ANTI-NOCICEPTIVE EFFECTS OF AN AQUEOUS EXTRACT OF THE AERIAL PARTS OF *PHYLLANTHUS MUELLERIANUS* (KUNTZE) EXELL. AND ITS MAIN CONSTITUENT, GERANIIN, IN RODENTS

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DECLARATION

The experimental work described in this thesis was carried out at the Department of Pharmacology, KNUST. This work has not been submitted for any other degree.

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ABSTRACT

Phyllanthus muellerianus has been used in folk medicine for several ailments including toothache, dysmenorrhea and general body pains. However, there is little scientific data supporting its use in pain management. This study therefore evaluated the antinociceptive potential and the acute toxicological profile of the aqueous extract of the aerial parts of *Phyllanthus muellerianus* (PME) as well as its dominant secondary metabolite, geraniin. Acetic acid-induced abdominal writhing and formalin–induced nociception tests were used to assess the anti-nociceptive effects of PME and geraniin. The involvement of the opioidergic, adrenergic, muscarinic, adenosinergic, serotonergic and nitric oxide pathways in anti-nociception were evaluated *in vivo* by selective antagonism of these pathways in ICR mice. An isobolographic analysis was performed using geraniin and the conventional analgesics; morphine and diclofenac. The formalin test was used to determine whether tolerance develops to the anti-nociceptive activity of PME and geraniin after chronic administration. The effect of the agents on naloxoneprecipitated morphine withdrawal signs was also evaluated. The acute toxicological profile of the extract was also assessed after both single and multiple administrations in mice and rats respectively. Oral administration of PME (30, 100, 300 mg kg^{-1}) and geraniin (3, 10, 30 mg kg^{-1}) produced significant anti-nociceptive effects in all models used. The anti-nociceptive effects of both the extract and geraniin were not antagonized by all the antagonists except naloxone which reversed the anti-nociceptive effects of only geraniin. Also, isobolographic analysis of geraniin/morphine as well as geraniin/diclofenac combinations indicated synergistic effects. PME and geraniin did not produce any tolerant effects. PME and geraniin did not induce withdrawal signs and significantly produced a reduction in the number of jumps of morphine dependent mice. In the toxicological study, acute administration of high doses of the extract did not produce any lethality with the Lethal Dose 50 (LD_{50}) of PME estimated to be above 5000 mg kg^{-1}. There were also no significant differences found in almost all of the hematological and serum biochemical parameters as well as organ/body weight ratio. In conclusion, the aqueous extract of the aerial parts of *P. muellerianus* and geraniin possess anti-nociceptive effects justifying the use of the plant in traditional use as an analgesic.

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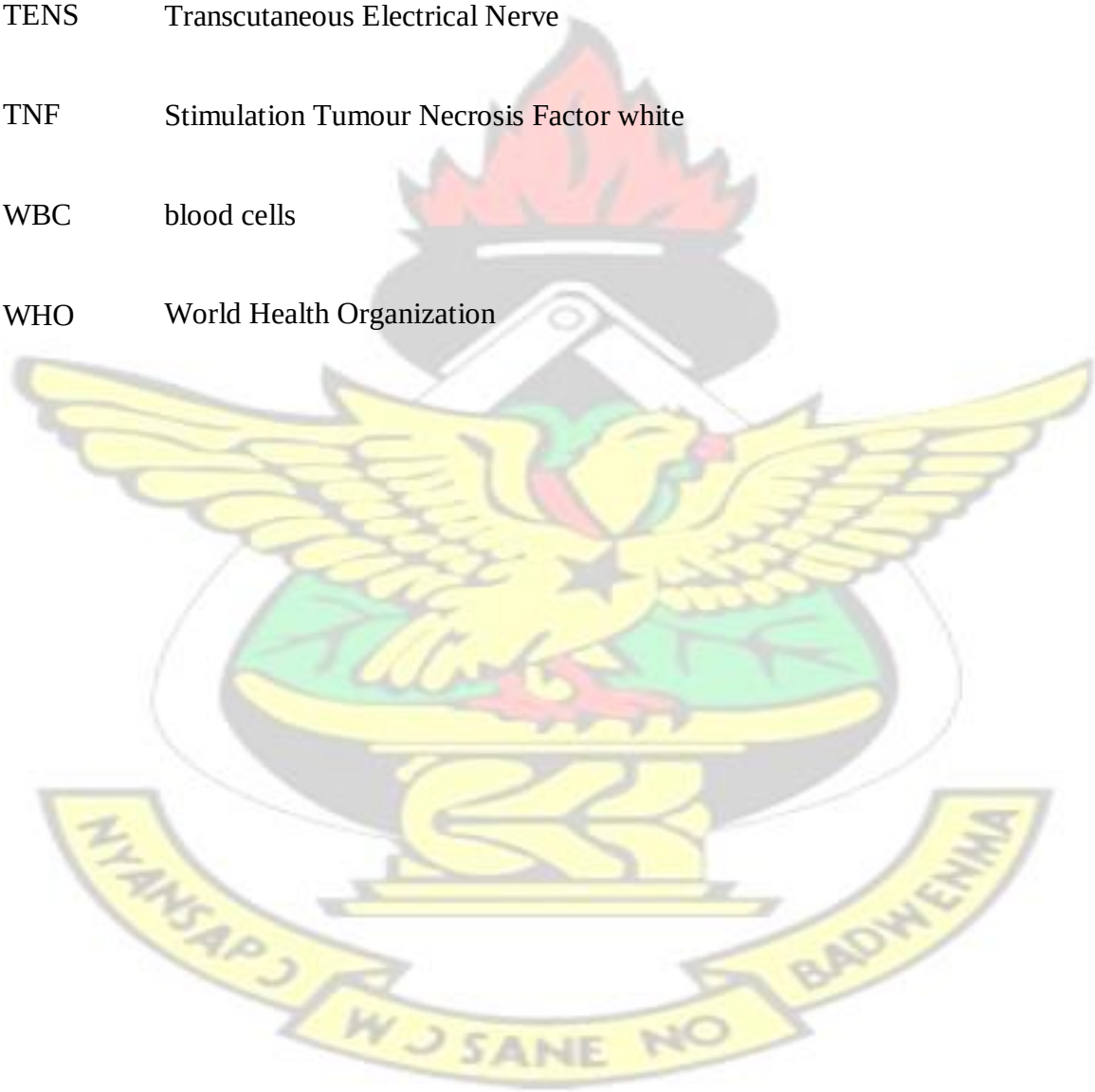
ABBREVIATIONS

AIDS	Acquired Immune Deficiency Syndrome
ALP	Alkaline Phosphatase
ALT	Alanine Transaminase
ANOVA	Analysis of variance
AST	Aspartate Transaminase
ATP	Adenosine Triphosphate
AUC	Area under the curve
D-BIL	Direct Bilirubin
I-BIL	Indirect Bilirubin
T-BIL	Total Bilirubin
BNF	British National Formulary
CL	Confidence Limit
CGRP	Calcitonin Gene Related Protein
CNS	Central Nervous System
COX	Cyclo-oxygenase

ED_{50}	Effective Dose 50
GGT	Gamma Glutamyl transferase
Hsp	Heat shock protein
HaCaT	Human adult high calcium low temperature
HCT	Hematocrit
HGB	Haemoglobin
HPLC	High Performance Liquid Chromatography
5-HT	5-hydroxytryptamine
ICR	Imprint Controlled
IL	Region Interleukin
i.p.	intraperitoneal injection
L-NAME	N^G-L-nitro-arginine methyl ester Lethal
LD_{50}	Dose 50 relative percent content of lymphocytes mean corpuscular haemoglobin mean corpuscular haemoglobin concentration
LYM %	
MCH	
MCHC	

MCV	mean corpuscular volume
MPV	mean platelet volume
MXD	% mixture of monocytes, basophils and eosinophils
NO	nitric oxide
NOAEL	No-observed-adverse-effect-level
NEUT %	relative percent content of neutrophils
NSAIDs	Non-Steroidal Anti-Inflammatory
NMDA	Drugs N-Methyl-D-Aspartate *per os*
p.o.	Platelet large cell ratio
P_LCR	Platelets
PLT	Platelet Distribution Width *Phyllanthus muellerianus* extract revolutions per minute red blood cells red blood cell distribution width (coefficient of variation)
PDW	
PME	
rpm	
RBC	
RDW_CV	

RDW_SD	red blood cell distribution width (standard
ROW	deviation) Relative Organ Weight subcutaneous
s.c.	injection
S.E.M.	Standard error of the mean
TENS	Transcutaneous Electrical Nerve
TNF	Stimulation Tumour Necrosis Factor white
WBC	blood cells
WHO	World Health Organization



Chapter 1 INTRODUCTION

1.1 GENERAL INTRODUCTION

The universal role of plants in the treatment of diseases is exemplified by their use in all the major systems of medicine (Evans, 2005). Current isolation procedures available and pharmacological testing procedures means that the newly discovered plant drugs are transformed into medicines in the pure but not in their crude forms. According to the World Health Organization, large proportions of the population in most developing nations rely on herbal medicines to maintain good health (WHO, 2008).

The interest in traditional knowledge and its importance is gradually becoming much more recognized in developmental policies, the media and scientific literature. The government of Ghana recognized this when in September 2012; clinical herbal medicine practice was integrated into the main healthcare delivery system by the ministry of health after piloting of herbal clinics in selected government hospitals nationwide commenced in 2011. The operation as at 2012 covered about 18 government health facilities nationwide.

As a result of the widespread use and relative effectiveness of medicinal plants, traditional medicine is now being considered by drug manufacturing companies as a source for the identification of lead compounds and biologically active agents that can be used in the preparation of orthodox medicines (Evans, 2005).

Pain is a subjective experience, very difficult to define precisely, although it is experienced by almost everyone. *Phyllanthus muellerianus* (Euphorbiaceae), a widely distributed scandent shrub with numerous stems from the base is used traditionally for various painful conditions

and for fever. The aqueous leaf extract has been shown to possess antiinflammatory activity (Boakye *et al*., 2013) and also various species in the Phyllanthus genus have been shown to possess anti-nociceptive activity (Santos *et al*., 2000). However, the anti-nociceptive properties of *Phyllanthus muellerianus* has not been scientifically established. Hence, the need for this study which seeks to determine if an aqueous extract of the aerial parts of *Phyllanthus muellerianus* as well as its main biological constituent, geraniin, exhibit anti-nociceptive properties in murine models.

1.2 PHYLLANTHUS MUELLERIANUS

Botanical name: *Phyllanthus muellerianus* (Kuntze) Exell.

Family: Euphorbiaceae

1.2.1 Description

A monoecious, scandent shrub with numerous stems from the base, or small tree up to 7.5 m tall. The leaves are nearly 8 x 4 cm, ovate or ovate-elliptic, with a rounded base and alternate along the lateral twigs. The flowers are unisexual and are greenish white or greenish yellow in appearance. Fruits have fleshy pericarps, green at first, later ripening through pink to reddish-brown or black. Seeds are minutely reticulate and shiny reddishbrown (Radcliffe-Smith, 1996; Burkill, 2000).

Figure 1.1: The aerial part of *Phyllanthus muellerianus* (Source: West African Plants: A Photo Guide)

1.2.2 *Ecological and geographical distribution*

Phyllanthus muellerianus is distributed widely in tropical and subtropical countries such as Ghana, Guinea, Sudan, Angola and Tanzania found in Africa (Radcliffe-Smith, 1996) as well as Brazil and the Carribeans (Calixto *et al.*, 1998). It is commonly found in dry evergreen forest and thicket as well as high rainfall woodlands and tall grassland (RadcliffeSmith, 1996).

1.2.3 Traditional uses

1.2.3.1 Non-medicinal uses

The fruits are edible and slightly acidic. In Cameroon, the bark is sometimes added to palm wine to make it intoxicating. In East Africa, brown dye obtained from the bark is used to dye mats and fishing lines. The leaves are also used as fodder. In Zambia, the wood is used

for construction work as well as basketry. In Gabon, the plant is used in magic to lift taboos (Schmelzer and Gurib-Fakim, 2008).

1.2.3.2 Medicinal uses

The use of the species of the genus Phyllanthus in traditional medicine for various ailments has been ongoing for decades. In Côte d'Ivoire, the leaf sap is used as a wash for fevers. In Zambia, a leaf infusion is also used to bathe the body in feverish conditions while in Cameroon, a section of the population use the decoction of *Phyllanthus muellerianus* stem bark in the management of tetanus (Brusotti *et al*., 2012). A root decoction is used for the treatment of gonorrhoea in Côte d'Ivoire and severe dysentery in Ghana. Freshly ground leaves are applied to boils and wounds and also used for treatment of menstrual disorders, fevers and skin eruptions in Sierra Leone, Ghana, Nigeria and Cameroon (Burkill, 2000; Agyare *et al*., 2009). In Côte d'Ivoire and Burkina Faso, twigs are sucked to prevent tooth ache while in DR Congo, a leaf decoction is used as a mouthwash for dental conditions. In Gabon, roasted powdered twigs are used to treat dysmenorrhea (Schmelzer and GuribFakim, 2008).

1.2.4 Previous studies on the activities of *Phyllanthus muellerianus*

The aqueous leaf extract has been shown to exhibit antimicrobial and antioxidant activity (Boakye and Agyare, 2013) as well as anti-inflammatory activity (Boakye *et al*., 2013). The aqueous leaf extract and stem bark exhibit antibacterial activity (Doughari and Sunday, 2008). The antimicrobial properties of *Phyllanthus muellerianus* stem bark essential oil have been proven against *Clostridium sporogenes*, *Streptococcus mutans*, *Streptococcus pyogenes* and *Trichophyton rubrum* (Brusotti *et al*., 2012). The aqueous leaf extract has been shown to increase significantly the synthesis of collagen (Agyare *et al*., 2011).

Additionally, the leaf extract has been found to possess moderate antiplasmodial activity and low cytotoxicity in mammalian cell lines. An aqueous leaf extract was found to cause sedation in young chicks while a chloroform extract has also been shown to exhibit high antifungal activity against *Candida albicans* (Schmelzer and Gurib-Fakim, 2008).

1.2.5 Identified chemical constituents

Several organic compounds of interest have been identified such as triterpenoids 22ß-hydroxyfriedel-ene and 1ß, 22ß-dihydroxyfriedelin (Schmelzer and Gurib-Fakim, 2008). Geraniin, furosin, corilagin, isoquercitrin, astragalin, rutin, phaselic acid, gallic acid, methylgallate, caffeic acid, chlorogenic acid, 3, 5-o-dicaffeoylquinic acid have been isolated from the aerial parts of *P. muellerianus* (Agyare *et al*., 2011). Doughari and Sunday (2008) have reported the secondary metabolites; alkaloids, anthraquinones, flavonoids and tannins are present in the leaves of *P. muellerianus*. An initial phytochemical screening of the leaves and stem bark also showed the presence of tannins and saponins (Schmelzer and Gurib-Fakim, 2008).

1.3 GERANIIN

Geraniin ($C_{41}H_{28}O_{27}$) is a dehydroellagitannin which occurs as two isomers in solution (Figure 1.2). A pale amorphous compound, it has a molecular weight of 952.64 g mol^{-1} and density of 2.26 g mL^{-1} (Agyare *et al.*, 2011). Geraniin was first isolated by Okuda *et al*. in 1977 as the major tannin in *Geranium thunbergii*, a popular Japanese folk medicine used in the management of diarrhea (Ishimaru and Shimomura, 1995). It has also been isolated from many medicinal plants in the tropical and subtropical regions belonging to the Euphorbiaceae family notably the Phyllanthus and Geranium genus (Luger *et al.*, 1998).

Geraniin has been isolated from the aqueous extract of aerial parts of *Phyllanthus muellerianus* (Kuntze) Exell. (Agyare *et al*., 2011), aqueous leaf extract of *Phyllanthus amarus* Thonn. and Schum., (Foo, 1993), ethanolic leaf and stem extract of *Phyllanthus sellowianus* Muller Arg. (Miguel *et al*., 1996) and other plants.

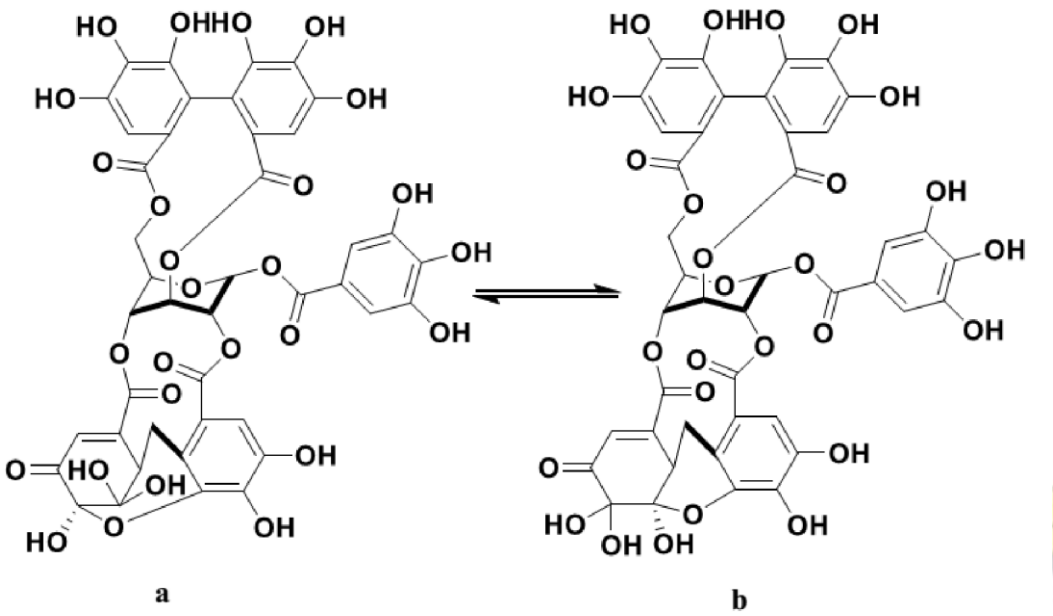


Figure 1.2: The chemical structures of the two isomers of geraniin (Adopted from Agyare *et al*., 2011)

1.3.1 Biological properties of geraniin

Geraniin has been shown to possess strong cellular proliferation effects using primary dermal fibroblasts and human adult high calcium low temperature (HaCaT) keratinocytes (Agyare *et al*., 2011). Geraniin has also been shown to possess antiviral activity (Ishimaru and Shimomura, 1995). It has been proven to possess high anti- HIV reverse transcriptase activity and angiotensin-converting enzyme inhibitory activity involved in diabetic complications (Ueno *et al*., 1988, Ogata *et al*., 1992, Unander, 1996) as well as the ability to inhibit heat shock protein 90 (Hsp 90), an effect which incapacitates the activity of

multiple proteins resulting in an attack on cellular oncogenic processes (Vassallo *et al.,* 2013).

1.4 PAIN

Pain is a complex sensory feeling which is intense and not so favourable. It may differ in terms of intensity, quality or even duration and usually includes personal experiences, cognitive and emotional components. As a result, pain management is usually supposed to be tailored to a particular patient (Woolf, 2004). In our everyday lives, usually pain is experienced when there is a potential or actual tissue damaging stimuli applied to the body even though pain has also been reported in patients who suffer from brain or nerve injury (Rang *et al.,* 2007). The direct correlation between this damaging stimulus and the feeling of pain causes us to avoid the behaviors and circumstances that might cause the pain (Schaible, 2006). The sensation of pain is useful when it warns us of immediate or impending injury and therefore allows for appropriate protective mechanisms to be initiated.

1.4.1 Classification of pain

Pain can be described as either being acute or chronic. Generally, acute pain has a rapid onset and most often associated with trauma or acute disease. It is usually self-limiting of short duration and lasts < 3 to 6 months. It is pain that is transitory or persists only until the cause has been removed (Skyba *et al*., 2004). Chronic pain on the other hand, usually lasts longer than 6 months (Schaible, 2006). Chronic pain arises from deep tissues such as muscle or joints and it is difficult to localize and hard to treat clinically (Skyba *et al*., 2004).

Pain may also be classified into two main categories: nociceptive or adaptive and neurogenic or maladaptive pain (Woolf, 2004, Marchand, 2008). Nociceptive pain can further be subcategorized into three different types: somatic, visceral and inflammatory pain while neurogenic pain can be subcategorized into neuropathic and functional pain.

1.4.1.1 Nociceptive pain

Nociceptive pain is generally transitory and as a result of a noxious stimuli (Marchand, 2008). This type of pain usually helps the organism to survive by either protecting it from hurting itself or helping the healing process if there is already an injury (Woolf, 2004). In spite of that, in some instances, the pain is still present after healing has occurred. Most of the time, clinicians are not able to predict as to which patients will develop chronic pain following this type of pain hence the importance of the early treatment of acute pain in the prevention of chronic pain.

The recommended treatments for nociceptive pain are the non-steroidal anti-inflammatory drugs (NSAIDs). Although very powerful in the management of pain and inflammation, most NSAIDs present with gastrointestinal adverse events, as a result of the decreased synthesis of mucosal prostaglandins by their inhibition of cyclo-oxygenase (COX) - 1 enzymes (Wolfe *et al*., 1999). Selective COX-2 inhibitors hardly produce this side effect but have an increased risk of thrombotic events and as such should only be used when specifically indicated and after assessing their cardiovascular risk (BNF 61, 2011). Opioids also have an important place in the treatment of acute pain, as peripheral opioid receptors are up-regulated following an inflammatory response. However, they may also present with severe side effects such as euphoria and sedation, respiratory depression, nausea, vomiting and reduced gastrointestinal motility causing constipation. Physical dependence and

tolerance to opiates may occur to some degree whenever opiates are administered for more than a few days (Rang *et al*., 2007).

It is necessary to differentiate somatogenic from viscerogenic nociceptive pain that may present a comparable clinical picture. Somatogenic pain usually is characterized by superficial (skin) or deep pain (muscle, fascia, tendon) such as in tissue injury while viscerogenic pain is characterized by constant or cramping, poorly localized pain as in cystitis or irritable bowel syndrome (Marchand, 2008).

Inflammatory pain is used to accomplish the goal of healing of an injured tissue after a lesion. In a situation where in spite of the defense mechanism in place, damage to tissue occurs, the body directs its 'resources' towards aiding the healing of the injured body part (Woolf, 2004). Inflammation is a natural protective reaction of the organism following an injury. When this happens, the affected body part is so sensitive that even a non-noxious stimuli tends to cause pain. Inflammatory substances are released into the periphery by cells in the area of damaged tissue and since most of these molecules released during inflammation are pro-nociceptive, the use of NSAIDs will reduce this nociceptive activity. Inflammatory pain usually diminishes as the injury and inflammatory response wane (Marchand, 2008). Even though this type of pain is not maladaptive, sometimes, elective injury is inflicted on purpose such as in surgery. In such situations, the inflammatory pain has to be managed effectively without removing or severely blunting the defense mechanism with the aim being to normalize pain sensitivity and not remove it (Woolf, 2004).

1.4.1.2 Neurogenic pain

This results as a direct effect of diseases which affect the somatosensory system. As there can be a peripheral component as well as a central component with regards to the mechanism, the treatments that can be used also depend on which pathways are involved.

However, neurogenic pain, even of a peripheral origin, is frequently associated with sensitization of the central nervous system (CNS) hence pharmacologic approaches aim to reverse or ameliorate the hyperactivity of the nociceptive neurons. Commonly used agents include opioids, anticonvulsants (gabapentin and phenytoin), antidepressants (such as tricyclic antidepressants), and N-Methyl-D-Aspartate (NMDA) receptor antagonists (Marchand, 2008).

The tricyclic antidepressants act centrally by inhibiting noradrenaline reuptake. Phenytoin acts on voltage-gated sodium channels. The target for gabapentin is the $\alpha_2\delta$ subunit of the L-type calcium channel. Ketamine, a dissociative anaesthetic that works by blocking NMDA receptor channels, has analgesic properties probably directed at the wind-up phenomenon in the dorsal horn (Rang *et al*., 2007).

