KWAME NKRUMAH UNIVERSITY OF SCIENCE AND TECHNOLOGY, KUMASI, GHANA

ANTINOCICEPTIVE, ANTI-INFLAMMATORY AND ANTICONVULSANT EVALUATION OF THE HYDROETHANOLIC LEAF EXTRACT OF *CALOTROPIS PROCERA* (AIT) R. BR. (APOCYNACEAE)

BY

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DECLARATION

I hereby declare that this submission is my own work and that, to the best of my knowledge and belief, it contains no material previously published or written by another person nor material which to a substantial extent has been accepted for the award of any other degree or diploma at Kwame Nkrumah University of Science and Technology, Kumasi or any other educational institution, except where due acknowledgment is made in the thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners. I understand that my thesis may be made electronically available to the public.

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ABSTRACT

Pain, inflammation, and epilepsy remain a real and currently, a major problem in clinical medicine which requires new agents with improved efficacy for more effective therapy. Plant sources can serve as a basis for the search for these novel drugs. The analgesic, anti-inflammatory and anticonvulsant effects of hydroethanolic extract 70% v/v of *Calotropis procera* (CPE*)* which is widespread in Ghana and other tropical areas in the subregion was evaluated. A primary neurological assessment revealed that CPE has some central nervous system depressant and analgesic effects. It also showed impairment on motor coordination in the rotarod test. The extract potentiated duration of sleeping time in the pentobarbitone interaction test and analgesic properties were also further confirmed in the tail immersion test while it delayed pentylenetetrazole-induced convulsions. The anti-inflammatory assessment showed that CPE was able to significantly reduce both carrageenan-induced ($F_{4,15}$=6.24, *P=0.0105*) and formalin-induced inflammation ($F_{4,15}$=100.9, *P<0.0001*) in rats. The analgesic effects were demonstrated in the acetic acid-induced writhing and formalin-induced paw licking tests. Writhing induced by acetic acid was significantly reduced ($F_{4,15}$=24.14, P<0.0001) on treatment with oral doses of CPE (30-300 mg/kg). The extract significantly inhibited both phase 1 and phase 2 states induced by injection of formalin ($F_{4,60}$=12.21, P<0.0001) comparable to morphine, the standard analgesic used. The extract significantly attenuated hyper-nociception induced by tumour necrosis factor-alpha, interleukin 1β, bradykinin, and prostaglandin E_2. The anti-nociceptive effect exhibited by CPE in the formalin test was reversed by systemic administration of naloxone and theophylline. The extract was further evaluated for anticonvulsant activity in rodents using animal models, picrotoxin–induced convulsions, strychnine-induced convulsions, and pilocarpine-induced *status epilepticus*. CPE (100–300 mg/kg) exhibited anticonvulsant effect against strychnine-induced clonic seizures by significantly reducing the duration ($F_{6,20}$=4.196, P=0.0068) and frequency ($F_{6,21}$=5.438, P=0.0016) of convulsions. The extract (100-300 mg kg^{-1}) caused a profound dose-dependent delay in the onset of clonic convulsions induced by picrotoxin ($F_{6,25}$=17.43, P<0.0001) and tonic convulsions ($F_{6,20}$=43.45, P<0.0001) in mice. The duration of convulsions was reduced significantly ($F_{6,19}$=41.71, P<0.0001). CPE (100-300 mg kg^{-1}), showed profound anticonvulsant effect and protected against death induced the pilocarpine. ED_{50} (~ 0.1007) and E_{max} values calculated from the dose-response curves demonstrated that the extract was less potent than diazepam in reducing the duration and onset of convulsions but had comparable efficacies. Flumazenil – a $GABA_A$ receptor antagonist, did not reverse the onset or duration of convulsions produced by the extract in the picrotoxin-induced seizure model. Overall, the hydroethanolic leaf extract of *Calotropis procera* possesses analgesic, anti-inflammatory, and anticonvulsant properties.

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5-HT	5-Hydroxytryptophan
5-HTP	5-Hydroxytryptamine
AEDs	Antiepileptic drugs
ALP	Alkaline phosphatase
ALT	Alanine aminotransferase
AMPA	Alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid
AMPT	Alpha-methyl-para-tyrosine
ANOVA	Analysis of variance
EXTRACT/CPE	70%v/v hydroethanolic *Calotropis procera* extract
BUN	Blood urea nitrogen
CNS	Central nervous system
C RP	C – reactive protein
DNA	Deoxyribonucleic acid
DOPA	3,4-dihydroxyphenylalanine
EEG	Electroencephalogram
ED_{50}	A dose of a drug which elicits 50% of the maximum response
EPM	Elevated plus-maze
GABA	Gamma-aminobutyric acid
GAD	General anxiety disorder
GAT-1	GABA transporter 1
GGT	Gamma-glutamyltranspeptidase
HCT	Haematocrit
HLEs	Hind limb tonic extensions
IASP	International Association for the study of pain
I.P.	Intraperitoneal
ICH	International Committee for Harmonization

ICR	Institute of Cancer Research
iGuR	ionotropic glutamate receptors
IL	Interleukin
ILAE	International League Against Epilepsy
KNUST	Kwame Nkrumah University of Science and Technology
MEST	Maximal electroshock test
mGuR	Metabotropic glutamate receptors
MPE	Maximal possible effect
NE	Norepinephrine
NET	Norepinephrine transporter
NMDA	N-methyl-D -aspartate
P.O.	*Per os*
PCPA	Para-chlorophenylalanine
PD	Panic disorder
PSTD	Post-traumatic stress disorder
PTZ	Pentylenetetrazole
PTX	Picrotoxin
ROS	Reactive Oxygen Species
S.C.	Subcutaneous
SE	*Status epilepticus*
SEM	Standard Error of Mean
SERT	Serotonin transporter
SSRI	Selective serotonin reuptake inhibitors
STR	Strychnine
TCAs	Tricyclic antidepressants
TNF-α	Tumour Necrosis Factor-α
WHO	World Health Organisation

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Chapter 1

INTRODUCTION

1.1 GENERAL INTRODUCTION

Ethnopharmacology deals with understanding the and other potential outcomes of drugs using concepts of ethology (Reyes-Garcia, 2010). Ethnopharmacology is described as the systematic exploration of naturally active agents which is historically used or discovered by man (Taylor *et. al*., 2001). The target of this area of research concerns authentication of folkloric formulations either by the separation of potent materials or via pharmacological discoveries. It is necessary to interpret the detection of organically effective agents in the original use and formulation of the plant material. This ought to include an investigation and chemical and pharmacological assessment of initial drug preparations to determine dose-effect associations for the utilisation of the treatment. Ethnopharmacological surveys can make a significant contribution to contemporary medicine and can lead to many new and helpful medicines. (Taylor *et. al*., 2001).

The research on the impacts of drugs on normal action patterns, and to behaviour showcase the elementary concepts of ethnopharmacology (Johnson and Sargent, 1990). Ethnopharmacology studies natural medicines derived from plants and other substances that have been traditionally used by groups of people to treat various human diseases. In traditional medicine, many modern drugs originate. Traditional Indian Medicine (Ayurveda) has a long history and is one of the excellent traditions of life. (Patwardhan, 2005). Ethnopharmacological knowledge is very common and is of most importance in the tribal populations. However, most of such information lacks scientific validation. There is a common use of plant resources in traditional medicine but only a few of these have had medicine bioassay analyses done on them to validate their medicinal properties to establish safety and effectiveness when used as folkloric medicines (Taylor *et al*, 2001). The ethnopharmacological information, its complete methodology backed by empirical data can provide a pioneering and formidable breakthrough for a novel, harmless as well as inexpensive drugs.

Traditional data on medicinal plants in many countries is fast eroding. The steady attrition of traditional knowledge has dire consequences as it reduces the independence of native

people by making them rely on urban societies. In the absence of basic healthcare amenities in rural areas, traditional medicine provides a good substitute for several people. However, it is of importance to get an impartial assessment of such practices to get the maximum benefit of the system. The native and contemporary systems of medicine are not conflicting but rather correlative, and using them together will be more affordable, reasonable as well as more workable. Traditional medicine and medical practices have positively contributed to the discovery of many medicines, such as morphine, digoxin, and reserpine (Elujoba *et. al.*, 2005). The traditional information about cinchona bark led to the discovery of quinine for treating malaria (Kumari, 2003). It is therefore imperative that records of all ethnobotanical information among the various traditional societies are kept before such data is lost. A lot of folkloric plant-based remedies are nowadays being reused and has found increasing application as potential therapeutic agents or as basic material for the expansion of semi-synthetic compounds (Gurib-Fakim, 2006). These may serve as leads for the discovery of newer compounds.

Herbal medicines have become more widespread in the management of many ailments due to the common credence that green remedy is harmless, easily accessible and have few unwanted effects. Indeed, public request of medicinal plant use has been very huge such that there is a big risk that these plants may move towards extermination or loss of hereditary characteristics (Olowokudejo *et al.*, 2008). According to the WHO, over 70% of the populace, mostly those from unindustrialized countries, depend on traditional medicines (principally herbs) for their health needs (Abdullahi, 2011). Therefore, usage of medicinal plants which are present in local settings is usually a cheaper alternative to costly western remedies. Thus, there is a huge global demand for it. Existing estimations suggested by Addo-Fordjour *et al*, 2011 show that around 70% of Ghanaians count on conventional homeopathic systems for their wellbeing.

Owing to the reliance on medicinal plants coupled with the potential for drug discovery, it has become important to explore for powerful, valuable and comparatively harmless medicines from plant sources. Systematic authentication of assertions concerning the medicinal value of plants is currently being used is also important in order to improve their safety and effectiveness.

Herbal products such as *Calotropis procera* are traditionally used worldwide for their pharmacological properties. *Calotropis procera* has been used for a wide range of

conditions such as cough, rhinitis, some skin infections, asthma, lymphatic filariasis, dizziness, fever, indigestion and sometimes, loose stools (Oloumi, 2014). This work seeks to evaluate the analgesic, anti-inflammatory and anticonvulsant properties of *Calotropis procera* in a bid to confirm its usefulness as recommended in folklore medicine.

1.2 CALOTROPIS PROCERA

1.2.1 *The plant*

Figure 1-1 A picture of the fresh leaves of *Calotropis procera.*

Calotropis procera erstwhile belonged to the family Asclepiadaceae, but now is a taxonomic category of Apocynaceae (Wong *et al.*, 2013). It is ordinarily found in tropical and sub-tropical countries of Asia and the African continent (Rahman and Wilcock, 1991) in dry and semi-arid climates and can tolerate extremely low annual rainfall and a dry period of up to about ten months in a year (Barbaosa *et. al.*, 2014).

This ligneous plant is commonly referred to as apple of Sodom, king's crown, rubber bush. It is regionally referred to as mpatu-asa (Twi), wolaporhu, wolapugo (Dagbani), blɔfo tɔtɔ, gbé'kɛ̃biì-awuɔ (Ga) and pòlípòlí (Guan-Gonja) (Burkill, 1985).

1.2.2 **Description and Distribution**

Morphologically, *Calotropis procera* (Ait). R. Br. is a multi-branched plant with xanthous barks having white, soft and corky crevices. Leaves are simple, opposite, subsessile, blade loosely obviates and egg-shaped. It has fairly few leaves, which are largely close to the elevated end. The perimeters of the foliage are almost dark green with just about white veins. They are 7 to 18 cm long and 5 to 13 cm wide, mildly leathery, with a good coat of smooth hair that rub off readily (Oloumil, 2014). Its fruit is simple, magnified and

contains numerous brown coloured seeds with white silken hairs. Matured fruits spew to scatter seeds which are widely circulated by wind and animals. (Azhar *et al*., 2014; Khairnar *et al*., 2012).

It is drought-impermeable, to a comparatively elevated degree salt-tolerant. It soon becomes a weed along degraded roads, lagoon edges and in overgrazed indigenous grasslands (Kumar, 2013). It is typically central in areas of low rainfall; assumed to be an indicator of over-cultivation. *C. Procera* is from India, Pakistan, and some other Asian regions, Kenya, Nigeria and Ghana. (Sharma., 2011; Kumar *et al*., 2013).

In a variety of soils and different environmental circumstances, the plant develops very well. The plant, *Calotropis procera* is one of the very limited plants which is not eaten by livestock. It can grow on impoverished soils, especially where overgrazing has removed native grass competition. Occasionally, this plant remains the sole survivor in some fields where nothing else grows (Kumar *et al*., 2013). The presence of latex, widespread branched root system and dense leaves with waxy texture makes the plant xerophytic. It is therefore scattered throughout the warm and humid regions of the globe (Ahzar *et al*., 2014; Khairnar *et al*., 2012).

1.2.3 **Traditional Uses**

In India, consistent with Ayurvedic medicine, the entire plant is employed to counter poison from snake bites or infections and cures leprosy, ulcers, and liver diseases. Indigenously, the juice has anthelmintic properties, acts as a laxative, and cures piles and the root bark cure respiratory illnesses and some venereal diseases (Meena et. al., 2011). The flowers of *Calotropis procera* are analgesic, astringent, cures inflammations and tumours and features a strong cytotoxic activity (Smit *et al.*, 1995). Historically, the plant has been used as an antifungal (Markouk *et al*., 2000), antipyretic (Al. Yahya *et al*., 1985) and analgesic agent (Mascolo *et al*., 1988).

The plant decoction is used in ancient Indian medicine to treat excruciating muscle spasms, dysentery, fever, rheumatism, asthma and as an expectorant and laxative (Basu and Chaudhuri, 1991). Furthermore, the leaves of this plant were used to relieve body and joint pain and cure cephalalgia, and reduce swelling (Dewan, 2000). Additionally, it is used as homeopathic therapy in Gwari populations in Central Nigeria for the treatment of ringworms. The pulverized root decoction of *Calotropis procera* has been described to

provide respite in some inflammatory diseases of the intestine which is usually characterised by severe diarrhoea and abdominal pain and utilised as a carminative to reduce flatulence associated with indigestion (Verma *et. al*., 2010). The fine-grained root extract of *Calotropis procera* are used by numerous tribes in parts of South Asia to cure jaundice (Dewan et al., 2000). Considering such uses and properties, this study evaluated the leaves of *Calotropis procera* for effects against pain, inflammation, and seizures possibly leading to the discovery of new agents in this area.

1.2.4 Previous Work on the Plant

Calotropis procera leaves contain mainly calotropagenin, calactin, calotoxin, calotropin, taraxasteryl acetate, β-sitosterol, α-amyrins, and β amyrins. The leaves as well contain organic carbonate and stigmasterol (Oloumi, 2014). Khairnar *et al*., (2012) also reported the pharmacological actions of *C. procera* as being hepatoprotective, anthelmintic, anti-inflammatory, anti-diarrheal, antioxidant and antidiabetic. They also depicted the plant as having a strong effect against myocardial infarction, it also has antifertility, schizonticidal, analgesic, anticancer and anticytotoxic, antioxidant and antibacterial activities. Studies that were conducted by Murti *et. al*., (2015) assessed the analgesic potential of the leaves of *Calotropis procera*. An aqueous leaf extract of *Calotropis procera* was demonstrated by Shenoy (2016) to possess anti-seizure potential using the pentylenetetrazole-induced seizure test model in rats.

1.3 EPILEPSY

1.3.1 Background

The term epilepsy is derived from the word *epilepsia*, which means to be taken, seized or attacked in Greek (Singh and Trevick, 2016). It is a chronic disorder characterized by the uncontrolled or excessive electrical activity of either a part or the entire central nervous system (Fisher *et al*., 2005). Epilepsy could affect individuals of different age groups. The outward signs of epilepsy are known as seizures which could occur spontaneously and in a recurrent manner. This abnormal electrical activity may result in a variety of events, including loss of consciousness, abnormal movements, atypical or odd behaviour, or distorted perceptions that are of limited duration but recur if untreated. The site of origin of

the abnormal neuronal firing determines the symptoms that are produced (Panayiotopoulos, 2010).

Convulsions are described as a transitory change in consciousness caused by uncontrollable occurrences or other signs or symptoms that may be due to brain malfunction. Epileptic seizures are often sparked by extreme, irregularly coordinated, concentrated locally or widespread electrical firing of neurons. (Fischer *et al.*, 2005). These electrophysiological assessments are usually assumed by indirect proof, although electrical readings are sometimes made during seizures (Cervenka, 2012). Epilepsy is one of the most common neurologic problems worldwide (De Boer *et. al.*, 2008). Roughly over three million individuals in the United States of America have epilepsy (Theodore *et. al.*,, 2006), and it is estimated that at some stage in their life, 3% of the general population will have epilepsy according to a prospective cohort study done by Walczak *et al.*, 2001. Significant progress has been generated in the diagnosis and therapy of seizure disorders in latest years (Moshé *et al.*, 2015). Nonetheless, the cellular and molecular processes by which epilepsy or epileptogenesis develops is not fully understood.

Epilepsy is a brain condition typified by more than one seizure with an ongoing predisposition to produce more seizures associated with neurobiological, behavioural, psychological and social disorders. (Schneider, 2009). It is the most common nervous system illness after cerebrovascular accident, with a incidence rate of 0.5%, and a 2–3% lifetime possibility of being diagnosed of epilepsy (Browne & Holmes, 2008). Epileptic seizures may be due to abnormal hypersynchronous discharges of neurones that may be caused by any pathological process that affects the brain (Bodalia *et al.*, 2013).

Prevalence of the condition in developing countries is generally higher than in the developed countries (Stafford, 2008). It is reported to be the second most common neurological disorder after stroke and is estimated that approximately 0.8% of the population is affected by some form of epilepsy (Pitkanen and Lukasiuk, 2009). In African communities, epilepsy is seen as a shameful disorder. It carries with it a stigma and has severe social implications. Sufferers are often shunned and discriminated against with respect to employment, marriage, family relationships and effectively reduces the overall quality of life (Baskind & Birbeck, 2005).

1.3.2 **Classification of epilepsy**

The term "epilepsy" involves several different syndromes whose prime feature is a tendency to persistent unprovoked seizures. Specific seizures can be classified according to their clinical features; complex partial seizures and generalized tonic-clonic seizures (Engel, 2006). Epilepsy syndromes can also be classified according to the type of seizure, the presence or absence of neurologic or developmental abnormalities, and electroencephalographic (EEG) findings (Verhelst *et al.*, 2005). Epilepsy syndromes fall into two broad categories: generalized and partial (or localization-related) syndromes (Benbadis *et al.*, 2001). In generalized epilepsies, the prevalent type of seizures begins in tandem in both cerebral hemispheres. Several categories of generalized epilepsy have a strong genetic component. In most of these cases, the neurologic function is usually normal. In partial epilepsies, by contrast, seizures originate in one or more localized foci, although they can spread to involve the entire brain. Most partial epilepsies are believed to be the result of one or more central nervous system insults, but in many cases, the nature of the insult is never identified (Englot *et al*., 2012)

1.3.3 **Neurobiology of epileptic seizures**

The paroxysmal depolarization shift (PDS) is the pathophysiological cellular phenomenon that is the underlining cause of all types of epileptic seizures and interictal epileptiform electroencephalography (EEG) abnormalities (spikes). A deep insight of the cellular and molecular processes by which epilepsy happens, or epileptogenesis, is however lacking. However, in recent years advances have been made in the diagnosis and treatment of epilepsy (Bromfield *et al.*, 2006). Several decades have been devoted to the study of the pathophysiology of epilepsy. Increasing knowledge in the field only contributed to a partial understanding of the underlying mechanisms. Nonetheless, a decent understanding of the pathophysiology of epilepsy and its fundamental histological and neurochemical modifications has aided the logical development approaches of modern antiepileptic drugs (AEDs).

Glucose and electrolyte abnormalities play significant roles in the pathophysiology of seizures. Increased extracellular potassium has been found to decrease neuronal hyperpolarization and promote seizure activity. Similarly, low extra-neuronal

concentrations of calcium or magnesium may increase synaptic excitability and may predispose individuals to seizures. The low levels of magnesium in the cell causes the stimulation of NMDA receptors, which are typically impeded by voltage-dependent magnesium block. Additionally, alterations in the levels of other ions within the extracellular environment of the neuron may as well have critical effects on the activity of voltage-gated ion channels (Vaughan and Jackson, 2014).

Inherent variations influence the ion channel or receptor function, and this may influence the generation of seizure episodes. It is being recognized that many seizures with unknown cause or mechanism of apparent spontaneous origin have a genetic origin (Berkovic *et. al.*, 2006). Recent studies have revealed an association between inflammation and epilepsy. Experiments performed on rodents have demonstrated that seizures induce high levels of inflammatory mediators in regions of the brain involved in the generation and propagation of epileptic activity. Moreover, inflammatory cytokines such as interleukin-1β, IL-6, and TNFα have been found to trigger a cascade of downstream inflammatory events that also involve neurons and endothelial cells of the blood-brain barrier (BBB) (Vezzani et. al., 2011). Cytokines have an effect on the permeability properties of the BBB either by disrupting the tight junctions or generating nitric oxide in endothelial cells. These alterations in BBB permeability result in albumin extravasation and this has been shown to favour hyperexcitability. Hence, there is a positive association between the frequency of seizures and seepage of the blood-brain barrier (Vezzani *et al.*, 2011). Further studies into the role of cytokines and other inflammatory mediators in epilepsy may offer insights into the mechanism of seizure generation and will possibly highlight prospective new targets for therapeutic intervention (Vezzani *et al.*, 2011).

Epileptic seizures result from an overly synchronous and continuous flow of a cluster of neurons. The sole characteristic of all epileptic syndromes is a relentless surge of neuronal excitability. Abnormal cellular discharges may be associated with a variety of causative factors such as trauma, oxygen deprivation, tumours, infection, and metabolic derangements. However, no specific causative factors are found in about half of the patients suffering from epilepsy (Engelborghs *et al.*, 2000). More than a hundred neurotransmitters or modulators have been shown to play a role in neuronal excitation. However, the major

excitatory neurotransmitter in the brain is L-glutamate and the major inhibitory neurotransmitter in the brain is gamma-aminobutyric acid (GABA) (Schwartz, 1988).

An increase in excitatory processes or a decrease in inhibitory processes can result in a seizure. Normal neurones discharge repetitively at a low baseline frequency. If neurones are damaged or suffer electrical or metabolic insult, a change in the pattern of discharge may develop. In the case of epilepsy, regular low-frequency discharges are replaced by frequent bursts of high-frequency discharges usually followed by periods of inactivity (Dhillon & Sander, 2003).

1.3.3.1.1 Role of GABA in epileptogenesis

Generation of seizures has been attributed to an imbalance between excitatory and inhibitory neurotransmission in the brain. Hence, it is important to highlight the role of neurotransmitters especially γ-aminobutyric acid (GABA) and glutamate in epileptogenesis, since they are the major inhibitory and excitatory neurotransmitters in the central nervous system. GABA is located primarily in short-axon interneurons that synapse on cell bodies and proximal axons and serves to counterbalance neuronal excitation. A perturbation of this balance causes seizures. Compounds that interfere with GABA-mediated inhibition have been shown to be convulsant (Treiman, 2001).

The task of gamma-aminobutyric acid (GABA) transmission in the management of convulsive epileptic attacks is measured from the viewpoint of the actions of drugs that strengthen GABA transmission in the brain. Specifically, the influence of a direct-acting $GABA_A$ receptor agonist, muscimol, is matched with the effects of a GABA-elevating agent, gamma-vinyl GABA (GVG, vigabatrin), in animal models of convulsive seizures. The direct stimulation of postsynaptic GABA receptors (by direct receptor agonists) circumvents typical mechanisms of synaptic transmission and can induce abnormal neurological indicators, whereas the augmentation of presynaptic accessibility of GABA escapes these difficulties. GVG plays a role in boosting the presynaptic GABA stores, which can then be exploited physiologically; this may account for the comparatively low occurrence of CNS-related unwanted effects with anticonvulsant doses of GVG. There is enough proof which reveals that there are some regions of the brain where greater GABA

transmission is considered anticonvulsant whereas, in other areas, blockade of GABA communication exerts anticonvulsant impacts. Also, there are brain zones where the effects of the agents, muscimol, and GVG are different from one another, due to a comparatively low level of endogenous GABA transmission in those parts (Gale, 1992).

Sivam *et. al.,* (1982) showed that muscimol, a GABA agonist, enhanced pentobarbital sleeping time in a dose-dependent manner. The GABA antagonists such as bicuculline and picrotoxin, and the CNS stimulant such as pentylenetetrazole, inhibited pentobarbital sleeping time. On the other hand, all the stated agents except picrotoxin generated less than 35% maximum inhibition. Picrotoxin, an agent which blocks the chloride ionophore of GABA-receptor complex, exhibited a parallel dose-response curve with respect to muscimol. The prolonged administration of pentobarbital by pellet implantation produced tolerance as evinced by reduced sleeping time; the tolerance waned steadily upon immediate removal of the agent. Muscimol enhanced pentobarbital sleeping time both in tolerant and withdrawal mice. They corroborated that the sodium-independent Gamma Amino Butyric Acid receptor binding, using [^{3}H] muscimol as a ligand, was enhanced following both acute and chronic pentobarbital injection; removal of the pentobarbital overturned the surge in receptor concentration.

GABA exerts its major inhibitory effect through $GABA_A$ receptor (which is a ligand-gated ion channel) by increasing neuronal membrane conductance for chloride ions causing membrane hyperpolarization, reduced neuronal excitability and most rapid inhibition in the brain (Pirker *et al.*, 2006). The $GABA_A$ receptor is, therefore, a target for many neuroactive drugs such as benzodiazepines and barbiturates. $GABA_A$ receptor consists of five subunits that form a chloride ion channel (Macdonald and Olsen, 1994). The subunits consist of various subtypes and studies have shown that individual subunits and subtypes confer different sensitivities to agents acting on $GABA_A$ receptors (Coulter, 2001).

1.3.3.2 ***Role of Glutamate in epileptogenesis***

Glutamate is the most important excitatory neurotransmitter of the central nervous system. Glutamate acts via two types of receptors – ionotropic glutamate receptors (iGuR) which are ligand-gated cation-specific channels and metabotropic glutamate receptors (mGluR) which are G-protein-coupled receptors. Ionotropic glutamate receptors are classified

according to their prototype agonists: NMDA (N-methyl-D -aspartate), AMPA (alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid) and kainite. The role played by metabotropic glutamate receptors depends on the type of receptors. Activation of type I is convulsant whereas activation of types II and III is anticonvulsant (Moldrich *et al.*, 2003).

Epilepsy may result from over-release of glutamate. Limbic seizures in experimental animals result in brain damage that resembles that due to glutamate toxicity. Similar changes are seen at autopsy in patients with intractable epilepsy. Reduction of excitatory glutamatergic neurotransmission is potentially important. AMPA receptor blockade probably contributes to the antiepileptic effect of drugs such as lamotrigine (Eid *et al.*, 2008).

1.3.3.3 *Role of ion channels in epilepsy*

Ion channels are membrane-spanning proteins forming selective pores for Na^+, K^+, Cl^-, or Ca^{2+}. During action potentials, precise control of ion channel gating is mediated by membrane voltage, during synaptic transmission by the binding of specific neurotransmitters, such as acetylcholine (ACh). Regarding these basic principles, two distinct and structurally conserved classes of ion channels emerged during evolution, the voltage-gated and the ligand-gated channels and mutations in such channels could cause epilepsy (Fletcher *et al.*, 1996). Calcium channels are key mediators of calcium entry into neurons in response to membrane depolarization, mediating several essential functions including the release of neurotransmitters and the regulation of neuronal excitability (Mizielinska, 2007).

1.3.4 **Diagnosis of epilepsy**

Correct diagnosis of an epilepsy syndrome can be complicated. It requires the application of multi-dimensional criteria and various investigations depending on the suspected disorder. The cornerstones of the diagnosis of epilepsy are neurological examinations and a good history. Laboratory investigations serve as supplementary tests (Stafstrom and Carmant, 2015)

1.3.4.1 *Medical history*

Medical history is the most important feature of clinical evaluation of an epilepsy patient. Medical history including symptoms and duration of the seizures helps in determining epilepsy and the kind of seizures present in the person. Moreover, an accurate eyewitness description can be a piece of valuable information that can help in the diagnosis of the condition (Kander *et al.*, 2012).

1.3.4.2 *Neurological examination*

The aim of neurological examinations is to assess focal signs that might implicate or localize cerebral pathology. Examinations conducted soon after an epileptic event could reveal if observed deficits are transient or not. Signs such as postictal weakness, aphasia, and sensory dysfunctions provide important data that assist physicians in diagnosing patients (Ahmed and Spencer, 2004).

1.3.4.3 *Laboratory investigations*

Seizures are sometimes caused by an acute underlining noxious or metabolic disorder in which case suitable therapy should be directed to the specific abnormality. Blood samples are often screened for disorders such as hyponatremia, hypoglycaemia, hypomagnesaemia, uraemia and hepatic encephalopathy that may be associated with seizures. Testing for electrolytes, blood glucose nitrogen, creatinine, and liver function tests may provide important clues in the diagnosis of epilepsy. Blood samples may also be tested for infections, lead poisoning, anaemia and diabetes that may be causing or triggering the seizure (Kandar *et al.*, 2012).

1.3.4.4 *Electroencephalogram (EEG)*

The EEG is used clinically to record the electrical activity of the brain. Due to its ability to demonstrate the physiological manifestations of abnormal cortical excitability, which is characteristic of epilepsy, it plays a central role in the diagnosis of the condition. Moreover, an EEG can help classify seizures and epilepsy syndromes, and hence the type of AED that should be used in treatment (Smith, 2005). The important parts of an EEG include its electrodes, amplifiers, and a display device. The electrodes are placed on the head and the electrical potentials to the amplifiers. The features of epilepsy are displayed as spikes (20 to

70 ms) and sharp waves (70 to 200 ms). It is crucial to note that a normal EEG does not necessarily exclude epilepsy and an abnormal EEG may also not indicate that a patient has a seizure disorder (Casson, 2018).

1.3.4.5 *Neuroimaging*

The significance of neuroimaging in the diagnosis of epilepsy is to identify a lesion that could explain a seizure. It may also be used to predict if a seizure could occur (Scharfman, 2017). Imaging also plays an important role in the evaluation of candidates for epilepsy surgery. Imaging techniques include magnetic resonance imaging (MRI), positron emission tomography (PET) and single-photon emission computed tomography (SPECT) (Pittau *et al.*, 2014)

1.3.5 **Management of Epilepsy**

1.3.5.1 *Pharmacological treatment*

The goal of pharmacotherapy is to achieve a seizure-free status without adverse effects. Antiepileptic drugs (AEDs) guard against seizures through exchanges with an array of cellular targets. By affecting the functional activity of these targets, AEDs suppress abnormal hypersynchronous activity in brain circuits, leading to protection against seizures. Antiepileptic drugs (AEDs) are categorized according to their mechanisms of action. The core classes include sodium channel blockers, calcium current inhibitors, gamma-aminobutyric acid (GABA) enhancing agents, carbonic anhydrase inhibitors, hormones, and some other drugs with unknown mechanisms (Rogawski, and Löscher, 2004).

