leaves were procured from Mampong Centre for Plant Medicine Research, Eastern Region, Ghana and were authenticated by a botanist at the Department of Botany, University of Ghana, and a voucher specimen (CPMR 314/17) kept.

3.2. Preparation of Plant Extract

The leaves were air-dried under shade for seven days and the dried leaves pulverized into fine powder with a hammer mill. About 6 kg of the powder was cold macerated with ethanol (70% v/v) in a stoppered container and allowed to stand at room temperature for a period of 72 hours. The mixture obtained after the 72 hours was strained, the damp solid material left behind was also pressed and the liquids combined. The resulting solution was concentrated to a syrupy mass using a rotary evaporator (Heidolph Elektro, GmbH, KG, Kcl Hcim, Germany) by removing the alcohol. This was done under reduced temperature (60°C) and pressure. The syrupy mass was then dried on a water bath to a solid mass and kept in a desiccator till it was ready to be used. The percentage yield was 5.18%. The final product yielded was called the extract or *Mallotus oppositifolius* extract (MOE) in this work.

3.3. Preliminary High-Performance Liquid Chromatography (HPLC) of MOE

The above procedure was carried out using a Perkin Elmer Flexar LC binary pump coupled to a PDA detector. The constituents of MOE were separated on a μ Bondapak C18 Column (3.9 \times 300 mm, 5 μ m). The mobile phase used was distilled water (A) and methanol (B). Gradient elution started with 99% distilled water for 5 minutes and gradually moved to 0% within 35

minutes. It stayed at 0% distilled water for 5 more minutes and then returned to 99% distilled water in 5.1 minutes, bringing the total runtime to 45.1 minutes. The flow rate of 1mL/min and a sample injection volume of 20 μ L was used. The wavelength was set at 272 nm and 278 nm. An initial blank reading using the solvent was taken before the analysis on MOE.

3.4. Preliminary Phytochemical Screening

This was performed on the freshly prepared *Mallotus oppositifolius* extract using methods described by Evans *et al.* (2009) and Kukuia (2012) aimed at the detection of tannins, flavonoids, alkaloids, reducing sugars, sterols, saponins and terpenoids.

3.4.1. Test for Tannins

About 0.5g of MOE was added to 25ml of water and boiled 5 minutes after which it was cooled and then filtered. The filtrate obtained was again topped up to 25 ml with water. To 1 ml of the filtrate, 10ml of distilled water followed by 2 drops of 1% ferric chloride was added and observed for the appearance of a blue-black or green precipitate.

3.4.2. Test for Alkaloids

About 0.1g of the extract was measured and transferred into a test tube. About 5.0 ml of 2.0M hydrochloric acid was added to the test tube containing the extract to giving acidified solution. This acidified extract solution was swirled vigorously whilst warming over a water bath for about 5 minutes and thereafter filtered. The filtrate was divided into three portions designated A, B and C. Dragendorff's reagent was added to portion A of the solution, to portion B, Mayer's reagent and to portion C, Wagner's reagent. The presence of a reddish-brown precipitate in tubes A and C and a yellowish precipitate in test tube B was an indication that alkaloids were present.

3.4.3. Test for Saponins

About 4 ml of distilled water was added to a small amount of the extract (0.2g). This was then shaken for a few seconds. The resulting mixture was observed for the presence of a froth which did not break readily when left to stand.

3.4.4. Test for Reducing Sugars

An amount of the extract (0.2 g) was boiled in 5 ml of water and the resulting mixture cooled and filtered. An equal quantity (5 ml) of Fehling's A and B solution was added. This mixture was then heated and observed for the formation of any red-brown precipitate.

3.4.5. Test for Sterols

About 5 ml of acetic anhydride was added to and mixed with about 0.1g of MOE and filtered. The filtrate was treated with Liebermann-Burchard reagent. The appearance of a dark green ring at the interphase was indicative of the presence of sterols and steroids in the extract (Zohra *et al.*, 2012).

3.4.6. Test for Terpenoids

Thin-layer chromatography (TLC) was carried out on the extract using acetone/petroleum in the ratio of 8:2 as the solvent system. The dried plates were sprayed on with Liebermann-Buchard reagent and placed in an oven for 10 minutes set at a temperature of 110°C. The plate was then examined under UV light for any pink spots. The presence of pink spots is indicative of the presence of terpenoids in the extract.

3.4.7. Salkowski's Test for Glycosides

About 0.5g of MOE was dissolved with 2 cm³ of chloroform. 2M sulphuric acid was carefully poured into the solution down the glassware's walls to form a lower layer. Glycosides were present when a reddish-brown colouration was formed at the interface.

3.4.8. Test for Anthraquinones and Anthracene Derivatives

Exactly 0.5g of the extract was dissolved in 30 cm³ of distilled water and filtered. The filtrate obtained was vigorously mixed with about 10 cm³ of benzene in a separating funnel. The benzene layer was transferred into a test tube and shaken with 5 cm³ of 2M ammonia solution. The presence of red colour in the ammonia layer was indicative of the presence of anthraquinones in the extract.

3.4.9. Test for Flavonoids (Shinoda's test)

About 0.1g of the extract was dissolved in 10.0 ml methanol in a test tube and filtered. Ten pieces of Magnesium turnings were added to the filtrate. Few drops of concentrated hydrochloric acid were added to the resulting solution. The presence of pink colorations in the test solution indicated the presence of flavonoids in the extract

3.5. Experimental Animals, Housing and Ethical Considerations.

Male ICR mice, weighing 20–40 g, and male Sprague-Dawley rats, weighing 150–350 g, were purchased from and kept at the Department of Animal Experimentation, Noguchi Memorial Institute of Medical Research, University of Ghana, where all the experimental procedures were undertaken. Experimental animals used were kept in stainless steel cages (dimensions: 47 × 34 × 18 cm) which had wood shavings as bedding and were fed with normal commercial feed (AGRIMAT, Madina), given water *ad libitum* and maintained under the required laboratory conditions of relative humidity 60-70%, temperature 23±2 °C and a 12-hour light-dark cycle. All animals used in this study were handled according to the Guide for the Care and Use of Laboratory Animals (National Research Council (U.S.A.) *et al.*, 2011). All animal experimentation was performed during the day from 9 am to 4 pm. Ethical clearance was received from the University of Ghana Institutional Animal Care and Use Committee, Noguchi Memorial Institute for Medical Research, University of Ghana (NIACUC-2018-02-2V).

3.6. Drugs and Chemicals Used

These are the drugs and chemicals used in this study: formalin [prepared from 40% formaldehyde solution (Research-Lab Fine Chem Industries, Mumbai, India)]; morphine (Fresenius Kabi Manufacturing SA (pty) Ltd, SA); diclofenac (KRKA, Slovenia); acetic acid (BDH, poole, UK); ondansetron (Novartis Pharmaceuticals Corp, UK); glibenclamide (Ernest Chemists Limited, Ghana); nifedipine (Lek Pharmaceuticals, Slovenia); yohimbine (Walter Ritter GmbH, Germany); atropine (Yanzhou Xierkangtai Pharma. Co. Ltd., China); naloxone (Duopharma Biotech Berhad, Malaysia); theophylline (Adcock Ingram Ltd, SA). All the drugs used were of high standard grade and solutions from these were freshly prepared daily before use.

3.7. Experimental Design

The models used in this study have been divided into acute models, chronic models and the 90-day sub-chronic oral toxicity study. The first acute model employed in this study was the formalin test which can demonstrate whether an agent has peripheral or central analgesic activity. To confirm the results from this experiment, the acetic acid-induced writhing test, a peripheral pain model was used. Again, to confirm the central activity of MOE, the hot plate test was employed. The formalin test was then employed to elucidate the possible mechanism(s) by which MOE exerts its antinociceptive effect.

In another experiment, effect of MOE in chronic models of pain was evaluated. Paclitaxel-induced peripheral neuropathy was the chronic model used. For peripheral neuropathy, two basic symptomologies exist; hyperalgesia and allodynia. For hyperalgesia, mechanical hyperalgesia was measured using the Randall-Selitto analgesiometer, whereas thermal hyperalgesia was evaluated using the hot plate. For allodynia, cold allodynia test using cold water at 4°C was employed.

Finally, the safety of MOE was assessed for extended use in the 90-day sub-chronic toxicity study.

3.8. Acute models of pain

3.8.1. Formalin Test

The formalin test was carried out as described by Hunskaar and Hole (1987) and Woode *et al.* (2009). ICR mice were divided into ten (10) groups (n=5); one negative control group, three (3) treatment groups and six (6) positive control groups. Every test animal was assigned one formalin test chamber (Perspex chamber of dimensions 15 x 15 x 15 cm) and then acclimatized for 1 hour before pre-treatments. Mice were then pre-treated with MOE, morphine, diclofenac and distilled water. Mice in the negative control group received distilled water *p.o.* (vehicle) by oral gavage. Those in the three (3) treatment groups were given the *Mallotus oppositifolius* extract at doses of 30, 100 or 300 mg/kg *p.o.* respectively, all by oral gavage. Finally, mice in the positive control groups were given morphine (0.3, 1, 3 mg/kg; *i.p.*) and diclofenac (3, 10 and 30 mg/kg; *i.p.*).

The right hind paws of the mice were injected subcutaneously with 10 μ L of 5% formalin, 30 minutes after pre-treatment with morphine or diclofenac (*i.p.* route) and 1 hour after pre-treatment with MOE (oral route) and immediately returned to individual testing chambers where their nociceptive behaviour from time zero (0) was captured for one (1) hour using a tripod-mounted video recorder (OnePlus™ A600 camera set at 1080p) placed opposite a mirror at the base of the test chamber positioned at an angle of 45°.

The nociceptive behaviour exhibited by the experimental animals was tracked using the BehaviorTracker™ Personal Edition software [version 1.6 (www.BehaviorTracker.com)]. The nociceptive scores were calculated in 5-minute time blocks as the product of the duration and

frequency of biting/licking of the right hind paw. The results were designated as first/neurogenic phase (0–10 minutes) and second/inflammatory phase (10–60 minutes). A time-course curve was plotted from nociceptive scores for each of the three drugs used as well as the vehicle. The area under the curve (AUCs) from both phases of the formalin test were calculated and plotted as well. ED₅₀ values for MOE, diclofenac and morphine were calculated and compared.

3.8.2. Acetic Acid-Induced Writhing Assay

This assay was carried out as earlier described by Woode *et al.* (2009); Woode *et al.* (2012). Thirty-five mice was divided randomly into seven (7) groups of five (n=5); one negative control group, three (3) treatment groups and three (3) positive control groups. Each animal was assigned and acclimatized to one test chamber for 1 hour prior to 0.6% acetic acid injections. After this, the ICR mice were pre-treated with MOE, diclofenac and distilled water. Mice in the negative control group received distilled water (vehicle) by oral gavage. Those in the three (3) treatment groups were given the *Mallotus oppositifolius* extract at doses of either 30, 100 or 300 mg/kg respectively, all by oral gavage. Finally, mice in the positive control group were given diclofenac (3, 10 and 30 mg/kg; *i.p.*).

Each animal was given an intraperitoneal administration of acetic acid (0.6%, 10 ml/kg), 30 minutes after pre-treatment with diclofenac (*i.p* route) and 1 hour after pre-treatment with MOE (oral route) and immediately returned to individual test chambers. The intraperitoneal administration of acetic acid induces a nociceptive behaviour known as writhing (abdominal constrictions, pelvic rotation, and hind limb stretching) (Cheng *et al.*, 2016).

The total time and frequency of writhes were captured for 30 minutes using a tripod-mounted video recorder (OnePlus™ A600 camera set at 1080p) placed opposite a mirror at the base of the test chamber positioned at an angle of 45°. The nociceptive behaviour exhibited by the

experimental animals was tracked using the BehaviorTracker™ Personal Edition software (www.BehaviorTracker.com). The nociceptive score was determined for each 5-minute time block and a time-course curve plotted. The total nociceptive scores calculated as AUCs from each treatment was determined and plotted as well. ED₅₀ values for both MOE and diclofenac were calculated and compared.

3.8.3. Hot Plate Test

The hot-plate test is used to evaluate the central analgesic activity of MOE and was conducted as previously described (Woode *et al.*, 2012; Simões *et al.*, 2018) with some modifications. ICR mice were sorted randomly into seven (7) groups of five; one negative control group, three (3) treatment groups and three (3) positive control groups. Before drug administrations, baseline measurements on the hot plate apparatus (Model DS37; Ugo Basile, Italy) were recorded for all the animals being used. After that, mice in the negative control group received distilled water (vehicle) by oral gavage. Those in the three (3) treatment groups were given the *Mallotus oppositifolius* extract at doses of 30, 100 or 300 mg/kg respectively, all by oral gavage, 1 hour before being placed on the hot plate apparatus. Finally, mice in the positive control group were given morphine (0.3, 1 and 3 mg/kg *i.p*) 30 minutes before being placed in the hot plate apparatus heated to 54±1°C.

Latency to withdraw paw (in seconds) was recorded using a stopwatch as the time between placing the animal on the hot plate and the appearance of symptoms of discomfort as licking or shaking of the hind paws or jumping off from the surface of the hot plate apparatus. A significant increase in the latency is considered as indicative of antinociceptive activity. This is evaluated at time points 0.5, 1, 1.5, 2 and 4 hours after the administration of MOE, vehicle or morphine. A cut-off of 30 seconds was used to prevent damage to mouse paw tissue. The percentage maximum effect (%MPE) was calculated from the formula below:

$$\%MPE = \frac{(Post\ drug\ latency) - (Pre\ drug\ latency)}{(Cut\ off\ latency) - (Pre\ drug\ latency)} \times 100$$

Time-course curves were plotted from the results. The total antinociceptive scores calculated as the area under the curve (AUCs) from each treatment was determined and plotted as well. ED₅₀ values for MOE and morphine were calculated and compared.

3.8.4. Possible Mechanism(s) of Antinociception

Formalin test (Tjølsen *et al.*, 1992; Woode & Abotsi, 2011) was employed to assess the possible involvement of certain pathways in the observed antinociceptive effect of MOE. Here, nineteen groups (19) of mice (n=5) were randomly selected and grouped (n=5) and then pre-treated with seven (7) different inhibitors/antagonists; naloxone (a nonselective opioid receptor antagonist), glibenclamide (an ATP sensitive potassium channel inhibitor), theophylline (a nonselective adenosine receptor antagonist), ondansetron (a 5-HT₃ receptor antagonist), yohimbine (a selective adrenoceptor antagonist), nifedipine (L-type voltage-gated calcium channel blocker) and atropine (a nonselective muscarinic receptor antagonist).

Briefly, mice were pre-treated with these antagonists 15 minutes (*i.p*) and 30 minutes (*p.o*) before the administration of either MOE (100 mg/kg *p.o*) or morphine (1 mg/kg *i.p*). Mice then received an intraplantar administration of 10 µL of 5% formalin to the right hind limb, 30 minutes (after morphine) and 1 hour (after MOE) and immediately returned to individual testing chambers where their nociceptive behaviour from time zero (0) was captured for one (1) hour using a tripod-mounted video recorder (OnePlus™ A600 camera set at 1080p) placed opposite a mirror at the base of the test chamber positioned at an angle of 45°. The doses of the various antagonists used were chosen based on literature (Woode & Abotsi, 2011; Asante *et al.*, 2019).

The nociceptive behaviour exhibited by the experimental animals was tracked using the BehaviorTracker™ Personal Edition software [version 1.6 (www.BehaviorTracker.com)]. The nociceptive scores were calculated in 5-minute time blocks as the product of the duration and frequency of biting/licking of the right hind paw. The results were designated as first/neurogenic phase (0–10 minutes) and second/inflammatory phase (10–60 minutes). A time-course curve was plotted from nociceptive scores for each of the three drugs used as well as the vehicle. The area under the curve (AUCs) from both phases of the formalin test were calculated and plotted as well.

3.8.4.1. Involvement of the Opioid System

Experimental animals were pre-treated with naloxone (2 mg/kg, *i.p.*) 15 minutes before the administration of MOE (100 mg/kg, *p.o.*) or morphine (1 mg/kg, *i.p.*). As described in section 3.8.4, their nociceptive responses were recorded for 1 hour after the pre-treatments.

3.8.4.2. Involvement of ATP-sensitive K⁺ Channels

Experimental animals were pre-treated with glibenclamide (8 mg/kg, *i.p.*) 15 minutes before the administration of MOE (100 mg/kg, *p.o.*) or morphine (1 mg/kg, *i.p.*). As described in section 3.8.4, their nociceptive responses were recorded for 1 hour after the pre-treatments.