Funtional neurogenic pain is as a result of a dysfunction in the pain modulation mechanisms rather than the presence of an anatomic lesion within the nervous system. This may occur as a result of central activation of endogenous excitatory systems that will amplify the nociceptive signal or by a dysfunction of endogenous inhibitory mechanisms. An example of such a pain state is fibromyalgia where there is a deficit of descending endogenous pain inhibitory mechanisms (Marchand, 2008).

Neuropathic pain results from injury to the peripheral nervous system or to the central nervous system (Woolf, 2004). The resulting pain in these cases may be as a result of a

noxious stimuli or it may even arise unchallenged from a non-noxious stimuli. Different pain behaviors are exhibited by patients suffering from this pain condition such as allodynia where pain is as a result of a non-noxious stimulus or hyperalgesia where the pain felt is enhanced and of a longer duration from a noxious stimulus (Woolf, 2004).

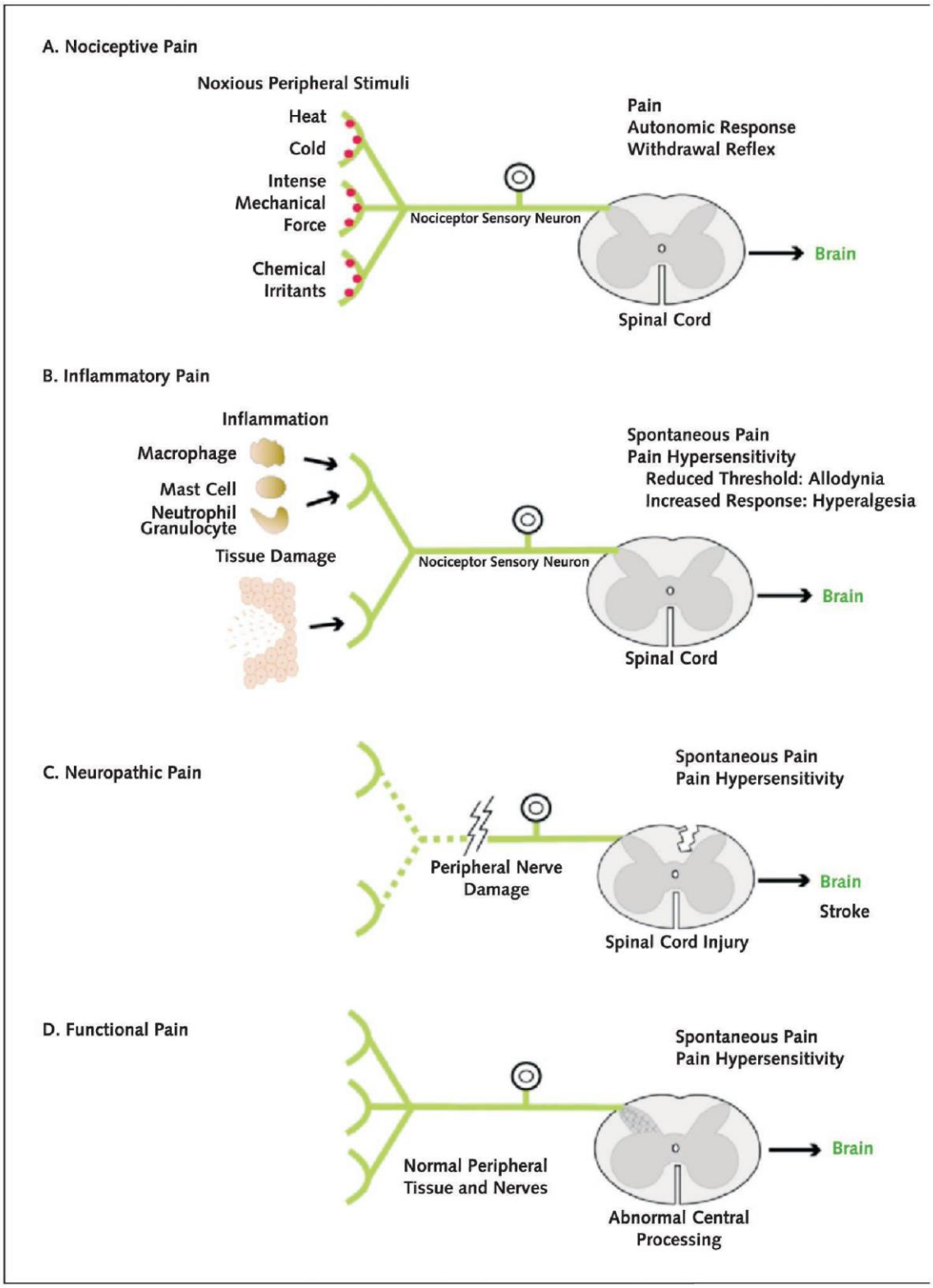


Figure 1.3: The schematic pathways of the four primary types of pain (Woolf, 2004)

1.4.2 Pathophysiology of pain

Understanding the physiology of pain requires an in-depth look at the nociceptive signal pathway from the periphery to the brain focusing on the integration and modulation of the pain signal in the CNS. Different types of stimuli may cause nociceptive stimulation which will lead to the recruitment of peripheral nociceptors that conduct the signal in the primary somatosensory neuron to the dorsal horn of the spinal cord. The afferent fibers are divided into three main groups namely: Aß fibers, C fibers, and Aδ fibers. The Aß fibers are large myelinated fibers that conduct at high speed and usually transmit non-nociceptive signals (Table 1.1). Nociceptor messages are mainly transmitted by the larger myelinated Aδ fibers and the thin unmyelinated C fibers. The larger the size of the fiber, the higher the speed of conduction of the stimulus. The Aδ fibers are responsible for the sharp localization of pain while the C fibers, which have a slow conducting velocity, are responsible for a slow and dull aching pain (Marchand, 2008).

Table 1.1: The characteristics of the nociceptive afferent fibers (Marchand, 2008)

	Aß fibers	**Aδ Fibers**	**C Fibers**
Myelination	Yes	Yes	No
Conduction (ms^{-1})	35 – 75	5 – 30	0.5 – 2
Role	Proprioception, Light touch	Temperature, Nociception (mechanical, thermal)	Nociception (mechanical, thermal and chemical)

A nociceptive stimulation will initiate a cascade of events. Pro-nociceptive inflammatory molecules will be released into the periphery from various blood cells (mastocytes, polymorphonuclear cells and platelets) and may include bradykinins, prostaglandins, histamine, serotonin, adenosine triphosphate, as well as from immune cells (interleukins, interferon and tumor necrosis factor). Substance P and calcitonin gene related protein (CGRP), which act as neurotransmitters in the CNS, are also released into the periphery and act as pro-inflammatory factors in the periphery, favoring neurogenic inflammation (Marchand, 2008).

Nociceptor activation produces action potentials that are transmitted by the peripheral nervous system along fibers to the spinal cord where they will have a first synaptic contact with secondary neurons that are principally located in the superficial zones of the dorsal horn of the spinal cord (substantia gelatinosa). Both nociceptive and non-nociceptive afferents to the spinal cord will also have synaptic contact with an important network of inhibitory and excitatory interneurones that modulate the nociceptive signal before the secondary neuron projects to superior centers. The secondary neuron travels to superior centers by two main pathways: the spinothalamic tract, which sends afferents to the lateral nuclei of the thalamus, and the spinoreticular tract, which sends afferents to the medial thalamus and nuclei of the brainstem, including the nucleus raphe magnus (NRM) and the periaqueductal grey (PAG) area which are involved in descending pain modulation. From the thalamus, pain signals pass to the cortex and other CNS centres to elicit the conscious sensation of pain and the emotional response as well as experience (Marchand, 2008).

As pain is a complex phenomenon, the nociceptive signal will be modulated at multiple levels of the CNS before pain is fully perceived. There are three main levels by which

modulation can be done. They include spinal mechanisms producing localized analgesia; descending inhibitory mechanisms from the brainstem producing diffuse inhibition and superior center effects that will either modulate descending mechanisms or change the perception of pain by reinterpreting the nociceptive signal (Marchand, 2008).

The gate control theory explains the modulation at the spinal cord and it hypothesizes that, among other mechanisms, selective activation of non-nociceptive afferent Aß fibers will recruit inhibitory interneurones in the substantia gelatinosa of the posterior spinal cord, producing a localized analgesia and decreasing pain perception. Again, the PAG area and the NRM have been identified as important serotoninergic and noradrenergic descending inhibitory pathways which then recruit enkephalinergic interneurones in the spinal cord to produce the analgesic response. Activation of certain cortical regions, including the primary and secondary somatosensory cortices, related to the sensory aspect of pain, and the anterior cingulate cortex and the insular cortex for the affective component of the pain experience has been shown to be effective in pain modulation (Marchand, 2008).

1.4.3 Risk factors for developing chronic pain

It has been proposed that three main factors play important parts in determining whether an individual will develop chronic pain. They include individual disposition, environmental and psychological factors. It has been postulated that taking note of these factors will help greatly in the management of patients suffering from this type of pain.

Personal disposition deals with the characteristics of a person either innate or acquired which influences the tendency of a person to develop chronic pain. The factors: gender and biological sex, age and the role of endogenous pain modulation responses have to be considered. It is generally known that women have a higher predisposition to chronic pain syndromes than men.

Sex hormones may play an important role here and animal studies in rats have supported this finding. It has been realized that the difference in pain sensitivity between men and women are seen only after puberty and disappear after menopause or andropause (Marchand, 2008). It is also widely known that aging increases one's chances of developing chronic pain and the widely recognized reason is the progressive musculoskeletal degeneration that is observed with the aged, however, a new theory is a decline in the efficacy of endogenous pain control systems in the elderly. Again, genetic predisposition influences the chances of an individual developing chronic pain and this also applies for response to certain treatments. It is well recognized that different patients respond differently to individual analgesic medications with regard to both efficacy as well as side-effect profile hence the need to tailor medications to patients (Marchand, 2008).

It has been realized that external stressors, history of pain or abuse are good forecasters for developing this type of pain. For instance, it has been shown that individuals born prematurely are more sensitive to pain later in life and deficits in their pain inhibitory mechanisms may account for this observation (Marchand, 2008).

Psychological factors such as anxiety and depression are very important predictors for developing chronic pain and not only that but also they predict the reactions of an individual to a pain experience, the individual's ability to cope with the pain as well as the evolution of the chronic pain symptoms. As a result of this, clinicians are advised to consider the role of psychological factors when managing individuals with chronic pain (Marchand, 2008).

1.4.4 Animal models of pain

The important mechanisms that make it possible for an organism to react to a stimulus, which might endanger its existence, exist throughout the animal kingdom, except perhaps

in arthropods and particularly in insects (Le Bars *et al*., 2001). Hence, the possibility of using rodents as models for the investigation of agents with possible analgesic effects. The absence of verbal communication in animals is perhaps one of the foremost obstacles to the evaluation of pain. There are situations where one can specifically tell that an animal is experiencing pain for instance when it is vocalizing its pain such as squealing or groaning. However, if an animal is not showing any typical physical signs or behaviors, there is no way to certify that an animal is not feeling pain. This is so because sometimes immobility and/or prostration are the only responses accompanying pain (Le Bars *et al*., 2001).

A common feature in pain research for both humans and animals is the lack of objectivity. One may not know exactly how rats feel pain, but this is also unknown in higher animals. In both species, we infer the presence of pain based on behavior (Mogil *et al*., 2010). Usually, pain in test animals is detected by the presence of basic motor responses, neurovegetative reactions with an increase in sympathetic tone (tachycardia, arterial hypertension, hyperpnea, mydriasis, etc.), and vocalization. Behavioral reactions (escape, avoidance, aggressiveness, etc.) or changes in behavior are also sometimes noticed (Le Bars *et al.,* 2001). Humans, however, have the distinct feature of being able to express themselves hence they are able to describe their experience. Mogil *et al*. in 2010 reported that in terms of science, they foresee no instance in the nearest future whereby true advances in pain research are possible without the usage of animal models. This is because animal models are important for the investigation of basic physiological mechanisms of pain as well as their ability to predict the efficacy of analgesics which may eventually lead to hospital use.

In animal studies of the mechanisms underlying acute pain, an appropriate stimuli has to be used to provoke the sensation. These stimuli have to be quantifiable, reproducible and noninvasive. The four primary stimuli used include electrical, thermal, mechanical and chemical stimuli. No matter the type of stimuli used, all nociceptive behavioral models should be specific, sensitive, valid, reliable and reproducible (Le Bars *et al.*, 2001).

1.5 JUSTIFICATION AND OBJECTIVES OF THE STUDY

1.5.1 Justification

Pain is usually a warning signal which provokes protective mechanisms but sometimes, this warning system becomes faulty and instead the pain becomes chronic and debilitating (Julius and Basbaum, 2001). According to a fairly recent study, pain is a significant menace that puts a burden on the population valued at $ 560 - $ 635 billion yearly. In the United States, millions suffer from this condition yearly and the consequences are seen clearly in high health care costs, rehabilitation and low work input as well as the emotional and financial burden on the community (Institute of Medicine of the National Academies Report, 2011).

Pain is reported in almost all medical conditions and it is most of the time, one of the prominent aspects of the disease which causes individuals to seek health care (Schim and Stang, 2004). It is also one of the cardinal markers for inflammation (Tracy, 2006). Although, people report often with complaints of pain, most often than not, patients return to their daily lives without their complaints beings resolved and this may be as a result of the improper usage of current analgesics (Mcmahon and Koltzenburg, 2006; Chen and Tang, 2011). Insufficient use of existing therapies can be due to the diverse and sometimes fatal side effects observed with the usage of some of these agents (Mirshafiey *et al.*, 2005,

Wolfe *et al*., 1999), such as gastric irritation with Non-Steroidal Anti-Inflammatory Drugs (NSAIDS), considerable gastrointestinal and renal damage with glucocorticoids and addiction and respiratory depression with opioids (Rang *et al*., 2007).

Due to the various adverse effects accompanying the usage of some of these analgesics as well as the widespread disappointment felt by some patients battling with pain, there is still the need for the discovery of more effective analgesics with safer toxicological profiles at therapeutic doses.

Since time immemorial, natural products have contributed greatly in the search for modern analgesics (Calixto *et al*., 2005). It is estimated that about 40 % of all medicines on the market today were derived either directly or indirectly from natural products with about 25 % being from plants. In fact, plants have been used traditionally for various painful conditions and some plants have even contributed to the development of modern analgesics such as *Papaver somniferum* (morphine, codeine), Salix species (salicylic acid) and *Cannabis sativa* (tetrahydrocannabinols) (Calixto *et al.*, 2005). This shows the importance of plants in the search of potential analgesics hence one of the reasons why this study focused on the use of *Phyllanthus muellerianus*, a monoecious shrub.

After potential analgesics are discovered, it is important to determine if the combination of drugs with established analgesic effects leads to better analgesic effects. Combination therapy has become one of the mainstays of several disease management including pain management and the combination of analgesics has become a common practice. This has been a popular practice because the use of these combinations might either lead to better pain management or even similar efficacies to individual drugs but with better side effect profiles.

Again, there is the need for all plant medicines to be evaluated for their safety and toxicity profile in order to determine the possible adverse effects on functional and physiological systems including central and peripheral nervous system, cardiovascular system, respiratory system and renal and gastrointestinal functions. This evaluation plays a key role in the safety assessment process of a drug (Vogel *et al.*, 2013).

Thus, the need for this study which seeks to investigate and establish the anti-nociceptive effects of the aqueous extract of the aerial parts of *Phyllanthus muellerianus* (Kuntze) Exell. and its main constituent, geraniin as well as determine its toxicological profile in rodents.

1.5.2 Aim and specific objectives

The main aim of this study is to provide pharmacological evidence to justify the traditional use of the aerial parts of *Phyllanthus muellerianus* in the management of pain as well as discover the potential of geraniin serving as a lead compound in the search for analgesics.

Specific objectives would include:

i) To evaluate PME and geraniin for their potential analgesic effects using animal models of chemical nociception

ii) To determine the possible sites and mechanism of action of PME and geraniin *in vivo* in mice.

iii) To determine the effect of geraniin on pain when combined with standard analgesics using isobolographic analysis.

iv) To determine whether PME and geraniin induce dependence or tolerance and their effects on naloxone – precipitated morphine withdrawal signs.

v) To evaluate the possible toxicological effects of PME after acute administration as well as after a 7 day administration schedule.

Chapter 2 MATERIALS AND METHODS

2.1 PLANT COLLECTION AND EXTRACTION

Fresh matured aerial parts of *Phyllanthus muellerianus* were identified and collected from uncultivated fields around KNUST in February, 2015. The plant was authenticated by Mr. Asare of the Herbal Medicine Department, KNUST. The fresh matured aerial parts of *P. muellerianus* were washed in water to completely remove foreign materials and air dried at room temperature (25 - 28 ºC) for seven days. The dried plant sample was then powdered with a lab mill machine (Christy and Norris, Chelmsford, England). Five hundred grams (500 g) of the powdered plant material was suspended in 5 L of sterile distilled water and heated at 90 ºC for 15 min. The mixture was centrifuged at 6000 ×g for 10 min and the supernatant lyophilized to obtain the powdered extract. The yield of the extract related to the dried powdered plant material was 12.08 % $^{w}/_{w.}$ The extract was then stored at 4 - 8 ºC in the refrigerator in air tight containers. This aqueous extract was subsequently referred to as PME in this study.

Geraniin (96 %$^{w}/_{w}$ HPLC grade) isolated from the aqueous extract of the aerial parts of *Phyllanthus muellerianus* (Kuntze) Exell. was kindly provided by Prof. Andreas Hensel, Institute of Pharmaceutical Biology and Phytochemistry, University of Muenster, Muenster, Germany through the help of Dr. Christian Agyare, Department of Pharmaceutics, Kwame

Nkrumah University of Science and Technology, KNUST, Ghana.

2.2 ANIMALS

Imprint Controlled Region (ICR) mice (30 ± 5 g) and Sprague Dawley rats (180 ± 20 g) were purchased from Noguchi Memorial Institute for Medical Research, University of Ghana, Legon, Ghana and housed in the *vivarium* of the Department of Pharmacology, KNUST. The animals were housed in groups of five in stainless steel cages (34 47 18 cm^3). Mice were fed with the normal mice chow (AGRICARE Ltd, Kumasi, Ghana) while rats were fed with normal pellet diet. Both species were given water *ad libitum* and maintained in a 12-hour light-dark cycle with soft wood shavings as bedding. All procedures and techniques used in these studies were in accordance with the Guide for the Care and Use of Laboratory Animals (Institute for Laboratory Animal Research, 2011). All protocols used were approved by the Departmental Ethics Committee.

2.3 DRUGS AND CHEMICALS

Diclofenac sodium was purchased from Troge Medical GmbH, Hamburg, Germany; morphine hydrochloride was obtained from BODENE (PTY) Limited Trading, Intramed, Port Elizabeth, South Africa; formalin, acetic acid and theophylline were purchased from British Drug Houses, Poole, England; N^G-Nitro-L-arginine methyl ester (L-NAME), yohimbine, D-tubocurarine, glibenclamide, ondansetron, nifedipine, naloxone and atropine were obtained from Sigma-Aldrich Inc., St. Louis, MO, USA and diazepam from Kilitch Drugs, India.

2.4 ANTI-NOCICEPTIVE EFFECTS

The anti-nociceptive effects of an aqueous extract of the aerial parts of *P. muellerianus*, a herb used in managing pain traditionally, and the major constituent, geraniin were evaluated in animal models of nociception.

2.4.1 Acetic acid-induced writhing test

The test was conducted as described by Amresh *et al.*, 2007. Eleven (11) groups of mice (n = 5) were used and they received vehicle (10 ml kg^{-1} of normal saline, i.p.), PME (10, 30, 100 and 300 mg kg^{-1}, *p.o.*), morphine (1, 3 and 10 mg kg^{-1}, i.p.) or diclofenac (10, 30 and 100 mg $kg^{-1,}$ i.p.) 60 min (*p.o.*) or 30 min (i.p.) before the intraperitoneal injection of 0.6 % acetic acid (10 ml kg^{-1}). Drugs were prepared such that no animal received more than 0.5 ml either orally or intraperitoneally. Mice were placed individually in a Perspex testing chamber (15×15 ×15 cm) and with the aid of a mirror inclined at 45° below the floor of the chamber and a camcorder placed directly opposite the mirror, the response of the mice after intraperitoneal injection of acetic acid was recorded. The response induced consists of a contraction of the abdominal muscle, together with a stretching of the hind limbs. The response of the mice after intraperitoneal injection of acetic acid was recorded for 30 min. Tracking of the behavior was done using the public domain software JWatcher™, Version 1.0 (University of California, LA, USA, and Macquarie University, Australia) to obtain the frequency and duration of writhes per 5 min. A nociceptive score was determined for each 5 min time block by multiplying the frequency and duration of writhes.

2.4.2 Formalin-induced nociception

The formalin test was carried out as described by Dubuisson and Dennis, 1977. Mice were acclimatized to the Perspex test chambers (15 15 15 cm) for thirty minutes before formalin injection. Thirteen groups of mice (n = 5) were then pre-treated with vehicle, PME (30 - 300 mg kg^{-1}, *p.o.*), geraniin (3 - 30 mg kg^{-1}, *p.o.*), morphine (1 - 10 mg kg^{-1}, i.p.) or diclofenac (10 - 100 mg kg^{-1}, i.p.) 60 min (*p.o.*) or 30 min (i.p.) before intraplantar injection of 10 µl of 5 % formalin. The mice were returned individually into the testing chamber after

the formalin injection and their nociceptive behaviors captured for 1 h for analysis in the same way as described previously in the writhing test above. A nociceptive score was determined for each 5 min time block by measuring the amount of time spent in the biting/licking of the injected paw. Tracking of the behavior was done using public domain software JWatcher™, Version 1.0. The average nociceptive score for each time block was calculated by multiplying the frequency and time spent in biting/licking and data were expressed as the mean ± S.E.M. of scores between 0–10 min (first phase) and 10–60 min (second phase) after formalin injection.

2.4.3 Assessment of the mechanism of anti-nociception of PME and geraniin in the formalin test

To determine the possible sites and mechanism of the analgesic activity of PME and geraniin, the formalin-induced nociception model was used employing various antagonists. The pathways investigated include the adenosinergic, opioidergic, adrenergic, serotoninergic, nitric oxide and muscarinic receptors or pathways. The involvement of the ATP sensitive potassium (K^+) channels and voltage gated calcium channels were also investigated. The doses of drugs were selected on the basis of previous literature data and in pilot experiments in the laboratory (Woode *et al.*, 2009; Woode and Abotsi, 2011).

2.4.3.1 The opioidergic pathway

Mice were pre-treated with naloxone (a non-selective opioid receptor antagonist; 2 mg kg^1, i.p.). Fifteen (15) minutes later, PME (100 mg kg^{-1}, *p.o.*), geraniin (10 mg kg^{-1}, *p.o.*) or vehicle (10 ml kg^{-1}, *p.o.*) were administered.

Groups of mice were also pre-treated with vehicle and after 15 min PME (100 mg kg^{-1}, *p.o.*), geraniin (10 mg kg^{-1}, *p.o.*) or vehicle (10 ml kg^{-1}, *p.o.*) were administered.

Formalin was injected one hour after oral administrations to induce nociception, the behavior of the mice was recorded for one hour and analysis was done as stated under section 2.4.2.

2.4.3.2 The nitric oxide pathway

Mice were pre-treated with L-NAME (N^G-L-nitro-arginine methyl ester, a NO synthase inhibitor, 10 mg kg^{-1}, i.p.) and after 15 min received PME (100 mg kg^{-1}, *p.o.*), geraniin (10 mg kg^{-1}, *p.o.*) or vehicle (10 ml kg^{-1}, *p.o.*). Nociception was induced as described under section 2.4.3.1.