1.3.5.1.1 Sodium channel blockers

Voltage-gated sodium channels perform a critical role in the induction and dissemination of action potentials in neurons. They are accountable for the depolarization of the nerve cell membrane and conduction of action potentials throughout the surface of neuronal cells. One of the most popular mechanisms of action of AEDs is a blockade of voltage-gated sodium channels. Examples of drugs in this group include phenytoin and carbamazepine. Voltage-gated sodium channels occur in one of three major conformational states: resting, open, and inactivated. During a single sequence of depolarization, channels cycle through these

conformational states in turn and are unable to respond to further depolarization until adequate numbers have reverted from the inactivated state to the resting state. Antiepileptic agents with sodium channel blocking properties have the highest affinity for the channel protein in the inactivated state and binding slows the conformational recycling process. Consequently, these drugs produce a distinctive voltage-dependent decline in channel conductance, which results in the limitation of rhythmic neuronal firing, with a modest effect on the initiation of single-action potentials (MacDonald and Kelly, 1995).

1.3.5.1.2 Calcium channel blockers:

Another important target for several antiepileptic agents is voltage-gated calcium channels. These channels mediate the influx of calcium ions that regulate intracellular signalling pathways and neuronal excitability. Blockade of calcium channel provides antiepileptic activity. A classic example of a drug that confers its antiepileptic activity through this mechanism is ethosuximide (Cain and Snutch, 2013).

1.3.5.1.3 GABA enhancers

GABA is the main inhibitory neurotransmitter in the brain. It is produced by intraneuronal decarboxylation of glutamate. GABA is catabolized by GABA transaminase. Inhibition of the enzyme increases the levels of GABA in the brain, and hence raises the threshold for a seizure to occur. Benzodiazepines such as diazepam and midazolam exert their anticonvulsant effect through this mechanism (Lasoń *et al.*, 2013).

1.3.5.1.4 Carbonic anhydrase inhibitors:

The acid-base balance and maintenance of local pH are critical to the normal functioning of the nervous system. Carbonic anhydrase is responsible for catalysing the bi-directional conversion of carbon dioxide and water to bicarbonate and hydrogen ions ($CO_2 + H_2O \leftrightarrow HCO_3^- + H^+$). The forward reaction is rapid, whereas the rate of the reverse reaction is relatively slow. Inhibition of carbonic anhydrase influences the latter more significantly, producing a localized acidosis and increased bicarbonate ion concentration. This, in turn, attenuates excitatory neurotransmission by reducing NMDA receptor activity and

enhancing inhibitory neurotransmission by facilitating the responsiveness of $GABA_A$ receptors. Acetazolamide is a carbonic anhydrase inhibitor which is used successfully as an antiepileptic agent (Kwan, 2001).

1.3.5.1.5 Drugs with unknown mechanism

A classic example of an antiepileptic agent in this group is sodium valproate. Although valproate is one of the most valuable drugs used in the management of epilepsy syndromes, the mechanism by which it protects against seizures is not completely understood. It has multiple pharmacologic actions, and it has been difficult to relate anyone mechanism to the drug's broad spectrum of activity. Therefore, it has been proposed that combined actions on several targets could account for its therapeutic properties. It is also believed that its pharmacological effects on GABA systems are among the most likely to be relevant to valproate's anti-seizure activity. At high concentrations, valproate affects voltage-gated sodium channels. However, there is not enough evidence to provide support for sodium channel block as a relevant mechanism to explain the clinical activity. There is also little support for its effects on T-type calcium channels. The pharmacologic effects of valproate relevant to its anti-seizure activity remain unclear (Rogawski and Cavazos, 2014).

1.3.5.2 ***Non-pharmacological treatment***

Non-pharmacological treatment of epilepsy may be used to complement the treatment of epilepsy with antiepileptic drugs, or in the treatment of medically intractable epilepsy. Non-pharmacological treatment of epilepsy include surgery, vagal nerve stimulation, ketogenic diet and other complementary therapies (Schuele and Lüders, 2008)

1.3.5.2.1 Surgery

A percentage of epileptic patients have seizures that cannot be controlled by available AEDs. For such patients, resection of the epileptogenic brain may control drug-resistant epilepsy. Resective surgery is an option in individuals whose seizures originate in a region that can be removed with minimal risk of disrupting normal neurologic and cognitive functions. The use of neuroimaging techniques such as MRI and PET are employed to reveal the origin of the seizures (Miller and Hakimian, 2013; Jehi *et al.*, 2016).

1.3.5.2.2 Vagal nerve stimulation

Vagal nerve stimulation (VNS) provides an adjunctive treatment option for children with refractory epilepsy in whom epilepsy surgery is not an option. It can be used to complement antiepileptic drugs. Studies have shown that VNS may be helpful in the desynchronizing electrocerebral activity. The exact mechanism through which VNS exerts anti-epileptic effects has not been completely elucidated yet. Although it has been demonstrated that type A fibres are the most excitable ones, followed by types B and C, respectively, it was once believed that all fibres should be stimulated to suppress seizures. Subsequently, scientists have found that C fibres are the ones responsible for the EEG desynchronization associated with epileptiform activity abolishment. However, the disadvantage of this treatment is the cost of the device and the need for its surgical implantation (Englot *et al.*, 2011).

1.3.5.2.3 Ketogenic diet

A ketogenic diet is a high fat, low carbohydrate and adequate protein regimen that has been used for almost a century in the treatment of medically refractory epilepsy. Numerous studies support the effectiveness of a ketogenic diet in the treatment of epilepsy. Ketogenic diets may be also effective for adult *status epilepticus* and adult epilepsy (Nabbout *et al.*, 2010; Smith *et al.*, 2011) and as a first-line treatment of seizures associated with glucose transporter 1 deficiency (Overweg-Plandsoen *et al.*, 2003). Although ketogenic diets have shown impressive results in its use for the treatment of epilepsy in children, there have been reports of serious adverse events including hypoproteinaemia, lipaemia, haemolytic anaemia, renal tubular acidosis, and elevated liver transaminases. Therefore, administration of such a diet requires coordinated care by a team of health professionals and a full commitment of parents (Sheth and Montouris, 2005).

1.3.6 **Anticonvulsant screening**

The first important neuropharmacological step in detecting the potential value of candidate anticonvulsant compounds is the classical maximal electroshock (MES) test in mice, introduced by Putnam and Merritt (1937). The MES is the most widely used animal model in AED discovery because seizure induction is simple and the predictive value for detecting clinically effective AEDs is high (Loscher, 2002). A powerful detection system is ensured when the MEST is combined with the pentylenetetrazole (PTZ) seizure test which is usually

regarded as an acute animal model. These are the two primary bioassays employed in the *in vivo* screening of new anticonvulsant compounds (Raza *et al*., 2001; Loscher, 2002). AEDs such as phenytoin, carbamazepine, valproic acid, that inhibit the hind limb tonic extension phase of the electroshock seizure in MEST are effective in the therapy of generalized tonic-clonic and partial seizures, whereas AEDs that hinder seizures induced by pentylenetetrazole (PTZ) in the PTZ test, examples being ethosuximide and phenobarbitone, are effective in the treatment of generalized myoclonic and absence seizures (White *et al.*, 1997; Raza *et al*., 2001).

The subcutaneous administration of bicuculline, picrotoxin and strychnine are also valuable tests to induce seizures and evaluate the effectiveness and mechanisms of anticonvulsant compounds (Raza *et al*., 2001). Although the MEST is often considered a mechanism-independent model (Bialer *et al*., 2001), it is particularly sensitive to drugs blocking sodium channels (Meldrum, 1997). This means that several clinically efficacious AEDs which act by other mechanisms (such as levetiracetam, vigabatrin, and tiagabine) and were initially not screened or detected by using MEST would have been missed using MEST as the only drug discovery model (Loscher, 2002). Furthermore, the PTZ test may possibly not be able to identify all antiepileptic drugs against non-convulsive seizures (Loscher, 2002). This is due to the fact that lamotrigine, which is very efficacious against non-convulsive seizures in patients, is ineffectual in the PTZ test, while vigabatrin and tiagabine, which are quite effective in the PTZ test, are not useful in patients and even aggravate non-convulsive seizures (Loscher, 2002). Chronic seizure models including kindling, genetic models such as Genetic Absence Epilepsy Rats from Strasbourg (GAERS) or lethargic mice have been used (Loscher, 2002). Also, even after the primary screening of anticonvulsants, advanced experiments on primate models and 'kindling in rodents' which may follow include monkey models of absence (petit mal) seizures, aluminium hydroxide-induced partial or secondary generalized (grand mal) seizures in monkeys, experimental temporal lobe epilepsy in monkeys and amygdala-kindled seizures in rats (Berlucchi, 1990).

1.4 INFLAMMATION

Inflammation is a response to cellular injury as a part of the biological response of the body's tissue to stimuli such as pathogens and infection, and this seeks to keep the immune

system safe. Inflammation can be chronic or acute. Acute inflammation is the primary reaction of the body to damaging stimuli which is attained by the enhanced movement of plasma and leukocytes from the blood into the injured tissues, with a sequence of biochemical reactions that mediate and develops the inflammatory response. The initial phase of acute inflammation consists of cellular influx coupled with the release of mediators like histamine and serotonin that is followed by the production of bradykinin and prostaglandins (Di Rosa *et al*., 1971). Acute inflammation is additionally associated with a systematic reaction known as the acute-phase response which is typified by the swift adjustment of the concentration of plasma proteins. Chronic inflammation which is mostly protracted is exemplified by the damage and restoration of the tissue from the inflammatory process (Giannoudis, 2003).

Inflammation is caused by physical factors such as burns, tremors, ionization radiations, foreign bodies, biological factors such as infections by pathogens, stress and hypersensitivity reactions and chemical causes such as toxins and alcohol. Common signs and symptoms of inflammation are pain, heat or temperature, swelling, redness, and loss of function. The redness and heat are due to increased blood flow to the site of injury in the process of vasodilation, swelling caused by the accumulation of fluid and the pain caused by the release of pain mediators such as bradykinin and histamine at the nerve endings that cause them to be stimulated. Loss of function is at the very severe stage having multiple causes (Jagadeesh, 2017).

These cardinal signs of inflammation reflect three major events of an inflammatory response which are vasodilation, increase in capillary permeability and influx of phagocytes. In vasodilation, there is an increase in the diameter of the blood vessels that carry blood away from the affected area and it constricts, resulting in engorgement of capillary networks. This is responsible for the redness and an increase in temperature. An increase in capillary permeability then occurs to facilitate the influx of fluids and cells from the engorged capillaries into tissues. The exudate has much higher protein content than the fluid that is normally released from the vasculature and this contributes to the swelling "oedema". Lastly, there is an influx of phagocytes which is facilitated by increased permeability of capillaries into the tissues. Phagocytes emigrating includes adherence of the

cells unto the cells of the endothelial walls of the blood vessels known as margination followed by movement between the capillary endothelial cells into tissues known as extravasation or diapedesis and finally their migration through the tissues to the site of injury known as chemotaxis. The phagocytic cells then accumulate at the site and phagocytise bacteria (Larsen and Henson, 1983; Gilroy *et al.*, 2004).

Traditionally, inflammation has been categorized into two. Acute and chronic inflammation. The acute inflammation is the rapid, short-lived (minutes to days). This response to an acute injury is characterized by the accumulation of fluids, plasma proteins, and granulocytic leukocytes. However, chronic inflammation is of longer duration and includes an influx of lymphocytes and macrophages and fibroblast growth (Ullah *et al*., 2014).

1.4.1 **Acute Inflammation**

Inflammation is initiated differently, and it depends on the nature and portal of entry of the foreign substance and, to some extent, the nature and condition of the individual. For instance, Pathogens can initiate inflammation through activation of the plasma protease systems by interaction with degradation products of the bacterial cell walls and by secretion of toxins that can activate the inflammatory response directly (Rahman, 2015). Injured cells themselves can release degradation products that initiate one or more of the plasma protease cascades and can augment expression of pro-inflammatory cytokines that promote the inflammatory process (Gambhire *et al.*, 2010). Irrespective of the initiating agent, the physiological changes associated with acute inflammation includes four main features. These are vasodilation, increased vascular permeability, neutrophil recruitment and activation, and fever (Ullah *et al*., 2014). Vasodilation is among the earliest physical responses to acute tissue injury. It is however preceded by brief vasoconstriction. The arterioles are the first to be involved, followed by the capillary beds, and this results in a net increase in blood flow. The increased blood flow results in the characteristic heat and redness (Frezza *et al.,* 2001).

In response to inflammatory stimuli, endothelial cells lining the venules contract, widening the intercellular junctions to produce gaps that permit passage of plasma proteins. This results in characteristic pain and swelling in the affected region (Ullah *et al*., 2014; Freeza

et al., 2001). Soon after inflammation begins, there is an invasion by phagocytes; neutrophils and macrophages and if the inflammation was provoked by pathogens, the complement system is activated and immune elements such as lymphocytes and antibodies also invade the site of injury and mount an immune response. Fever is induced as a result of the pyrogens released from leukocytes in response to specific stimuli, including bacterial endotoxin. Pyrogens act on the thermoregulatory set point of the hypothalamus, increasing body temperature (Chandra *et al.*, 2012)

The role of prostaglandins in inflammation cannot be ignored. Prostaglandins are oxidized derivatives of the fatty acid arachidonate that mediate the cardinal signs of inflammation, including fever, pain, and vascular permeability (Matsuoka *et al*., 2000). The major sources of prostaglandin in acute inflammation include mononuclear phagocytes, endothelial cells, and platelets. Prostaglandin synthesis is augmented during inflammation by several stimuli, including bacterial endotoxin, immune complexes, complement component C3a, bradykinin, and IL-1, and they mediate their pro-inflammatory effects through specific receptors (Matsuoka *et al*., 2000).

1.4.2 **Chronic Inflammation.**

When acute inflammation persists, either through incomplete clearance of the initial inflammatory focus or as a result of multiple acute events occurring in the same location, it becomes chronic inflammation (Matsuoka *et al*., 2000). In contrast to acute inflammation, which is characterized primarily by neutrophil influx, chronic inflammation includes accumulation of macrophages and lymphocytes and the growth of fibroblasts and vascular tissue. These result in tissue scarring that is typically seen at sites of prolonged or repeated inflammatory activity (Kumar *et al.*, 2009)

Among the most interesting aspect of chronic inflammation is the formation of tissue granulomas. A granuloma is a collection of inflammatory cells mostly macrophages and lymphocytes, which are eventually surrounded by a fibrotic wall that forms in tissues as part of the inflammatory response to a persistent irritant (Chensue *et al.*, 1995).

Chronic inflammatory diseases, such as asthma, COPD, rheumatoid arthritis and inflammatory bowel disease, involve the infiltration and activation of many inflammatory and immune cells, which promote the release many inflammatory mediators that interact and stimulate structural cells at the location of the inflammation. The pattern of

inflammation evidently contrasts between these diseases, with the participation of many different cells and mediators (Barnes, 2006), but all are epitomised by an increased expression of multiple inflammatory proteins, some of which are common to all inflammatory diseases, whereas others are more explicit to an actual disease.

The increased expression of most of these inflammatory proteins is regulated at the level of gene transcription through the activation of proinflammatory transcription factors, such as nuclear factor-kB (NF-kB) and activator protein-1 (AP-1). These pro-inflammatory transcription factors are activated in all inflammatory diseases and play a critical role in amplifying and perpetuating the inflammatory process. Thus, NF-kB is activated in the airways of asthmatic patients and COPD patients (Di Stefano *et al*., 2002; Mathy-Hartet *et. al.*, 2003) and is activated in the joints of patients with rheumatoid arthritis (Muller-Ladner *et al*., 2002) and the vessels of patients with atherosclerosis (Monaco *et. al.*, 2004). The molecular pathways involved in regulating inflammatory gene expression are now being delineated and it is now clear that chromatin remodelling plays a critical role in the transcriptional control of genes. Stimuli that switch on inflammatory genes do so by changing the chromatin structure of the gene, whereas corticosteroids reverse this process (Barnes, 2006).

1.4.3 **Role of Reactive Oxygen Species (ROS)**

Reactive oxygen species (ROS) are a chemically reactive chemical group containing oxygen. Examples of such species include peroxides, superoxide, hydroxyl radical, singlet oxygen (Hayyan *et al.*, 2016). Biologically, reactive oxygen species are produced as a natural by-product of the natural breakdown of oxygen and they have crucial roles in the cell signalling process and also homeostasis (Tilak *et al.*, 2004).

Oxygen-derived free radicals and their products are known to play an important role in the pathogenesis of chronic inflammatory disorders (Sapirstein and Bonventre, 2000). These activated oxygen intermediates together with secondarily formed radicals, like the hydroxyl radicals (OH^-) can destroy membrane lipids, proteins, DNA, hyaluronic acid, and cartilage (Valko *et al.*, 2007). The significance of oxygen free radicals and its associated activated

oxygen intermediates in the pathogenesis of rheumatoid arthritis has been ascertained (Valko *et al.*, 2007).

In such situations of oxidative stress, polymorphonuclear leukocytes and macrophages are activated, causing the production of inflammatory mediators including a large amount of superoxide and hydrogen peroxide (Gulluce *et al.*, 2007). These free radicals and, particularly, superoxide radicals cause cellular disturbance as a result of the peroxidation of membrane lipids (Gulluce *et al.*, 2007).

The production of free radicals is essential for normal metabolism, but they can be destructive if their activity is not controlled by intra/extracellular defence mechanisms. A range of intracellular antioxidant systems limits the toxic potential of intermediates formed during the electron reduction of oxygen to water (Sriram *et al.*, 1997). Of huge importance is the enzyme superoxide dismutase which catalyses the dismutation of O_2 to H_2O_2. Once again, the cell is protected from the potential toxicity of H2O2 either by the haeme enzyme catalase or the enzyme glutathione peroxidase. The other non-enzymatic antioxidants include reduced glutathione, vitamin E, vitamin C and uric acid (Salvemini *et al.*, 2006).

1.4.4 **Fever in inflammation**

Fever is one of the most obvious systemic manifestations of acute inflammation, especially when inflammation is linked with infection (Romanovsky *et al.*, 2005). It relies on humoral signals from the body and is coordinated by the hypothalamus involving the orchestration of a wide range of behavioural, endocrine and autonomic systems (Saper and Breder, 1994). The components of this purported acute-phase reaction include endocrine and metabolic processes. This entails the exudation of acute-phase proteins by the liver (including C-reactive protein) which increases the production of glucocorticoids and activates a stress response. This decreases vasopressin secretion, thus reducing the volume of body fluid required to be warmed (Ohsugi, 2007). Autonomically, there is redirection in blood flow from cutaneous to deep vascular beds, thus minimizing heat loss through the skin and increased pulse and blood pressure, and decreased sweating. Behavioural responses include rigors (shivering), chills (search for warmth), anorexia, somnolence, and malaise. The principal manifestation of fever is an elevation of body temperature, usually by 1 to 4°C. This elevation in temperature may improve the efficiency of leukocyte killing and probably impairs the replication of many offending microorganisms (Lehrnbecker *et al.*, 2012).

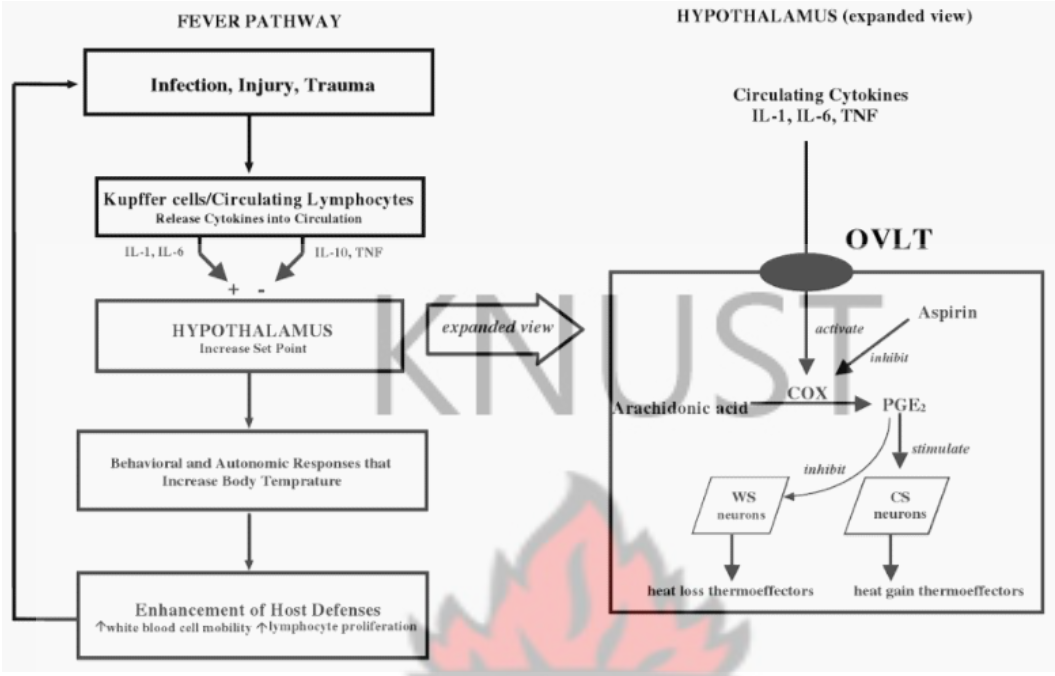

Figure 1-2 Mechanism of fever (https://epomedicine.com/clinical-medicine/fever-definition-mechanism-types/)

1.4.5 **Role of cytokines in inflammation**

A key function of macrophages is to get rid of cellular debris created during normal tissue function while following tissue injury or infection. Macrophages can respond promptly to a variety of "alarm" signals generated from inflamed sites and become triggered to release pro-inflammatory mediators (Lehrnbecker *et al.*, 2012). Cytokines play a key role in signalling a fever (Conti *et al.*, 2004). IL-1, IL-6, and TNF-alpha are produced by leukocytes (and other cell types) in response to infectious agents or immunologic and toxic reactions and are released into the circulation. IL-1 acts directly and by inducing IL-6, which has essentially similar effects in producing the acute-phase reactions. Among the cytokines, IL-1, IL-6, TNF-α, and the interferons can cause fever, therefore act as primary endogenous pyrogens (Wong *et al.*, 1996). Peripheral cytokines signal the brain through four mechanisms to cause fever (Conti *et al.*, 2004): (1) They can enter the brain through regions lacking a blood-brain barrier (specialized areas along the cerebral ventricular surface); (2) they can cross the blood-brain barrier by specific transport mechanisms; (3) they can transmit a signal to the brain via the vagus nerve; and (4) they can activate brain vasculature stimulating release of mediators such as prostaglandins (PGE), NO, or cytokines (IL-1beta), which act on brain parenchymal cells. In contrast, during more

significant sepsis, circulating cytokine levels are high, and the vascular route to brain activation becomes more prominent. Once generated, the signal is transmitted from the anterior through the posterior hypothalamus to the vasomotor centre to induce the responses (Conti *et al.*, 2004).

1.4.6 **Experimental models of acute inflammation**

There are several ways of inducing acute inflammatory episodes including injection of pro-inflammatory agents such as heat-killed *E. coli*, zymosan (Lucas *et al.*, 2003; Araico *et al.*, 2007), vasoactive agents like platelet-activating factor and histamine (Vasudevan *et al.*, 2007), arachidonic acid (Kang and Weylandt, 2008), carrageenan (Jeon *et al.*, 2008), yeast (Ushiyama *et al.*, 2008; Zakaria *et al.*, 2007), dextran (Lima *et al.*, 2007; Melgar *et al.*, 2008), and latex (Shivkar and Kumar, 2003) into various parts of the body. The effect of such injections can then be measured by responses such as the increase in foot volume produced by oedema (e.g. in the rat's paw), detection of plasma markers in skin, the local rise in skin temperature, measurement of inflammatory mediators in plasma exudates, hyperaemia, lymphocyte accumulation, monocyte infiltration, polymorphonuclear leukocyte accumulation, quantization of hemorrhage, platelet deposition and thrombosis using several techniques (Issekutz, 1989). Of these models, the carrageenan-induced acute oedema in chicks (Nonato *et al.,* 2012) was employed in this study. Carrageenan, which was used in this study, is a substance extracted from red and purple seaweeds, consisting of a mixture of polysaccharides. Widely used in the food industry, it is the agent of choice for testing anti-inflammatory drugs as it is known to be antigenic and is devoid of apparent systemic effect (Di Rosa *et al.*, 1971; Kaur *et al.*, 2004). It causes the release of more than one inflammatory mediator which makes it a useful tool in testing for anti-inflammatory effect. Carrageenan has been used because of its ability to induce an intense and reproducible inflammatory action and its sensitivity to inhibition by various anti-inflammatory drugs (Winter *et al.*, 1965; Kaur *et al.*, 2004).

1.4.7 **Experimental models of chronic inflammation**

Polyarthritis induced in the rat with Mycobacterium (Freund's adjuvant arthritis) is the most frequently studied models of chronic inflammation (Adam *et al.*, 1989; Brand, 2005; Danquah *et al.,* 2011). Adjuvant-induced arthritis (AIA) in rodents, a chronic inflammatory

disease which is depicted by permeation into the synovial membrane and it is accompanied by the destruction of the joints which resembles rheumatoid arthritis in humans (Behar and Porcelli, 1995; Kumar *et al.*, 2006). The arthritis is induced by heat-killed cells of *Mycobacterium tuberculosis* and it mimics the immunological and biochemical features of rheumatoid arthritis where self-antigens are recognized as foreign bodies (Aota *et al.*, 1996; Ramprasath *et al.*, 2006). Adjuvant arthritis in rats serves as an animal model for rheumatoid arthritis. Other models of arthritis have been developed over the past decade including the polyarthritis induced by type II collagen in rats and mice (Bajtner *et al.*, 2005; Wang *et al.*, 2007; Yanaba *et al.*, 2007), a condition resembling gout may be produced by the injection of urate crystals into the synovial fluids of joints (Kannan *et al.*, 2005; Martinon *et al.*, 2006). Chronic inflammatory reactions can be produced by subcutaneous implantation of cotton wool pellets; these may be subsequently removed and weighed to determine the extent of granulation (Khanna and Sharma, 2001). Also, injection of turpentine oil into pleural cavity or subcutaneous pouch offers a long-standing inflammatory reaction (Singh *et al.*, 2007).

1.4.8 **Anti-inflammatory drugs**

In healthy states, inflammation is self-limiting; with many cell types and tissues involved in the initiation and termination of the acute phase (Schwab and Serhan, 2006). However, inflammation often results in tissue injury due to direct destructive action or the activation of a reparative process that alters tissue functions (Schmid-Schönbein, 2006). Anti-inflammatory drugs are substances that reduce swelling and inflammation. The anti-inflammatory drugs specifically the Nonsteroidal Anti-inflammatory Drugs (NSAIDs) are a chemically diverse class of drugs having antipyretic, analgesic and anti-inflammatory properties that work by inhibiting the fatty acid cyclooxygenases COX-1 and COX-2 responsible for the initiation of the biosynthesis of prostaglandins and thromboxane that create inflammation in a whole thus the NSAIDs reduce or eliminate pain.

The NSAIDs are a chemically diverse class of drugs that have anti-inflammatory, analgesic, and antipyretic properties. They are among the most frequently prescribed drugs **(**Freeza *et al.*, 2001). They contain carboxylic acid including salicylate derivatives (acetylsalicylic acid), Propionic acid derivatives (ibuprofen, ketoprofen, flurbiprofen), phenylacetic acid derivatives (diclofenac), and heterocyclic and carboxylic acid derivatives (indomethacin).

These drugs act by binding to the receptors of the cyclooxygenase (COX) enzyme thereby having an inhibitory effect on COX. Since this COX is responsible for the synthesis of prostaglandins, thromboxane (which are pain and inflammatory mediators from arachidonic acid), the inhibitory action of these drugs prevents the synthesis of pain and inflammatory mediators (Freeza *et al*., 2001). Despite the anti-inflammatory potential of NSAIDs, the non-selectivity of some of them and the side effect on long term use poses a threat to users. For instance, COX 1 is involved in the protection of the gastrointestinal tract (GIT), platelet function, kidney function and the regulation of blood flow, however, COX 2 is involved in the mediation of pain, inflammation, and fever. Hence, NSAIDs which inhibits COX non-selectively poses adverse side effects such as platelet dysfunction, gastric ulcer, renal impairment among others (Adinortey *et al.*, 2013).

Corticosteroids are the most valuable anti-inflammatory therapy for various chronic inflammatory diseases, such as asthma but are relatively ineffectual in other disorders such as chronic obstructive pulmonary disease (COPD) (Barnes, 2006). Chronic inflammation is marked by the amplified expression of several inflammatory genes that are regulated by proinflammatory transcription factors, such as nuclear factor-kappa B and activator protein-1, that bind to and activate coactivator molecules, which then acetylate core histones to switch on gene transcription (Barnes, 2006). The drugs (corticosteroids) repress the various inflammatory genes that are triggered in chronic inflammatory diseases. They mostly do this by reversing histone acetylation of the activated inflammatory genes through binding of liganded glucocorticoid receptors (GR) to coactivators and recruitment of histone deacetylase-2 (HDAC2) to the activated transcription complex (Barnes, 2006; Barnes, 2009). At higher corticosteroid concentrations, the glucocorticoid receptor homodimers also interact with DNA recognition spots to activate transcription of anti-inflammatory genes and to hinder transcription of several genes. This is linked to observed corticosteroid side effects (Barnes, 2006). In patients with COPD and acute asthma, and in asthmatic patients who smoke, the expression and activity levels of histone deacetylase-2 is significantly reduced due to oxidative or nitrative stress, as such, the inflammation associated with such conditions develops resistance to the actions of corticosteroids administered (Barnes, 2006). The corticosteroids principally used therapeutically are dexamethasone, hydrocortisone, and prednisone (Barnes, 2006).

The new biologic response modifiers infliximab and etanercept are proving to be extremely effective for some people whiles others fail to respond to them (Efthimiou and Markenson, 2005; Mahajan *et al.*, 2006; Moreland, 2004; Ruderman and Pope, 2006). Studies show that these new treatments are more effective at slowing joint damage when used in combination with methotrexate, a conventional disease-modifying antirheumatic drug (DMARD), than methotrexate alone (Fleischmann *et al.*, 2005). Combination treatment with etanercept and methotrexate or infliximab and methotrexate has been found even more effective than either of the new treatments alone (Hisadome *et al.*, 2004; Nordstrom *et al.*, 2006). Current therapies are often effective at relieving symptoms, although this benefit is attended by a significant risk of toxicity (Ruderman, 2005). It is, therefore, necessary to develop new agents that are effective for preventing joint destruction, as well as synovial inflammation, in rheumatoid arthritis which will be less toxic.