3.8.4.3. Involvement of the Adenosinergic System

Experimental animals were pre-treated with theophylline (10 mg/kg, *i.p.*) 15 minutes before the administration of MOE (100 mg/kg, *p.o.*) or morphine (1 mg/kg, *i.p.*). As described in section 3.8.4, their nociceptive responses were recorded for 1 hour after the pre-treatments.

3.8.4.4. Involvement of 5-HT₃ Receptors

Experimental animals were pre-treated with ondansetron (0.5 mg/kg, *i.p.*) 15 minutes before the administration of MOE (100 mg/kg, *p.o.*) or morphine (1 mg/kg, *i.p.*). As described in section 3.8.4, their nociceptive responses were recorded for 1 hour after the pre-treatments.

3.8.4.5. Involvement of α 2-adrenoceptors

Experimental animals were pre-treated with yohimbine (3 mg/kg, *i.p.*) 15 minutes before the administration of MOE (100 mg/kg, *p.o.*) or morphine (1 mg/kg, *i.p.*). As described in section 3.8.4, their nociceptive responses were recorded for 1 hour after the pre-treatments.

3.8.4.6. Involvement of Voltage-gated Calcium Channels

Experimental animals were pre-treated with nifedipine (10 mg/kg, *i.p.*) 15 minutes before the administration of MOE (100 mg/kg, *p.o.*) or morphine (1 mg/kg, *i.p.*). As described in section 3.8.4, their nociceptive responses were recorded for 1 hour after the pre-treatments.

3.8.4.7. Involvement of the Muscarinic Cholinergic System

Experimental animals were pre-treated with atropine (5 mg/kg, *i.p.*) 15 minutes before the administration of MOE (100 mg/kg, *p.o.*) or morphine (1 mg/kg, *i.p.*). As described in section 3.8.4, their nociceptive responses were recorded for 1 hour after the pre-treatments.

3.9. Assessing the Effect of MOE in Chronic Pain Models

Paclitaxel-induced peripheral neuropathy in ICR mice was performed as previously described (Ameyaw *et al.*, 2014; Amoateng *et al.*, 2017) with some modifications. ICR mice were divided randomly into seven (7) groups of five ($n=5$) and allowed to acclimatize to the test environment on every testing day, 1 hour or more before drug administrations. Baseline measurements of thermal, mechanical and cold stimuli were performed on day zero (0), before paclitaxel injections.

3.9.1. Induction of Peripheral Neuropathy

ICR mice were injected intraperitoneally with paclitaxel (2 mg/kg) dissolved in distilled water on 5 consecutive days (days 1–5) as described by Amoateng *et al.* (2017). The cumulative paclitaxel dose was 10 mg/kg. Behavioural testing for thermal, mechanical and cold stimuli

were performed on day 1, 3 and 5, starting one hour after paclitaxel injections for those days. After confirmation of paclitaxel-induced peripheral neuropathy from the behavioural tests, mice were assigned to groups as follows: one negative control group, three (3) treatment groups and three (3) positive control groups. Mice in the negative control group received distilled water (vehicle) by oral gavage. Those in the three (3) treatment groups were given the *Mallotus oppositifolius* extract at doses of 30, 100 or 300 mg/kg respectively, all by oral gavage. Finally, mice in the positive control group were given pregabalin (10, 30 and 100 mg/kg; *p.o.*). The effect of MOE and pregabalin on paclitaxel-induced peripheral neuropathy was evaluated in the hot plate test, the Randall-Selitto test and cold allodynia test for 5 consecutive days starting from day 7 post paclitaxel injections.

3.9.2. Thermal Hyperalgesia (Hot Plate test)

To assess the effect of MOE (30, 100 and 300 mg/kg), pregabalin (10, 30 and 100 mg/kg) and vehicle on thermal hyperalgesia the hot-plate test was employed. The test was conducted daily for 5 days as has been described in previous studies (Ameyaw *et al.*, 2014; Amoateng *et al.*, 2017). ICR mice were divided into seven (7) groups (n=5); one negative control group, three (3) treatment groups and three (3) positive control groups. Before drug administrations, baseline measurements on the hot plate were recorded for all the animals being used. After that, mice in the negative control group received distilled water (vehicle) by oral gavage. Those in the three (3) treatment groups were given the *Mallotus oppositifolius* extract at doses of 30, 100 or 300mg/kg respectively, all by oral gavage. Finally, mice in the positive control group were given pregabalin (10, 30 and 100 mg/kg *p.o.*) 1 hour (*p.o.*) before being placed in the hot plate apparatus. Test was carried out as described in section 3.8.3.

3.9.3. Mechanical Hyperalgesia (Randall-Selitto test)

To assess the effect of MOE (30, 100 and 300 mg/kg), pregabalin (10, 30 and 100 mg/kg) and vehicle on mechanical hyperalgesia the Randall-Selitto paw pressure test was employed. The test was conducted daily for 5 days as has been described in previous studies (Ameyaw *et al.*, 2014; Amoateng *et al.*, 2017) with a few modifications. Mice were divided randomly into seven (7) groups of five (n=5); one negative control group, three (3) treatment groups and three (3) positive control groups. Before drug administrations, baseline measurements were recorded for all the animals being used. After that, mice in the negative control group received distilled water (vehicle) by oral gavage. Those in the three (3) treatment groups were given the *Mallotus oppositifolius* extract at doses of 30, 100 or 300 mg/kg respectively, all by oral gavage. Finally, mice in the positive control group were given pregabalin (10, 30 and 100 mg/kg *p.o.*).

In this test, the hind paw of mice was placed in the paw pressure analgesimeter (model 7200, Ugo Basile, Varese, Italy) and a steadily increasing pressure stimulus was applied (cut-off of 250g) to the dorsal surface of the hind paws until there was withdrawal or vocalization. The force (g) reading was then recorded for each animal as the nociceptive threshold value. The test was performed on both hind paws to obtain two readings. The final value is presented as the mean of both hind paw values. The %MPE was calculated and plotted as described in section 3.8.3.

3.9.4. Cold Allodynia

The antinociceptive effect of *Mallotus oppositifolius* extract (30-300 mg/kg), pregabalin (10, 30, 100 mg/kg) and vehicle on cold allodynia was investigated daily for 5 days as has been described in previous studies but with minor modifications (Ameyaw *et al.*, 2014; Amoateng *et al.*, 2015). ICR Mice were divided into seven (7) groups (n=5); one negative control group, three (3) treatment groups and three (3) positive control groups. Before drug administrations,

baseline measurements were recorded for all the animals being used. After that, mice in the negative control group received distilled water (vehicle) by oral gavage. Those in the three (3) treatment groups were given the *Mallotus oppositifolius* extract at doses of 30, 100 or 300mg/kg respectively, all by oral gavage. Finally, mice in the positive control group were given pregabalin (10, 30 and 100 mg/kg *p.o.*).

For this experiment, cold allodynia was investigated by immersing mice tail into cold water maintained at $4^{\circ}\text{C}\pm 0.5$ 1 hour after drug administrations. The time it takes for a mouse to withdraw its tail from the second it was immersed in cold water was measured with a timer. The cut-off point was set to 20 seconds. The %MPE was calculated and plotted as described in section 3.8.3.

3.9.5. Sub-chronic Oral Toxicity (90-Day)

Sprague-Dawley rats were divided randomly into four groups (n=5); one control group and three (3) treatment groups. Rats in the control group received distilled water (vehicle) by oral gavage. Those in the three (3) treatment groups were given the *Mallotus oppositifolius* extract at doses of 30, 100, or 300 mg/kg *p.o.* respectively, all by oral gavage. MOE was freshly prepared daily with distilled water for the 90-day period. Administration was repeated daily for 90 days. On each day, the rats are weighed and observed for mortality and clinical signs of toxicity or abnormality.

On day 91, blood samples were collected from all animals via abdominal aorta puncture into Eppendorf tubes containing EDTA for haematological analyses to evaluate levels of haemoglobin (HGB), red blood cells (RBC), white blood cells (WBC), mean corpuscular volume (MCV), mean corpuscular haemoglobin concentration (MCHC), mean corpuscular haemoglobin (MCH) and platelets (PLT), granulocytes (Gran), and monocytes (MONO) with an automated haematology analyser (KX-2IN, Sysmex Corporation, Japan). Alongside, blood

samples for biochemical analyses were collected into BD-serum separator tubes and centrifuged at 3000 rpm for 10 minutes. The serum obtained was analysed for aspartate aminotransferase (AST), alanine aminotransferase (ALT), albumin (ALB), total protein (TP), alkaline phosphatase (ALP), triacylglycerol (TG), urea, creatinine (CREA), cholesterol (CHO).

The rats were euthanized in a chloroform chamber on the 91st day. The organs of interest were excised, weighed and visually examined. Principal vital organs (liver, kidney, brain, heart and spleen) were preserved in sterile containers with 10% buffered formalin and transported to a pathology laboratory for processing and examination.

Portions of the organs were selected into labelled tissue processing cassettes and processed into paraffin blocks. Each was passed through ascending grades of alcohol (70%, 80%, 90% and absolute) and two further changes of absolute alcohol for dehydration, cleared in three changes of xylene and finally infiltrated and embedded in paraffin wax. Five-micron sections were cut from each block, mounted on microscope slides and stained by the haematoxylin and eosin method and observed under a microscope. The method is outlined below.

1. Sections on slides were put in two changes of xylene for 15 minute each
2. Section through three changes of absolute ethanol for 5 minutes each
3. Sections to 70% ethanol for 15 minutes
4. Sections to 50% ethanol for 15 minutes
5. Sections to tap water for 30 minutes
6. Sections to distilled water for 2 minutes
7. Sections to Mayer's haematoxylin stain for 15 minutes
8. Sections to tap water for 30 minutes
9. Sections to working eosin solution for 5 minutes
10. Sections to 80% ethanol for 5 minutes

11. Sections to two changes of absolute ethanol for 5 minutes
12. Sections to three changes of hemode for 5 minutes each
13. Sections mounted in DPX

3.10. Statistical Analysis

GraphPad Prism 8.0.2 (San Diego, CA) for Windows was the software used to perform all statistical analyses and ED₅₀ calculations, and a p-value of ≤ 0.05 was considered statistically significant for all analyses. Mean \pm standard error of mean (SEM) was used in presenting all data. The time-course curves in the study were analysed using two-way (treatment x time) analysis of variance (ANOVA) followed by a Bonferroni's *post hoc* test. The total (anti-)nociceptive score for each treatment was calculated as the area under the curve (AUC). One-way ANOVA followed by Dunnett's multiple comparisons test was used to determine differences between treatment groups (areas under the curves). Percentage inhibition was calculated using the following formula:

$$\%Inhibition = \left(\frac{AUC_{control} - AUC_{treatment}}{AUC_{control}} \right) \times 100\%$$

The ED₅₀s for each drug was calculated by using an iterative computer least squares method, with the following non-linear regression (three-parameter logistic) equation.

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

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 midpoints (ED₅₀s) of the curves were compared statistically using the F-test. All graphs in this study were plotted using SigmaPlot Version 14.0 (Systat Software, Germany).

Chapter Four

RESULTS

4.1. HPLC Analysis of MOE

A total of 12 constituents were observed beginning at the elution time of 4.78 min to 22.14 min with varied peaks (Table 4.1). No constituents were detected after this time period. Figure 4.1 shows the HPLC chromatogram of the hydroethanolic extract of *M. oppositifolius*. Three major constituents were observed at retention times 4.78, 13.25 and 20.88 min (Table 4.1). The percentage composition of these constituents was calculated to be 33.61%, 15.63% and 11.96% respectively, using the area under the curves (AUCs). These three constituents make up about 62% of the extract.

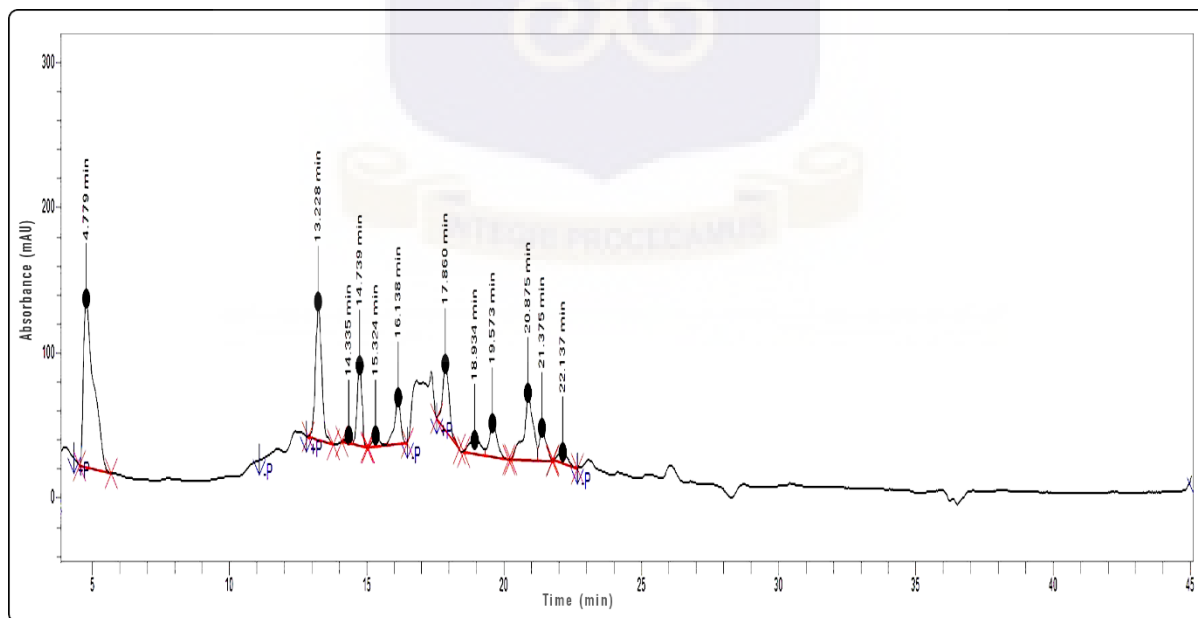


Figure 4.1. HPLC chromatogram of MOE monitored at 272, 278nm

Table 4.1. Report on the HPLC analysis of MOE

Peak No.	Retention Time	Area	Height	Percentage (%)
1	4.779	3,017,600.60	115,731.90	33.61
2	13.228	1,402,988.10	94,766.10	15.63
3	14.335	77,362.80	5,172.70	0.86
4	14.739	669,320.60	55,659.90	7.46
5	15.324	137,361.80	8,034.10	1.53
6	16.138	510,770.20	31,901.60	5.69
7	17.86	773,004.80	45,825.80	8.61
8	18.934	290,487.30	9,878.60	3.24
9	19.573	467,553.90	22,967.80	5.21
10	20.875	1,073,238.10	46,666.00	11.96
11	21.375	364,867.40	22,232.80	4.06
12	22.137	192,440.70	7,702.40	2.14
TOTAL		8,976,996.30		

4.2. Phytochemical Screening

Phytochemical screening of the hydroethanolic (70%) extract of *M. oppositifolius* revealed the presence of secondary metabolites including saponins, alkaloids, flavonoids, glycosides, sterols, tannins and reducing sugars (Table 4.2).