2.4.3.3 The ATP-sensitive potassium channels

Mice were pre-treated with glibenclamide (an ATP-sensitive K^+ channel inhibitor, 8 mg kg^{1}, *p.o.*). 30 min later, PME (100 mg kg^{-1}, *p.o.*), geraniin (10 mg kg^{-1}, *p.o.*) or vehicle (10 ml kg^{-1}, *p.o.*) were administered. Nociception was induced as described under section 2.4.3.1.

2.4.3.4 The adenosinergic pathway

To investigate the role played by the adenosinergic system in the anti-nociception exhibited by the extract and geraniin, mice were pre-treated with theophylline (5 mg kg^{-1}, i.p., a nonselective adenosine receptor antagonist). After 15 min, the mice received PME (100 mg kg^{1}, *p.o.*) or geraniin (10 mg kg^{-1}, *p.o.*) or vehicle. Nociception was induced as described under section 2.4.3.1.

2.4.3.5 The adrenergic pathway

To assess the possible involvement of the α_2 receptor/system, mice were pre-treated with yohimbine (an α_2 receptor antagonist 3 mg kg^{-1} *p.o.*) 30 min before PME (100 mg kg^{-1},

p.o.), geraniin (10 mg kg^{-1}, *p.o.*) or vehicle administration. Nociception was induced as described under section 2.4.3.1.

2.4.3.6 The serotoninergic system

To assess the possible involvement of 5-HT_3 receptors to the anti-nociceptive effects caused by the agents, animals were pre-treated with ondansetron (0.5 mg kg^{-1}, i.p., a 5-HT_3 receptor antagonist) and after 15 min received PME (100 mg kg^{-1}, *p.o.*), geraniin (10 mg kg^{1}, *p.o.*) or vehicle administration. Nociception was induced as described under section 2.4.3.1.

2.4.3.7 The voltage-gated calcium channels pathway

Mice were pre-treated with nifedipine (10 mg kg^{-1}, *p.o.*, L-type calcium channel blocker) and after 30 min received PME (100 mg kg^{-1}, *p.o.*), geraniin (10 mg kg^{-1}, *p.o.*) or vehicle administration. Nociception was induced as described under section 2.4.3.1.

2.4.3.8 The cholinergic (muscarinic) system

Mice were pre-treated with atropine (non-selective muscarinic antagonist, 5 mg kg^{-1} i.p.) 15 min before PME (100 mg kg^{-1}, *p.o.*), geraniin (10 mg kg^{-1}, *p.o.*) or vehicle administration. Nociception was induced as described under section 2.4.3.1.

2.5 ISOBOLOGRAPHIC ANALYSIS OF GERANIIN/ MORPHINE AND GERANIIN/DICLOFENAC COMBINATIONS

The formalin test was employed because of its high predictiveness of acute pain and it being a valid model of clinical pain (Le Bars *et al*., 2001). The isobolographic analysis was performed similarly as described by Pinardi *et al*., 2005, Miranda *et al.*, 2002 and Woode *et al*., 2015. The ED_{50}s of geraniin, morphine and diclofenac were determined by obtaining

the dose–response curves for the administration of these agents in the formalin test for isobolographic analysis. Dose response curves were also obtained and analyzed after the coadministration of geraniin with morphine or with diclofenac in fixed ratio (1:1) combinations based on the following fractions $^1/_2$, $^1/_4$, $^1/_8$ of their respective ED_{50} for the formalin test (Miranda *et al*., 2013).

To obtain the experimental ED_{50} for geraniin/morphine as well as geraniin/diclofenac coadministration for isobolographic analysis, animals were grouped into thirteen (n=5) for each drug combination and were treated as follows in the formalin test:

Table 2.1: Groupings for isobolographic analysis

Groups	Geraniin and Morphine	Geraniin and Diclofenac
I	Morphine 0.14 mg kg^{-1} i.p. (ED_{50}) for phase I analysis	Diclofenac 14.51 mg kg^{-1} i.p. (ED_{50}) for phase I analysis
II	Morphine 0.33 mg kg^{-1} i.p. (ED_{50}) for Phase II analysis	Diclofenac 21.30 mg kg^{-1} i.p. (ED_{50}) for phase II analysis
III	Geraniin 0.94 mg kg^{-1} *p.o.* (ED_{50}) for phase I analysis	----------
IV	Geraniin 1.02 mg kg^{-1} *p.o.* (ED_{50}) for phase II analysis	----------
V - VIII	[Morphine + Geraniin]: (0.14 + 0.94) mg kg^{-1}, (0.14 + 0.94)/2 mg kg^{-1}, (0.14 + 0.94)/4 mg kg^{-1}, (0.14 + 0.94)/8 mg kg^{-1} for phase I analysis	[Diclofenac + Geraniin]: (14.51 + 0.94) mg kg^{-1}, (14.51 + 0.94)/2 mg kg^{-1}, (14.51 + 0.94)/4 mg kg^{-1}, (14.51 + 0.94)/8 mg kg^{-1} for phase I analysis
IX - XII	[Morphine + Geraniin]: (0.33 + 1.02) mg kg^{-1}, (0.33 + 1.02)/2 mg kg^{-1}, (0.33 + 1.02)/4 mg kg^{-1}, (0.33 + 1.02)/8 mg kg^{-1} for phase II analysis	[Diclofenac + Geraniin]: (21.30 + 1.02) mg kg^{-1}, (21.30 + 1.02)/2 mg kg^{-1}, (21.30 + 1.02)/4 mg kg^{-1}, (21.30 + 1.02)/8 mg kg^{-1} for phase II analysis
XIII	mice were treated with vehicle (10 ml kg^{-1})	mice were treated with vehicle (10 ml kg^{-1})

After the different groups received their respective treatments, formalin was administered and the resultant behavior was recorded for either 10 min for phase I or 60 min for phase II analysis ignoring the first 10 min during analysis.

Isobologram (a cartesian plot of pairs of doses that, in combination, yield a specified level of effect) was then built by connecting the theoretical ED_{50} of morphine or diclofenac plotted on the ordinate with that of geraniin plotted on the abscissa to obtain the additivity line. For each drug mixture, the ED_{50} (experimental) and its associated 95 % confidence intervals were determined by linear regression analysis of the log dose–response curve (and compared by a 't'-test to a theoretical additive ED_{50}) obtained from the calculation;

Z_{add} = f (ED_{50}) of morphine + (1-f) (ED_{50}) of geraniin

Where f is the fraction of each component in the mixture and the variance (Var) of Z_{add} was calculated as:

Var Z_{add} = f^2 (Var ED_{50} of morphine) + $(1-f)^2$ Var ED_{50} of geraniin

From these variances, S.E.M.'s were calculated and resolved according to the ratio of the individual drugs in the combination. A supra-additive or synergistic effect is defined as the effect of a drug combination that is higher and statistically different (ED_{50} significantly lower) than the theoretically calculated equieffect of a drug combination in the same proportion. If the ED_{50}'s are not statistically different, the effect of the combination is additive and additivity means that each constituent contributes with its own potency to the total effect. The degree of interaction was calculated using fractional analysis by dividing the experimental ED_{50} (Z_{mix}) by the theoretical ED_{50} (Z_{add}). A value close to 1 was considered as additive interaction. Values lower than 1 are an indication of the magnitude

of supra-additive or synergistic interactions ($Z_{mix}/Z_{add} < 1$), and values higher than 1 correspond to sub-additive or antagonistic interactions (Ameyaw *et al.*, 2015).

2.6 TOLERANCE AND DEPENDENCE STUDIES

2.6.1 Tolerance studies

The formalin test was used to ascertain whether, after chronic treatment, tolerance develops to the anti-nociceptive activity of PME, geraniin and morphine and if there is morphineinduced tolerance cross-generalization with the extract or geraniin. Mice were divided randomly into nine groups (n = 5) and for the first eight days were treated as follows:

Groups I – IV - Vehicle.

Groups V – VI - PME (200 mg kg^{-1} *p.o.*) and geraniin (20 mg kg^{-1} *p.o.*) respectively.

Groups VII - IX- Morphine 6 mg kg^{-1}, i.p.

On Day 9, these groups were treated in the following manner:

Group I – vehicle

Groups II – IV- PME (100 mg kg^{-1}), geraniin (10 mg kg^{-1}) and morphine (3 mg kg^{-1})

Groups V - VI- PME (100 mg kg^{-1}) and geraniin (10 mg kg^{-1}) respectively

Group VII - Morphine (3 mg kg^{-1})

To ascertain the possibility of morphine-induced tolerance cross-generalizing with extract or geraniin, these groups were treated as follows:

Groups VIII – IX – PME (100 mg kg^{-1}) and geraniin (10 mg kg^{-1}) respectively

Sixty min after geraniin and PME administration and 30 min after morphine administration, formalin was injected into the right paw and the behaviors of the mice were recorded for 60 min just as previously described.

2.6.2 Assessment of the ability of PME and geraniin to induce withdrawal syndromes of dependence

The method as described by Hosseinzadeh and Nourbakhsh, 2003 was used to determine whether PME or geraniin induces withdrawal signs similar to that produced by morphine administration. Mice were grouped (n = 5) and received the following drug treatments,

For the first three days,

Group I - morphine (50, 50 and 75 mg kg^{-1} s.c. at 1100, 1400 and 1700 h, respectively)

Group II – vehicle (10 ml kg^{-1} s.c. at 1100, 1400 and 1700 h, respectively)

Group III – PME (1500, 1500 and 2250 mg kg^{-1} *p.o.* at 1100, 1400 and 1700 h, respectively)

Group IV – geraniin (150, 150 and 225 mg kg^{-1} *p.o.* at 1100, 1400 and 1700 h respectively)

On the fourth day, Group I received morphine (50 mg kg^{-1}, s.c.), Group II received vehicle (10 ml kg^{-1}, s.c.), Group III received PME (1500 mg kg^{-1}, *p.o.*) and Group IV received geraniin (150 mg kg^{-1}, *p.o.*). Two hours later, naloxone (5 mg kg^{-1}, s.c.) was administered to all the animals and mice were immediately placed in a glass cylinder (30 cm high, 20 cm in diameter). The number of jumping episodes (withdrawal symptoms) was recorded for 30 min.

2.6.3 Assessment of the effect of PME and geraniin on the withdrawal syndrome of morphine dependence

To determine whether PME and geraniin can ameliorate withdrawal signs produced by naloxone precipitated morphine dependence (Hosseinzadeh and Nourbakhsh, 2003), mice were randomly assigned to 8 groups (I - VIII) and received morphine (50, 50 and 75 mg kg^{-1} s.c. at 1100, 1400 and 1700 h, respectively) for 3 days. On the 4th day, group I received vehicle (10 ml kg^{-1}, *p.o.*), groups II - IV received PME (30 – 300 mg kg^{-1}, *p.o.*), groups V – VII received geraniin (3 - 30 mg kg^{-1}, *p.o.*) and group VIII was treated with diazepam (5 mg kg^{-1}, i.p.). Thirty minutes (for i.p.) or one hour (for *p.o.*) later, all animals received morphine (50 mg kg^{-1}, s.c.) and then 2 h later they were all treated with naloxone (5 mg kg^{-1}, s.c.) to precipitate morphine withdrawal. The number of jumps was recorded for 30 min and scored with JWatcher.

2.7 ASSESSMENT OF THE SAFETY AND TOXICITY PROFILE OF PME

2.7.1 Motor co-ordination test

Naive mice were trained on three successive days on the rota-rod (Ugo Basile, model 7600, Comerio, Varese, Italy) at a speed of 25 rpm. On the test day (day 4), five groups of mice (n = 5) were administered the extract (30 - 300 mg kg^{-1}, *p.o.*), D-tubocurarine (0.1 mg kg^{-1}, i.p.) or vehicle. The animals were then repeatedly tested for their motor co-ordination performance on the rota-rod (cut off time 120 s) at 0.5, 1, 1.5, 2, 2.5 and 3 h after drug administration. The maximum time that the animals were able to spend on the rota rod was recorded (Gareri *et al.*, 2005).

2.7.2 Acute toxicity

Mice (25 - 30 g) were put into six groups of five mice each. They were fasted for 3 h and doses of *P. muellerianus* extract (0.1, 0.3, 1, 3, and 5.0 g kg^{-1}) were orally administered. Distilled water was administered to the control group and the mice were observed for 24 h after treatment for any toxic effects or death. Mice were also observed for two weeks for any lethality.

2.7.3 Sub-acute toxicity studies of the extract

To determine whether *Phyllanthus muellerianus* produced toxic effects after continuous administration of the extract, male Sprague-Dawley rats, 5 per group, were treated orally with *P. muellerianus* (100, 300, 1000 mg kg^{-1}) daily, for 7 consecutive days (Bamisaye *et al.*, 2015). Group A, the control, received 10 ml kg^{-1} *p.o* of distilled water twice daily. Group B, C and D were also treated with twice daily doses of the extract. The animals were monitored closely for signs of toxicity by assessing their appearance and behaviour pattern.

2.7.3.1 Preparation of serum and isolation of organs

The rats were sacrificed on the eighth day by cervical dislocation, the jugular vein was cut for blood to flow freely. Approximately 1.5 ml of blood was collected into vacuum tubes containing ethylene diamine tetra acetic acid (EDTA) as an anticoagulant for haematological assay and 3.5 ml of the blood was collected into sample tubes without anticoagulant for biochemical analysis. The blood without the anticoagulant was allowed to clot before centrifugation (4000 rpm at 4 ℃ for 10 min) to obtain serum, which was collected and stored at -20 ºC until assayed for biochemical parameters. After blood collection, the rats were dissected and the organs (liver, kidney and stomach) removed,

freed of fat and connective tissue, blotted with clean tissue paper and then weighed on a balance.

2.7.3.2 Effect of PME on haematological and biochemical parameters

Haematological parameters including haemoglobin (HGB), red blood cells (RBC), white blood cells (WBC), haematocrit (HCT), platelets (PLT), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH) and mean corpuscular haemoglobin concentration (MCHC) were determined by an automatic analyzer (Sysmex KX-21N™ Automated Hematology Analyzer, Sysmex America, Inc., Lincolnshire, IL, U.S.A.). Biochemical analyses were performed on serum for the determination of the following parameters: electrolytes, aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), total bilirubin (T-BIL), direct bilirubin (D-BIL), indirect bilirubin (IBIL), total protein, albumin, urea and creatinine. All analyses were carried out using an automated clinical chemistry analyser (Vital Scientific Flexor Junior®, AC Dieren, The Netherlands).

2.7.3.3 Effect of extract on body and organ weights

Body weights (in grams) of the rats were taken on days 0 and 8. The relative organ weights (ROW) of each organ was calculated as follows:

$$\mathbf{ROW} = \frac{\mathbf{Absolute\ Organ\ Weight\ (g)}}{\mathbf{Rat\ body\ weight\ on\ sacrifice\ day\ (g)}} \times \mathbf{100}$$

2.7.3.4 Histopathological examination

Portions of the tissue from liver, kidney and stomach were used for histopathological examination. Tissues were fixed in 10 % neutral buffered formalin (pH 7.2) and dehydrated through a series of ethanol solutions, embedded in paraffin and routinely processed for

histological analysis. Sections of 2 μm thickness were cut and stained with haematoxylineosin for examination. The stained tissues were observed with a Leica microscope (Leica DMD 108, Germany).

2.8 STATISTICS

In all experiments, a sample size of n = 5 were used. All data are presented as mean ± S.E.M. The time-course curves were subjected to two-way (*treatment* × *time*) repeated measures analysis of variance (ANOVA) followed by Dunnett's multiple comparisons test. Total anti-nociceptive effect for each treatment was calculated in arbitrary unit as the area under the curve (AUC). Differences in total anti-nociceptive effect were analyzed using one-way ANOVA followed by Tukey's multiple comparisons test with drug treatment as a between subject factor for data which were normally distributed. For data which were not normally distributed, differences in total anti-nociceptive effect were analyzed using Kruskal-Wallis test followed by Dunn's multiple comparison test. To determine the percentage inhibition for each treatment, the following equation was used

$$\%\ Inhibition = \frac{(AUCcontrol - AUCtreatment)}{AUCcontrol} * 100\ \%$$

Doses for 50 % of the maximal effect (ED_{50}) for each drug were determined by using an iterative computer least squares method, with the following nonlinear regression (three parameter logistic) equation

$$Y = \frac{a + (b - a)}{(1 + 10^{(LogED_{50} - x)})}$$

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 (ED_{50}s) of the curves were compared statistically using *F* test.

Isobolographic calculations were performed with the program Pharm Tools Pro (version 1.27, the McCary Group Inc.). Results are presented as mean ± S.E.M. or as ED_{50} values with 95 % confidence limits. The statistical analyses of the isobolograms were performed according to Tallarida (Tallarida, 2006) and the statistical difference between experimental and theoretical values was assessed by the Student's 't' test for independent means. Levels of significance were represented with * and †. $P \leq 0.05$ was considered statistically significant

Chapter 3

RESULTS

3.1 ANTI-NOCICEPTIVE EFFECTS

3.1.1 Acetic acid-induced writhing test

PME, morphine and diclofenac significantly reduced the writhing in the mice as shown by the time-course curve (Figure 3.1a, c and e). Two-way ANOVA (*treatment x time*) revealed a significant (PME: $F_{4, 20}$ = 1.397, $P < 0.0001$; morphine: $F_{3, 15}$ = 6.519, $P < 0.0001$ and diclofenac: $F_{3, 15}$ = 5.221, $P < 0.0001$) effect of drug treatments on the acetic acid-induced abdominal constrictions. PME (10 – 300 mg kg^{-1}) significantly (P = 0.0156) reduced the number of abdominal writhes over 30 min with the highest dose of 300 mg kg^{-1} giving an increase in total anti-nociceptive effect of 86.67 % compared to the control (Figure 3.1b). Morphine and diclofenac also produced a significant and dose-dependent (P = 0.0095; P = 0.0036 respectively)(Fig. 3.1d, f) increase in total anti-nociceptive effects with the highest doses of 10 mg kg^{-1} and 100 mg kg^{-1} producing significant increases of 92 % and 94.1 % respectively in total anti-nociceptive effect. Morphine (ED_{50}: 0.11 mg kg^{-1}) was however the most potent of the three agents used, followed by diclofenac (ED_{50}: 4.24 mg kg^{-1}) and then PME (ED_{50}: 22.60 mg kg^{-1}) (Fig. 3.5a and Table 3.1).

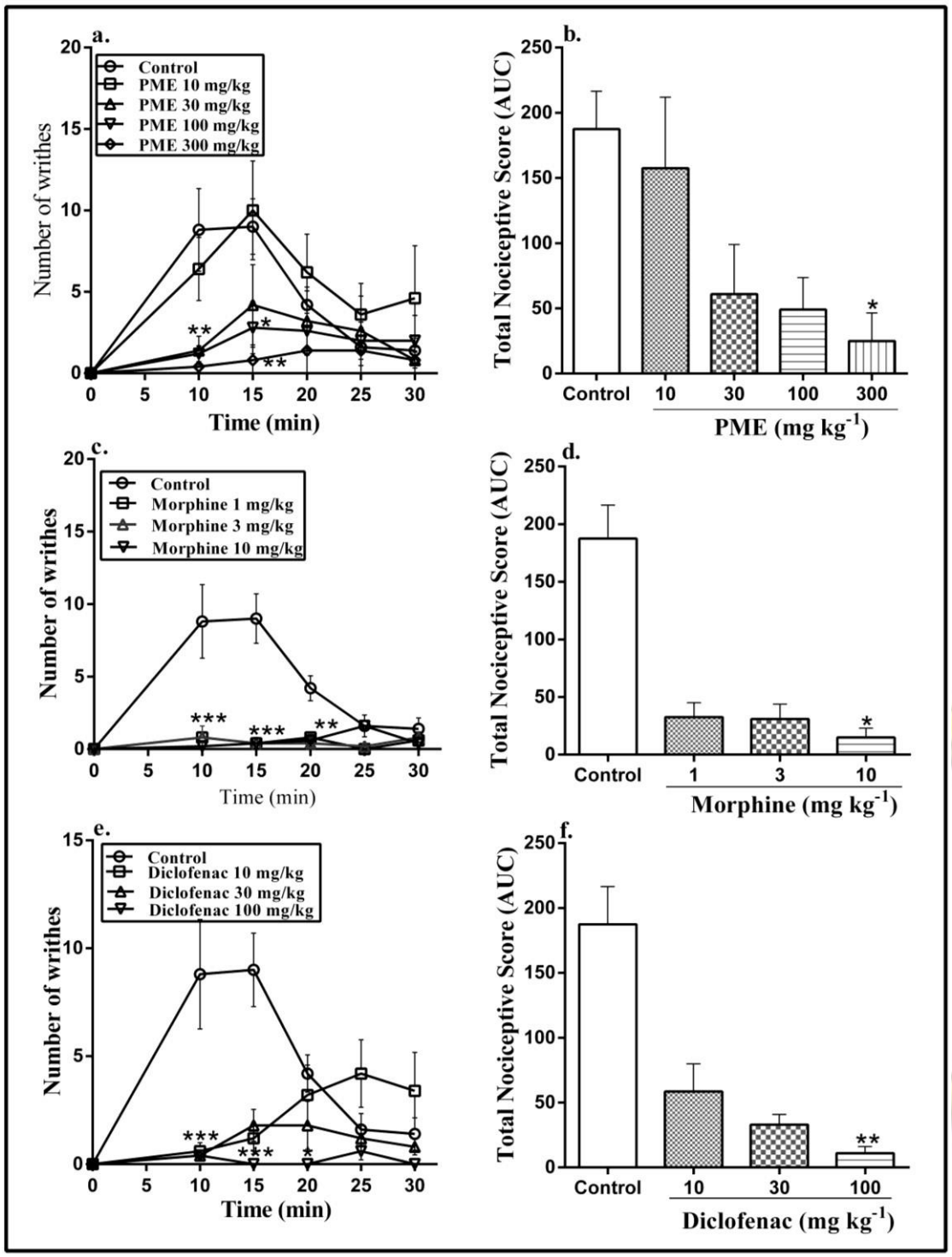


Figure 0.1: Effects of PME (10 – 300 mg kg^{-1}) (a,b), morphine (1 - 10 mg kg^{-1}) (c, d) and diclofenac (10 – 100 mg kg^{-1}) (e, f) on acetic acid-induced writhing. Data points are group means ± S.E.M. Significantly different from control: * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$. Two-way ANOVA followed by Bonferroni posthoc test and differences in AUCs analysed by Kruskal-Wallis test followed by Dunn's multiple comparison test.

3.1.2 Formalin-induced nociception

Injection of formalin produced a biphasic nociception response as shown in Figs. 3.2 - 3.4. Administration of PME (30 – 300 mg kg^{-1}), geraniin (3 - 30 mg kg^{-1}), diclofenac (10 - 100 mg kg^{-1}) and morphine (1 – 10 mg kg^{-1}) to the mice significantly attenuated the nociception induced by formalin as shown by the time-course curves (Fig. 3.2). Two-way ANOVA (*treatment x time*) revealed a significant (PME: $F_{36, 204} = 3.427$, $P < 0.0001$; geraniin: $F_{36, 204} = 2.962$, $P < 0.0001$; morphine: $F_{36, 204} = 3.352$, $P < 0.0001$; diclofenac: $F_{36, 204} = 2.897$, $P < 0.0001$) effect of drug treatments on the formalin induced nociception. PME dose dependently and significantly suppressed paw licking time in both the first phase ($P = 0.0032$) and second phase ($F_{3, 15} = 29.79$, $P < 0.0001$) (Fig. 3.3b and 3.4b). However, the highest increase in anti-nociception of 99.68 % as compared to the control was observed in the second phase at a dose of 300 mg kg^{-1} (Fig. 3.4b). Geraniin also significantly and dose dependently suppressed paw licking time in both the first phase ($P = 0.0041$) and second phase ($P = 0.0046$) (Fig. 3.3a and 3.4a). Percentage increases in anti-nociceptive effects of 98.45 % and 99.24 % as compared to the control were observed in the first and second phases respectively at a dose of 30 mg kg^{-1} (Fig. 3.3a and 3.4a).