1.5 PAIN

Pain is termed as an obnoxious sensory and emotional experience related to actual or potential tissue damage or described in terms of such damage (Merskey *et al.*, 1994). It is usually the main factor that alerts the patient to seek medical care and forms a good component of most diseases (Schim and Stang, 2004). Pain can also be described as "whatever the experiencing person says it is, existing whenever s/he says it does" (Herr *et al.*,2006). This indicates that pain is subjective and as such, the one going through the pain is the only dependable indication of the intensity and quality of the pain (Clancy and Mcvicar, 1992; Carr *et al.*, 1997).

Pain has many forms. It warns against damage to the body, which is important for avoiding injuries and consequently for survival. The way of pain is received varies from time-to-time in the same individual. The intensity of pain is difficult to measure, and an individual's perception of pain depends on his emotional state, circumstances under which the pain was acquired, and whether it is perceived as a threatening signal. Pain perception depends on factors such as arousal, attention, distraction, and expectation (Świeboda *et al.*, 2013). The negative impact of pain on the quality of life is huge as it affects several aspects of health and well-being including relationships, cognitive abilities and the capacity to work (Wilhelm *et al.*, 2009). Despite the frequency of pain symptoms, individuals often do not obtain satisfactory relief of pain and the reason for this is attributable to inappropriate or

insufficient use of existing remedies (McMahon and Koltzenburg, 2006; Chen and Tang, 2011).

Pain and inflammation are important components of the body's immune system which help in preventing physical injury. Therefore, individuals with congenital insensitivity to pain are prone to serious health conditions such as corneal scarring, auto-amputation, and self-mutilation (Axelrod *et al.*, 2003). The behavioural changes produced by sickness are like those induced by pain. There is a clear-cut similarity existing between the functional consequence of pain and febrile illness. Thus, just as analgesia may be an important component of an animal's defensive behavioural repertoire so too may pain be an important component of an animal's recuperative behavioural repertoire. With pain now considered the fifth vital sign, its assessment is equally important as obtaining a patient's temperature, pulse, blood pressure, and respiratory rate (Chapman, 2005). It is therefore important that the needed assessments and appropriate pain-relieving treatments are put in place for patients who are unconscious, developmentally delayed children, people who have impaired communication skills, as well as patients who are non-native to caregivers' language and culture as all these people, cannot communicate effectively the complexities involved in their pain experiences (Craig, 2006; Craig, 2009).

1.5.1 **Classifications of pain**

Different classifications of pain exist. Pain has been classified by anatomic location, body system, duration, severity, frequency, and aetiology (Cole, 2002). However, the most common categorization of pain is according to its aetiology, duration, and location.

1.5.1.1 ***Classification based on aetiology***

This categorisation is the most common and it classifies pain as nociceptive, inflammatory or neuropathic according to their pathogenesis and is displayed in Fig. 1-3 on page 32.

1.5.1.1.1 Nociceptive pain

Nociception is defined as the neural processes of encoding and processing noxious stimuli and this activity is initiated by nociceptors, (also called pain receptors), that can detect mechanical, thermal or chemical changes, above a certain specified threshold. Once

stimulated, a nociceptor transmits a signal along the spinal cord to the brain (Loeser and Treede, 2008). Nociceptive pain is the type of pain that arises from the stimulation of such nociceptors from somatic and visceral structures. It occurs when pain receptors found on non-damaged tissues are triggered in the absence of sensitization. It causes withdrawal reflexes which prevent further tissue damage. The pain produced is highly constant, well-localized and often throbbing or aching (Smith, 2003). The sensory input is mediated through nociceptors such as 5-HT_3, bradykinin and vanilloid receptors coupled to excitatory, cation-permeable ion channels. This causes sensitization of nocisponsive peripheral afferent fibres by the engagement of intracellular transduction systems including the activation of adenylyl cyclase and phospholipase C leading to a further increase in the intensity and duration of nociceptive impulse discharges (Mcnally, 1999). Nociceptive pain can be described in terms of visceral (the pain of organs, in the thoracic or abdominal cavities), superficial (activation of nociceptors in the skin or other superficial tissue), somatic (experienced in the skin, muscles, bones, and joints) or deep somatic pain (stimulation of nociceptors in ligaments, tendons, blood vessels, fasciae). This distinction is important because it does not only reflect the cause of pain but also it gives an insight into its treatment (Cohen and Mao, 2014).

1.5.1.1.2 Inflammatory Pain

Inflammatory pain mostly occurs as a result of exaggerated sensitivity to pain due to injury. A feature of this type of pain is the development of hypersensitivity such that stimuli, which would normally be innocuous, begins to do so resulting in the production of a broad range of inflammatory mediators by inflammatory cells. These mediators act on and alter the properties of high-threshold primary-sensory neurons. The three major features underlying this type of pain are peripheral sensitization (the peripheral sensitivity of high-threshold nociceptors is altered), phenotypic switch (changes in properties and function of the chemical makeup of neurons) and central sensitization (up-regulation in the sensitivity of the neurons in the CNS) (Woolf, 2004).

Inflammatory pain results from the activation and sensitization of nociceptors by inflammatory mediators, caused for example, by an inflammatory synovial response to cartilage damage of the facet joint (Grace *et al.*, 2014). Elevated levels of inflammatory cytokines (e.g. interleukin [IL]-1 or IL-6), as well as increased capsular vascularization

and inflammatory cells, are present in degenerate facet joints (Inquimbert *et al*., 2012). Several inflammatory markers (e.g. IL-1α, tumour necrosis factor [TNF]-α, transforming growth factor-β) have been found in herniated discs, and increased levels of discogenic cytokines correlate with increased pain levels. Tissue damage or inflammation results in local release of the intracellular content of injured cells and of inflammatory signaling molecules from immune cells, such as prostaglandins, growth factors (e.g. nerve growth factor [NGF]) and cytokines (IL-6, IL-1β, TNF-α) (Julius and Basbaum, 2001; Gold and Gebhart, 2010).

Peripheral inflammation induces not only changes in the nociceptor but also in the central nervous system. An example is a marked increase of COX-2 in spinal cord neurons after peripheral inflammation in response to systemically acting cytokines such as IL1-β and this seems key to the development of mechanical hyperalgesia in the inflamed anatomical area, whereas the local expression of COX-2 at the inflamed site drives heat hypersensitivity. COX-2 inhibitors with well documented blood-brain barrier penetration (e.g. celecoxib) might, therefore, be more efficacious in conditions with marked mechanical inflammatory pain hypersensitivity due to such a central COX-2 induction (Vardeh *et al.,* 2016).

1.5.1.1.3 Neuropathic Pain

Neuropathic pain is caused by abnormal signal processing in the central or peripheral nervous system (Kaiko *et al.*, 1983). This type of pain is mainly a chronic syndrome associated with damage to or an alteration in the function of the somatosensory systems which are involved in the pain signalling (Treede *et al.*, 2008). Particularly, neuropathic pain suggests nervous system injury or damage. Popular triggers of neuropathic pain comprise trauma, inflammation, metabolic diseases (e.g., diabetes), some infections (e.g., herpes zoster), tumours, toxins, and primary neurological diseases (Woolf, 2004). Some examples of this type of pain overlap with those that are responsible for inflammatory pain, but many of them are quite distinct and the distinction is important because different approaches will be required for their management (Woolf, 2004). In recognition of the prominent inflammatory component in neuropathic pain, several inflammatory markers have been identified as potential therapeutic targets. As an example, TNF-a is elevated in the periardicular epidural fat in patients with radiculopathy from herniated disc disease

(Olmarker *et al.*, 2004) and infusion with a TNF-α neutralizing antibody is reported to result in pain reduction for up to 3 months in patients with severe sciatic pain due to disc herniation (Mulleman *et al.*, 2004).

Neuropathic pain can be broadly categorized as peripheral or central in origin (Kaiko, *et al.*, 1983). These categories can be further sub-classified as painful peripheral mononeuropathy and polyneuropathy, deafferentation pain, sympathetically maintained pain, and central pain. (Chen, 2004). Neuropathic pain is sometimes called "pathologic" pain because it serves no purpose (Woolf, 2004). A chronic pain state may occur when pathophysiologic changes become independent of the inciting event (Portenoy, 1996). Sensitization plays an important role in this process and although central sensitization is relatively short-lived in the absence of continuing noxious input, nerve injury triggers changes in the CNS that can persist indefinitely (Covington, 2000). Thus, central sensitization explains why neuropathic pain is often disproportionate to the stimulus, examples being hyperalgesia (increased sensitivity to pain) and allodynia (pain due to a stimulus that's not usually painful) or occur when no identifiable stimulus exists (e.g., persistent pain, pain spread). Neuropathic pain could be continuous or episodic and is apparent in many ways (e.g., burning, numbness, prickling, shooting, electric shock-like, stabbing, squeezing, deep aching, spasm, or cold) (Haddox *et al.*, 1997).

1.5.1.1.4 Functional Pain

This is described as hypersensitivity to pain resulting from altered function of the nervous system or abnormal central processing of normal input. In this type of pain, there is no clear peripheral pathology, nor can any pathology in the nervous system itself be identified. There is also no obvious lesion to either the peripheral or central nervous system and yet the patient experiences pain. Some examples of this type of pain are the myofascial syndromes, fibromyalgia, non-cardiac chest pain, tension-type headache and gastrointestinal hypersensitivity disorders such as irritable bowel syndrome (Woolf, 2004).

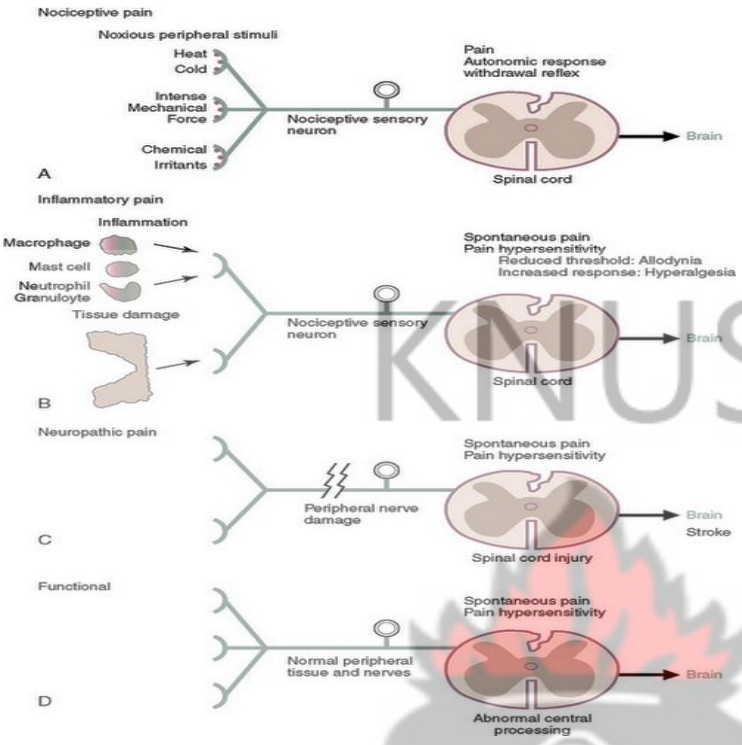

Figure 1-3 Principal categories of pain based on aetiology (courtesy Woolf, 2004)

1.5.1.2 *Classification based on the duration*

Cole (2002) classified pain under duration as acute or chronic.

1.5.1.2.1 Acute Pain

Acute pain was previously described merely in terms of duration. It is presently thought of as a "complex, unpleasant encounter with emotional and cognitive, as well as sensory, elements that occur in response to tissue trauma" (Berry *et. al.*, 2006). Acute pain lasts briefly and typically displays in ways that can be simply explained and examined. It can be described as achieving physiological roles by its intrinsic protecting nature (Millan, 1999). Though short-lasting, it is felt instantly after injury and is very severe in intensity (Thienhaus and Cole., 2002). In contrast to chronic pain, relatively high levels of pathology usually accompany acute pain, and the pain resolves with the healing of the underlying injury. Acute pain is mostly nociceptive but may well be neuropathic. Common sources of

acute pain include trauma, surgery, labour, medical procedures, and acute disease states. Acute pain serves an important biological function, as it warns of the potential for or extent of the injury. Several protective reactions (e.g., withdrawal of a damaged limb, muscle spasm, autonomic responses) often complement it. However, the "stress hormone response" prompted by acute injury also can have adverse physiologic and emotional effects (Jacox *et al.* 1994). Even brief intervals of painful stimulation can induce suffering, neuronal remodelling, and chronic pain; associated behaviours (e.g., bracing, abnormal postures, excessive reclining) may further contribute to the development of chronic pain. Therefore, increasing attention is being focused on the aggressive prevention and treatment of acute pain to reduce complications, including progression to chronic pain states (Dworkin, 2001). The intensity of acute pain is from mild to severe and lasts less than 3 to 6 months (Calvino *et al.*, 1992; Schim and Stang, 2004; Abdel-Salam and El-Batran, 2005).

1.5.1.2.2 Chronic Pain

Chronic pain was once defined as pain that extends 3 or 6 months beyond onset or beyond the expected period of healing (Turk and Monarch, 2002). However, new-found classifications distinguish chronic pain from acute pain focused on more than just time. Chronic pain is now recognized as pain that extends beyond the period of healing, with levels of identified pathology that often are low and insufficient to explain the presence and/or extent of the pain (Sheir-Neiss *et al.*, 2003). Chronic pain is also defined as persistent pain that "disrupts sleep and normal living, ceases to serve a protective function, and instead degrades health and functional capability" (Chapman and Stillman, 1996). This type of pain is often portrayed as pain that persists beyond the usual healing time of a particular sickness and as such, lacks the severe warning function associated with physiological acute pain (Treede *et al.*, 2015). Therefore, unlike acute pain, chronic pain serves no adaptive purpose.

Chronic pain may be nociceptive, neuropathic, or both and caused by injury (e.g., trauma, surgery), malignant conditions, or a variety of chronic non-life-threatening conditions (e.g., arthritis, fibromyalgia, neuropathy). In certain cases, chronic pain occurs anew with no apparent cause. Although injury often initiates chronic pain, factors pathogenetically and physically remote from its cause may perpetuate it (Turk and Monarch, 2002).

Environmental and affective factors also can exacerbate and perpetuate chronic pain, leading to disability and maladaptive behaviour.

1.5.1.3 *Classification based on Location*

Pain classification due to location involves schemes that relate the pain to the specific anatomy and/or body system known to be involved. Anatomic pain identifies sites of pain as viewed from a regional perspective such as lower back pain, headache, pelvic pain among others. However, the body system pain focuses on classical body systems including neurologic, vascular musculoskeletal pain. This classification system based on location is one-dimensional (why or where does the patient hurt) and may eventually fail to sufficiently explain the neurophysiology of the pain (Cole, 2002).

1.5.2 **Neurophysiology of pain**

1.5.2.1 *Central transmission of pain*

The central system of the physiological state of analgesia entails three key components: the periaqueductal grey matter, the nucleus raphe magnus and the nociception inhibitory neurons in the dorsal horns of the spinal cord, which act to impede nociception-transmitting neurons which is also situated in the spinal dorsal horn (Andary *et al.*, 1997). There are three types of fibres that carry pain signals to the brain namely, Aβ, Aδ, and C-fibres. The Aβ, Aδ are evolutionarily modern fibres that are myelinated (insulated) and carry nerve impulses rapidly to the cortical regions of the brain (Andary *et al.*, 1997). Pain transmission through the spinal cord involves the lateral spinothalamic tract pathway. The lateral spinothalamic tract encompasses two paths for nociceptive information to get to the brain: the neospinothalamic tract for "fast spontaneous pain" and the paleospinothalamic tract for "slow increasing pain".

The neospinothalamic tract is responsible for fast pain which travels through type Aδ fibres to terminate on the dorsal horn of the spinal cord where they synapse with the dendrites of the neospinothalamic tract. The axons of those neurons move up the spine to the brain and traverse the plane through the anterior white commissure. This then goes upwards in the contralateral anterolateral columns and ends on the ventrobasal complex of the thalamus. At this stage, it synapses with the dendrites of the somatosensory cortex. The paleospinothalamic tract is involved in slow pain transmission via slower type C

fibres to laminae II and III of the dorsal horns, together known as the substantia gelatinosa. Impulses are then transmitted to nerve fibres that terminate in lamina V, also in the dorsal horn, synapsing with neurons that join fibres from the fast pathway, crossing to the opposite side through the anterior white commissure, and travelling upwards through the anterolateral pathway. These neurons terminate throughout the brain stem, with a tenth of fibres halting within the neural structure of the thalamus and also the rest terminating within the medulla, pons and periaqueductal gray of the midbrain tectum (Millan, 2002; Kivell and Prisinzano, 2010). Neurotransmitters including aspartate and glutamate which are excitatory amino acids, as well as substance P, are released by Aδ and C fibres to activate dorsal horn neurons which contain pharmacologically ionotropic glutamate receptors such as AMPA, kainite and NMDA receptors (Traynelis *et al.*, 2010). The binding of glutamate activates these receptors to allow the inflow of Na^+, K^+, and Ca^{2+} resulting in an excitatory postsynaptic current. This depolarizing may trigger an action potential which propagates the excitatory signals along the axon ascending to supraspinal structures. Inhibitory neurons in the dorsal horn are also activated by the firing of the Aδ, C and Aβ fibres. After stimulation, gamma-aminobutyric acid (GABA) – the main inhibitory neurotransmitter in the CNS, is released by the inhibitory neurons to modulate nociception by interacting with GABA receptors in the projection neurons and the primary afferents.

The $GABA_A$, $GABA_B$, and $GABA_C$ are the gamma-aminobutyric acid (GABA) receptor subtypes. It is established that activation of $GABA_C$ receptors induces anti-nociception (Reis *et al.*, 2007). The $GABA_C$ receptors, however, are mainly expressed in the retina and play an important role in visual signalling (Qian and Ripps, 2009). After binding to $GABA_A$ and $GABA_C$, chloride-permeable ion channels are activated to hyperpolarize neurons and to impair the propagation of excitatory signals. Activation of $GABA_B$ receptors results in an increase in K^+ conductance resulting in cell hyperpolarization (Bormann, 2000; Chen *et al.*, 2005). Triggering of $GABA_A$ and probably $GABA_C$ receptors generate inhibitory postsynaptic potentials while $GABA_B$ receptors have a role in both postsynaptic and presynaptic inhibition (Yang *et al.*, 2001; Lemke, 2007; Labrakakis *et al.*, 2009).

1.5.2.2 *Peripheral transmission of Pain*

Nociceptors are neurons which are located peripherally and are preferentially sensitive to a noxious stimulus or to a stimulus that would become noxious if prolonged. (Dubin and Patapoutian, 2010). They are distributed broadly in the skin and deep tissues. Some of these nociceptors are activated by a specific type of noxious stimulus which could be mechanical, thermal or chemical whereas most of them are activated by multiple types of noxious stimuli. The two main types of nociceptive fibres involved in pain transmission are Aδ and C. Aδ fibres produce a well-defined and sharp pain which is stimulated usually by a physical blow, an electrical shock or a cut. Due to myelination of the Aδ fibres, an action potential can pass through them towards the central nervous system at a faster rate of 20 m/s. The fast transmission through Aδ fibres causes the withdrawal of the affected part of the body before pain is perceived (Julius and Basbaum, 2001).

1.5.2.3 *Inflammatory mediators of Pain*

Tissue or nerve injury leads to the production and release of several chemical mediators including bradykinin, serotonin and prostaglandins, endothelin, substance P, sympathetic amines and nerve growth factors (NGF) which can originate locally or from cells that infiltrate the site of injury. A mixture of these agents known as inflammatory 'soup' contributes to changes in vascular permeability, resulting in erythema and oedema. These mediators facilitate the electrical activity of the neuronal membrane by acting on neuronal receptors directly resulting in the activation of some molecular mechanisms. It is normally recognized that the inflammatory mediators trigger G-protein coupled receptors to cause the activation of the enzyme adenylate cyclase which in turn lead to the production of cAMP. The activation of cAMP A triggers a group of protein kinases A and C leading to the phosphorylation of ion channels in the membrane. This subsequently facilitates inward sodium current by tetrodotoxin-resistant Na^+ channels and inward Ca^{2+} currents albeit outward K^+ currents are inhibited. This is a basic peripheral mechanism that may account for the hyper nociception induced by the stimulation of sub-threshold chemical, thermal, or mechanical stimuli (Verri Jr *et al.*, 2007; Linley *et al.*, 2010; Schaible *et al.*, 2011). The inflammatory 'soup' also sensitizes peripheral nociceptors primarily C fibres by initiating a cascade of events that change ionic conductance of the peripheral nociceptor terminal. The nociceptive input is further processed spinally (Ito *et al.*, 2001).

1.5.2.4 ***Role of cytokines***

Cytokines are little secreted proteins discharged by cells have a specific impact on the interactions and communications between cells. Proinflammatory cytokines are generated largely by activated macrophages and are implicated in the up-regulation of inflammatory reactions. There is abundant evidence that certain pro-inflammatory cytokines such as IL-1β, IL-6, and TNF-α are involved in the process of pathological pain (Zhang *et al.,* 2007). Cytokines are important mediators of peripheral sensitization. Intradermal administration of cytokines such as keratinocyte-derived chemokine (KC), TNF-α, interleukins IL-1β, IL-8, IL-12, IL-15, and IL-18 have produced intense and sustained mechanical sensitization and hyper nociception in rodents (Stein *et al.*, 2009). Previous studies had led to the proposal of the following sequence of events in hyper nociception: in the first place, TNF-α is stimulated by carrageenan, lipopolysaccharide or the antigen itself which, in turn, induces IL-1β and IL-6, thus activating the synthesis of COX products (PGE_2). Again, TNF-α can induce another cytokine, IL-8, thus stimulating the local production of sympathetic amines which subsequently produces hyper nociception. Also, IL-18 and IL-12 activate endothelin-1 by resulting in hyper nociception (Ferreira, 1993; Sugamura *et al.,* 1996). The figure below (Fig. 1-4) shows mechanical hyper nociception in rats is mediated by inflammatory stimulus-induced cytokine cascades.

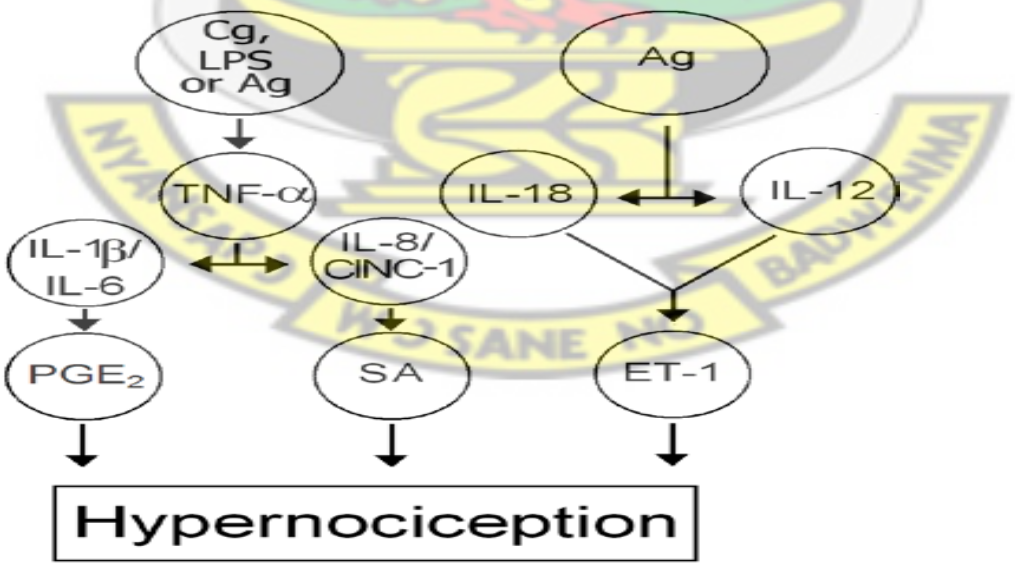

Figure 1-4 Inflammatory mediators coordinated release initiated by different stimuli. Cg (carrageenan), Ag (antigen), CINC-1 (cytokine-induced neutrophil chemoattractant-1), IL (Interleukin), ET-1 (endothelin-1), LPS (lipopolysaccharide), SA (sympathetic amines) PGE_2 (prostaglandin E_2) culled from (Verri *et al.*, 2007).

1.5.3 **Experimental Models of Pain**

There is at least four basic experimental animal pain model described by Mogil (2009). These are nociceptive, inflammatory, neuropathic and clinically oriented pain models.

1.5.3.1 ***Nociceptive models of Pain***

To gain deeper insight and understanding into the mechanism of pain in order to develop safer and more effective analgesics, various animal models have been developed to elucidate the mechanisms of these painful states and to gauge analgesics for exploring effective treatment. The animals' response to noxious stimuli either through reflex or voluntary behaviours are employed as indicators of pain. Nociceptive pain models are grouped into two based on the site of application of the noxious stimuli as visceral or somatic pain tests (Millan, 1999; Niyom, 2013).

1.5.3.1.1 Visceral Pain Models

Many visceral pain models have been developed to assess nociceptive responses in the viscera of animals. The writhing test was the earliest attempt to induce visceral pain in animals by intraperitoneal injection of irritants, such as acetic acid (Siegmund *et al.*, 1957; Singh *et al.*, 1983). After the administration of such irritants, the number of abdominal constrictions (writhing) which is known as a nociceptive behaviour are counted for a period. This method, however, non-selective on the viscera and causes suffering of the animals involved. Consequently, newer methods were invented to apply a finite noxious stimulation specific to each organ such as colon, urinary bladder, stomach, uterus, and ovary (Ness *et al.*, 2001; Christianson *et al.*, 2007).

1.5.3.1.2 Somatic Pain Models

Some tests considered as somatic pain models include the tail-flick and hot plate test which were developed to measure pain sensation in rats (D'Amour and Smith, 1941; Le Bars *et al.*, 2001). In the tail-flick test, radiant noxious heat is stimulated on the tail of the animals and

this leads to a withdrawal reflex by the defensive movement of the tail and the reaction time is noted as tail-flick latency. The hot plate test employs a heat source of stimulus in pain induction. The paw of the animal is heated by contact with a hot plate (50 – 56 °C) and the latency time to paw licking or withdrawal is measured (Kamal and Abdulrahman, 2002). A similar approach to do this is by immersing the tail into hot water known as a tail-immersion test (Sewell *et al.*, 1976; Statile *et al.*, 1988). A closely related test involves the direction of a high-intensity radiant beam of light at the hind paws of freely moving rodents and the reaction time recorded (Hargreaves *et al.*, 1988). Also, in attempting to improve the specificity, reliability, and sensitivity in the assessment of pain, a number of mechanical threshold testing devices in animals have been developed where these devices apply an increasing measurable pressure at the tail or paw until withdrawal reflex is observed (Hargreaves *et al.*, 1988). The Randall Selitto test employs the application of linearly increasing pressure (range: 0 – 450 g) between the third and fourth metatarsals of the hind paw using a blunt Perspex cone until the rat vocalises or withdraws the paw. This value is recorded as the threshold for withdrawal from a mechanical stimulus (Ito *et al.*, 2001).

1.5.3.2 ***Inflammatory Models of Pain***

The aim of the models of this category is to induce a painful condition that mimics inflammatory pain. The pain arising from acute inflammation act as a physiological function to prevent further tissue damage and it mostly ceases after removal of the noxious stimulus. Chronic inflammatory pain, however, happens when healing persists beyond the expected time, due to an ongoing inflammatory process. These models have helped researchers to understand the underlying mechanism of inflammatory pain and as such provides a guide in the development of potential treatments. Chronic inflammatory conditions produce a state of hyperalgesia that is evident from a few hours to days after peripheral injection of chemical irritants such as carrageenan, dextran, formalin, yeast, turpentine, CFA, and mustard oil (Northover and Subramanian, 1962; Di Rosa *et al.*, 1971; Shivkar and Kumar, 2003). To induce inflammation, irritating substances or the inflammatory mediators such as beer yeast croton oil, complete Freund's adjuvant (CFA), formalin, or carrageenan is injected into the body part of an animal such as the hind paws (Chipkin *et al*., 1983; Gilfoil et al., 1963; Winter and Flataker, 1965). Generally, tissue inflammation lowers the nociceptive threshold, and/or reduces the latency period of paw

withdrawal. The Formalin test also considered as a persistent model of animal nociception employs injection of a dilute solution of formalin (1–5%) into the hind paw. This produces two distinct phases of pain-like behaviour. The first phase results from activation of primary afferent fibres, while the second is believed to represent both inflammation-evoked sensory activity and facilitatory processes in the spinal cord resembling prolonged injury-induced hyperalgesia states (Millan, 1999; Ito *et al.*, 2001; Kamal and Abdulrahman, 2002).

1.5.3.3 ***Neuropathic Pain Models***

Several chemotherapy-induced peripheral neuropathy models have been developed and are used to study the underlying mechanisms of neuropathic pain. Although all these models feature some degree of direct neuronal damage, they differ in the time course and pathophysiology that is associated with the development of hyperalgesia. Examples include pain associated with traumatic injury – phantom limb pain after amputation (Kim and Chung, 1992; Decosterd and Woolf, 2000), chemically induced nerve damage – neuropathy caused by cancer chemotherapies. An example of the chemotherapy-induced neuropathic pain is vincristine-induced neuropathy. The development of vincristine-induced neuropathy is a formidable clinical problem as it is stubbornly resistant to existing pharmacotherapy, such as the NSAIDs, opioids, and steroids (Higuera and Luo, 2004). Other experimental animal models for neuropathic pain, such as diabetic neuropathy, inflammatory neuropathy, nerve-injured neuropathy, and neuropathic herpes pain share common characteristics with vincristine-induced neuropathy. For experimental animal models for diabetic neuropathy, streptozotocin-induced diabetic animals are used and also use is made of spontaneous diabetic BB/Wor rat (Courteix *et al.*, 1998; Zhang *et al.*, 2002).

There are three major experimental animal models for the nerve-injured neuropathy, Seltzer model, Bennett model, and Chung model. In Seltzer model also known as the partial ligation model, half of the sciatic nerve is tightly ligated, while the whole sciatic nerve is loosely and constrictively ligated in the Bennett model also known as the chronic constriction injury model. In contrast, the segmental spinal nerve (especially L5 or L6) is ligated in the Chung model (Mizoguchi *et al.*, 2009).

