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ANALGESIC EFFECTS OF *ANNONA MURICATA* LEAF EXTRACT IN PACLITAXEL AND STREPTOZOTOCIN-INDUCED DIABETIC NEUROPATHY IN MURINE MODELS

BY

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DECLARATION

DECLARATION BY THE CANDIDATE

I hereby declare that this is the product of my own research undertaken under supervision and has neither been presented in whole nor in part for another degree elsewhere. I am solely responsible for any residual flaws in the work.

Signature……. Date 01/08/2022

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DECLARATION BY SUPERVISORS

We hereby declare that the principal work and presentation of the thesis were supervised by us in accordance with guidelines on supervision of thesis laid down by the University of Ghana.

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ABSTRACT

Background: *Annona muricata* have demonstrated antinociceptive and anxiolytic effects in animal models through its leaf extracts. This study evaluates the aqueous leaf extract of the plant for possible analgesic properties in hyperalgesia and allodynia associated with paclitaxel-induced neuropathy in mice and streptozotocin (STZ)- induced diabetic neuropathy in rats.

Methods:10kg of the coarse crushed leaves was soaked in 3 liters of distilled water to make a decoction, cooled, filtered and freeze dried for use. Sub-acute toxicity test was carried out for 14-days after which blood samples were taken and examined for haematological analysis. Phytochemistry of the extract was conducted and analgesic property was accessed using hot plate test. Irwin test was also conducted to observe alterations in behavior and physiological activity, neurotoxicity and mortality. Diabetic- induced neuropathy in Sprague-Dawley rats was accomplished by injecting 55mg/kg body weight of STZ followed by 120mg/kg body weight of nicotinamide to achieve type 2 diabetes mellitus. Paclitaxel-induced neuropathy was also achieved by injecting ICR mice with 2mg/kg body weight body weight of paclitaxel continuously for 5 days. Parameters which include cold allodynia mechanical hyperalgesia and thermal hyperalgesia were measured before the administration of paclitaxel and on day 1 – 5 and after the administration of paclitaxel. In STZ-induced diabetic neuropathy experiment parameters were measured before the administration of STZ and after the administration of STZ on day 2, 4, 6, 8, 10, 12 and 14. These animals were then treated with *Annona muricata* extract (AME) (30, 100 and 300 mg/kg body weight), pregabalin (10, 30 and 100 mg/kg body weight) and distilled water as a vehicle daily for 5 days and 14 days continuously in paclitaxel- and diabetic-induced peripheral neuropathy respectively. Pain thresholds were measured on day 1, 2, 3 and 5 in paclitaxel-induced neuropathy experiment and that of STZ-induced - diabetic neuropathic experiment, it was measured from day

1-7.

Results: *Annona muricata* Extract (AME) showed no toxicity as no death were observed during the 14-day study period in sub-acute toxicity studies. Preliminary phytochemical screening of the extract indicated the presence of secondary metabolites which includes alkaloids, saponins, flavonoids, tannins, glycosides, triterpenoids and sterols. The extract showed analgesic property during the hot plate test. CNS safety pharmacology using Irwin test indicated no mortality when experimental animals were observed for 24 hours after various treatment doses were employed. Observable physiological/ pharmacological effects were noted which include straub tail, defecation, sniffing among others. Relative organ weight of the experimental animals also indicated no obvious abnormally when compared to the control during Irwin's test. AME and pregabalin produced analgesic properties which was exhibited in paclitaxel and STZ-induced - neuropathy as increased paw withdrawal latencies to mechanical, cold-water stimuli and thermal hyperalgesic tests.

Conclusions: The findings from this study suggest that aqueous extract of *Annona muricata* is sub acutely safe with observable CNS physiological effect and no observable CNS toxicity. Again, the extract possesses an analgesic property as seen in both paclitaxel- and STZ-induced diabetic neuropathy in animal models which may contribute to its traditional use in managing neuropathic pain.

DEDICATION

This work is dedicated to the Almighty God and the Department of Pharmacology and Toxicology, University of Ghana.

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LIST OF ABBREVIATIONS

ABTS	2, 2'-Azino-Bis-3-Ethylbenzothiazoline-6-Sulfonic Acid
AME	Annona muricata extract
AST	Aspartate aminotransferase
ATP	Adenosine triphosphate
AUC	Area Under Curve
CIPN	Chemotherapy induced peripheral neuropathy
CNS	Central nervous system
DIPN	Diabetic-induced peripheral neuropathy
DN	Diabetic neuropathy
DPN	Diabetic Peripheral Neuropathy
DSP	Diabetic sensorimotor polyneuropathy
DNA	Deoxyribonucleic acid
DRG	Dorsal root ganglion
ED	Effective dose
ED_{50}	Effective dose for 50%

$FeCl_3$	Ferric (III) Chloride
ORAC	Oxygen Radical Absorbance Capacity
HCT	Hematocrit
HDL	High-density lipoprotein
HGB	Hemoglobin
IASP	International Association for the Study of Pain
ICR	Imprint control region
IL	Interleukin
Ip.	Intraperitoneal
IV	Intravenous
K3Fe (CN)6	Potassium ferricyanide
L-NAME	NG-nitro-L-arginine methyl ester
LD	Lethal dose
LDL	Low density lipoprotein
LYM	Lymphocytes
MCH	Mean corpuscular hemoglobin
MCHC	Mean corpuscular hemoglobin concentration
MCV	Mean corpuscular volume
MPE	Maximal possible effect
MPV	Mean platelet volume
MOR	Morphine
NO	Nitric oxide
PIPNE	Paclitaxel-induced peripheral neuropathy experiment

PGB	Pregabalin
ROS	Reactive oxygen species
SNRI	Serotonin-Norepinephrine reuptake inhibitors
STZ	Streptozotocin
T1DM	Type 1 Diabetes Mellitus
T2DM	Type 2 Diabetes Mellitus
USDA-ARS	United States Department of Agriculture-Agricultural Research Service
WHO	World Health Organisation

CHAPTER ONE

INTRODUCTION

1.1 Background

The development of peripheral neuropathy in patients with long term paclitaxel usage and diabetes mellitus have different symptoms, mechanism of neurologic involvement, conduit, risk covariates, pathologic modification and underlying mechanisms (Tesfaye *et al.*, 2010). Neuropathic pain is as a result of a damage in the peripheral nerves or central nervous system (Daousi *et al.*, 2004). Neuropathic pain causes may include diabetes mellitus (Pop-Busui *et al.*, 2017), shingles (Woolf. *et al.*, 1999), multiple sclerosis (Mori *et al.*, 2010), spinal cord injuries (Wilmshurst *et al.*, 2019) stroke (Ocean & Vahdat, 2004) HIV infection (Lefaucheur *et al.*, 2017), cancer and cancer treating drugs (Rang, 2003).

Peripheral neuropathy and neuropathic pain are used interchangeably. It is estimated that 25% of patients suffering from diabetes might experience neuropathy (Boulton *et al.*, 2004). According to Van Hecke *et al.* (2013) with reference to paclitaxel-induced peripheral neuropathy, 6.9% to 10% of the world's population suffers from it. The clinical signs are distal, symmetrical and are usually with nocturnal exacerbations such as deep aching electric-like shock and a burning sensation with hyperalgesia and often allodynia upon examination (Boulton *et al.*, 2004). Major treatment options normally entail treating the underlying cause and managing the pain symptomatically. For instance, lowering hyperglycemia or halting the use of paclitaxel usage could be the first line treatment approach when it comes to diabetic-induced peripheral neuropathy (DIPN) and paclitaxel-induced peripheral neuropathy (PIPN) respectively (Finnerup *et al.*, 2015). Relief from pain is often between 30% and 50% even in patients taking higher doses of pain relief medications (Attal, 2019). Uncontrolled diabetes caused by type-1 diabetes or type-2 diabetes is primarily one of the major

contributions to diabetic complications in various organs and systems including the somatosensory nervous system (Backonja, 2004). Type-1 diabetes mellitus arises as a result of total inability of the pancreatic beta cells to secrete insulin (Casellini & Vinik, 2007) whiles type-2 diabetes is caused by the resistance of beta cells to insulin (Huang *et al.*, 2016). It can also be caused by loss of pancreatic beta cells as a result of infections mostly of viral source or toxic damage producing insulin insufficiency. Hyperglycemia-induced oxidative and nitrosative stress serves as a major link between diabetes and diabetic complications (Casellini & Vinik, 2007), this release free radicals from autoxidation and glycosylation of glucose and proteins respectively (Zimmermann, 2001).

Paclitaxel, an anticancer agent possessing tubulin-stabilizing effect, is usually used in diseases such as ovarian cancer, breast cancer, non-small cell lung carcinoma and stomach cancer.

However, the use of paclitaxel is often limited by incidence of severe adverse reactions which includes peripheral neuropathy characterized by frequently occurring sensory neuropathies like dysesthesias, numbness, pain and thermo hyperesthesia in the feet and hands and usually mild motor neuropathies including muscle weakness and reduction of motor skill for instance buttoning a shirt (Ocean & Vahdat, 2004).

1.2 Problem Statement and Justification

Aside acute kidney shock and depression of the bone marrow, one of the major reasons for not complying with anti-cancer therapy or changing the dose regimen can be largely attributed to the neurotoxic side-effects of some common chemotherapeutic agent. A typical side effect associated with these agents includes neuropathic pain. Diabetes mellitus affects approximately 132 million people as of 2010 with 1.9% of the population having one of its developing complications as peripheral neuropathy (Van Acker *et al.*, 2009). Peripheral neuropathy as caused by paclitaxel usage

or long-standing diabetes mellitus complication can significantly interfere with function of peripheral nerves and can compromise the quality of life of individuals who suffers from it. Usually, the symptoms show a predominant sensory or sensory-motor neuropathy which sometimes occur together with the dysfunction of the autonomic nervous system.

There is currently no cure for this type of nerve damage. Management is tailored symptomatically and is normally treated with the use of antidepressants, opioid analgesics, anticonvulsants among others (Attal *et al.*, 2006). Pharmacovigilant study of these drugs used clinically have been indicative of some undesirable adverse effects and sometimes unsafe when employed long term for pain management. Some of these undesirable effects includes; drowsiness, constipation, dependence, dizziness, dry mouth, headaches, heart burn, and palpitations, insomnia, cardiac myopathies among others. (Attal *et al.*, 2006). They are also very expensive for the average individual to afford especially in Ghana (Amoateng *et al.*, 2017).

Considering the above setback in the treatment of neuropathy, there is a need to find agent(s) with high safety profile, cheaper and of better therapeutic value compared to the existing drugs on the market for clinical use. In identifying new agent/s to curb the existing burden, medicinal plants usage has empirically been identified to be a promising area in disease management where conventional medicine seems to be struggling, especially with respect to pain management. A review by Fatemah Forouzanfar *et al* in April 2018 have suggested common medicinal plants employed in the management of neuropathic pain. These include *Acorus calamus, Artemisia dracunculus, Butea monosperma*, among others. *Annona muricata* which is one of the readily available plant in the tropics has undergone quite a number of studies as far as pain and the nervous system is concern, its folkloric use and animal studies has been channeled in the line of anti-depressant, anticonvulsant, analgesic, anti-diabetic as well as anti tumour activities (Alali *et al.*, 1999). Its anti-inflammatory

effect is same as the action of indomethacin, a non-steroidal anti-inflammatory drug (De Sousa *et al.*, 2010; Poma *et al.*, 2011). The antinociception of the hydro-ethanolic extract has also been demonstrated using different chemical and thermal nociceptive models (Hamid *et al.*, 2012). Anxiolytic and anti-stress effects have also been found (Oviedo *et al.*, 2009). Traditionally it has also been used as a sedative (Hasrat *et al.*, 1997). In Brazil Martinique, Mexico and Nicaragua the leave stock is also used as an analgesic (Coria-Tellez *et al.*, 2018). The above research findings indicate clearly that *A. muricata* have a nervous system effect and possesses an analgesic property making it a suitable candidate for other forms of neurological screening especially that of peripheral neuropathy. Several agents from plant origin with potential therapeutic properties in the treatment of neuropathic pain have been identified unsuitable in the line of safety pharmacology, (Sengupta *et al.*, 2012). In this regard, the plant of concern will go through sub-acute toxicological studies and subsequent Irwin's test to ascertain its neurological effect.

To a very large extend this research will focus on safety *Annona muricata* aqueous leaf extract and it potential in the management of paclitaxel and diabetic-induced peripheral neuropathy in animal models.

1.3 Hypothesis

H_1: The Aqueous leaf extract of *Annona muricata* possesses an effect on the nervous system and will be useful in the management of peripheral neuropathy caused by paclitaxel and diabetes mellitus using animal models.

1.4 Aim

Evaluate the analgesic effect of aqueous extract of *Annona muricata* on paclitaxel and diabetic-induced peripheral neuropathy using animal models.

1.5 Specific Objectives

1.To conduct sub-acute toxicity studies on *A. muricata*.

2. To perform preliminary phytochemical screening on the extract of *A. muricata*

3. To investigate the general CNS safety pharmacology using Irwin test.

4. To demonstrate the effect of the aqueous extract of *A. muricata* on paclitaxel-induced neuropathy.

5. To investigate the effect of aqueous leaf extract of *A. muricata* on diabetic-induced peripheral neuropathy.

CHAPTER TWO

LITERATURE REVIEW

2.1 Background

Sensation of pain resulting from a lesion or disease of the somatosensory nervous system have been attributed to several causes including a variety of systemic, metabolic and toxic agents. Some of the commonest treatable causes are diabetes mellitus, hypothyroidism, drugs and nutritional deficiencies (Baron *et al.*, 2010). Accurate diagnosis involves careful clinical assessment, judicious laboratory testing and electrodiagnostic studies or nerve biopsy. Systematic investigation begins with localization of the lesion to the peripheral nerves, identification of fundamental etiology and the exclusion of potential causes which are treatable (Azhary *et al.*, 2010). Analysis on cerebrospinal fluids and lumbar puncture have been useful in diagnosing Guillain-Barre syndrome and chronic inflammatory demyelinating neuropathy (Sainaghi *et al.*, 2010). Electrodiagnostic analysis such as nerve conduction studies and electromyography contribute to the differentiation of axonal and demyelinating or mixed neuropathy. Treatment targets fundamental disease processes, nutritional deficiencies and provide symptomatic pharmacological intervention (Chichkova & Katzin, 2010). Diabetes which is characterized by persistence high blood glucose affects the peripheral nervous system. Its neuropathic complication is largely involved in almost all of impairments occurring in the peripheral nerves. Diabetic sensorimotor polyneuropathy (DSP) and diabetic neuropathy (DN) are used synonymously. Patients with DSP typically have numbness, tingling, pain and/or weakness that begin in the feet and spread proximally in a length-dependent fashion (stocking and glove distribution) (Bouhassira *et al.*, 2013). It has been estimated that diabetic peripheral neuropathy (DPN) occurs between 10% and 20% of patients suffering from diabetes whiles about 40% to 60% experience neuropathy (Callaghan, 2012).

However, these statistics may be underrated because about 12% of diabetic patients suffering from DPN do not inform their health care providers about this condition. Clinical symptoms observed during examination are allodynia and hyperalgesia (Brown & Asbury, 1984). Some neuropathic pains are differentiated based on electric-stabbing sensations with insensitivities or without insensitivities. Chemotherapeutic agents can also induce toxic action on peripheral nerves. The gravity can span from loss of sensory activity and lenient paresthesia to neuropathic pain, extreme ataxia and frailty resulting in conspicuous disability (Zilliox, 2017). Autonomic nerve fiber activity with orthostatic hypotension, impotence and incontinence can worsen the quality of life of suffers (Jost, 2003). Several cytotoxic agents are outlined as neurotoxic however only a few like the paclitaxel has its peripheral neuropathy as a dose-limiting side-effect (Cata *et al.*, 2006).

Paclitaxel, an antineoplastic drug obtained from the bark of the western yew tree *Taxus brevifolia,* is active against various tumors such as carcinoma of the ovary, breast, lung and the head and the neck. Paclitaxel exhibits antitumor action by fostering microtubule convergence (Park *et al.*, 2014) making neuropathy one of its untoward effects. Peripheral sensory neuropathy is habitually reported neurotoxic effect of paclitaxel which restricts treatment with high and cumulative doses when administered singly or together with other neurotoxic antineoplastic drugs such as cisplatin.

2.2 OVERVIEW OF PAIN

Pain can be described as unpleasant sensation which is often a response to external or internal stimulus having the potential to cause tissue damage. *Pain* has its origin from the Latin word '*poena'* which means punishment and it depicts the damaging consequences that can be imposed on the body. Pain is defined by the World Health Organization (WHO) and International Association for the Study of Pain (IASP) as any unpleasant experience either sensory or emotional connected to actual or potential tissue damage or described in terms of such damage" (Taxonomy, 2014). Even though

pain can be unpleasant, overbearing or present as a symptom to numerous medical conditions, it is an essential, adaptive warning sign that provides protection. Alerting the patient by the stimulation of immune function promotes healing and prevents further damages to the tissue (Tripathi *et al.*, 2016). It is usually an uninterrupted reaction to an outward activity often associated with harm to tissues caused by infection, injury, inflammation, cancer among others. Sometimes it is with no known cause (e.g., trigeminal neuralgia).

Clinically, pain has been added to the four different vital sign assessment which are temperature, pulse, blood pressure and respiratory rate (Fitzgibbon *et al.*, 2010). Unfortunately, there isn't any objective test to measure pain making it sometimes difficult for clinicians to assess it. (McCaffery, 1990). Pain evaluation is a vital phase when offering clinical intervention (Lacroix *et al.*, 2017). There are several suggestions and instructions which determine what appropriate pain assessment protocols must include. Unfortunately, some of these protocols seem impractical. It is essential then that health care professionals select the adequate characteristics of pain assessment in relation to the presented clinical situation.

2.2.1 Neurophysiology of pain

Pain is beyond the nociceptive neuronal transmission from injury site to where it is generated and perceived which is the brain. Pain involves numerous physiological processes including the somatosensory and limbic systems. It is also subjective because every person experience pain in different ways (Muthuraman *et al.*, 2008). Numerous painful conditions are linked with an impairment of normal physiological pain pathway. This pathway is composed of the peripheral and central processes. The peripheral involves activities influencing nerve terminals whiles central pathway influences transmission in the synapse along the dorsal horn to the brain (Rang, 2003).

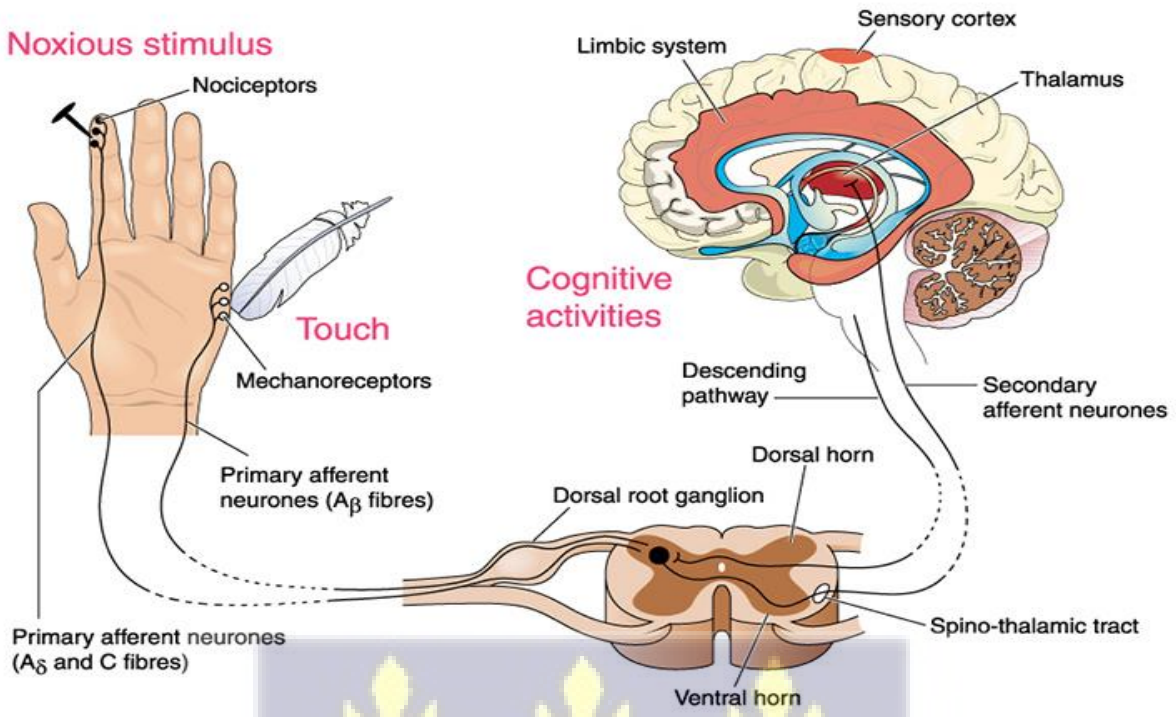


Figure 2.1: Illustration of the processes of pain initiating from the periphery to the brain (Reddi *et al*. 2013).

Briefly in figure 2.1, primary afferent neurons transmit noxious stimulus via the spinothalamic tract to the sensory cortex of the brain for interpretation and adequate response.

2.2.2 Peripheral pain processes

2.2.2.1 Pain transmission in the dorsal horn of the spinal cord

The dorsal horn of the spinal cord contains Aδ and C fibers synapse with secondary afferent neurons. Histologically, the dorsal horn has ten layers known as Rexeed laminae. The synapse fibers Aδ and C sends information to Rexeed lamina I, II and other laminae via nociceptive-specific neurons. (Reddi *et al.*, 2013). Primary afferent terminals secrete and release different excitatory neurotransmitters, calcitonin gene-related peptide (CGRP) and somatostatin. Diverse reactions involving inter-neurons, afferent neurons as well as descending modulatory pathways occur inside

dorsal horn (Reddi *et al.*, 2013). These reactions influence activities in the dorsal horn including processes of the secondary afferent neurons. Laminae II of the dorsal horn has been found to be the primary source of inhibition or facilitation of the transmission of pain.

2.2.2.2 Pain transmission in the ascending tracts in the spinal cord

Second-order brain cells travels through contra-laterally in the spinothalamic, spinoreticular and spinomesencephalic tracts and transmits information to the supraspinal centers; hypothalamus, thalamus, periaqueductal grey, locus coeruleus and cerebral cortex. Here nociceptive signals are localized and generated together with sympathetic, thermoregulatory and arousal responses. They then synapse with third-order neurons in the somatosensory cortex (Meriaux *et al.*, 2018; Reddi *et al.*, 2013).

2.2.3 Central processing of pain

Pain processing in the central nervous system is mediated by nucleus raphe magnus and periaqueductal grey matter. In addition, nociceptive inhibition of neurons in the dorsal horns which block nociception-transmitting neurons also contributes to the central processing of pain. Transmission of pain via the spinal cord is promoted by the lateral spinothalamic tract pathway for nociceptive information to reach the brain. The lateral spinothalamic tract pathway is divided into two different pathways. One of which is the neospinothalamic tract for "fast spontaneous pain". This pathway controls fast pain which travels through type Aδ fibers to discontinue on the dorsal horn of the spinal cord where they form a synapse with the dendrites of the neospinothalamic tract (Millan, 1999). The axons of these neurons pass through the spine to the brain and cross the midline via the anterior white commissure, through the contralateral anterolateral columns and then stops on the ventrobasal complex of the thalamus which synapses with the dendrites of the somatosensory cortex.

The second pathway is the paleospinothalamic tract. This pathway slowly advances pain through type C fibers. It also transmits the sensation of pain to the dorsal horn through laminae II and III. Type C fiber together with laminae II and III are called the substantia gelatinosa. Electrical impulses from nerve fibers passes through lamina V in the dorsal horn. Lamina V synapses with brain cells that connect with fibers from the fast pathway. The fast pathway transmits the impulse to the opposite side through anterior white commissure. This then travels up anterolateral pathway. These brain cells or neurons are linked to one-tenth of fibers in brain regions including the thalamus and medulla (Kivell & Prisinzano, 2010; Millan, 1999).

2.2.4 Inhibiting pain mediation

Processes work to restrict pain mediation in the spinal cord and through descending blocking from higher centers.