In a similar manner, morphine administration resulted in a significant reduction of response time in the early ($P = 0.0059$) and the late ($F_{3, 13} = 26.76$, $P < 0.0001$) phases of formalininduced licking with maximal inhibitions of 99.02 % and 99.88 % of the first and second phase respectively (Figure 3.3c and 3.4c). Diclofenac also significantly suppressed paw licking time in both the first phase ($P = 0.0012$) and second phase ($F_{3, 15} = 22.66$, $P <$

0.0001) (Fig. 3.3d and 3.4d). Percentage increases in anti-nociceptive effects of 99.86 % and 99.87 % as compared to the control were observed in the first and second phases respectively at a dose of 100 mg kg^{-1} (Fig. 3.3d and 3.4d). In both phases, morphine was however the most potent, followed by geraniin, PME and then diclofenac (Fig. 3.5b, 3.5c and Table 3.1).

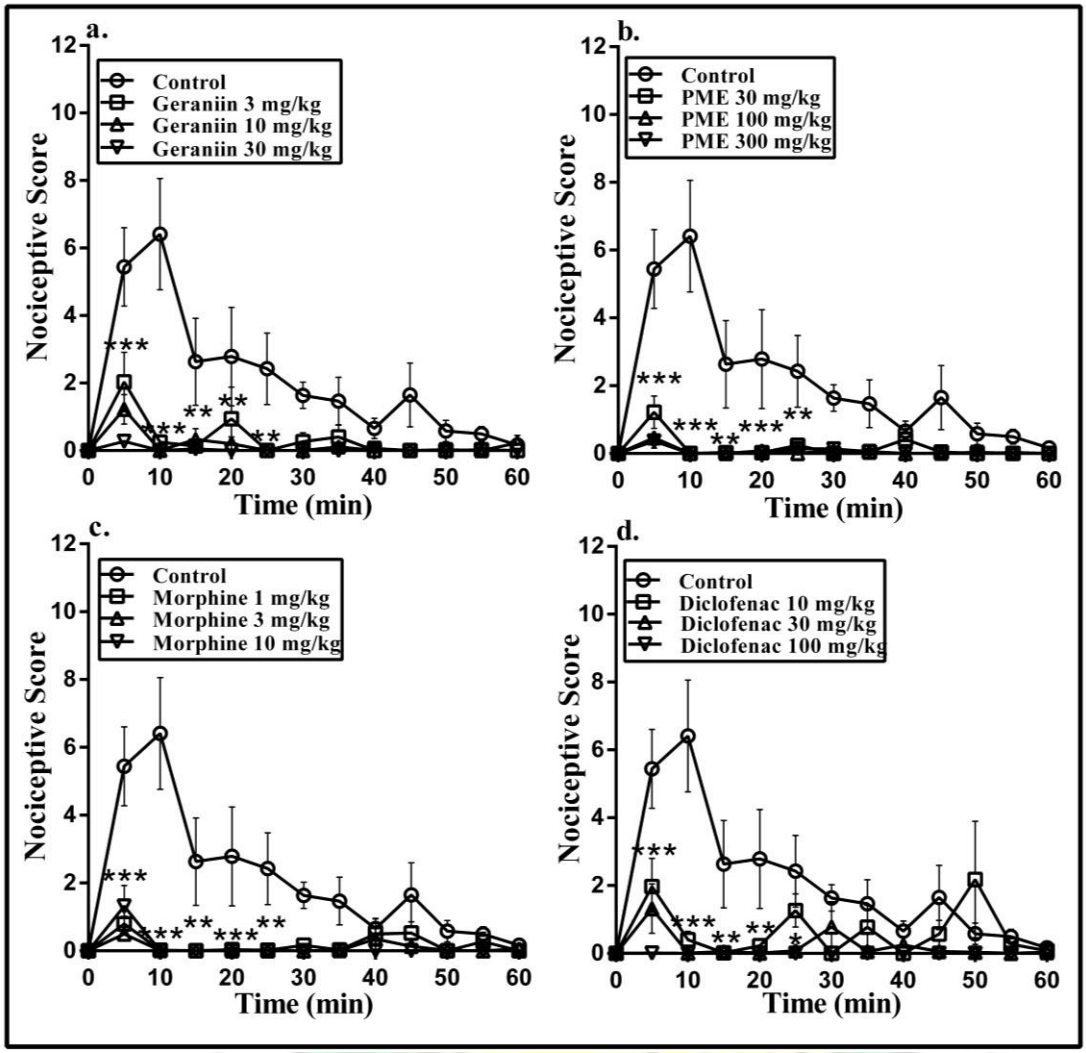


Figure 0.2: The time-course effects of geraniin (3 – 30 mg kg^{-1}), PME (30 - 300 mg kg^{-1}), morphine (1 - 10 mg kg^{-1}) and diclofenac (10 – 100 mg kg^{-1}) on formalin-induced nociception in mice. ** $P \leq 0.01$, *** $P \leq 0.001$ (Two-way ANOVA followed by Bonferroni posthoc test).

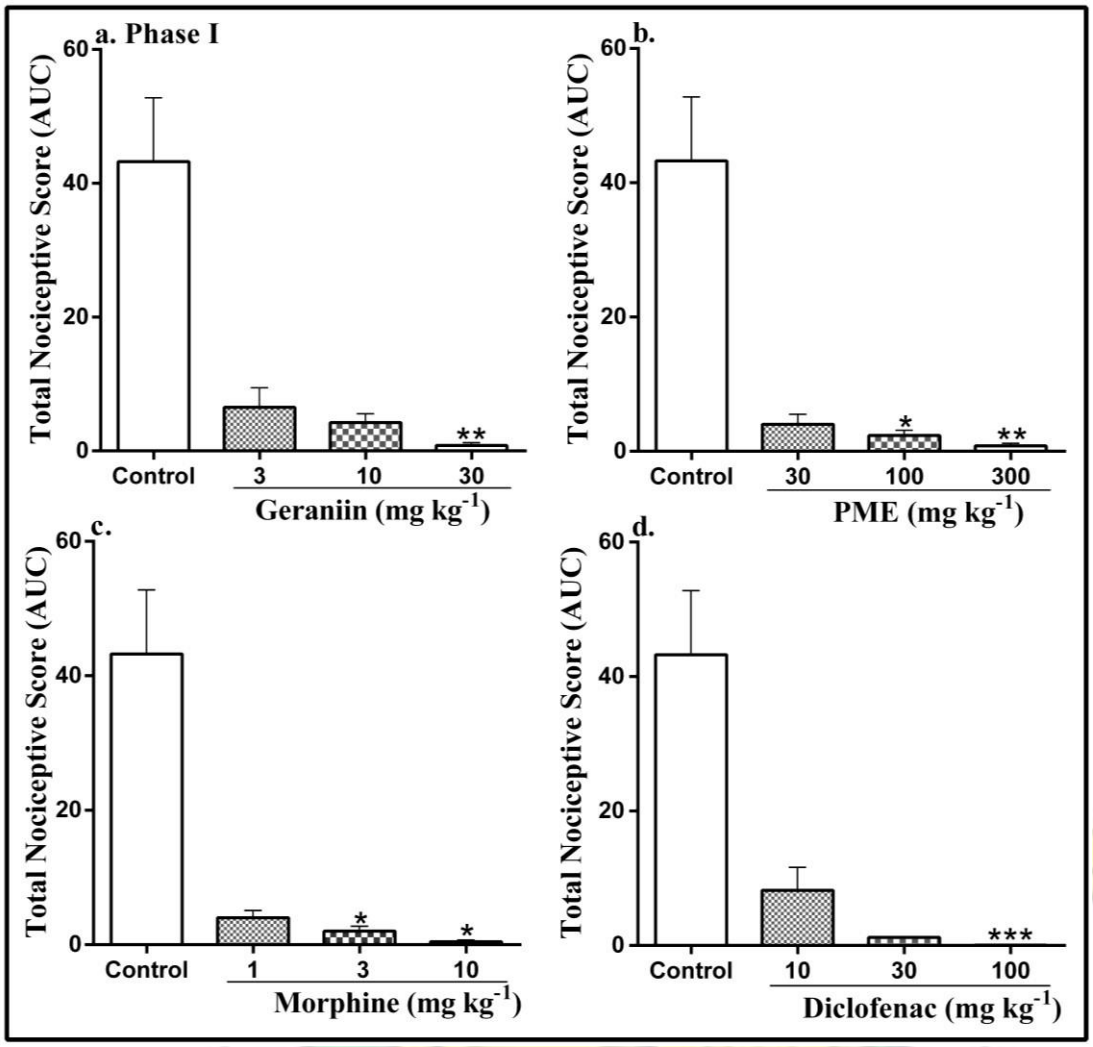


Figure 0.3: The total anti-nociceptive effects of geraniin (3 – 30 mg kg^{-1}), PME (30 - 300 mg kg^{-1}), morphine (1 - 10 mg kg^{-1}) and diclofenac (10 – 100 mg kg^{-1}) in the phase of the formalin-induced nociception. * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$

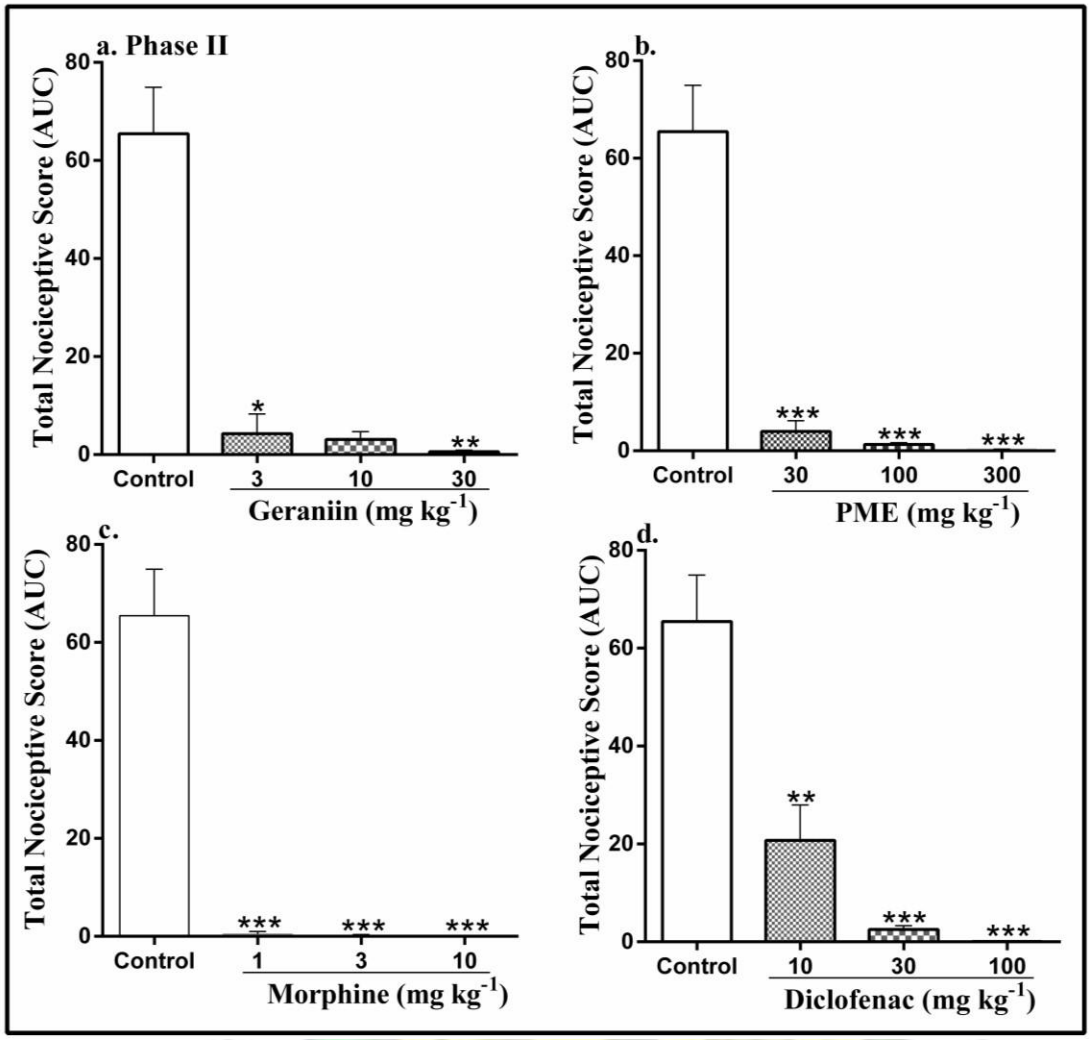


Figure 0.4: The total anti-nociceptive effects of geraniin (3 – 30 mg kg^{-1}), PME (30 - 300 mg kg^{-1}), morphine (1 - 10 mg kg^{-1}) and diclofenac (10 – 100 mg kg^{-1}) in the second phase of the formalininduced nociception in mice. * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$.

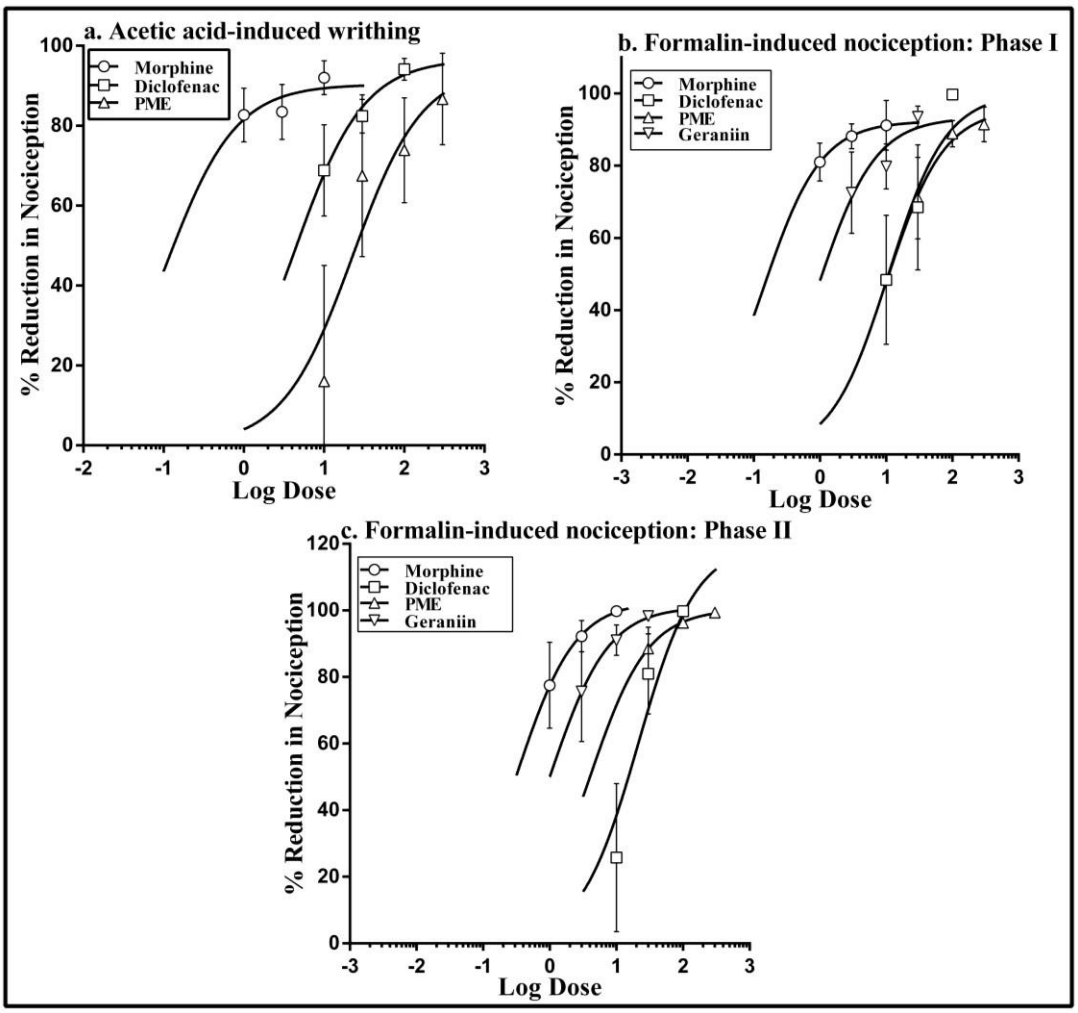


Figure 0.5: Dose-response effects of PME (30 - 300 mg kg^{-1}), geraniin (3 – 30 mg kg^{-1}) morphine (1 - 10 mg kg^{-1}) and diclofenac (10 – 100 mg kg^{-1}) in the acetic acid-induced writhing and formalininduced nociception models. Data points represents mean ± S.E.M. (n = 5).

Table 3.1: The potencies of the various agents used in the models of nociception

Drugs	ED$_{50}$s (mg kg^{-1})

	Acetic acid-induced writhing	Formalin-induced ociception	
		Phase I	Phase II
Morphine	0.11	0.14	0.33
Diclofenac	4.24	14.51	21.30
PME	22.60	10.13	4.05
Geraniin	****	0.94	1.02

****: Geraniin was not used in the acetic acid-induced writhing test.

3.1.3 Mechanism of Action

The effect of naloxone, glibenclamide, L-NAME, yohimbine, atropine, theophylline, ondansetron and nifedipine on the anti-nociceptive effects of the extract (Fig. 3.6a) and geraniin (Fig. 3.6b) were investigated. All the antagonists except naloxone did not affect significantly the anti-nociceptive activity of PME and geraniin. Naloxone reduced the antinociceptive effect of geraniin in the second phase by 55.42 % (Fig. 3.6b) but such a significant reversal of anti-nociceptive effect was not observed in the first phase.

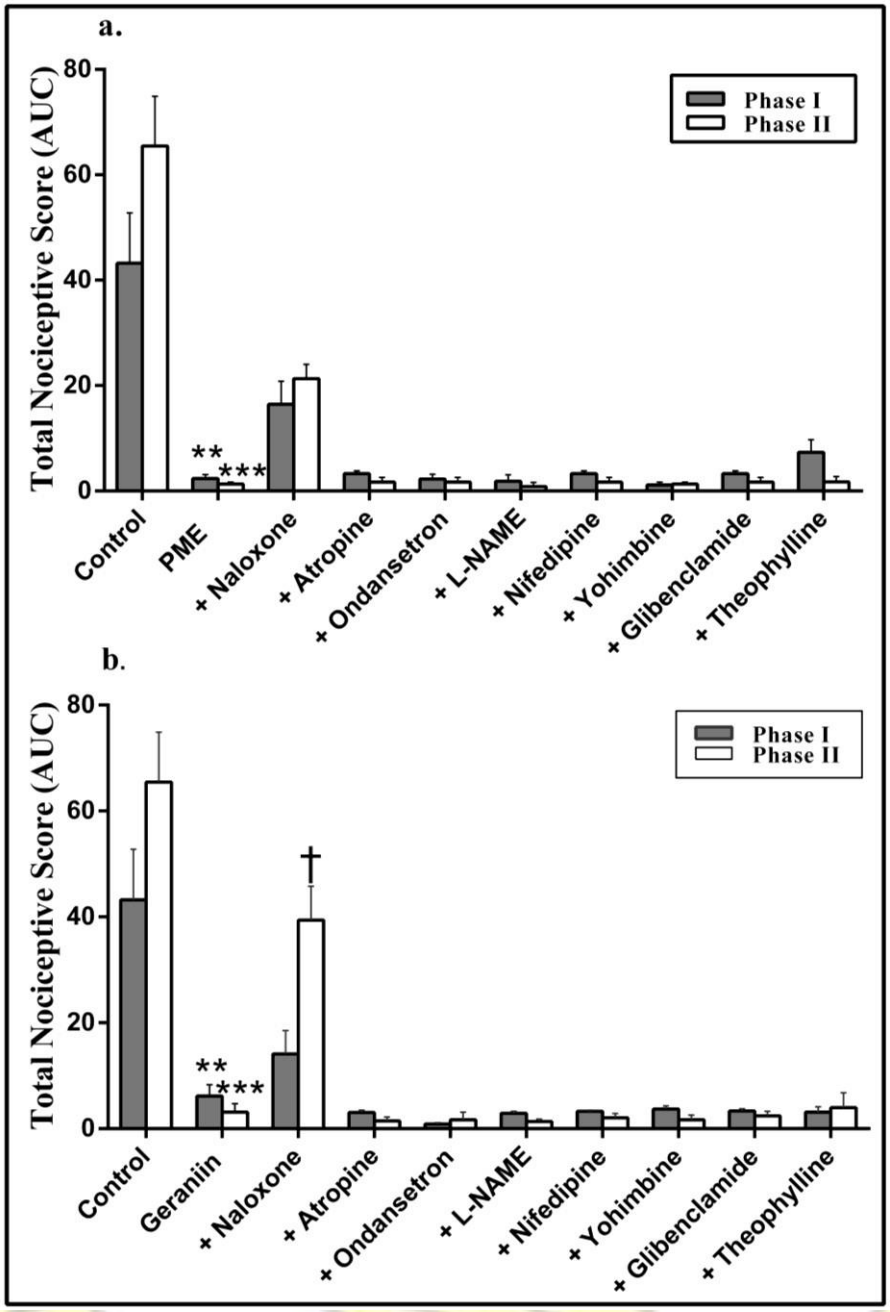


Figure 0.6: Effect of different antagonists on the anti-nociceptive effect of (a) PME (100 mg kg^{-1}) and (b) geraniin (10 mg kg^{-1}) for phase I and phase II of formalin-induced nociception. Each column represents the mean ± S.E.M. ** $P \leq 0.01$, *** $P \leq 0.001$, † $P \leq 0.05$ compared to respective controls.

3.2 ISOBOLOGRAPHIC ANALYSIS

3.2.1 Isobolographic analysis of geraniin and morphine

Geraniin, morphine and the fractions of geraniin and morphine combinations inhibited both phases in the formalin-induced nociception model (Fig. 3.7a). However the inflammatory pain inhibitions of both morphine and geraniin were higher than the neurogenic pain inhibition. Geraniin significantly inhibited neurogenic pain by 91.30 ± 9.76 % for phase I and 99.86 ± 0.29 % for phase II respectively (Fig. 3.7b and c). Morphine also inhibited neurogenic pain by 87.63 ± 7.84 % for phase I and 98.21 ± 2.79 % for phase II respectively (Fig. 3.7b and c). All the combinations significantly inhibited the biting and licking behavior induced in the mice after formalin injection with the highest change in response to the control being observed for $Z_{mix}/8$ in the second phase (Fig. 3.7c). Morphine was however more potent than geraniin in both phases (Table 3.2).

Table 3.2: Potencies of morphine and geraniin for both phases of formalin-induced nociception.