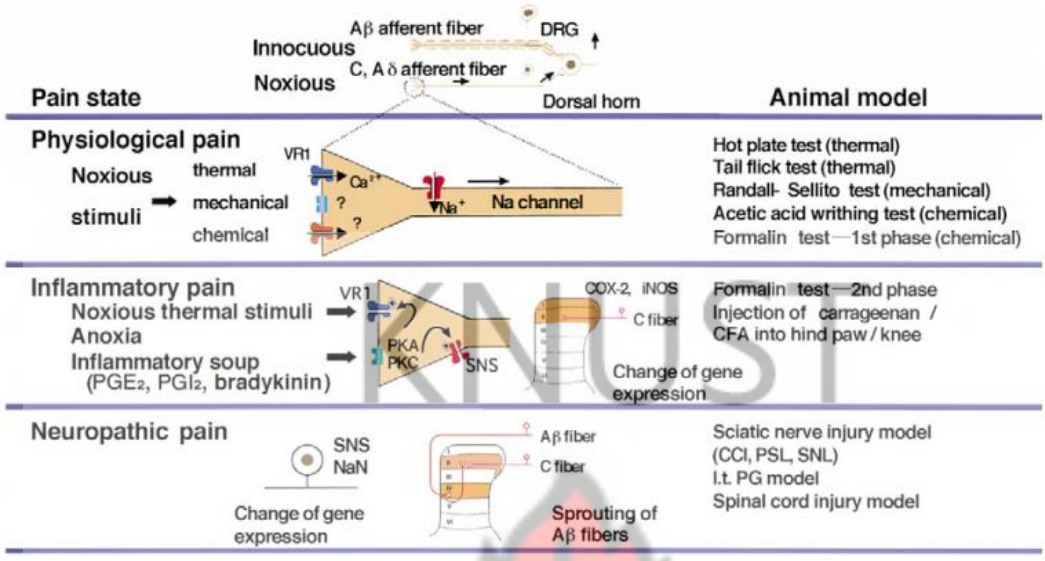

Figure 1-5 Summary of pain states and animal models of assessment (Ito *et. al.*, 2001)

1.5.4 **Management of Pain**

Strategies for pain management or treatment can be broadly categorized as pharmacologic and nonpharmacologic. The correct diagnosis and proper treatment of pain is an important public health concern and it requires the intervention of all disciplines in a holistic approach because pain affects the patient's physical, psychological, social, and spiritual well-being (Bryan, 2012).

1.5.4.1 ***Non-pharmacological management of pain***

The optimum pain management similarly includes mental, physical rehabilitative, and in certain cases, surgical treatment. As part of the management of acute pain, recommendation is made for cognitive-behavioural approaches, examples being, patient education, simple relaxation, imagery, hypnosis and physical therapeutic agents and modalities via superficial heat or cold, massage, exercise, immobility, electroanalgesia and virtual reality (Hoffman *et al.*, 2001; Gillanders, 2012). These strategies should, however, supplement, but not totally replace, the use of medications. In addition to augmenting the pain-mitigating outcomes of analgesics, non-pharmacologic approaches offer other advantages. This includes the ability to improve mood, reduce anxiety, increase a patient's sense of control, strengthen coping abilities, assist with sleep, relax muscles, and ultimately improving the quality of life (Dysvik, 2005). Physical back pain management strategies can be divided into passive therapies (e.g. manipulation and massage) and active therapies like patient-directed

exercise, stretching, and core-stability techniques. There is some evidence for such therapies working in the short to medium-term (Kizhakkeveettil *et al.*, 2017). Factors that influence the choice of a nonpharmacologic approach to pain management include the pain type, duration, and severity; the patient's preferences, coping skills, and capabilities; family and community and cost.

1.5.4.2 *Pharmacological management*

Pharmacological pain relief in clinical practice often represents an empirical journey up an analgesic ladder (nonsteroidal anti-inflammatory drugs, ''neuropathic'' agents, other adjunct medications including antiepileptic drugs, and finally opioid medications), and is more often influenced by patient-centred factors rather than targeting a pain mechanism. Due to difficulties in identifying mechanisms clinically, one strategy is to postulate the mechanism based on pharmacological response (*ex juvantibus)* (Vardeh *et al.*, 2016).

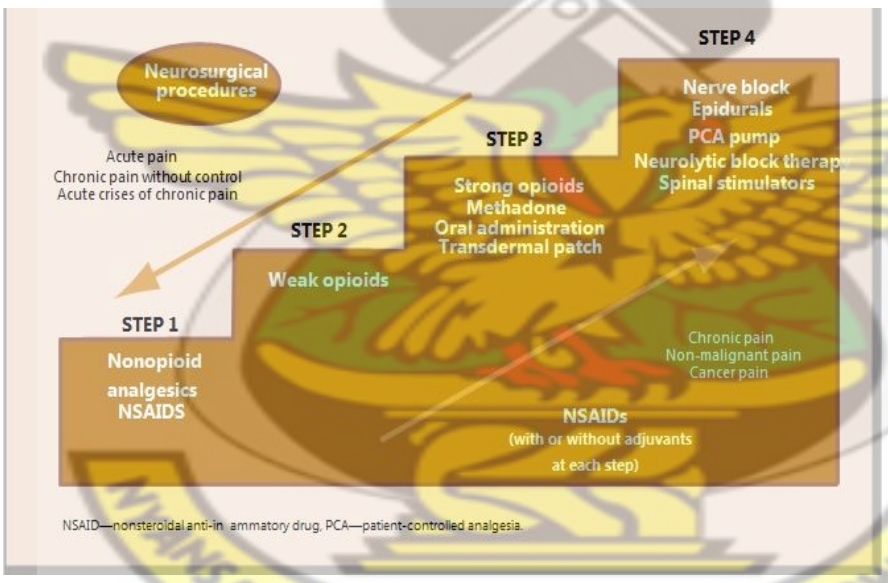

Figure 1-6 New adaptation of the WHO analgesic ladder (culled from Vargas-Schaffer, 2010).

1.5.4.2.1 Non-Steroidal Anti-Inflammatory Drugs (NSAIDs)

The primary mechanism of action of NSAIDs is the inhibition of the enzyme cyclooxygenase (COX) which blocks prostaglandin synthesis (Coyle and Layman-Goldstein, 2005). The analgesic effect of NSAIDs is prompt, effects are usually seen within few minutes to hours, whereas the anti-inflammatory effect may take longer time,

sometimes after about one to two weeks or even longer (Murray and Brater, 1993). This longer-lasting impact can indirectly alleviate some pain by diminishing tissue swelling. The somewhat recent discovery that COX has two isoforms, COX-1 and COX-2, has advanced NSAID pharmacology. COX-1 is constitutively expressed in nearly all normal tissues but plays a particularly important role in the gastrointestinal (GI) tract, kidneys, and platelets; COX-1 primarily produces prostaglandins with beneficial effects (e.g., control of blood movement to the gastric mucosa and kidneys). In contrast, COX-2 is normally not present but may be induced in response to inflammatory stimuli; COX-2 primarily produces prostaglandins that activate and sensitize nociceptors (NPC, 2001).

Although effective in relieving pain and inflammation, traditional nonsteroidal anti-inflammatory drugs (NSAIDs), are associated with a significant increase in the risk for gastrointestinal haemorrhage, ulcers, and renal damage, because of the non-selective inhibition on cyclooxygenase (COX)-1 and (COX)-2 (Samad *et al.*, 2001; Wong ***et al.***, 2013; Carter *et al.*, 2014). The recently introduced COX-2 selective inhibitors (or "coxibs") selectively inhibit COX-2 without affecting COX-1 at therapeutic doses. Thus, these offer the advantage of efficacy comparable to that of nonselective NSAIDs, with a reduced risk of certain gastrointestinal side effects but have also been reported to precipitate adverse cardiovascular events with chronic use and higher doses. The coxibs affect COX-2 both centrally and peripherally (Clemett and Goa, 2000; Solomon *et al.*, 2005).

1.5.4.2.2 Opioid Analgesics

Opioids bind to opioid receptors in the central nervous system (CNS) to either, inhibit the transmission of nociceptive input from the periphery to the spinal cord, activate descending inhibitory pathways that modulate transmission in the spinal cord and alter limbic system activity to modify sensory and affective aspects of pain. They are classified into either weak (e.g. codeine and dihydrocodeine) or strong (e.g. morphine, fentanyl, methadone, and oxycodone) and are useful in managing moderate to severe pain (Świeboda *et al.*, 2013). They interact with μ, δ and κ opioid receptors (G_1 protein-coupled), to mimic the activities of endogenous opioid peptides. As the opioids bind to their receptors, they open to inwardly rectifying calcium-dependent potassium channels, shut N-type voltage-operated calcium channels which in turn lead to a decrease in neuronal excitability and subsequent

hyperpolarization. They also act by modulating the release of substance P and other nociceptive neurotransmitters through the reduction in intracellular cyclic AMP (Bovill, 1997). Opioid drugs avoid the peripheral toxicity induced by NSAIDs, but their long-term use is also limited by side effects, such as nausea, constipation, confusion, respiratory depression, sedation and tolerance, clouding of consciousness and drug dependence (Dray and Urban, 1996; Carter *et al.*, 2014).

1.5.4.2.3 Antiepileptic Drugs (AEDs)

Antiepileptic drugs such as carbamazepine, phenytoin, and valproic acid are types of adjuvant analgesics approved for the management of trigeminal neuralgia, migraine prophylaxis, and neuropathic pain. The increasing use of AEDs for neuropathic pain is possibly based on their ability to reduce membrane excitability and suppress abnormal discharges in pathologically altered neurons (Landmark, 2008). However, the precise origin of their analgesic properties is unclear. It does not, however, seem to be explicitly related to their antiepileptic activity. Other drugs that suppress seizures (e.g., barbiturates) do not relieve pain, and AEDs with effective antiepileptic activity do not necessarily have good analgesic activity (Patsalos and Perucca, 2003).

1.5.4.2.4 Antidepressants

Antidepressants exhibit analgesic properties in animal models of nociceptive, inflammatory, and neuropathic pain and some relieve chronic and neuropathic pain in humans (Sawynok *et al.*, 2001). Tricyclic antidepressants (TCA), especially amitriptyline, are useful in the treatment of neuropathic pain and are also of immense benefit for patients with chronic musculoskeletal pain problems, such as fibromyalgia and low back pain (Maizels and McCarberg, 2005). The analgesic effect is generally thought to be mediated by central mechanisms and may be due to the ability of some antidepressants to block the reuptake of serotonin and norepinephrine in the CNS, hence boosting the action of endogenous pain-controlling pathways. A supraspinal effect in the brain is also suspected to be involved (Anjaneyulu and Chopra, 2004; Anjaneyulu and Chopra, 2006; Marchand *et al.*, 2003). However, some studies have demonstrated a peripheral action of antidepressants, which might be of primary relevance to localized delivery methods such as topical application (Sawynok, 2003). Their analgesic actions do not depend on antidepressant activity

(O'Malley *et al.*, 1999), and antidepressants are equally effective in patients with or without depression. While analgesia may occur at lower doses and sooner than antidepressant activity, maximum efficacy may require high antidepressant doses and trial duration (Raja *et al.*, 2002).

1.5.4.2.5 Local anaesthetics

Local anaesthetics are another type of adjuvant analgesic. Examples are bupivacaine and lidocaine. These drugs act by blocking sodium channels and inhibits the generation of abnormal impulses by damaged nerves to exert their peripheral analgesic effects. These compounds are used to prevent or reduce the firing of nociceptive fibres resulting in pain relief (Niyom, 2013). When used systemically, they do not produce conduction block (anaesthesia) as they do with local injection and topical application but may suppress aberrant electrical activity in structures associated with pain (Kauppila, 1998).

1.5.4.2.6 Other Agents in development

Compounds such as TRPV1 antagonists (Willis, 2009), nerve growth factor (NGF) antagonists (Watson *et al*, 2008; Cattaneo, 2010) and selective Na^+ channel blockers (Jarvis *et al*, 2007), interleukin 1β receptor antagonists, etc. are currently being investigated to augment already available analgesics. Bradykinin receptors (B_1 and B_2) are other targets of interest for novel analgesics (Rodger, 2009).

1.5.4.2.7 Analgesics from medicinal plants

Clinically relevant analgesic drugs such as morphine, salicylic acid, capsaicin and tetrahydrocannabinol (THC) were obtained from plants such as *Papaver somniferum, Salix species, Capsicum species,* and *Cannabis sativa* respectively. These drugs have also contributed to the development of new analgesics and are very important in the understanding of the complex pathways related to the electrophysiological and molecular mechanisms associated with pain transmission (Calixto *et al*, 2005). Plant-derived secondary metabolites have greatly contributed to the current understanding of important mechanisms related to the process of pain transmission and treatment. They have also permitted the characterization of receptor types and identification of endogenous ligands

involved in the mechanism of nociception (Calixto et al, 2005). Recently, several secondary metabolites such as alkaloids, terpenoids, and flavonoids are reported to possess potent anti-nociceptive activities. For instance, flavonoids such as hesperidin, apigenin, luteolin, and quercetin are reported to possess analgesic effects (Kumar and Pandey, 2013). Also, Woode *et al.* (2012), established the analgesic properties of the ethanol fruit extract of *X. aethiopica* and its diterpene, xylopic acid in several murine models of pain.

1.6 PROBLEM STATEMENT AND JUSTIFICATION

Many diseases are now known to have an inflammatory element as part of the pathophysiology (Serhan, 2004). Pain accompanies almost every disease state and it is usually the major factor of the disease that alerts the patient to seek medical attention (American Pain Society, 2000; Corrigan, 2011). Pain is also the most common reason for absenteeism from work, which could lead to unemployment and results in a huge financial loss to individuals and countries (Baigi and Stewart., 2015). Aside from the cost of pain treatment, it also complicates the treatment of other ailments (Gaskin and Richard, 2012). Most of the drugs currently used as anti-inflammatory and analgesic agents include the non-steroidal anti-inflammatory drugs (NSAIDs), opioids, steroids and some others including antidepressants and anticonvulsants; which have all proven very effective but are associated with numerous and life-threatening side effects (Loewen, 2002, Mirshafiey *et al.*, 2005). There is still the rigorous search for more effective anti-inflammatory and analgesic agents with minimal or no side effects at therapeutic doses. Also, currently, pharmacotherapy for central nervous system disorders like epilepsy is far from optimum despite the availability of many antiepileptic drugs (AEDs) with a good number of patients experiencing refractoriness and unacceptable AED-related adverse effects (Brodie, 2005).

Various medicinal properties have been attributed to natural herbs. The story of plants being utilized for medicinal intent is probably as old as the history of mankind. The use of medicinal plants in modern cultures has been tracked to the extraction and development of several drugs from these plants as well as from traditional medicine (Shrikumar & Ravi, 2007). Indigenous herbal medicines are widely used throughout the African continent, despite an apparent lack of scientific evidence for their quality, safety, and efficacy (Johnson *et al.*, 2007). According to World Health Organization (WHO, 2012), medicinal

plants would be the best source to obtain a variety of drugs and is advocating for the incorporation of traditional medicine in the primary healthcare of developing nations.

Plant extracts are some of the most attractive sources of new drugs and have been shown to produce promising results for the treatment of several ailments. One of such useful medicinal plant is *Calotropis procera* has been extensively used globally as medication for various diseases (Al-Qura'n, 2009). However, most of these claims of the therapeutic success of the plant in the treatment of these ailments are based on its traditional uses and have not been verified scientifically. The core aim of this study was, therefore, to assess the leaves of *Calotropis procera* as an alternate anti-inflammatory, analgesic and antiepileptic agent from a plant source.

1.7 AIMS OF THE STUDY

The overall aim of the study is to evaluate the antinociceptive, anti-inflammatory and anti-epileptic effect of the hydroethanolic leaf extract of *Calotropis procera* (Apocynaceae).

1.7.1 Specific objectives

The specific objectives of this study are:

- To perform phytochemical analysis on the extract
- To carry out neuropharmacological screening of the hydroethanolic extract using
    - Irwin Test (Irwin, 1968; Williams *et al.*, 2007)
    - Activity Meter Test (Anon, 2000)
    - Pentobarbitone-induced sleeping time
    - Tail Withdrawal Test (Janssen *et al.*, 1963; Steinmiller and Young, 2008)
    - Convulsive Threshold Test (PTZ-induced seizures) (Vellucci and Webster, 1984)
    - Tail immersion test (Janssen *et al.*, 1963)
- To evaluate the anti-inflammatory and analgesic properties of the extract in

- *In vitro* models – hypotonic solution-induced haemolysis, heat-induced haemolysis, albumin denaturation assay, bovine serum albumin (BSA) model (Rahman *et al.*, 2015)
- *In vivo* models – carrageenan-induced paw oedema (Nonato et al, 2012) and formalin-induced paw oedema (Choudhary *et al*., 2014)
- the acetic acid-induced writhing test (Tang *et al*, 2007) and formalin-induced paw licking models (Malmberg and Yaksh, 1995).

➢ To evaluate some of the possible nociceptive pathways involved in the antinociceptive effects using:

- specific antagonists to the opioid and adenosine receptors, ATP sensitive K^+ channels, serotonergic, muscarinic, adrenergic and voltage-gated calcium channel.
- specific pain mediators such as prostaglandin E_2, bradykinin, tumour necrosis factor-α, and interleukin - 1β

❖ To determine of anticonvulsant effect using

- strychnine -induced seizure model (Bogdanov *et al.*, 1997),
- pilocarpine-induced *status epilepticus* model (Turski *et al*., 1989),
- picrotoxin – induced seizure model (Vellucci and Webster, 1984) and

❖ To determine the involvement of GABAergic mechanisms in the anticonvulsant effect of the hydroethanolic leaf extract of *C. procera* if any.

Chapter 2

PLANT COLLECTION, EXTRACTION, AND PHYTOCHEMICAL ANALYSIS

2.1 INTRODUCTION

Medicinal plants have remained the major sources of drugs. In fact, many of the currently available drugs were derived either directly or indirectly from them. The approach to new drugs through natural products has proved to be the single most successful strategy for the discovery of new drugs. Medicinal plants are rich source of novel drugs that forms the ingredients in traditional systems of medicine, modern medicines, nutraceuticals, food supplements, folk medicines, pharmaceutical intermediates, bioactive principles and lead compounds in synthetic drugs In the past decade, research has been focused on scientific evaluation of traditional drugs of plant origin for the treatment of various diseases. One of the most important medicinal plants, which are widely used in the traditional system of medicine, is *Calotropis procera* (Al-Qura'n, 2009). This drives the need to screen medicinal plants for novel bioactive compounds as plant-based drugs are biodegradable and safe (Ramakrishna *et. al.,* 2000). A natural product plays an important role in the field of new drugs research and development because of its low toxicity, easy availability and cost-effective.

The primary metabolite like chlorophyll, amino acids, nucleotides, simple carbohydrates or membrane lipids and they play recognized roles in photosynthesis, respiration, solute transport, translocation, nutrient assimilation and differentiation (Tariq and Reyaz, 2013). Secondary metabolites are synthesized by the plants as part of the defense system of the plant and may also perform other roles in the plant metabolism (Phan *et. al.,* 2001). Searching for new therapeutic agents is a big challenge for the scientist of the present modern era and plants are the biggest source of these agents. Screening of plants for their pharmacological properties with the hope of finding safe and effective agents is very essential. Many synthetic compounds are available but due to their environmental pollution and adverse effect on the human body, their use is restricted (Parihar and Balekar, 2016). To find the safe, effective, and environmentally friendly agent from a plant source,

Calotropis procera is a plant that may present as an effective one. Thus, a phytochemical screening for the presence or absence of the potential metabolites can explain some of the pharmacological properties of the plant.

2.2 PLANT COLLECTION AND EXTRACTION

2.2.1 Collection of plant material

Fresh leaves of *Calotropis procera* were collected from Iture (CC-271-5408), a town near the University of Cape Coast (UCC), during the months of August to December 2015. The leaves were identified by a botanist at the School of Biological Sciences Herbarium, the University of Cape Coast and its voucher specimen (UCC/SBSH/15/M044) was deposited in the Herbarium.

2.2.2 Plant Extraction

Leaves of the *Calotropis procera* were dried in the shade for seven days and pulverised into a fine powder. The powder was extracted by cold maceration with 70% (v/v) ethanol in water over a period of 72 h and the resulting extract concentrated in a rotary evaporator (Rotavapor R-215 model, BÜCHI Labortechnik AG, Flawil, Switzerland) into a syrupy mass under reduced pressure at 50°C. It was further dried in a hot air oven at 50°C for a week and kept in a refrigerator and used when required. The yield was 4.1% (w/w). In this study, the crude extract is subsequently referred to as CPE or extract when reconstituted using distilled water.

2.3 PHYTOCHEMICAL TESTS

Calotropis procera hydroethanolic leaf extract was tested for the presence of tannins, alkaloids, carbohydrates, glycosides, reducing sugars, flavonoids, saponins, proteins, amino acids and steroids by simple qualitative methods.

2.3.1 Test for Tannins

An amount of 0.5 g of CPE was boiled with 25 ml of water for 5 min, cooled and filtered. The volume of the filtrate was adjusted to 25 ml with water. To 1 mL of the filtrate was added 10 mL of water and 5 drops of 1 % ferric chloride and observed for a blue-black or

green precipitate formation. The procedure was repeated using 5 drops of 1 % lead acetate and observed for any change in colour or formation of a precipitate (Sofowora, 1993).

2.3.2 Test for Alkaloids

An amount of 0.5 g of the extract was boiled with 10 mL of dilute hydrochloric acid in a test tube for 5 min. The supernatant was filtered into another test tube and 3 drops of Dragendorff's reagent (potassium bismuth iodide solution) was added to 1 mL of the filtrate, which was shaken and observed for the appearance of an orange-red spot and precipitate formation (Sofowora, 1993).

2.3.3 The general test for Glycosides (Reducing Sugars)

An amount of 0.2 g of CPE was boiled in 5 mL dilute H_2SO_4 on a water bath for 2 min. The mixture was cooled, filtered and rendered distinctly alkaline with 2 to 5 drops of 20 % NaOH. 1 mL each of Fehling's A and B solutions were added to the filtrate, heated on a water bath for 2 minutes and observed for a red-brown precipitate (Sofowora, 1993).

2.3.4 Test for Steroids (Liebermann-Burchard test)

To 0.2 g of the extract, 2 mL of acetic acid was added. The solution was cooled well in ice followed by the addition of conc. H_2SO_4 carefully. Colour development from violet to blue or bluish-green indicated the presence of a steroidal ring i.e. aglycone portion of cardiac glycoside (Sofowora, 1993).

2.3.5 Test for Saponins (Frothing test)

A small amount (0.2 g) of the extract was shaken with 5 mL of water in a test tube and the mixture observed for the presence of a froth which does not break readily upon standing (Trease and Evans, 2002).

2.3.6 Test for Flavonoids (Ferric chloride test)

About 0.5 of CPE was boiled with distilled water and then filtered. To 2 mL of the filtrate, two drops of 10% ferric chloride solution was then added. A green-blue or violet colouration indicated the presence of a phenolic hydroxyl group (Trease and Evans, 2002).

2.3.7 Test for Carbohydrates (Molisch's test)

Five drops of Molisch's reagent was added to 0.5 g of the extract dissolved in distilled water. This was then followed by the addition of 1 mL of conc. H_2SO_4 by the side of the

test tube. The mixture was then allowed to stand for 2 min and then diluted with 5 mL of distilled water. Formation of a red or dull violet colour at the interphase of the two layers was a positive test (Sofowora, 1993).

2.3.8 **Test for Proteins (Biuret's test)**

An amount of 0.5 g of the extract was dissolved in 10 mL of distilled after which 2 mL of it was treated with one drop of 2 % copper sulphate solution. To this, 1 mL of ethanol (95%) was added, followed by an excess of potassium hydroxide pellets, pink colour in the ethanolic layer indicated the presence of proteins (Trease and Evans, 2002).

2.3.9 **Terpenoids (Salkowski test)**

An amount of 0.5 g of the extract was extracted with 2 mL of chloroform in a test tube followed by the addition of 1 mL of concentrated sulphuric acid. The reddish-brown coloration at interface shows the presence of terpenoids (Sofowora, 1993).

2.3.10 **Amino acids (Ninhydrin test)**

Two drops of ninhydrin solution (10 mg of ninhydrin in 200 mL of acetone) was added to two mL of extract. A characteristic purple colour indicated the presence of amino acid (Sofowora, 1993).

2.4 RESULTS

Phytochemical analysis of the hydroethanolic leaf extract of *Calotropis procera* revealed the presence of the tannins, glycosides, amino acids, flavonoids, alkaloids, saponins, reducing sugars, steroids, proteins, and terpenoids.

Table 0-1 Phytochemical analysis of the ethanolic extract of the leaves of *C. procera*

TEST	***RESULTS***
Tannins	Present
Alkaloids	Present
Reducing sugars	Present
Steroids	Present
Saponins	Present
Flavonoids	Present
Carbohydrates	Present
Proteins	Absent
Terpenoids	Present
Amino acids	Present

2.5 DISCUSSION

The result of the phytochemical screening of hydroethanolic leaf extract of *Calotropis procera* revealed the presence of alkaloids, tannins and saponins, flavonoids, reducing sugars, carbohydrates, sterols, amino acids, glycosides, and terpenoids. It, however, demonstrated the absence of proteins. This confirms earlier phytochemical tests performed on the plant, *Calotropis procera* by Tiwari *et al*., 2014. As has been reported by several authors, the presence of many biologically active phytochemicals such as flavonoids, triterpenes, alkaloids, steroids, tannins and glycosides in various plant extracts may be responsible for their respective pharmacological properties (Singh *et al.*, 2002; Yokosuka and Mimaki, 2009; Gomes *et al.*, 2009; Liu *et al.*, 2010, Pérez-Amador *et al.*, 2010, Maganha *et al*., 2010). For instance, plants rich in saponins have immune-boosting, anti-inflammatory, anticonvulsant, antidepressant, anti-inflammatory, anxiolytic, sedative, cytotoxic and analgesic properties (Nemmani and Ramarao, 2002; Gurib-Fakim, 2006; Wei *et al*., 2007; Jiang *et al*., 2007; Zhou *et al*., 2010; Xiang *et al.*, 2011). Flavonoids, also exhibit sedative, anticonvulsant and antidepressant effects (Medina *et al.*, 1998; Wolfman *et al.*, 1998; Machado *et al*., 2008; Yi *et al.*, 2010; Cho *et al.*, 2012).

Tannins, aside from their usefulness as astringents, have anti-inflammatory, antioxidant and anti-nociceptive, antiulcer, antimicrobial, antiviral and antitumour properties (Akinpelu *et al.*, 2006; Souza *et al.*, 2007; Koleckar *et al.*, 2008). Alkaloids have an outstanding range of pharmacological activities including antidepressant, anxiolytic, anticonvulsant, sedative, analgesic, anti-inflammatory and anti-cancer activities (Flausino *et al*., 2007; O'Malley *et al*., 2007; Gomes *et al*., 2009; Bhutada *et al*., 2010).

2.6 CONCLUSION

The hydroethanolic leaf extract of *Calotropis procera* contains tannins, glycosides, amino acids, flavonoids, alkaloids, saponins, reducing sugars, steroids, carbohydrates, and terpenoids.

Chapter 3

PRIMARY NEUROPHARMACOLOGICAL EVALUATION

3.1 INTRODUCTION

As part of the continuing search for plants with central nervous system activity, this chapter evaluated the potential of *Calotropis procera* (Ait) R. Br. (Apocynaceae) to affect CNS using mice. Even though the plant has widespread use as anti-inflammatory, anticancer, antimicrobial, analgesic agent (Dewan *et al.*, 2000), very little scientific information exists about its effects on the CNS although it's used traditionally in the management of epilepsy and some mental disorders in the northern part of Ghana and also parts of Burkina Faso (Kinda *et al*, 2017). Also, until just over a decade ago, the brain was regarded as an 'immune-privileged' organ, which was not susceptible to inflammation or immune activation and was thought to be largely unaffected by systemic inflammatory and immune responses. This view has been revised significantly and it has been found that pro-inflammatory cytokines and other mediators play an essential role in CNS inflammation through the induction of chemokines and adhesion molecules which is at play in some diseases of the CNS (Lucas *et al*, 2006).

The methods employed in this study were adapted from the core battery of assessment of the central nervous system as proposed by the International Conference on Harmonization (ICH) S7A Guideline for Safety Pharmacology (Anon, 2000). It recommends the testing of novel compounds on the central and peripheral nervous system and on the cardiovascular system as part of the "core battery" of assessment (Williams *et al*., 2007). These are generally simple tests employed in safety assessment and are frequently applied at the very beginning of the discovery process to screen for substances with a potential for CNS benefit or risk. These include protocols for measuring gross behavioural signs (Irwin test), effects on spontaneous locomotor activity (Activity meter test), neuromuscular coordination (Rotarod test), convulsive threshold (PTZ seizure test), sleep-enhancing or reducing effect (Barbiturate interaction test) and on the pain threshold (Tail immersion test). Due to their use early in the safety evaluation process, such studies are conducted almost exclusively in the rodent (Porsolt *et al.*, 2002).

Rodents are mainly used as the species of choice for detection of behavioural and neurological effects. The mouse shares many anatomical, cellular, biochemical and molecular features with humans and some functions such as memory, sexual behaviour, and emotional responses are also similar. Due to these similarities, murine models are therefore employed to approximate human behavioural responses in disease states (Van Meer and Raber, 2005). Thus, this study involved the use of *in vivo* methods in freely moving conscious mice.

3.2 MATERIALS AND METHODS

3.2.1 Animals

Male ICR (Institute for Cancer Research) mice weighing 20 – 25 g were purchased from the Noguchi Memorial Institute for Medical Research (NMIMR), Univesity of Ghana, Accra. They were kept in the animal house of the Department of Pharmacology, KNUST for seven days to acclimatize before the experiments. The animals were housed in cages (34×47×18 cm^3) with softwood shavings as bedding and were maintained at a 12 h light-dark cycle. They had free access to food and water. The studies conducted were in accordance with NIH Guidelines for Care and Use of Laboratory Animals with approval from Department of Pharmacology Ethics Committee.

3.2.2 Drugs and Chemicals

Morphine was purchased from Phyto-Riker (Accra, Ghana). Pentobarbitone, caffeine, *d*-tubocurarine, pentylenetetrazole, and diazepam were obtained from Sigma-Aldrich (St Louis, MO, USA).

3.2.3 Irwin Test

The effects of *Calotropis procera* extract (CPE) on gross behaviour and physiological function were investigated using the original procedure described by Irwin (1968). Mice were randomly distributed to six groups (n=7) and left to acclimatize for 24 h. Animals were fasted overnight but had free access to water. They were treated with oral doses of either CPE 30, 100, 300, 1000, 3000 mg kg^{-1}, or distilled water 10 ml kg^{-1}. The animals were subsequently observed for changes in behaviour and physiological function (e.g.

spontaneous activity, response to touch, gait, posture, righting reflex, convulsions, analgesia, etc) and death at 0 to 15, 30, 60, 120, 180 min and at 24 h.

3.2.4 **Activity Meter Test**

The effects of CPE on spontaneous locomotion was evaluated with the Ugo Basile activity cage (model 7401, Comerio, VA, Italy). Mice were distributed randomly to seven groups (n=7) and treated orally with either extract (30, 100, 300, 1000 mg kg^{-1}), diazepam (8 mg kg^{-1}), caffeine (16 mg kg^{-1}) or distilled water (10 mL/kg, *p.o.*). After 1 h, the animals were individually placed in the activity meter cage and their activities scored in 5 min blocks for 30 min. Diazepam and caffeine were used as CNS depressant and stimulant respectively. Total activity in 30 min was computed as AUC of the time-course curve.