2.2.4.1 Gate control theory of pain

The gate control theory of pain was described by Melzack and Wall in 1965. It illustrates a mechanism showing that controlling pain at the spinal cord level is possible (Reddi *et al.*, 2013). The theory also explains the reason banging our head feels better when we rub it against an obstacle and states that Aβ fibers inhibitory interneurons activation in the dorsal horn blocks the transmission of pain signals through the C fibers (Reddi *et al.*, 2013)

2.2.4.2 Descending inhibition

Parts or regions of the brain that has an impact on descending inhibitory are the periaqueductal grey and rostral ventromedial medulla (RVM). These areas have numerous endogenous opioids as well as opioid receptors demonstrating why opioids are considered as analgesics. Some pathways descend

through the dorsal horn which contains adrenergic, serotoninergic and opioid receptors inhibiting pain medication. Since the pathways involves noradrenaline and serotonin it can be concludes that they are monoaminergic (Reddi *et al.*, 2013).

2.2.5 Classification of pain

Pain is classified into nociceptive and neuropathic. Other types of pain are classified according to their duration, which are the acute and chronic pain. In addition, some other types of pain also exist outside these classification e.g. pain from fibromyalgia (Rang, 2003)

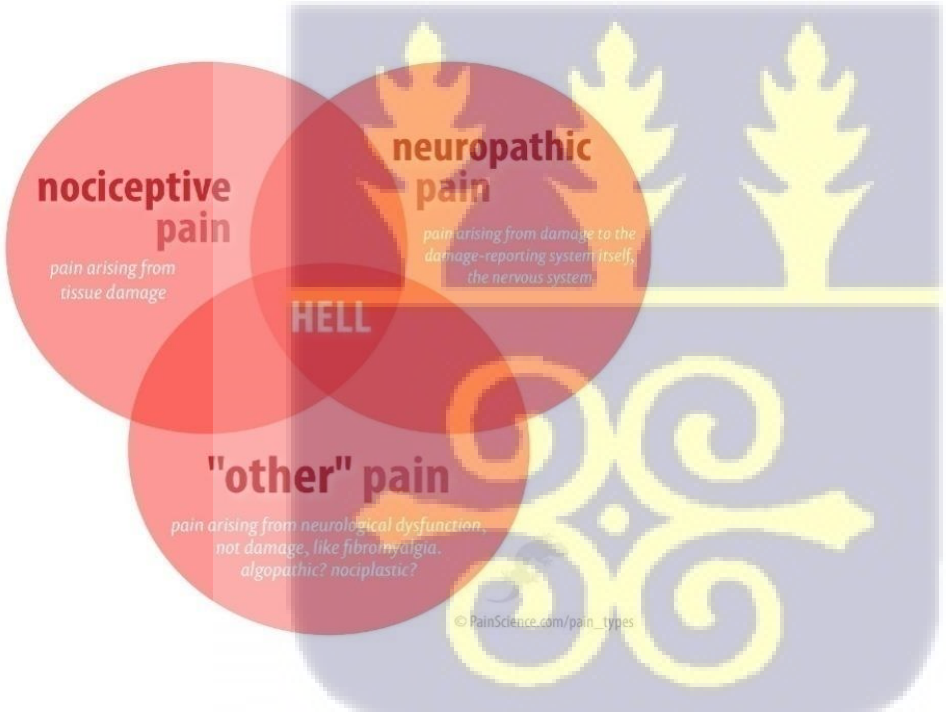


Figure 2.2: An illustration of the types of pain (www.Painscience.com)

Figure 2.2 indicates three main pain classification which includes nociceptive pain, neuropathic pain and pain arising from other noxious stimuli. They all exhibit different classical signs but some of their clinical characteristics appears to be the same description in all pain types.

2.2.6 Nociceptive pain

Nociceptive pain refers to pain caused by activities in the neuronal pathways which are secondary to tissue damage or stimuli that are harmful to tissues. It is activated by factors such as inflammation or diseases. Examples of nociceptive pain are arthritic or surgical pain or lower bac pain (Rang, 2003). Nociceptors are involved in the identification of dangerous stimuli and conveying them into electrical impulses they are transmitted to the brain. Nociceptors are free nerve endings sensory receptors of core sensory afferent neurons possessing a single cell body and found inside dorsal root ganglion. There are three main groups of afferent neurons which are as follows; Group A, further classified into α, β, γ, δ, group B, and group C. Noxious or harmful stimuli are responded to by the primary sensory afferent fibers such as tiny diametric, unmyelinated C-polymodal fibers and thinly myelinated, small diametric (Aδ) fibers. These primary sensory afferent fibers are connected to nociceptors and are triggered by stimuli including pressure, chemicals and heat (Koltzenburg, 2000). Damaged tissues also release inflammatory mediators such as cytokines, bradykinin and H+ which stimulates or sensitizes nociceptors by decreasing the threshold for their activation (Reddi *et al.*, 2013).

The expression of pain is two-fold. It's either first pain or second pain. First pain is conducted through Aδ nociceptors. Consequently, its fast, sharp, localized and short-lived. the second pain is conducted and transmitted by the C-fiber, polymodal nociceptors. It is slow, diffuse, persistent, burning and not short-lived like first pain but long-lived and can even last after termination of the stimulus (Ma & Zhang, 2010).

2.2.7 Neuropathic pain

Neuropathic pain may be induced by lesions or diseases to the nerves of the somatosensory neurons. The injury to these nerves can be attributed to factors such as an infection, trauma, diseases like

diabetes mellitus, surgery or chemotherapy. Neuropathic pain is more likely to be spontaneous and is experienced as a burning or 'like an electric shock'. It is experienced in response to a stimulus which might not necessarily cause pain (allodynia),or may be experienced as an exaggerated response to a painful stimulus (hyperalgesia) (Colloca *et al.*, 2017). Pain generated by peripheral nerve damage is interrelated with neuropathic pain and as such they are used synonymously even though it may include central pain linked to injuries to the CNS. Neuropathic pain changes the patient's quality of life by interrupting the mental wellness. Due to the chronic nature of neuropathic pain it presents a setback in clinical setting other factors that may contribute to this setback includes the gravity and reduced efficacy of some classical analgesics (Backonja, 2004). With reference to the manifestations of this type of pain, mechanical allodynia consists of marked disruption in sensation. In addition, cold allodynia may be prominent particularly in sympathetically stimulating episodes (Bennett & Xie, 1988).

2.2.7.1 Etiology of neuropathic pain

Neuropathic pain has been classified into two. The first classification is based on the etiology of injury to the nervous system whiles the second is based on its anatomical distribution. This classification is helpful in differential diagnosis and disease-modifying treatment. However, it does not have a framework for clinical management of the pain. The connections between its etiology, mechanisms, and symptoms is very intricate (Devor *et al.*, 1994). The manifestation of pain in various disease states may occur through common mechanisms. These mechanisms are the consequence of a particular disease process. Even though, few patients are affected by neuropathic pain there are no predictors for its development. Some fundamental causes of neuropathic pain are excessive intake of alcohol, surgery on the spinal cord, chemotherapy (Boland *et al.*, 2010), diabetes

mellitus, facial nerve problems, HIV/AIDS infection and syphilis (Sharif-Alhoseini *et al.*, 2012).

2.2.7.2 Symptoms of neuropathic pain

Most people suffering from neuropathic pain shows continuous or paroxysmal pain without a stimulus. This type of pain may be striking, piercing or burning and is determined by the type of activity the sympathetic nervous system may be involved in. Spontaneous activity in the fibers of nociceptor C causes a burning sensation and sensitizes neurons of the dorsal horn. Spontaneous activity in large, myelinated A fibers is often linked with stimulus-independent paresthesia and the sensitization of the central nervous system to pain and dysesthesias. Pain which is induced by a stimulus is characterized by peripheral nerve impairment attributed to hyperalgesia and allodynia. Allodynia in isolation does not implicate a specific process it is therefore essential to view as a subset of hyperalgesia so that the clinical manifestations can be diagnosed based on the mechanism involved. Stimulus-induced hyperalgesia has been divided into mechanical, thermal or chemical. Mechanical hyperalgesia can occur as dynamic, static or punctate hyperalgesia.

2.3 Paclitaxel-induced Peripheral Neuropathy

2.3.1 Paclitaxel and peripheral neuropathy

Taxanes, an antineoplastic drug works on microtubules by interrupting sensory dominant nerves by acting on sensory fibers with small diameters. Manifestation usually involves microtubule depolymerization and repolymerization which causes damage. Such damages include dysesthesias, paresthesias, changes in proprioception, numbness and loss of dexterity in the toes and fingers. sometimes other localizations including effect on motor and autonomic activity may occur. Clinical manifestations usually begin just few days after the first dose and its dose dependent. The effect ameliorates when treatment ceases. For some people symptoms can last for up to 3 years after completing therapy however for some it continues the entire life. Examples include paclitaxel,

docetaxel and cabazitaxel. These drugs are approved for treating cancer such as cancer of the breast, ovary and prostate. The occurrence of taxanes can causes chemotherapy-induced peripheral neuropathy (CIPN) which may rise between the range of 11% and 87% with Paclitaxel recording the greatest rate. Paclitaxel fostered neuropathy is marked by various sensory alterations for instance the occurrence of mechanical allodynia where light pressure or touch usually seen as harmless elicits pain. The exact pathobiology of CIPN is not fully clear, however, current studies points to "terminal arbor degeneration" (Bobylev *et al.*, 2015), oxidative stress (Han & Smith, 2013), mitochondrial impairment and mitotoxicity (Ocean & Vahdat, 2004).

2.3.2 Pathogenesis of paclitaxel-induced peripheral neuropathy

Paclitaxel causes microtubule interference, which inhibits axonal transport and results in Wallerian degeneration. This affects the action of ion channels such as sodium and potassium causing excitability of peripheral neurons. Mitochondrial injury caused by Paclitaxel increases the levels of reactive oxygen species which causes impairment in calcium homeostasis of neurons and damages to enzymes, proteins and lipids. This causes apoptotic alteration and demyelination of peripheral nerves. Paclitaxel also activates microglia and astrocytes which in turn attracts and activates immune cells. It also causes the secretion and elevation of pro-inflammatory cytokines which can lead to nociceptor sensitization and ultimately to the development of neuroinflammation.

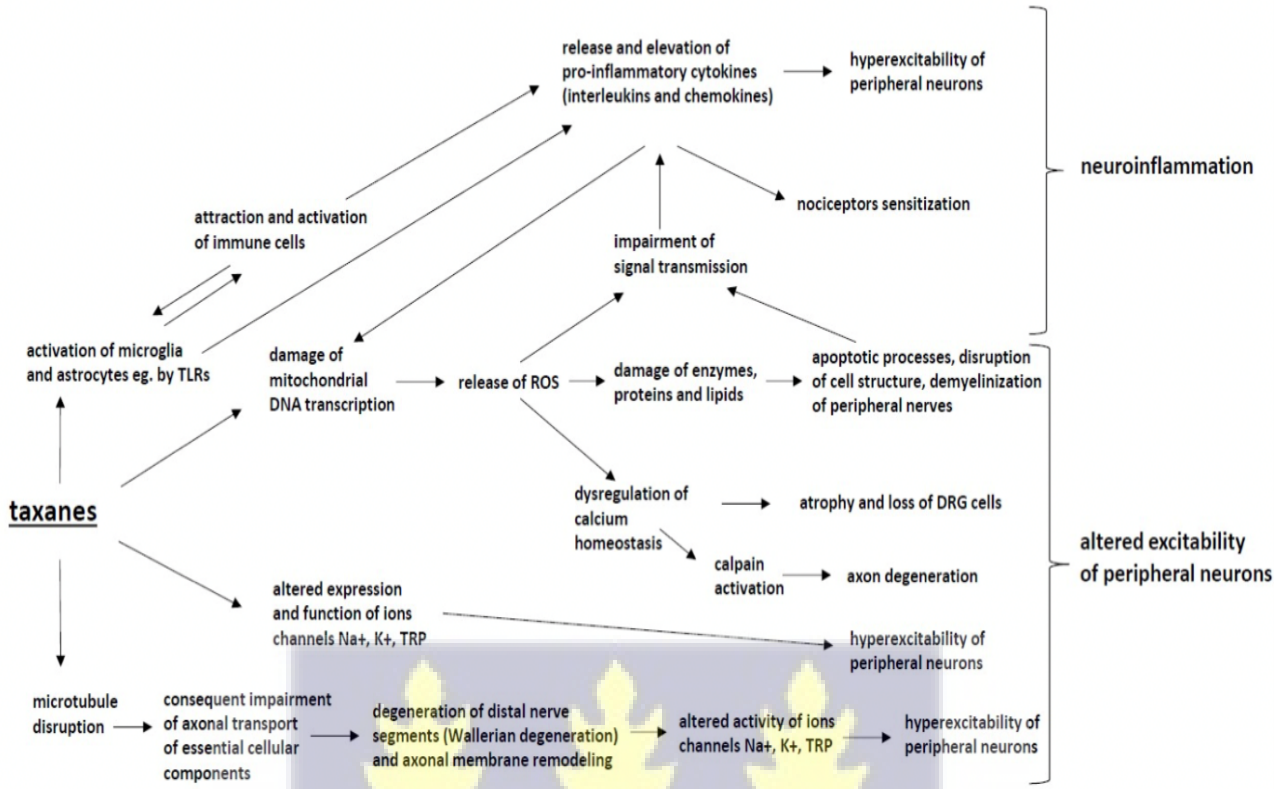


Figure 2.3: A diagrammatic representation of mechanism of action of taxanes causing peripheral neuropathy (Zajączkowska et al., 2019).

Figure 3 illustrates the MOA of taxanes causing neuropathic pain, briefly taxanes causes peripheral neuropathy via two major pathways which includes microtubule disruption and activation of microglia and astrocytes which eventually results in neuroinflammation and altered excitability of peripheral neurons resulting in peripheral neuropathy.

2.3.3 Paclitaxel and microtubule interference

Microtubule interference is the primary mode of activity of taxanes and it accounts for their antineoplastic effect, it is also linked with the evolution of peripheral neuropathy (Gornstein, 2017). Its assembling and packaging of the microtubules results in changes in cell shape and cell strength

which inhibits transport of synaptic vesicles in the axons (Scott *et al.*, 2011).

2.3.4 Paclitaxel and mitochondrial dysfunction

Mitochondria damage in neurons and other cells causes oxidative stress and produces reactive oxygen species (ROS) including hydroxyl radicals and superoxide. Axonal transport of important cellular components and mRNA deficits (Bobylev *et al.*, 2015) to distal neuronal regions because of microtubule interruption may have notable influence on this mechanism. Elevated levels of ROS are found in the spinal cord and sensory neurons. These elevated levels can activate apoptotic pathways and can disrupt cell structure and demyelination. These activities inhibit signal transmission and immune response activation leading to increased secretion of cytokines (pro-inflammatory cytokines). This mechanism amplifies itself in that the above mechanisms can initiate further mitochondrial injury. (Bulua, 2011;Areti, 2014). In recent studies, paclitaxel is known to cause swelling of the mitochondria as well as vacuolation and loss of mitochondrial structure (Gilardini *et al.*, 2012).

2.3.5 Paclitaxel and axon degeneration

Numerous studies have stated that the administration of paclitaxel causes damage to peripheral nerves, decreases neuronal fibers and leads to. The disruption of microtubule and the resulting impairment in axonal transport of some cellular components can lead to the degeneration of distal nerve segments (Wallerian degeneration) and the restructuring of axonal membrane (Bober, 2015). Boyette et al. demonstrated that there is a reduced number of intra-epidermal fibers in paclitaxel-induced CIPN murine models (Boyette-Davis, 2012). Ferrari also showed that there is a restricted corneal innervation in these murine models. Signaling of cytokines and chemokines may also be implicated in the degeneration of axons as Zhang et al. have revealed that a decrease in the levels of chemokine MCP1/CCL-2 decreases nerve degeneration and CIPN behaviors in a murine model

(Ferrari, 2013).

2.3.6 Paclitaxel and calcium homeostasis

An impairment in Ca^{2+} hemostasis is involved in PIPN pathogenesis. An impairment in intracellular Ca^{2+} has been noticed in models illustrating paclitaxel neuropathy. Endoplasmic reticulum and mitochondria are rich in intracellularly Ca^{2+}. Paclitaxel administration can lead to the release of Ca from the mitochondria through the activation of mitochondrial permeability transition pore (mPTP). This can cause a rapid depolarization in the mitochondria (Kidd, 2002). The is a high probability that in the ER paclitaxel can cause the release of Ca^{2+} through the regulation of 1,4,5-trisphosphate receptor (IP3R) (Boehmerle, 2006). This process leads to an increased expression of CaV3.2 channels in rats. The repression of these process leads to an overturn of hyperalgesia (Peltier & Russell, 2002).

2.3.7 Paclitaxel and peripheral nerve excitability

Modification in the expression and activity of NaV, TPR and KV ion channels is an alternate process that can account for the development of PIPN. A reduction in the expression of K^+ ion channel was found in the DRG of paclitaxel evoked CIPN model. This caused a spontaneous function of nociceptors. Activation of TRPV1 and TRPA1 cation channels plays a significant role in pain signaling and are identified in DRG neurons (Materazzi, 2012). TRPA1 antagonists are known to relieve paclitaxel-induced inflammation, cold allodynia and hyperalgesia. Paclitaxel also increases the levels of NaV1.7 contributing to the development of CIPN (Hara, 2013) and hence restricting this channel reduces hyperalgesia in rats.

2.3.8 Paclitaxel, immune processes and neuroinflammation

Paclitaxel increases pro-inflammatory cytokines (TNF alpha and IL-1 beta) and reduces anti-

inflammatory cytokines (IL-4 and IL-10) (Doyle, 2012; Areti, 2014) . This mechanism attract and activate immune cells consequently leading to neuroinflammation (Krukowski, 2016). Krukowski (2016) demonstrated that IL-10 decreases paclitaxel-induced CIPN. Paclitaxel also activates microglial and astrocyte as well as increasing macrophage number of DRG in neuronal and non-neuronal cells (Gornstein, 2017).

2.3.9 Genetics and paclitaxel-induced peripheral neuropathy

It has been found in recent studies that low frequency variants of EPHA6, EPHA5 and EPHA ephrin gene receptors and its associated severity in taxanes induced neuropathy (Apellániz-Ruiz *et al.*, 2015; Leandro-García *et al.*, 2012)**.** Genes playing an essential role in paclitaxel-induced neuropathy includes glycogen synthase kinase-3β gene (GSK3β) (Park *et al.*, 2014), Charcot-Marie-Tooth disease gene ARHGEF10 (Boora *et al.*, 2016) and VAC14 which codes for parts of a trimolecular complex regulating the levels of phosphatidylinositol 3,5-bisphosphate (Komatsu *et al.*, 2015).

2.4 Diabetic-Induced Peripheral Neuropathy

2.4.1 Diabetes and diabetic neuropathy

The effect of diabetes mellitus on the peripheral nervous system manifests diversely. Diabetic neuropathy is largely involved in damages to the peripheral nerves. Symptoms of diabetic neuropathy include tingling sensation, numbness, pain with/without weakness. These symptoms generally start from the feet and travels to the fingers (Albers & Pop-Busui, 2014). These symptoms correspond with sensory manifestations which are more conspicuous than motor involvement. Majority of patients experience a condition of insensibility and excruciating sensitivity simultaneously. However, this condition varies within patients (Callaghan *et al.*, 2012).

Diabetic-induced neuropathic pain and numbness results in imbalance making patients fall. This

makes it a major factor that increases the number of falls in patients suffering from diabetic neuropathy (D'Silva *et al.*, 2016). It has been discovered that patients suffering from diabetic neuropathy have increased susceptibility to falls than patients suffering from diabetes without neuropathy (Peltier *et al.*, 2014). In severe cases, patients are more likely to acquire foot ulcers and lower extremity amputations in disease state (Dabkana *et al.*, 2018). Diabetes is the leading cause of lower extremity amputations, which is more highly probable to occur in patients also suffering from neuropathic pain (Al-Rubeaan *et al.*, 2015). Many patients with diabetic foot ulcers undergo lower extremity amputations globally (Ferreira-Chamorro *et al.*, 2018). Diabetes affects the health standard of patient and becomes worse in patients also suffering from neuropathic pain (Alleman, 2015). DN incapacitates patients and because there are no therapeutic agents to treat it makes patient more devastated (Goldman & Appell, 1999). In the diabetic population, about 20% of them suffer from diabetic neuropathy whiles 40% to 60% have reported neuropathy (Vincent *et al.*, 2011). These figures may be under-recorded since 12% of patients with DNP informs their healthcare provider about their situation. The symptoms of DNP are similar to other forms of neuropathic pain but are distinguished by electric-stabbing sensations with or without insensibilities (Brown & Asbury, 1984). Diabetic neuropathy is based on the length of axon and begins in toes travelling upwards to the calf and finally to the fingertips (Edwards *et al.*, 2008).

Figure2.4: An illustration of the features of diabetic peripheral neuropathy

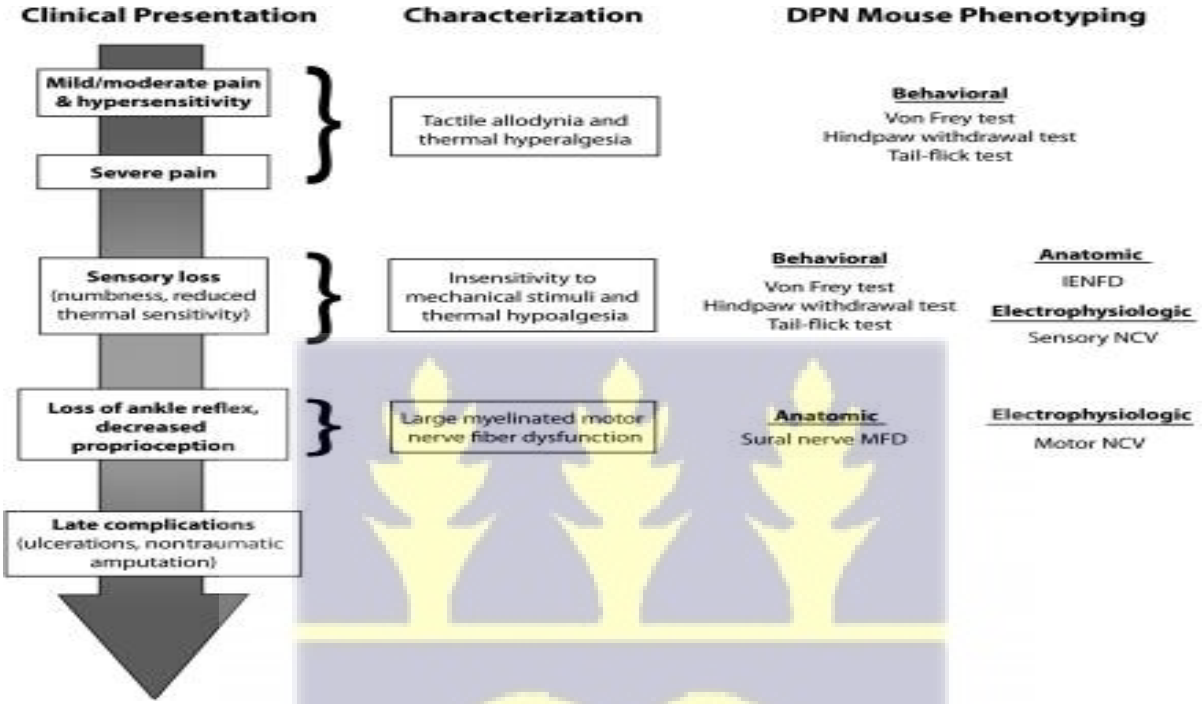


Figure 2.4 illustrates the clinical manifestation of diabetes peripheral neuropathy which is characterized by allodynia and hyperalgesia and can be illicited using Von Frey test, hindpaw withdrawal test and tail flick test in animal modules. (Azhary, H. *et al* 2010)

Figure 2.5: Stocking Glove Configuration of DPN. (easd.org)

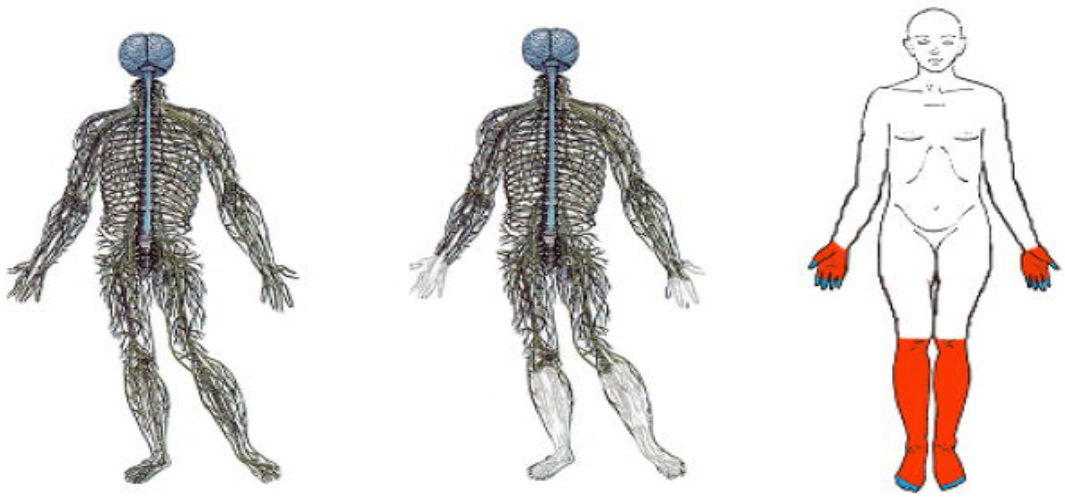


Figure 2.5 illustrates the pattern with which diabetes peripheral neuropathy affects certain specific body areas. It is realized that the pattern of neuropathy manifestations is with respect to the extremities(limbs).