Drugs	Phase I ED_{50} (mg kg^{-1})	Phase II ED_{50} (mg kg^{-1})
Morphine	0.14 ± 0.10	0.33 ± 0.19
Geraniin	0.94 ± 0.60	1.02 ± 0.67

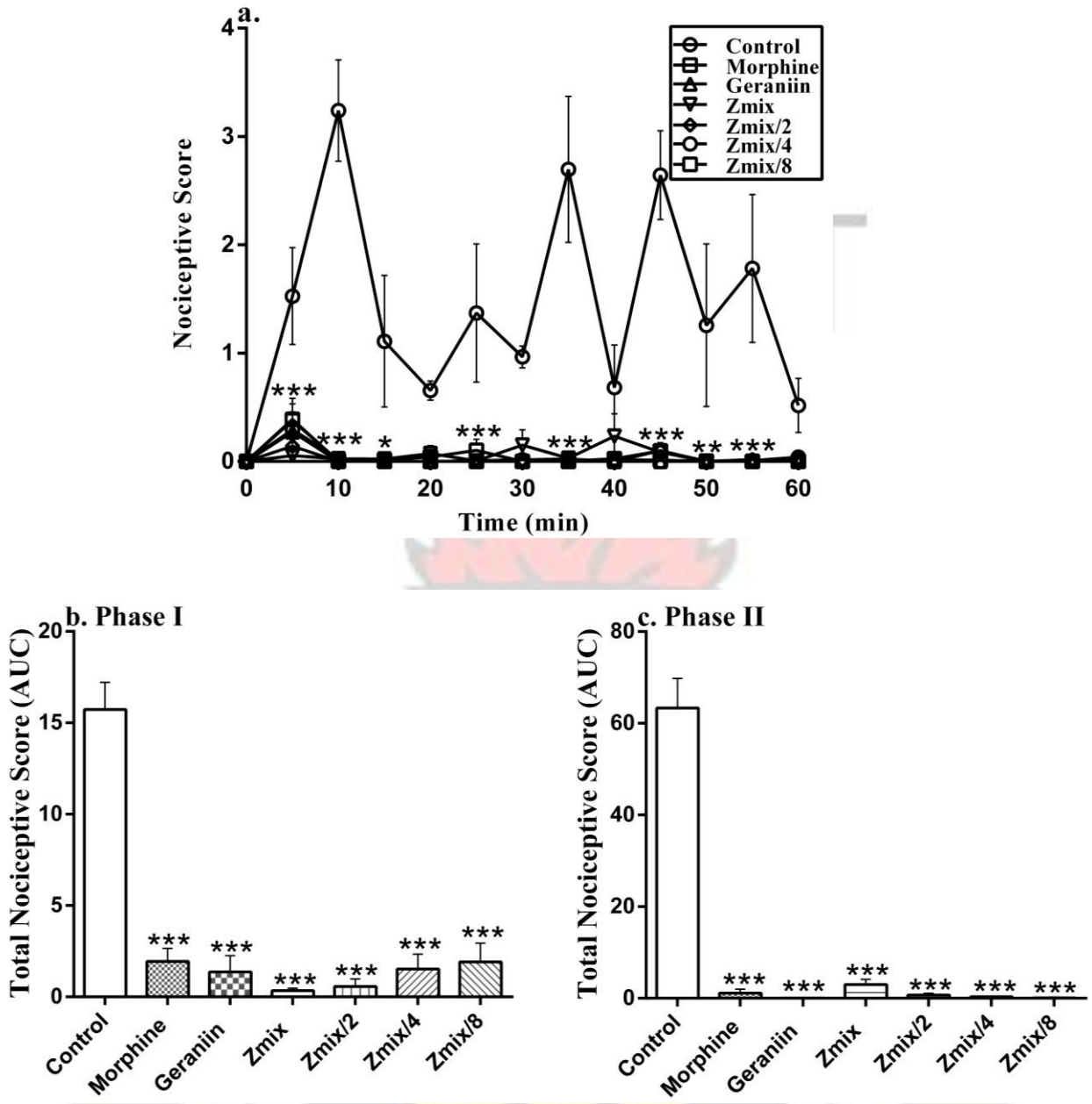


Figure 0.7: The time-course and total anti-nociceptive effects of geraniin, morphine and fractions of their combination on formalin-induced nociception. Each point represents the mean ± S.E.M, * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$ compared to respective controls (Two-way ANOVA followed by Dunnett's multiple comparisons test; One-way ANOVA followed by Dunnett's multiple comparisons test).

3.2.1.1 Isobologram of geraniin and morphine

The experimental ED_{50} (Z_{mix}) obtained by non-linear regressional analysis for phase I (Fig. 3.8a) was 0.022 ± 5.01 x 10^{-3} mg kg^{-1} and 0.0027 ± 1.89 x 10^{-3} mg kg^{-1} for phase II (Fig.

3.8b) indicating synergism of the anti-nociceptive effect of the combination as comparing the experimental ED_{50} to the theoretical ED_{50} using the Student's 't' test for independent means, revealed significantly smaller values for the experimental ED_{50}s ($p < 0.05$ for phase I and $p < 0.01$ for phase II).

This synergistic effect was confirmed further by the calculation of the interaction index by fractional analysis for both phases and also graphically represented as Z_{mix}'s lying below the line of additivity (Fig. 3.8 c and d respectively) of the isobologram.

Table 3.3: Theoretical and experimental potencies of geraniin and morphine for both phases of formalin-induced nociception with their computed interaction indices.

Combinations	**geraniin/ morphine**	**geraniin/morphine**
	Phase I	**Phase II**
Theoretical ED_{50} (mg kg^{-1})	0.54 ± 0.19	0.68 ± 0.14
Experimental ED_{50} (mg kg^{-1})	0.0220 ± 5.01 x 10^{-3}*	0.0027 ± 1.89 x 10^{-3}**
Interaction Index	0.040	0.004
Drugs ratio	6.71 : 1	3.09 : 1

*$P \leq 0.05$ compared experimental ED_{50} to theoretical ED_{50}. Values are expressed as mean ± S.E.M.

a. Phase I **b. Phase II**

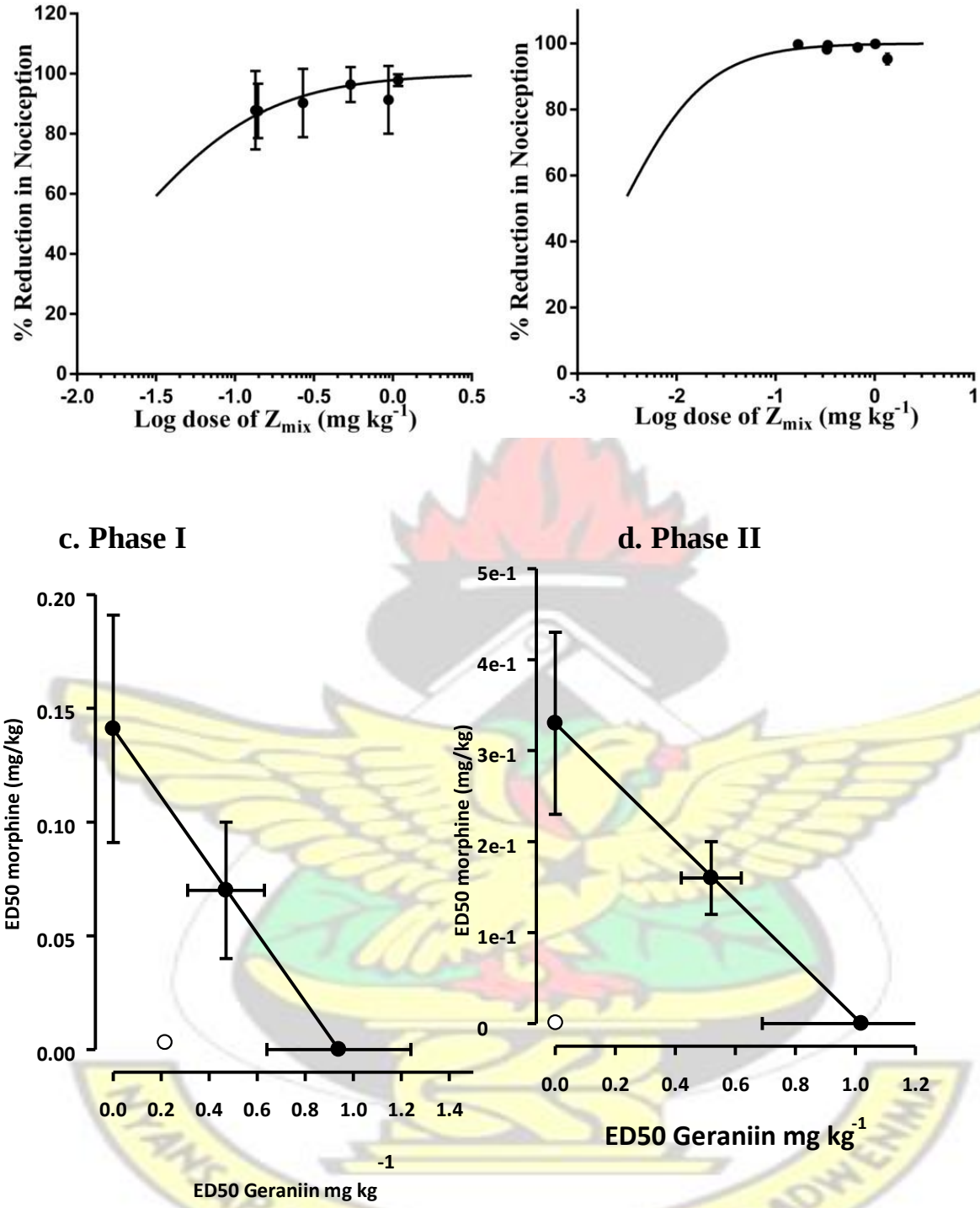


Figure 0.8: Dose-response curves for geraniin and morphine and fractions of their combination for (a) phase I and (b) phase II of formalin-induced nociception respectively. Isobolograms for the combination of morphine and geraniin in (c) phase I and (d) phase II of formalin-induced nociception in mice found below the curves. Filled circles (●) are the theoretical ED_{50}'s ± S.E.M. and open circles (o), the experimental ED_{50}'s ± S.E.M.

3.2.2 Isobolographic analysis of geraniin/diclofenac combination

Geraniin, diclofenac and the fractions of geraniin and diclofenac combinations inhibited both neurogenic and inflammatory pain in the formalin test (Fig. 3.9a). However the inflammatory pain inhibitions by both diclofenac and geraniin were higher than the neurogenic pain inhibitions. Geraniin significantly inhibited neurogenic pain by 91.30 ± 9.76 % for phase I and 99.86 ± 0.29 % for phase II respectively (Fig. 3.9b and c). Diclofenac also inhibited neurogenic pain by 93.17 ± 5.79 % for phase I and 98.21 ± 2.79 % for phase II respectively (Fig. 3.9b and c). Again, all the combinations significantly inhibited the biting and licking behavior induced in the mice after formalin injection with even the lowest combinations of the doses showing significant anti-nociceptive effects ($p < 0.001$) in both phases (Fig. 3.9b, c). Geraniin was however more potent than diclofenac in both phases (Table 3.4).

Table 3.4: Potencies of diclofenac and geraniin for both phases of formalin-induced nociception.

Drugs	**Phase I ED_{50} (mg kg^{-1})**	**Phase II ED_{50} (mg kg^{-1})**
Diclofenac	14.51 ± 10.03	21.30 ± 15.17
Geraniin	0.94 ± 0.60	1.02 ± 0.60

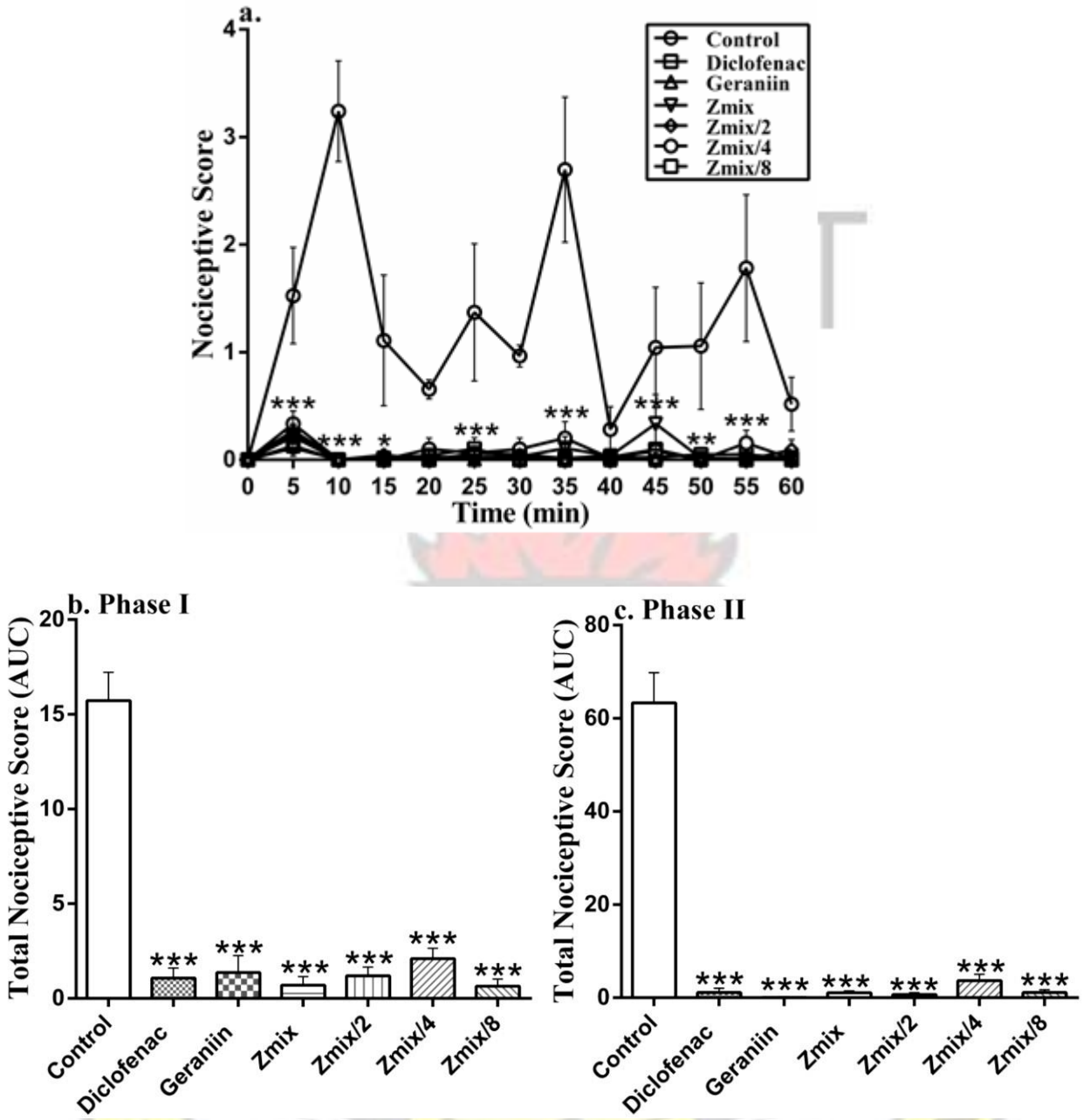


Figure 0.9: The time-course and total anti-nociceptive effects of geraniin, diclofenac and fractions of their combination on formalin-induced nociception. Each point represents the mean ± S.E.M, * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$ compared to respective controls (Two-way ANOVA followed by Dunnett's multiple comparisons test; One-way ANOVA followed by Dunnet's multiple comparisons test).

3.2.2.1 Isobologram of geraniin and diclofenac

The theoretical additive ED_{50} (Z_{add}) was computed as 7.73 ± 2.00 mg kg^{-1} for phase I (Fig. 3.10c) and 11.16 ± 3.08 mg kg^{-1} for phase II (Fig. 3.10d) and (Table 3.5).

The experimental ED_{50} (Z_{mix}) obtained by non-linear regressional analysis for phase I (Fig. 3.10a) was 0.13 ± 0.044 mg kg^{-1} and 0.019 ± 0.016 mg kg^{-1} for phase II (Fig. 3.10b) indicating a synergistic anti-nociceptive effect of the combination as comparing the experimental ED_{50} to the theoretical ED_{50} using the Student's 't' test for independent means, revealed significant smaller values for the experimental ED_{50}s ($p < 0.01$ for phase II for both phases).

This synergistic interaction was confirmed by the calculation of the interaction index by fractional analysis for phase I and phase II and also graphically displayed as Z_{mix}'s lying below the line of additivity (Fig. 3.10c and d respectively) of the isobologram.

Table 3.5: Theoretical and experimental potencies of geraniin and diclofenac for both phases of formalin-induced nociception with their computed interaction indices.

Combinations	**geraniin/diclofenac** **Phase I**	**geraniin/diclofenac** **Phase II**
Theoretical ED_{50} (mg kg^{-1})	7.73 ± 2.00	11.16 ± 3.08
Experimental ED_{50} (mg kg^{-1})	0.130 ± 0.044**	0.019 ± 0.016**
Interaction Index	0.017	0.002
Drugs ratio	1 : 0.065	1 : 0.048

* $P \leq 0.05$ compared experimental ED_{50} to theoretical ED_{50}. Values are expressed as mean ± S.E.M.

a. Phase I **b. Phase II**

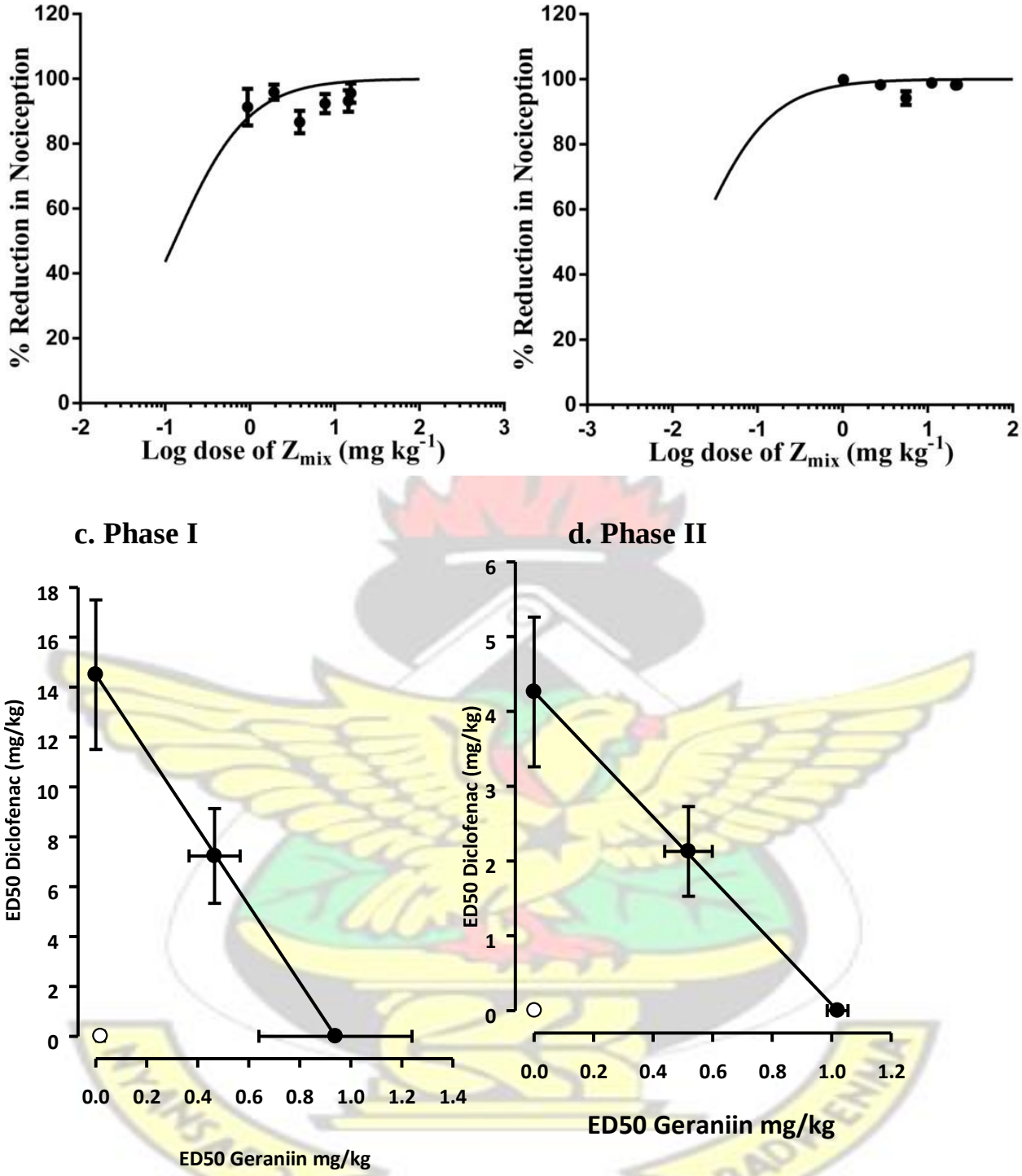


Figure 0.10: Dose-response curves for geraniin and diclofenac and fractions of their combination for (a) phase I and (b) phase II of the formalin-induced nociception respectively. Isobolograms for the combination of morphine and geraniin in (c) phase I and (d) phase II of formalin test in mice found below the curves. Filled circles (●) are the theoretical ED_{50}'s ± S.E.M. and open circles (o), the experimental ED_{50}'s ± S.E.M.

3.3 TOLERANCE AND DEPENDENCE STUDIES

3.3.1 Tolerance Studies

Morphine (3 mg kg^{-1}, i.p.) significantly reduced ($F_{2,12}$ = 51.15, P < 0.0001 Phase I; $F_{2,12}$ = 82.99, P < 0.0001 Phase II) the nociceptive responses in the neurogenic and inflammatory phases of formalin-induced nociception in mice which were treated with vehicle from day 1 - 8. However, the same dose of morphine administered on day 9 in mice treated with morphine from day 1 - 8 did not show a similar effect suggesting the incident of tolerance (Figs. 3.11c, 3.12). In contrast, oral administration of PME (100 mg kg^{-1}) showed a comparable anti-nociceptive activity ($F_{3,16}$ = 52.78, P < 0.0001 Phase I; $F_{3,15}$ = 151.7, P < 0.0001 Phase II) in mice given chronic treatment of either PME (200 mg kg^{-1}, *p.o.*) or vehicle, indicating lack of tolerance development. Furthermore, PME (100 mg kg^{-1}, *p.o.*) still demonstrated anti-nociceptive activity in mice chronically treated with morphine (6 mg kg^{-1}, i.p.), indicating that no cross-tolerance exists with morphine (Figs. 3.11a, 3.12). Again, oral administration of geraniin (10 mg kg^{-1}) showed a comparable anti-nociceptive activity ($F_{3,16}$ = 53.72, P < 0.0001 Phase I; $F_{3,14}$ = 137.2, P < 0.0001 Phase II) in mice given chronic treatment of either geraniin (20 mg kg^{-1}, *p.o.*) or vehicle, indicating lack of tolerance development. Furthermore, geraniin (10 mg kg^{-1}, *p.o.*) still demonstrated anti-nociceptive activity in mice chronically treated with morphine (6 mg kg^{-1}, i.p.), indicating that no crosstolerance exists with morphine (Figs. 3.11b, 3.12).