Table 4.2. Preliminary phytochemical screening of MOE

PHYTOCHEMICALS	INFERENCE
Saponins	Present
Alkaloids	Present
Flavonoids	Present
Terpenoids	Absent
Glycosides	Present
Sterols	Present
Tannins	Present
Reducing sugars	Present

4.3. The Effect of MOE in Acute Models of Pain

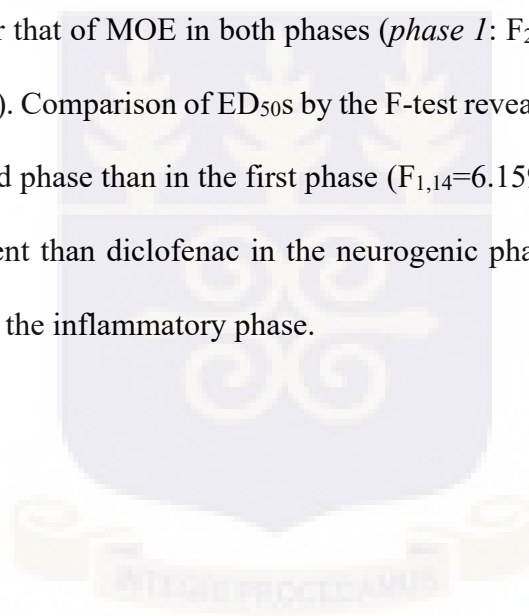
4.3.1. Formalin Test

The intraplantar injection of 10 μ L of 5% formalin of the right hind paw of mice induced a nociceptive response characterized by the biting and licking of the injected paw. Figure 4.3.1 shows the effect of the pre-treatment of mice with MOE, diclofenac and morphine in the formalin test. As depicted by the time course curves (Figure 4.3.1A,C,E) all the drug-treated groups displayed a significant reduction in nociceptive scores when compared to the vehicle (VEH) treated group [(MOE: $F_{3,16}=32.52$, $P<0.0001$; diclofenac: $F_{3,16}=41.81$, $P<0.0001$ morphine: $F_{3,16}=34.46$, $P<0.0001$ (two-way ANOVA)]. Two-way ANOVA revealed that the oral administration of MOE (30, 100, 300 mg/kg) one hour before the intraplantar injection of formalin significantly suppressed the formalin-induced nociceptive behaviour in mice in both the neurogenic ($F_{3,16}=7.91$, $P=0.002$) and inflammatory ($F_{3,16}=24.28$, $P<0.0001$) phases (Figure 4.3.1B). Analysis of the AUCs (Figure 4.3.1B) for the doses used revealed that MOE attenuated formalin-induced nociceptive behaviour by 58.30 to 73.74% in the early/neurogenic phase and 53.60 to 93.17% in the late/inflammatory phase. Also, one-way ANOVA followed by Dunnett's multiple comparisons test revealed a significant decrease in total nociceptive scores in both phases in the presence of MOE ($F_{3,16}=7.87$, $P=0.001$; and $F_{3,16}=23.45$, $P<0.0001$; respectively) (Figure 4.3.1 B).

Similarly, the intraperitoneal administration of the positive analgesic control used, morphine (0.3, 1, 3 mg/kg), a centrally acting opioid, 30 minutes before formalin injection resulted in a significant and dose-dependent reduction in nociceptive scores (Figure 4.3.1F) in both the early ($F_{3,16}=6.67$, $P=0.004$) and late ($F_{3,16}=27.50$, $P<0.0001$) phases (two-way ANOVA). Analysis of AUCs (Figure 4.3.1F) show that morphine produced a maximum inhibition of 66.43% in the neurogenic phase ($F_{3,16}=6.77$, $P=0.004$) and 91.69% in the inflammatory phase ($F_{3,16}=26.65$, $P<0.001$).

Also, an intraperitoneal administration of the reference drug, diclofenac (3-30 mg/kg) a peripherally acting analgesic, given 30 minutes before formalin administration dose-dependently inhibited the inflammatory phase ($F_{3,16}=36.90$, $P<0.0001$ two-way ANOVA followed by Bonferroni's multiple comparisons test) but was not active in the neurogenic phase ($F_{3,16}=0.7226$, $P=0.55$) as shown in Figure 4.3.1D. The percentage inhibitions calculated from the AUCs in Figure 4.3.1.1D were 84.54 to 97.24% in the late phase.

The ED_{50} values of the three drugs used were calculated using non-linear regression (Table 4.3.1) for both phases of the formalin test. ED_{50} s for Diclofenac and morphine were significantly different for that of MOE in both phases (*phase 1*: $F_{2,42}=22.13$, $P<0.0001$; *phase 2*: $F_{2,42}=47.20$, $P<0.0001$). Comparison of ED_{50} s by the F-test revealed that MOE was 1.5 times more potent in the second phase than in the first phase ($F_{1,14}=6.159$, $P=0.03$). Also, MOE was four (4) times more potent than diclofenac in the neurogenic phase and about 40 times less potent than diclofenac in the inflammatory phase.



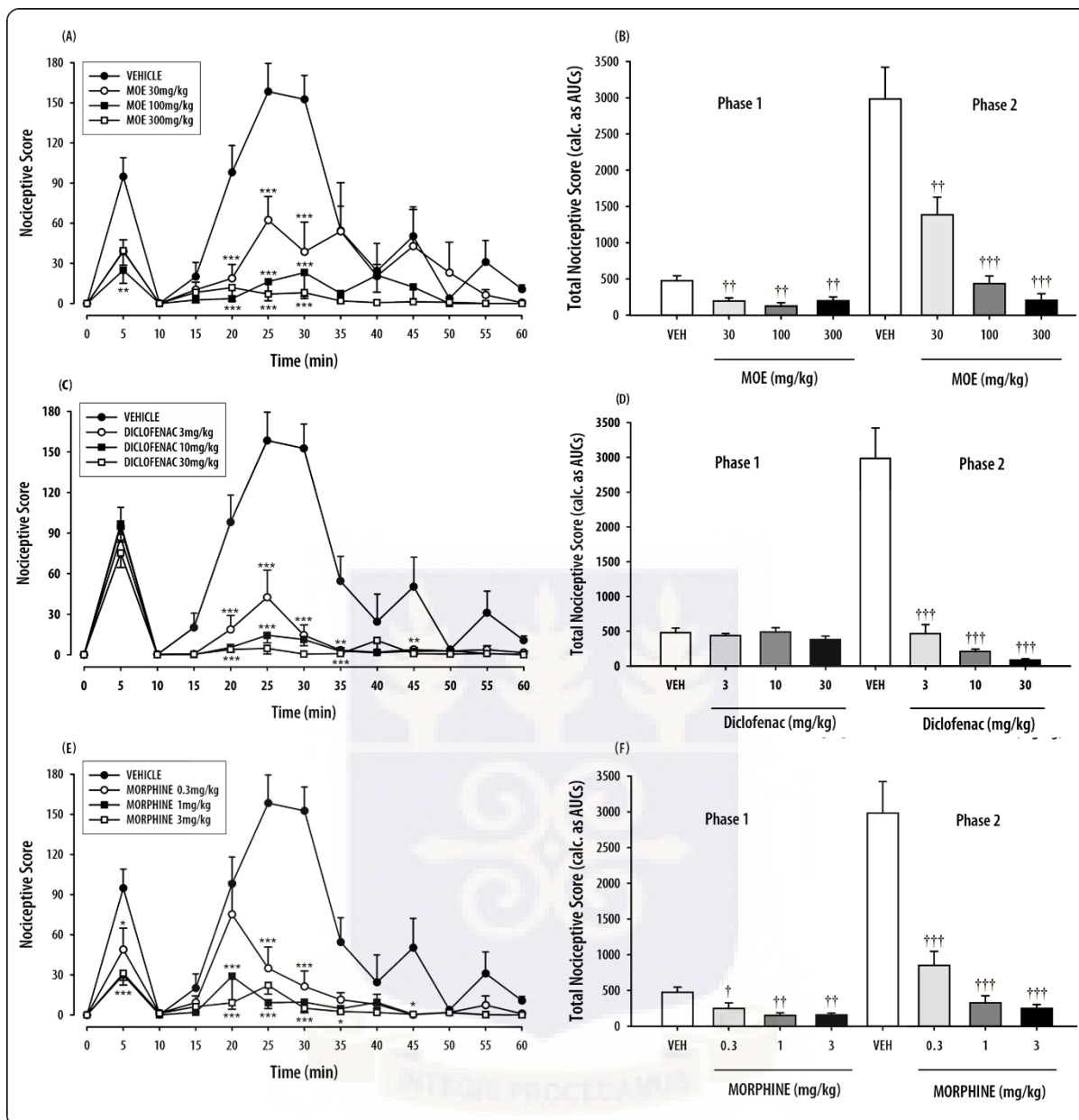


Figure 4.3.1: Effect of MOE (30, 100 and 300 mg/kg), diclofenac (3, 10 and 30 mg/kg) and morphine (0.3, 1 and 3 mg/kg) on the time-course events (A, C, E respectively) and the total nociceptive score (calculated as AUCs) (B, D, F respectively) on formalin-induced nocifensive behaviour in ICR mice.

Nociceptive scores are shown in 5-minute time blocks up to the 60th minute post formalin injection. Plotted data are mean \pm SEM (n = 5). *P<0.05, **P<0.01 and ***P<0.001 compared to the vehicle-treated group (two-way ANOVA followed by Bonferroni's *post hoc* test). †P<0.05, ††P<0.01, †††P<0.001 compared to the vehicle-treated group (one-way ANOVA followed by Dunnett's multiple comparisons test).

Table 4.3.1.1. ED₅₀s for MOE, diclofenac and morphine in the formalin test

DRUG	ED ₅₀ (mg/kg)	
	Phase 1	Phase 2
MOE	34.33 ± 13.77	22.91 ± 4.01
Diclofenac	137.42 ± 91.04	0.58 ± 0.10
Morphine	0.49 ± 0.17	0.02 ± 0.03

4.3.2. Acetic Acid Induced Writhing Test

The intraperitoneal injection of mice in this experiment with acetic acid (0.6%, 10 mL/kg) induced a characteristic nociceptive behaviour known as writhing (abdominal constrictions, pelvic rotation, and hind limb stretching). Figure 4.3.2 shows the effect (nociceptive score) of the pre-treatment of mice with MOE and diclofenac on mice in the acetic acid induced writhing test over the 30-minute observation period. The oral administration of MOE (30-300 mg/kg) one hour before the administration of 0.6% acetic acid significantly inhibited abdominal writhes over the 30-minute period as compared to the control group [$F_{3,15}=13.92$, $P<0.001$ (two-way ANOVA)] (Figure 4.3.2A). Analysis of the AUCs shows that MOE at 30, 100 and 300 mg/kg significantly ($F_{3,15}=14.16$, $P<0.001$) and dose-dependently attenuated acetic acid induced writhes by 73.14% ($P=0.003$), 92.92% ($P<0.0001$) and 96.49% ($P<0.0001$).

In a similar way, the intraperitoneal administration of the peripherally acting non-steroidal anti-inflammatory drug (NSAID), diclofenac (3, 10 and 30 mg/kg), 30 minutes before in a 0.6% acetic acid administration significantly reduced acetic acid induced writhes [$F_{3,16}=17.54$, $P<0.0001$ (two way ANOVA)] (Figure 4.3.2C) in comparison to the negative control. Analysis of the AUCs shows that diclofenac at 3, 10 and 30 mg/kg significantly reduced acetic acid induced writhes by 95.13% ($P<0.0001$), 97.01% ($P<0.0001$) and 99.69% ($P<0.0001$).

ED₅₀s were calculated by non-linear regression method (table 4.3.2). Comparison with the F-test revealed that diclofenac was more potent than MOE in the writhing test (P<0.001).

Table 4.4. ED₅₀s for MOE and diclofenac in the writhing test

DRUG	ED₅₀ (mg/kg)
MOE	10.41 ± 1.14
Diclofenac	0.17 ± 0.06



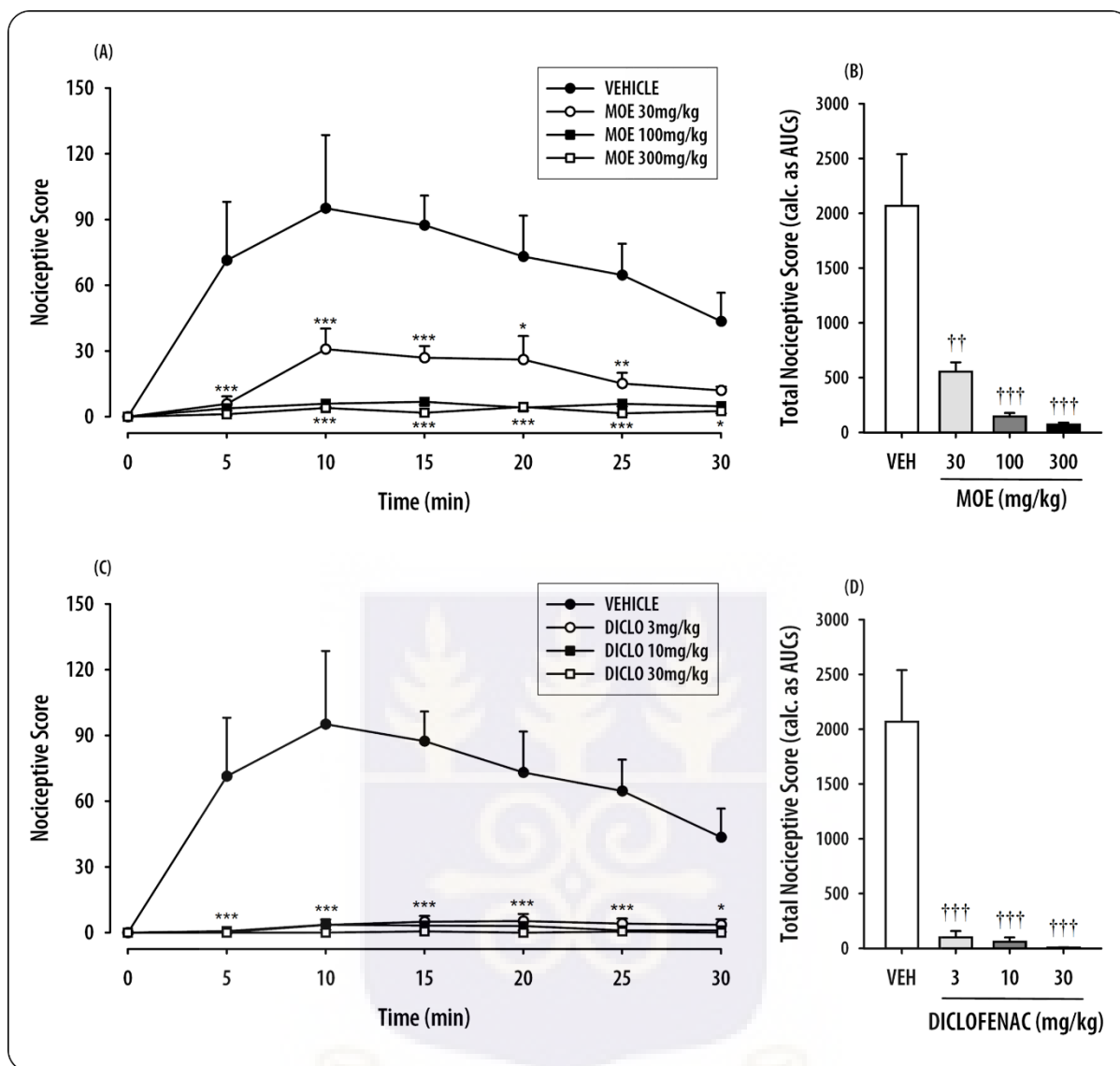


Figure 4.3.2. Effect of MOE (30, 100 and 300 mg/kg) and diclofenac (3, 10 and 30 mg/kg) on the time-course events (A and C respectively) and the total nociceptive score (calculated as AUCs) (B and D respectively) on acetic acid induced writhing behaviour in ICR mice.

Nociceptive scores are shown in 5-minute time blocks up to 30 minutes post acetic acid injection. Plotted data are mean \pm SEM (n=5) *P<0.05, **P<0.01 and ***P<0.001 compared to the vehicle-treated group (two-way ANOVA followed by Bonferroni's *post hoc* test). †P<0.05, ††P<0.01, †††P<0.001 compared to the vehicle-treated group (one-way ANOVA followed by Dunnett's multiple comparisons test).

4.3.3. Hot Plate Test

Figure 4.3.3 shows the effect of MOE (30-300 mg/kg) and morphine (0.3-3 mg/kg) on paw withdrawal latencies calculated as %MPE in the hot plate test. As depicted by the time course curve in Figure 4.3.3A, the oral administration of MOE significantly increased paw withdrawal latencies ($F_{3,16}=6.23$, $P=0.005$ [two-way ANOVA]) compared to the distilled water treated group over the 3-hour test period. Analysis of the AUCs (Figure 4.3.3B) shows that MOE significantly ($F_{3,16}=6.48$, $P=0.004$ [one-way ANOVA]) increased total paw withdrawal thresholds with a maximum effect ($P=0.002$) [one-way ANOVA] at the highest dose (300 mg/kg). However, MOE at 30 mg/kg (Figure 4.3.3B) was not statistically significant ($P=0.21$ [one-way ANOVA]).

In a very similar way, the intraperitoneal injection of the positive control drug used, morphine (Figure 4.3.3C) the centrally acting opioid analgesic, 30 minutes before the hot plate testing displayed a significant and dose-dependent increase in paw withdrawal latencies ($F_{3,16}=6.23$, $P=0.005$ [two-way ANOVA]), compared to the distilled water treated group over the 3 hour test period. AUC analysis (Figure 4.3.3D) revealed that overall, morphine significantly ($F_{3,16}=14.92$, $P<0.001$ [one-way ANOVA]) and dose-dependently increased total paw withdrawal thresholds with a maximum effect at the highest dose (3 mg/kg; $P<0.001$; [one-way ANOVA]).