2.4.2 Free radicals and diabetic neuropathy

Reactive oxygen species which includes hydroxyl radicals, peroxide, superoxide and stress triggered by single oxygen are actively involved in the development of diabetic neuropathy. Oxidative stress induced by long-term hyperglycemia is a direct linkage that presents a unified mechanism involving tissue damage (Negi, 2011). Biological markers of oxidative stress undergo significant alteration in DN. Also, there is a nerve dysfunction which may be caused by the over expression of superoxide and peroxynitrite in sciatic nerves (Pacher *et al.*, 2005). The over expression of superoxide decreases vascular activity which can be a hindrance to nutrient supply to the sciatic nerve. A reduction in glutathione and antioxidant enzymes has also been found to contribute to diabetic neuropathy (Yagihashi *et al.*, 2011). DNA fragmentation has been identified in peripheral nerve sections of

animals induced with diabetic neuropathy (Sullivan *et al.*, 2007).

In addition, natural cell death of the dorsal root ganglion (DRG) and vagus ganglion was recorded in streptozotocin-induced diabetic animals (Guo *et al.*, 2004). However, similar report was not recorded in peripheral neurons of models showing elevated ROS from chronic hyperglycemia (Zherebitskaya *et al.*, 2009). It has been stated by Zherebitskaya and his team that elevated levels of blood glucose decreases the activity of antioxidant enzymes. This created distortions in DRG which was regulated by ROS but there was no recorded natural cell death. The occurrence of natural cell death in peripheral nerves and diabetes has not been fully understood. However, a correlation between chronic hyperglycemia and elevated ROS has been established. Therefore, oxidative biological markers can be used as indicators for accurate diagnoses and progression of neuropathy in diabetes (Yagihashi *et al.*, 2011).

2.4.3 Anatomy of diabetic neuropathic pain

The sensation of pain indicates the presence of actual or potential tissue injury caused by a stimulus. Sensory afferent nerves transmit pain through myelinated fibres from the skin and other parts of the body. Large, myelinated fibres such as A-alpha and A-beta are involved in limb proprioception and the transmission of sensations from limb proprioception respectively. Also, large, myelinated A-delta fibres and small C unmyelinated fibres transmit nociceptive sensations. Pain that is superficial, producing stinging or pricking sensation is transmitted by A-delta fibres whiles pain that is deep-seated, burning and itching travels through slow, unmyelinated C fibres. This type of pain is often followed by hyperalgesia and allodynia. Damage to tissues causes the secretion and release of inflammatory chemicals such as prostaglandins, cytokines, bradykinins, and histamines at the site of inflammation. This leads to the depolarization of nociceptors causing an action potential (Willis & Westlund, 1997). Consequently, action potential transmits nociceptive sensation via dorsal root

ganglion (DRG) to the spinal cord through its dorsal horn. The release of glutamate and substance P causes transmission of nociceptive sensations through spino-thalamic tract, thalamus and the cortex. Pain is perceived and intercepted in the cortex (Willis & Westlund, 1997). Neuropathic pain is a consequence of lesion or disease attacking the somatosensory system not necessarily by a stimulus. It can be classified as peripheral neuropathic pain or central pain. Peripheral neuropathic pain is marked by the activation of pathways of pain in the peripheral nerves and posterior roots. Central pain is marked by the activation of pain pathway in the spinal cord and brain (Treede *et al.*, 2008). Conversely, nociceptive pain is a reaction to damages to tissues such as skin and muscles caused by a stimulus. It usually stops when the injury is healed. Based on the nature of origin pain and its etiology, the symptoms of pain can be focal, multifocal or generalized (Aslam *et al.*, 2014).

2.4.4 Pathophysiology of diabetic neuropathy

The development of Diabetic Peripheral Neuropathy (DPN) remains unclear. However, recent studies show DPN is as a result of an impact exerted by physiologic and metabolic dysfunction in somatosensory nerves. The effect of diabetes impacts these nerves and the microvasculature supplying the nerves (vasa nervorum) (Van Dam *et al.*, 2013). Chronic hyperglycemia, characterized by an increase in plasma glucose levels (Britland *et al.*, 1992) plays a vital role in nerve injury. It initiates aberrant biochemical mechanisms such as dyslipidemia (Vincent *et al.*, 2013;Hinder *et al.*, 2013), secretion of advanced glycated end products (Jack & Wright, 2012), protein kinase C (Geraldes & King, 2010), inflammation (Pučić *et al.*, 2011) dysfunction of insulin signaling (Kim & Feldman, 2012), production of ROS by the mitochondria (Sena & Chandel, 2012), hyperactivity of the polyol pathway (Oates, 2002) and ER stress (Lupachyk *et al.*, 2013). These abnormalities interfere cellular homeostasis and promote the development of diabetic neuropathy eventually progresses. Vascular disorders are known to cause neuropathy in diabetic animal models (Tesfaye

et al., 2010). As DPN progress, neuronal impairment is linked to the development of endoneurial microangiopathy (Malik *et al.*, 1994), vascular irregularities promote reduced oxygen tension and hypoxia resulting in neuronal ischemia.

Recent findings shows that DPN in both type-1 and type-2 diabetic patients may be inherently separate disorders (O'Brien *et al.*, 2014a). Another clinical study evaluated the effectiveness of regulating glucose on the prevalence of DPN. It has been found that regulating glucose lessened the onset and progression of DPN in type-1 diabetes mellitus (T1DM). This has not been recorded in people suffering from type-2 diabetes mellitus (T2DM). This shows that DPN in T1DM and T2DM are different from each other (Callaghan *et al.*, 2012).

2.4.5 Mechanisms leading to the development of DNP

2.4.5.1 Polyol Pathway

Aldose reductase is an enzyme that reduces toxic aldehydes into inactive alcohols and its the main enzyme in polyol pathway. When a cell becomes extremely hyperglycemic, glucose is converted to sorbitol by aldose reductase. Sorbitol is then oxidized to fructose. This process involves a cofactor of aldose reductase, nicotinamide adenine dinucleotide phosphate (NADPH). NADPH produces glutathione which reduces intracellular oxidative stress (Brownlee, 2005). In a study conducted for a period of five years, aldose reductase inhibitor administered to diabetic dogs resulted in an impairment of nerve conduction velocity as its observed in diabetic patients. It was reported that the induced diabetic impairment in the velocity of nerve conduction was ameliorated after treatment (Engerman *et al.*, 1994).

2.4.5.2 Advanced glycated end products (AGE)

Damages to cells by the precursors of AGE involves three different mechanisms. Firstly, endothelial cell which is its main target changes intracellular proteins for instance proteins taking part in gene transcription. Secondly, the AGE diffuses from cells and changes the molecules of extracellular matrix altering signaling of the cells and matrix which causes cellular impairment. This process is identified by the cross linkage of collagen, consequent tendon and ligament pathology. Lastly, products of AGE disperse from cells and changes proteins that are circulating in the blood including albumin. These proteins trigger AGE's resulting in the secretion of inflammatory cytokines as well as growth factors creating a vascular dysfunction (Winocour *et al.*, 1988).

2.4.5.3 Protein Kinase C Pathway

Protein Kinase C is triggered by an increase in the synthesis of diacylglycerol by hyperglycemia. This actuates protein kinase C cofactors. PKC produce an effect on the expression of genes such as causing a reduction in endothelial nitric oxide (NO). It also increases vasoconstrictor endothelin-1 and modifies cellular metabolism and axonal flow of Schwann cells (Greene *et al.*, 1990).

2.4.5.4 Hexosamine Pathway

Hexosamine pathway is the final pathway hastened by high blood glucose which is metabolized by glycolysis. Metabolites like fructose 6-phosphate from glycolysis is transported into signaling pathway and converted into Serine and threonine by GFAT enzyme. Serine and threonine are then bound by uridine phosphate (UDP) N-acetyl glucosamine causing alterations to gene expression and subsequent nerve irregularities (Nawroth *et al.*, 2018).

2.4.6 Clinical features

2.4.6.1 Microvascular ischemic changes

Pathological alteration of diabetic nerves include the thickening of capillary basement membrane, endothelial cell hyperplasia, neuronal ischemia and even neuronal death (Pallas & Larson, 1996).

2.4.6.2 Advanced glycosylation end products

Chronic intracellular hyperglycemia releases glycating agents known as advanced glycosylation end products. Advanced glycosylation end products can form inside and around peripheral nerves. They can disrupt axonal transport, resulting in decreased velocity of nerve conduction. Advanced glycosylation end products can also decrease NADPH through the activation of NADPH oxidase. This process can produce hydrogen peroxide and oxidative stress (Zochodne, 1999)

2.4.6.3 Inflammatory microvasculopathy

Numerous studies have suggested different forms of diabetic neuropathies such as asymmetrical neuropathies, mononeuritis multiplex and diabetic amyotrophy can be induced by inflammatory vasculopathy. Diabetic nerves emerge having high responsiveness to immune factors (cellular and humoral), which involves removal of immunoglobulin, actuation of lymphocytes and complement trigger (Greene *et al.*, 1990).

2.4.6.4 Growth factor and insulin deficiency

To keep the structure of the nerves, its activities and restoration following an injury, neurotrophic factors are needed. Decrease in the number of nerve growth factor and insulin-like growth factors 1 have been connected to serious forms of diabetic neuropathy in murine models. Insulin have

demonstrated neurotrophic activities and its insufficiency can account for the evolution of neuropathy (Brown & Asbury, 1984).

2.4.6.5 Neuronal membrane ion channel activity

Aberrant calcium channel action is implicated in cellular damage and death observed in different diseases. Elevated functions of voltage-gated calcium channels are illustrated in diabetic neuropathy that results in tissue damage. Sodium channel defect plays a vital function in initiating painful neuropathy, a frequent observation in diabetic sufferers.

2.4.6.6 Essential fatty acids

Essential fatty acid from linolenic acid, prostaglandins and thromboxane have been found to be unbalanced in diabetic patients. This causes several cellular abnormality like anomaly in membrane fluid, cell membrane modifications of red blood and decreased levels of prostaglandin E2 (Edwards *et al.*, 2008).

2.4.6.7 Distal predominant sensory polyneuropathy

Distal predominant sensory polyneuropathy is the commonest type of diabetic neuropathy that occurs because of "dying-back" axopathy. a length-dependent procedure. Paresthesias and numbness begins stealthily and continues steadily, starting from the feet and proceeds with time. Loss of touch usually precedes loss of perception and vibration. Serious forms of neuropathy and uncoordinated movements can occur. Sensory deficits can result in complexities like unhealing sores and neuropathic arthropathy. Motor nerves are often impacted late, resulting in muscle weakness and reduction in tissues. Notable motor frailty signals a coincidental neuropathy from a different source such as chronic inflammatory demyelinating polyradiculoneuropathy (CIDP) (Peltier *et al.*, 2014).

2.4.6.8 Autonomic neuropathy

Autonomic neuropathy is rarely reported in diabetes but occurs. Some of its clinical symptoms are distal anhidrosis, orthostatic hypotension, and erectile weakness in men. Comorbidity with cardiac disease may possibly account for death in diabetic suffers because of silent myocardial ischemia and arrhythmia. Gastrointestinal defects such as serious constipation, or diarrhea may be observed in some cases.

2.4.6.9 Compressive mononeuropathy

compressive injury has a higher incidence in diabetic nerves. Carpal tunnel syndrome occurs in one-third of patients. Likewise, ulnar nerve disorders occur across the elbow whiles nerve damage occur across the fibular head. Results from diabetic patients with carpal tunnel syndrome can be successful after surgery with clear focal modification of demyelination in the median nerve across the carpal tunnel (Callaghan *et al.*, 2012).

2.4.6.10 Noncompressive focal and multifocal neuropathies

Diabetic amyotrophy, truncal neuropathies, cranial neuropathies, mononeuropathies, and mononeuropathy multiplex are examples of clinical spectrum of multifocal neuropathies in diabetes. These may be as a result of nerve ischemia and occlusion of the vasa nervorum. Diabetes is connected to accelerated atherosclerosis, which may be implicated in evolution of neuropathy. Current studies have indicated that mononeuropathy multiplex and diabetic amyotrophy are as a result of inflammatory vasculopathy which acts on vasa nervorum, as such, immune therapy has been recommended. Similar pathogenetic mechanisms may be the cause for the development of other noncompressive focal neuropathies. Unfortunately, pathological examinations in this regard

are not sufficient to draw any relevant conclusions (Tesfaye *et al.*, 2010).

2.4.6.11 Diabetic amyotrophy

Diabetic amyotrophy is uncommon but disabling neuropathy that shows in type-2 diabetics. The manifestations are usually sub-acute with pain and asymmetric frailty and atrophy of proximal lower limb muscles. Muscles and limbs such as distal lower extremity muscles and infrequently upper limb muscles respectively may be affected. Whiles the resulting pain is severe, sensory impairments are comparatively less severe. Unintended reduction in weight at the beginning is a frequent occurrence. Symptoms can be present for about 6 months after which there may be a steady improvement over 2-3 years. Some experiments indicate that immunosuppressant therapy can be helpful, especially in early treatment. For instance, pulse corticosteroid shows fast pain relief and rapid motor recuperation. The treatment is with less adverse events and does not induce hyperglycemia (Al-Rubeaan *et al.*, 2015). It was found in a study that 7 out of 8 people who took a 2-hour glucose tolerance test had an increased glucose level in their serum in the deficient glucose tolerance range. They also had normal levels of fasting blood glucose and HbA1c. This shows that sporadic hyperglycemia and glucose tolerance deficiency may be implicated in cases where diabetes is absent.

2.4.6.12 Mononeuritis multiplex and multifocal neuropathy

Mononeuropathy multiplex is a condition of more than two peripheral nerve trunks causing multifocal sensory motor impairment. Sensory motor impairment often occurs in collagen vascular disorders. it can also occur as a symptom of nonsystemic vasculitis in peripheral nerves. Mononeuritis multiplex unlike diabetic amyotrophy shows muscle extremity activity, stepwise progression and sensory-motor multifocal irregularities. Diabetic amyotrophy however, proceeds quickly involving predominant proximal and striking motor. Inflammatory vasculopathy with

multifocal axon loss are fundamental in the pathogenesis of mononeuritis multiplex in diabetes. In one study 3 out of 4 patients on corticosteroids and chlorambucil demonstrated dramatic and faster amelioration.

2.4.6.13 Cranial neuropathies

Cranial neuropathy is frequently seen as oculomotor nerve palsy in diabetes. It usually produces pupil-sparing third nerve palsy. Some cranial nerves have been implicated. These neuropathies occurring suddenly have fine course with steady sudden recovery.

2.4.6.14 Other non-compressive focal neuropathies

Truncal neuropathy shows sudden initiation of pain in the truncal nerve. Truncal neuropathy may occur independently or cofound with diabetic amyotrophy or mononeuritis multiplex. Unintended reduction in weight can take place. Ulnar, peroneal, femoral, and sciatic mononeuropathies may occur acutely or sub acutely. Pathophysiological of these conditions are not completely understood but may be connected to axon loss, possibly because of loss of oxygen to nerves. Treatment of these focal neuropathies includes pain control and regulation of hyperglycemia; the results are usually good (Woolf. *et al.*, 1999).

2.4.6.15 CIDP in diabetes

CIDP is often seen in patients exhibiting sub-acute and severe motor neuropathy with proximal frailty not like the "typical" diabetic neuropathy which usually is sensory and length dependent. Diabetes in CIDP and vice versa are very common in the general population. The frequent occurrence of nerve conduction irregularities together with axon loss and demyelination have made the diagnoses of CIDP in diabetics difficult. This difference is essential, as patient's response to

immune treatments can be comparable to patients with CIDP but not diabetic (Callaghan *et al.*, 2012)

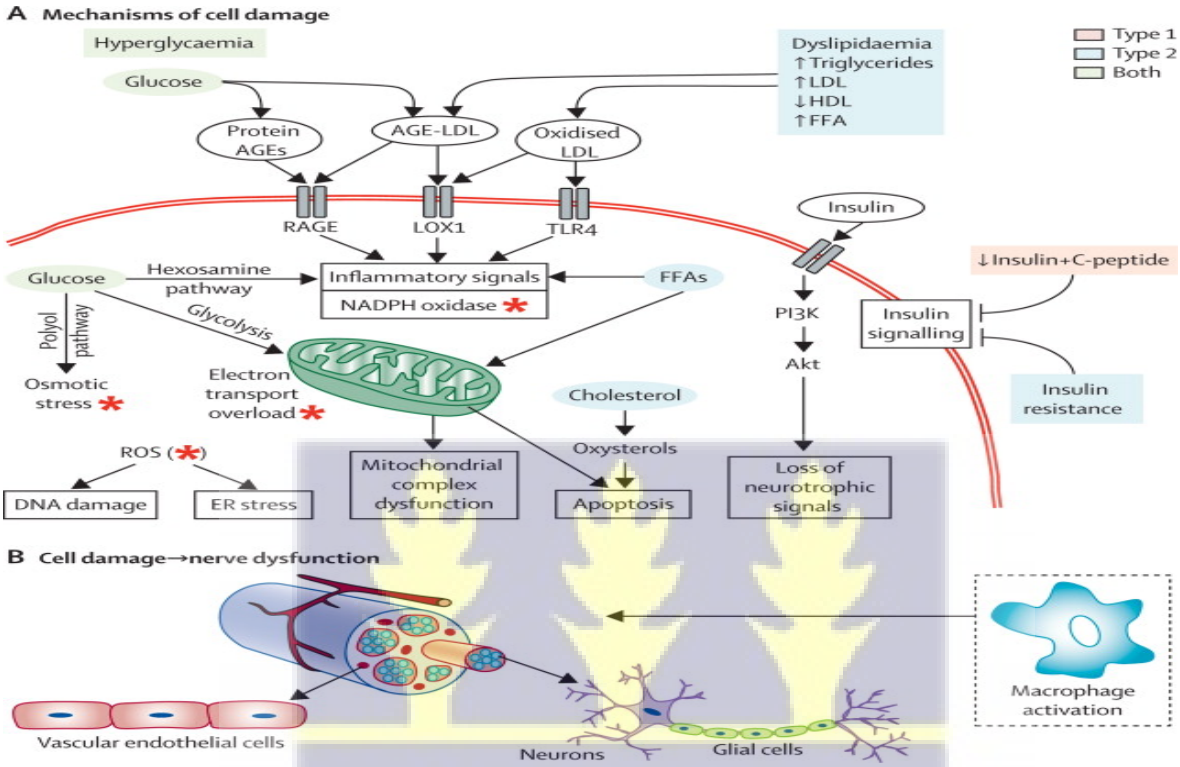


Figure 2.6: Mechanisms of diabetic neuropathy (https://ars.els-cdn.com/content/image/1-s2.0-S1474442212700650-gr3.jpg)

Figure 2.6 illustrates the MOA of diabetic neuropathy. Briefly hyperglycaemia can cause neuropathy via osmotic stress resulting in ROS hence causing cell damage including nerve cells.

2.5 Pharmacotherapy of Diabetes and Paclitaxel-induced Neuropathic Pain

2.5.1 Antidepressants

Antidepressant also produce analgesic effects because of their influence on modulatory inhibitory controls. Several antidepressants are used to treat NP. Such antidepressants include tricyclic antidepressants (TCAs) and monoamine oxidase inhibitors (MAOI). Duloxetine through numerous studies have been recommended to treat pain from diabetic neuropathy. Its use is not only limited to diabetic neuropathy but also to other forms of NP including paclitaxel-induced neuropathy (Attal & Bouhassira, 2015). Some common adverse effects attributed mostly to TCA are somnolence, constipation, dry mouth and dizziness. In addition, tertiary amine TCA produce sedation, postural hypotension and anticholinergic effects. Duloxetine on the other hand is more associated with nausea (Attal *et al.*, 2016).

2.5.2 Tramadol

Tramadol which is of the opioid group possess analgesic property. They exert their influence by inhibiting serotonin and norepinephrine reuptake like tapentadol. These drugs are often misused and abused with a high probability of dependency compared to other opiods. Tramadol has been efficiently used to treat peripheral NP. However, it is contraindicated in geriatrics because it may cause confusion and in patients already on antidepressants because they may cause serotonin syndrome (Vinik *et al.*, 2014).

2.5.3 Cannabinoids

It has been demonstrated that treatment with Oro-mucosal cannabinoids (2.7 mg delta-9-tetrahydrocannabinol and 2.5 mg cannabidiol) is effective against pain associated with multiple sclerosis and also against refractory peripheral NP with allodynia (Rog *et al.*, 2007; Attal *et al.*,

2016). However, negative results have been observed in some studies (Attal *et al.*, 2016). Side effects such as dizziness, fatigue and nausea are known to occur. Cannabis in some situations can increase the severity of psychiatric conditions therefore, it is contraindicated in patients suffering from psychiatric disorders (Zilliox, 2017).

2.5.4 Opioids

Opioids such as oxycodone and morphine are averagely effective against peripheral Neuropathic pain (Eisenberg *et al.*, 2005). Side effect from these opioids include constipation, nausea and somnolence. After several years of administering high doses of opioids there is a high probability of its abuse. It may also cause cognitive deficits, modification of endocrine and immunologic functions (Provenzano & Viscusi, 2014; Pujol *et al.*, 2018). Some other factors that call for attention to increased prescription of opioid are overdose mortality, morbidity and misuse (Zilliox, 2017). Daily dose of morphine equivalence must be carefully evaluated especially when patients require higher daily doses.

2.5.5 Antiepileptic drugs

It has been found in animal studies that the analgesic effect of pregabalin and gabapentin are linked with decreased central sensitization and nociceptive transmission through alpha-2- delta subunit of calcium channels (Luo, 2002; Wijayasinghe *et al.*, 2016). They are highly effective against peripheral or central NP even though negative results have been detected in some recent studies (Simpson *et al.*, 2010). Extended release of gabapentin formulations (enacarbil) produced similar results as gabapentin used in clinical trials, which was administered twice daily. Similar activity in comparison with TCA has been stated with frequent side effects such as somnolence and weight gain (Attal *et al.*, 2016). Anti-epileptics like pregabalin and gabapentin have not been consistent with its treatment against Neuropathic pain. However, that has not been the case with carbamazepine

in trigeminal neuralgia (Attal *et al.*, 2016).

2.6 Use of Plants as Analgesic Agents in the Management of Pain

Plants are good sources of bioactive substances which are useful in treatment and management of different diseases. These bioactive compounds accounts for the reason why 90% of drugs are from plant sources (Vittalrao *et al.*, 2011). Medications from plant sources are noted to be relatively safer (Sengupta *et al.*, 2012). Plants contain several compounds which have been found to possess analgesic properties and with lower toxic profiles and higher therapeutic effects (Sengupta *et al.*, 2012). Examples of these natural compounds are tannins, alkaloids, flavonoids and saponins, (Jain *et al.*, 2011; Ranjan *et al.*, 2010; Zulfiker *et al.*, 2010). Plants like *Manilkara zapota*, *Scoparia dulcis* L, *Ficus racemose*, *Allium stracheyi*, *Murraya paniculate* have analgesic effect and are traditionally used to treat pain (Borikar *et al.*, 2009; Jain *et al.*, 2011).