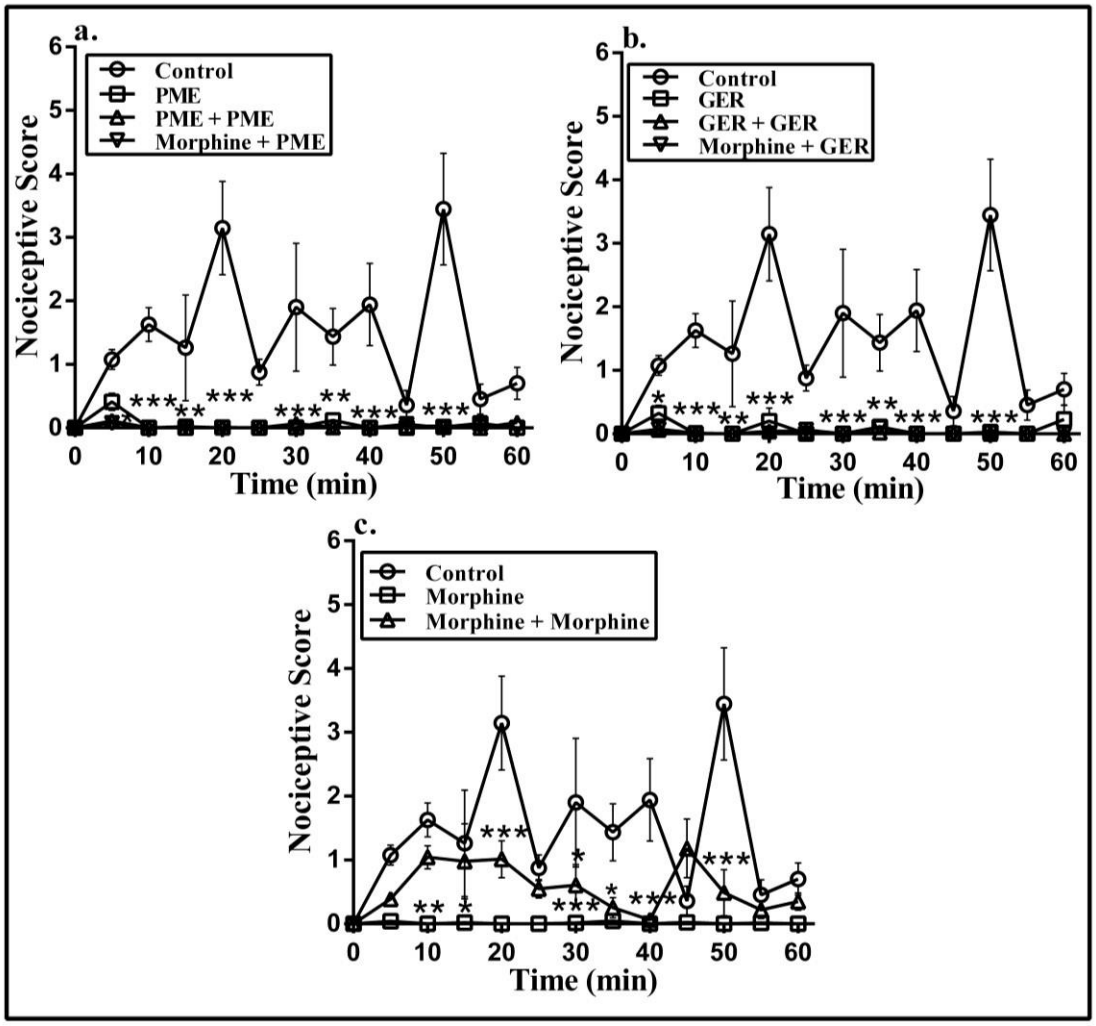


Figure 0.11: The time-course effects of (a) PME (100 mg kg^{-1}), (b) geraniin (10 mg kg^{-1}) and (c) morphine (3 mg kg^{-1}) challenge on formalin-induced nociception in mice chronically treated with vehicle, PME (200 mg kg^{-1}), geraniin (20 mg kg^{-1}) or morphine (6 mg kg^{-1}) for 8 days. Each point represents the mean ± S.E.M. * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$ compared to respective controls (Two-way ANOVA followed by Dunnett's multiple comparisons test).

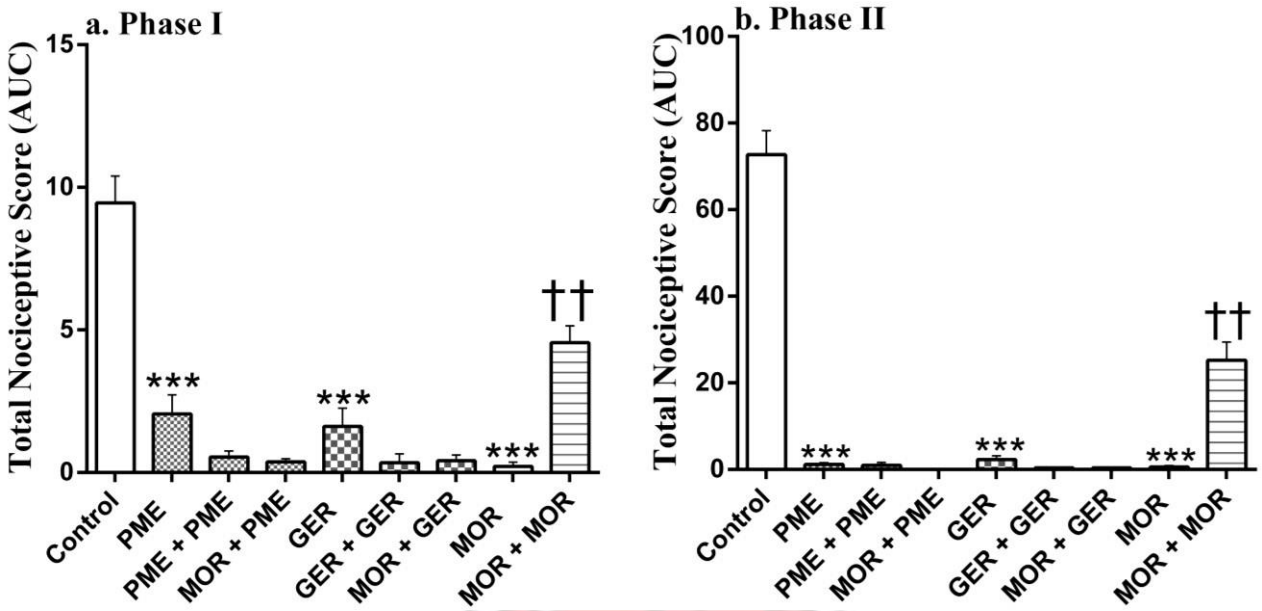


Figure 0.12: The total anti-nociceptive effects of PME (100 mg kg^{-1}), geraniin (10 mg kg^{-1}) and morphine (3 mg kg^{-1}) challenge on formalin-induced nociception in mice chronically treated with vehicle, PME (200 mg kg^{-1}), geraniin (20 mg kg^{-1}) or morphine (6 mg kg^{-1}) for 8 days. Each column represent the mean ± S.E.M. *** $P \leq 0.001$ compared to respective controls, †† $P \leq 0.01$ compared to individual treatments (One-way ANOVA followed by Tukey's multiple comparisons test).

3.3.2 Assessment of the ability of PME and geraniin to induce withdrawal syndromes of dependence

Withdrawal signs notably jumping, writhing and diarrhea were observed after naloxone was administered to the morphine treated animals. However, these effects were not observed in the PME, geraniin or vehicle treated animals. The incidence of jumping episodes were quantified and used to assess the extent of withdrawal (Fig. 3.13). Two – way analysis of variance revealed a significant incidence ($F_{3, 16} = 17.21$, $P < 0.0001$) (Fig. 3.13a); $P = 0.0077$ (Fig. 3.13b) of jumping episodes for morphine compared to the vehicle treated animals. There was however no significant difference between the number of jumps for the PME, geraniin and vehicle treated animals as depicted in the area under the curve graph.

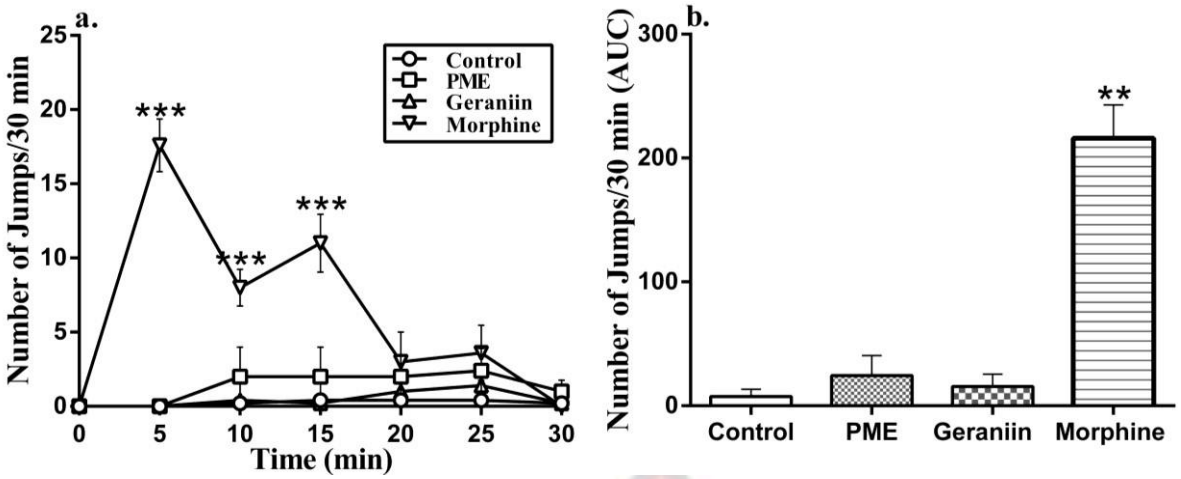


Figure 0.13: Effect of PME, geraniin, vehicle and morphine in inducing withdrawal signs in mice depicted as a) a time course curve b) an area under the curve graph respectively. Data represented as mean ± S.E.M. ** $P \leq 0.01$, *** $P \leq 0.001$ compared to the control.

3.3.3 Assessment of the effect of PME and geraniin on the withdrawal syndrome of morphine dependence

Administration of PME and geraniin after naloxone significantly suppressed ($F_{4,\ 13}$ = 121.70, $P < 0.0001$ and $F_{4,\ 15}$ = 62.34, $P < 0.0001$ respectively) the jumping behaviour in mice dependent on morphine (Fig. 3.14a; 3.15a). PME and geraniin at the highest doses used blocked the morphine-dependent withdrawal effect by 96.22 % and 97.90 %, respectively (Figure 3.14b; 3.15b). The i.p. administration of diazepam (30 min before the final dose of morphine) also significantly reduced the jumping reaction by 97.90 % (Figure 3.14; 3.15).

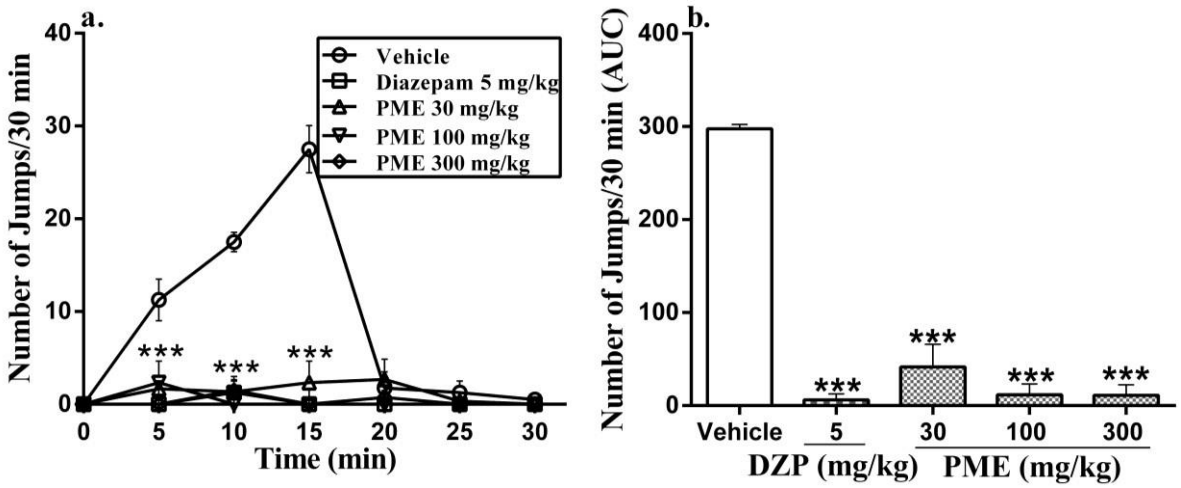


Figure 0.14: Effect of PME, diazepam (DZP) and vehicle on the naloxone-precipitated withdrawal syndrome of morphine dependence in mice depicted as a) the time course curve b) area under the curve respectively. Data represented as mean ± S.E.M. *** $P \leq 0.001$ compared to the vehicle treated group.

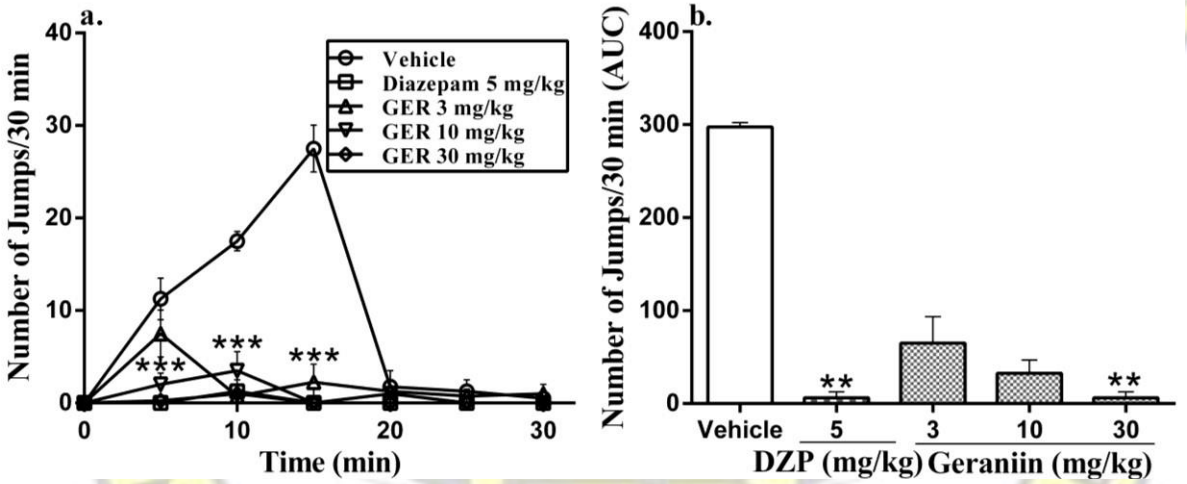


Figure 0.15: Effect of geraniin, diazepam (DZP) and vehicle on the naloxone-precipitated withdrawal syndrome of morphine dependence in mice depicted as a) the time course curve b) area under the curve respectively. Data represented as mean ± S.E.M. ** $P \leq 0.01$, *** $P \leq 0.001$ compared to the vehicle treated group.

3.4 TOXICITY AND SAFETY

3.4.1 Motor co-ordination test

PME (30 - 1000 mg kg^{-1}) at all doses used did not cause any significant change in the time spent on the rota rod as shown in Fig. 3.16a and b. The non-depolarising neuromuscular blocker D-Tubocurarine (D-TC, 0.1 mg kg^{-1}) however caused a significant reduction in the time spent on the rota rod (Fig. 3.16a and b).

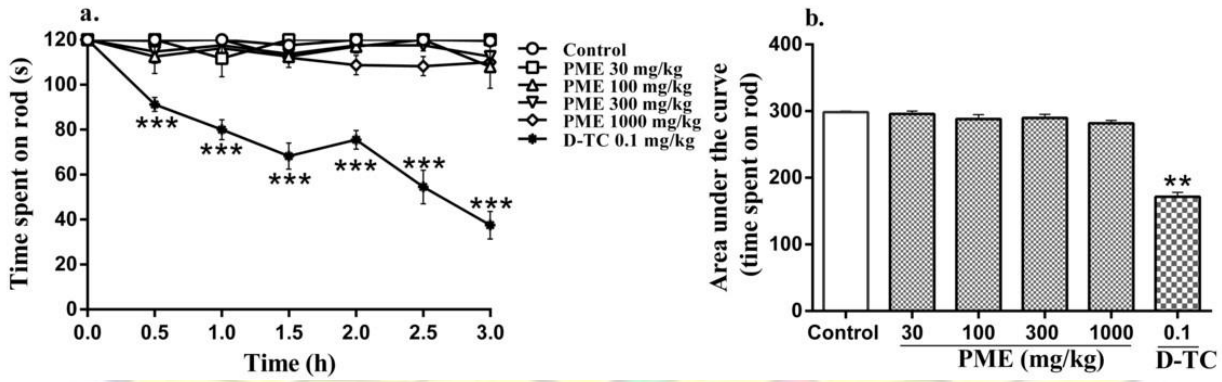


Figure 0.16: Effects of PME (30 - 1000 mg kg^{-1}), D-Tubocurarine (D-TC, 0.1 mg kg^{-1}) and vehicle on time spent on rods shown as a) time course curves and b) area under the curve. Time course curve analysed by a two way ANOVA followed by Dunnett's multiple comparison test while AUC was analysed by Kruskal-Wallis test followed by Dunn's multiple comparison test. ** $P \leq 0.01$, *** $P \leq 0.001$.

3.4.2 Acute toxicity

There was no lethality recorded during this study. During observation, the animals treated with the doses of 100 - 300 mg kg^{-1} (NOAEL) did not exhibit any toxic signs (Table 3.6). However at 1000, 3000 and 5000 mg kg^{-1}, the mice displayed increased defecation and reduced grip strength as compared to untreated controls.

Table 3.6: Observations in the acute toxicity test after oral administration of PME in mice

	Mortality		
Dose (mg kg^{-1})	D/T	Latency (min)	Toxicity Signs
0	0/5	-	None
100	0/5	-	None
300	0/5	-	None
1000	0/5	-	Increased defecation
3000	0/5	-	Aggression, hyperactivity, increased defecation
5000	0/5	-	Reduced activity, increased defecation, reduced grip strength

3.4.3 Sub-acute toxicity

No lethality was also recorded among the rats used for the 7 day study period. There were no significant changes noted in behaviour, activity, posture, or external appearance in all rats that received the extract.

3.4.3.1 Effect of extract on haematological parameters

There were generally no significant differences noted between vehicle and extracted treated groups for the parameters measured (Table 3.7).

3.4.3.2 Effect of extract on serum clinical biochemistry parameters

There were no statistically significant differences between vehicle and extracted treated groups for the biochemical parameters measured except for a decrease in the level of creatinine ($F_{3,8} = 6.2680$; $P < 0.05$) and an increase in the level of urea ($F_{3,8} = 8.3090$; $P < 0.01$)(Table 3.8) but these effects were not dose dependent.

Table 3.7: Haematological values of rats treated with PME and vehicle for 7 days.

	PME (mg kg^{-1})					
Parameters	0	100	300	1000	*F*	*P* value
WBC (×10^3/µL)	12.57±2.19	5.67±1.56	8.50±0.51	12.50±4.02	$F_{3,8}$ = 1.9040	0.2075
RBC (×10^6/µL)	6.53±0.93	7.76±0.57	7.42±0.10	7.92±0.57	$F_{3,8}$ = 1.0210	0.4329
HGB (g/dL)	12.80±0.64	12.67±0.97	12.33±0.09	13.30±0.59	$F_{3,8}$ = 0.3784	0.7713
HCT (%)	48.27±2.94	49.13±1.54	48.47±0.87	52.27±2.23	$F_{3,8}$ = 0.8247	0.5162
MCV (fL)	76.03±7.33	63.77±3.61	65.33±1.14	66.20±1.85	$F_{3,8}$ = 1.7280	0.2383
MCH (pg)	20.83±4.39	16.33±0.10	16.63±0.15	16.87±0.52	$F_{3,8}$ = 0.9211	0.4733
MCHC (g/dL)	26.90±3.40	25.73±1.48	25.43±0.29	25.43±0.26	$F_{3,8}$ = 0.1686	0.9146
Platelets (× 10^3/µL)	394.00±105.30	682.50±128.50	703.00±96.00	450.00±24.76	$F_{3,6}$ = 2.9760	0.1185
LYM %	64.27±21.43	85.10±4.86	88.27±4.25	86.33±3.93	$F_{3,8}$ = 0.9762	0.4505
MXD %	3.60±0.58	2.35±0.35	2.87±0.84	3.63±0.78	$F_{3,7}$ = 0.6460	0.6098
NEUT %	11.25±1.95	10.50±2.82	8.87±3.42	10.03±3.15	$F_{3,7}$ = 0.0970	0.9592
RDW_SD	41.80 ±4.25	45.43±2.03	42.17±1.48	39.13±1.21	$F_{3,8}$ = 1.0310	0.4290
RDW_CV	15.37±1.84	21.87±3.42	17.17±0.38	15.47±0.96	$F_{3,8}$ = 2.3060	0.1534
PDW (fL)	10.90±1.55	8.50±0.10	9.00±0.45	9.80±0.29	$F_{3,8}$ = 1.6210	0.2598
MPV (fL)	8.40±1.11	7.07±0.13	7.30±0.32	7.60±0.15	$F_{3,8}$ = 0.9886	0.4456
P_LCR (%)	8.75±0.85	8.23±0.77	9.17±1.30	11.13±1.39	$F_{3,8}$ = 1.2300	0.3682

Values are presented as mean ± S.E.M. (n = 3). *$P \leq 0.05$ compared to vehicle-treated group (one-way ANOVA followed by Dunnett's multiple comparison test).

Table 3.8 : Clinical biochemistry values of rats treated with PME and vehicle for 7 days

	PME (mg kg^{-1})					
Parameters	0	100	300	1000	*F*	*P* value
Albumin (g/L)	28.27±1.44	28.70±2.17	31.47±2.15	36.50±3.01	$F_{3,8}$ = 2.8100	0.1078
Total Protein (g/L)	54.40±3.25	57.43±9.19	63.70±3.99	69.07±0.85	$F_{3,8}$ = 1.5350	0.2786
ALP (U/L)	590.70±93.76	705.0±58.76	679.70±75.07	504.00±73.08	$F_{3,8}$ = 1.4410	0.3012
ALT (U/L)	137.30±12.60	143.30±17.34	148.00±12.58	119.00±14.01	$F_{3,8}$ = 0.7945	0.5305
AST (U/L)	230.30±44.37	243.30±37.81	233.30±35.53	304.70±11.67	$F_{3,8}$ = 1.0180	0.4340
GGT (U/L)	6.47±4.90	4.73±2.42	10.10±3.01	3.20±2.39	$F_{3,8}$ = 0.7886	0.5333
T-BIL (µmol/L)	3.00±1.00	2.67±0.67	2.33±0.33	3.00±0.00	$F_{3,8}$ = 0.2619	0.8510
D-BIL (µmol/L)	1.67±0.33	1.33±0.33	1.00±0.00	2.33±0.88	$F_{3,8}$ = 1.2960	0.3406
I-BIL (µmol/L)	1.10±1.95	1.07±0.56	1.20±0.06	1.05±0.37	$F_{3,8}$ = 0.0217	0.9953
Globulins (g/L)	26.09±1.95	28.74±7.17	32.27±2.10	32.56±2.95	$F_{3,8}$ = 0.5577	0.6575
Creatinine (µmol/L)	37.43±0.95	28.23±2.02**	34.83±2.03	33.00±0.70	$F_{3,8}$ = 6.2680	0.0170
Urea (mmol/L)	6.98±0.56	7.92±1.46	7.52±1.10	13.32±0.73 **	$F_{3,8}$ = 8.3090	0.0077
Uric acid (µmol/L)	705.70±161.40	1161±415.00	827.00±61.13	1984±929.90	$F_{3,8}$ = 1.2460	0.3557
Sodium (mmol/L)	123.40±11.70	134.20±3.52	136.00±4.19	136.80±0.62	$F_{3,8}$ = 0.9235	0.4723
Potassium (mmol/L)	6.60±0.94	6.96±0.92	7.17±0.24	8.02±0.82	$F_{3,8}$ = 0.5791	0.6450
Chloride (mmol/L)	86.27±8.79	92.53±3.44	96.13±2.60	93.83±0.98	$F_{3,8}$ = 0.7369	0.5589

3.4.3.3 Effect of extract on body and organ weights of rats

In general, there was an increase in body weight over the course of this study for all the rats. The percentage changes in body weights were slightly higher in the extract treated groups (100, 300 and 1000 mg kg^{-1}) as compared to the control treated group but these differences were not statistically significant ($P > 0.05$) (Fig. 3.17). There was also no statistically significant differences in relative organ weights (ROW, %) between treated and control groups (Fig. 3.18).