ED_{50} s were calculated by non-linear regression method (table 4.3.3). Comparison with the F-test revealed that diclofenac was more potent than MOE in the writhing test ($P<0.001$).

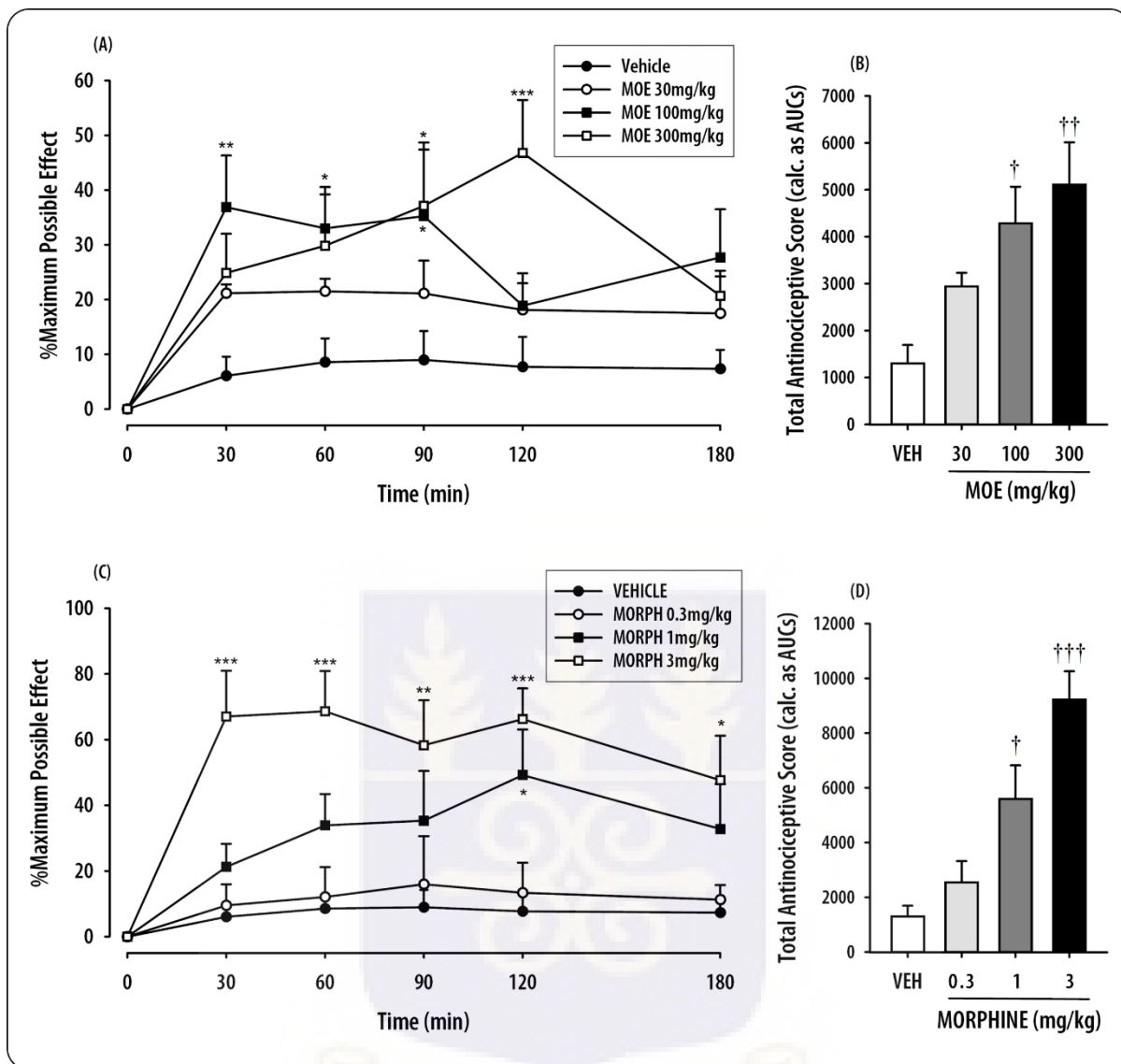


Figure 4.3.3. Effect of MOE (30, 100 and 300 mg/kg) and morphine (0.3, 1 and 3 mg/kg) on the time-course events (A and C respectively) and the total antinociceptive score (calculated as AUCs) (B and D respectively) on thermally induced jumping and paw licking behaviour in ICR mice.

Nociceptive scores are shown from 0 to 180 minutes post MOE administration (*p.o.*). Plotted data are mean \pm SEM (n = 5). * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ compared to the vehicle-treated group (two-way ANOVA followed by Bonferroni's *post hoc* test). † $P < 0.05$, †† $P < 0.01$, ††† $P < 0.001$ compared to the vehicle-treated group (one-way ANOVA followed by Dunnett's multiple comparisons test).

Table 4.5. ED₅₀s for MOE and morphine in the hot plate test

DRUG	ED ₅₀ (mg/kg)
MOE	380.41 ± 91.28
Morphine	1.33 ± 0.35

4.3.4. The Possible Mechanism(s) of Antinociception

Figure 4.4.1-4.4.3 shows the effect of the pre-treatment of mice with 7 different antagonists on the antinociceptive activity of MOE (100 mg/kg *p.o*) and morphine (1 mg/kg *i.p*) in the formalin test.

Systemic pre-treatment of ICR mice with naloxone (2 mg/kg), glibenclamide (8 mg/kg), theophylline (10 mg/kg), ondansetron (0.5 mg/kg), yohimbine (3 mg/kg), nifedipine (10 mg/kg) and atropine (5 mg/kg) could not significantly block the antinociceptive activity of MOE in the first phase of the formalin test (Figure 4.4.3) [naloxone (P=0.17), glibenclamide (P=0.99), theophylline (P=0.19), ondansetron (P<0.24), yohimbine (P>0.99), nifedipine (P=0.16) and atropine (P=0.39) (One-way ANOVA followed by Dunnett's multiple comparisons test)]. Similarly, naloxone, glibenclamide, theophylline, ondansetron, yohimbine, nifedipine and atropine could not significantly reverse the antinociceptive effects of MOE in the second phase of the formalin test (P=0.37; P=0.95; P=0.95; P=0.64; P=0.92; P=0.15 and P=0.73 respectively [Figure 4.4.3]).

In the positive control tests, morphine (1 mg/kg) was used as the reference drug (Figure 4.4.2). Pre-treatment of ICR mice with naloxone and theophylline significantly blocked the antinociceptive activity of morphine in both the first phase (P<0.001 for both) and the second phase (P<0.01 and P<0.03 respectively) of the formalin test exhibited as an increase in the

frequency and time of formalin-induced paw licking in mice (Figure 4.4.3). Similarly, pre-treatment of mice with ondansetron, a 5-HT₃ receptor antagonist, significantly reversed the antinociception caused by morphine in both phase one ($P < 0.001$) and phase two ($P < 0.03$) of the formalin test. In contrast, pre-treatment with glibenclamide, yohimbine, nifedipine and atropine did not significantly reverse the antinociceptive activity of morphine in the first phase ($P > 0.99$, $P > 0.99$, $P = 0.43$, $P > 0.99$ respectively) and second phase ($P = 0.97$, $P > 0.99$, $P = 0.93$, $P = 0.72$ respectively) of the formalin test in mice (Figure 4.4.3).



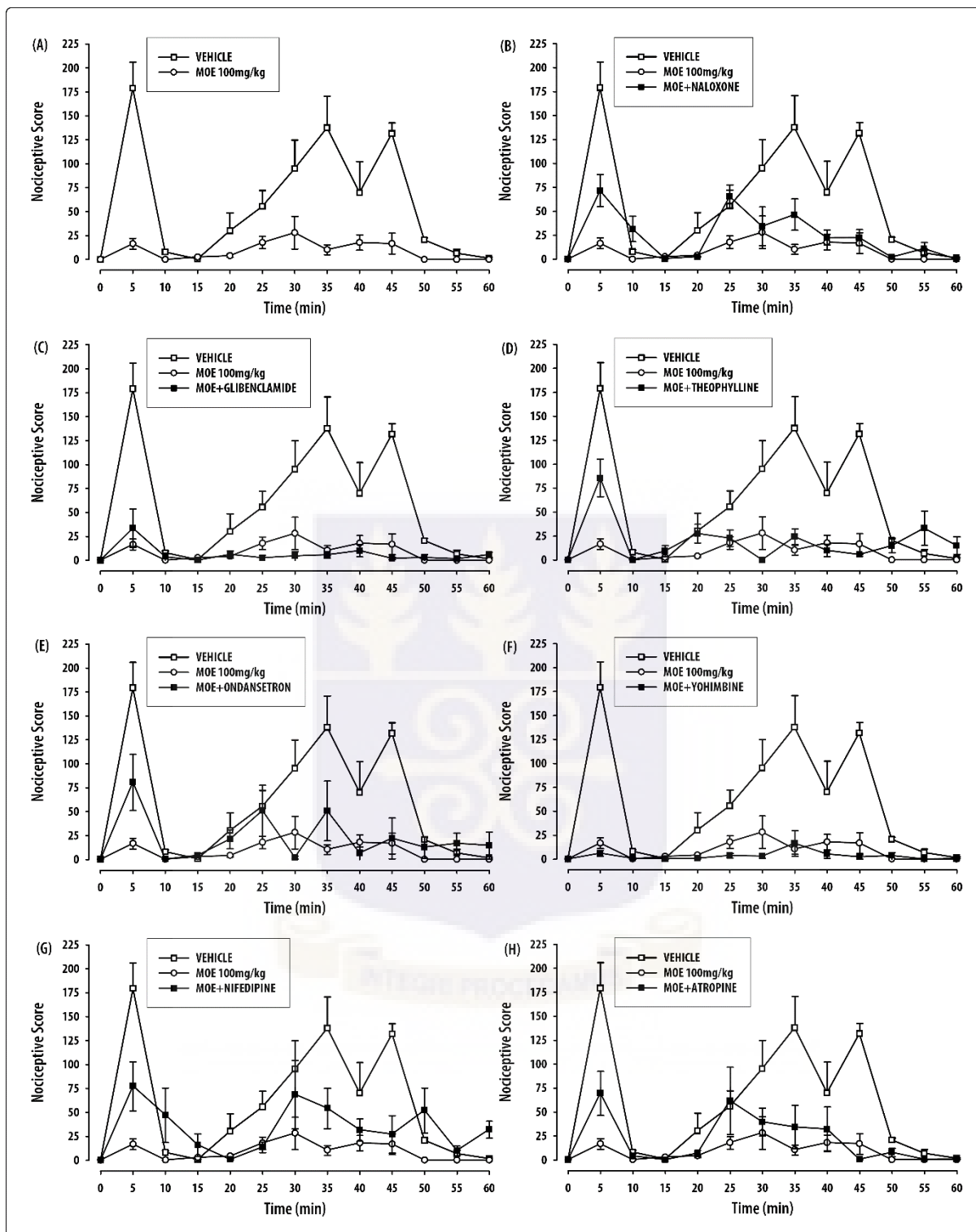


Figure 4.4.1. The effect of the pre-treatment of ICR mice with (B) naloxone (2 mg/kg), (C) glibenclamide (8 mg/kg), (D) theophylline (10 mg/kg), (E) ondansetron (0.5 mg/kg), (F) yohimbine (3 mg/kg), (G) nifedipine (10 mg/kg) and (H) atropine (5 mg/kg) on the antinociceptive activity of MOE (100 mg/kg *p.o.*) in the first and second phases of formalin-induced nociception.

Nociceptive scores are shown in 5-minute time blocks up to the 60th minute post formalin injection. Plotted data are mean \pm SEM (n = 5).

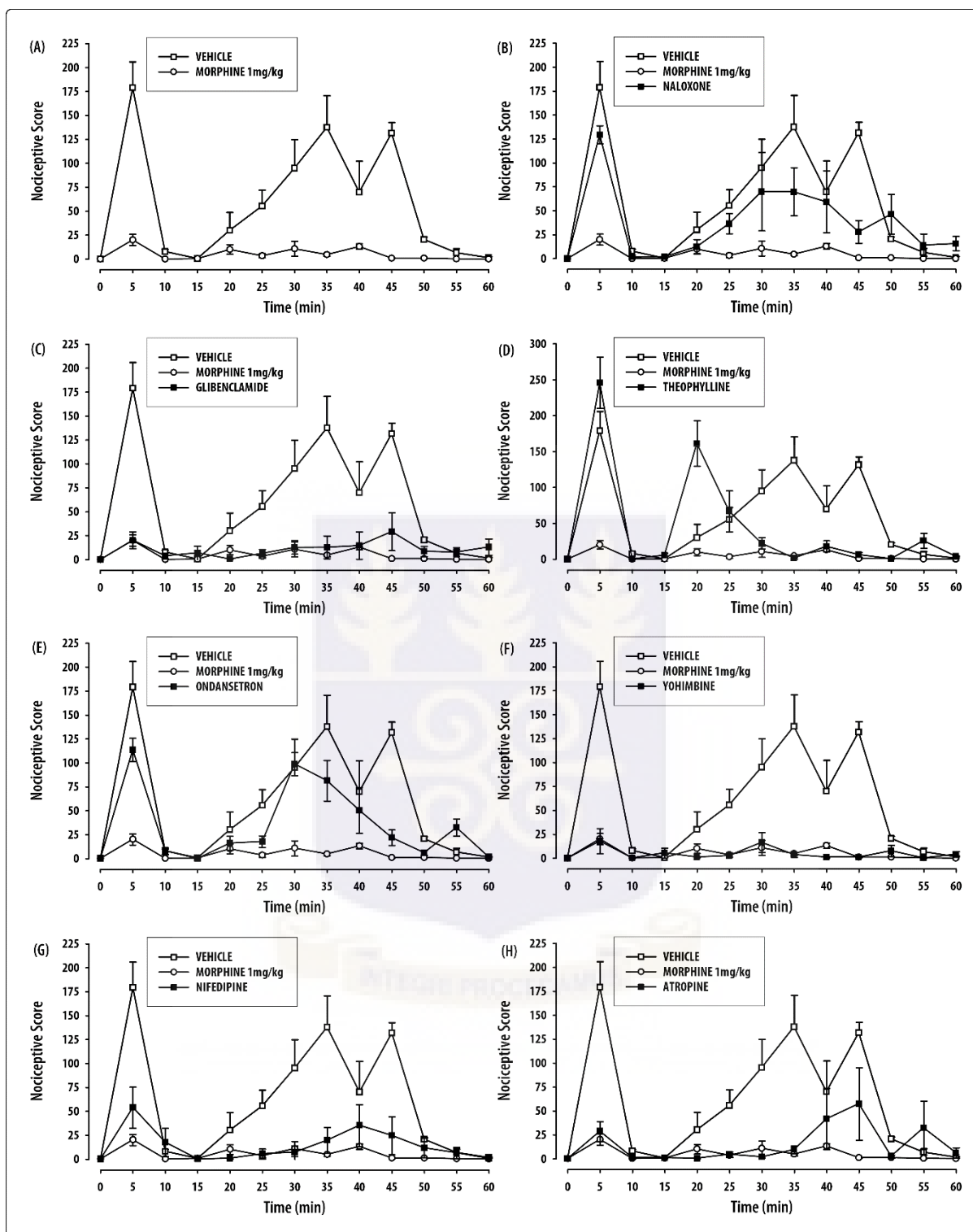


Figure 4.4.2. The effect of the pre-treatment of ICR mice with (B) naloxone (2 mg/kg), (C) glibenclamide (8 mg/kg), (D) theophylline (10 mg/kg), (E) ondansetron (0.5 mg/kg), (F) yohimbine (3 mg/kg), (G) nifedipine (10 mg/kg) and (H) atropine (5 mg/kg) on the antinociceptive activity of morphine (1 mg/kg *i.p.*) in the first and second phases of formalin-induced nociception.

Nociceptive scores are shown in 5-minute time blocks up to the 60th minute post formalin injection. Plotted data are mean \pm SEM (n = 5).