2.6.1 *Annona muricata*

Annona muricata also called soursop, 'Graviola' in Portuguese and 'Guana´bana' in Latin America. In Ghana, Akans call it "Aluguintuguin". Taxonomically it is classified; Kingdom: Plantae, Division: Angiosperms Magnoliophyta: Class: Magnolids, Order: Magnoliales, Family: Annonaceae, Genus: Annona, Species: Muricata.

2.6.1.1 Botanical description of *A. muricata*

Annona muricata is always green and has a height of about 5–8 m, 15-83 cm in diameter with low branches, features an open, roundish canopy with large, glossy, dark green leaves (Coria-Tellez *et al.*, 2018). It has large fruits, heart-shaped and green in color. The diameter of the fruits varies between 15 and 20 cm. It flowers and fruits all year round but has some definite seasons during which it blossoms (Pinto *et al.*, 2005). The plant commonly located in West Africa, Central and

South America and also in Southeast Asia. The plant grows in regions with altitudes less than 1200 m above sea level, temperatures between 25 and 28°C, relative humidity between 60 and 80% and annual rainfall above 1500 mm. Its fruit is edible and dark green in color, the average weight is 4 kg in some countries (Pinto *et al.*, 2005) but in México (Arenas- Ocampo *et al.*, 2003), Venezuela (Ojeda *et al.*, 2007) and Nicaragua (Coria-Tellez *et al.*, 2018). The seeds in each fruit are about 55-170 when fresh and become light brown when dry (Awan *et al.*, 1980). It has white and creamy flesh with pleasant fragrance and flavor (Pinto *et al.*, 2005).

Figure 2.7: Diagram showing leaves and fruits of Annona muricata

2.6.1.2 Geographical distribution

The exact origin *A. muricata* is unknown (Vijayameena *et al.*, 2013) but may be native to Central America and Northern South America (Hanelt *et al.*, 2001). It is cultivated in the warm lowlands of the Caribbean, East Africa, West Africa, temperate and tropical Asia, North America and South-

Central Pacific Islands (USDA ARS-2014). It been stated as exotic to the Caribbean and West Indies including Puerto Rico (Acevedo-Rodríguez. P. & Strong, 2012). It has also been listed as native to Puerto Rico by United States Department of Agriculture-Agricultural Research Service (USDA ARS-2014).

2.6.1.3 Phytochemical constituents

Studies have shown that *A. muricata* is made of 212 bioactive compounds. The notable ones are acetogenins, alkaloids, and phenols. Most of the phytochemicals have been isolated from organic extracts but lately the direction has been on aqueous extracts (Orwa *et al.*, 2009) also mentioned the presence of carbohydrates and essential oils.

2.6.1.3.1 Acetogenins

Studies have shown that there are about 120 acetogenins found in the ethanolic, methanolic or other organic extracts of different parts of the plant (Beg *et al.,* 2011). Pulp and fruit peel acetogenins can be identified by their aliphatic chains of about thirty-five to thirty-eight carbon atoms with γ-lactone α ring attached (Alali *et al.*, 1999). A large portion of acetogenins in *A. muricata* has tetrahydrofuran ring with two THF rings. Acetogenins are found predominantly in leaves and fruits (Liaw & Wiener, 2002; Höllerhage *et al.*, 2009), some are in seeds (Wu *et al.*, 1995). Acetogenins are the main bioactive compounds found in Annonaceae family (Alali *et al.*, 1999). It has been demonstrated through research that acetogenins are more cytotoxic comparted to alkaloids and rotenone (synthetic cytotoxic compound). Acetogenins and alkaloids are much researched on because of their therapeutic and neurotoxic effects (Mohanty *et al.*, 2008).

2.6.1.3.2 Alkaloids

Alkaloids such as reticuline and coreximine are found in *A. muricata* (Nawwar *et al.*, 2012). The leaves of *Annona muricata* has the highest alkaloid content (Matsushige, 2012). Alkaloids have been identified its stems, roots (Nawwar *et al.*, 2012) and fruits (Hasrat *et al.*, 1997). They are usually in the form of isoquinoline, aporphine and protoberberine (Mohanty *et al.*, 2008). *In vitro* analysis exhibit that the alkaloids have high affinity for 5-HT1A receptors and are involved in the synthesis of dopamine (Hasrat *et al.*, 1997). This suggests that they may be responsible for the plant's antidepressant-like effect (Hasrat *et al.*, 1997). It has also been proven to have cytotoxic impact (Matsushige, 2012). Some alkaloids may have neurotoxic activity which may cause them to evoke neuronal death via apoptosis (Mohanty *et al.*, 2008).

2.6.1.3.3 Phenolic compound

A. muricata has about thirty-seven phenolic compounds with the essential ones being, quercetin (Nawwar *et al.*, 2012), gallic acid, flavonoids and lipophilic compounds (Correa *et al.*, 2012). Asare and his team in 2014 suggested that there are differences in the quantity of total phenols extracted when using organic or aqueous extracts. This is vital reason been that habitual uses of medicinal plants are in aqueous infusion coupled with the fact that most phenols are known to be water soluble. Phenolic compounds are believed to be responsible for plant free radical scavenging activity (Asare *et al.*, 2015).

2.6.1.3.4 Other compounds

The leaves, seeds and pulp of *A. muricata* contains vitamins and carotenoids (Correa *et al.*, 2012; Vijayameena *et al.*, 2013). Amide N-p-coumaroyl tyramine and cyclopeptides seen in seeds possesses anti-inflammatory and anti-cancer activities (Wu *et al.*, 1995; Wélé *et al.*, 2004). The pulp of *A. muricata* has 37 volatile compounds. Almost all of these volatile compounds are aromatic and

aliphatic esters (Cheong *et al.*, 2011). Some essential oils that are predominantly sesquiterpenes derivates from the leaves produce cytotoxic effect against human breast carcinoma cell line (MCF-7) (Jaramillo *et al.*, 2000; Kossouoh *et al.*, 2007).

2.7 Ethnobotanical uses of *Annona muricata*

The plant has been screened and found to possess numerous medicinal properties (Badrie & Schauss, 2009; Gbaguidi *et al.*, 2017). Traditionally, the bark, root, seed or leaf has different applications. In Indonesia (Boyom *et al.*, 2011a), the Caribbean islands (Boulogne *et al.*, 2011) and South Pacific countries, its leaves treat skin ailments (Cano & Volpato, 2004). In countries like Mauritius, New Guinea and Ecuador the leaves are applied topically (Sreekeesoon & Mahomoodally, 2014). In Brazil Martinique, Mexico and Nicaragua the leave stock is used as an analgesic (Coria-Tellez *et al.*, 2018), while in several countries like Benin, the Caribbean, Cuba (Joyeux *et al.*, 1995) and México (Waizel-Bucay & Waizel-Haiat, 2009) they are useful in curing colds, flu and asthma. In Malaysia leaves of *A. muricata* are efficient in healing infections of cutaneous external and internal parasites. In Ghana, the plants is decocted into a mixture and used to bath for therapeutic purposes (Asare *et al.*, 2015). The fruit is not only used as food but used to treat diarrhea, heart and liver pathologies as well. In South America, it is used in the management of Intestinal parasites (Badrie & Schauss, 2009). Recently, the leaves of *A. muricata* is being used in the treatment of hypertension (Badrie & Schauss, 2009), diabetes and cancer (Alonso-Castro *et al.*, 2011). The unripe fruit, seeds, leaves and roots of the plant are being used as pesticides, insecticides and insect repellents (Isman & Akhtar, 2007). The plant has also been recommended for the control lepidopteran larvae, aphids and thrips (Falistocco & Ferradini, 2020).

2.7.1 Pharmacological studies of *Annona muricata*

2.7.1.1 Cytotoxic effect

The anticancer property have been mentioned by Asare and others and it is believed to influence the cytotoxic property of *A. muricata* (Asare *et al.*, 2015). Some extracts have shown toxic action to cancer cell lines (Betancur-Galvis *et al.*, 1999). It has been found through research that a concentration of 1.6 µg/ml and 50µg/ml hydroethanolic leaf extract of the plant increases the viability of non-cancerous cells. However, 100µg/ml of the hydroethanolic leaf extract left their viability unchanged. In tumor cells, the healing period of the plant increased whiles in rodents the healing time of induced wound is reduced (Paarakh *et al.*, 2009). Hydroethanolic extract of the plant influence results obtained; organic solvents such as pentanolic and ethanolic, were the most effective plant extracts against cancer cells grown *in vitro*. The effect is higher in pentanolic and ethanolic extracts than that of the aqueous extract (Ménan *et al.*, 2006). Extracts with LC50 less than10 µg/ml are highly cytotoxic but extract from plants with LC50 values ≤20 µg/ml can be used to treat cancer (Falistocco & Ferradini, 2020). Ethyl acetate leaf extract of the plant possess an inhibitory property against U-937 cell line. According to Osorio and his colleagues *A. muricata* extracts exhibits good cytotoxic action however, there are more plants with potent cytotoxic activity, for instance, *Thevetia ahouai* has LC50 <1µg/ml (Calderón *et al.*, 2007). Hexane leaf extract from the plant have high levels of flavonoids and much efficient in inhibiting cell proliferation relative to methanol or chloroform extracts. Moghadamtousi *et al*., 2015 has stated that the mechanism of action of plant extract shows interference of mitochondrial membrane to suppress apoptosis and cells in G0/G1 phase.

2.7.1.2 Anti-protozoal activity

The plant exhibits antiprotozoal activity against different protozoans such as genera *Plasmodium* (Boyom *et al.*, 2011a), *Leishmania* , *Biomphalaria* (Luna *et al.*, 2006) *Trypanosoma*, and *Entamoeba* (Shivas *et al.*, 2015). The antiplasmodial action is especially important because of the need to find antimalarial agents in tropical regions. Methanolic extract showed activity against the parasites in vitro even though it was not as potent as chloroquine or artemisinin (Boyom *et al.*, 2011b). The most therapeutic effect are its seed extracts as the alkaloids (Fofana *et al.*, 2013), acetogenin, anonaine, and gallic acid screened from the plant has antiplasmodial action (Yamthe *et al.*, 2015). Phenolic compounds inhibit enzymes involved in fatty acid biosynthesis in *P. falciparum* thereby disrupting their growth (Tasdemir *et al.*, 2006). In FabG, phenols such as luteolin are noncompetitive inhibitors to FabG conversely in FabZ, luteolin acts as competitive inhibitor of the substrate crotoyl-CoA (Tasdemir *et al.*, 2006).

Methanolic and ethyl acetate peel extracts of the plant has increased antileishmanial compared to Glucantime® which is an anti-protozoa commercial compound (Jaramillo *et al.*, 2000). In addition, various solvent extracts from the plant has trypanocidal effect even though it is100 times less effective compared to benznidazole (Osorio *et al.*, 2007). Plant extracts also have antiparasitic activity and it is used to treat adult forms of metazoan or helminth. For instance, extract from the plant has been used to treat *Haemonchus contortus*, which is a gastrointestinal parasite found in sheep. It demonstrated similar results as levamisole (Ferreira *et al.*, 2013).

Isoquinoline alkaloids block the antioxidant enzyme trypanothione reductase of *Leishmania* and *Trypanosoma*. The enzyme shields the parasites from reactive oxygen species produced by the host (TTempone *et al.*, 2005).

2.7.1.4 Insecticidal, larvicidal and repellent activity

Extracts from the seeds, leaves, barks, stems, roots and flowers of *A. muricata* have insecticidal action (Bobadilla *et al.*, 2005). Ethanolic extracts of the plant inhibited the larvae of insects such as *Aedes aegypti* (Bobadilla *et al.*, 2005), *Anopheles albimanus* (Thomas, 2011). It also inhibit insects that affect other plants such as *Spodoptera litura* (Leatemia & Isman, 2004), *Callosobruchus maculatus* and *Plutella xylostella* (Predes Trindade *et al.,* 2011). *S*eed extracts of the plant produce the most effective insecticidal activity (Bobadilla *et al.*, 2005; Ravaomanarivo *et al.*, 2014). This can be attributed to the presence of alkaloids, fatty acids and acetogenins. Current technologies such as nanotechnology is being used to study a safer, effective, cheap and easy to use insecticides. Consequently, nanoparticles from aqueous crude extract of the plant exhibited toxicity against larvae of *Aedes aegypti* (Santhosh *et al.*, 2015).

2.7.1.5 Antioxidant activity

The plant have been screened for several antioxidant activity (Coria-Tellez *et al.*, 2018). Its antioxidant activity has been demonstrated in fresh and frozen pulp as well as in juice and fresh or dried leaves. The pulp antioxidant activity quantified by 2,2'-azino-bis 3-ethylbenzothiazoline-6-sulfonic acid (ABTS), Fluorescence Recovery After Photobleaching (FRAP) and Oxygen Radical Absorbance Capacity (ORAC) shows antioxidant compounds from the plant are mainly lipophilic and the mechanism of action is by donating hydrogen (Correa Ribeiro *et al.*, 2016). The solvent used for the extraction influences the constituents of the extract. This can be seen in the different antioxidant property of methanolic, ethanolic, n-butanolic and aqueous leaf extracts as shown by 2,2-diphenyl-1-picrylhydrazyl (DPPH). The aqueous fresh leaf extract of the plant was found to be a thousand times less active compared to the commercial butylated hydroxytoluene antioxidant

(Alitonou *et al.*, 2013). According to George *et al.*(2012) there is a strong correlation between the antioxidant action and the total polyphenol content of the plant.

2.7.1.6 Antibacterial and antiviral properties

The plant has antibacterial influence on both gram-positive and gram-negative bacteria, similar to that of streptomycin. Its antibacterial action is based on the type of solvent. It has been shown that both ethanolic and methanolic extracts possess antibacterial effect against *Staphylococus aureus,* however, the aqueous extract of the peel does not produce such effect. Combining the ethanolic extract of the plant with commercial antibiotics potentiates antibiotic activity which is effective against multidrug-resistant strains of *E. coli* and *S. aureus* (Bento, 2013; Vieira *et al.*, 2010). Ethanolic extracts of the stem and bark of the plant produces antiviral effect *in vitro* against *Herpes simplex* virus (Padma *et al.*, 1998). Antimicrobial action of the plant is contributed to by the presence of flavonoids, steroids and alkaloids. Its mechanism of action is therefore concerted effort by these compounds. Some alkaloids bind to DNA of microorganisms and block transcription (Mohanty *et al.*, 2008). Flavonoids work by restricting both cytoplasmic membrane activity and replication. For example, quercetin binds GyrB subunit of the DNA of *E. coli.* This prevents the activity of ATPase enzyme. Phenylphenol has been found to bind hydrogen or membrane protein with microbial enzymes and prevents changes in activities (Olugbuyiro *et al.*, 2018).

2.7.1.7 Hypoglycemic activity

The leaf extract of the plant possess hypoglycemic effect (Adewole & Caxton-Martins, 2006). It was found in a study that the aqueous and methanolic leaf extracts of the plant lowers blood glucose in diabetic rats which were induced by streptozotocin (STZ). Histological and biochemical analysis of their pancreas did not show any change in pancreatic β-cells of rats that was used as control unlike

in diabetic rats. Increased antioxidant activity as well as insulin content were seen in pancreatic serum. After a month of treating rats daily with the plant extract it was found that glucose levels, body weight, food intake, water intake, lipid profile and oxidative defense was returning to standard levels. This is an indication that the deleterious effect of STZ can be ameliorated by the antioxidant and protective effect of pancreatic β-cells (Adewole & Caxton-Martins, 2006). The positive correlation of tannins, flavonoids and triterpenoids and their inhibition of α-glucosidase were also mentioned. Flavonoids exert their effect by inhibiting α-glucosidase through hydroxylation bonding and substitution at β ring. This decreases carbohydrate hydrolysis, absorption of glucose and prevents carbohydrates from metabolizing into glucose (Sovia *et al.*, 2016).

2.7.1.8 Anti-cancer activity

Ethyl acetate extract of the plant reduced the development of cancer in azoxymethane-induced colonic deviating crypt foci in rats (Moghadamtousi *et al.*, 2015). The plant extract increases Bax protein, decreases PCNA and Bcl-2 proteins and replaces antioxidant enzymes. High levels of reactive oxygen species lead to malondialdehyde (MDA) formation in patients with colorectal cancer however treatment with the plant extract decreased the generation of MDA in colon tissues, demonstrating protection against oxidative stress (Moghadamtousi *et al.*, 2015; Pedras *et al.*).

2.7.1.9 Anti-tumorigenic activity

Anti-tumor activity of the plant as well some isolated acetogenins from it has been researched on (Asare *et al.*, 2015). The ethanolic leaves extract displayed higher anti-tumor effect in mice than curcumin, a known cancer reducing agent. It was found to be a protective agent against morphological modification in generated colorectal carcinogenesis and in biochemical analysis. Aqueous extract of the leaf and stem of the plant exerted anti-metastatic and anti-tumorigenic

activity on pancreatic tumors in murine models. Treating with fruit extract of the plant for 5 weeks was found to be effective against tumors in the breast (Dai *et al.*, 2012). The mode of action is through the blockage of pathways controlling metastasis, metabolism and induction of necrosis (Rosdi *et al.*, 2015). It was also shown that bullatacin lowered tumor induction about 300 folds in rodents which is higher than the commercial drug Taxol (Amoateng *et al.*, 2017). Annonacin also decreased tumor size in mice and the results are similar to cisplatin and adriamycin (Wang *et al.*, 2009). It has been revealed by Yang *et al.* (2015) that crude leaf extract of the plant demonstrated a higher *in vitro* inhibition of the proliferation of prostate cancer and more efficient in preventing the growth of relative to extracts rich in flavonoid. This is an indication enhanced activity of crude extract can be attributed to the synergistic interplay between flavonoids and acetogenins.

2.7.1.10 Anxiolytic and anti-stress activities

The anti-stress activity of extracts from the plant can be due to the contribution of alkaloid compounds evidence of which is seen in the two isolated alkaloids (annonaine and asimilobine) which are known to possess relaxing effect. These compounds may exert their influence through the 5HT1A receptor on central nervous system. This receptor binds to serotonin which regulates emotions (Hasrat *et al.,* 2011). This action may explain the brain behind its use as a sedative.

2.7.1.11 Hypotensive activity

Leaf extract of the plant induced a dose-dependent decreasing effect on mean arterial pressure in normal blood pressure (Nwokocha *et al.*, 2012). The mechanism of action of this aqueous extract of the plant does not involve endothelial or nitric oxide-dependent pathways. The extracts reduced blood pressure by inhibition of calcium ion channel, and is shown by the potential to loosen contractions caused by increased potassium ions (Nwokocha *et al.*, 2012). The presence of alkaloids

and some essential oils like β- caryophyllene may be responsible for the hypotensive activity of the extract (Nwokocha *et al.*, 2012).

2.7.1.12 Wound healing

Extracts of the leaves and bark have shown wound healing characteristics (Padma *et al.*, 2020). Wound healing consists of coagulation, inflammation, proliferation and maturation. The extract increases the process at some of these stages. During inflammation the expression of heat shock proteins (Hsp70) are essential for successful treatment because of their cell proliferative activity. It caused an increase in Hsp70 within tissues of wounds. Inflammatory cells produce high levels of cytokines and free radicals leading to the production of lipid peroxidation in wounds. Tissues treated with the plant extract enhanced the effect of catalase, glutathione and superoxide dismutase shielding tissues against oxidative harm and promoting the process of wound healing. The plant extract also decreased MDA, which causes dysfunctional endothelial cells, fibroblast and collagen metabolism important for wound healing. During maturation, collagen accumulate, and fibroblast proliferation transpired. Extracts from the plant increases the accumulation of collagen fibers in the wound as observed in histological examinations (Moghadamtousi *et al.*, 2015).

2.7.1.13. Analgesic properties.

Scientific studies have showed anti-inflammatory and analgesic properties in the leaves of *Annona muricata* plant (Roslida *et al.*, 2010; De Sousa *et al.*, 2010). The antinociceptive and anti-inflammatory activities of the ethanol extract of *Annona muricata* leaves in animal models have been indicated in recent studies conducted by De Sousa and colleagues in 2010.

CHAPTER 3

MATERIALS AND METHODS

3.1 Reagents/Drugs/Apparatus

The chemicals used in the experiment are morphine, (Morphine sulphate, Sterop Belgium), hot plate apparatus, Randall Sellito apparatus, ice, oral gavage, syringes, needles, paclitaxel (Intaxel® purchased from Fresinius Kabi, oncology Baddi India), STZ and pregabalin (Lyrica® purchased from Pfizer pharmaceutical, Germany)

3.2 Experimental Animals and Housing Conditions

Male ICR mice weighing between 20 and 30g and Sprague-Dawley rats weighing between 150 and 300g were obtained from and maintained at Nogouchi Memorial Institute for Medical Research (NMIMR, University of Ghana). Mice and rats were kept in separate stainless-steel cages (34 cm x 47 cm x 18 cm) in groups of five. The animals were fed with commercial pellet purchased form NMIMR and soft wood shavings used as bedding. Water was provided for them *ad libitum.* They were housed under laboratory conditions at a room temperature of 25±2 °C and at a relative humidity between 60-70% and a 12-hour light-dark cycle. All procedures and techniques in this study has been scientifically reviewed by the department of Pharmacology and Toxicology under and the Animal Experimental Department at NMIMR all under University of Ghana. All experiments and procedures were performed in accordance with the guidelines outlined by the Organisation for Economic Co-operation and Development (OECD).

3.3 Time of Experimentation

This research was carried out in the light cycle between with experimentally naïve mice and rats.

3.4 Plant Collection and Extraction

The plant was collected from Centre for Plant Medicine Research Akuapem Mampong in the Eastern part of Ghana (N 5.914752, E-0.137005). Identification and authentication of the plant was done at the Centre's herbarium where a voucher specimen was lodged **(CPMR 4896).** The plant was dried for twenty-one days in a well-ventilated room and then crushed into coarse aggregates. About 10kg of the coarse crushed leaves was soaked in 3 liters of distilled water. The mixture was boiled for one hour, allowed to cool and filtered using filter paper. The liquid sample was then freeze dried and stored under room temperature for future use.

3.5 Toxicity Studies of Annona *muricata* Extract

3.5.1 Sub-Acute Toxicity

Rats were grouped into four with 5 animals in each group. Three out of the four groups were treated with AME (100 mg/kg body weight, 300 mg/kg body weight and 1000 mg/kg body weight). The remaining group received normal saline (10 ml). For 14 days the rats were monitored daily and sacrificed on the 15th day for postmortem evaluation. Blood samples from animals was obtained by cardiac puncture and stored in an EDTA tube (1 ml) for haematological studies. Blood from the animals was analyzed using haematology analyzer (KX-2IN, Sysmex Corporation, Japan) whiles their organs and tissues of interest was stored in formalin and macroscopically examined. (Ameyaw, E. O. *et al* 2013).

3.6 Parameters Investigated

3.6.1 Clinical observations and body weights

Morbidity and mortality were observed twice daily aside the abnormal physical and behavioral examination of the animals. They were also examined based on changes to their skin, fur, autonomic

activity, eyes and mucus membrane. The autonomic activity of the animals included lacrimation, piloerection, size of pupil and unusual pattern of breathing. Furthermore, other behavioral modifications including posture, response to handling, adjustments in gaits and stereotypic activities such as extreme grooming and circling was analyzed. Intensity, duration and the onset of these modifications was recorded as exhibited by the animals. Ocular examinations on animals were conducted before the beginning of the experiments and the day before euthanasia. The body weight of animals was recorded at intervals prior to the initiation of treatment and during the investigation period.

Mean body weights of the animals and change in their mean body weight were calculated on day 0, 2, 5, 9, 14 and a day before the scheduled necropsy. (Ameyaw, E. O. *et al* 2013).

3.7 Clinical Pathology

3.7.1 Haematology

In haematology, the indices analyzed were hemoglobin concentrations, packed cell volume, platelet, red and white blood cell count, mean corpuscular volume, blood clotting time, total leukocyte count, differential leukocyte count, activated partial prothrombin time and reticulocyte time. All the various parameters analysis were done in Accra, Ghana.