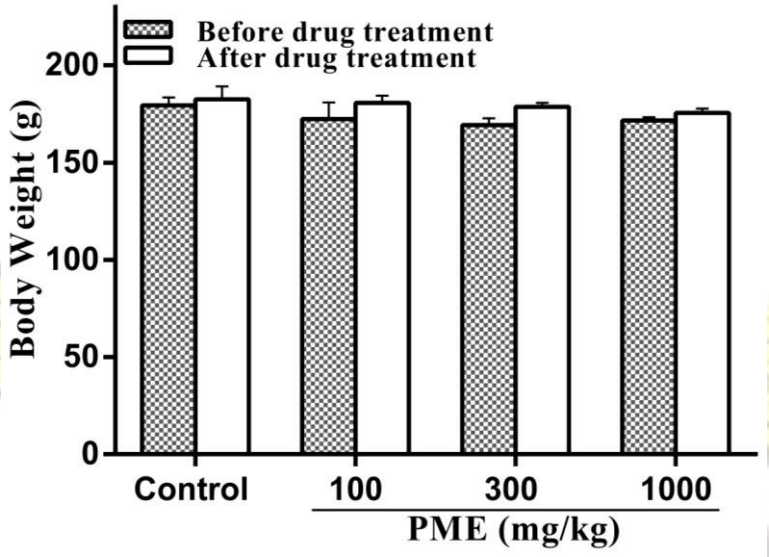


Figure 0.17: Effect of oral administration of PME on the body weight of rats in the sub-acute toxicity test. Data are expressed as mean ± S.E.M. (n=5). Treated groups were compared to their respective controls before drug administration using the Student's 't' test for independent means.

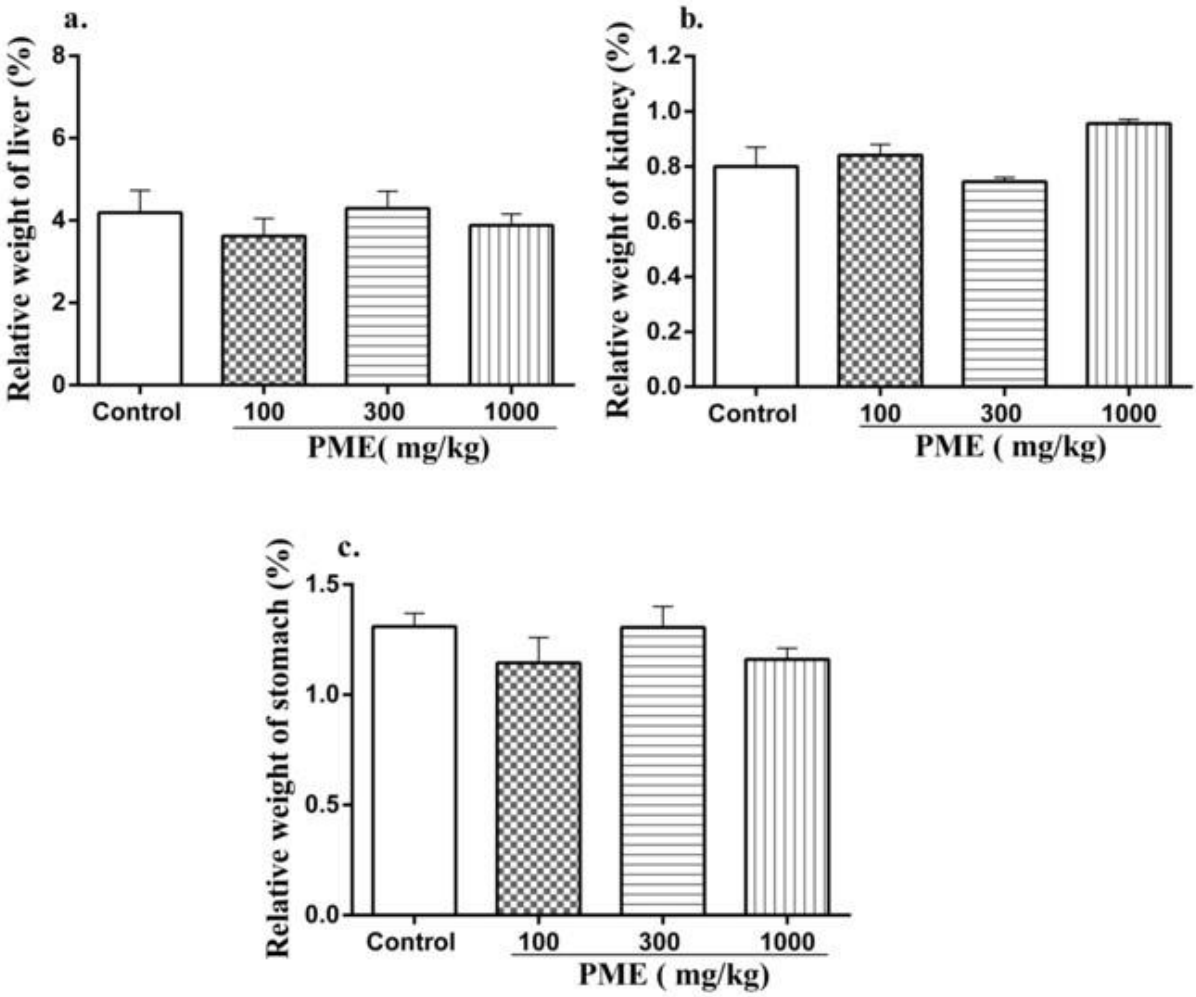


Figure 0.18: Effect of oral administration of PME on the relative organ weights (ROW) of: (a) liver, (b) kidney and (c) stomach. Data are expressed as mean ± S.E.M. Treated groups were compared to controls using a one-way ANOVA followed by Dunnett's multiple comparison test.

3.4.3.4 Histopathological analysis

The photomicrographs obtained from sections of the liver, kidney and stomach of rats that were treated with vehicle and PME (100 – 1000 mg kg^{-1}) for the 7-day study period are shown in Plates 3.1 – 3.3.

The results revealed no significant PME-related changes for the kidney, liver and stomach. No significant alterations were also observed in the organs of the control animals.

Comparison of liver morphological structure in extract-treated rats to controls (Plate 3.1) showed no remarkable abnormalities. The micrograph showed normal hepatocytes radiating around the sinusoids. This arrangement shows normal configuration within normal limits.

Damage to the kidneys are usually determined by examining the glomeruli (to determine the cellularity), tubules (for dilatation) and the vessels. The kidney samples showed glomeruli exhibiting round configuration with intact capsule and normal cellularity. The tubules were normal and intestitium had normal vasculature (Plate 3.2).

All stomach samples showed the fundic gastric mucosa with normal glands. Samples obtained from animals treated with 1000 mg kg^{-1} of the extract showed gastric pits infiltrated by neutrophil polymorphs but this was within normal limits (Plate 3.3).

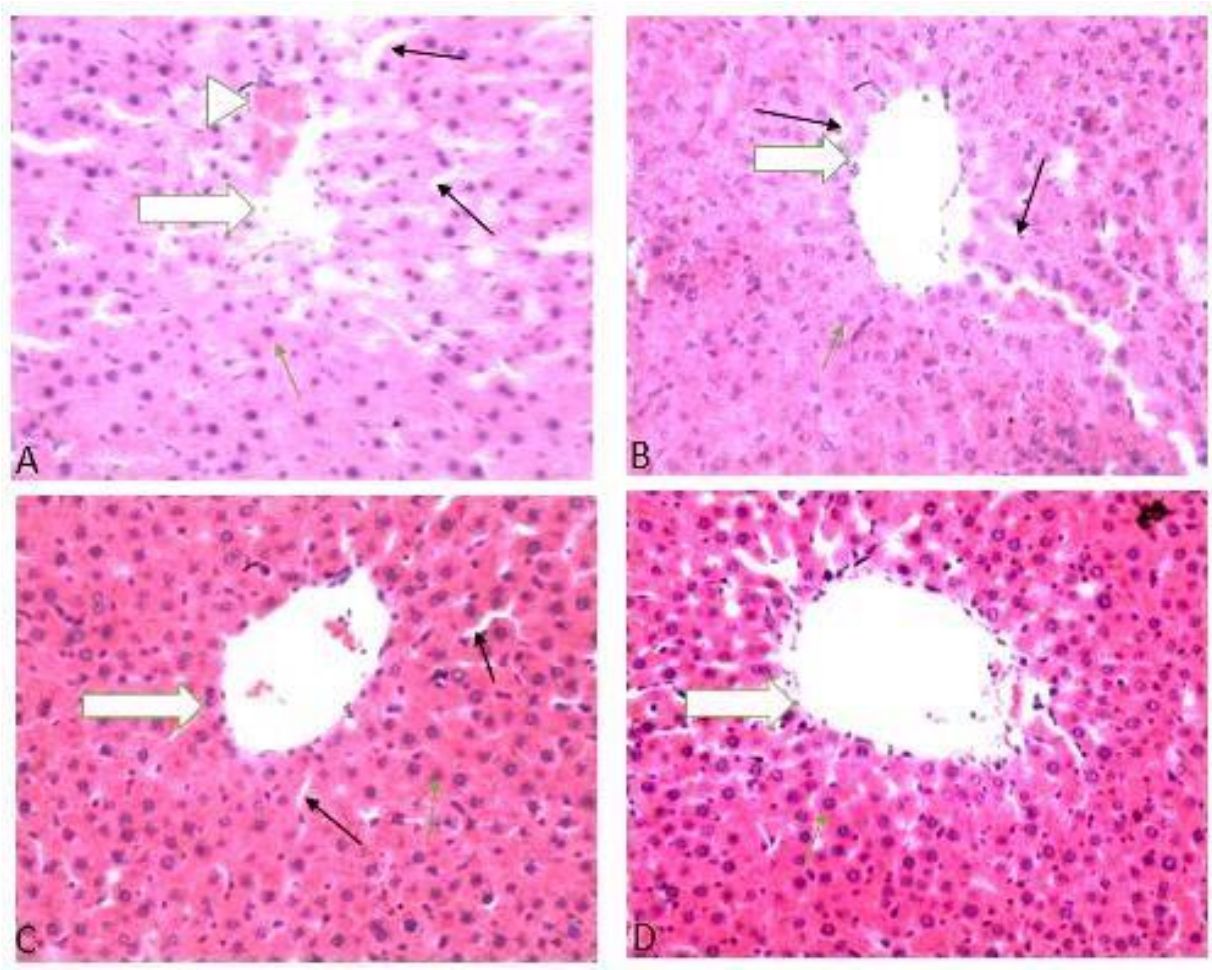


Plate 0.1: Photomicrograph of the liver sections in (A) vehicle treated rats, rats treated with (B) 100 mg kg^{-1}, (C) 300 mg kg^{-1} and (D) 1000 mg kg^{-1} of PME (H & E stain, ×200). All the micrographs showed normal hepatocytes (green arrow) radiating around the sinusoids (black arrow). Blood from the sinusoids drain into the central vein (white arrow). Arrow head represents degenerating red blood cells,

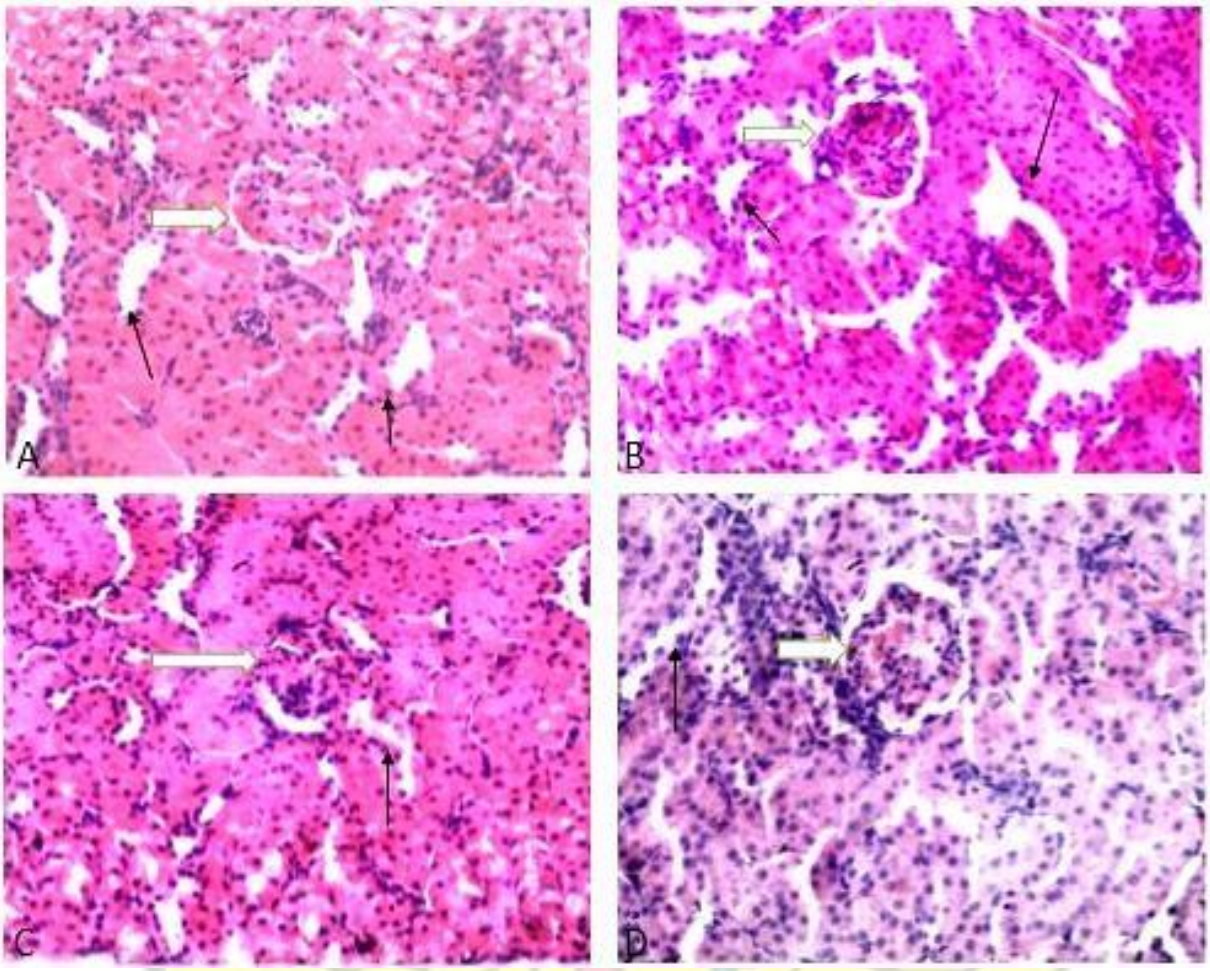


Plate 0.2: Photomicrograph of the kidney sections in (A) vehicle treated rats, rats treated with (B) 100 mg kg^{-1}, (C) 300 mg kg^{-1} and (D) 1000 mg kg^{-1} of PME (H & E stain, ×200). All the kidney samples showed glomeruli (white arrows) exhibiting round configuration with intact capsule and normal cellularity as well as normal tubules (black arrows).

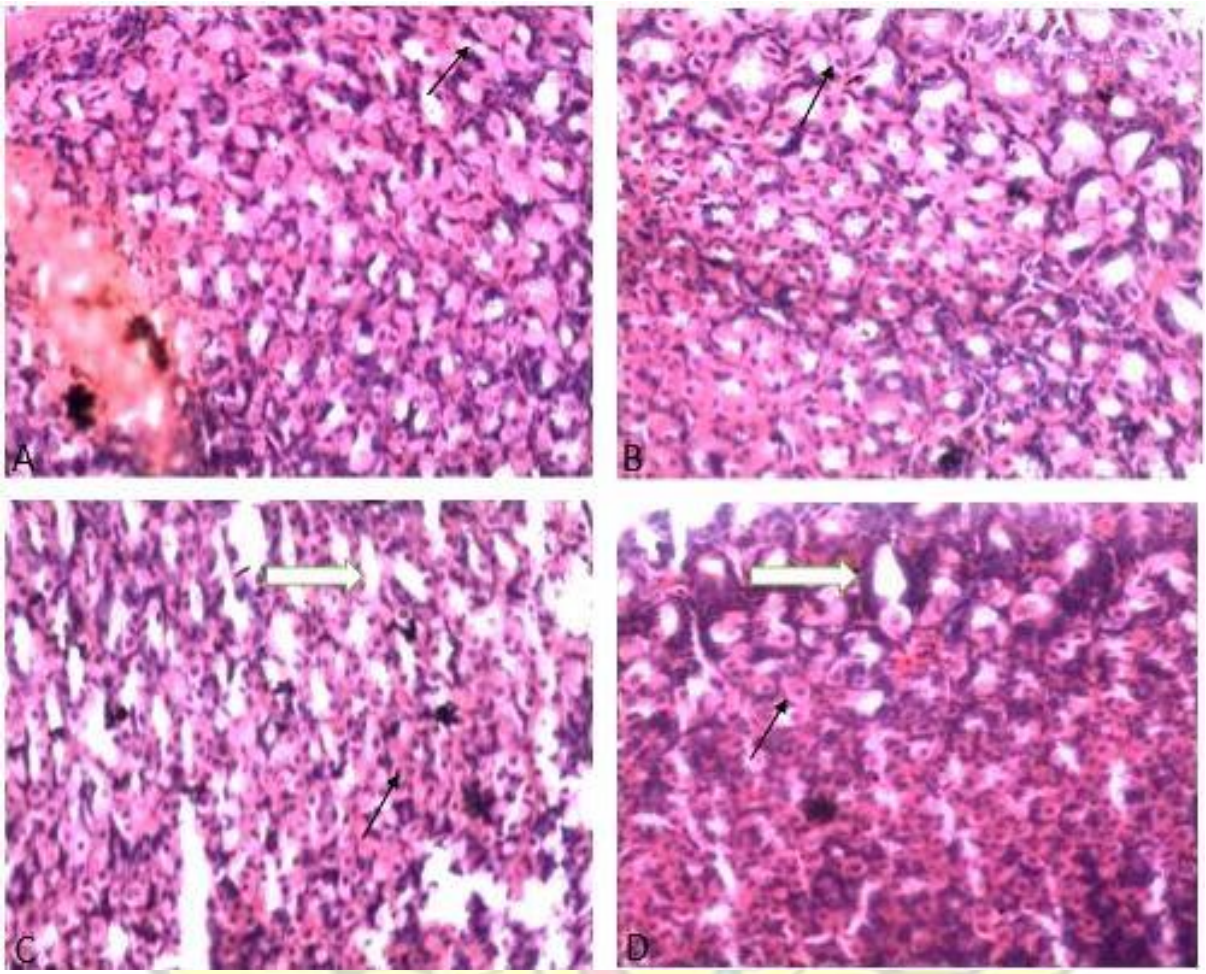


Plate 0.3: Photomicrograph of the gastric mucosa in (A) vehicle treated rats, rats treated with (B) 100 mg kg^{-1}, (C) 300 mg kg^{-1} and (D) 1000 mg kg^{-1} of PME (H & E stain, ×200). All stomach samples showed the fundic gastric mucosa with normal glands. Gastric pits are represented by the white arrows while parietal cells are shown by the black arrows.

Chapter 4

DISCUSSION

This study aimed at establishing the anti-nociceptive properties of the aqueous extract of the aerial parts of *Phyllanthus muellerianus* using chemical models of nociception. The acetic acid-induced writhing test is a very sensitive model as it allows for evidence to be obtained for all major and minor analgesics (Le Bars *et al*., 2001). Abdominal writhing, a

reflexive response due to the contraction of the dorso-abdominal muscles, was observed upon the intraperitoneal injection of acetic acid. This effect is due to increased level of prostanoids; particularly PGE_2, PGF_2, bradykinin, serotonin and lipoxygenase products which are released in response to activation of chemosensitive nociceptors (Bhattacharya *et al.*, 2014). The nociceptive activity of acetic acid has also been attributed to the reduction in pH as well as the release of cytokines like TNF-α, IL-1β, IL-8 by macrophages and mast cells present in the peritoneum (Ribeiro *et al.*, 2000). Diclofenac, an NSAID can reduce the frequency as well as the duration of writhing in this model by inhibiting cyclooxygenase, thereby disrupting the mechanism of transduction in primary afferent nociceptors by inhibiting the production and/or release of inflammatory mediators (Panthong *et al.*, 2007). The anti-nociceptive effects of morphine in this model appear to be mediated both centrally and peripherally by the activation of opioid receptors (Smith *et al.*, 1982). From the results obtained from the acetic acid-induced writhing model, the anti-nociceptive effects of the extract could be due to an interaction with some of the mediators released such as prostaglandins or cytokines like IL-1β, IL-8 or TNF-α as geraniin has been shown to inhibit TNF-α (Fujiki *et al.*, 2003). The action could also be through a novel mechanism yet to be elucidated.

Even though the acetic acid-induced writhing model is very sensitive, it lacks specificity (Le Bars *et al.*, 2001). However, as the extract also showed anti-nociceptive effect in the formalin model, the positive results from the acetic acid-induced writhing model can be said to be truly an analgesic effect. The results from the formalin-induced nociception test demonstrated that PME and geraniin, in a dose-dependent manner, significantly decreased the duration of licking and biting in the neurogenic (first phase) as well as inflammatory (second phase) pain responses of the formalin-induced nociception model, with the effect in the second phase being more notable as compared to the first phase for PME. The

formalin test, a tonic model of continuous pain, is a convenient model, particularly for the screening of new potential analgesic agents, since the nociception evoked is associated with inflammatory, neurogenic and central components (Ellis *et al*., 1998). Again, this test was used to determine the anti-nociceptive properties of PME and geraniin as it is accepted to be the most predictive of acute pain and a more authentic model for clinical pain (Dubuisson and Dennis, 1977; Tjolsen *et al.*, 1992). The initial phase which is transient is caused by the activation of the transient receptor potential ankyrin subtype 1 receptors (TRPA 1), a member of the Transient Receptor Potential family of cation channels that is highly expressed by a subset of C-fiber nociceptors, by formalin (McNamara *et al*., 2007) while the longer subsequent phase is associated with the combination of an inflammatory reaction in the peripheral tissue. This reaction causes a release of nociceptive mediators which subsequently cause sensitization of the central neurons leading to changes in central processing of pain (Santa-Cecilia *et al*., 2011). While it is a well-known fact that centrallyacting drugs such as morphine inhibit nociception in both phases equally (Hunskaar and Hole, 1987) some studies have also shown that diclofenac can also inhibit the neurogenic and inflammatory phases of the formalin-evoked nociception (Asomoza-Espinosa *et al*., 2001; Santos *et al.*, 1998) as was observed in this study.

Taken together the ability of PME and geraniin to produce anti-nociceptive effects in the acetic acid-induced abdominal writhing test and in both phases of the formalin-induced paw licking test showed that the two agents may be acting both centrally and peripherally. It also denotes that the extract possesses activity against both neurogenic and inflammatory pain.