3.2.5 **Rotarod Test**

This test was performed to elucidate the effect of *Calotropis procera* extract on neuromuscular coordination. The rotarod consisted of a rotating rod (diameter: 3 cm) rotating at a constant speed of 25 revs/s with individual compartments for each mouse (Ugo Basile model 7600, Comerio, VA, Italy). Mice were trained for three days before the test day to stay on the rotating rod for 180 s. On the test day (24 h after the last training session) the animals received orally CPE (30, 100, 300 and 1000 mg kg^{-1}), diazepam (8 mg kg^{-1}), *d*-tubocurarine (0.01 mg kg^{-1}) or distilled water (10 ml kg^{-1} *p.o.*) and placed on the rod to walk. Latency to fall off the rotating rod within a maximum cut off time of 180 s was determined at 0, 1, 1.5, 2 h and 3 h post drug administration (LeDoux, 2005; Meredith and Kang, 2006).

3.2.6 **Pentobarbitone-Induced Sleeping Time**

The effect of CPE on pentobarbitone-induced sleeping time was investigated in the pentobarbitone interaction test. The method described by Lovell (1986) and modified by Adongo *et al.*, 2014 was adopted. Mice in seven groups (n=7) received either CPE (30, 100, 300 and 1000 mg kg^{-1}, *p.o.*), diazepam (8 mg kg^{-1}), caffeine (16 mg kg^{-1}) or distilled water (10 mL kg^{-1}, *p.o.*) orally. Sodium pentobarbitone (50 mg kg^{-1}) was administered intraperitoneally one hour after the respective drug treatments. Latency to sleep (time

between pentobarbitone injection and loss of righting reflex) and duration of sleep (time between loss of and regaining of righting reflex) were recorded.

3.2.7 **Convulsive Threshold Test**

Mice were randomly assigned to five groups and administered either CPE (100, 300, 1000 mg kg^{-1}), diazepam (10 mg kg^{-1}) or distilled water (10 ml kg^{-1}) *p. o.* 1 h after drug treatment, the seizure was induced by a single subcutaneous dose of pentylenetetrazole (85 mg kg^{-1}) at the nape and the mice were placed in plastic cages (Perspex chamber, 15×15×15 cm) for observation (Stone, 1970). Latency to convulse as well as frequency and duration of convulsions were observed through video recording (Sony-Handycam, model: HDRCX675/B, Tokyo, Japan) for 30 min and quantified with the behavioural analysis software, JWatcher TM version 1.0 (University of California, Los Angeles, USA and Macquarie University, Sydney, Australia. Available at http://www.jwatcher.ucla.edu). Clonic seizures were characterized as the appearance of facial myoclonus, forepaw myoclonus and forelimb clonus and tonic seizures were characterized as explosive clonic seizures with wild running and tonic forelimb and hind limb extension. The latency for the onset, frequency and duration of the convulsive episodes (clonic or tonic) were recorded as indicators of pro- or anti-convulsive effect of the substances.

3.2.8 **Tail Immersion Test**

The test was carried out according to the method described by Janssen *et al.* (1963) with some modifications. The distal part (2-3 cm) of the tail of the mice was immersed in a water bath maintained at 50.0±1.0°C. The time in seconds to deflect or withdraw the tail out of the water was taken as the reaction time (T). A cut off time of tail immersion was taken as 10 s, and thereafter the measurement was stopped to avoid any tissue injury. Withdrawal latency was taken after 0.5, 1, 2, and 3 h intervals following administration of CPE (30, 100, 300 mg kg^{-1}, *p.o.*), or morphine (10 mg kg^{-1}, *p.o.*). Prior to the tail immersion test, the animals were screened by immersing their tail in hot water (50.0±1.0°C) and only those animals that showed tail withdrawal latency of <5 s were selected for the experiment. Increase in tail withdrawal latency was the measure of anti-nociception. It was calculated as:

$$\%\ Maximal\ Possible\ Effect\ (MPE) = \frac{[(T_1 - T_0)]}{[(T_2 - T_0)]} \times 100$$

where T_0 and T_1 are defined as the latencies obtained before and after drug treatment respectively, and T_2 is the cut-off latency.

3.2.9 Data Analysis

All results are presented as mean ± SEM. Except otherwise stated, data was analysed as a one-way analysis of variance (ANOVA). When ANOVA was significant, multiple comparisons between treatments were performed using the Holm-Sidak *post hoc* test. GraphPad Prism for Windows Version 7 (GraphPad Software, San Diego, USA) was used for all statistical analyses.

3.3 RESULTS

3.3.1 Irwin Test

The drug, CPE (30 – 3000 mg kg^{-1}) did not have any lethal effects on the animals during the 24-h period of observation. CPE showed decreased reactivity to touch, analgesia and sedation at all doses administered compared to control.

Table 3-1 Effects of *Calotropis procera* extract in Irwin test

DOSE (mg kg^{-1})	*Calotropis procera* extract (CPE)
0	No observed change
30	Decreased activity and reactivity to touch, analgesia at 15 – 180 min
100	Decreased activity and reactivity to touch, analgesia at 15 – 180 min
300	Decreased activity and reactivity to touch, analgesia at 15 – 180 min
1000	Decreased activity and reactivity to touch, analgesia at 15 – 180 min
3000	Decreased activity and reactivity to touch, analgesia at 15 – 180 min

****** *No death was recorded within the 24 h period the test was carried out.*

3.3.2 **Activity Meter Test**

CPE significantly reduced spontaneous locomotion at 30 – 1000 mg kg^{-1} as did diazepam (8 mg kg^{-1}), the reference CNS depressant ($F_{6,42}$=23.66 *P<0.0001*). Caffeine, the reference CNS stimulant at 16 mg kg^{-1} increased activity significantly (Fig 3-1).

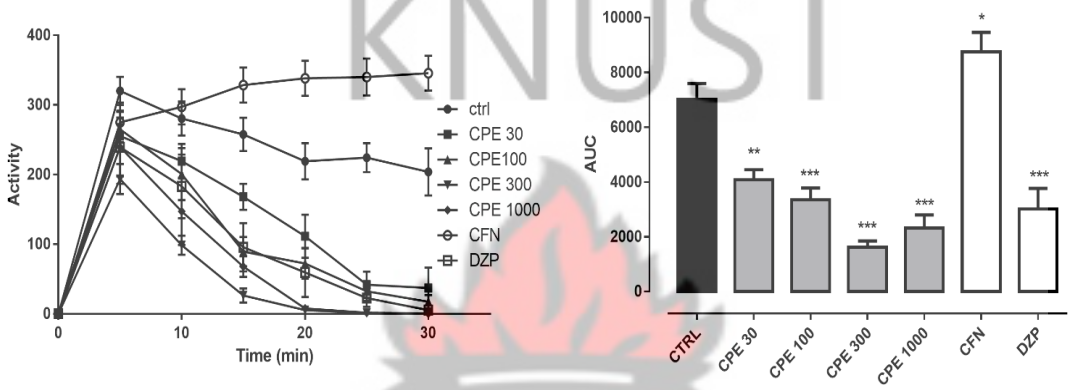

Figure 3-1 Time course curve of activity of mice administered CPE, Caffeine (CFN) and diazepam (DZP) in the activity meter test (a) and AUC of total activity (b) over 30 min test period in the activity meter test. One-way Analysis of Variance (ANOVA) followed by Holm-Sidak *post hoc* test in (b). Data are mean ± SEM, n=7, ****P<0.0001*, ***P<0.001 and * P <0.05* compared to control.

3.3.3 **Rotarod Test**

CPE reduced significantly the time spent on the rod only at doses 100 – 1000 mg kg^{-1} ($F_{6,39}$=73.36 *P*<0.0001). The reference muscle relaxants; diazepam (8 mg kg^{-1}), *d*-tubocurarine (0.01 mg kg^{-1}) also significantly reduced time spent on the rod (Fig 3-2).

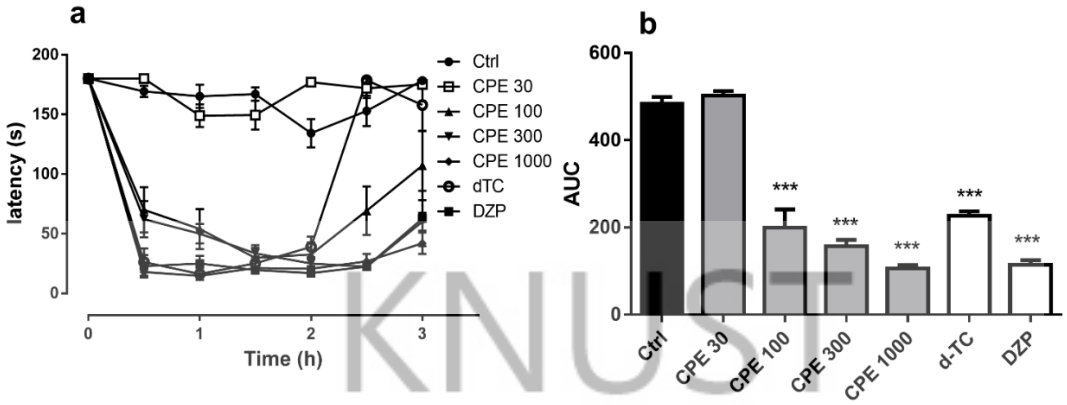

Figure 3-2 Effect of *Calotropis procera* extract (CPE), diazepam (DZP) and d-tubocurarine (d-Tc) on neuromuscular coordination in mice in the rotarod test. Time course curve of duration spent on the rod (a) and total latency (AUC) (b) over 3 h. Data are presented as mean ± SEM, n=7, *** *P <0.0001* compared to control.

3.3.4 **Pentobarbitone-Induced Sleeping Time Test**

The extract did not significantly affect the latency to sleep, however, it significantly prolonged sleep duration at doses 100 to 1000 mg kg^{-1} ($F_{6, 41}$ = 7.804 *P<0.0001*). Diazepam the reference CNS depressant increased the duration of sleep while caffeine significantly decreased latency sleep duration (Fig. 3-3).

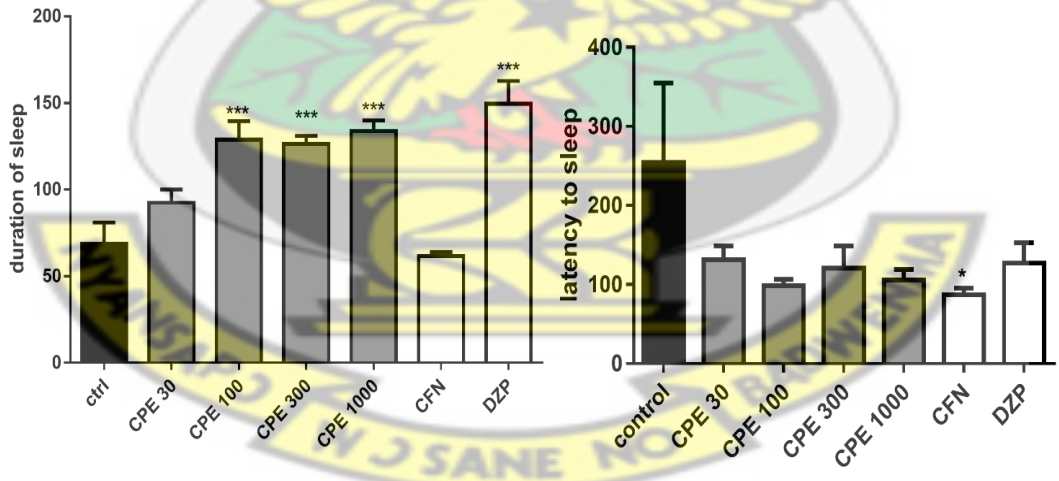

Figure 3-3 Effect of *Calotropis procera* extract on (a) duration of sleep and (b) latency to sleep in mice in the pentobarbitone-induced sleeping time test. One-way ANOVA followed by Holm-Sidak *post hoc* test. Data are mean ± SEM, n=7, ****P<0.0001*, ***P<0.001 and * P <0.05* compared to control.

3.3.5 **Convulsive Threshold Test**

CPE (100 – 1000 mgkg^{-1}) was able to significantly reduce the frequency ($F_{4, 30}$=11.79 *P<0.0001*) and duration ($F_{4, 30}$=14.05 *P<0.0001*) of clonic convulsions. However, only 1000 mgkg^{-1} of CPE was able to significantly increase the latency of the clonic convulsions ($F_{4, 30}$=6.479 *P<0.0007*). This is shown in Fig. 3-4. The extract (100 – 1000 mg kg^{-1}) also significantly reduced the duration ($F_{4, 27}$=6.297 *P=0.0010*) and delayed the onset of tonic convulsions ($F_{4, 27}$=4.882 *P=0.0043*). Lower doses of CPE were unable to reduce the frequency of tonic convulsions; however, the 1000 mg kg^{-1} dose of the extract significantly reduced the frequency of tonic convulsions ($F_{4, 27}$=2.997 *P=0.0247*) (Fig. 3-5). Overall, CPE was able to reduce the total frequency ($F_{4, 30}$=10.33 *P<0.0001*) and duration ($F_{4, 30}$=12.93 *P<0.0001*) of convulsions significantly (Fig. 3-6). The reference anticonvulsant used (diazepam) was also able to reduce the frequency and duration of the convulsions as well as delay the onset of tonic convulsions.

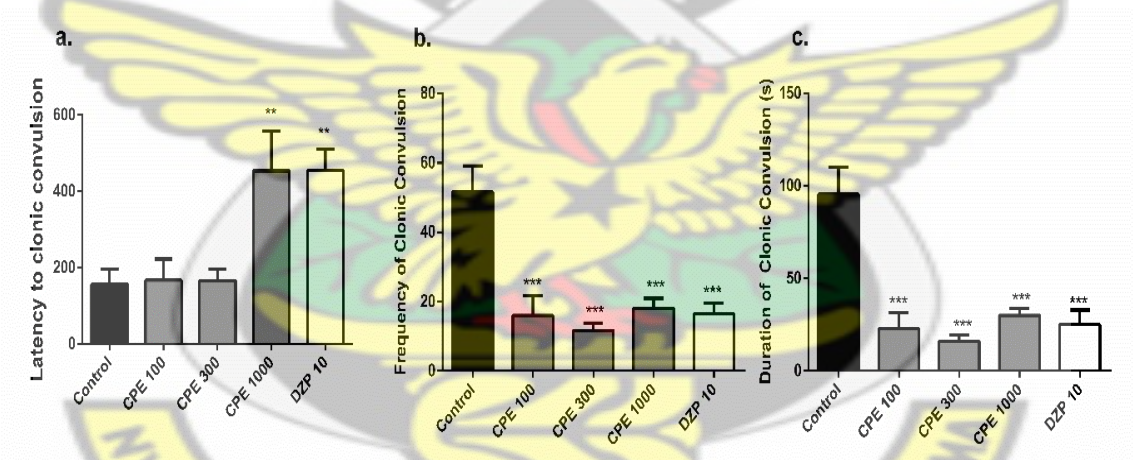

Figure 3-4 Effect of *Calotropis procera* extract on (a) latency (b) frequency and (c) duration of clonic convulsions in mice. One-way ANOVA followed by Holm-Sidak *post hoc* test. Data are mean ± SEM, n=7, ***$P<0.0001$, **$P<0.001$ and * $P<0.05$ compared to control.

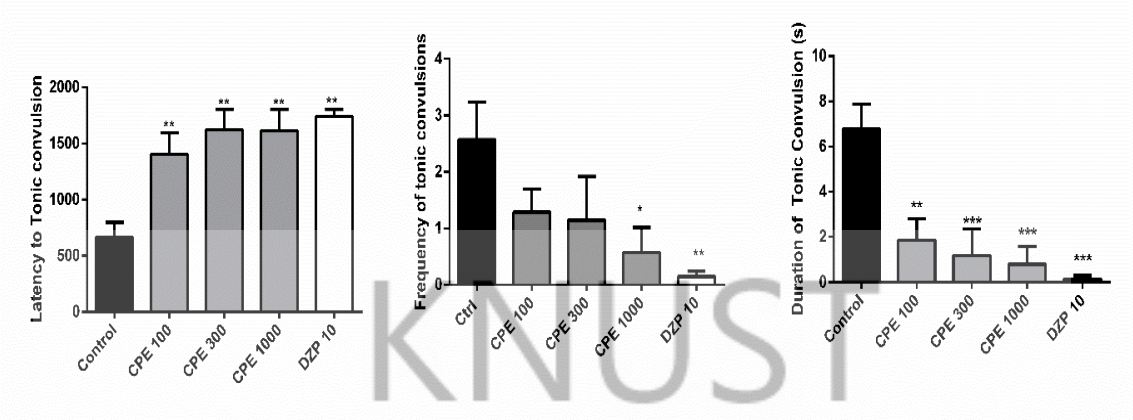

Figure 3-5 Effect of *Calotropis procera* extract on (a) latency (b) frequency and (c) duration of tonic convulsions in mice. One-way ANOVA followed by Holm-Sidak *post hoc* test. Data are mean ± SEM, n=7, ***P<0.0001, **P<0.001 and * P <0.05 compared to control.

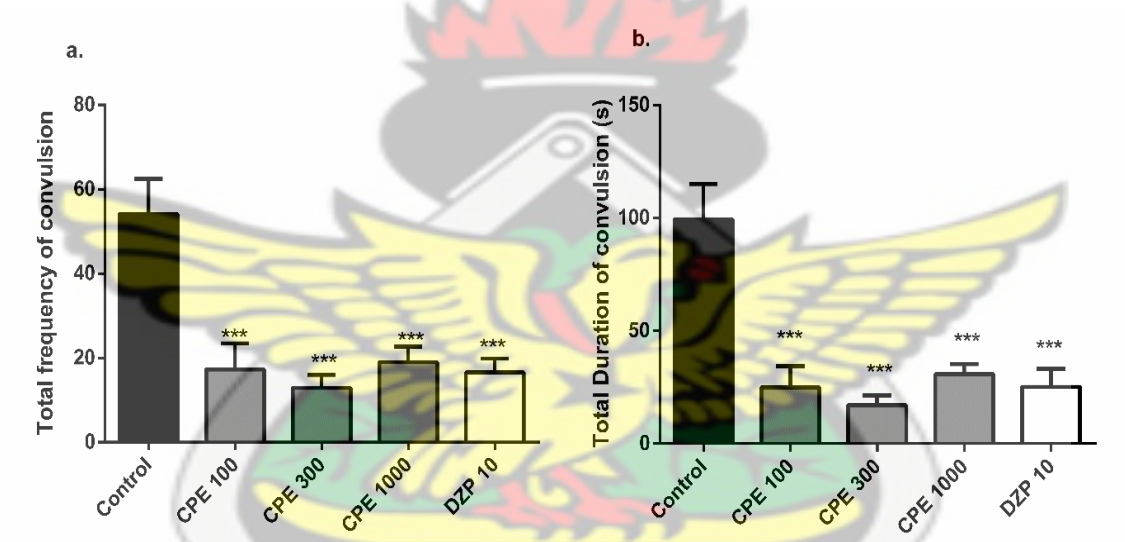

Figure 3-6 Effect of CPE on the (a) total frequency and (b) total duration of convulsions in mice. One-way ANOVA followed by Holm-Sidak *post hoc* test. Data are mean ± SEM, n=7, ****P<0.0001, **P<0.001 and * P <0.05* compared to control.

3.3.6 **Tail Immersion Test**

From the time-course curves in Figure 3-7, two-way ANOVA (*treatment versus time*) revealed a significant effect of drug treatments on the tail withdrawal latencies calculated as a percentage of the maximum possible effect (% MPE) ($F_{5,15}$=55.06, P<0.0001). CPE (30-300 mgkg^{-1}, *p.o.*) significantly increased tail withdrawal latency in a dose-dependent manner ($F_{4,14}$=17.13, P=0.0001). Morphine (10 mgkg^{-1}, i.p.), the standard analgesic drug used, also showed a significant increase in the withdrawal latency (P<0.05).

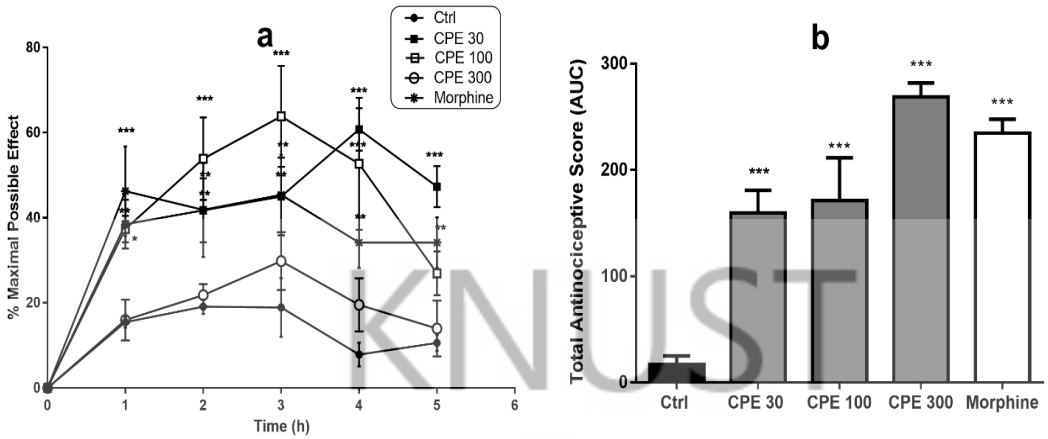

Figure 3-7 Effect of CPE (30-300 mg kg^{-1}, *p.o.*) and morphine (10 mg kg^{-1}, i.p.) on the time course curve (a) of the tail immersion test and the AUC (b) in mice. Data are presented as mean ± S.E.M. **P<0.05; **P<0.001; ***P<0.0001* compared to the control group (Two-way ANOVA followed by Holm-Sidak *post hoc* test for the time-course curve or one-way ANOVA followed by Bonferroni *post hoc* test for AUC).

3.4 DISCUSSION

This present study has shown that the hydroethanolic leaf extract of *Calotropis procera* possesses some CNS depressant, anticonvulsant, analgesic activity and muscle relaxant effects in the animal models used.

The mice treated with CPE showed signs of sedation and analgesia, suggesting possible central depressant and analgesic effects. The Irwin test can furnish a first but pertinent orientation towards a specific therapeutic indication, a specific mechanism of action or a specific physiological function as such presence of sedation in the test is suggestive of a possible anxiolytic, antipsychotic, or anticonvulsant activity (Roux *et al.*, 2005). Irwin test also assesses the minimum lethal dose of a test substance and the primary effects on behaviour and physiological functions as data from this test is also used to assess the safety pharmacology of drugs (Irwin, 1968; Porsolt *et al.*, 2002). Over the course of 24 h, the plant extract used caused no mortality and caused no apparent toxicity even at a relatively high dose of 3000 mg kg^{-1}. This result suggests that CPE is relatively non-toxic since Obici *et al.*, 2008 stated that substances with an LD_{50} above 1000 mg kg^{-1} by the oral route are regarded as being safe or of low toxicity.

The activity meter test quantified the decreased activity observed in the Irwin test and assessed the effects of *Calotropis procera* extract on spontaneous locomotion. A decrease in spontaneous locomotion is predictive of sedation (Green *et al.*, 2001) although neuromuscular impairment may confound the results (Bohlen *et al.*, 2009). The rotarod test was used to elucidate the cause of the decreased activity observed (Green *et al.*, 2001). The extract decreased the activity of mice in the activity meter test; a confirmation of the sedative effects observed in Irwin's test.

From the results, it was clear that there was significant motor impairment at the doses of the extract that reduced spontaneous activity in the activity meter test. Many CNS depressant compounds can cause a reduction in spontaneous locomotor activity in laboratory animals. Nearly all the neuroleptic agents used in psychiatry diminish spontaneous locomotor activity in all species including man (Simon *et al*., 2000; Kinkead and Nemeroff, 2002). It was observed in the experiment as expected that, diazepam, the positive control, a CNS depressant, reduced spontaneous locomotive activity and impaired motor coordination at

the doses used while caffeine, a CNS stimulant, which also served another positive control, increased the locomotor activity (Solinas *et al*., 2002; Dunne *et al.*, 2007).

Sleep-enhancing effect of substances can readily be detected in the barbiturate induced sleeping time test by substances, which do not induce sleep even at high doses when administered alone (Porsolt *et al*., 2002; Nayak *et al*., 2004). It has been observed that there is a high correlation between the effects observed in this procedure and those observed in some other complex tests and also in man (Zhao *et al.*, 2006). In the present study, though the extract did not affect the latency to sleep, however, it profoundly prolonged sleep duration, this was not apparent in the Irwin test. The potentiation of the pentobarbitone-induced sleep further supports the central depressant activity of the extract (Silva *et al.*, 2007).

The *Calotropis procera* extract produced significant inhibition of PTZ-induced seizures which confirms its traditional use in epilepsy management (Kinda *et al.*, 2017). It was observed that at doses above 30 mg kg^{-1}, the extract delayed the onset of clonic and tonic convulsions; and reduced the frequency and duration of the clonic and tonic convulsions. One of the generally accepted mechanisms by which pentylenetetrazole exerts its action is by acting as an antagonist at the $GABA_A$ receptor complex (Katzung, 2017). GABA is the major inhibitory neurotransmitter in the central nervous system in humans (Katzung, 2017). Inhibition of pentylenetetrazole-induced seizures could indicate that the potential anticonvulsant effects of CPE may be linked with GABA activity modulation in the central nervous system. This is probable as it has demonstrated significant central depressant properties.

Finally, in the tail immersion test, CPE caused a prolonged latency period in all the doses tested, indicating an increase in the nociceptive threshold. This test can differentiate between central opioid-like analgesics and peripheral analgesics (de Mesquita Padilha *et al*., 2009). The response to the tail-immersion test is a spinal reflex but may also involve higher neural structures and are used to evaluate central analgesic activity (Pavin *et al.*, 2011). The antinociceptive effect of CPE shown in this test is a further confirmation of analgesia observed in the Irwin test.

3.5 CONCLUSION

From the core CNS battery tests, the *Calotropis procera* extract exhibited significant CNS depressant, anticonvulsant and analgesic effects. The extract reduced spontaneous activity and impaired motor coordination. The LD_{50} is estimated to be above 3000 mg kg^{-1}. Due to the antinociceptive and anti-inflammatory activities of the extract observed in the core battery test, the extract was comprehensively evaluated for its antinociceptive and anti-inflammatory properties.

Chapter 4

ANTI-INFLAMMATORY AND ANALGESIC EFFECTS

4.1 INTRODUCTION

The *Calotropis procera* extract showed significant analgesic activity in the primary neurological assessment performed. This extract is also used locally as an antipyretic and as an agent in the treatment of pain and also inflammation (Al. Yahya *et al*., 1985; Mohsin *et al.*, 1989; Khairnar *et al*., 2012) Inflammation is the response of the body to injury or infection characterised by redness, pain, swelling, heat and the loss of function (Chandra *et al.*, 2012). This response by the body against the aetiological agents is mediated by activated inflammatory cells such as neutrophils, macrophages, plasma cells and lymphocytes (Chirisa *et al.*, 2016). The key components of inflammation include the blood vessels, circulating leukocytes, connective tissue cells (mast cells, resident macrophages, and fibroblasts) and chemical mediators produced from plasma and leukocytes (Ullah *et al.*, 2014).

Steroidal and non-steroidal anti-inflammatory drugs (NSAIDs) such as diclofenac, aspirin, and indomethacin are the mainstay in the management of inflammatory disorders. Other classes include the corticosteroids and the disease-modifying anti-inflammatory agents. However, the use of these drugs is limited by their numerous deleterious side effects such as gastric ulceration (Carter *et al.*, 2014) which limit their use. This underscores the need to search for newer, more effective and safer drugs.

Even though there is widespread usage of *C. procera* in folk medicine for the treatment of several inflammatory conditions, there is still a paucity of scientific report validating its usage. The few anti-inflammatory reports on the plant used the latex of the plant (Kumar & Baso, 1994; Arya & Kumar, 2005), which is far from its traditional usage where aqueous or alcoholic leaf extracts are used. It is therefore imperative that additional anti-inflammatory activity tests on the leaf extract are conducted using several *in vitro* and *in vivo* models to authenticate the folkloric usage and give insight for the potential mechanisms of action of this plant. A significant anti-inflammatory effect in a battery of anti-inflammatory models in addition to multi-faceted mechanisms of action would be a strong justification for further research on the plant. This may lead to the discovery of novel phytoconstituents that can

serve as scaffolds for novel therapeutic agents that can be used to treat arthritis and other inflammatory diseases.

4.2 MATERIALS AND METHODS

4.2.1 Animals

Sprague-Dawley rats (170 – 250 g) and ICR mice (20 – 25 g) of both sexes were bought from Noguchi Memorial Institute for Medical Research, University of Ghana, Legon-Ghana. They were kept in stainless steel cages (34×47×18 cm^3) in groups of five at the animal facility of the Department of Biomedical Sciences, University of Cape Coast. The animals were given normal commercial pellet diet (Agricare, Kumasi, Ghana) and provided water *ad libitum.* The animals were kept under normal laboratory conditions with regards to room temperature and humidity. All the techniques and protocols used in the study were done in accordance with established public health guidelines in "Guide for Care and Use of Laboratory Animals" (Garber *et al.,* 2011). Also, all protocols used in the study were approved by the Department of Pharmacology Ethics Committee.

4.2.2 Drugs and Chemicals

Diclofenac sodium, formalin, acetic acid, carrageenan, bovine serum albumin used were of analytical grade and were purchased from Sigma-Aldrich Inc, St. Louis, MO, USA.

4.2.3 Collection of Blood Samples

To obtain erythrocytes for the various *in vitro* experiments, whole blood was collected from a healthy volunteer who had not taken any non-steroidal anti-inflammatory drug (NSAID) for two weeks prior to the experiment and the blood kept in heparinized vacutainer. The blood was washed three times with 0.9% saline and centrifuged simultaneously for 10 min at 3000 rpm. The packed cells were washed with 0.9% saline and 10% $^{v}/_{v}$ RBC suspension was made using isotonic by keeping it in 0.9% NaCl solution.