3.7.2 Macroscopic and microscopic examinations

Necropsy was carried out by examining the external surface, orifices and the cranial, thoracic, abdominal and pelvic cavities and viscera. Organs from the animals were put in 10% neutral-buffered formalin. Organs such as prostate, kidney, liver, and brain were weighed. (Ameyaw, E. O. *et al* 2013).

3.7.3 Histological examination of isolated organs

The isolated hearts, livers and kidneys were preserved in 10% neutral buffered formalin solution for 7 days and washed with water. Tissues were cut with a disposable microtome blade, into approximately 3 mm thick slices. Three slices each were obtained from each of the organs under investigation. Dehydration of the organs was done using ethanol. This was followed by immersion in xylene to remove the alcohol and allow infiltration with paraffin wax. The tissues were then embedded in paraffin. Paraffin wax blocks of the tissues were prepared, and tissue blocks were cast using a molten wax dispenser, plastic cassettes and mould boxes. The tissue blocks were sectioned at 4µm, using a rotary microtome. They were then mounted onto microscopic slides and then dried overnight. The slides were later observed under a light microscope after being staining with hematoxylin and eosin (H&E) dyes. The slides were identified with codes written on the frosted sides of the slides.

3.8 Phytochemical Screening of *Annona muricata Extract*

The plant was screened for the presence of phytochemicals according to what was described by Trease and Evans (2002). The chemicals used are flavonoids (NaOH and HCl), alkaloids with Mayer's and Dragendoff`s reagents, saponins (frothing test), tannins ($FeCl_3$), glycosides (Glacial acetic acid and Fehling`s solutions A and B) cardiac glycosides (Salkowski test), anthraquinones (Borntrager`s reaction) phenols ($FeCl_3$ and K_3Fe (CN) $_6$), steroids (Chloroform, H_2SO_4) and terpenoids (Chloroform and H_2SO_4).

3.9 Primary Observation and Safety Pharmacology Assessment Using Irwin Test

Behavioral and neuroactive activity of plant extract were evaluated in accordance with the standard proposed by Irwin (1968). The mice were randomly grouped into six with each group containing 5 mice maintained and allowed to acclimatize for a week. The animals were denied food overnight,

but water was accessible *ad libitum*. They were treated orally with the plant extract in doses of 30, 100, 300, 1000, and 3000 mg/kg body weight body weight or with 10 ml of normal saline. The mice were examined at 0, 15, 30, 60, 120 and 180 min, up to 48 hours following treatment. During these times, the animals were observed based on their behavioral and physiological activities. Neurotoxicity, mortality and autonomic activities was also observed. The purpose of these tests was to determine the safety, pharmacological activity and the selection of the doses which had pharmacological effect on the experimental animals for subsequent experimental study (Ameyaw *et a*l., 2014)

3.10 Establishing the Analgesic Activity of AME Using Hot Plate Test

The procedure proposed by Eddy and Leimbach (1953) was used in this experiment but with few modifications. Mice were dropped on a hot plate (Model 7280, Ugo Basile Inc., Milan, Italy) that was heated at 52 ± 0.5 °C for some few minutes. The baseline reaction time of the animals to the hot plate was recorded. Nociceptive responses by the animals such as jumping, licking and shaking of their paws were measured as baseline reaction latency. The animals were given AME (100, 300, 1000 mg/kg body weight, *p.o*) and morphine (0.3 1, 3, 10 mg/kg body weight, *i.p*). Their reaction was observed and recorded at an interval of 30 minutes, 1 hour and 2 hours following a latency period of 30 minutes post vehicle (10 ml/kg, *p.o*) administration. To prevent injury to tissues in the foot of the animals, a 20-second cutoff reaction time was set. The percentage maximal possible effect (MPE) of each mouse was quantified as:

$$\% \text{ MPE} = \frac{\textbf{Post-treatment latency-pre-treatment latency}}{\textbf{cut-off-pre-treatment latency}} * 100$$

Area under the curve (AUC) was calculated and graphically plotted as the total nociceptive score against the dose in mg/kg body weight.

Table 3: Animal groupings and mode of drug administration

No. of groups	Animals per group	Drugs Dose	Mode of Administration
7	5	AME:10mg/kg body weight, 300mg/kg body weight 100mg/kg body weight	Oral
		Morphine:1mg/kg body weight, 3mg/kg body weight	*i.p*
		Vehicle: 10ml/kg	oral

3.11 Investigating the Effect of AME on Paclitaxel-induced Neuropathic Pain

3.11.1 Inducing peripheral neuropathy using paclitaxel

Clinical observation of the mice was conducted to rule out complications such as alopecia, diarrhoea or weight loss among others. The animals received intraperitoneal injection of paclitaxel of 2mg/kg body weight for five days during the induction experiment. Three sets of experiments were performed in order to ascertain progress of peripheral neuropathy. These include Randall Sellito, cold allodynia tail flick assay and hot plate test.

3.11.1.1 Mechanical hyperalgesia using Randall Selitto test

Hind paws of rats were shortly put inside a pressure applicator and an increasing pressure was applied to the dorsal surface of their paws until there is either a vocalization or withdrawal. The pain threshold was consequently determined by taking two separate recordings of each hind paw and calculating their average (force). (Ameyaw *et a*l., 2014; Görlitz & Frey, 1972)

3.11.1.2 Cold allodynia (cold water at 4 °C) tail flick assay

About 3 - 4 cm of the distal portion of rats' tail was placed inside cold water (<4°C) until there is a withdrawal and the duration recorded. A cut-off time of 20 s was used. (Rautenberg *et al.*, 1978).

3.11.1.3 Thermal hyperalgesia using hot plate test

The animals were gently placed on a pre-heated hot plate (55°C) and the latency of paw withdrawal was recorded using a timer. The timer was stopped the moment the rat withdraws its paw. The shaking or licking of their paws was recorded with a cut-off latency of 20 seconds.

Latencies to reaction times following treatment were recorded during the hot plate, tail flick and Randall Sellito tests. This was done on day 1, 3 and 5 following first paclitaxel administration. A cutoff reaction time of 20 seconds was employed to avert injury to foot and tail tissues. (Muthuraman *et al*., 2011; Thiagarajan *et al*., 2013).

3.11.2 Treatment with AME, pregabalin and saline on paclitaxel - Induced neuropathic ICR mice

In the evaluation of behavioural neuropathic pain, three different sets of experiment were used to study the effect of AME, pregabalin and normal saline on paclitaxel-induced peripheral neuropathy in mice. These include the following:

3.11.2.1 Cold allodynia test

The tail-immersion/ flick test was used to investigate the analgesic activity of the vehicle, AME (30, 100 and 300 mg/kg body weight) and pregabalin (10, 30, 100 mg/kg body weight) on cold allodynia (Kim *et al.*, 2005). The distal portion of the tail (3 - 4 cm) of rats was immersed in cold water at a temperature of < 4°C until the rat withdraws its tail. The duration of immersion was recorded, and a cut-off time of 20 s was set. (Rautenberg *et al*., 1978).

3.11.2.2 Mechanical hyperalgesia

Mice received AME (30, 100 and 300mg/kg body weight), pregabalin (10, 30 and100 mg/kg body weight) and normal saline (10 ml). The effect of the drugs on mice were evaluated by mechanical hyperalgesia in which Randall-Selitto paw pressure analgesimeter was used (IITC Life Science Model 2888 Woodland Hills, CA, USA) (Ameyaw *et al.*, 2013). The dorsal surface of the hind paw of rats was subjected to increasing pressure through a pressure applicator (cut-off of 200 g) until the

rat withdrew its paws or vocalization was heard. Pain threshold was determined by taking the average of two recordings for each hind paw. (Ameyaw *et a*l., 2014; Görlitz & Frey, 1972)

3.11.2.3 Thermal hyperalgesia/Hot plate test

Mice were subjected to hotplate analgesimeter as previously described (Muthuraman *et al.*, 2011). They were then gently placed on a pre-heated hot plate (55°C). Using a timer, the paw withdrawal latency of mice was recorded immediately mice was placed on preheated plate. The timer was stopped when withdrawal, shaking or licking of either hind paw was seen and cut-off latency of 20 seconds. The latency was recorded before drug or extract administrations and time 30 min after drug or extract administration.

3.12 Investigating the Effect of AME on Diabetic-Induced Peripheral Neuropathy.

3.12.1 Inducing diabetic neuropathy using STZ

Streptozotocin (STZ) was freshly dissolved in 0.1mol/L Citrate buffer (pH 4.4) and intraperitoneally injected into animals at a dose of 55 mg/kg body weight. 120mg/kg body weight of nicotinamide was injected after 30 minutes of STZ administration to induce type-2 diabetes. Seven days after STZ injection coupled with 120mg/km body weight of nicotinamide blood glucose was measured using blood from their veins at the tail (OneTouch select plus, China). Rats that had 11mmol/l or more blood glucose levels were used for this study. After the induction of diabetes, baseline measurements for hot plate, Randal Sellito and cold allodynia test were performed and used as an indication for neuropathic pain assessment. (Junod. *et al.*, 1967)

3.12.2 Extract/drug treatment of streptozotocin-induced neuropathic pain

After neuropathic pain was induced with streptozotocin (STZ), AME (30, 100 and 300 mg/kg body weight body weight, *p.o*), PGB (10, 30 and 100 mg/kg body weight, *p.o*) and vehicle (normal saline) was administered daily in rats for 7 days.

3.13 Statistical Analysis

Data was presented as mean ± standard error mean (S.E.M.). The time-course curves were analysed with two-way analysis of variance (ANOVA) and a post-hoc Bonferroni test. Total nociceptive score was done for each treatment and calculated in arbitrary unit as area under the curve (AUC). Analysis on the differences in AUCs was done using one-way ANOVA. Further comparisons were made between vehicle- and drug-treated groups using Bonferroni post-hoc test. Iterative computer least squares method was used to determine doses for 50% of the maximal effect (ED_{50}) for each drug. (De Sousa, O. V. *et al* 2010)

CHAPTER FOUR

RESULTS

4.1 Toxicity

4.1.1 Sub-acute toxicity

4.1.1.1 Mortality, body weights and clinical observations

Before the scheduled necropsy, no death was recorded in any of the groups during the 14-day period. There were also no dose-dependent side effects observed during the physical and behavioral examinations in the animals. In addition, there was no significant increase or decrease in body weight of the groups treated with AME compared to the control group. However, there was an increase in body weight in animals treated with AME at 300 and 1000 mg/kg body weight. The body weight was statistically significant (P=0.0417*). Necropsy evaluation did not indicate any gross pathological abnormalities in the rats compared to the control there was no significant change in absolute and relative organ weights in mice treated with AME (Table 4.6). This demonstrates that AME had no significant adverse effects on organ weight.

Table 4.1: The effects of AME on the relative weights of major organs (g) isolated from rats in a 14-day sub-acute toxicity study

Organ	Vehicle	AME 100mg/kg body weight	AME 300mg/kg body weight	AME 1000mg/kg body weight	P value
Prostate	0.03493± 0.001294	0.03597± 0.002292	0.03162± 0.001740	0.03393± 0.001829	0.4006

Brian	0.007832± 0.0004366	0.007521± 0.0003645	0.007253± 0.0003542	0.007816± 0.0003074	0.6510
Kidney	0.003211± 0.0001503	0.003327± 0.0001747	0.002978± 9.447e-005	0.003186± 0.0002339	0.5545
Lungs	0.006486± 0.0004460	0.006347± 0.0005107	0.006092± 0.0004915	0.006703± 0.0004656	0.8346
Heart	0.003601± 0.0001398	0.003966± 0.0002523	0.003487± 0.0002689	0.003523± 0.0003133	0.5317
Liver	0.02874± 0.0008232	0.02920± 0.001687	0.02749± 0.001757	0.02801± 0.002004	0.8830

4.1.1.2 Haematology

Haematology parameters investigated indicated no significant dose-related adverse effects in the groups studied compared to the control (Table 4.2). These results show that treating rats with AME at doses up to 1000 mg/kg body weight per day does not negatively affect their haematological indices.

Table 4.2: Haematological analysis of AME (100, 300 and 1000 mg/kg body weight) after a 14-day observation period

Day	Vehicle	AME 100mg/kg body weight	AME 300mg/kg body weight	AME 1000mg/kg body weight	P values

WBC (th/cmm)	6.824 ± 1.165	6.708 ± 0.1745	5.168 ± 1.526	7.960 ± 0.7928	0.3380
LYM ($\times 10^{12}$/L)	44.9 7± 1.770	41.76 ± 0.7448	41.36 ± 0.7185	47.12 ± 5.829	0.5195
MON ($\times 10^{12}$/L)	5.048 ± 0.9301	6.452 ± 0.8482	6.652 ± 1.886	5.620 ± 0.9165	0.7745
NEU ($\times 10^{12}$/L)	24.13 ± 8.364	23.01 ± 7.573	37.21 ± 7.082	16.49 ± 6.233	0.2811
EOS ($\times 10^{12}$/L)	4.920 ± 0.7889	5.504 ± 0.6986	4.260 ± 0.1860	4.444 ± 0.4096	0.4437
LYM (%)	1.993 ± 0.6076	2.558 ± 0.7820	1.934 ± 0.2050	1.848 ± 0.2252	0.7646
MON (%)	0.2904 ± 0.2002	0.6902 ± 0.2450	0.6902 ± 0.2450	0.6900 ± 0.4000	0.6881
NEU (%)	2.290 ± 0.4328	1.827 ± 0.3711	1.368 ± 0.1150	1.622 ± 0.2777	0.2616
EOS (%)	0.5872 ± 0.1135	0.4814± 0.08749	0.5956 ± 0.1287	0.7458 ± 0.1077	0.4304
RBC, (mi/cmm)	7.340 ± 0.6889	8.000 ± 0.1225	8.680 ± 0.3992	8.260 ± 0.2015	0.1825
HGB (gm/dl)	14.30 ± 0.8809	15.10 ± 0.9397	15.70 ± 0.8228	14.60 ± 0.8849	0.6994
HCT (%)	47.54 ± 1.423	46.30 ± 1.544	45.90 ± 1.927	42.50 ± 1.554	0.1944
MCV (fL)	54.20 ± 1.123	52.62 ± 0.4212	53.22 ± 0.3760	53.20 ± 0.3674	0.4175
MCH (pg/rbc)	17.94 ± 0.3970	19.50 ± 0.9165	19.74 ± 0.8256	19.78 ± 0.8139	0.3059
MCHC (g/dl)	34.12 ± 0.7677	34.50 ± 0.7225	33.86 ± 0.6720	33.44 ± 0.6400	0.7529
RDW (%)	0.8180 ± 0.1820	1.016± 0.01600	1.216 ± 0.2160	0.9760 ± 0.0240	0.2994
RDW SD (%)	33.08 ± 0.7473	32.40 ± 0.2449	32.88 ± 0.5713	31.96 ± 0.3600	0.4448
PLT, (th/cumm)	835.6 ± 11.81	830.4 ± 8.606	830.0 ± 8.735	831.6 ± 7.928	0.9729

MPV, (fL)	5.600 ± 0.2000	6.200 ± 0.3000	6.200 ± 0.3000	6.300 ± 0.2449	0.2618
PDWV,	14.90 ± 0.4313	14.20 ± 0.8198	13.80 ± 0.7694	13.32 ± 0.5851	0.4195
PCT (%)	0.3960 ± 0.04966	0.3900 ±0.04970	0.4320 ± 0.05426	0.3340 ±0.09331	0.7580

Data is presented as Mean± SEM. WBC= White Hemoglobin, HCT= Hematocrit, MCV= Mean corpuscular volume, MCH= Mean corpuscular hemoglobin, MCHC= Mean corpuscular hemoglobin concentration, PLT= Platelet. LYM = lymphocytes. Values obtained were not significantly different from the control (One-way ANOVA follow by Dunnet's post hoc test). Blood cells, RBC= Red blood cells, HBG= Hemoglobin, HCT= Hematocrit, MCV= Mean corpuscular volume, MCH= Mean corpuscular Hemoglobin, MCHC= Mean corpuscular hemoglobin concentration, PLT= Platelet. LYM = lymphocytes. Values obtained were not significantly different from the control (One-way ANOVA follow by Dunnet's post hoc test).

4.1.1.3 Clinical pathology

4.1.1.3.1 Macroscopic Examinations

There were no dose-related macroscopic findings at the scheduled necropsy after daily spontaneous and/or related in nature, but not to the treatment. These results suggest that administration of AME at dose levels up to 1000 mg/kg body weight to rats for 14 days has no adverse macroscopic effects.

Table 4.3: The effects of AME (100, 300 and 1000 mg/kg body weight) on the change in body weights

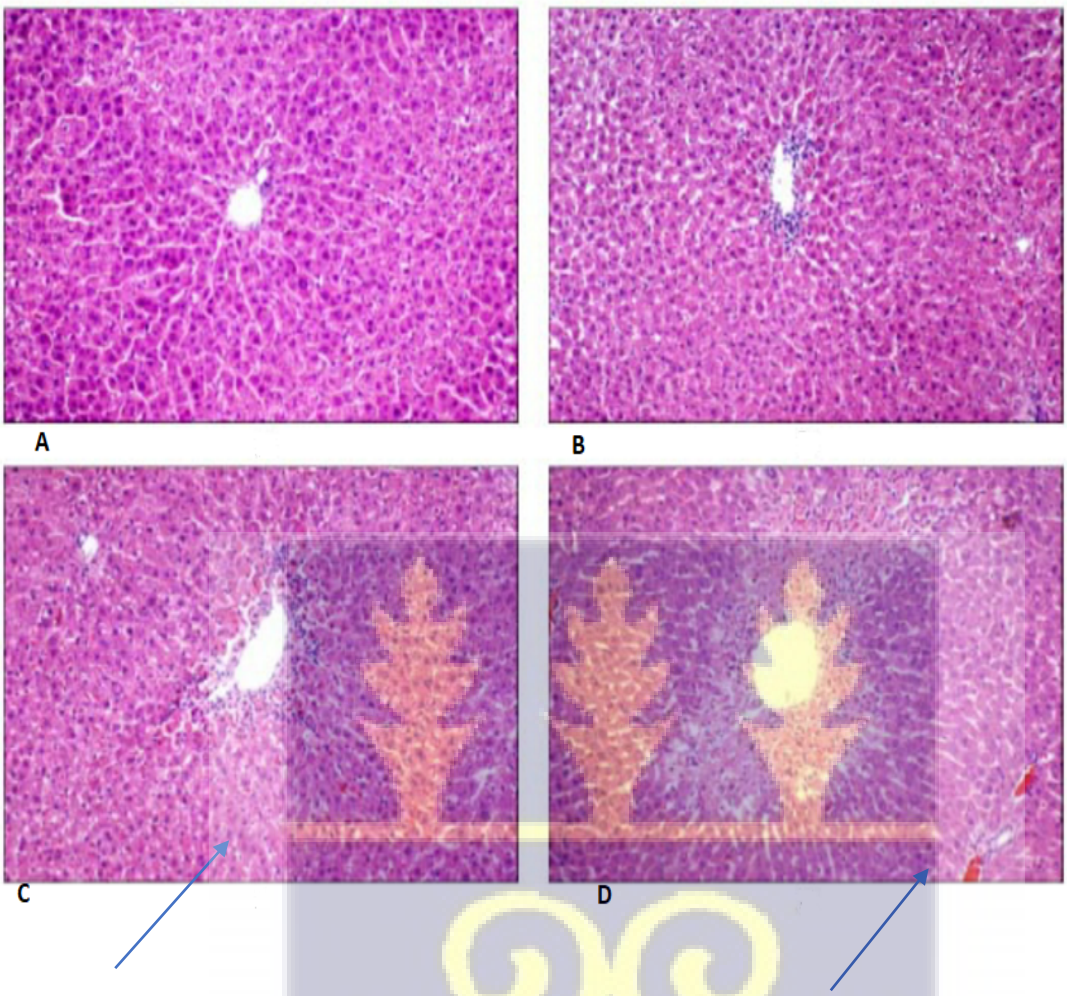
	Vehicle	AME 100mg/kg body weight	AME 300mg/kg body weight	AME 1000mg/kg body weight	P value
0	183.6 ± 10.67	199.2 ± 6.711	179.2 ± 19.73	187.2 ± 1.855	0.6656
2	195.2 ± 12.94	218.4± 4.167	209.2 ± 10.09	186.8 ± 1.855	0.0771
5	174.8 ± 5.389	208.0 ±7.797	221.6 ±18.89*	194.8 ± 3.720	0.0417
9	203.2 ± 12.72	219.2 ±8.913	221.6 ±10.53	202.4 ± 2.713	0.3555
14	205.0 ± 11.40	217.6 ±9.579	220.4± 9.368	9.2± 8.237	0.6583

4.1.1.3.2 Microscopic Examinations

Histological studies on the various organs isolated from the animals are as follows:

4.1.1.3.2.1 Liver

Histopathological studies on the liver showed regular hepatocytes and no ballooning or giant cell formation. The portal tracts in the liver were intact even though in some instances there was mild chronic inflammation in their lymphocytes and eosinophils. There was no significant cholestasis, portal- or central-to-portal fibrosis. These histological results were not different from the control group (Fig 4.1A - Fig 4.1D).



Micrograph of Liver (x10)

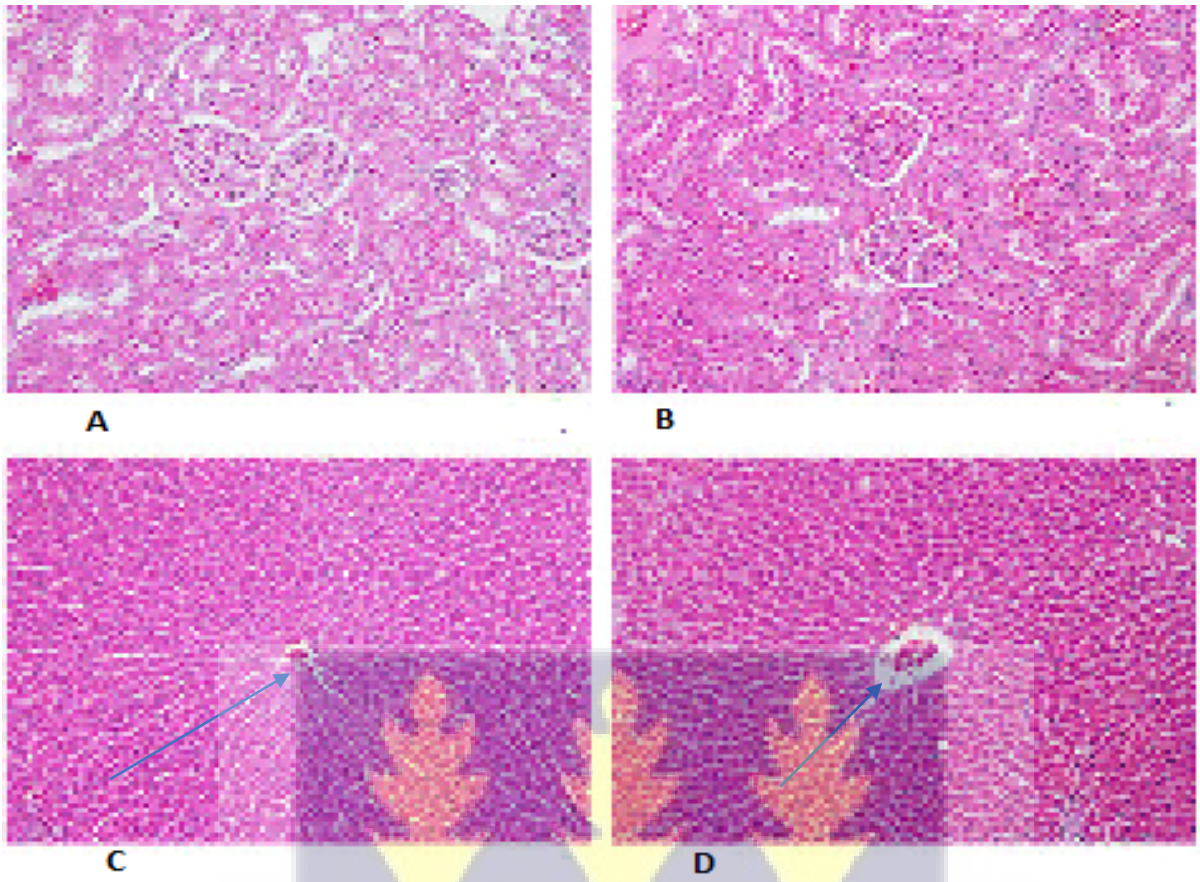
Figure 4.1: Photomicrographs of the livers isolated from of Sprague-Dawley rats after AME treatment at various doses. (A) control group showed normal hepatocytes, nuclei, sinusoids and central vein; (B) 100 mg/kg body weight AME treatment showed hepatic tissue with normal hepatocytes, nuclei, sinusoids and central vein; (C) 300 mg/kg body weight AME treatment showed normal hepatocytes with nuclei, central vein and portal vein with slight dilatation of sinusoids (D) 1000 mg/kg body weight AME treatment showed normal hepatocytes with nuclei, central vein and portal vein. Sinusoids appear slightly dilated, H and E staining.