Various receptors and pathways have been identified to be involved in pain modulation. In an attempt to determine the possible site(s) and mechanism of action of PME and geraniin,

several antagonists were chosen based on their interaction in the nociceptive pathway. LNAME, a NO synthase inhibitor, was used to activate the nitric oxide- cyclic GMP pathway at peripheral and/or central levels of the nociceptive pathway. The analgesic effect from this activation has been explained in literature to be dependent on an intracellular signaling pathway involving the activation of protein kinase G and the subsequent opening of K^+ channels which stabilizes membrane potentials and reduces neuronal excitability with release of neurotransmitters which can sensitize the nociceptors (Cury *et al*., 2011). To verify the involvement of spinal 5-HT_3 receptors in the anti-nociceptive effects of PME and geraniin, ondansetron was used to antagonize the 5-HT_3 receptors. These receptors have been shown to mediate anti-nociception, possibly via GABA release (Alhaider *et al.*, 1991; Millan, 2002). The role of L-type calcium channels in helping with the release of neurotransmitters from sensory neurons in the spinal cord has been well studied and thus the role of nifedipine, an antagonist at these receptor sites may modulate analgesia (Dogrul *et al*, 2001). Activation of ATP sensitive potassium channels have been shown to modulate the electrical activity of the dorsal horn neurons by reducing neuronal excitability with a subsequent decrease in the release of neurotransmitters such as Substance P and CGRP (Xia *et al*., 2014). Using glibenclamide, a K_{ATP} channel antagonist, was therefore prudent to check the involvement of this pathway in the observed anti-nociceptive effects of PME and geraniin. Activation of adenosine A_1 receptors in the periphery as well as within the spinal cord has been shown to mediate pain suppression hence the choice to use theophylline which is an adenosine A_1 and A_2 antagonist (Sawynok, 1998). $M_1 – M_4$ receptors have been implicated in mediating the analgesic effects of muscarinic agonists at both spinal and supraspinal levels (Ghelardini *et al.*, 2000). Peripheral activation of M_2 receptors is also likely to contribute to analgesia via reduced CGRP release (Bernadini *et al*., 2002; Wess *et al*., 2003). Based on the importance of alpha-2 receptors in peripheral, spinal and

supraspinal pain modulation, yohimbine was used to assess the influence of alpha-2 receptors on the observed anti-nociceptive effects of PME and geraniin. In the spinal cord, noradrenaline released from descending pathways suppresses pain by inhibitory action on α_{2A}-adrenoceptors on central terminals of primary afferent nociceptors (presynaptic inhibition) (Pertovaara, 2006). The endogenous opioid system is greatly associated with the central regulation of pain, as well as in the action of opioid-derived analgesics (Sakurada *et al*., 2005) as activation of these receptors inhibits the release of neurotransmitters that are involved in nociceptive transmission. This was illustrated in the present study when the anti-nociceptive effect of geraniin was reversed by the non-selective opioid receptor antagonist, naloxone. All the other agents did not significantly reverse the effects of either PME or geraniin while naloxone did not have a significant effect on the activity of PME. This results is not so surprising as previous studies on other species of Phyllanthus have shown that the anti-nociceptive action of the extract are unrelated to the interaction with opioid, serotonin or L-arginine-nitric oxide pathways (Calixto *et al*., 2000). A possible reason why naloxone may not have had a significant effect on the anti-nociceptive effect of the extract is that, the extract is known to have several components apart from geraniin such as furosin, corilagin, gallic acid and caffeic acid (Agyare *et al*., 2011) which may also play a part in the analgesic activity of PME but may not mediate their effects through the pathway investigated. For instance, it has been demonstrated that the anti-nociceptive response caused by gallic acid ethyl ester depends on the activation of Gi/o protein sensitive to pertussis toxin and involves both small- or large-conductance Ca^{2+}-activated K^+ channels and ATP-sensitive K^+ channels mechanisms (Calixto *et al*., 2000). Hence, the mechanism of action of the PME could not have been determined as it may have numerous sites of action due to its various constituents or even through a novel mechanism

Administration of geraniin/morphine and geraniin/diclofenac combinations produced antinociceptive effects in the formalin-induced nociception test which were greater than that achieved for the individual drugs separately. The formalin test was used because of previously stated reasons about its usefulness and validity (Tjolsen *et al.,* 1992, Ellis *et al.,* 1998). The co-administration of geraniin and morphine resulted in a synergistic effect in the neurogenic as well as the inflammatory phase of the formalin-induced nociception test. However, the interaction was more effective in the inflammatory phase rather than the neurogenic phase. From the initial studies done, it was realized that one of the mechanisms by which geraniin exerts its anti-nociceptive effect is via the opioidergic pathway while the muscarinic, serotoninergic, adrenergic and nitric oxide pathway did not show any significant contribution. However, this pathway may not be the only pathway through which geraniin exerts its effect as a synergistic effect instead of an additive effect depicts that not only the opioidergic pathway is involved in the anti-nociceptive effect of geraniin.

One reason why the interaction could have been more effective in the inflammatory phase rather than the neurogenic phase could be that a lot more mechanisms may be involved in the alleviation of the inflammatory pain in the second phase of the formalin test. Morphine and geraniin may also share a major of the common mechanisms needed to alleviate neurogenic pain rather than the inflammatory pain. Even though, the exact mechanism of the interaction has not been elucidated, this study has proven that there is a lot of merit in administering the two agents together in smaller amounts, and this could possibly lead to a better side effect profile.

Diclofenac is a non-steroidal anti-inflammatory agent which is known to act by the inhibition of cyclo-oxygenase enzymes and thus the inhibition of prostaglandin synthesis (Santa-Cecilia *et al.,* 2011). The co-administration of diclofenac and geraniin also resulted

in a synergistic effect in both phases with a greater effect seen in the inflammatory phase. This could be as a result of geraniin and diclofenac having different but efficient mechanisms of inhibiting the release and/or effect of nociceptive mediators such as serotonin and histamine which subsequently cause sensitization of the central neurons leading to changes in central processing of pain (Santa-Cecilia *et al*., 2011).

The usefulness of opioid analgesics in clinical settings is often impeded by the development of tolerance that requires increase in dose irrespective of the disease advancement (Chen *et al*., 2008). Tolerance produced by sustained morphine treatment has been associated with regulation of Transient Receptor Potential Vanilloid 1 (TRPV1) receptor, which is induced by chronic morphine application via Mitogen Activated Protein Kinases (MAPK) signaling pathways (Chen *et al*., 2008). The mitogen-activated protein kinases (MAPKs) which include extracellular signal-regulated kinases 1 and 2 (ERK1/2), c-Jun amino-terminal kinases 1 to 3 (JNK1 to -3), p38 (α, β, γ, and δ), and ERK5 families are protein Serine/Threonine kinases that regulate diverse cellular processes by relaying extracellular signals to intracellular responses (Cargnello and Roux, 2011). Results from this study have shown that, unlike morphine and other centrally acting analgesics, geraniin and PME, at doses tested; do not induce tolerance after chronic administration in the formalin test of nociception. Moreover, in animals that showed significant tolerance to the analgesic effects of morphine, PME and geraniin were still able to exhibit analgesic effects. These observed effects of the agents could be due to interactions or blockade of the TRPV1 receptor or the MAPK signaling pathway.

Development of physical dependence which is manifested by a unique withdrawal syndrome with diverse behavioral and physiological signs is also a limiting factor to the usefulness of most of the opioid analgesics including morphine (Suzuki *et al*., 1996).

Withdrawal is usually observed following sudden cessation of morphine administration or induced by administering an opioid antagonist (Iliya *et al*., 2015). Several withdrawal behaviors have been reported in rodents including jumping, exploratory rearing, body shakes, ptosis, weight loss and diarrhea (Suzuki *et al*., 1996). However, jumping has been considered the most responsive and dependable measure of withdrawal intensity and thus routinely used to assess the extent of morphine withdrawal (Kest *et al.,* 2001). Again, jumping is widely used as jumps are easily counted and the frequency of jumps increases when dependence increases (Alemy *et al.,* 2012).

Physical dependence signs associated with opioid withdrawal have been attributed to a rebound hyperactivity of both the dopaminergic and adrenergic systems in the CNS (Tabatabai *et al*., 2014). Benzodiazepines, via $GABA_A$ receptors, have been shown to have a suppressive effect on the dependence of morphine (Suzuki *et al*., 1996) and this was illustrated in this study when diazepam at a dose of 5 mg kg^{-1} significantly reduced the jumping episodes in morphine-dependent mice. Proposed reasons for the effectiveness of benzodiazepine in suppressing the development of physical dependence on morphine include their continuous activation of benzodiazepine binding sites during chronic morphine treatment and the inhibition of the increase in Ca^{2+} level which results from chronic morphine treatment (Suzuki *et al.,* 1996).

In this study, both PME and geraniin showed inhibitory effect against withdrawal signs of morphine dependence. With geraniin being a polyphenolic compound, these results are in agreement with previous reports citing the inhibitory effects of polyphenolic compounds on naloxone precipitated morphine withdrawal (Capasso *et al*., 1998, Ghannadi *et al*., 2012). Even though, the extent of its inhibitory effect was equivalent to that of diazepam at the highest dose, the mechanism of action cannot be emphatically stated to be via the same pathway as diazepam.

Some oxidant species including nitric oxide (NO), superoxide anions and malondialdehyde (MDA) have been implicated in the development of opiate abstinence symptoms (Zarrindast *et al.*, 2003, Pinelli *et al.*, 2008). As such, some agents with significant antioxidant activity have proven effective in reducing opioid withdrawal signs (Tabatabai *et al.*, 2014). Both PME and geraniin have been reported previously as very potent antioxidants with the ability to reduce malondialdehyde levels (Boakye and Agyare, 2013) which could also contribute to the effectiveness of the agents in attenuating naloxoneprecipitated morphine withdrawal signs in mice.

In assessing the toxicity profile of PME, its effect on motor co-ordination was determined. The rota-rod test was used to determine if PME altered motor performance in mice. This was done because it has been found that some drugs such as adrenergic blockers, antihistamines, muscle relaxants, monoamine oxidase inhibitors and neuroleptics which do not necessarily have analgesic activity test positive for the acetic acid writhing test based on their ability to inhibit contraction of the dorso-abdominal muscles (Le Bars *et al.*, 2001). However, the mice did not seem to have altered motor co-ordination after the administration of the PME.

From the acute toxicity study, adverse effects were not observed with mice treated with low oral doses of PME. However, with higher doses of the extracts, defecation, aggression, reduced activity and reduced grip strength were observed although these effects were all reverted within 24 h after PME was given. These effects suggest that the extract may have an effect on the nervous system both the peripheral nervous system and the central nervous system. The LD_{50} of PME can be estimated to be above 5000 mg kg^{-1} from the results obtained. This indicates that PME is relatively non-toxic based on the recommendations of the Organisation for Economic Co-operation and Development (OECD) for chemical

labelling and classification of acute systemic toxicity based on oral LD_{50} values.

Even though, acute toxicity studies provide relevant data, they have limited clinical application as toxic effects of drugs do not only occur with the administration of single large doses and also the cumulative toxic effects at very low doses occur in only multiple dose administration (Hemalatha and Hari, 2014). Hence, the need to carry out a sub-acute toxicity study.

Daily cage side observations were made after the administration of the extract to determine any signs of toxicity such as changes in posture or locomotion, increased defecation, presence of secretions from the body orifices, alopecia or pallor in the eyes. Increased defecation was the only observation which was noted in the group of rats which received 1000 mg kg^{-1} of PME but this effect was reverted within 24 h. Generally, body weight changes are an index of adverse effects, after exposure to toxic substances as usually surviving animals should not lose more than 10 % of the initial body weight (Feres *et al*., 2006, Hemalatha and Hari, 2014). High increases in body weight may be due to drugs which stimulate appetite in the animals or even agents which affect the lipid profile of the animals probably causing fat deposition while decrease in weight could also be due to the anorexic effect of certain drugs. From the results obtained, since no significant differences in the body weight between the extract-treated groups and the control group were observed, the effect observed cannot be said to be drug related.

The Society of Toxicologic Pathology suggests that organ weights be included routinely in toxicity studies for multi-dose drugs administered in durations from 7 days to 1 year (Sellers *et al*., 2007) and this is because organ weight changes are often as a result of treatments administered. Generally, an increase in relative organ weights is likened to inflammation while a decrease is attributed to cellular constriction (Moore and Dalley, 1999). In this

study, there were no significant differences in the relative organ weights between the animals treated with vehicle and those that were treated with the various doses of PME suggesting that PME had no effect on organ weight.

Blood acts as a pathological reflector of the status of exposed animals to toxicant and other conditions (Etim *et al*., 2014) hence it is very important to conduct haematological analyses which deal with the study of the number and morphology of the cellular elements of the blood in toxicological studies. All the extract treated animals did not show any significant variations in the hematological parameters. According to Olson *et al*., 2000, blood parameters analysis is very important in assessing the risk of a drug causing toxicity as it has a higher predictive value for toxicity in humans (91 %). Being a transport medium, the blood carries many drugs and xenobiotics, as a result, the blood components such as red blood cells are forced to initially high amounts of the xenobiotic which may be toxic. However, in the present investigation, PME did not have any significant effect on the hematological parameters measured, suggesting the non-toxic nature of the PME on blood and its components.

For the biochemical analysis, among the parameters evaluated, AST, ALT, ALP, GGT, albumin and bilirubin are considered liver function markers, the analysis of which is important since it has been shown that phytotherapeutic products may cause damage to the liver (Feres *et al*., 2006). The AST, ALT, and alkaline phosphatase tests are used often to differentiate between hepatocellular and cholestatic disease. However, as there were not any significant changes in the levels of these enzymes, it can be said that PME did not cause hepatocellular injury or obstructive disease and this finding correlates positively with the histopathological analysis of the liver as there were no cellular lesions and no significant damage to the liver observed.

The fact that the total bilirubin, albumin, globulin and total protein levels were not altered by the extract suggest that the secretory function of the liver was not impaired (Ashafa *et al.*, 2009). In this study, there were no significant differences in the extract treated groups as compared to the control which suggests that the function and integrity of the liver may not have been compromised by the PME extract.

Creatinine and urea determinations are vital as these are indicators of kidney function. A slight increase in urea level at the dose of 1000 mg kg^{-1} and a decrease in creatinine level at the dose of 100 mg kg^{-1} were observed in this study. Even though these parameters are indicative of the function of the kidney, a significant clinical correlation cannot be made using these values alone. Again, histopathological assessment revealed no significant changes in the kidney ultrastructure. This implies that the changes in serum levels of creatinine and urea were not significant enough to affect kidney function and can be considered as clinically unimportant.

Urea, a by-product of protein catabolism, is one of the most abundant nitrogenous wastes produced in the body by the liver. One of the possible reasons as to an increase in urea could be that there is an increase in protein metabolism. Other reasons could be a decrease in its renal excretion, high protein diet, intestinal haemorrhage, dehydration, severe haemorrhage, shock, etc. (Adedapo *et al*., 2009). However, in this case, none of the reasons can be specifically attributed to the increase in urea levels.

Creatinine is produced by the catabolism of creatine phosphate. "Serum creatinine is usually used to detect damage to the kidney or impairment in the function of the kidney (Nankivell, 2001). The decrease in creatinine levels here cannot however be related directly to the function of the kidney. It could be as a result of a reduction of creatinine production. The levels of creatinine may vary as its production may not be due to just a product of muscle

mass but affected by function and composition of the muscle as well as the activity, diet and health status of the organism involved. Generally, decreased values are noticed with glomerulonephritis, congestive heart failure, polycystic kidney disease and dehydration (Gowda *et al.*, 2010) but the results obtained does not suggest any of the abovementioned reasons.

PME treated rats did not show any significant difference in all the other biochemical parameters investigated against the normal control group suggesting that PME does not have any effects on the electrolytes. Also, histopathological analysis of the stomach did not reveal any mucosal lesions or damage. All the parameters observed were without any significant changes which suggest that PME may not have any gastric toxicity properties.

Chapter 5 CONCLUSION

This study has provided pharmacological data to support the use of the aerial parts of *Phyllanthus muellerianus* in painful conditions. Furthermore, it has illustrated that the analgesic effect of the aerial parts was due to the presence of geraniin, the most dominant constituent in the aqueous extract. The findings from the study include

- the aqueous extract and geraniin possess both peripheral and central antinociceptive effects in the animal models of chemical nociception:
- the effect of geraniin may be mediated through the opioidergic pathway.
- geraniin/morphine and geraniin/diclofenac combination produced synergism for both combinations when an isobolographic analysis was performed.
- PME and geraniin do not have any tolerant effects, do not induce withdrawal signs of dependence and also alleviates the signs associated with naloxone-precipitated morphine withdrawal.
- the aqueous extract of the aerial parts of *Phyllanthus muellerianus* is safe in rodents and more importantly, it does not affect the integrity of the stomach and the kidneys like some of the conventional analgesics.

Chapter 6

RECOMMENDATIONS

6.1 RECOMMENDATIONS FOR FURTHER WORK

The following are recommendations for further research work on the extract and geraniin

i) The effect of the extract and geraniin in other models of pain such as neuropathic pain models as well as the mechanism of action of both agents should be further evaluated by using other antagonists and algesic agents such as bradykinin, glutamate, epinephrine and capsaicin.

ii) Radioligand binding studies of geraniin and the extract could also be done to determine the exact receptors involved in the anti-nociceptive effects of these two agents.

iii) The other major components of the extract should be isolated and their antinociceptive activity evaluated individually and in combinations.

iv) The toxicological profile of the extract should be evaluated after chronic administration to determine the possible reproductive, genetic and carcinogenic adverse effects of the extract.

6.2 GENERAL RECOMMENDATIONS

This work sought to give scientific credence to back the usage of the plant, *Phyllanthus muellerianus,* in the traditional setting for various painful conditions. From the results obtained, the plant has been proven to show analgesic properties in rodents but the data cannot be directly extrapolated to humans so care should still be taken with the usage of the plant.

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APPENDIX

DRUG PREPARATION AND ADMINISTRATION

The extract and geraniin concentrations were prepared by dissolving in sterile distilled water. Drug concentrations were prepared so that the required dose was equivalent to the body weight of each rat. Also, drug concentrations were given in volumes not exceeding a total volume of 0.5 mL for all administrations.

DETAILED OBSERVATIONS IN THE IRWIN'S TEST

Dose (mg kg^{-1})	**0**							**100**							**300**						
Time (min)	**0-15**	**15**	**30**	**60**	**120**	**180**	**1440**	**0-15**	**15**	**30**	**60**	**120**	**180**	**1440**	**0-15**	**15**	**30**	**60**	**120**	**180**	**1440**
Death	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Convulsions	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Tremor	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Straub tail	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Activity	#	0	0	0	0	0	0	#	0	0	0	0	0	0	#	0	0	0	0	0	0
Jumps	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Muscle tone	#	0	0	0	0	0	0	#	0	0	0	0	0	0	#	0	0	0	0	0	0
Reactivity to touch	#	0	0	0	0	0	0	#	0	0	0	0	0	0	#	0	0	0	0	0	0
Aggression	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Fore paw threading	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Head twitch	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Head movements	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Chewing	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Sniffing	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Scratching	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Catalepsy	#	0	0	0	0	0	0	#	0	0	0	0	0	0	#	0	0	0	0	0	0
Akinesia	#	0	0	0	0	0	0	#	0	0	0	0	0	0	#	0	0	0	0	0	0
Abnormal gait (rolling)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Abnormal gait (tip toe)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Motor incoordination	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Loss of balance	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Loss of righting reflex	#	0	0	0	0	0	0	#	0	0	0	0	0	0	#	0	0	0	0	0	0

Loss of corneal reflex	#	0	0	0	0	0	0	#	0	0	0	0	0	0	#	0	0	0	0	0	0
Fear	#	0	0	0	0	0	0	#	0	0	0	0	0	0	#	0	0	0	0	0	0
Abdominal muscle tone	#	0	0	0	0	0	0	#	0	0	0	0	0	0	#	0	0	0	0	0	0
Writhing	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Analgesia	#	0	0	0	0	0	0	#	+	+	0	0	0	0	#	+	+	+	0	0	0
Ptosis	#	0	0	0	0	0	0	#	0	0	0	0	0	0	#	0	0	0	0	0	0
Exophthalmia	#	0	0	0	0	0	0	#	0	0	0	0	0	0	#	0	0	0	0	0	0
Piloerection	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Salivation	#	0	0	0	0	0	0	#	0	0	0	0	0	0	#	0	0	0	0	0	0
Urination	#	0	0	0	0	0	0	#	0	0	0	0	0	0	#	0	0	0	0	0	0
Lacrimation	#	0	0	0	0	0	0	#	0	0	0	0	0	0	#	0	0	0	0	0	0

Defecation	#	0	0	0	0	0	0	#	0	0	0	0	0	0	#	0	0	0	0	0	0
Respiration	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Limb tone	#	0	0	0	0	0	0	#	0	0	0	0	0	0	#	0	0	0	0	0	0

Provoked biting response	#	0	0	0	0	0	0	#	0	0	0	0	0	0	#	0	0	0	0	0	0
Loss of grip strength	#	0	0	0	0	0	0	#	0	0	0	0	0	0	#	0	0	0	0	0	0

Finger approach response	#	0	0	0	0	0	0	#	0	0	0	0	0	0	#	0	0	0	0	0	0

Dose (mg kg^{-1})	**1000**	**3000**	**5000**

Time (min)	**0-15**	**15**	**30**	**60**	**120**	**180**	**1440**	**0-15**	**15**	**30**	**60**	**120**	**180**	**1440**	**0-15**	**15**	**30**	**60**	**120**	**180**	**1440**
Death	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Convulsions	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Tremor	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Straub tail	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Activity	#	-	0	0	0	0	0	#	0	0	0	0	0	0	#	-	0	0	0	0	0
Jumps	0	0	0	0	0	0	0	0	+	0	0	0	0	0	0	0	0	0	0	0	0
Muscle tone	#	0	0	0	0	0	0	#	0	0	0	0	0	0	#	0	0	0	0	0	0
Reactivity to touch	#	0	0	0	0	0	0	#	0	0	0	0	0	0	#	+	0	0	0	0	0
Aggression	0	0	0	0	0	0	0	0	+	0	0	0	0	0	0	0	0	0	0	0	0
Fore paw threading	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Head twitch	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Head movements	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Chewing	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Sniffing	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Scratching	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Catalepsy	0	0	0	0	0	0	0	0	0	0	0	0	0	0	#	0	0	0	0	0	0
Akinesia	#	0	0	0	0	0	0	#	0	0	0	0	0	0	#	0	0	0	0	0	0
Abnormal gait (rolling)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Abnormal gait (tip toe)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Motor incoordination	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Loss of balance	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Loss of righting reflex	#	0	0	0	0	0	0	#	0	0	0	0	0	0	#	0	0	0	0	0	0
Loss of corneal reflex	#	0	0	0	0	0	0	#	0	0	0	0	0	0	#	0	0	0	0	0	0
Fear	#	0	0	0	0	0	0	#	0	0	0	0	0	0	#	+	+	+	0	0	0
Abdominal muscle tone	#	0	0	0	0	0	0	#	0	0	0	0	0	0	#	0	0	0	0	0	0
Writhing	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Analgesia	#	+	+	+	+	0	0	#	+	+	+	+	0	0	#	+	+	+	+	0	0
Ptosis	#	0	0	0	0	0	0	#	0	0	0	0	0	0	#	0	0	0	0	0	0
Exophthalmia	#	0	0	0	0	0	0	#	0	0	0	0	0	0	#	0	0	0	0	0	0
Piloerection	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Salivation	#	0	0	0	0	0	0	#	0	0	0	0	0	0	#	0	0	0	0	0	0
Urination	#	0	0	0	0	0	0	#	0	0	0	0	0	0	#	0	0	0	0	0	0

Lacrimation	#	0	0	0	0	0	0	#	0	0	0	0	0	0	#	0	0	0	0	0	0
Defecation	#	0	+	+	0	0	0	#	0	+	+	0	0	0	#	0	+	+	0	0	0
Respiration	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Limb tone	#	0	0	0	0	0	0	#	0	0	0	0	0	0	#	0	0	0	0	0	0
Provoked biting response	#	0	0	0	0	0	0	#	0	0	0	0	0	0	#	0	0	0	0	0	0
Grip strength	#	0	0	0	0	0	0	#	0	0	0	0	0	0	#	0	0	0	-	-	0
Finger approach response	#	0	0	0	0	0	0	#	0	0	0	0	0	0	#	0	0	0	0	0	0

PME was administered at 100. 300, 1000, 3000 and 5000 mg kg^{-1}, *p.o.* Data is presented as the number of animals showing symptoms during the test. Observations were performed at 15, 30, 60, 120, 180 min and 1440 min after administration. The symptoms that did not necessitate handling were also observed up to 15 min immediately following administration. # Parameters not measured. + = slight increase, - = slight decrease, # = Parameters not measured