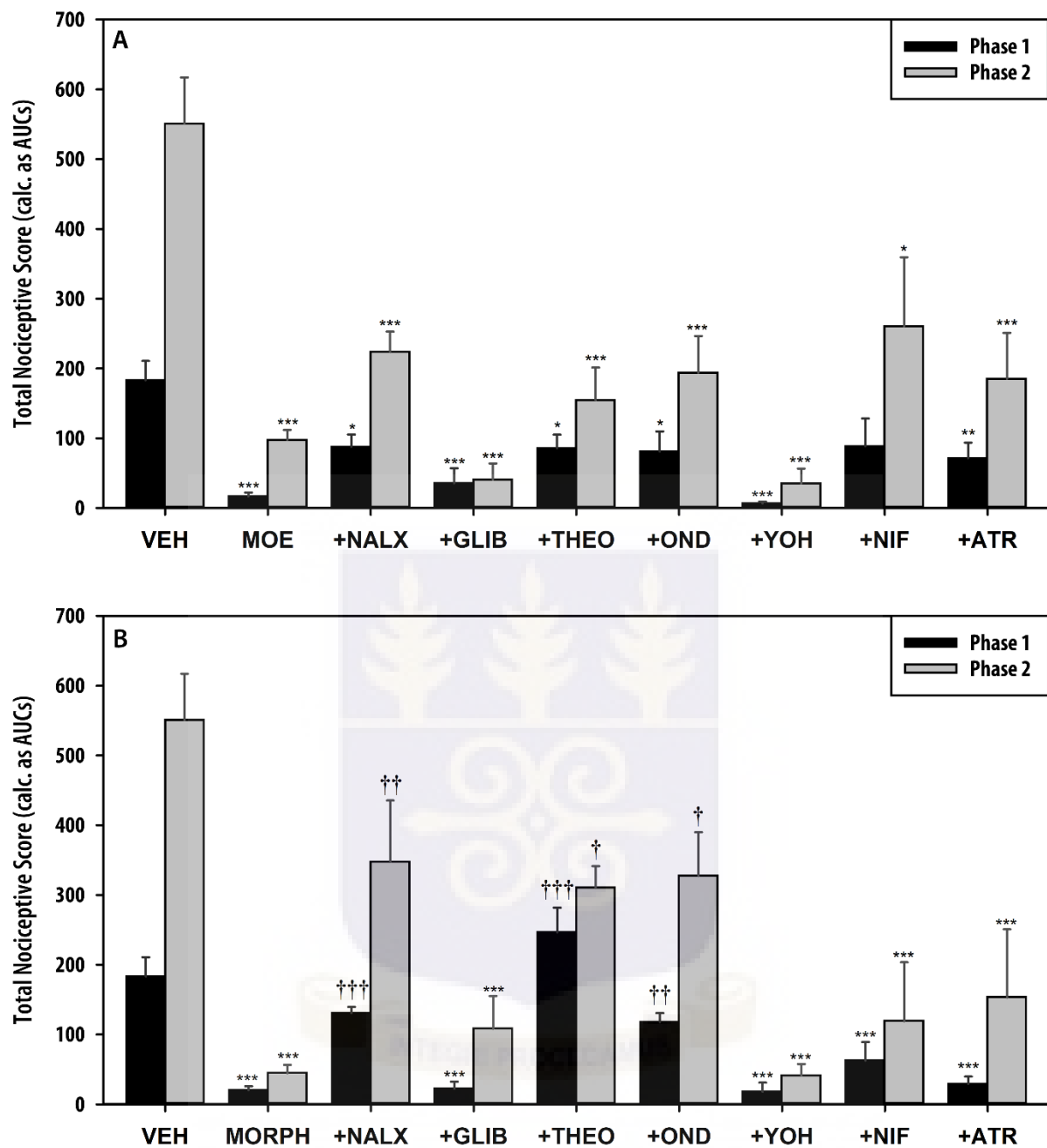


Figure 4.4.3. The effect of the pre-treatment of ICR mice with naloxone (2 mg/kg), glibenclamide (8 mg/kg), theophylline (10 mg/kg), ondansetron (0.5 mg/kg), yohimbine (3 mg/kg), nifedipine (10 mg/kg) and atropine (5 mg/kg) on the antinociceptive activity of (A) MOE (100 mg/kg *p.o.*) and (B) morphine (1 mg/kg *i.p.*) in the first and second phases of formalin-induced nociception.

Each column represents mean \pm SEM. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ compared to the vehicle-treated group (two-way ANOVA followed by Bonferroni's *post hoc* test). † $P < 0.05$, †† $P < 0.01$, ††† $P < 0.001$ compared to the either MOE- or morphine-treated group (one-way ANOVA followed by Dunnett's multiple comparisons test).

4.4. The Effect of MOE in Paclitaxel-induced Peripheral Neuropathy

4.4.1. Induction of Peripheral Neuropathy

Assessment of thermal hyperalgesia, cold allodynia and mechanical hyperalgesia during the 5-day induction (day 1 to 5) of peripheral neuropathy with a cumulative paclitaxel dose of 10 mg/kg yielded a gradual decline in latencies and nociceptive thresholds within the groups used, from day 0 to day 5 as depicted by the time course curves (Figure 4.5.1 A,C,E).

Two-way ANOVA followed by Bonferroni's *post hoc* test showed a significant decline in latencies and paw withdrawal thresholds in the thermal hyperalgesia test ($P < 0.0001$) and in the mechanical hyperalgesia test ($P < 0.0001$) respectively, comparing day 0 (baseline) to day 5 (day of final paclitaxel administration) (Figure 4.5.1A-D). This suggests a reduced pain threshold and confirms the induction of peripheral neuropathy. Analysis of the responses between day 0 and day 5 in the cold allodynia test (Figure 4.5.1E-F) revealed a significant reduction in tail withdrawal latencies (group 1: $P = 0.003$; group 2: $P = 0.04$; group 3: $P = 0.04$; group 6: $P = 0.001$; group 7: $P = 0.03$). However, decline in latencies for group 4 ($P = 0.051$) and group 5 ($P = 0.23$) did not reach statistical significance.

Two deaths were recorded on day 0, pre-paclitaxel administration. No deaths were recorded during the induction period. However, two deaths were recorded on day 2, two on day 3, two on day 4 and one on day 5 due to cannibalism by cage members.

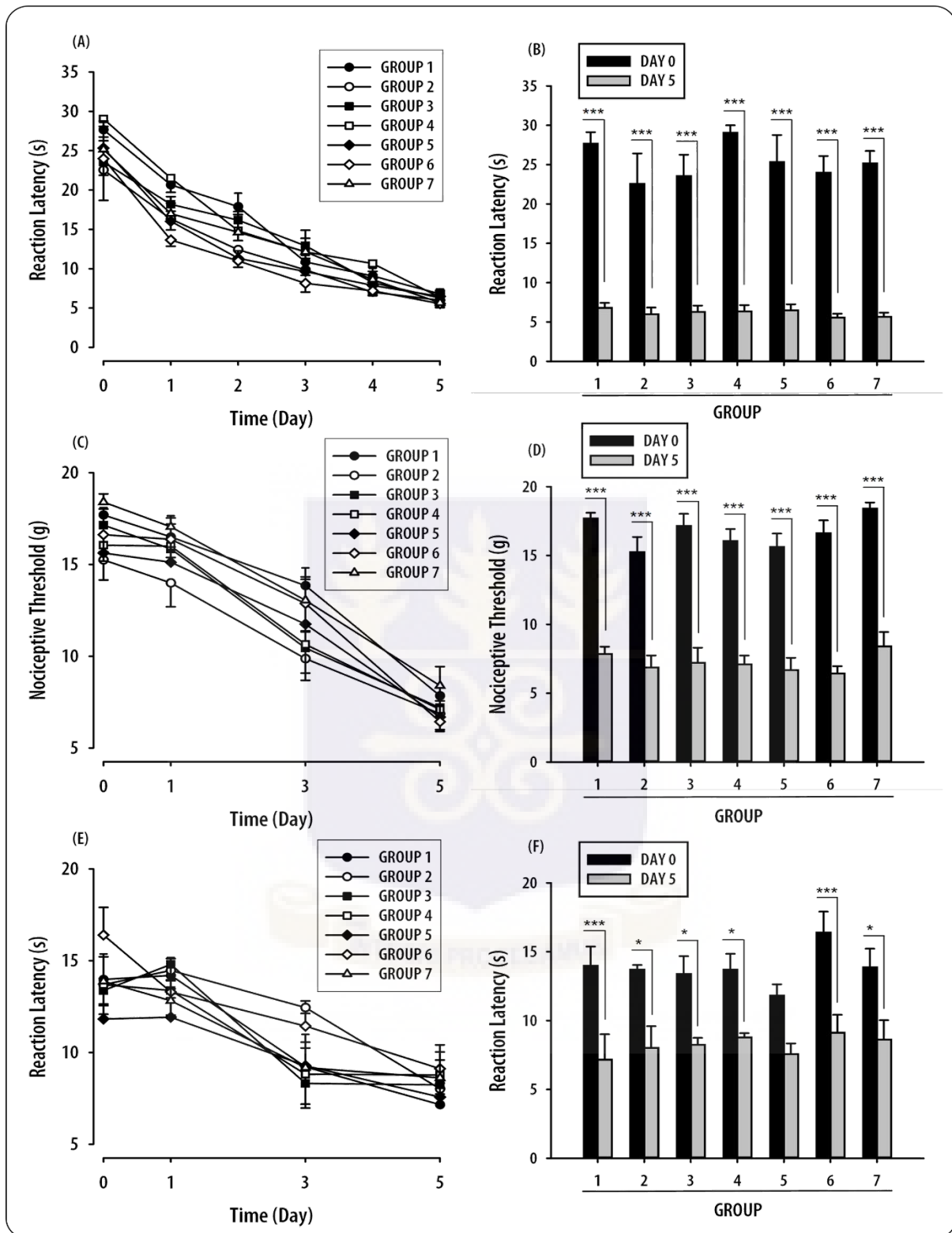


Figure 4.5.1: Effect of the daily intraperitoneal administration of paclitaxel (2 mg/kg) on thermal hyperalgesia (A-B), mechanical hyperalgesia (C-D) and cold allodynia (E-F) tests in ICR mice for 5 days.

A, C, E: Time-course event from day 0 to 5. **B, D, F:** A comparison of reaction latency on day 0 and day 5. Plotted data are mean \pm SEM (n = 5). *P < 0.05, **P < 0.01 and ***P < 0.001 compared to the vehicle-treated group (two-way ANOVA followed by Bonferroni's *post hoc* test).

4.4.2. Thermal Hyperalgesia (Hot Plate test)

As depicted in Figure 4.5.2, sustained thermal hyperalgesia was observed in the mice in the vehicle treated group from day 1 through to day 5. MOE (30-300 mg/kg) and pregabalin (10-100 mg/kg) produced significant and dose dependent reversal of thermal hyperalgesia (MOE: $F_{3,65}=82.6$, $P<0.0001$; pregabalin: $F_{3,66}=33.38$, $P<0.0001$) displayed as an increase in latency to nocifensive behaviour on the hot plate apparatus. Treatment of mice with MOE at the doses of 100 mg/kg and 300 mg/kg (Figure 4.5.2A, B) produced significant reversal of thermal hyperalgesia each day from test day one to day five. However, results obtained when mice were treated daily with MOE at 30 mg/kg produced significant reversal of thermal hyperalgesia on day 5 ($P=0.009$). Analysis of AUCs revealed that overall, MOE significantly ($F_{3,15}=4.86$; $P=0.01$; [one-way ANOVA]) and dose-dependently reversed thermal hyperalgesia with a maximum effect at the highest dose (300 mg/kg; $P<0.001$; [one-way ANOVA]).

Similarly, treatment of mice with pregabalin at the dose of 100 mg/kg (Figure 4.5.2C-D) produced a significant reversal of thermal hyperalgesia in the hot plate test, daily, from test day one to day five. The pregabalin dose at dose 30 mg/kg yielded significant analgesic effect from day 2 to day 5. Treatment of mice with the lowest dose of pregabalin yielded no statistically significant results except on day 5 ($P=0.03$). AUC analysis revealed that pregabalin significantly inhibited thermal hyperalgesia at the doses of 100 mg/kg ($P<0.001$) and 30 mg/kg ($P<0.001$).

The ED_{50} s (mg/kg) obtained by non-linear regression for MOE and pregabalin are 150.1 and 42.43 respectively. Thus, MOE was about 3.5 times less potent than pregabalin in the thermal hyperalgesia test.

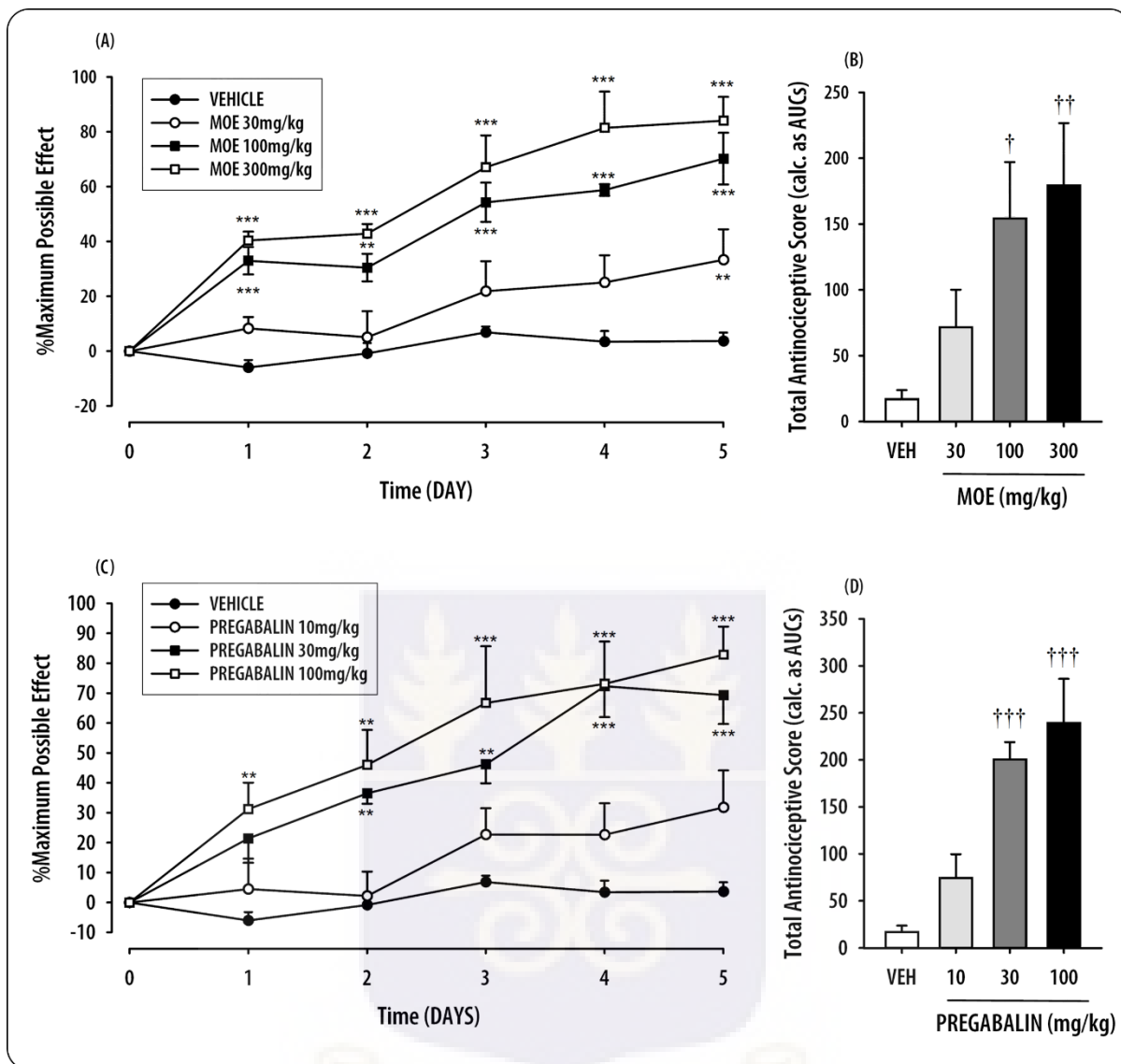


Figure 4.5.2: Effect of MOE (30, 100 and 300 mg/kg) and Pregabalin (10, 30 100 mg/kg) on thermal hyperalgesia in paclitaxel induced neuropathy in ICR mice. The left panels represent time-course effects of MOE (A) and pregabalin (C) from day 0 to day 5 after neuropathy was confirmed.

The right panels represent the total antinociceptive effects (calculated as AUCs) of MOE (B) and pregabalin (D). Plotted data are mean \pm SEM (n = 5). **P<0.01 and ***P<0.001 compared to the vehicle-treated group (two-way ANOVA followed by Bonferroni's *post hoc* test). †P<0.05, ††P<0.01, †††P<0.001 compared to the vehicle-treated group (one-way ANOVA followed by Dunnett's multiple comparisons test).