4.2.4 *In vitro* Models of Inflammation

4.2.4.1 *Hypotonic Solution-Induced Haemolysis Test*

A method earlier described by Rahman *et al*. (2015) was used. The test sample was made up of 0.5 mL stock erythrocyte (RBC) suspension mixed with 5 mL of hypotonic solution (0.45 % NaCl) and varying concentrations of *Calotropis procera* extract (100, 300 and 1000 µg/mL). The negative control was made of 0.5 mL RBC suspension mixed with hypotonic buffered solution alone. The positive control was made of 0.5 mL of the RBC suspension, 5 mL of the hypotonic solution and diclofenac (100, 300 and 1000 µg/mL). The mixtures were incubated for 10 min at room temperature, centrifuged for 10 min at 3000 rpm and haemoglobin content of the supernatant was measured spectrophotometrically (PG Instruments, UK) at 540 nm. The experiment was carried out in triplicate. The percentage inhibition of haemolysis was calculated using the following equation:

$$\%\ Inhibition = 100 \times \left[\left[\frac{Absorbance\ of\ sample}{Absorbance\ of\ negative\ control} - 1\right]\right]$$

4.2.4.2 *Heat-Induced Haemolysis Test*

The test was performed as described by Rahman *et al*. (2015). The test sample consisted of 2.0 mL of 10% RBC suspension and 2.0 mL of CPE (100, 300 and 1000 µg/mL). The negative control consisted of 2.0 mL of 10% RBC suspension and 2.0 mL of normal saline. The positive control samples were made of 2.0 mL of 10% RBC and 2.0 mL of diclofenac (100, 300 and 1000 µg/mL). The experiment was carried out in triplicate. The samples were heated at 56°C for 30 min then cooled to room temperature followed by centrifugation at 2500 rpm for 10 min. The supernatant was collected, and absorbance was measured at 560 nm. Percent of membrane stabilization was calculated by the method of Rahman *et al*., (2015) as follows:

$$\%\ Inhibition = 100 \times \left[\left[\frac{Absorbance\ of\ sample}{Absorbance\ of\ negative\ control} - 1\right]\right]$$

4.2.4.3 *Albumin Denaturation Assay*

The test was performed as described by Rahman *et al*. (2015) previously. The reaction mixture (5 mL) for the test sample consisted of 0.2 mL of egg albumin (from a fresh egg), 2.8 mL of phosphate-buffered saline (PBS, pH 6.4) and 2.0 mL CPE (100, 300 or 1000 µg/mL). The negative and positive control samples contained the same volume of the egg albumin and PBS, but the extract was replaced with 2.0 mL of distilled water and 100, 300 or 1000 µg/mL diclofenac respectively. The mixtures were incubated at 37 ± 2°C for 15 min and then heated at 70°C for 5 min. After cooling, the absorbance was measured at 660 nm. The experiment was performed in triplicate. The percentage inhibition of protein denaturation was calculated with the formula:

$$\%\, Inhibition = \left(\frac{Absorbance\ of\ sample}{Absorbance\ of\ negative\ control} - 1\right) \times 100$$

4.2.4.4 *Bovine Serum Albumin (BSA) Denaturation Assay*

The test was performed using the method described by Rahman *et al*. (2015). The reaction mixture of the test sample consisted of 0.5 mL of 1% BSA fraction and 0.5 mL CPE (100, 300 or 1000 µg/mL). The negative and positive control samples contained the same volume of 1% BSA and 0.5 mL of normal saline and diclofenac (100, 300 or 1000 µg/mL) respectively. The samples were incubated at 37°C for 20 min and then heated at 51°C for 20 min. The samples were cooled, and the turbidity was measured spectrophotometrically at 660 nm. The percentage inhibition was calculated using the formula:

$$\%\, Inhibition = \left(\frac{Absorbance\ of\ sample}{Absorbance\ of\ negative\ control} - 1\right) \times 100$$

4.2.5 *In Vivo* Models of Inflammation

4.2.5.1 *Carrageenan-Induced Paw Oedema*

The Sprague – Dawley rats were randomly divided into 5 groups (n=5). Carrageenan-induced paw oedema was performed according to the method described by Winter *et al.*, (1965). The paw diameters of each rat were measured initially as the baseline using the electronic digital callipers (Hexagon Manufacturing Intelligence, UK) and recorded. The rats were then treated orally as follows: negative control (normal saline, 10 mL/kg, *p.o*),

positive control (diclofenac, 10 mg/kg, *p.o*) and CPE (30, 100 or 300 mg/kg, *p.o.*). All rats were intraplantarly injected with 0.1 mL of 1% $^{w}/_{v}$ carrageenan in sub-plantar tissues and the paw diameters measured at times 0 h, 1 h, 2 h, 3 h, 4 h, and 5 h post-carrageenan treatment. Percentage inhibition of oedema was calculated using the relation;

$$\%\ Inhibition\ of\ Paw\ Oedema = \left(\frac{Paw\ diameter\ at\ time\ T}{Paw\ diameter\ at\ time\ 0}\right) \times 100$$

Where time T = time of measurement of paw and time 0 = baseline reading of paw thickness

4.2.5.2 ***Formalin-Induced Paw Oedema***

The test was performed as described previously by (Choudhary *et al*., 2014). Twenty-five (25) male Sprague-Dawley rats randomly assigned to five groups (n=5) were used in this study. Rats in three of the groups were administered with CPE 30, 100 or 300 mg/kg orally. Diclofenac was administered to one group as the positive control group (10 mg/kg, *p.o*) whereas the negative control received distilled water (10 mL/kg *p.o*). Prior to drug administration, the left hind paw diameters of all animals were measured and recorded using electronic digital callipers (Hexagon Manufacturing Intelligence, UK). 1 h after the drug administration, 0.05 mL of 1% $^{v}/_{v}$ solution of formalin was injected into the left hind paws of each animal. 3 h after each formalin injection, the paw diameters were measured again and thereafter, every 24 h for 10 consecutive days. Results were expressed as percentage inhibition in paw thickness at various intervals in comparison to the initial values as follows:

$$\%\ Inhibition\ of\ Paw\ Oedema = \left(\frac{Paw\ diameter\ at\ tme\ T}{Paw\ diameter\ at\ time\ 0}\right) \times 100$$

Where time T = time of measurement of paw and time 0 = baseline reading of paw thickness

4.2.6 **Evaluation of antinociceptive properties of CPE**

4.2.6.1 ***Acetic Acid-Induced Writhing***

The test was carried out as described earlier by Tang *et al.,* 2007. The mice (20 – 25 g), selected in this experiment were weighed individually and randomly grouped into treatment, positive control and negative control groups each of 5 mice. The positive control

group received morphine sulphate (3 mg/kg), the negative control group, normal saline (10 mL/kg) and the treatment groups were given the CPE (30, 100 and 300 mg/kg) orally. After 30 min, all the animals were treated intraperitoneally (i.p) with 0.6% acetic acid for induction of writhing. Mice were then placed individually in a testing chamber (Perspex chamber, 15×15×15 cm). A mirror inclined at 45° below the floor of the chamber allowed a complete view of the mice. Injection of acetic acid-induced a nociceptive behaviour, writhing, an exaggerated extension of the abdomen combined with the outstretching of the hind limbs. Responses were captured for 30 min for analysis by a video recorder ((Sony-Handycam, model: HDRCX675/B, Tokyo, Japan) placed directly opposite the mirror and attached to a computer. The behaviour was tracked using the software, JWatcher™, Version 1.0 (University of California, LA, USA, and Macquarie University, Sidney, Australia, available at http://www.jwatcher.ucla.edu/) to obtain the frequency and duration of abnormal constrictions (writhes) per 5 min, starting 5 min after acetic acid administration. A nociceptive score was determined for each 5-min time block by multiplying the frequency and duration of writhes. These data were expressed in a time course which enabled the observation of changes in the writhing induced and from which the areas under the curves (AUCs) were calculated.

4.2.6.2 *Formalin-Induced Paw Licking*

The formalin test first described by Dubuisson and Dennis (1977) was carried out as described by Malmberg and Yaksh, (1995). The mice (20 – 25 g) used were randomly assigned to five different experimental groups each containing five mice. They were allowed to acclimatize for 15 – 30 min. The test group was given the extract orally (30, 100 and 300 mg/kg), the positive control group with morphine sulphate (3 mg/kg) and the negative control group with normal saline (10 mL/kg) an hour before formalin administration. The 5% formalin (10µL) was given by intraplantar injection into the dorsal surface of the right hind paw of each mouse. The animals were immediately returned individually into the testing chamber and their nociceptive behaviours (paw licking) captured 60 min for analysis in the same way as described previously in the writhing test above. The pain response was scored for 60 min, starting immediately after formalin injection. A nociceptive score was determined for each 5-min time block by measuring the amount of time spent by each animal biting or licking of the injected paw. Tracking of the

behaviour was done using public domain software JWatcher™, Version 1.0. The average nociceptive score for each time block was calculated by multiplying the frequency and time spent in biting/licking. The first phase was recorded as the time spent in licking/biting the injected paw between 0 – 10 min of observation and the second phase is the time spent in licking between 10 – 60 min of observation and time-course curves were plotted and the areas under the curve for each phase and each treatment determined and plotted.

4.2.7 Data Analysis

Data were expressed as mean ± SEM. GraphPad® Prism for Windows Version 7.0 (GraphPad Software, San Diego, CA, USA, 2016) was used for all statistical analysis. $P < 0.05$ was considered statistically significant for all tests. A sample size of five rats or mice per group was used in all *in-vivo* tests. All time-course curves in the study were analysed using a two-way analysis of variance (ANOVA) with Holm-Sidak's *post hoc* test. One - way ANOVA with Holm-Sidak' *post hoc* test was used to determine differences between treatments groups (areas under curves). The equation below was used to calculate the percentage inhibition for each treatment:

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

4.3 RESULTS

4.3.1 In Vitro Anti-Inflammatory Tests

4.3.1.1 *Effect of CPE on Hypotonic solution-induced haemolysis*

CPE significantly protected the membranes of human red blood cells against haemolysis with a maximum mean inhibitory effect of 85.09% at a concentration of 1000 µg/mL ($F_{6,14}$= 120.1 P<0.0001). Similarly, diclofenac exhibited significant protection of the membranes of human red blood cell by a mean inhibition of 84.21 % at 1000 µg/mL as shown in and Table 4-1 and Fig 4-1.

Table 4-1 *In vitro* anti-inflammatory activity of CPE in the hypotonic solution-induced haemolysis

Treatment	Concentration(µg/ml)	% Inhibition
Control	-	-
CPE	100	57.89±1.52
CPE	300	70.18±3.82
CPE	1000	85.09±2.32
Diclo	100	67.54±0.88
Diclo	300	71.93±2.32
Diclo	1000	84.21±1.52

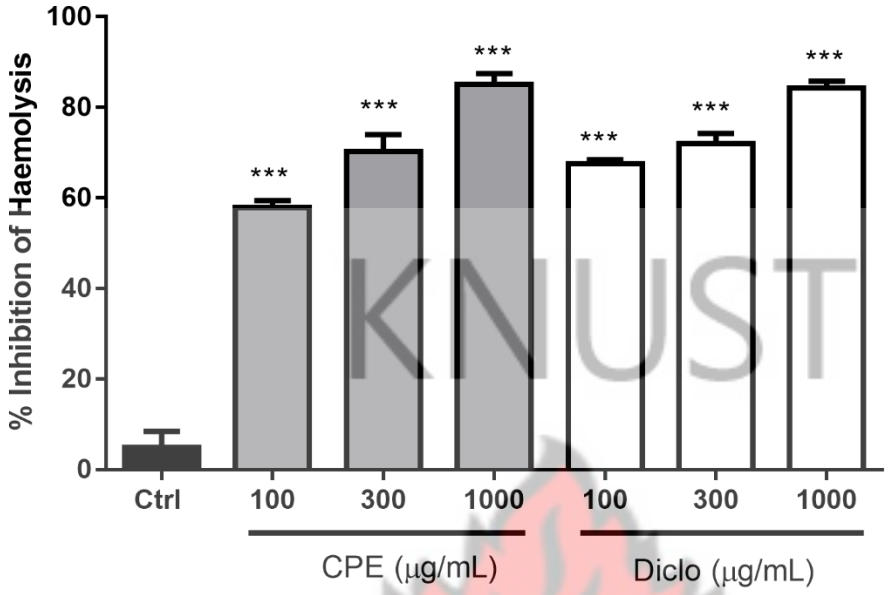

Figure 4-1 Effect of *Calotropis procera* extract (100, 300 and 1000 µg/mL, *p.o.*) and diclofenac (100 - 1000 µg/mL, *p.o.*) on % inhibition of hypotonic solution-induced haemolysis. Data expressed as mean ± SEM (n=5). Statistical differences between means evaluated using One-Way Analysis of Variance (ANOVA) followed by Dunnet's Multiple Comparison Test. *P < 0.05; **P<0.01; ***P <0.001 compared to negative control group.

4.3.1.2 ***Heat-Induced Haemolysis***

CPE markedly protected the membranes of human red blood cells against haemolysis ($F_{6,14}$= 82.15 P<0.0001) with a maximum mean inhibitory effect of 69.24% at a concentration of 1000 µg/mL. Also, diclofenac exhibited similar protection of the membranes of human red blood cell by a mean inhibition of 84.05% at 1000 µg/mL as depicted in Table 4-2 and Fig 4-2.

Table 4-2 *In vitro* anti-inflammatory activity of CPE in the heat-induced haemolysis

Treatment	Concentration(µg/ml)	% Inhibition
Control	-	-
CPE	100	38.62±4.62
CPE	300	59.26±3.87
CPE	1000	69.24±3.73
Diclo	100	66.26±2.24
Diclo	300	80.23±1.51
Diclo	1000	84.05±0.60

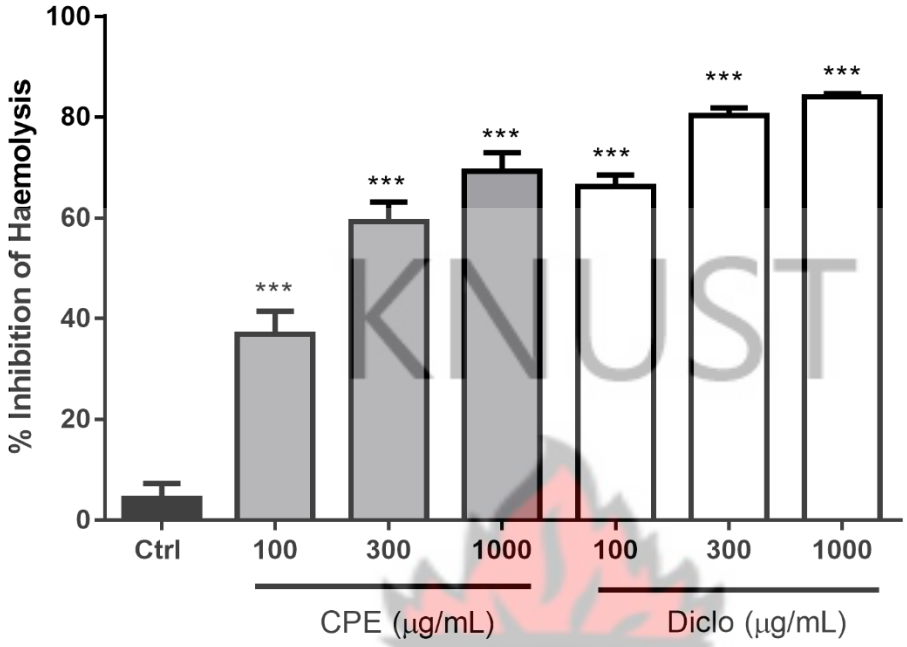

Figure 4-2 Effect of *Calotropis procera* extract (100, 300 and 1000 µg/mL, *p.o.*) and diclofenac (100 - 1000 µg/mL, *p.o.*) on % inhibition of heat-induced haemolysis. Data expressed as mean ± SEM (n=5). Statistical differences between means evaluated using One-Way Analysis of Variance (ANOVA) followed by Dunnet's Multiple Comparison Test. **P < 0.05; **P<0.01; ***P <0.001* compared to negative control group

4.3.1.3 *Effect of CPE on Albumin Denaturation*

CPE protected the proteins in fresh egg albumin against denaturation by exhibiting a maximum mean inhibitory effect of 87.80 % at a concentration of 1000 µg/mL ($F_{6,14}$= 107.9 P<0.0001). Also, diclofenac similarly exhibited significant protection of the proteins in the fresh egg albumin by a mean inhibition of 72.23 % at 1000 µg/mL as shown in Table 4-3 and illustrated in Fig 4-3.

Table 4-3 *In vitro* anti-inflammatory activity of CPE in the albumin denaturation test

Treatment	Concentration(µg/ml)	% Inhibition
Control	-	--
CPE	100	54.61±3.02
CPE	300	60.87±2.23
CPE	1000	87.8±3.18
Diclo	100	37.79±1.86
Diclo	300	54.48±2.34
Diclo	1000	72.23±3.15

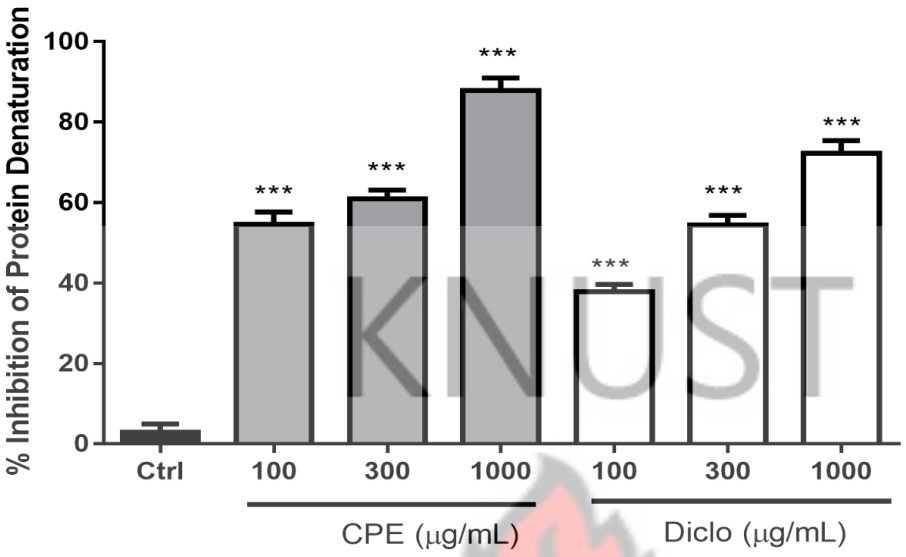

Figure 4-3 Effect of *Calotropis procera* extract (100, 300 and 1000 µg/mL, *p.o.*) and diclofenac (100 - 1000 µg/mL, *p.o.*) on % inhibition of protein denaturation. Data expressed as mean ± SEM (n=5). Statistical differences between means evaluated using One-Way Analysis of Variance (ANOVA) followed by Dunnet's Multiple Comparison Test. **P < 0.05; **P<0.01; ***P <0.001* compared to negative control group.

4.3.1.4 ***Effect of CPE on Bovine Serum Albumin Denaturation***

Results are shown in Table 4-4 and Fig 4-4 which indicates that CPE could protect the proteins in the bovine serum albumin against denaturation ($F_{6,14}$ = 95.74 P<0.0001) by exhibiting a maximum mean inhibitory effect of 96.86 % at a concentration of 1000 µg/mL. Also, diclofenac similarly exhibited significant protection of the proteins in the bovine serum albumin by a mean inhibition of 98.06% at 1000 µg/mL.

Table 4-4 *In vitro* anti-inflammatory activity of CPE in the bovine serum albumin assay

Treatment	Concentration(µg/ml)	% Inhibition
Control	-	-
CPE	100	45.49±0.52
CPE	300	90.73±7.94
CPE	1000	96.86±4.38
Diclo	100	64.2±2.50
Diclo	300	93.57±0.91
Diclo	1000	98.06±1.35

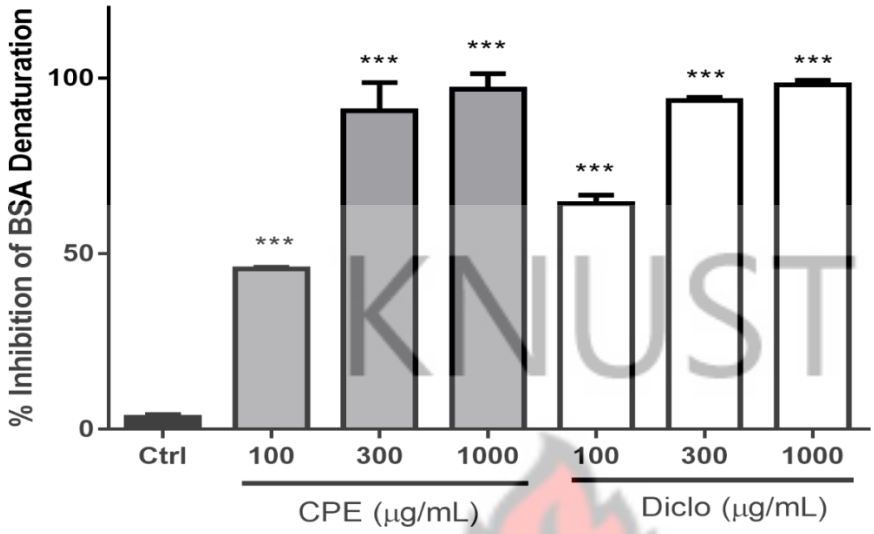

Figure 4-4 Effect of *Calotropis procera* extract (100, 300 and 1000 µg/mL, *p.o.*) and diclofenac (100 - 1000 µg/mL, *p.o.*) on % inhibition of BSA denaturation. Data expressed as mean ± SEM (n=5). Statistical differences between means evaluated using One-Way Analysis of Variance (ANOVA) followed by Dunnet's Multiple Comparison Test. **P < 0.05; **P<0.01; ***P <0.001* compared to negative control group.

4.3.2 *In Vivo* Anti-Inflammatory Tests

4.3.2.1 *Carrageenan-Induced Paw Oedema Model*

Results presented in Fig 4-5(a) represents the time-course curve of the percentage change in paw oedema after the administration of CPE (30, 100 and 300 mg/kg), diclofenac (10 mg/kg) and normal saline 10 mL/kg. CPE at 300 mg/kg significantly (p<0.05) reduced paw oedema produced by carrageenan at 2 h which continued to the 5 h. Diclofenac (10 mg/kg) however significantly (p<0.01) decreased paw oedema at the first hour and was sustained throughout the entire duration of the experiment. Fig 4-5(b) shows total oedema calculated as AUC of the time course curve of the various treatment groups. Diclofenac 10 mg/kg produced the smallest total oedema compared to the control group (Ctrl). The percentage inhibition produced by the various treatments was found to be 28.499 %, 28.547 %, 39.663 % and 53.465 % respectively for CPE 30, 100, 300 mg/kg and diclofenac (10 mg/kg) which showed that CPE 30, 100 and 300 mg/kg also respectively reduced total oedema significantly ($F_{(4,15)}$ = 6.24; p=0.0105*)* in a dose-dependent fashion.

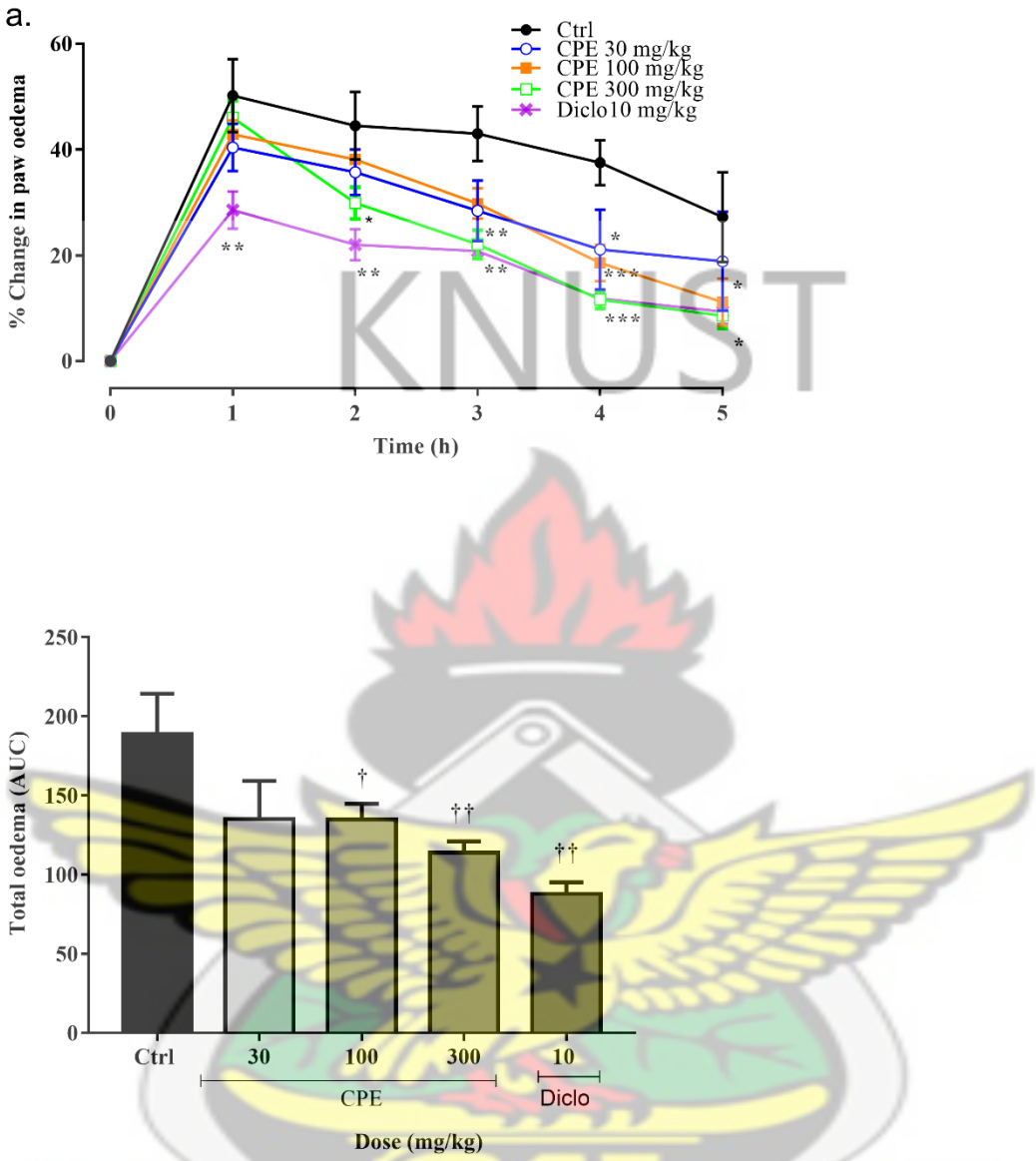

Figure 4-5 Effect of *Calotropis procera* extract (30, 100 and 300 mg/kg, p.o.) and diclofenac (10 mg/kg, *p.o.*) on (a) % change in paw oedema and (b) total oedema in carrageenan-induced acute inflammation model in rats. Data expressed as mean ± SEM (n=5). Statistical differences between means evaluated using Two-way ANOVA followed by Bonferroni's *post hoc* test, *$P < 0.05$; **$P<0.01$; ***$P <0.001$ compared to negative control group One-Way Analysis of Variance (ANOVA) followed by Dunnet's Multiple Comparison Test, $^{\dagger}P < 0.05$; $^{\dagger\dagger}P<0.01$; $^{\dagger\dagger\dagger}P <0.001$ compared to negative control group.

4.3.2.2 ***Formalin-Induced Paw oedema***

Intraplantar injection of formaldehyde into rat's hind paw produced a marked increase in paw diameter in all animals (Fig 4-6a and b). This was indicated by marked paw oedema

on the first day which was sustained in the negative control group throughout the entire duration of the experiment (day 1 to 10). CPE (30, 100 and 300 mg/kg, *p.o.*) significantly reduced paw diameter by 19.60% 35.88% and 47.65% respectively while diclofenac (10 mg/kg) produced 85.15% inhibition (Fig 4-6a). CPE also produced a significant and dose-dependent anti-oedematous effect at the 3 doses tested ($F_{4,15}$=100.9; p<0.0001) (Fig 4-6b) where the total anti-oedematous effect (AUC) of the control was 93, CPE (30, 100, 300 mg/kg) was determined to be 200.25, 371.42, 485.22 and diclofenac (10 mg/kg) was 555.75.

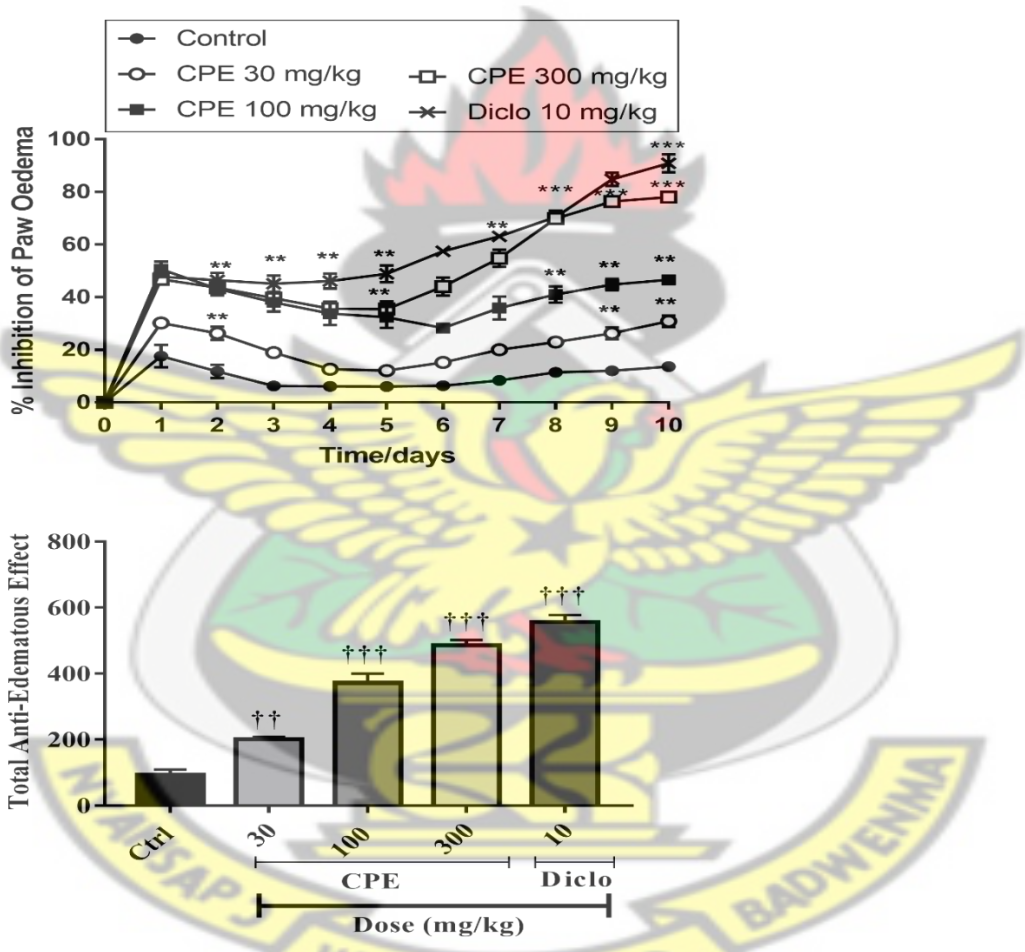

Figure 4-6 The effect of *Calotropis procera* extract (30, 100 and 300 mg/kg, p.o.) and diclofenac (10 mg/kg, *p.o.*) on (a) % inhibition of paw oedema and (b) total anti-oedematous effect in the formalin-induced inflammation model in rats. Data expressed as mean ± SEM (n=5). Statistical differences between means evaluated using Two-way ANOVA followed by Bonferroni's *post hoc* test, **P < 0.05; **P<0.01; ***P <0.001* compared to negative control group; One-Way Analysis of Variance (ANOVA) followed by Dunnet's Multiple Comparison Test, *††P<0.01; †††P <0.001* compared to negative control group.