Objective magnification: ×10

4.1.1.3.2.2 Kidney

Histological studies on the kidney showed regular glomeruli. Furthermore, there was no sclerosis, mesangial cell proliferations, basal membrane activity and any inflammatory activity in the glomeruli. Fibrin deposition in the glomeruli was not observed. There were no significant differences in the proximal and distal tubules between the treated rats and the control. The interstitium of treated rats were normal as there was no observable fibrosis or increased inflammatory activity. Also, the vessels in the kidney were normal (Fig 4.2A - Fig 4.2D)

These results were either similar or the same to lesions recorded in healthy rats of the same age and strain like those used in this experiment. It was also observed that administering AME at different doses up to 1000 mg/kg body weight for a period of 14 days has no adverse effect on the histology of the kidneys.



Micrograph of Kidney (x 40)

Figure 4.2: Photomicrographs of the kidneys harvested from Sprague-Dawley rats after treatment with various doses of AME: (A) control group showed normal kidney tissue appearance, with normal of glomerular, urinary space, Bowman's capsule, proximal convoluted tubule and distal convoluted tubules; (B) 100 mg/kg body weight AME treatment showed normal sized glomerulus, nuclei, Bowman's capsule, distal and proximal tubules. (C) 300 mg/kg body weight AME treatment showed normal renal architecture with normal cell distribution and cellular integrity; (D) 1000 mg/kg body weight AME treatment showed normal renal architecture i.e., renal corpuscles and renal tubules H and E staining. Objective magnification: ×40 82 PT.

4.2 Phytochemical Screening of AME

Preliminary report on qualitative phytochemical screening of AME shows that it contains alkaloids, saponins, flavonoids, tannins, glycosides, triterpenoids and sterols.

4.3 Irwin Test

Mice in all groups did not show any symptom of toxicity for the 48-hour period of observation. The same report cannot be said for the initial sedation (characterized by sluggish movement) of mice at 100mg/kg body weight, 300mg/kg body weight 1000mg/kg body weight 3000mg/kg body weight body weight. Mice treated at 100mg/kg body weight, 300mg/kg body weight and 1000mg/kg body weight body weight demonstrated sniffing, analgesia and defecation There were no signs of hypothermia, hyperthermia and convulsions in all the treated groups. After 48 hours of extract treatment there was no mortality and mice were physically active at the 24th hour.

Table 4.4: Physiological and pharmacological effect of AME in Irwin test

D/T – DEAD/ TREATED

Dose (mg/kg body weight)	Mortality (d/ t)	Latency (min)	Observable physiological/ pharmacological effect
			↑ sniffing, ↑ scratching
30	0/7	60	↑↑ sniffing, chewing, ↑ scratching, ↑analgesia
100	0/7	30	↑Sedation, ↑analgesia
300	0/7	30	↑ straub tail, ↑↑ sniffing ↑↑analgesia
1000	0/7	30	↑↑ straub tail, ↑ defecation, ↑sedation
3000	0/7	30	↑ straub tail, ↑↑sedation, ↑↑analgesia,

↑ - mild; ↑↑- moderate; ↑↑ - intense

Table 4.5: The effects of AME (10, 30, 100, 300, 1000 and 3000 mg/kg body weight body weight) on relative weights of major organs (g) from the mice in a preliminary pharmacological study using Irwin test

Organ	Saline	30	100	300	1000	3000	P-value
Lung	0.04± 0.03	0.02± 0.001	0.02± 0.001	0.02± 0.001	0.02± 0.001	0.02± 0.002	0.4438
Liver	0.07± 0.003	0.1± 0.003	0.1± 0.01	0.1± 0.004	0.1± 0.01	0.1± 0.01	0.3755
Heart	0.01± 0.001	0.01± 0.001	0.01± 0.001	0.01± 0.001	0.01± 0.001	0.01± 0.001	0.5744
Spleen	0.01± 0.001	0.01± 0.001	0.01± 0.001	0.01± 0.001	0.01± 0.001	0.01± 0.001	0.4947
Right Kidney	0.01± 0.001	0.01± 0.00	0.01± 0.001	0.01± 0.001	0.01± 0.001	0.01± 0.00	0.8146
Left Kidney	0.01± 0.001	0.01± 0.001	0.01± 0.001	0.01± 0.001	0.01± 0.001	0.01± 0.001	0.2879
Brain	0.03± 0.001	0.03± 0.002	0.03± 0.003	0.03± 0.002	0.03± 0.001	0.03 ± 0.002	0.6459

4.4 Assessing the Analgesic Effect *Annona muricata* using Hot Plate Test

Overall AME significantly increased the percentage maximal possible effect (%MPE) (increased paw withdrawal latency) in the hot plate test of the AME treated rats as compared to the vehicle treated group of rats ($P= 0.0030$, $F_{(4, 20,)} =8.458$; two-way ANOVA, Figure 4.4A). The significant effect was seen for AME 300 and 1000 mg/kg body weight from 20 mins and was sustained till 180 mins. Total MPE calculated as AUC for AME showed significant dose-dependent increase (100, 300 and 1000 mg/kg body weight) ($P<0.0001$, $F_{(4, 5)} = 5.341$; one-way ANOVA, Figure 4.4B). MOR also significantly increased the %MPE (0.3-10 mg/kg body weight ($P<0.0001$, $F_{(4, 20)} = 31.35$; Figure 4.4C) in the MOR treated rats as compared to vehicle. The significant effect for MOR was seen for doses 0.3- 10.0 mg/kg body weight from 20 mins to 180 mins. Total MPE calculated as

AUC for MOR demonstrated a significant increase dose- dependently (0.3-10 mg/kg body weight ($P<0.0001$, $F_{(4,5)} = 24.63$; Figure 4.4D).

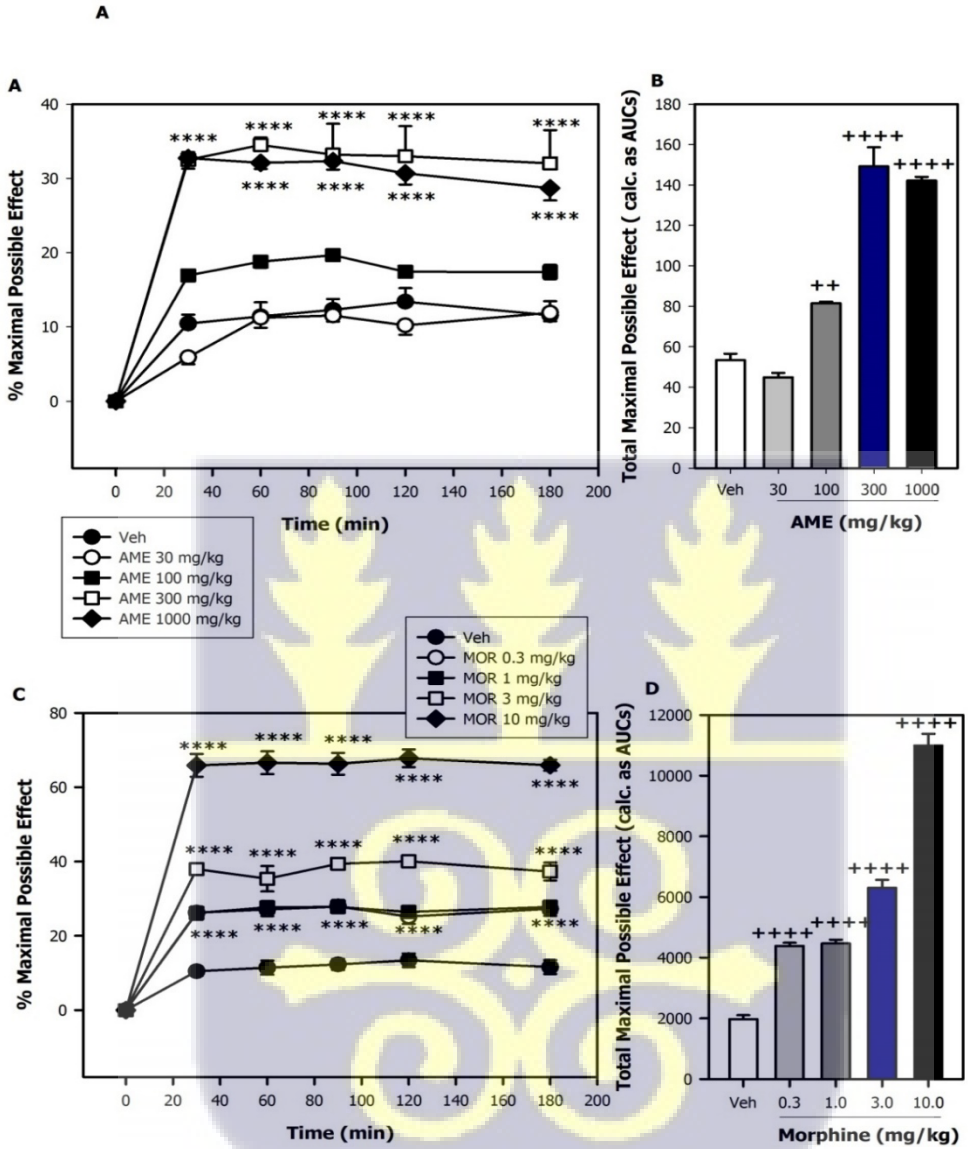


Figure 4.3: Effect exhibited by AME (30 -1000 mg/kg body weight, *p.o*), morphine (0.3-10mg/kg body weight body weight) and the Vehicle (veh) on the evaluation of analgesia using %MPE (A) and AUC (B) in the hot plate test. Data were presented as mean ± SEM (n=5). **P≤ 0.01, ****P≤ 0.001 compared with vehicle (one-way ANOVA) followed by a Dunnett's multiple comparison in a post hoc test.

Table 4.6: ED_{50} of AME and MOR in the Hot plate experiment confirming the extract's analgesic effect

Drug	Hot plate test
AME	167.4 ± 0.05
MOR	1.388 ± 0.08

4.5.1 Paclitaxel induced peripheral neuropathy experiment

4.5.1.1 inducing neuropathy using paclitaxel (cold allodynia test)

Significant difference was recorded during the tail withdrawal test on day 1 and day 5. The difference was shown by two-way ANOVA followed by a Bonferroni 's post-hoc test; (cage 1 $P<0.001$), (cage 2, $P < 0.05$), (cage 3 $P < 0.05$), (cage 4 $P<0.001$), (cage 5 $P<0.001$), (cage 6, $P > 0.05$), (cage 7, $P<0.01$) An overall significant gradual decline of latencies was observed from day 1 to day 5 (Figure 4.4).

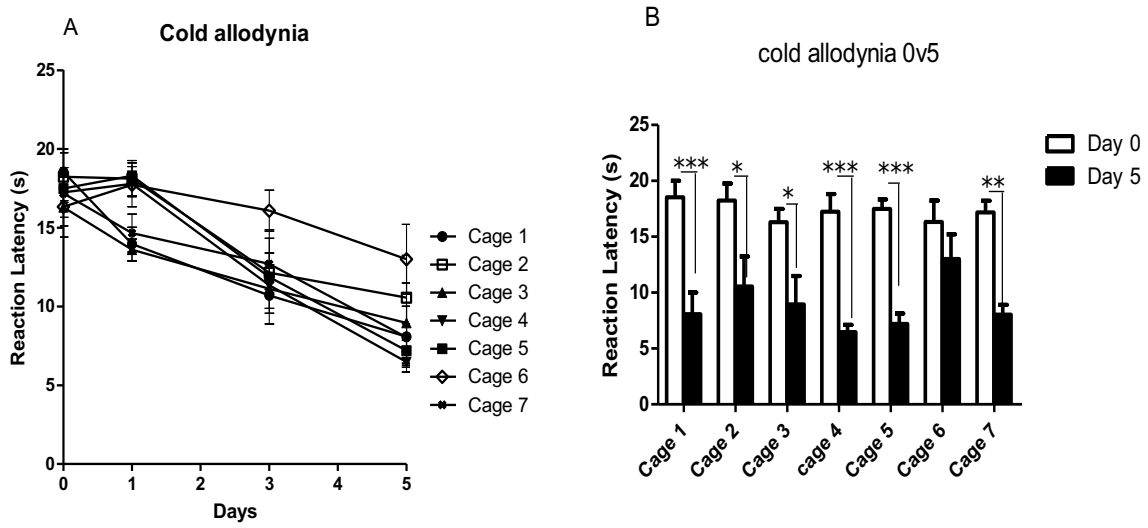


Figure 4.4: A comparison of tail withdrawal as cold allodynia) on day 1 and day 5 post paclitaxel-induced neuropathy. Data are presented as mean ± SEM (n=5). *P≤ 0.05, **P≤ 0.01, ***P≤ 0.001 compared with the vehicle (one-way ANOVA followed by a Dunnett's multiple comparison post hoc test).

4.5.1.2 Inducing neuropathy using paclitaxel (hot plate test)

An assessment of the paw withdrawal latencies to thermal pain during the induction of neuropathy using paclitaxel yielded a gradual decline in paw withdrawal latencies from day 1 to day 5. Two-way ANOVA followed by a Bonferroni's post-hoc test demonstrated significant difference between days 1 and 5) (cage 1 P<0.001), (cage 2, P<0.001) (cage 3, P<0.001), (cage 4, P<0.001) (cage 5 P<0.001), (cage 6 P<0.001), (cage 7 P<0.001). An overall significant increase in paw withdrawal latencies was observed between day 1 and day 5 (Figure 4.5).

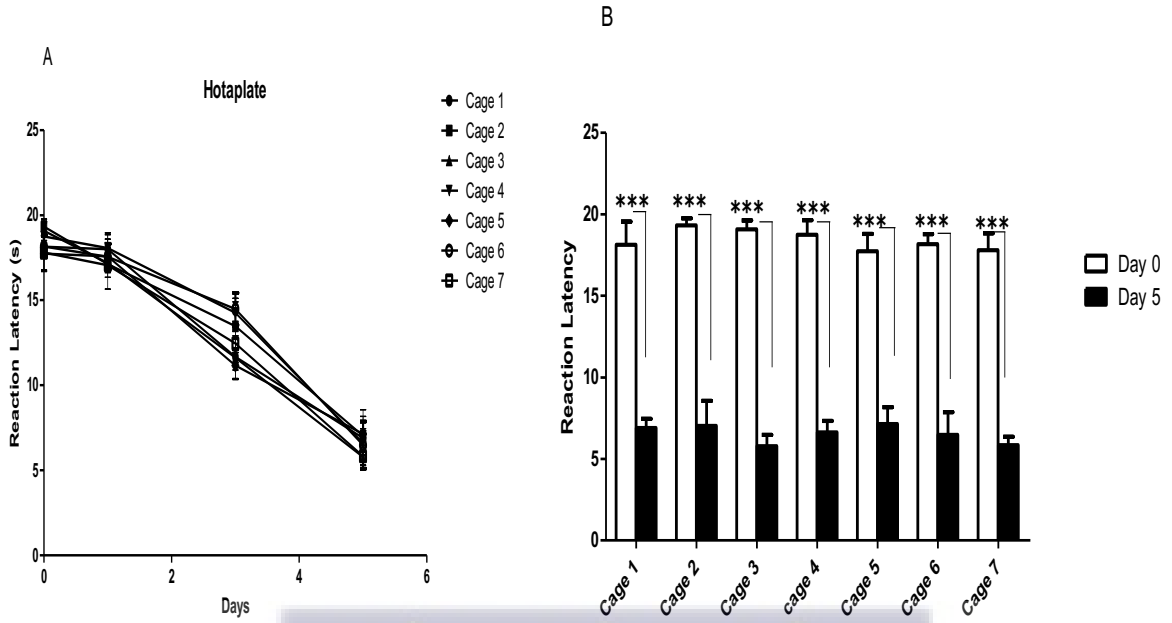


Figure 4.5: A comparison of paw withdrawal (thermal hyperalgesia) on day 1 and day 5 after paclitaxel-induced neuropathy. Data are presented as mean ± SEM (n=5). *P≤ 0.05, **P≤ 0.01, ***P≤ 0.001 compared with vehicle group (one-way ANOVA followed by a Dunnett's multiple comparison post hoc test).

4.5.1.3 Inducing neuropathy using paclitaxel (mechanical hyperalgesia test)

Reaction latencies (reduced paw withdrawal latency) of mice was significantly reduced in the Randall Sellito test from day 1- 5. Two-way ANOVA followed by Bonferroni's post-hoc test demonstrated significant difference (cage 1 P<0.001), (cage 2, P<0.001) (cage 3 P<0.01), (cage 4, P<0.01), (cage 5 P<0.001) (cage 6, P<0.001) (cage 7, P<0.001). An overall gradual decline in reaction latencies was observed from day 1 to 5.

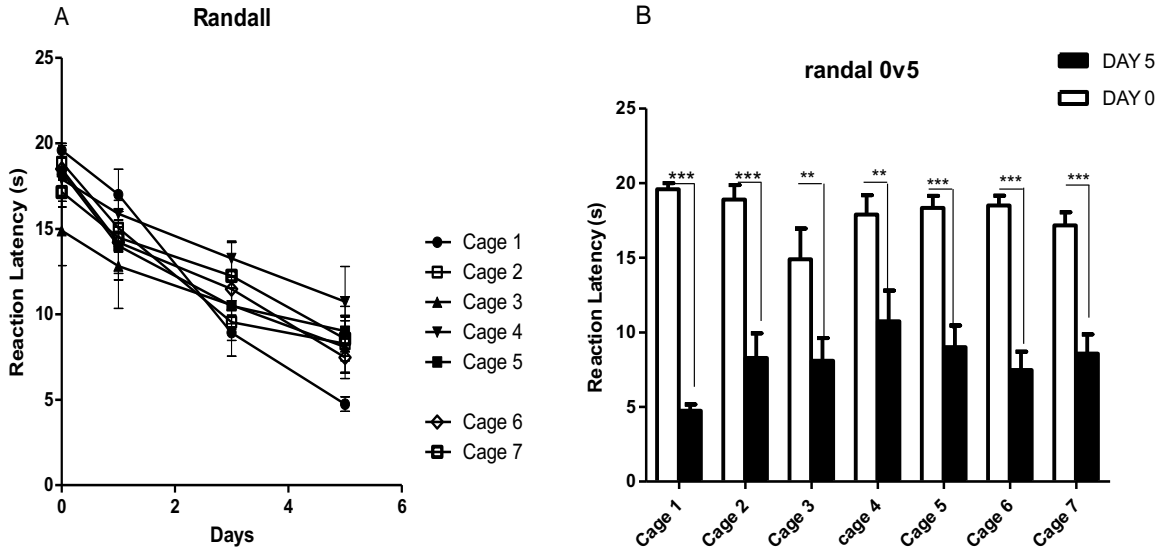


Figure 4.6: A comparison of paw withdrawals as a measure of the onset of mechanical hyperalgesia on day 1 and day 5 after paclitaxel administration. Data are presented as mean ± SEM (n=5). *P≤ 0.05, **P≤ 0.01, ***P≤ 0.001 compared with vehicle group (one-way ANOVA followed by a Dunnett's multiple comparison post hoc test).

4.5.1.4 Effects of AME on cold allodynia in PIPNE

Overall AME showed a significant increase in %MPE (increased tail withdrawal latency) in the cold allodynia test in AME treated mice as compared to the vehicle ($P<0.01$ and $P < 0.05$). The significant effect was seen in doses 100 and 300 mg/kg body weight from day 2 and this was sustained through day 5. That of 30mg/kg body weight dose exhibited significant difference from day 3 to day 5 ($P<0.01$). Total MPE calculated as AUC for AME showed significant increase (30, 100 and 300 mg/kg body weight) ($P= 0.0160$, $F_{(3, 14)} = 1.633$, One-way ANOVA Figure 4.7B). PGB (10, 30 and 100 mg/kg body weight) significantly increased %MPE (increased tail withdrawal latency) in mice from day 1- 5 dose dependently compared to the vehicle. The highest effect was seen in doses 30 and 100 mg/kg body weight ($P=0.0004$, $F_{(3, 14)} = 56.05$, two-way ANOVA, figure 4.7C) Total MPE

calculated as AUC for PGB demonstrated a significant increase (10, 30, 100 mg/kg body weight) ($p=0.0004$, $F_{(3, 14)} = 56.05$, one-way ANOVA, Figure 4.7D).

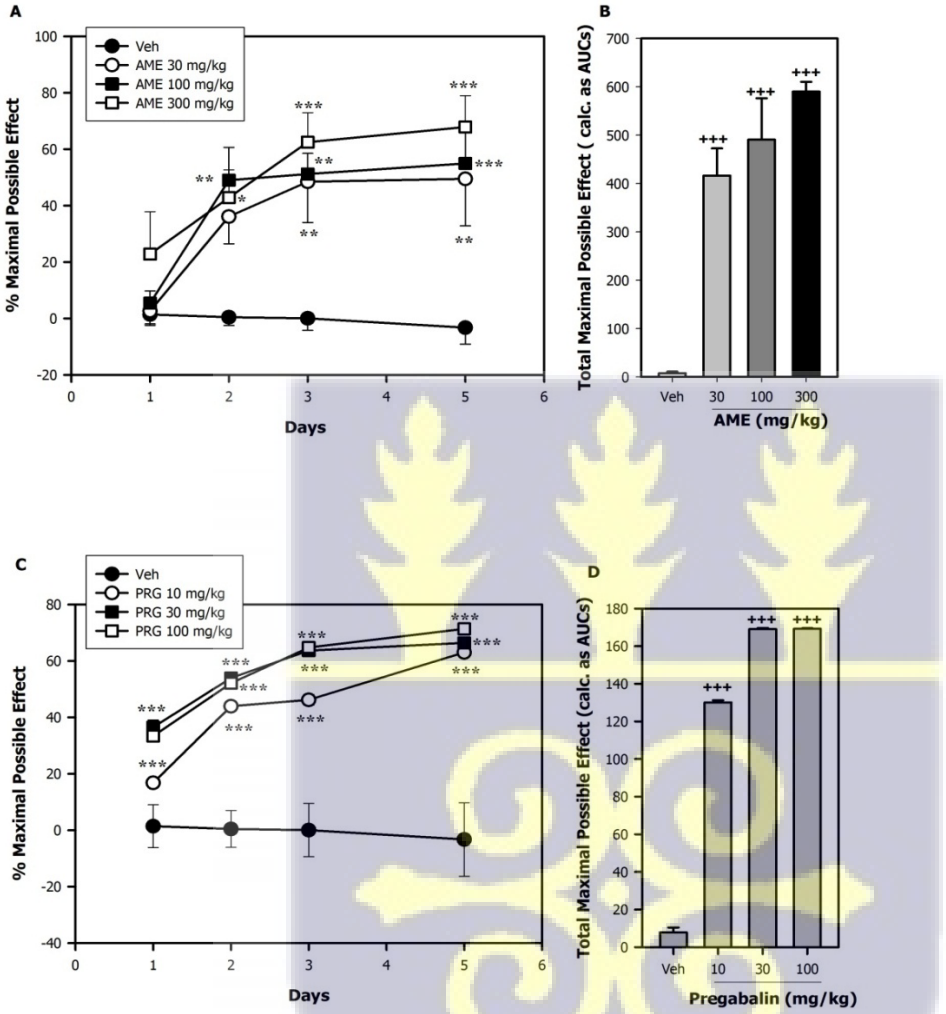


Figure 4.7: The effect of AME (30, 100, 300 mg/kg body weight, *p.o*) and PGB (10, 30, 100 mg/kg body weight, p.o) on cold allodynia in paclitaxel-induced neuropathic rats. The left panels (A and C) represent a time-course effects of AME (A) and PGB (C) after the induction of neuropathic pain. The right panels (B and D) also represent the total anti-cold allodynic effects (calculated from the AUCs) of AME (B) and PGB (D). Data are presented as mean ± SEM (n=5). *P≤ 0.05, **P≤ 0.01,

***P≤ 0.001 compared with vehicle group (one-way ANOVA followed by a Dunnett's multiple comparison post hoc test).

4.5.1.5 Effects of AME on hot plate test in PIPNE

An overall significant increase in the percentage maximal possible effect (%MPE) in the hot plate test was observed for AME treated mice animals as compared to the vehicle treated group. The significant effect was observed in all doses from days 1-5. The highest effect was seen in 100 and 300mg/kg body weight ($p < 0.0001$, $F_{(3, 14)} = 7.297$, two-way ANOVA, Fig 4.8A). Total MPE calculated as AUC for AME showed significant increase in a dose dependent manner ($P<0.001$, $F_{(3, 14)} = 56.05$, one-way ANOVA, Fig 4.8B). PGB treated mice significantly increased the %MPE in a dose dependent fashion from day 1 to 5 as compared to vehicle. The effect was highest for the 100 mg/kg body weight group ($P < 0.0001$ $F_{(3, 14)} = 6.856$, two-way ANOVA, Fig 4.8C). Total MPE calculated as AUC for PGB demonstrated a significant increase dose- dependently ($P < 0.0001$ $F_{(3, 14)} = 52.51$, one-way ANOVA, Fig 4.8D).