Table 4.5.2. ED₅₀s for MOE and pregabalin in the thermal hyperalgesia test

DRUG	ED₅₀ (mg/kg)
MOE	150.1 ± 58.03
Pregabalin	42.43 ± 9.94



4.4.3. Mechanical Hyperalgesia (Randall-Selitto test)

Both mice hind paws demonstrated distinct mechanical hyperalgesia in baseline measurements taken on day 0 in this experiment. From figure 4.5.3, sustained mechanical hyperalgesia was observed in the vehicle-treated mice. MOE (30-300 mg/kg) and pregabalin (10-100 mg/kg) produced significant and dose dependent reversal of mechanical hyperalgesia (MOE: $F_{3,65}=53.75$, $P<0.0001$; pregabalin: $F_{3,65}=81.56$, $P<0.0001$; two-way ANOVA). Treatment of mice with MOE at the doses of 100 mg/kg and 300 mg/kg (Figure 4.5.3A) significantly reversed mechanical hyperalgesia from day one to five (MOE 300 mg/kg) and day two to five (MOE 100 mg/kg). Treatment with MOE at the dose of 30 mg/kg significantly reversed mechanical hyperalgesia on day 5 ($P=0.05$). One-way ANOVA followed by Dunnett's multiple comparisons test of the AUCs (Figure 4.5.3B) shows that MOE significantly and dose-dependently reversed mechanical hyperalgesia at the doses of 100 mg/kg ($P=0.03$) and 300 mg/kg ($P=0.01$).

As depicted by figure 4.5.3C, daily treatment of mice with pregabalin at the doses of 30 mg/kg and 100 mg/kg significantly ($P<0.0001$) reversed mechanical hyperalgesia from day one to five. However, inhibition of mechanical hyperalgesia by pregabalin at 10 mg/kg was not statistically significant on any of the test days as revealed by two-way ANOVA analysis followed by Bonferroni's multiple comparisons test. Analysis of the AUCs (figure 4.5.3D) shows that overall, pregabalin dose-dependently and significantly ($F_{3,13}=25.54$, $P<0.001$ [one-way ANOVA]) reversed mechanical hyperalgesia in both hind paws of the mice from day 1 to 5 with maximum inhibitions ($P<0.001$) occurring at 30-100 mg/kg dose levels.

The ED_{50} s (mg/kg) obtained by non-linear regression for MOE and pregabalin in this test are 112.4 and 25.11 respectively (Table 4.5.3). Thus, MOE was about 4.5 times less potent than pregabalin in the mechanical hyperalgesia test.

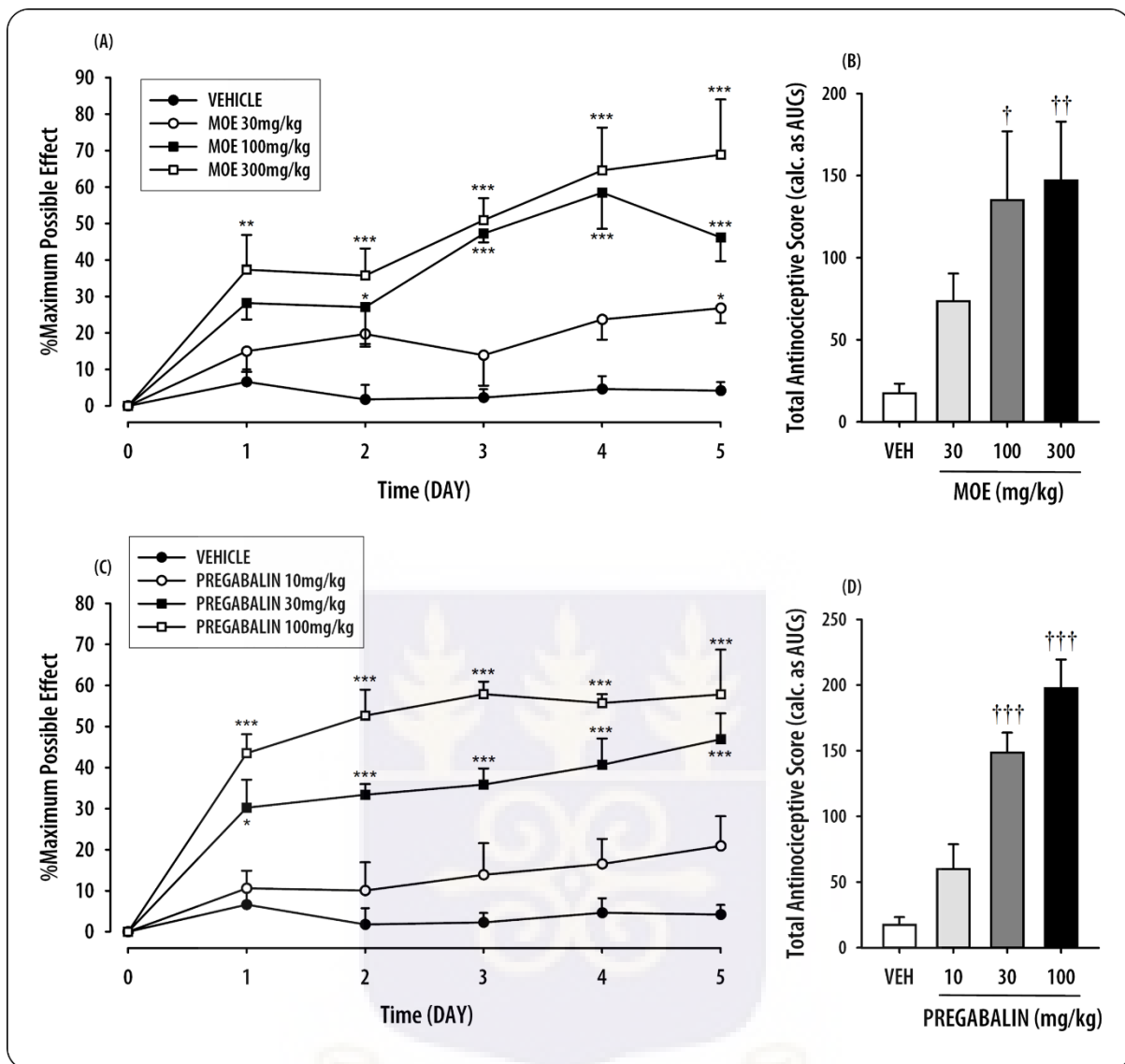


Figure 4.5.3: Effect of MOE (30, 100 and 300 mg/kg) and Pregabalin (10, 30 100 mg/kg) on mechanical hyperalgesia in paclitaxel induced neuropathy in ICR mice. The left panels represent time-course effects of MOE (A) and pregabalin (C) from day 0 to day 5 after neuropathy was confirmed.

The right panels represent the total antinociceptive effects (calculated as AUCs) of MOE (B) and pregabalin (D). Plotted data are mean \pm SEM (n = 5). *P<0.05, **P<0.01 and ***P<0.001 compared to the vehicle-treated group (two-way ANOVA followed by Bonferroni's *post hoc* test). †P<0.05, ††P<0.01, †††P<0.001 compared to the vehicle-treated group (one-way ANOVA followed by Dunnett's multiple comparisons test).

Table 4.5.3. ED₅₀s for MOE and pregabalin in the mechanical hyperalgesia test

DRUG	ED ₅₀ (mg/kg)
MOE	112.4 ± 45.72
Pregabalin	25.11 ± 6.05

4.4.4. Cold Allodynia

The daily administration of MOE to mice from treatment day 1 to 5 (figure 4.5.4A) yielded significant anti-cold allodynic effects at the dose of 300 mg/kg on the fourth (P=0.01) and fifth (P=0.005) days (two-way ANOVA) exhibited in the test as an increased latency to tail withdrawal from cold water at 4°C. Reversal of cold allodynia when mice were treated daily with MOE at 30 mg/kg and 100 mg/kg was not statistically significant on any of the 5 test days. Analysing the AUCs with one-way ANOVA followed by Dunnett's multiple comparisons test (figure 4.5.4B) reveals that overall inhibition of cold allodynia was not significant (F_{3,13}=0.93, P=0.45) when compared to the AUC of the vehicle treated group [(MOE 30: P>0.99; MOE 100: P=0.89; MOE 300: P=0.34).

From figure 4.5.4C pregabalin at the dose of 100 mg/kg produced significant analgesic effect in the cold allodynia test on the 4th (P<0.001) and 5th (P<0.001) days of administration and testing. Analgesic effect of pregabalin at 30 mg/kg produced significant antiallodynic effect on the 4th day (P<0.03) alone. Daily treatment with the dose of 10 mg/kg did not yield any significant anti-allodynic effects on any of the test days. Analysis of AUCs in figure 4.5.4D reveals that overall, pregabalin at 100 mg/kg produced significant antiallodynic effect (P=0.03) in the test conducted. Overall, analgesic effects of the doses of pregabalin at 10 mg/kg (P=0.42) and 30 mg/kg (P=0.12) did not reach statistical significance in the test.

The ED₅₀s (mg/kg) obtained by non-linear regression for MOE and pregabalin in this test are 584.8 and 58.07 respectively (Table 4.5.4). Thus, MOE was about 10 times less potent than pregabalin in this cold allodynia test.

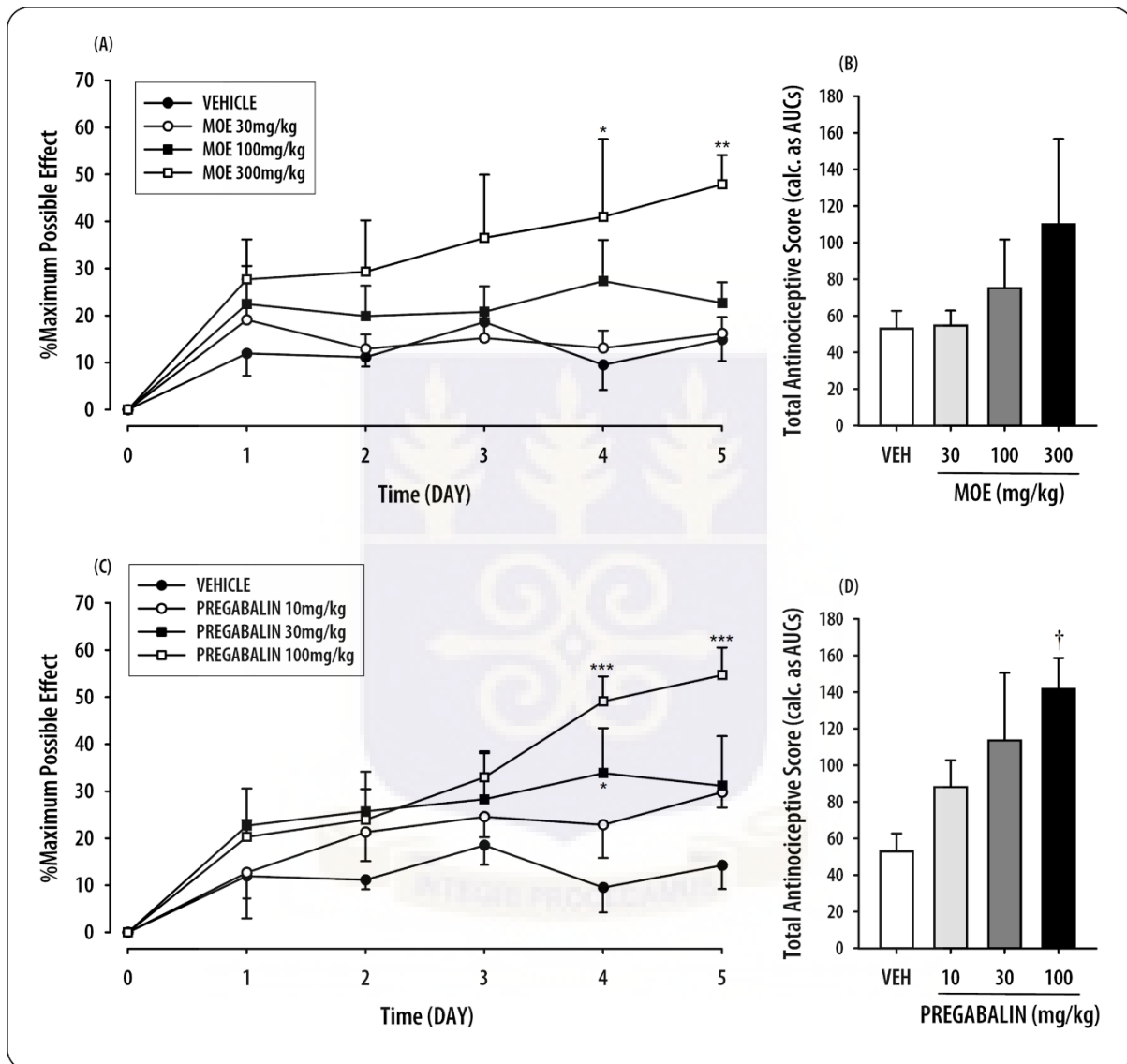


Figure 4.5.4. Effect of MOE (30, 100 and 300 mg/kg) and Pregabalin (10, 30 100 mg/kg) on cold allodynia in paclitaxel induced neuropathy in ICR mice.

The left panels represent of MOE (A) and pregabalin (C) from day 0 to day 5 after neuropathy was confirmed. The right panels represent the total antinociceptive effects (calculated as AUCs) of MOE (B) and pregabalin (D). Plotted data are mean \pm SEM (n = 5). *P<0.05, **P<0.01 and ***P<0.001 compared to the vehicle-treated group (two-way ANOVA followed by Bonferroni's *post hoc* test). †P<0.05, ††P<0.01, †††P<0.001 compared to the vehicle-treated group (one-way ANOVA followed by Dunnett's multiple comparisons test).

Table 4.5.4. ED₅₀s for MOE and pregabalin in the cold allodynia test

DRUG	ED₅₀ (mg/kg)
MOE	584.8 ± 395.41
Pregabalin	58.07 ± 23.86



4.5. Sub-chronic Oral Toxicity (90-Day)

Two Sprague-Dawley rats from the control group, the low dose group and the median dose group died. Two more died from the high dose group. This was due to oral administration problems and affected rats were isolated.

4.5.1. Necropsy and clinical signs

For the rats that were not isolated, no death or serious clinical signs were found in any of the groups all through the 90-day experimental period. Daily visual observation of the MOE (30, 100 and 300 mg/kg; group 1-3) treated rats reported no signs of toxicity on their skin, fur or eyes. They didn't exhibit any behavioural changes, coma, tremors or salivation. Rats in the vehicle treated groups produced similar results on routine checks. LD₅₀ is estimated above 3000 mg/kg.

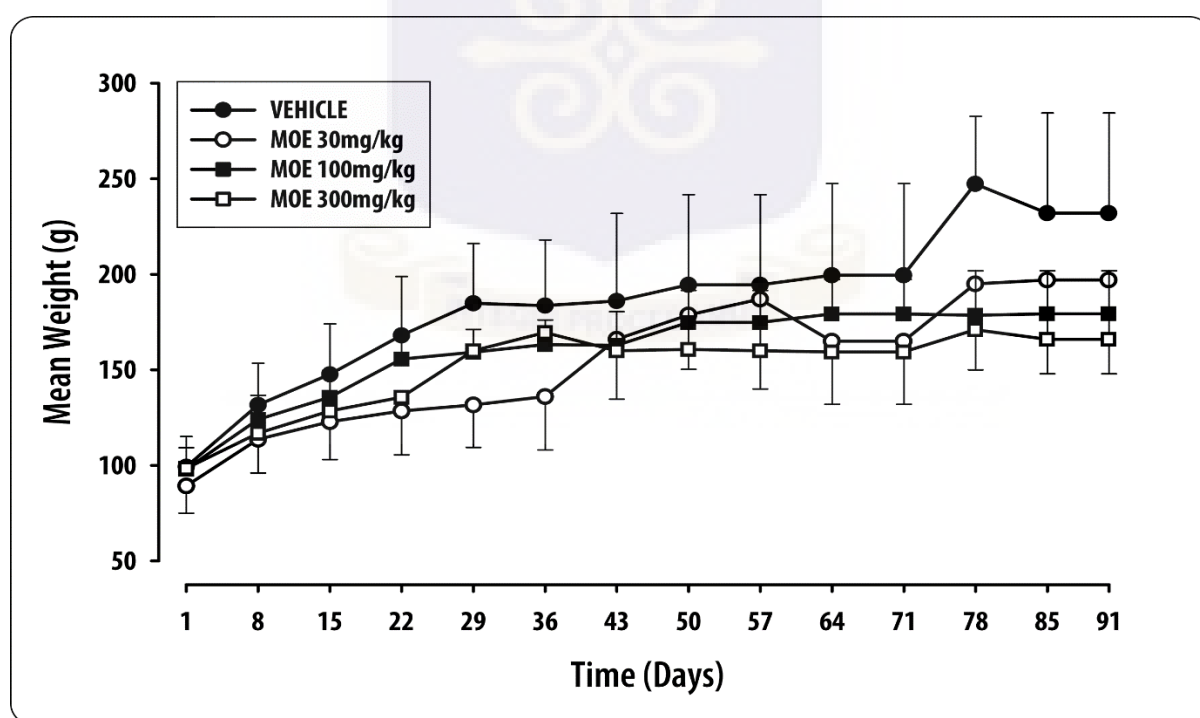


Figure 4.6.1. Mean weights of Sprague-Dawley rats for all groups(n=5) from day 1 to day 91.