4.3.3 **Evaluation of antinociceptive properties of CPE**

4.3.3.1 ***Acetic Acid-Induced Writhing Test***

The acetic acid injected intraperitoneally produced writhing, seen as an extension of the abdomen with simultaneous stretching of at least one of the hind limbs. Mice pre-treated with distilled water exhibited the highest number of writhes. The extract and morphine significantly suppressed the time-course of acetic acid-induced writhes. Treatment with doses of CPE (30, 100 & 300 mg/kg) showed significant ($F_{4,15}$ = 24.14, P<0.0001) inhibition of writhes respectively in the mice like that of morphine. Analysis of the area under the time-course curves of CPE (30,100,300 mg/kg) and morphine revealed a dose-dependent effect (Fig 4-7b).

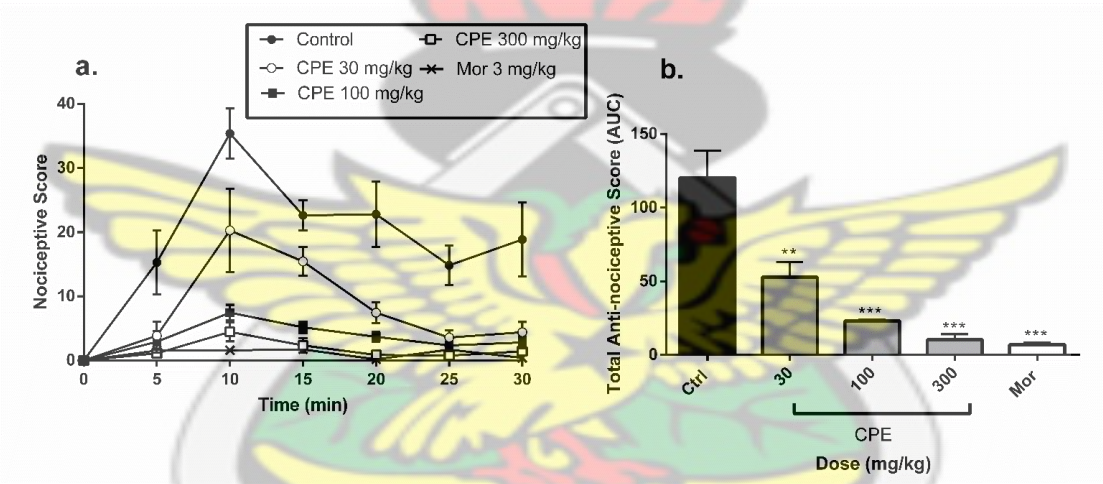

Figure 4-7 Antinociceptive activity of hydroethanolic extract of *Calotropis procera* (30-300 mg/kg) and morphine 3 mg/kg on (a) time course of writhing and (b) AUC of acetic acid-induced writhing in ICR mice. Data expressed as mean ± SEM (n=5). Statistical differences between means evaluated using One-Way Analysis of Variance (ANOVA) followed by Dunnet's Multiple Comparison Test. **P < 0.05; **P<0.01; ***P <0.001* compared to negative control group

4.3.3.2 ***Formalin-Induced Paw Licking***

The response to pain was biphasic with an initial intense response to pain immediately after formalin injection into the plantar surface of the hind leg within the first 10 min (first phase). This intense response rapidly waned and was replaced by a slowly rising but longer-lasting response 10 – 60 min with peak effects observed 20 – 30 min after the injection (second phase). Administration of the extract at doses 30 and 100 mg/kg significantly

inhibited both the first and second phases of formalin-induced nociception similar to the effects of morphine with the administration of CPE (300 mg/kg) showing inhibition of greater significance ($F_{4,20}$ = 12.21, P <0.0001). Analysis of the area under the time-course curves of CPE (30, 100, 300 mg/kg) and morphine (3 mg/kg) revealed a dose-dependent effect of the extract.

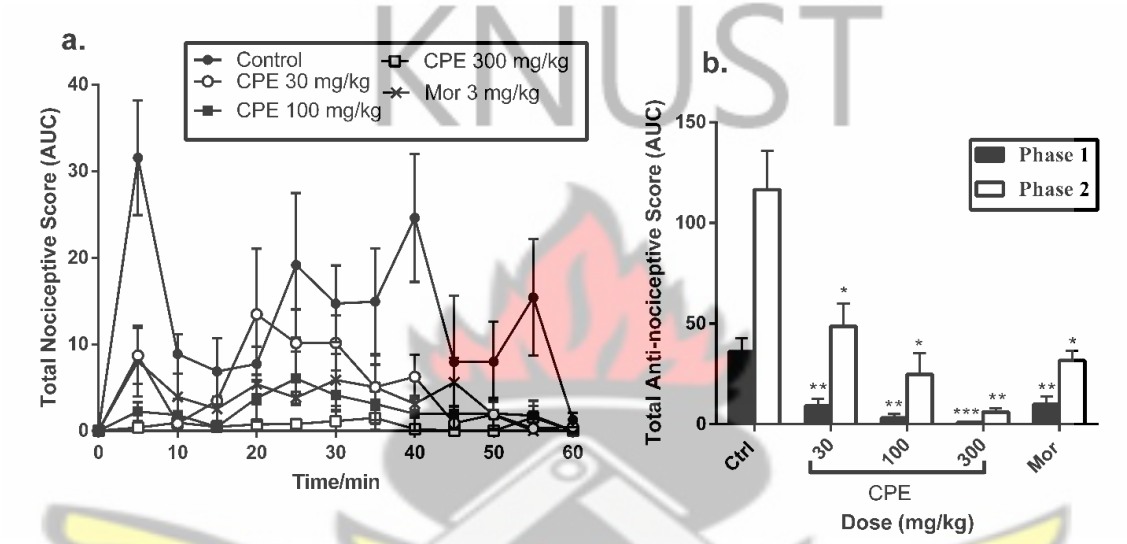

Figure 4-8 Antinociceptive activity of hydroethanolic extract of *Calotropis procera* (30-300mg/kg) and morphine 3 mg/kg showing (a) time course and (b) AUC on formalin-induced paw licking in ICR mice. Data expressed as mean ± SEM (n=4). Statistical differences between means evaluated using One-Way Analysis of Variance (ANOVA) followed by Dunnet's Multiple Comparison Test. **P < 0.05; **P<0.01; ***P <0.001* compared to negative control group.

4.4 DISCUSSION

Results of the present study confirm the anti-inflammatory and antinociceptive effects of *Calotropis procera* and indicate other possibly new mechanisms by which the reported and anti-inflammatory effects could be mediated.

The human red blood cell membrane stabilization method has been employed as one of the fastest *in vitro* methods for the rapid screening and identification of the anti-inflammatory properties of newly discovered plants or molecules (Kumari *et al.,* 1993*)*. The test is based on red blood cell because the membrane of the red blood cell is highly analogous to the membrane of lysosomes which are involved in the inflammatory processes (Marliyah *et al.,* 2015). The ability of the crude plant extract to stabilize the membrane of red blood cells shows that extract will be able to stabilize the membrane of lysosomes as well (Marliyah *et al.,* 2015). Stabilizing the membrane of lysosomes is very crucial in limiting inflammatory response since this process prevents the release of lysosomal contents of activated neutrophils which cause further tissue inflammation and damage following extracellular release (Chandra *et al*., 2012; Marliyah *et al.,* 2015). The extracellular activity of these enzymes is said to be related to acute or chronic inflammation.

The non-steroidal anti-inflammatory drugs (NSAIDs) used in the treatment of inflammation and its related disorders act either by inhibiting these lysosomal enzymes or by stabilizing the lysosomal membrane. The hydroethanolic *C. procera* extract at concentrations of 100, 300 and 1000 µg/mL exhibited membrane stabilization activity by inhibiting membrane lysis when RBCs were exposed to heat and hypotonic solution. Since the red cell membrane can be likened to that of lysosomes (Chandra *et al*., 2012; Rao *et al.*, 2005), its stabilization by CPE in both heat and hypotonic solution induced membrane destabilization indicates CPE may be postulated to as well stabilize the membrane of lysosomes thereby preventing the likelihood of inflammation.

Protein denaturation is the process by which proteins lose their tertiary and secondary structure by the application of external stress or compounds such as strong acids and bases, a concentrated organic or inorganic salt, organic solvent and heat (Chandra *et al.*, 2012). Denaturation of proteins is a well-documented cause of inflammation and rheumatoid arthritis (Chandra *et al.*, 2012) hence inhibition of protein denaturation may prevent or control inflammation. It is probably due to alteration in the hydrogen, hydrophobic,

electrostatic and disulphide bonding which also results in the production of autoantigens that stimulate auto-immune responses leading to some inflammatory disorders such as rheumatoid arthritis (Rahman *et al.*, 2015; Osman *et al.*, 2016). With this mechanism of inflammation in mind, the present study investigated the anti-inflammatory potential of *C. procera* by assessing its effects on the disruption of proteins from fresh egg albumin and bovine serum albumin (BSA). The hydroethanolic *C. procera* extract at concentrations of 100, 300 and 1000 µg/mL inhibited the disruption of the protein structures dose-dependently. Since protein denaturation is a well-documented cause of inflammation (Chandra *et al.*, 2012), it is speculated that CPE's anti-inflammation properties could be due in part to the inhibition of protein denaturation and the sequelae that follows it.

To confirm the results obtained from the *in vitro* studies and provide further evidence to support the use of *C. procera* traditionally for the management of inflammatory conditions, the carrageenan-induced paw oedema model was also used. This is a useful model to assess the contribution of inflammatory mediators involved in acute inflammation. The development of oedema in the rat hind paw following the injection of carrageenan has been described as a biphasic event with a late third phase in which various mediators operate in sequence to produce this inflammatory response. The initial phase of oedema (0 – 1 h) is attributed to the release of histamine and serotonin (Dongmo *et al.*, 2003) while the late accelerating phase of swelling (2 – 6 h) correlates with the elevated production of bradykinins, prostaglandins, and leukotrienes (Adedapo *et al.*, 2009). The extract possibly inhibited the release of inflammatory mediators such as bradykinin and prostaglandins which are implicated in the second phase of inflammation in these models for anti-inflammatory agents.

To further investigate the anti-inflammatory effect of the extract, the formalin-induced paw oedema model was also employed. Intraplantar injection of formalin is known to produce a biphasic inflammatory response – early and late phase (Hunskaar & Hole, 1987). In the early phase, histamine, bradykinin, and other inflammatory mediators are released and this occurs within two hours after intraplantar injection of formalin. The second phase which occurs after two hours involves the release of prostaglandins, cytokines, bradykinins, etc. The effect produced by these mediators last throughout the ten days of the test. CPE (30,

100, 300 mg/kg) ameliorated oedema like the standard analgesic drug used, diclofenac (10 mg/kg). Diclofenac has been known to exhibit this activity via inhibition of cyclooxygenase metabolism of arachidonic acid to produce prostaglandins and the extract may have also exhibited its anti-oedematous activity through a similar pathway. This view is supported by Arya and Kumar (2005) who reported earlier that the latex extract of the plant exhibited its anti-inflammatory activity through inhibition of prostaglandins and other inflammatory mediators such as bradykinin, serotonin, and histamine.

Pain is a key indicator in the inflammatory process (Zhang and An, 2007) and certain noxious stimuli, be it thermal, electrical, mechanical or chemical, are painful and reflex movements or behaviours resulting from such stimuli are indicative of a pain threshold (Shetty *et al.*, 2011). Acetic acid-induced writhing and formalin induced pain was used to demonstrate that the leaf extract of *Calotropis procera* possessed analgesic activity suggestive of both centrally and peripherally mediated mechanisms.

The acetic acid-induced abdominal writhing reflex model is a sensitive method for screening the analgesic effects of compounds (Bentley, 1983). The intraperitoneal administration of an agent that irritates the serous membranes cause a stereotypical behaviour in mice which is characterized by abdominal contractions, whole-body movements, twisting of the dorsal abdominal muscles, and a reduction in the motor activity and coordination (Singh *et al*, 2009). Acetic acid achieves writhing in the animals by activation of the chemo-sensitive nociceptors and the reduction in the number of abdominal contortions indicates the level of analgesia in the acetic acid-induced writhing model (Omeh and Ezeja, 2010). The sensitivity of the acetic acid-induced writhing model, makes it suitable for screening peripherally acting analgesics and the response is thought to involve local peritoneal cells and mediated through the prostaglandin pathway because acetic acid acts indirectly by inducing the release of prostaglandins (PGE_2 and $PGF_{2\alpha}$) as well as lipoxygenase products into the peritoneum which stimulate the nociceptive neurons sensitive to non-steroidal anti-inflammatory drugs and induces capillary permeability (Choi, 2007). Hence the test is useful for the evaluation of mild analgesic non-steroidal anti-inflammatory compounds.

Findings from the present study showed that the hydroethanolic leaf extract of *C. procera* produced a significantly attenuated acetic acid-induced abdominal constrictions in mice compared with the control group. The inhibition at the highest dose (300 mg/kg) produced by the extract was also comparable to that produced by morphine and this could indicate marked evidence of analgesic property. Therefore, the result of the acetic acid-induced writhing strongly suggests that the mechanism of this action may be linked partly to it interfering with the mechanism of transduction in primary afferent nociceptors. This inhibition may be mediated due to some phytoconstituents in the plant extract like flavonoids, steroids, and saponins which are present in the extract as shown in the phytochemical tests conducted.

The extract of *Calotropis procera* at the doses given produced a significant inhibition of the formalin-induced nociceptive behaviours of licking and biting of the injected hind paw in both the neurogenic and inflammatory phases respectively. The animals given the highest dose, (300 mg/kg), expressed greater inhibition compared to the reference drug morphine. This may have been due to the fact that the extract was crude, that is; it contains many phytochemicals: glycosides, proteins, steroids, flavonoids (Obese *et al.*, 2018) which can act in synergy to cause a slightly greater analgesic response compared to that of morphine which is a pure compound.

The early (neurogenic) phase is as a result of direct stimulation of nociceptors in the paw which ends in centrally mediated pain with the release of substance P, while the late phase is due to the release of histamine, serotonin, bradykinin, and prostaglandins (Oyebanji *et al.*, 2013). Drugs that act primarily on the central nervous system, such as narcotics, inhibit both phases equally while peripherally acting drugs such as non-steroidal anti-inflammatory drugs (NSAIDs) and steroidal anti-inflammatory only inhibit the late phase (Lopez-Garcia, 2004). *Calotropis procera* extract effectively inhibited only the late phase of the formalin response in this study thus reinforcing inhibition of prostaglandin synthesis as its possible mechanism of action.

4.5 CONCLUSION

The inhibition of red cell haemolysis and protein denaturation and the reduction of oedema and pain by the hydroethanolic leaf extract of *Calotropis procera* provides further evidence to the ethnomedicinal use of *C. procera* for pain and inflammatory conditions.

Chapter 5

ASSESSMENT OF POSSIBLE MECHANISMS OF ANTINOCICEPTIVE ACTIONS

5.1 BACKGROUND

The traditional use of the *Calotropis procera* extract as an analgesic agent has been established in the previous chapters using chemical models of pain. Formalin test, (Ellis *et al.*, 2008) was used to further assess the possible involvement of the opioid, adenosine, ATP sensitive K^+ channels, nitric oxide, serotonergic, muscarinic, adrenergic and voltage-gated calcium channel pathways in the observed anti-nociceptive effect of the extract. In the first phase of the formalin test, some cytokines like substance P and bradykinin are involved in the nociceptor's sensitization while histamine, serotonin, prostaglandins, nitric oxide, bradykinin, TNF α, IL-6, noradrenaline, Ach and other pro-inflammatory pain mediators were involved in the late phase of the formalin test. (Dalbo *et al.*, 2006; Lu *et al.*, 2007). Some receptors (serotoninergic, opioid, adrenergic, adenosine, muscarinic receptors voltage-gated calcium channels) and pain mediators (TNF-α, IL-1β, PGE_2, and bradykinin) involved in the pain mediation were also investigated using different antagonists to help predict the possible mechanism of action of the extract in this context.

5.2 MATERIALS AND METHOD

5.2.1 Animals

For this experiment, ICR mice (20 - 25 g) and Sprague-Dawley rats (150 – 200 g) of both sexes were used and cared for as previously described in section 4.2.1.

5.2.2 Drugs and Chemicals

Formalin, theophylline, carrageenan and acetic acid were obtained from British Drug House, Poole, England; morphine hydrochloride from Phyto-Riker, Accra, Ghana; granisetron hydrochloride was purchased from Corepharma LLC, Middlesex, New Jersey, USA; glibenclamide (Daonil®), from Sanofi-Aventis, Guildford, UK; yohimbine hydrochloride (Procomil®), from Walter Ritter GmbH + Co. KG, Germany); Atropine sulphate was purchased from E. Merck AG-Darmstadt, Germany; naloxone hydrochloride

was obtained from Sigma-Aldrich Inc., St. Louis, MO, USA; nifedipine from Denk Pharma, Germany); prostaglandin E_2 (PGE_2), bradykinin acetate salt, interleukin-1β (IL-1β) and tumour necrosis factor-alpha (TNF-α) were all purchased from Sigma-Aldrich Inc., St. Louis, MO, USA.

5.2.3 Assessment of the possible mechanism of antinociception of the extract using the formalin test

The mechanism of analgesic action of the *Calotropis procera* extract was investigated using various antagonists in the formalin test. The receptor pathways investigated were the adenosine, opioid, adrenergic, serotoninergic, ATP sensitive K^+ channels, and muscarinic receptors/pathways. The doses of antagonist, agonist and other drugs were carefully selected based on existing literature data and in pilot experiments in the laboratory (Woode and Abotsi, 2011).

5.2.3.1 *Involvement of the adenosinergic system*

To investigate the role played by the adenosinergic systems in the anti-nociception caused by extract, mice were pre-treated with theophylline, a non-selective adenosine receptor antagonist (10 mg kg^{-1}, i.p.,). After 15 min the mice received extract (100 mg kg^{-1}, *p.o.*), morphine (3 mg kg^{-1}, i.p.) or vehicle (10 mL/kg, *p.o.*). The nociceptive response to the intraplantar injection of formalin was recorded 60 min after administration of the extract or vehicle and 30 min after morphine administration. The formalin test was then performed as described under section 4.2.6.2.

5.2.3.2 *Involvement of ATP-sensitive K^+ channels*

Mice for this experiment were pre-treated with glibenclamide (an ATP-sensitive K^+ channel inhibitor, 8 mg kg^{-1}, *p.o.*) or vehicle and after 30 min received CPE (100 mg kg^{-1}, *p.o.*), morphine (3 mg kg^{-1}, i.p.) or vehicle (10 mL/kg, *p.o.*) and the nociceptive responses to formalin was recorded as previously described under section 4.2.6.2.

5.2.3.3 *Involvement of the serotonergic system*

In order to determine the contribution of $5HT_3$ receptors/pathway in the antinociceptive effects of the extract, mice were pre-treated with the $5HT_3$ inhibitors, ondansetron (0.5 mg

kg^{-1}, i.p.) and granisetron (2 mg kg^{-1}, *p.o.*) or saline 30 min before extract (100 mg kg^{-1}, *p.o.*), morphine (3 mg kg^{-1}, i.p.) or vehicle (10 mL/kg, *p.o.*) administration. The nociceptive responses to formalin were recorded for 60 min after 1 h (*p.o.*) or 30 min (i.p.) as described earlier under section 4.2.6.2.

5.2.3.4 ***Participation of the muscarinic system***

To determine the involvement of the muscarinic system, mice were pre-treated with atropine (5 mg kg^{-1}, i.p.) which is non-selective muscarinic antagonist 30 min before extract (100 mg kg^{-1}, *p.o.*), morphine (3 mg kg^{-1}, i.p.) or vehicle (10 mL/kg, *p.o.*). After 1 h (*p.o.*) or 30 min (i.p.), the nociceptive responses to formalin were recorded for 60 min and analysed as previously described under section 4.2.6.2.

5.2.3.5 ***Adrenergic pathway involvement***

The possible participation of the adrenergic system in antinociception was evaluated. Mice were pre-treated with yohimbine (an α_2 receptor antagonist 3 mg kg^{-1} i.p.) 30 min before extract (100 mg kg^{-1}, *p.o.*), morphine (3 mg kg^{-1}, i.p.) or vehicle (10 mL/kg, *p.o.*). The nociceptive responses to formalin were recorded as earlier described under section 4.2.6.2.

5.2.3.6 ***Involvement of the voltage-gated calcium channel***

To investigate the participation of the voltage-gated calcium channel (VGCC), mice were pre-treated with nifedipine (10 mg/kg, *p.o.*, L-type VGCC blocker) or vehicle and after 30 min., received CPE (100 mg/kg, *p.o.*), morphine (3 mg/kg, i.p.) or vehicle (10 mL/kg, *p.o.*). The nociceptive responses to formalin were recorded after 1 h (*p.o.*) or 30 min (i.p.) and analysed as described earlier under section 4.2.6.2.

5.2.3.7 ***Assessment of opioidergic pathway involvement***

Assessment of the opioid receptors or pathway involvement was done by pre-treating mice intraperitoneally with naloxone (2 mg kg^{-1}), a non-selective opioid receptor antagonist. After 15 min the animals received extract (100 mg kg^{-1}, *p.o.*), morphine (3 mg kg^{-1}, i.p.) or vehicle (10 mL/kg, *p.o.*). The nociceptive response to intraplantar injection of formalin was recorded as described earlier under section 4.2.6.2.

5.2.4 **Tumour Necrosis Factor-alpha (TNF-α) – induced hyper nociception**

Mechanical hyper nociception induced by TNF- α after pre-treatment of rats with CPE or morphine was performed as previously described in the Randall-Selitto test (Randall and Selitto, 1957; Vale *et al*., 2004; Stohr *et al*., 2006) by using an analgesimeter (Model No.15776, Ugo Basile, Comerio, Varese, Italy). The analgesimeter was used to apply a linearly increasing pressure, by means of a blunt Perspex cone, to the dorsal region of the right hind paw of the rat until the rat vocalized or withdrew the paw. Rats (n = 5) received pre-treatment with either vehicle (10 mL/kg, *p.o.*), CPE (30 - 300 mg/kg *p.o.*) for 1 h or morphine (3 mg/kg, i.p.) for 30 min. before intraplantar injection of TNF-α (2.5 pg/paw; 20 µL) into the right hind paws. The pressure was gradually applied to the right hind paw and paw withdrawal thresholds (PWTs) were assessed as the pressure (grams) required to elicit paw withdrawal. A 250 g cut-off point was chosen as the maximum weight to apply to prevent any damage to the tissue in the paw of the rats. Hyper nociception was measured in the injected paws at 1, 2, 3, 4 and 5 h the change was calculated as a percentage of the maximum possible effect (% MPE). The maximal possible effect (% MPE) was calculated according to the formula:

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

Where PWT: paw withdrawal threshold and CT: control threshold

5.2.5 **Bradykinin–Induced hyper nociception**

To evaluate the effect of CPE pre-treatment on mechanical hyperalgesia induced by bradykinin in rats, a previously described method was used (Wilhelm *et al*., 2009). Five groups of rats (n=5) were pre-treated with vehicle (10 mL/kg, *p.o.*), CPE (30 - 300 mg/kg, *p.o.*) for 1 h or morphine (3 mg/kg, i.p.) for 30 min. before the intraplantar injection of bradykinin (500 ng/paw; 20 µL) into their right hind paw. Hyperalgesia was measured in the injected paws at 1, 2, 3, 4 and 5 h after bradykinin injection using an analgesimeter in a similar manner as described earlier in section 5.2.4. The rats were pre-treated with captopril, 5 mg kg^{-1} s.c. (an angiotensin-converting enzyme inhibitor) 1 h before the experiments to prevent bradykinin degradation by angiotensin-converting enzymes.

5.2.6 **Prostaglandin E_2 (PGE_2) – hyper nociception**

The effect of pre-treatment of rats with CPE on prostaglandin E_2-induced hyper nociception was investigated like that described by Wilhelm *et al.*, 2009. Rats (n = 5) were pre-treated with either vehicle (10 mL/kg, *p.o.*), CPE (30 - 300 mg/kg, *p.o.*) for 1 h or morphine (3 mg/kg, i.p.) for 30 min before intraplantar injection of PGE_2 (100 ng/paw; 20 µL) into their right hind paw. Hyper nociception was measured in the injected paws at 1, 2, 3, 4 and 5 h after PGE_2 injection using an analgesimeter in a similar manner as described earlier in section 5.2.4.

5.2.7 **Interleukin 1 – beta (IL-1β) – induced hyper nociception**

Assessment of mechanical hyper nociception induced by IL-1β after pre-treatment of rats with CPE or morphine was performed as previously described by Vale *et al.*, (2004). Rats (n = 5) received pre-treatment with either vehicle (10 mL/kg, *p.o.*), CPE (30 - 300 mg/kg, *p.o.*) for 1 h or morphine (3 mg/kg, i.p.) for 30 min. before intraplantar injection of IL-1β (1 pg/paw; 20 µL) into the right hind paws. Hyper nociception was measured in the injected paws at 1, 2, 3, 4 and 5 h using an analgesimeter in a similar manner as described earlier in section 5.2.4.

5.2.8 **Data analysis**

All data are presented as Mean ± standard error of the mean (SEM). GraphPad® Prism Version 7.0 (GraphPad Software, San Diego, CA, USA) for Windows was used to perform all statistical analysis with $P < 0.05$ considered statistically significant for all tests. All time-course curves in the study were analysed using a two-way analysis of variance (ANOVA) with Holm-Sidak's *post hoc* test for the formalin tests and Tukey's *post hoc* test for the hyperalgesia models. One - way ANOVA with Tukey's *post hoc* test was used to determine differences between treatments groups (areas under curves). The equation below was used to calculate the percentage inhibition for each treatment:

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

5.3 RESULTS

5.3.1 Assessment of the possible mechanisms of antinociception of the extract

Results presented in Figures. 5-2 and 5-3 demonstrate the effect of naloxone, glibenclamide, nifedipine, yohimbine, atropine, theophylline, ondansetron and granisetron on the antinociceptive effects of the extract and morphine. Pre-treatment of mice with 2 mg kg^{-1} naloxone and 5 mg kg^{-1} theophylline significantly ($F_{6,20}$ = 11.61 $P \leq 0.0001$) reversed both phase 1 and phase 2 antinociception of CPE (100 mg kg^{-1}, p.o., Fig. 5-2b). However, prior treatment of the animals with other agents like yohimbine (3 mg kg^{-1} p.o.), nifedipine (10 mg kg^{-1} p.o.), ondansetron (0.5 mg kg^{-1} i.p.), granisetron (2 mg kg^{-1} i.p..), glibenclamide (8 mg kg^{-1}, p.o.) and atropine (5 mg kg^{-1} i.p.) was not able to significantly reverse the antinociception produced by the extract. Morphine (3 mg kg^{-1} i.p.), the standard analgesic agent had its effects being reversed by all the antagonists used for the experiment. As shown in figure 5-1, naloxone (opioid antagonist) and theophylline (adenosine antagonist) did not show and antinociceptive effect when they were administered alone to the test animals.

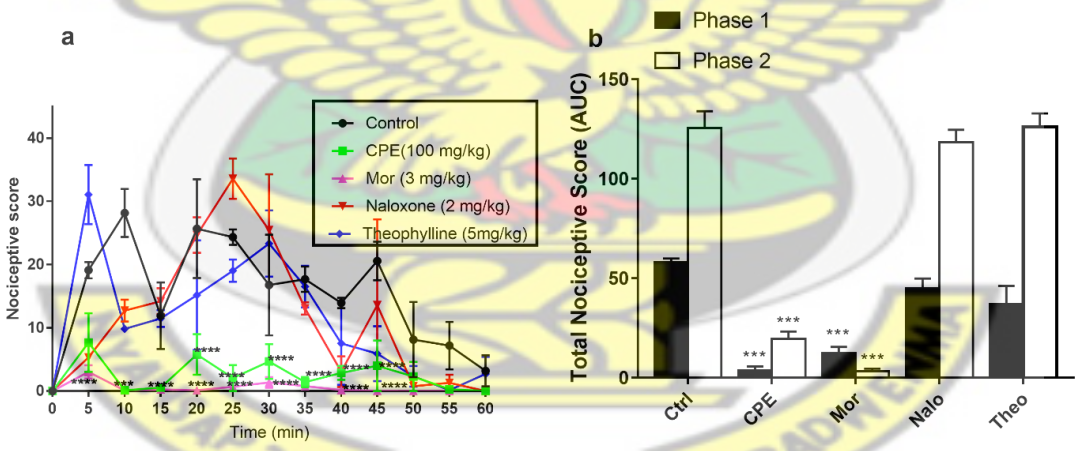

Figure 5-1 Time course curve (a) and total nociceptive score (AUC) (b) for phase 1 and phase 2 of formalin-induced nociception of CPE (100 mg kg^{-1}), Morphine (3 mg kg^{-1} i.p.), Naloxone alone (2 mg kg^{-1} i.p.) and theophylline (5 mg kg^{-1} i.p.). Each point represents the mean ± S.E.M. $*P \leq 0.05$, $**P \leq 0.01$, $***P \leq 0.001$ compared to the control (Two-way ANOVA followed by Bonferroni *post hoc* test for the time-course curves while one-way ANOVA followed by Bonferroni *post hoc* test for the AUC analysis).