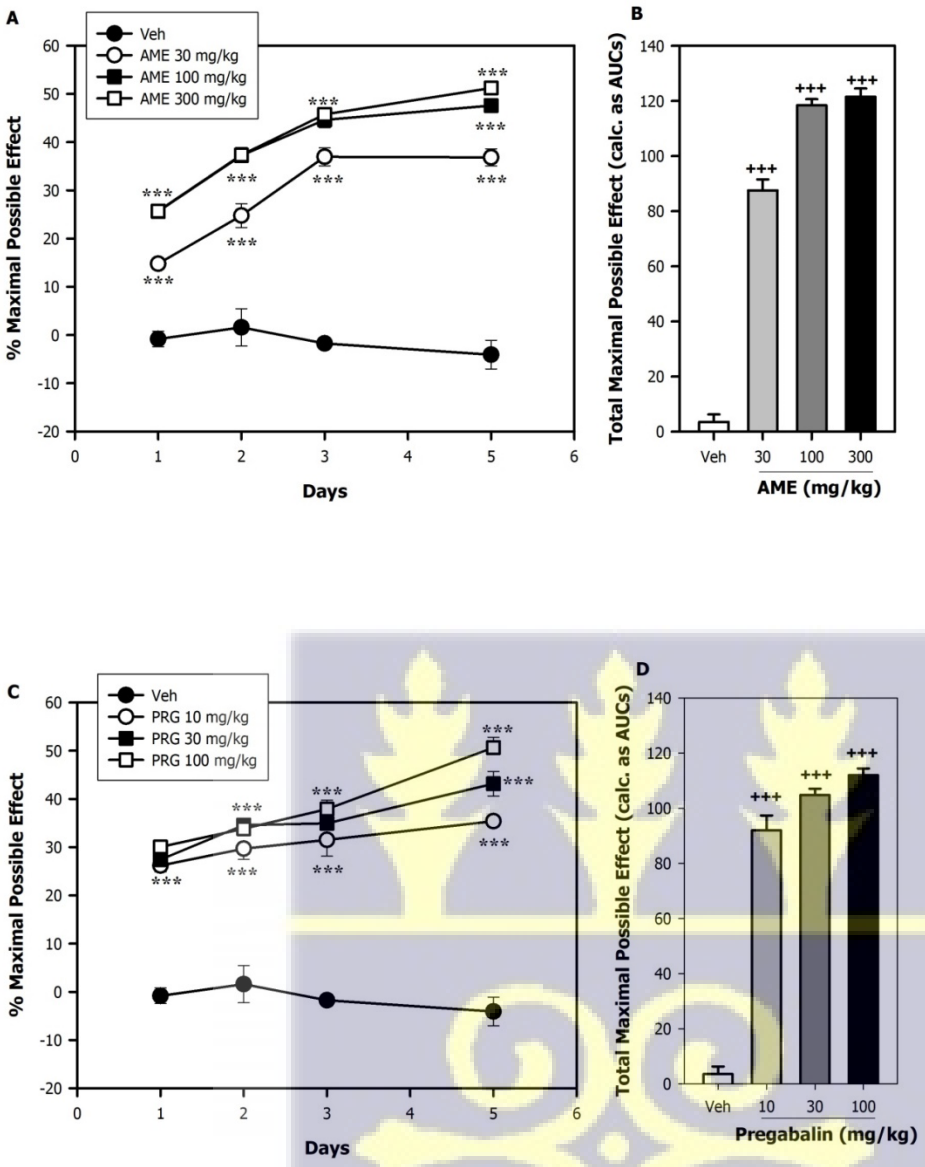


Figure 4.8: The effect of AME (30, 100, 300 mg/kg body weight, *p.o*) and PGB (10, 30, 100 mg/kg body weight, *p.o*) on thermal hyperalgesia in paclitaxel neuropathic rats. The left panels (A and C) represent a time course effects of AME (A) and PGB (C) after the induction of neuropathic pain. The right panels (B and D) also represent the total anti-nociceptive effects of AME (B) and PGB (D). Data are presented as mean ± SEM (n=5). *P≤ 0.05, **P≤ 0.01, ***P≤ 0.001 compared with vehicle group (one-way ANOVA followed by a Dunnett's multiple comparison post hoc test).

4.5.1.6 Effects of AME on mechanical hyperalgesia in PIPNE

An overall significant increase was observed dose dependently in the mechanical hyperalgesia test MPE (increased paw withdrawal) for the AME treated animals in comparison with the vehicle treated group. The significant effect was seen in the 300 mg/kg body weight on day 2 and increased through day 5. The 100 mg/kg body weight dose showed significant difference from day 3 which further increased on day 5. The 30 mg/kg body weight group did not show any significance till day 5 ($P= 0.0007$, $F_{(3, 14)} =5.990$, two-way ANOVA, Fig 4.9A). The total MPE was significant for all doses of AME. PGB also significantly and dose-dependently increased the %MPE (increased paw withdrawal) from day 1-5 ($P= 0.0008$, $F_{(3, 24)} = 16.51$, two-way ANOVA, Figure 4.9C). The total MPE was significant for all doses of PGB (10, 30 and 100 mg/kg body weight, one-way ANOVA, Figure 4.9D).

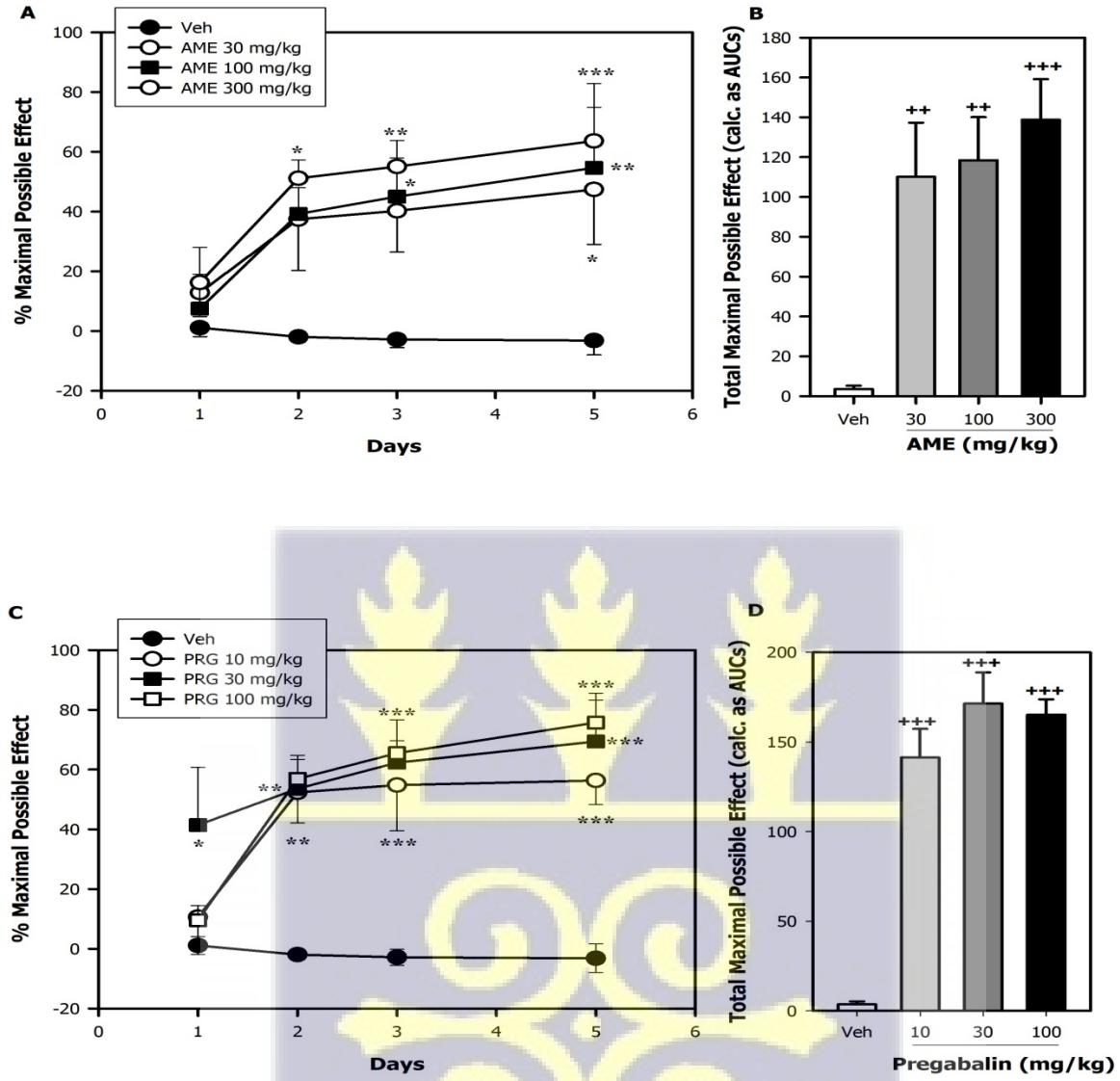


Figure 4.9: The effect of AME (30 – 300 mg/kg body weight, *p.o*) and PGB (10 – 100 mg/kg body weight, *p.o*) on mechanical hyperalgesia in paclitaxel-induced neuropathic mice. The left panels (A and C) represent a time course effects of AME (A) and PGB (C) after the induction of neuropathic pain. The right panels (B and D) also represent the total anti-nociceptive effects (calculated from the AUCs) of AME (A) and PGB (C). Data are mean ± SEM (n = 5). * P ≤ 0.05, ** P ≤ 0.01 and ***p ≤0.001 compared to vehicle group (two-way ANOVA followed by a Bonferroni's post-hoc test.

Table 4.7: ED_{50} of AME and PRG in paclitaxel-induced neuropathy experiment

Drug	Cold allodynia test	Hot plate test	Randall-Sellito test
AME	19.07 ± 0.36	16.68 ± 0.07	25.08 ± 0.78
PRG	7.557 ± 0.03	1.921± 0.29	4.55±0.25

4.5.2 Diabetic-Induced Peripheral Neuropathy

4.5.2.1 STZ-induced diabetic neuropathy (cold allodynia Test)

In the time course graph showing injection of STZ (55 mg/kg body weight) to induce peripheral diabetic neuropathy from days 1-14 showed a decrease in reaction latencies in hot plate and cold plate test. Cage 1 P<0.001, cage 2 P<0.001, cage 3, P<0.001, cage 4, P<0.001, cage 5, P<0.001 cage 6, P<0.001, cage 7, P<0.001, cage 8, P<0.001 (Figure 4.8

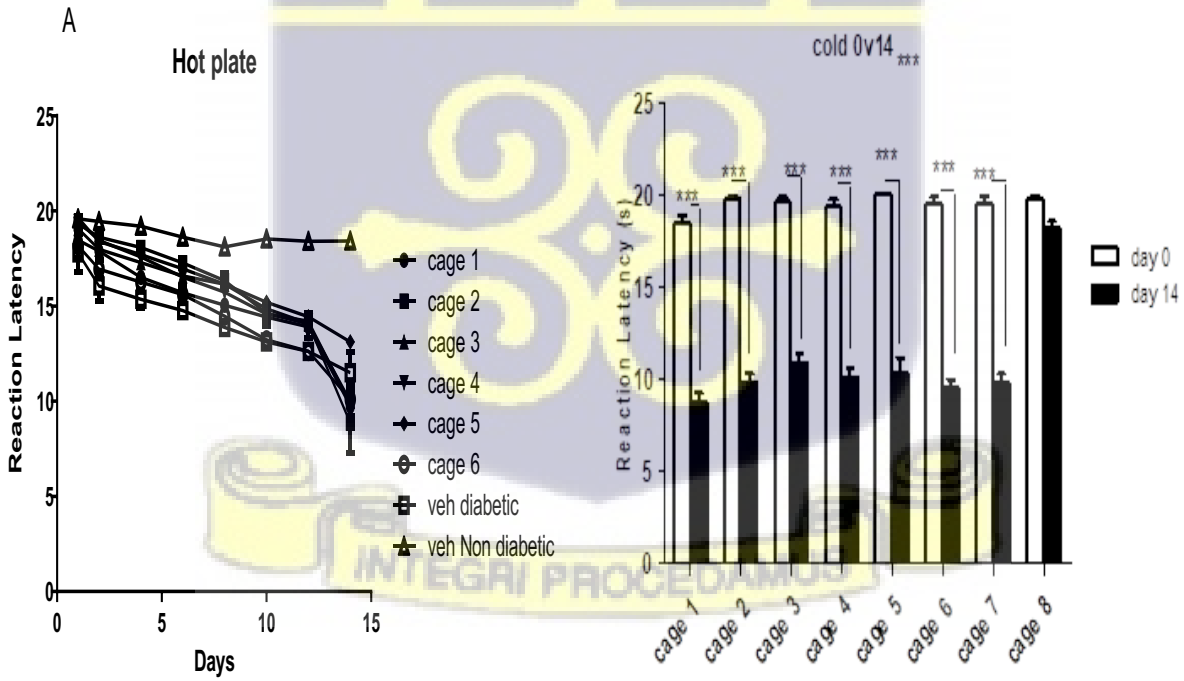


Figure 4.10: A comparison of tail withdrawal (as a measure of cold allodynia) on day 1 and day 14 after STZ-induced diabetes. Data are presented as mean ± SEM (n=5). *P≤ 0.05, **P≤ 0.01, ***P≤ 0.001 compared with vehicle group (one-way ANOVA followed by a Dunnett's multiple comparison post hoc test).

4.5.2.2 STZ-induced diabetic neuropathy (hot plate)

An assessment of the reaction latencies to thermal pain during the induction of diabetic neuropathy yielded a gradual decline in reaction latencies from day 1 to day 14. A two-way ANOVA followed by a Bonferroni's post-hoc test showed a significant difference between days 1 and 14 (cage 1 $P < 0.05$), (cage 2, $P > 0.05$) (cage 3, $P<0.001$), (cage, $P<0.001$) (cage 5 $P<0.001$) (cage 6 $P<0.001$) (cage 7 $P<0.001$). Total reaction latency showed decline in reaction latencies between day 1 and 14

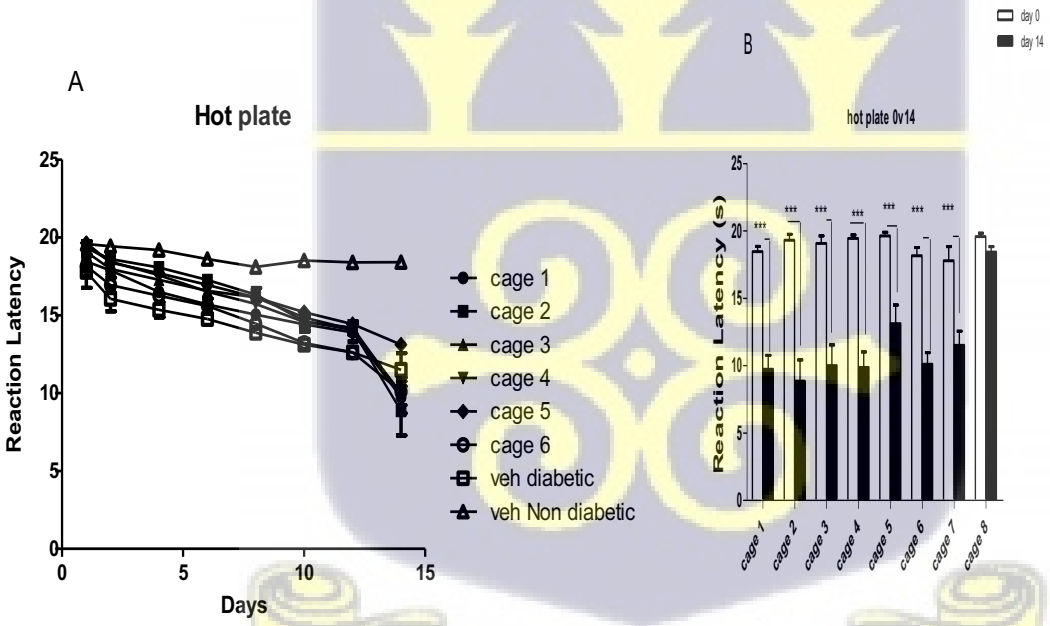


Figure 4.11: A comparison of paw withdrawals (as a measure of the onset of thermal hyperalgesia) on day 1 and day 7 after STZ administration. Data are presented as mean ± SEM (n=5). *P≤ 0.05,

**P≤ 0.01, ***P≤ 0.001 compared with vehicle group (one-way ANOVA followed by a Dunnett's multiple comparison post hoc test).

4.5.2.3 STZ-induced diabetic neuropathy (Mechanical hyperalgesia)

An assessment of the reaction latencies (paw withdrawal latencies) during the induction of diabetic neuropathy demonstrated a gradual decline in paw withdrawal latencies from day 1- 14 (cage 1 P < 0.05), (cage 2, P > 0.05) (cage 3, P<0.001), (cage 4, P<0.001) (cage 5 P<0.001) (cage 6 P<0.001) (cage 7 P<0.001). The total significant decline in paw withdrawal latencies was observed in all groups.

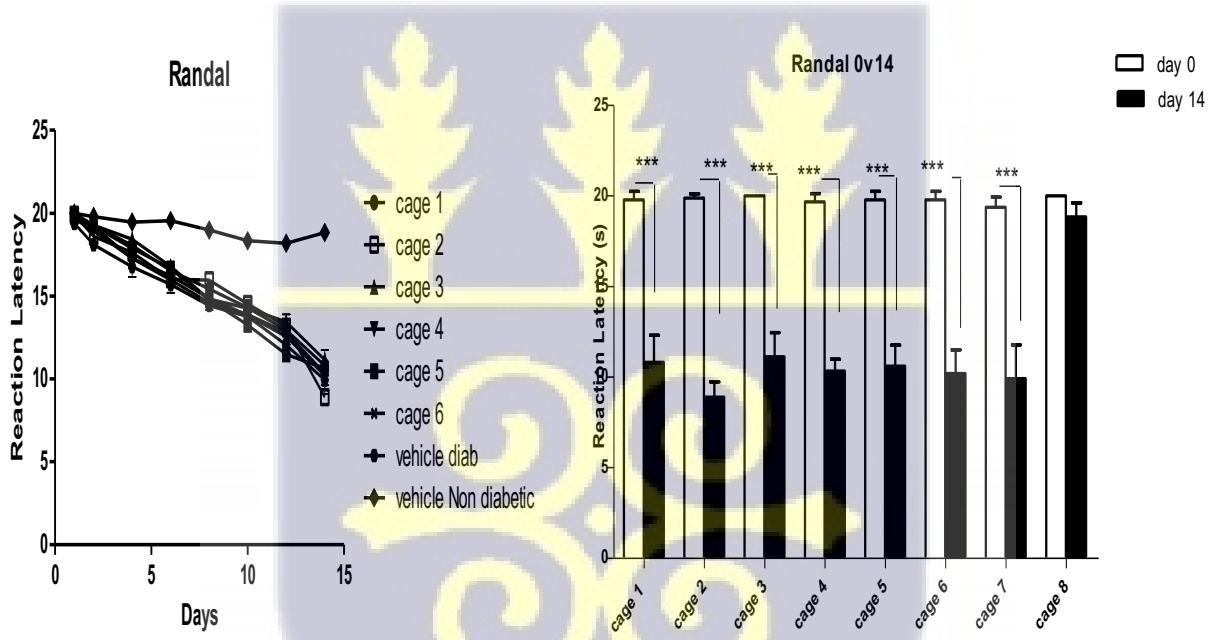


Figure 4.12: A comparison of paw withdrawals (as a measure of the onset of mechanical hyperalgesia) on day 1 and day 7 after STZ administration. Data are presented as mean ± SEM (n=5). *P≤ 0.05, **P≤ 0.01, ***P≤ 0.001 compared with vehicle group (one-way ANOVA followed by a Dunnett's multiple comparison post hoc test).

4.5.2.4 Effects of AME on Cold allodynia (DIPN)

Overall AME significantly increased %MPE (increased tail withdrawal latency) in cold allodynia test of the AME treated animals in comparison with the vehicle treated group. The effect was highest for the 300 mg/kg body weight dose. The significant difference was from day 1 to day 7. The significant difference for 30 and 100 mg.kg started from day 4 to 7 ($P< 0.0001$, $F_{(3, 14)} = 12.54$, two-way ANOVA, figure 4.13A). The total MPE produced a significant increase (increased tail withdrawal latency) ($P < 0.0001$, $F_{(3, 14)} = 200.1$, one-way ANOVA, Figure 4.13B). PGB significantly increased %MPE (increased tail withdrawal latency) from days 1-7. The highest significance was seen in the 100 mg/kg body weight dose. The effect started on day 1 through day 7. The 10 and 30 mg/kg body weight started showing significant difference from days 2 through 7 ($P<0.0001$, $F_{(3, 14)} = 11.67$, two-way ANOVA, figure 4.13C). The total MPE for PGB (increased tail withdrawal latency) in a dose dependent manner ($P< 0.0001$, $F_{(3, 14)} = 118.5$, one-way ANOVA, figure 4.13D).