4.5.2. Effect of daily MOE administration on weight change

Figure 4.6.1 is a graphical representation of the change in mean weights for all groups from day 1 to day 91. There was consistent weight gain over time in all the groups ($P < 0.001$). Rats in all groups demonstrated rapid weight gain during the first 5 weeks and a slower rate thereafter. However, compared with the vehicle treated group, there were no dose-specific patterns in body weight related to drug treatment ($F_{3,160} = 0.47$, $P = 0.70$).

4.5.3. Effect of daily MOE administration on relative organ weights

The relative organ weights of the rats are shown in Tables 4.6.1. The relative organ weight for each organ (liver, kidney, heart, spleen, brain) recorded at necropsy for rats in the extract treated groups did not show any significant difference compared to the control ($P > 0.5$).

Table 4.6.1. The relative organ weights of rats treated with MOE over the 90-day period

Organs	Control	MOE (mg/kg)		
		30	100	300
Liver	3.93±0.06	4.43±0.08	3.13±0.79	3.70±0.44
Kidneys	0.32±0.02	0.43±0.02	0.37±0.01	0.37±0.09
Heart	0.39±0.01	0.44±0.05	0.40±0.02	0.48±0.03
Spleen	0.30±0.05	0.30±0.01	0.27±0.04	0.31±0.04
Brain	0.80±0.19	0.90±0.16	1.02±0.14	1.27±0.50

The values are presented as Means ± SEM

4.5.4. Effect of daily MOE administration on RBC Indices

The influence of the daily administration of MOE on haematological indices for the male Sprague-Dawley rats after the 90-day sub-chronic toxicity study are displayed on Table 4.6.2. Analysis of the RBC indices for all groups revealed that the MOE treated groups showed no

significant changes/differences in any of the RBC indices ($F_{3,48}=1.545$, $P=0.22$) compared to the vehicle treated group.

4.5.5. Effect of daily MOE administration on WBC Indices

Table 4.6.2 shows the effect of MOE on the levels of lymphocytes, neutrophils, eosinophils, basophils, monocytes and the total WBC in the 90-day toxicity study. No significant differences were observed for the levels of monocytes, eosinophils and basophils when compared to the vehicle treated group. However, the levels of neutrophils for the group treated with the median dose (100 mg/kg) was significantly higher ($P=0.01$) compared to the vehicle treated group. Overall, differences in neutrophil levels was not dose-dependent. Percent lymphocyte levels were also significantly lower ($P=0.03$) for the median dose group. Overall, treatment with MOE had no significant dose dependent effect on the WBC indices ($F_{3,36}=0.1205$, $P=0.95$).

4.5.6. Effect of daily MOE administration on Platelet Indices

Analysis of the platelet indices displayed on Table 4.6.2 revealed a significant decrease in platelet count (PLT) for the 30 mg/kg dose $P=0.01$ and the 100 mg/kg dose ($P=0.02$) compared to the control group. However, there were no significant difference in the platelet ratio (PCT) and size (PDW and MPV) for any of the 3 MOE treated groups compared to the vehicle treated group.

Table 4.6.2. The haematological indices of rats treated with MOE over the 90-day period

Parameter	Unit	Control	MOE (mg/kg)		
			30	100	300
<i>RBC indices</i>					
RBC	10 ¹² /L	9.63±0.34	9.59±0.21	10.54±0.99	9.41±0.16
HGB	g/dL	16.23±0.49	15.4±0.30	16.77±1.05	15.10±0.50
HCT	%	40.97±1.53	38.7±0.90	43.00±3.44	38.45±0.75
MCV	fL	42.6±1.22	40.45±0.05	40.97±0.60	40.90±0.10
MCH	g/dL	16.83±0.50	16.00±0.00	15.93±0.47	16.00±0.30
MCHC	g/dL	39.33±0.33	40.00±0.00	39.33±0.67	39.50±0.50
RDW-SD	fL	35.23±1.03	33.3±1.20	34.47±1.36	35.05±0.55
RDW-CV	%	12.6±0.46	12.9±0.00	12.73±0.70	12.75±0.05
<i>WBC indices</i>					
WBC	10 ⁹ /L	12.69±3.55	4.18±0.15	16.29±3.51	15.68±7.92
LYM	%	63.91±3.44	73.2±0.29	42.29±14.04*	52.41±8.25
MON	%	8.65±1.60	9.59±0.73	9.61±1.62	8.72±1.80
NEU	%	25.7±4.72	15.32±1.57	46.38±14.65*	37.03±10.37
EOS	%	1.59±0.36	1.78±0.64	1.51±0.34	1.67±0.23
BASO	%	0.16±0.10	0.13±0.08	0.22±0.03	0.17±0.09
<i>Platelet indices</i>					
PLT	10 ⁹ /L	1272.67±70.05	818.5±6.5*	882±297.76*	1021.5±125.5
MPV	fL	3.83±0.13	4.15±0.15	3.87±0.09	4.05±0.05
PDW	fL	6.30±0.32	5.40±0.00	5.47±0.62	6.05±0.35
PCT	%	0.48±0.03	0.34±0.01	0.34±0.12	0.41±0.06

The values are presented as Means ± SEM **P* < 0.05

4.5.7. Effect of daily MOE administration on serum biochemistry parameters

Displayed on Table 4.6.3 are the results for the biochemical parameters at the end of the 90-day treatment period. The AST levels for the group treated with the median dose (100 mg/kg)

were significantly lower ($P=0.03$) compared to the vehicle treated group. Significantly lower values ($P<0.001$) were also recorded for ALP at the dose of 30 mg/kg. Differences were also recorded for other parameters such as the blood urea levels but these were not significant when compared to the vehicle treated group ($P>0.05$).

Table 4.6.3. Serum biochemistry parameters of rats treated with MOE over the 90-day period

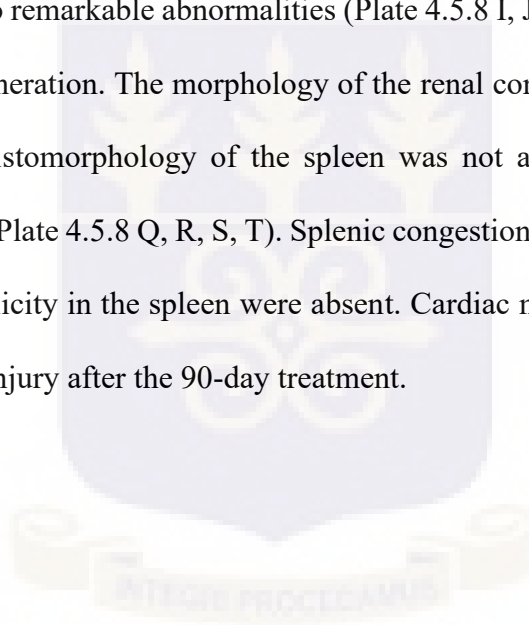
Parameter	Unit	Control	MOE (mg/kg)		
			30	100	300
Liver					
TBil	$\mu\text{mol/L}$	5.10 \pm 1.01	9.55 \pm 2.34	5.95 \pm 0.88	7.63 \pm 3.15
DBil	$\mu\text{mol/L}$	1.79 \pm 0.33	2.16 \pm 1.85	3.00 \pm 1.70	2.86 \pm 0.63
IBil	$\mu\text{mol/L}$	3.31 \pm 0.94	7.40 \pm 0.50	2.95 \pm 0.84	4.77 \pm 2.52
ALT	U/L	71.63 \pm 6.08	72.45 \pm 4.45	58.45 \pm 3.53	77.1 \pm 12.4
AST	U/L	148.53 \pm 27.96	116.85 \pm 6.85	94.9 \pm 10.42*	116.75 \pm 15.95
ALP	U/L	265.13 \pm 7.00	129.6 \pm 74.6*	303.85 \pm 64.87	238.11 \pm 4.11
GGT	U/L	4.00 \pm 2.31	5.50 \pm 3.50	9.33 \pm 0.88	12.50 \pm 6.50
Total Protein	g/L	70.47 \pm 1.43	64.70 \pm 1.30	65.7 \pm 2.43	59.20 \pm 3.80
ALB	g/L	27.37 \pm 1.62	27.50 \pm 1.10	23.93 \pm 1.36	23.55 \pm 3.35
GLB	g/L	43.10 \pm 3.04	37.20 \pm 0.20	41.77 \pm 1.34	35.65 \pm 0.45
A/G		0.65 \pm 0.08	0.74 \pm 0.03	0.57 \pm 0.03	0.66 \pm 0.09
Renal					
UREA	mmol/L	14.80 \pm 0.80	7.30 \pm 1.30	19.87 \pm 8.88	7.25 \pm 4.15
CREAT	$\mu\text{mol/L}$	71.05 \pm 7.64	74.72 \pm 18.09	68.9 \pm 12.54	70.32 \pm 14.04

The values are presented as Means \pm SEM

* $P < 0.05$

4.5.8. Histopathology

The photomicrographs obtained from sections of the brain, kidney, liver, heart and spleen from Sprague-Dawley rats treated with vehicle and MOE (30, 100 and 300 mg/kg) for the 90-day sub-chronic toxicity study period are shown in Plate 4.5.8. Histopathological evaluation of the organs revealed no significant extract-related changes when compared to the control animals except for some changes in the kidney for only one animal in the group receiving the low dose of 30 mg/kg which showed acute mononuclear cell inflammation and glomerulopathy. Comparison of liver morphological structure for the MOE treated groups to the distilled water treated group revealed no remarkable abnormalities (Plate 4.5.8 I, J, K, L). There was no tissue necrosis or cellular degeneration. The morphology of the renal corpuscles and hepatic lobules were all normal. The histomorphology of the spleen was not affected by the 90-day oral administration of MOE (Plate 4.5.8 Q, R, S, T). Splenic congestion or haemorrhage considered important features of toxicity in the spleen were absent. Cardiac myocytes (Plate 4.5.8 M, N, O,P) did not suffer any injury after the 90-day treatment.



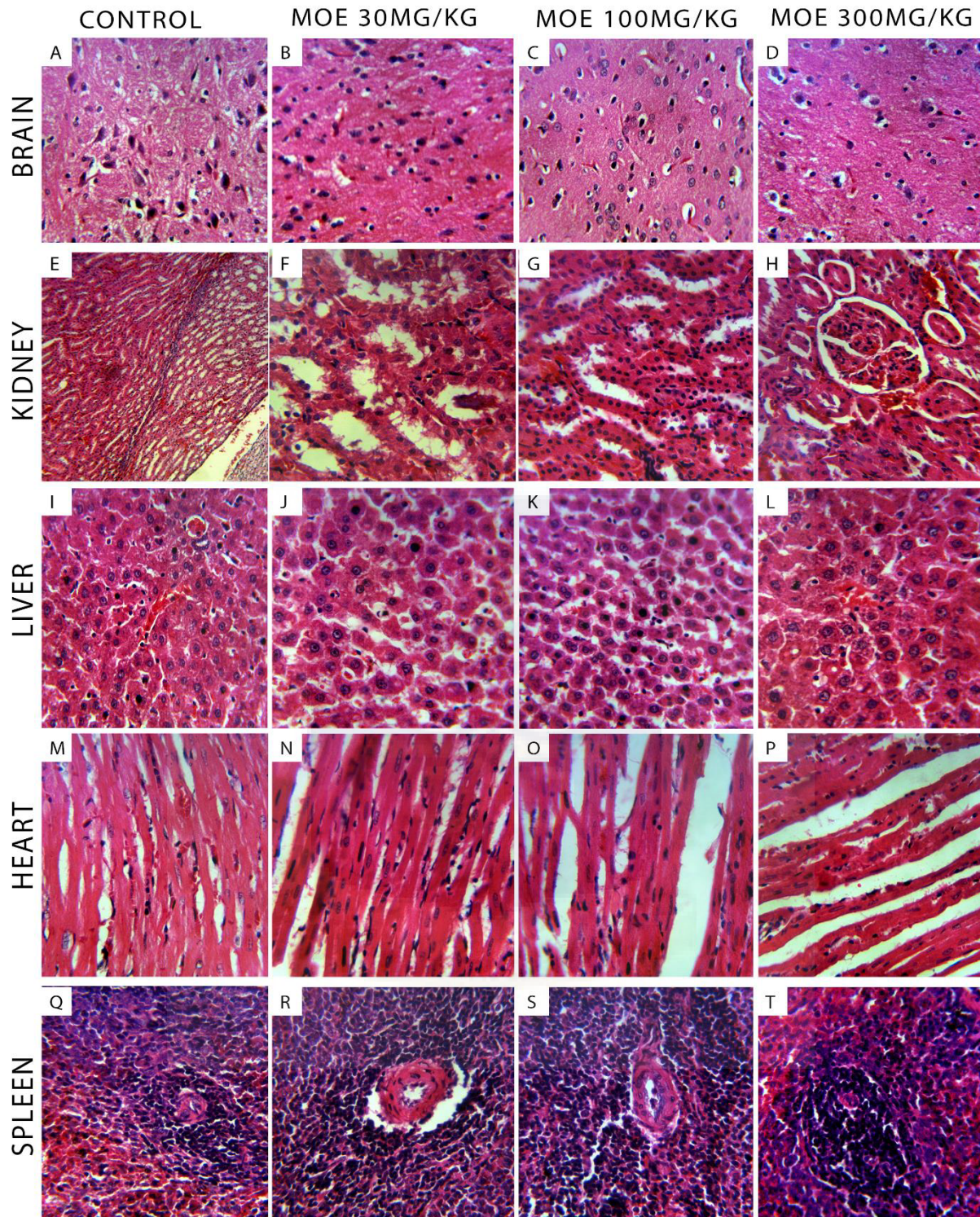


Plate 4.5.8. Photomicrographs of the brain, kidney, liver, heart and spleen in distilled water treated rats and rats treated with MOE (30, 100 and 300 mg/kg) daily over the 90-day sub-chronic toxicity study period (H & E, $\times 400$).

Chapter 5

DISCUSSION, CONCLUSION AND RECOMMENDATIONS

5.1. DISCUSSION

This study was aimed at assessing the possible antinociceptive activity of the hydroethanolic leaf extract of *Mallotus oppositifolius* (Geisler) Mull. Arg. using acute and chronic pain models in rodents. The findings obtained clearly suggest that the *Mallotus oppositifolius* extract has potent anti-nociceptive properties. They also clearly suggest that MOE possesses anti-hyperalgesia activity against thermally and mechanically induced hyperalgesia in paclitaxel-induced peripheral neuropathy mice. Findings clearly confirm an earlier report of the analgesic activity of the hydroethanolic leaf extract of *Mallotus oppositifolius* by Woode *et al.* (2012).

The secondary metabolites detected in preliminary phytochemical analysis was consistent with previous findings from work done on the hydroethanolic leaf extract by Woode *et al.* (2012), Adekunle and Ikumapayi (2006) and Farombi (2003). Several alkaloids and their derivatives have been isolated from plant sources and have been used for their analgesic effect. Examples are morphine, which was isolated from *Papaver somniferum* and is used clinically for the management of pain, and vincristine, which was isolated from *Vinca roseus* and is used as an anticancer agent (Staerk *et al.*, 2002; Norn *et al.*, 2005). Sterols are known to lower blood cholesterol levels and boost the immune system (Kukuia, 2012; Harcombe & Baker, 2014). Saponins have been cited to have antinociceptive properties, anti-inflammatory properties and anticancer properties (Waller & Yamasaki, 1996; Kukuia, 2012; Dong *et al.*, 2019; Jega *et al.*, 2019). Flavonoids are compounds known to possess anti-inflammatory activity (Liang *et al.*, 1999). It has been reported that some flavonoids block both the lipoxygenase and cyclooxygenase pathways of the arachidonate cascade at relatively high concentrations, and block only the lipoxygenase pathway at lower concentrations (Di Carlo *et al.*, 1999). Certain

flavonoids are known to exert their antinociception via the activation of opioid receptors (Rajendran *et al.*, 2000; Otuki *et al.*, 2005). According to Middleton *et al.* (2002) flavonoids also exhibit inhibitory effects against phospholipase A2 and phospholipase C pathways. The pharmacological activity of the extract may be due to the presence of one or more of these secondary metabolites.

MOE was evaluated in three murine models of pain to get a clearer picture of its analgesic activity. In all the tests conducted, MOE showed significant activity against all noxious stimuli. It significantly and dose-dependently reduced nociceptive scores in both phases of the formalin test suggesting that it possesses both central and peripheral analgesic activity. This was confirmed with follow-up tests in the acetic acid induced writhing test which showed that MOE possessed peripheral analgesic effect, and in the hot plate test which confirmed that MOE has central analgesic activity.

The formalin test is widely known as a highly predictive pain model and mimics acute clinical pain due to tissue injury (Abotsi *et al.*, 2017). In this experiment, we measured nociception continuously over a specific time period. This pain model makes it possible to assess the onset and duration of action of analgesics (Dubuisson & Dennis, 1977; Abotsi *et al.*, 2017). In this chemically induced pain model, the antinociceptive effect demonstrated by MOE was significant and comparable to that of diclofenac and morphine. However, both morphine and diclofenac were more potent. Intraplantar injection of formalin after pre-treatment with MOE, diclofenac or morphine produced a biphasic response, the result of two different nociceptive pathways. The first/neurogenic phase occurs almost immediately after formalin injections and is elicited by the direct chemical activation of central nociceptive afferent terminals of the A δ fibres. The second/inflammatory phase is an inflammatory response due to central sensitization in the dorsal horn and the direct stimulation of chemical nociceptors which results in an

increase in the input from C fibres (Hunskar & Hole, 1987; Ameyaw *et al.*, 2016; Abotsi *et al.*, 2017; Afify *et al.*, 2017). It is known that centrally acting analgesics like the reference drug used, morphine, inhibit both the neurogenic and inflammatory phases of the formalin test. The extract significantly inhibited both phases of the formalin test as well, which infers a central analgesic activity. Also, peripherally acting analgesics which includes some NSAIDs only effectively inhibit the late or inflammatory phase. Inhibition of the second phase by MOE suggests that it possesses peripheral and anti-inflammatory activity. The later has been confirmed in previous studies (Farombi, 2003; Chukwujekwu *et al.*, 2005; Nwaehujor *et al.*, 2014).

The acetic acid-induced writhing test was conducted in this study to confirm findings from the formalin test. This model has been used frequently to screen compounds for peripheral antinociceptive activity (Dhouibi *et al.*, 2018). It is a sensitive peritoneovisceral pain model, and is a convenient test for assessing the antinociceptive activity of compounds (Abotsi *et al.*, 2017). The visceral pain induced by the intraperitoneal injection of 0.6% acetic acid is perceived via C fibres (Koster *et al.*, 1959). The writhing response results from the activation of certain acid-sensitive ion channels and the transient receptor potential vanilloid 1 (TRPV1) located in the afferent primary fibres (Cavalcante-Silva *et al.*, 2014; Abotsi *et al.*, 2017). Additionally, the local irritation caused by acetic acid injection causes the release of pro-inflammatory cytokines such as interleukin 1 β , interleukin 8 and TNF- α by resident peritoneal macrophages and mast cells as well as prostaglandins (PGE₂ and PGF_{2 α}), bradykinin, substance P, serotonin, histamine and sympathomimetic amines (Pavao-de-Souza *et al.*, 2012; Abotsi *et al.*, 2017; Dhouibi *et al.*, 2018) which stimulate nociceptive neurons that are sensitive to NSAIDs and narcotics (Dhouibi *et al.*, 2018). The endogenous chemicals released stimulate nerve endings and peritoneal receptors and incite abdominal constriction responses attributable to receptor sensitization to prostaglandins (Hassan *et al.*, 2015; Dhouibi *et al.*, 2018). MOE

significantly and dose-dependently inhibited the abdominal writhes induced by acetic acid suggesting peripheral mediated analgesic activity which may result from the inhibition of the synthesis of arachidonic acid metabolites. This confirms the activity of the extract seen in the inflammatory phase of the formalin test. The effect of MOE seen in this model could be attributed to an interference with the stimulation of nociceptive fibres by the released endogenous nociceptive mediators or the suppression of the sensitization effect of the pro-inflammatory cytokines (Abotsi *et al.*, 2017). The extract probably exerts its effect by inhibiting the biosynthesis, release and action of prostaglandins. According to Nwafor *et al.* (2007) acetic acid also causes inflammatory pain by inducing capillary permeability and in part by sensitisation of peritoneal chemosensitive nociceptors by prostaglandins, particularly PGE₂ and PGF_{2α} as well as lipoxygenase products (Bentley *et al.*, 1983). These results then suggest that in part, MOE may exert its effect through the cyclooxygenase and/or the lipoxygenase system.

The hot plate test employs the use of a thermal noxa to induce pain by the stimulation of the Aδ fibers (Dhouibi *et al.*, 2018). This test is specific for centrally mediated activity therefore selective for centrally acting analgesics (Afify *et al.*, 2017). In this model, the hot plate is maintained at a constant temperature and this is known to produce two nociceptive behavioural responses, paw licking and jumping, which are measured as how long it takes for these behavioural responses to appear. Both of these behaviours are considered as supra-spinal mediated responses (Milind & Monu, 2013). According to Le Bars *et al.* (2001), the paw licking behaviour is affected by only opioids whereas the latency to jumping response is equally increased by less powerful analgesics such as paracetamol or aspirin. The pre-treatment of the mice with MOE and morphine significantly increased latency to paw licking and jumping. However, morphine was far more potent than MOE. *M. oppositifolius* demonstrated some central antinociceptive activity by increasing the latency to reaction to thermal stimulus

and may in part act like centrally active drugs. Some of these centrally active drugs exert their effect by stimulating the periaqueductal grey matter to release endogenous peptides (i.e., endorphins, enkephalins) (Pathan & Williams, 2012). The endogenous peptides travel to the synapse in the dorsal horn and inhibit the transmission of the thermal pain impulses (Woolf, 2004; Benzon & Raj, 2008).

With MOE demonstrating antinociceptive effects in the three different pain models, further studies to assess the possible mechanism(s) of antinociception were carried out. The formalin test was used as it is more specific for site of action and mechanism of action. In all the tests, the antinociceptive effect of MOE was not significantly reversed by any of the antagonists used. It is therefore necessary that further tests be carried out on the possible mechanism of antinociception of MOE and the doses of antagonists used in this study be modified.

This study also demonstrated that MOE possesses potent analgesic activity in paclitaxel-induced peripheral neuropathy in mice further confirming the results from the nociceptive pain models in this research work. Daily administration of paclitaxel produced significant peripheral neuropathy demonstrated by a lowering in the mechanical and thermal pain thresholds as well as a reduction in the latency to tail withdrawal from cold water at 4°C from day 0 to day 5.

Peripheral neuropathy (a chronic condition) is a common side effect of paclitaxel administration and is manifested as allodynia or hyperalgesia with direct action on the processing and secretion of proinflammatory mediators, which include TBARS, IL-1b and glutathione (Miranda *et al.*, 2018). Neuropathic pain is characterized by spontaneous pain, hyperalgesia and allodynia. These remain without adequate treatment and compromise the quality of life (Woolf, 2004; Costa *et al.*, 2011). The onset of hyperalgesia is known to occur within a few hours to 7 days after paclitaxel administration and can last several weeks (Dina *et al.*, 2001; Polomano *et al.*, 2001; Cata *et al.*, 2006). Similarly, thermal and mechanical

hyperalgesia markedly increased from day 1 to day 5 during the induction period and lasted throughout the test period for mice that were not treated with MOE or pregabalin.

Though the exact mechanism of pathogenesis by paclitaxel is yet to be fully elucidated, many findings have come up to suggest the involvement of several molecules and systems. Some findings suggest that paclitaxel may induce nerve damage by disrupting the action of microtubules which are needed for transport in axons (Pourmohammadi *et al.*, 2012), and cause the swelling of mitochondria in axons (Fidanboylu *et al.*, 2011; Ameyaw *et al.*, 2014) leading to the loss of certain intracellular functions. Consequently, the calcium levels in the axons are altered as well as an initiation of apoptosis (Flatters & Bennett, 2006; Amoateng *et al.*, 2017). The allodynia that develops from the administration of paclitaxel has been attributed to apoptosis in the dorsal root ganglion neurons (Ameyaw *et al.*, 2014). In a study by Zhang *et al.* (2012) they demonstrated that paclitaxel treatments induced the activation of spinal astrocytes and not microglia. They also showed that the involvement of spinal astrocytes in the pathogenesis of paclitaxel-induced neuropathy was possibly by downregulating glial glutamate transporters GLAST and GLT-1 but not by releasing proinflammatory cytokines such as TNF α , IL-1b, and IL-6 in spinal dorsal horn. GLAST and GLT-1 are two types of glial glutamate transporters that are predominantly expressed on spinal astrocytes for the uptake of glutamate (Liaw *et al.*, 2005; Yaster *et al.*, 2011). Blocking spinal GLAST and GLT-1 induces spontaneous nociceptive behaviours and hypersensitivity to both peripheral mechanical and thermal stimuli (Liaw *et al.*, 2005). Blockage leads to enhanced neuronal activity and excessive activation of post-synaptic AMPA and NMDA receptors in the dorsal horn (Weng *et al.*, 2007; Nie & Weng, 2009). The downregulation of GLAST and GLT-1 in the dorsal horn caused by damage to peripheral nerves is known to facilitate the development of neuropathic pain which has been reversed when the activity of these transporters were restored (Sung *et al.*, 2003; Zhang *et al.*, 2012).

The neuropathic mice were treated with MOE and pregabalin for 5 consecutive days. Much like pregabalin, the extract significantly and dose-dependently inhibited mechanical and thermal hyperalgesia in the mice. However, pregabalin was more potent. Pregabalin is effective both experimentally and clinically in the management of chemotherapy induced neuropathic pain (Ameyaw *et al.*, 2014; Mangaiarkkarsi *et al.*, 2015). It is known that its action is as a result of antagonist effects on $\alpha_2\text{-}\delta_1$ Ca^{2+} channel subunit of N-type voltage dependent calcium channels (Mangaiarkkarsi *et al.*, 2015) which consequently leads to a reduction in neuronal excitability and inhibition of other cellular enzymatic reactions that lead to pain sensation (Kumar *et al.*, 2010; Ameyaw *et al.*, 2014; Amoateng *et al.*, 2017).

The exact mechanism by which MOE attenuates mechanical and thermal hyperalgesia in this model of peripheral neuropathy is yet to be established and will be investigated in future studies. However, earlier reports (Kukuia *et al.*, 2014; Kukuia *et al.*, 2016) suggest that the analgesic effect of MOE in this model may be associated with an inhibitory effect on glutamatergic neurotransmission and inhibition of the excessive activation of NMDA receptors, as this neurotransmitter system and its associated NMDA receptors are implicated in paclitaxel-induced neuropathic pain (Liaw *et al.*, 2005; Nie & Weng, 2009; Yaster *et al.*, 2011).

Sub-chronic studies are designed to assess the undesirable effects of the continuous or repeated exposure to drugs, compounds and plant extracts over an extended time period in experimental animals (Zhang *et al.*, 2016). It provides very useful information on the adverse effects associated with these molecules and how this affect specific target organs (Yuet Ping *et al.*, 2013). In this study, the sub-chronic toxicity of MOE was evaluated in Sprague-Dawley rats at doses of 30, 100 and 300 mg/kg for 90 days.

No significant and dose-dependent changes in the weights of the harvested organs were observed, suggesting that the extended oral administration of MOE has no effect on normal growth. In general, body weight change is an index of adverse effects after exposure to toxic substances and animals that survive this exposure should not lose any more than 10% of their initial body weight (Hemalatha & Hari, 2014). A high increase in body weight may be attributable to appetite stimulating drugs or agents which affect the lipid profile of the animals causing fat deposition. On the other hand, decrease in body weight could be attributed to the anorexic effect of certain drugs (Gura & Ciccone, 2010; Koumba Madingou *et al.*, 2016).

Organ weights are very sensitive to toxicity, enzyme induction, acute injury and so on making them useful in toxicological studies (Zhang *et al.*, 2016). As such, they are frequently targeted in toxicity studies (Michael *et al.*, 2007). The Society of Toxicologic Pathology has recommended that organ weights be included in toxicity studies especially when multiple dose levels are given in durations from one week to one year (Sellers *et al.*, 2007). Results from this study reveals that there was no significant and treatment related differences in relative organ weights between the distilled water groups and any of the MOE treated groups, suggesting that MOE had no effect on organ weights.

Analysis of blood parameters/indices is very important when assessing the toxicity of a drug as they have a high predictive value for toxicity in humans (Olson *et al.*, 2000; Gautam & Goel, 2014). Blood is a transport medium and as such carries many drugs and xenobiotics that enter the body. Inadvertently, blood components are forced to interact with high amounts of xenobiotics which may be toxic. As such, evaluating haematological indices can be useful in pointing out the extent of toxic effect of a given plant extract (Olson *et al.*, 2000). In the study, there were no significant differences in RBC indices between the vehicle treated groups and the extract treated groups indicating that the extract has no significant dose-dependent effects

on erythropoiesis, morphology or osmotic fragility of the RBCs. Total WBC count for the drug treated groups was not significantly different when compared to the vehicle group. However, the group treated with the median dose showed a significantly high neutrophil count suggestive of an increase in their production in the bone marrow which may be attributed to stress or an ongoing infection or inflammation. This observation was however not dose dependent.

Serum biochemistry analyses were conducted to assess possible extract related effects on hepatic and renal functions which are very important in the evaluation of toxicity as it has been demonstrated in certain studies that phytotherapeutic products may cause liver or kidney damage (Féres *et al.*, 2006).

Among the parameters measured, ALT, ALP, AST, GGT, bilirubin and albumin are considered liver function markers. Drug metabolism and elimination occur majorly in the liver (Zhang *et al.*, 2016). Elevated ALP, AST and ALT levels are usually reported in liver diseases or hepatotoxicity and are often used to differentiate between hepatocellular and cholestatic diseases (Féres *et al.*, 2006). Another typical function of the liver is the production and maintenance of TP and ALB levels. A decrease in their levels is indicative of reduced synthesis function of the liver attributable to an hepatocellular impairment or injury (Yuet Ping *et al.*, 2013). There were no significant differences in biochemical parameters for treatment groups and vehicle groups when compared suggesting that liver function was not impaired. However, the median dose treated group (MOE at 100 mg/kg) had significantly low AST levels. Low AST levels are expected in such tests and are normal. On the other hand, high AST levels may suggest an ongoing inflammatory event in the liver or even a blockage of bile ducts (Zhang *et al.*, 2016). The group treated with the MOE at 30 mg/kg recorded significantly low ALP levels. This can be attributed to low levels of serum magnesium and zinc as well as vitamin C and B12 deficiency (Lum, 1995; Ray *et al.*, 2017). From the histopathological analysis, acute

inflammation was observed from two different liver sections from only two rats from the median dose group with one showing high mononuclear cell infiltration (Plate 4.5.8 K). This was not apparent from the biochemistry parameters measured. This phenomenon will be investigated in subsequent studies.

Comparison of kidney parameters for the treated group to the control did not show any significant difference. However, histopathological analysis revealed acute mononuclear cell inflammation and glomerulopathy in only one of the test subjects treated with MOE at 30 mg/kg. Histological analysis of isolated organs did not show any dose dependent toxicity pattern.

5.2. CONCLUSION

The oral administration of a hydro-ethanolic leaf extract of *Mallotus oppositifolius* produces dose-dependent antinociceptive effects in formalin, acetic acid-induced and thermal acute pain tests, and also significantly inhibited mechanical and thermal hyperalgesia in paclitaxel induced peripheral neuropathy in mice. There was no MOE dose-dependent toxicity in rats following a 90-day daily oral administration. The findings support the traditional uses of the plant in alleviating pain.

5.3. RECOMMENDATIONS

The extract should be fractionated to isolate the compounds responsible for the observed antinociceptive effects.

This study should be repeated in non-human primates in order to assess and/or confirm the scientific information gathered or discover those that were not apparent in this study.

Other pain models should be used to elucidate the possible mechanism(s) of action.

Budget should be increased in further chronic studies to cover the histopathological investigation of more organs in the gastrointestinal tract.



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