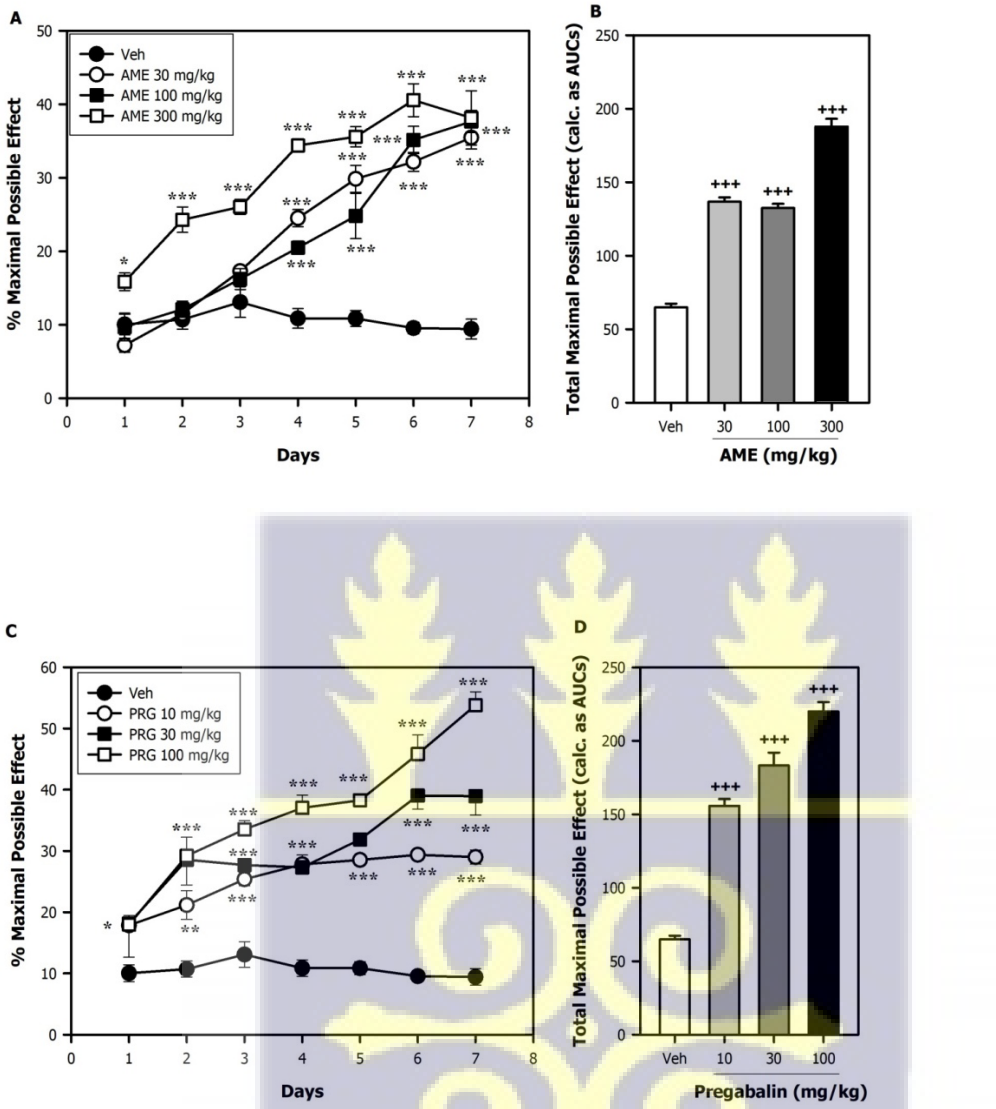


Figure 4.13: The effect of AME (30, 100, 300 mg/kg body weight, *p.o*) and PGB (10, 30, 100 mg/kg body weight, *p.o*) on cold allodynia in diabetic neuropathic rats. The left panels (A and C) represent a time-course effects of AME (A) and PGB (C) after the induction of neuropathic pain. The right panels (B and D) also represent the total anti-cold allodynic effects (calculated from the AUCs) of AME (B) and PGB (D). Data are presented as mean ± SEM (n=5). *P≤ 0.05, **P≤ 0.01, ***P≤

0.001 compared with vehicle group (one-way ANOVA followed by a Dunnett's multiple comparison post hoc test).

4.5.2.5 Effects of AME on Thermal hyperalgesia (DIPN)

AME (30, 1004, 300 mg/kg body weight) significantly increased %MPE (increased paw withdrawal latency) in thermal hyperalgesia test in comparison with the vehicle group from days 1-7. The highest effect was observed in the 300 mg/kg body weight group from day 1 to day 7. The significant difference for 30 and 100 mg/kg body weight was from day 3 to day 7 ($P< 0.0001$ $F_{(314)} = 28.31$, two-way ANOVA, figure 4.14 A). The total MPE produced a significant increase (increased paw withdrawal latency) dose-dependently ($P<0.0001$, $F_{(3, 14)} = 122.1$, one-way ANOVA, Fig 4.14 B). PGB (10, 30 and 100 mg/kg body weight) significantly increased %MPE (increased paw withdrawal latency). The significant effect was observed from day 1 to day 7 for all doses ($P<0.0001$ $F_{(314)} = 7.571$, two-way ANOVA, figure 4.14 C). The total MPE (increased paw withdrawal latency) in a dose dependent manner ($P< 0.0001$, $F_{(314)} = 195.0$, one-way ANOVA, Fig 4.14 D).

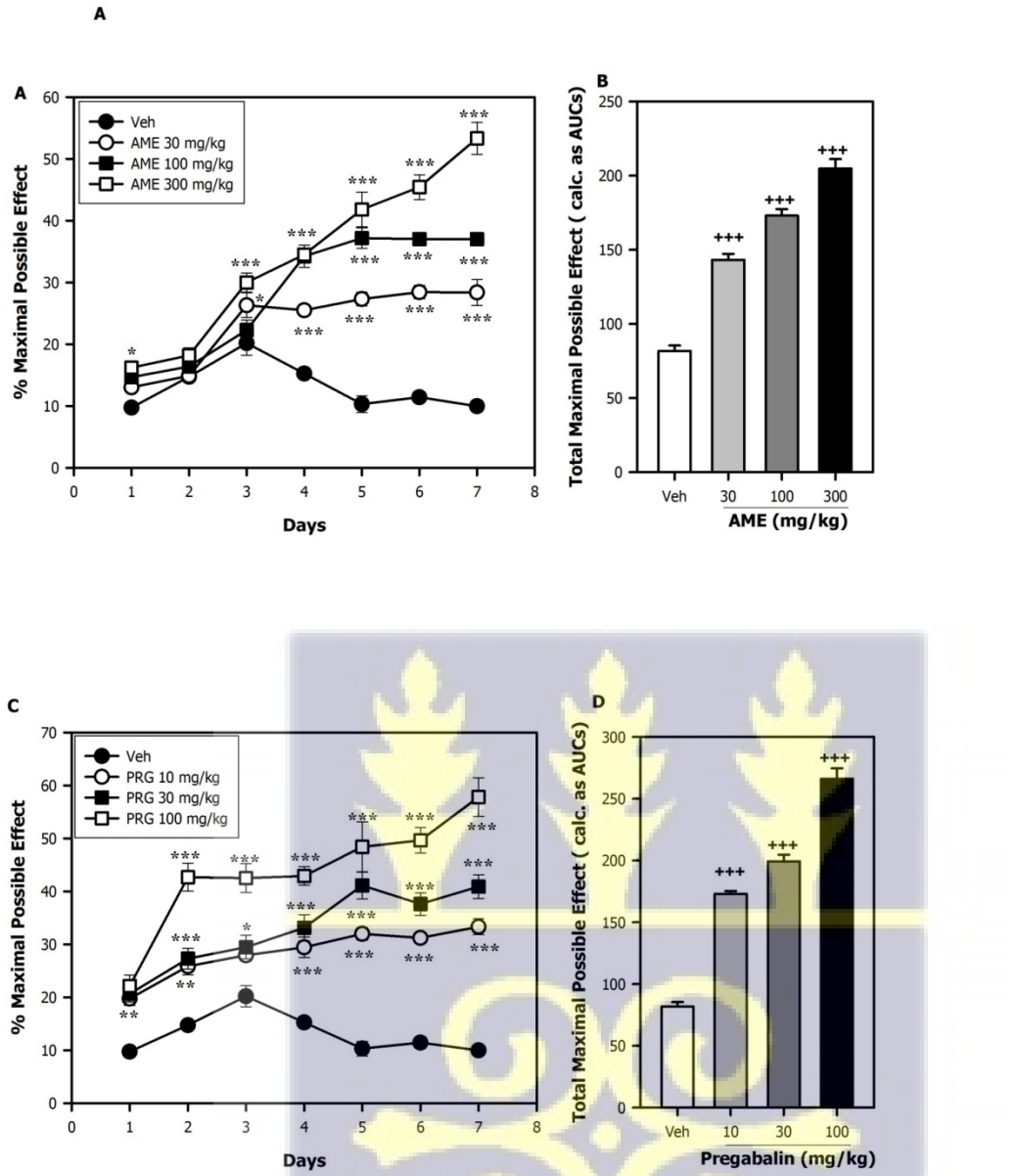


Figure 4.14: The effect of AME (30,100, 300, mg/kg body weight, *p.o*) and PGB (10, 30, 100 mg/kg body' weight, *p.o*) on thermal hyperalgesia in diabetic neuropathic rats. The left panels (A and C) represent a time course effects of AME (A) and PGB (C) after the induction of neuropathic pain. The right panels (B and D) also represent the total anti-nociceptive effects of AME (B) and PGB (D). Data are presented as mean ± SEM (n=5). *P≤ 0.05, **P≤ 0.01, ***P≤ 0.001 compared with vehicle group (one-way ANOVA followed by a Dunnett's multiple comparison post hoc test)

4.5.2.6 Effects of AME on mechanical hyperalgesia (DIPN)

Generally, AME significantly increased %MPE (increased paw withdrawal latency) in Randall-Sellito mechanical hyperalgesia test from days 2-7 in the AME treated animals as compared to the vehicle group. the 300 mg/kg body weight dose showed the highest significant difference starting from day 2 through day 7. Doses 30 and 100 mg/kg body weight showed similar significant differences when compared to vehicle from day 2 to day 7 ($P<0.0001$, $F_{(3, 14)} = 14.40$, two-way ANOVA, fig 4.16A). The total MPE produced a significant increase (increased paw withdrawal latency) dose dependently ($P<0.0001$, $F_{(3, 14)} = 277.7$, one-way ANOVA, Fig 4.15B). There was a significant increase in paw withdrawal latency (%MPE) in animals treated with PGB (10, 30 and 100 mg/kg body weight). Doses 30 and 100 mg/kg body weight showed similar significant differences from day 1 to day 7. The effect was sustained through to Day 7. Dose 10 mg/kg body weight also showed significant differences from day 1 which increased from day 2 to 7 ($P<0.0001$, $F_{(3, 14)} = 8.40$, two-way ANOVA, fig 4.15C). The total MPE (increased paw withdrawal latency) in a dose dependent manner ($P= 0.0373$, $F_{(3, 14)} = 3.585$, one-way ANOVA, Fig 4.15D).

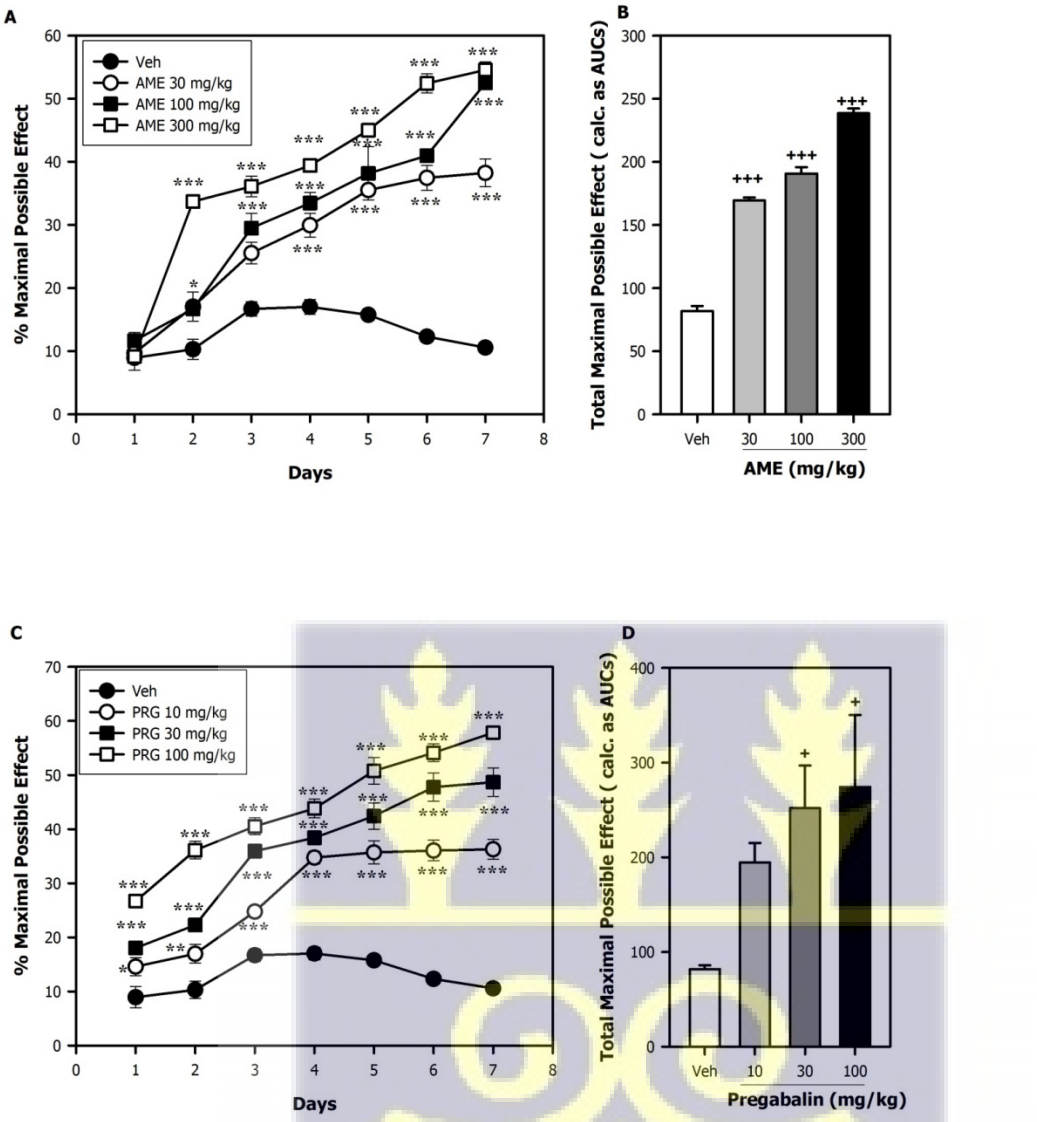


Figure 4.15: The effect of AME (30 – 300 mg/kg body weight, *p.o*) and PGB (10 – 100 mg/kg body weight, *p.o*) on mechanical hyperalgesia in diabetic neuropathic rats. The left panels (A and C) represent a time course effects of AME (A) and PGB (C) after the induction of neuropathic pain. The right panels (B and D) also represent the total anti-nociceptive effects (calculated from the AUCs) of AME (A) and PGB (C). Data are presented as mean ± SEM (n=5). *P≤ 0.05, **P≤ 0.01, ***P≤ 0.001 compared with vehicle group (one-way ANOVA followed by a Dunnett's multiple comparison post hoc test).

Table 4.8: ED_{50} of AME and PRG in STZ-induced diabetic neuropathic experiment

Drug	Cold allodynia test	Hot plate test	Randall-Sellito test
AME	39.32 ± 0.14	40.04 ± 0.06	30.06 ± 0.07
PRG	9.017 ± 0.09	16.13 ± 0.06	81.14 ± 0.51

CHAPTER FIVE

DISCUSSION, CONCLUSION AND RECOMMENDATIONS

5.1 Discussion

Traditionally, *Annona muricata* is used for the treatment of cancer, skin diseases and parasitic infections. The fruit is used for arthritic pain, neuralgia, diarrhea, diabetes, headaches and insomnia (Moghadamtousi *et al.*, 2015). Unfortunately, the effects of this plant in averting hyperalgesia and allodynia in paclitaxel-induced neuropathy and streptozotocin-induced diabetic neuropathy have not been investigated. The study therefore sought to determine the anti-hyperalgesic and anti-allodynic effect of the aqueous leave extract of this plant in STZ-induced diabetic and paclitaxel-induced peripheral neuropathy. Aside having analgesic, anti-hyperalgesic and anti-allodynia properties, it was revealed that the extract exerts some of its influence on the CNS in murine models. The extract also possesses a high safety profile which reflected in the toxicity studies conducted. The anti-allodynic and anti-hyperalgesic property of AME provide a gateway for further clinical drug trials in finding potent medication(s) with lesser adverse effect in the management of peripheral neuropathy as caused by diabetes or long standing taxanes usage. The efficacy of most phytomedicines may be attributed to its secondary metabolites. These phytochemicals present in most plants may be fundamentally involved in the pharmacological activities of plants through their influence in various pathways (Singh *et al.*, 2002; Gomes *et al.*, 2009).

Phytochemical screening of AME indicated the presence of flavonoids, triterpenoids, saponins, acetogenins, polyphenols, anthocyanins, alkaloids and tannins from the leaf extract (Awan *et al.*, 1980). Irwin test was used to examine the broad action of the agent on the central nervous system (CNS), the dose which causes death in 50% of the population which is noted as the minimum lethal dose of the test substance and the main impact on behaviour and physiological activities (Porsolt *et*

al., 2002; Roux *et al.*, 2004). Based on findings from this study, AME (30 mg/kg body weight, 100 mg/kg body weight and 300 mg/kg body weight) exhibited initial signs of sedation followed by analgesic effect on murine models. There was also an alteration to reactivity to touch and straub tail phenomenon which increased in murine models with doses of 100 mg/kg body weight and 300 mg/kg body weight. These findings from Irwin's test implicated its activity on the central nervous system and made it a potential in its it effects with respect to other CNS activities. The straub tail activity in murine models are often examined in response to opioids because of the involvement of μ receptor in its regulation (Nath *et al.*, 1994; Houshyar *et al.*, 2000). Some agents or drugs that influence the pathway of μ receptor which is seen in opiods are also known to influence the serotoninergic pathway (Zarrindast *et al.*, 2001; Diaz & Maroteaux, 2011). This effect was observed in the murine models at doses of 30, 100, 300, and 1000 mg/kg body weight indicating the possible influence of AME on opioidergic or serotoninergic pathways implying it potential use as an analgesic when opioidergic pathway is desired. The hot plate test which was used to examine the analgesic property of AME through central pain shows significant ($p< 0.0001$) increase in latency or reaction time indicating its effect involving higher centers of the brain.

Chemotherapy-evoked peripheral neuropathy (CIPN) is a recurring adverse event that may account for poor patient compliance and in some instances leads to suffers abandoning the treatment (Fidanboylu *et al.*, 2011). Chemotherapeutic drug, paclitaxel is used for the treatment of tumours (Zhang *et al.*, 2014). Even though the exact pathway through which paclitaxel is shown to induce peripheral neuropathy remains unclear, current studies posits that frequent paclitaxel use evokes serious peripheral neuropathy marked by thermal, mechanical hyperalgesia and allodynia, likely because of "atypical (swollen and vacuolated) mitochondria in peripheral sensory axons of both the C-fiber and myelinated axons, nerve injury by interruptive generation of microtubules necessary for

axonal transport in the dorsal root ganglia, axons and Schwan cells (Scripture *et al.*, 2006) and a loss of intra-epidermal nerve fibers resulting in further to loss of cellular activity (Fidanboylu *et al.*, 2011). Compared to the vehicle, AME was found to significantly decrease paclitaxel-induce hyperalgesia. Similar results was also observed between AME- and paclitaxel-treated groups as well as in animals treated with pregabalin (Luo, 2002). The analgesic and anti-epileptic property of pregabalin has been linked to its antagonistic effect on α2–δ1 subunit of N-type voltage-dependent calcium channels (Mangaiarkkarasi *et al.*, 2015).

Intraperitoneal injection of 55mg/kg body weight of streptozotocin (STZ) in a single dose coupled with 120mg/kg body weight body weight of nicotinamide treatment induced type-2 diabetes in rats with FBS > 11mmol/l. After two weeks of inducing diabetes in rats they developed diabetic complications (diabetic peripheral neuropathy). These rats were found to experience decreased latencies to thermal, cold and mechanical substances in the pain assessment test confirming a successful induction of neuropathic pain. Beta-cells from the pancreas of rats exposed to streptozotocin were found to be damaged because of the cytotoxicity of streptozotocin. This effect of STZ was observed within seventy-two hours following treatment. However, its onset and degree of damage is dose-dependent and ultimately resulting in increased blood pressure (Junod. *et al.*, 1967). AME significantly ameliorated thermal and mechanical hyperalgesia and exhibited antiallodynic property on STZ-induced diabetic neuropathic pain. The effect of pregabalin in the treatment of hyperalgesia and allodynia confirms its clinical efficacy in treating neuropathic pain (Blommel & Blommel, 2007).

There was a significant increase in weight of AME treated animals as compared to that of pregabalin and the control treated animals. This may be as a result of the extract's neurological effect on the hypothalamus hence influencing food consumption and weight gain.

Plant-based medicines are used world-wide in primary healthcare even though they are not entirely safe (Vaghasiya *et al.*, 2011). International opinion and regulations relating to human health has stated that any novel therapeutic agent must be examined on its safety before its introduction into patients (Klaassen, 1991). Animal models have been commonly used to assess the safety of therapeutic agents. In animal models treated with AME, haematological and histopathological parameters were similar as compared to the vehicle. This confirms the safety of AME. Sub-acute toxicity is usually observed by severe modifications to physiological, behavioral or biochemical processes in murine models. This modification may cause changes in autonomic activity such as lacrimation, pupil size, response to handling as well as changes in the nature and color of skin and fur or death (Pramyothin *et al.*, 2006). Neither of these effects were seen in AME treated groups even at a dose of 3000 mg/kg body weight. This further confirms its safety, since therapeutic agents with an LD_{50} value of 1000 mg/kg body weight when administered orally are regarded as safe (Obici *et al.*, 2008).

A comparable body weight differences between treated animals and the vehicle in a research is indicative of no significant effect of that agent on weight (Zimmermann, 2001). In comparison with the vehicle, the extract did not show any adverse effect on the weight of animals. Furthermore, change in body and organ weight of rodents is a reflection of an exposure to a toxic compound (Auletta, 1995). The weight of an organ is one of the few fundamental parameters which reflects the impact of a toxic compound on rodents, specifically in their physiological and pathological states (Raza *et al.*, 2011). Some of these organs include the brain, heart, liver, kidney and prostate (Dybing *et al.*, 2002). There was no significant change in weight of an organ from AME-treated animals compared to the vehicle.

The susceptibility of hematopoietic system to toxic compounds makes it one of the few indices to

consider in evaluating toxicity on physiological basis (Adeneye *et al.*, 2006). Full blood count is usually the test performed to assess the hematopoietic system. It gives an indication of an inflammation, infection, clotting factors, anaemia or the development of cancer (Ajeigbe *et al.*, 2013). Some of the indices analyzed under this test includes WBC, differential RBC, platelet and hematocrit count, the concentration of hemoglobin is also analyzed among other parameters. The extract after two-weeks treatment showed no significant difference in all these parameters compared to the vehicle. Even though AME did not significantly affect the hematopoietic system during the two-weeks period, further studies must be conducted to assess its chronic administration.

Histological examination is widely used to evaluate pathological alterations of organs and tissues (Sherif *et al.*, 2017). Therefore, histological analysis on the cells of the organ was analyzed. In this study, histopathological studies on the organs indicates that AME did not produce any adverse effect on the cells or the morphology of rat organs. The haematological analysis together with body and organ weight shows there was no structural damage by the extract to the organs analyzed. The liver is one of the main organs susceptible to acute toxicity because of its role in the metabolism of drugs (Bakoma *et al.*, 2013). Histology on the liver in this study exhibited normal hepatocytes with no defect in both the treated groups and the vehicle. This is indicative of no significant alteration to the structure or cells of the liver. Histological examination of the kidneys showed regular glomeruli without mesangial cell proliferation or basal membrane activity. There was no inflammation of the Bowman's capsule nor was there any in the renal tubules. In addition, serum levels of urea, creatinine and electrolytes such as Na^+, K^+ and Cl^- which are indicators for how the kidney is functioning, was all normal just as the vehicle. Therefore, during the two-weeks treatment with AME, the morphology and histology analysis on the kidney of rats showed no significant adverse effect.

With respect to all the above experiments, male ICR mice and rats were used. This is because

literature indicated no distinctive difference in gender with regards to results obtained in its analgesic property (Ishola I.O *et al* 2014) anxiolytic property (Hasrat *et al* 2013), hypoglycaemic property (Sovia *et* al 2016) and antioxidant property (Correa R. *et al* 2016). Moreover, the stress associated with induction of peripheral neuropathy were more tolerable with male experimental animals as compared to female ones. (Boland, B. A. *et al* 2016).

5.2 CONCLUSION

This study revealed AME possess analgesic property against streptozotocin-induced diabetes neuropathy as well as paclitaxel-induced neuropathic pain in murine models with a high safety. Also, AME may be a good therapeutic candidate that can be used in the management of various types of pain including that which is caused by diabetes as well as taxane usage.

Furthermore, secondary metabolites found in AME such as alkaloids, saponins, tannins and flavonoids could be the reason for the pharmacological activity of the extract. These findings contribute to the studies that has revealed the analgesic abilities of the AME and experimentally corroborate its use as an analgesic.

5.3 RECOMMENDATIONS

1. The active compound(s) in AME which is (are) responsible for its analgesic property should be isolated and characterized

2. Further studies should be conducted on the chronic toxicity of AME to ascertain its safety.

3. Different models should be used to evaluate the analgesic property of AME and its mechanism of action.

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