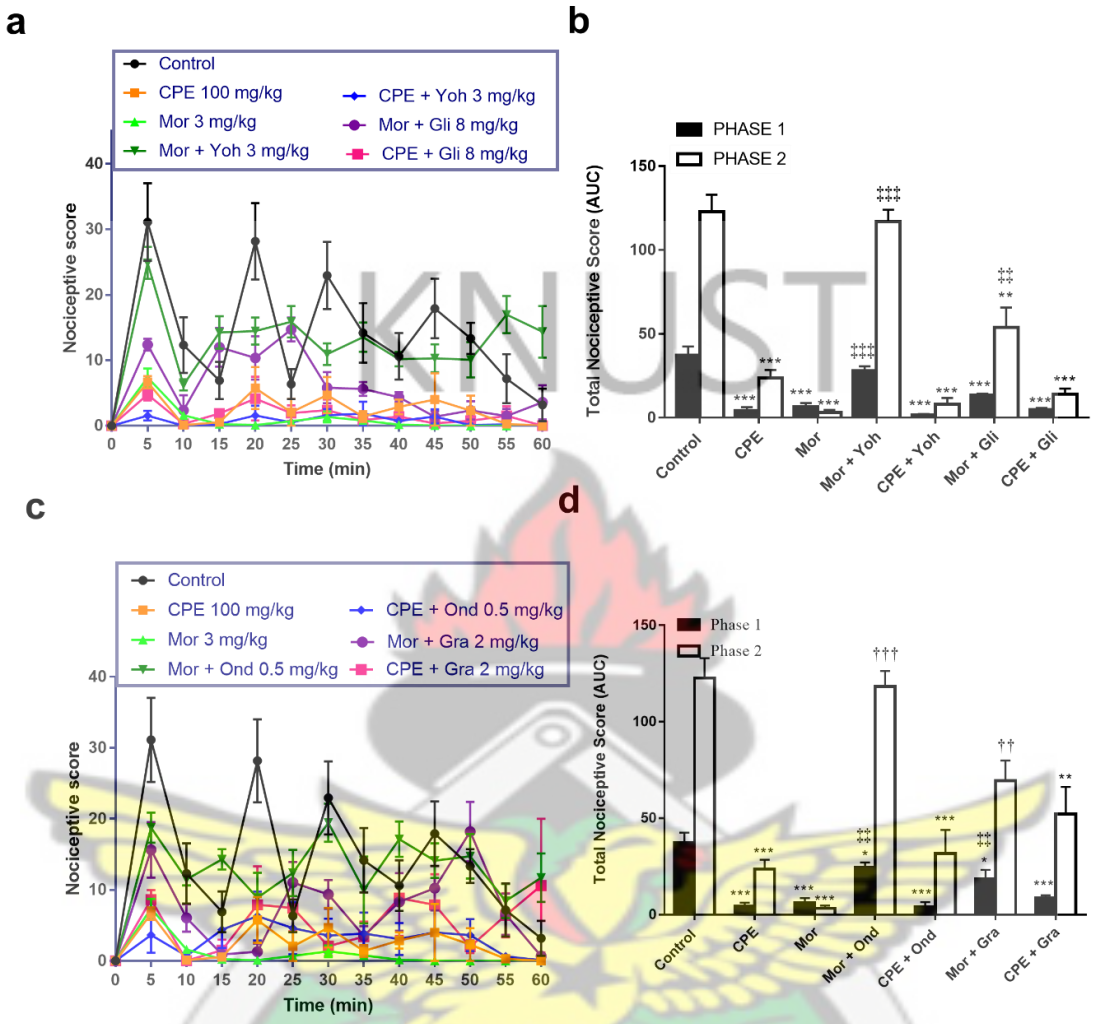

Figure 5-2 Time course curves (a, c) and total nociceptive score (AUC) for phase 1 and phase 2 of formalin-induced pain (b, d) or yohimbine (3 mg kg^{-1} i.p.), glibenclamide (8 mg kg^{-1} i.p.), ondansetron (0.5 mg kg^{-1} i.p.) and granisetron (2 mg kg^{-1} i.p.) on the antinociceptive effect of CPE (100 mg kg^{-1} p.o) in the formalin-induced paw licking test.. Each point represents the mean ± S.E.M. $^{*}P \leq 0.05$, $^{**}P \leq 0.01$, $^{***}P \leq 0.001$, $^{\dagger}P \leq 0.05$, $^{\dagger\dagger}P \leq 0.01$, $^{\dagger\dagger\dagger}P \leq 0.001$, $^{\ddagger\ddagger\ddagger}P < 0.001$, $^{\ddagger\ddagger}P < 0.01$, compared to control. (Two-way ANOVA followed by Bonferroni *post hoc* test for the time-course curves while one-way ANOVA followed by Bonferroni *post hoc* test for the AUC analysis).

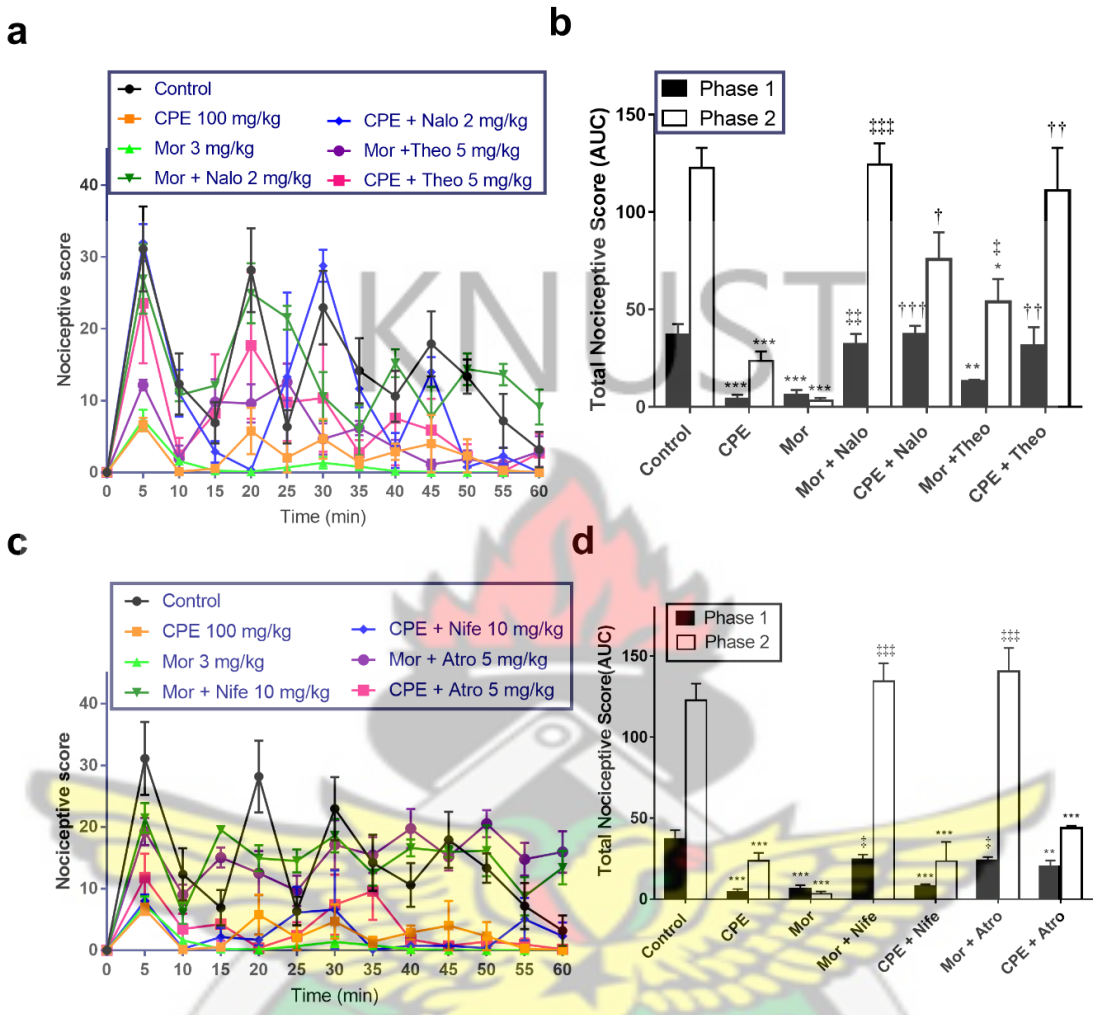

Figure 5-3 Time course curves (a, c) and total nociceptive score (AUC) for phase 1 and phase 2 of formalin-induced pain (b, d) of theophylline (5 mg kg^{-1} i.p.), naloxone (2 mg kg^{-1} i.p.), nifedipine (10 mg kg^{-1} i.p.) and atropine (5 mg kg^{-1} i.p.) on the antinociceptive effect of CPE (100 mg kg^{-1} *p.o*) in the formalin-induced licking test. Each column represents the mean ± S.E.M. $^{*}P \leq 0.05$, $^{**}P \leq 0.01$, $^{***}P \leq 0.001$, $^{\dagger}P \leq 0.05$, $^{\dagger\dagger}P \leq 0.01$, $^{\dagger\dagger\dagger}P \leq 0.001$, $^{\ddagger\ddagger\ddagger}P < 0.001$, $^{\ddagger\ddagger}P < 0.01$, compared to respective controls (Two-way ANOVA followed by Bonferroni *post hoc* test for the time-course curves while one-way ANOVA followed by Bonferroni *post hoc* test for the AUC analysis).

5.3.2 **Tumour necrosis Factor-alpha (TNF-α) – induced hyperalgesia**

Hyperalgesia was induced in all the animals treated with TNF-α. The change in hyperalgesia state was calculated as a percentage of the maximum possible effect. The results presented in Fig. 5-4 (a and b) show that administration of CPE (100 – 300 mg kg^{-1}, *p.o.*) and morphine markedly reversed hyperalgesia induced by intraplantar injection of TNF- α. A significant decrease in the paw withdrawal latencies of rats ($F_{5, 71}$ = 12.69, *P< 0.0001*) and the corresponding antinociceptive score ($F_{4, 12}$ = 7.863, *P = 0.0024*) was observed. Maximum possible mechanical anti-hyperalgesia induced by bradykinin was observed at the highest dose of CPE (300 mg kg^{-1}) which performed better than morphine (3 mg kg^{-1}).

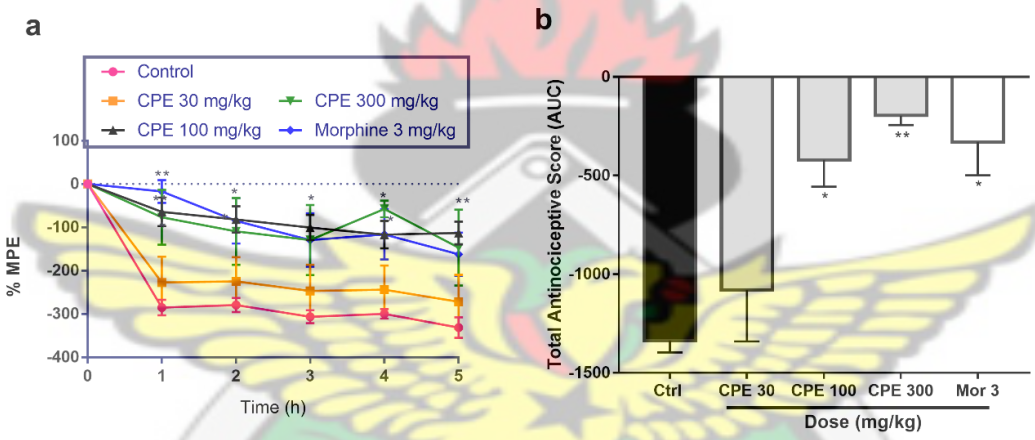

Figure 5-4 Effect of pre-treatment of rats with CPE (30-300 mg/kg, p.o.) and morphine (3 mg/kg, i.p.) on TNF-α - induced hyper-nociception. Each data represents the mean ±S.E.M (n=5). Data represents (a) the time-course curves and (b) total antinociceptive effects (AUC) of the treatments given. ** *P < 0.01*, **P < 0.05* (two-way ANOVA followed by Tukey's post hoc test for the time course curves and one - way ANOVA followed by Tukey's *post hoc* test for the AUC analysis).

5.3.3 **Bradykinin – induced hyper-nociception**

Mechanical pressure applied to the rats' right hind paws after intraplantar injection of bradykinin resulted in an increase in paw withdrawal reflexes. Pre-treatment of the animals with either CPE (100 – 300 mg/kg, *p.o.*) or morphine (3 mg/kg, i.p.) led to a significant increase in paw withdrawal latencies ($F_{5, 88} = 27.82$, $P < 0.0001$, Fig. 5-5a) and total anti-nociceptive score ($F_{4, 14} = 5.636$, $P = 0.0064$, Fig. 5-5b). again, in this model, the highest dose of the extract used showed a relatively increased effect than morphine, which was the standard drug used.

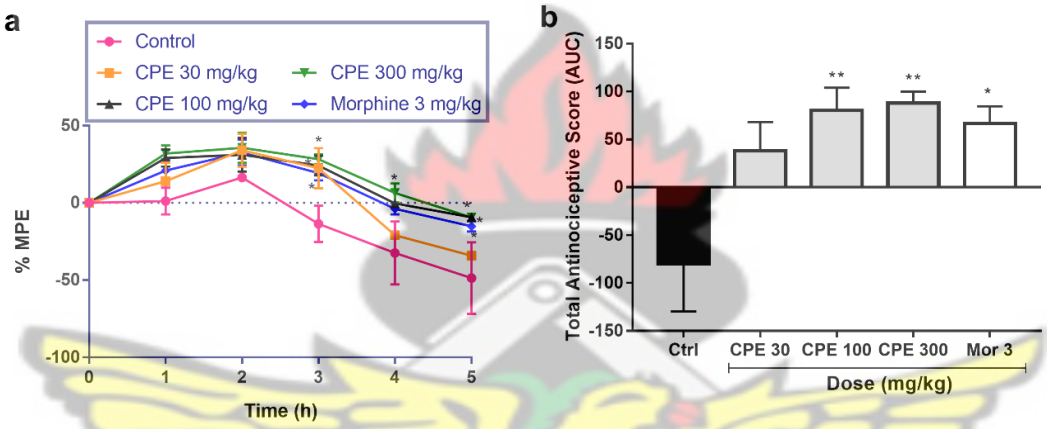

Figure 5-5 Effect of pre-treatment of rats with CPE (30-300 mg/kg, p.o.) and morphine (3 mg/kg, i.p.) on bradykinin-induced hyper-nociception. Each data represents the mean ±S.E.M (n=5). Data represents (a) the time-course curves and (b) total antinociceptive effects (AUC) of the treatments given. ***P < 0.01*, **P < 0.05* (two-way ANOVA followed by Tukey's post hoc test for the time course curves and one - way ANOVA followed by Tukey's *post hoc* test for the AUC analysis).

5.3.4 **Prostaglandin E_2 – induced hyper-nociception**

Intraplantar administration of prostaglandin E_2 caused a painful state which was assessed using a mechanical source of stimulus. All the animals exhibited some degree of mechanical hyperalgesia after intraplantar prostaglandin E_2 injection which was attenuated by the extract and morphine. The hyperalgesia produced was lowered significantly after the extract (30 – 300 mg/kg, *p.o.*) and morphine (3 mg/kg, i.p.), ($F_{5, 89} = 17.43$, $P < 0.0001$, Fig. 5-6a) and total anti-nociceptive scores ($F_{4, 11} = 8.875$, $P = 0.0019$, Fig. 5-6b).,

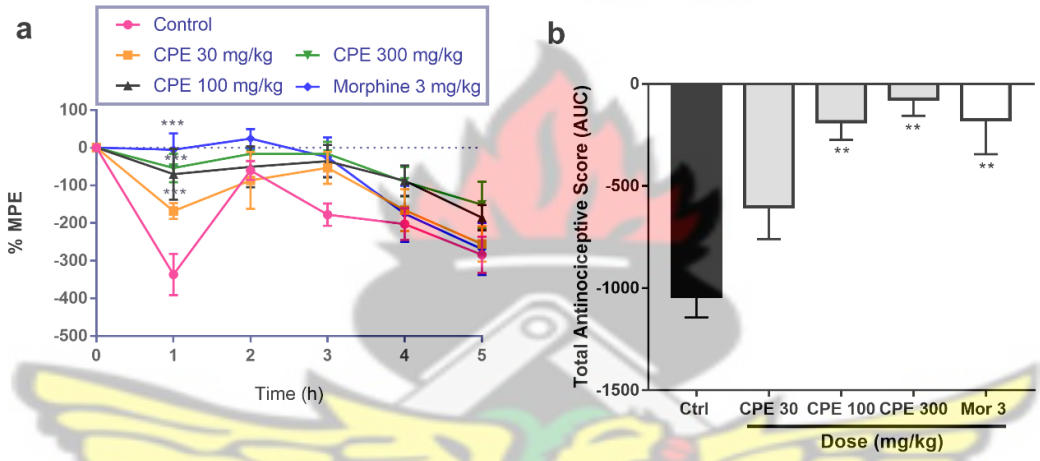

Figure 5-6 Effect of pre-treatment of rats with CPE (30-300 mg/kg, p.o.) and morphine (3 mg/kg, i.p.) on prostaglandin E2-induced hyper-nociception. Each data represents the mean ± S.E.M (n=5). Data represents (a) the time-course curves and (b) total antinociceptive effects (AUC) of the treatments given. ***P < 0.001, **P < 0.01, (two-way ANOVA followed by Tukey's *post hoc* test for the time course curves and one - way ANOVA followed by Tukey's *post hoc* test for the AUC analysis).

5.3.5 **Interleukin 1 – beta (IL-1β) – induced hyper-nociception**

The results presented in Fig. 5-7 (a and b) show that the injection of IL-1β prominently decreased rats' paw withdrawal thresholds in the Randall-Selitto test compared to baseline readings. Pre-treatment of the animals with either CPE (100 – 300 mg/kg, *p.o.*) or morphine (3 mg/kg, i.p.) significantly reversed the hyper-nociception by increasing paw withdrawal thresholds ($F_{5, 82}$ = 3.238, P = 0.0102, Fig. 5-7a) and total anti-nociceptive score ($F_{4, 11}$ = 8.875, P = 0.0025, Fig. 5.7b).

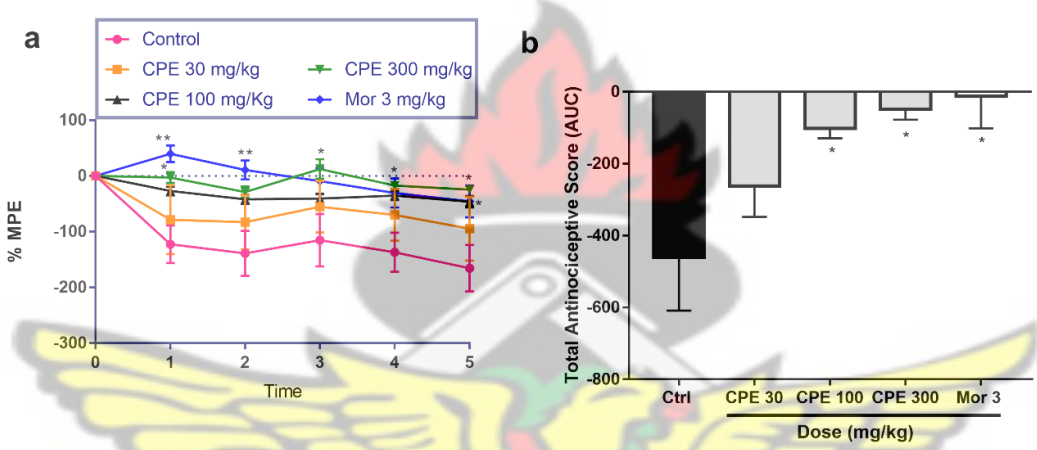

Figure 5-7 Effect of pre-treatment of rats with CPE (30-300 mg/kg, p.o.) and morphine (3 mg/kg, i.p.) on IL-1β – induced hyper-nociception. Each data represents the mean ± S.E.M (n=5). Data represents (a) the time-course curves and (b) total antinociceptive effects (AUC) of the treatments given. **P < 0.05* (two-way ANOVA followed by Tukey's post *hoc test* for the time course curves and one - way ANOVA followed by Tukey's *post hoc* test for the AUC analysis).

5.4 DISCUSSION

This study also looked at the possible mechanism(s) of action by which the *Calotropis procera* extract mediated its analgesic activity. The formalin test was selected for this study, since it is more specific and due to its ability to identify two distinct phases of nociception (Yin *et al.*, 2003; Basile *et al.*, 2007) and also since it reflects different pathological processes and allows the elucidation of the possible mechanism involved in analgesia (Tjølsen *et al*., 1992). The anti-nociceptive effect of the extract was assessed in the presence of various antagonists including naloxone, theophylline, glibenclamide, atropine, nifedipine, ondansetron, granisetron, and yohimbine.

Systemic administration of the opioid receptor antagonist naloxone inhibited the anti-nociceptive effects of the extract in both phases of the formalin test. This strongly suggests an opioidergic involvement in the actions of the extract. A classical opioid analgesic, morphine, has a high affinity for μ (compared to κ and δ) receptors acting as an agonist and resulting in analgesia. The opioid receptors are G_i protein-coupled and are responsible for mediating their analgesic effect. Opioids act by opening calcium-dependent inwardly rectifying potassium channels whiles closing N-type voltage-gated calcium channels leading to a decline in neuronal excitability and hyperpolarization. By so doing, intracellular cyclic AMP is reduced which in turn reduces nociceptive neurotransmitter release (Bovill, 1997; Kiran and Sinha, 2015).

The anti-nociceptive effects of the extract were reversed by pre-administration of theophylline implicating the involvement of adenosinergic pathway in their actions. Adenosine acts at several P1 receptors (A_1, A_{2A}, A_{2B}, and A_3) all of which are coupled to G proteins (Fredholm *et al.*, 2001). Adenosine A_1, located peripherally, is activated to produce pain suppression, while adenosine A_2 receptor activation produces pain enhancement (Sawynok, 1998). Within the spinal cord, adenosine A_1 receptor activation produces anti-nociception (Sawynok, 1998). Adenosine A_3 receptor activation produces pro-nociception in the CNS (Pedata *et al.*, 2010) and peripherally – secondary to mast cell degranulation and release of histamine and 5- hydroxytryptamine (5-HT) to exert nociceptive actions at sensory nerve terminal (Sawynok, 1998). Since theophylline blocks adenosine A_1 and A_2 receptors non-specifically, the antinociceptive effects may be due to activation of A_1

receptors and/or an increment in endogenous adenosine either centrally or peripherally. The observation from this study that both opioidergic and adenosinergic mechanisms are possibly involved in the anti-nociceptive effects of the extract is probable since both opioid and adenosine receptor agonists are known to share some similar anti-nociceptive mechanisms (Aley *et al.*, 1995; Sawynok, 1998). The A_1 receptor has been proposed to exist as part of a multi-receptor complex, in association with μ-opioid and α_2-adrenergic receptors on the basis of a demonstrated cross antagonism, cross-tolerance and cross withdrawal between these systems (Aley and Levine, 1997; Sawynok, 1998) and activation of one of these receptors may affect the rest (Suh *et al.*, 1997).

Despite availability of strong evidence that indicate the involvement of muscarinic (Wess *et al.*, 2003; Jones and Dunlop, 2007), adrenergic (Pertovaara, 2006), calcium channels (Park, 2010), ATP-sensitive K^+ channels (Ameyaw *et al.*, 2016) and serotonergic pathways (Fields *et al.*, 1991; Millan, 2002) in nociception, the results obtained revealed that their respective antagonists could not significantly reverse the anti-nociceptive effect of the extract. This suggests that CPE may not be acting through those pathways.

The extract inhibited bradykinin-, prostaglandin E_2, -interleukin 1β- and TNF-α – induced hyperalgesia. Bradykinin, an important peripheral mediator of pain, elicits nociception or hyperalgesia by direct stimulation of the nociceptors Aδ and C-fibres. Several inflammatory and algogenic substances such as products derived from arachidonic acid pathways, cytokines and nitric oxide as well as neuropeptides such as calcitonin gene-related peptide and substance P may also be released by bradykinin (Calixto *et al.*, 2001). The mechanical hyperalgesia induced by bradykinin involves a B2 receptor-mediated direct activation of protein kinase C and the indirect activation of the protein kinase A (Ferreira *et al.*, 2004; Meotti *et al.*, 2006). Prostaglandin E_2 acts through EP receptors to activate cAMP/PKA and PKC pathways leading to hyperalgesia (Khasar *et al.*, 1999; Villarreal *et al.*, 2009). By binding to G protein-coupled receptors, prostaglandin E_2 increases cAMP which subsequently activates protein kinase A (PKA) in cells. This pathway enhances the excitability of neurons by sensitizing ion channels in membranes such as TRPV1 receptors and Na^+ channels (Schaible *et al.*, 2011). On the other hand, the mechanical hyper-nociception induced by bradykinin involves an indirect activation of PKA and a direct

activation of B_2 receptor-mediated phospholipase C (PLC) which in turn leads to the production of PKC resulting in the sensitization of sensory ion channels which subsequently leads to hyper-nociception (Ferreira *et al.*, 2004; Linley *et al.*, 2010). The extract may have acted directly or indirectly to inhibit B2 or EP receptors or PKA and/or protein kinase C (PKC) pathways.

Furthermore, it has been proposed that intraplantar injection of TNF-α stimulates IL-1β and IL-6 production while also increasing the endogenous levels, thus inducing the secretion of cyclo-oxygenase (COX) products (PGE_2). A common feature of the inflammatory response produced by these mediators when injected intraplantar is increased pain sensitivity. These mediators (TNF-α and IL-1β) by acting directly on their respective receptors or targets to decrease pain thresholds, resulting in hyper-nociception (Binshtok *et al.*, 2008; Jin and Gereau, 2006). The *Calotropis procera* extract significantly and dose-dependently reversed the hyper-nociception induced by intraplantar injection of TNF-α and IL-1β suggesting a possible blockade of their receptors or inhibitory effect on their release peripherally and/or centrally.

5.5 CONCLUSION

The antinociception mechanism of the extract was mediated through the adenosinergic and opioidergic pathways. The extract additionally inhibits hyper-nociception induced by TNF-α, IL-1β, bradykinin, and prostaglandin E_2

Chapter 6

ANTICONVULSANT EFFECTS OF *CALOTROPIS PROCERA* EXTRACT

6.1 BACKGROUND

The *Calotropis procera* extract showed significant CNS depressant effect and the ability to delay and reduce the frequency of seizures in the primary neuropharmacological screening test coupled with its traditional use for the treatment of epilepsy and other CNS disorders (Kinda *et al.*, 2017). It has also been shown to have significant anti-inflammatory and antinociceptive properties as observed in experiments carried out in previous chapters and since pain and inflammation are acutely manifested in epilepsy (Vezzani, 2011; Marchi *et al.*, 2014), the anticonvulsant effect of the extract can be assessed to further confirm it's anticonvulsant properties.

Epilepsy is a chronic disorder of the brain which affects individuals of different age groups characterized by the uncontrolled or excessive electrical activity of either a part or all the central nervous system. The outward signs of epilepsy are known as seizures which could occur spontaneously and in a recurrent manner. Depending on the part of the brain affected, the characteristics of the seizures may vary. Most often they last for a few minutes. The seizures may be caused by different conditions including stroke, brain tumour, head injury or central nervous system infection (WHO, 2018). It is estimated that about fifty million individuals worldwide currently live with epilepsy. This disorder is responsible for one percent of the global burden of diseases (Shakirullah *et al*., 2014)

Despite the introduction of new antiepileptic drugs for the treatment of epilepsy with improved standard of care for a large number of patients in the form of reduced adverse events, a lower propensity for drug-drug interactions, and improved efficacy (Hachad *et al*., 2002; Bialer, 2006), there still remains a good proportion of patients with refractory epilepsy (Brodie, 2001; Sander, 2003). There is a need for the development of newer, better and safer antiepileptic drugs (AEDs) with improved clinical profiles.

Plant extracts are some of the most attractive sources of new drugs and have been shown to produce promising results for the treatment of epilepsy. Examples include *Passiflora incarnata*, *Antiaris toxicaria*, *Pseudospondias microcarpa*, *Mallotus oppositifolius* (Nassiri-Asl *et al.*, 2007; Mante *et al.*, 2013; Adongo *et al.*, 2014; Kukuia *et al.*, 2016). Since, it is estimated that about 80% of the population in the developing nation use herbal remedies for primary health care needs (Spinella, 2001) conditions like epilepsy, pain, etc. are usually treated using herbs.

The present study was conducted to further explore the anticonvulsant potentials of the hydroalcoholic extract of the leaves of *C. procera* by employing acute seizure models using agents such as picrotoxin and strychnine. The pilocarpine model of *status epilepticus,* a human temporal lobe epilepsy model was also employed in characterizing the anticonvulsant effect of the extract. The possible involvement of benzodiazepine/GABA receptor complex in the mechanism of action of the extract was investigated.

6.2 MATERIALS AND METHODS

6.2.1 Animals

Male ICR mice (20 – 25 g) were obtained from the Noguchi Memorial Institute for Medical Research and were cared for in the Departmental Animal House. Animals were handled as described in previous chapters.

6.2.2 Drugs and chemicals

Diazepam (DZP), picrotoxin (PTX), strychnine (STR) and pilocarpine were purchased from Sigma (USA). Flumazenil (FLU) was purchased from Roche (Brazil).

6.2.3 Strychnine – induced seizure test

This method has been described previously (Bogdanov *et al.*, 1997). Briefly, strychnine (STR) seizures were induced in male ICR mice (n=7) by the intraperitoneal injection of 0.5 mg kg^{-1} of the strychnine nitrate, 1 h after administration of the extract (30 - 300 mg kg^{-1}, *p.o.*) or 30 min of diazepam (0.1 – 1.0 mg kg^{-1}, i.p.) administration. The latency to myoclonic jerks, the frequency, and duration of convulsions was recorded for extract-

treated groups and the diazepam group compared with the control group (distilled water). Latency to convulse as well as frequency and duration of convulsions were observed through video recording (Sony-Handycam, model: HDRCX675/B, Tokyo, Japan) for 30 min and quantified with the behavioural analysis software, JWatcher^{TM} version 1.0 (University of California, Los Angeles, USA and Macquarie University, Sydney, Australia. Available at http://www.jwatcher.ucla.edu).

6.2.4 **Picrotoxin – induced seizure model**

The procedure used was the same as in the case of pentylenetetrazole-induced seizure test described in section 3.2.7 except that mice (n=7) in this experiment were administered picrotoxin, 3 mg kg^{-1} intraperitoneally (Mante *et al.*, 2013) 30 min and 1 h after treatment with diazepam and CPE, respectively. Control animals received distilled water (10 mL kg^{-1}, *p.o.*). The latency to myoclonic jerks, latency to tonic convulsions and the frequency and duration of tonic convulsions were recorded from the videos for each mouse as described in section 6.2.3.

6.2.5 **Pilocarpine – induced *status epilepticus***

This experiment followed a procedure previously described by Turski *et al.*, 1989. Seizures were induced by injection of pilocarpine (300 mg kg^{-1}, i.p.) to male ICR mice (n=7). *Calotropis procera* extract (30 – 300 mg kg^{-1}, *p.o.*) or diazepam (1 - 10 mg kg^{-1}, i.p.) was administered 1 h or 30 min respectively before pilocarpine administration. To counter the peripheral autonomic effects produced by pilocarpine, the mice were pre-treated with n-butyl-bromide hyoscine (1 mg kg^{-1}, 30 min before pilocarpine administration). After the injection of the pilocarpine, the animals were placed separately into the transparent plexiglass testing chamber and latency to and duration of clonic-tonic seizures observed through video recordings (Sony-Handycam, model: HDRCX675/B, Tokyo, Japan) and tracked using The JWatcher software version 1.0 (University of California, Los Angeles, USA and Macquarie University, Sydney, Australia. Available at http://www.jwatcher.ucla.edu). The ED_{50} (a measure of anticonvulsant potency) and E_{max} (a measure of efficacy) were calculated from the dose-response curves plotted.

6.2.6 **Involvement of GABAergic mechanism**

To investigate the probable involvement of $GABA_A$ receptors in the mechanism of anticonvulsant action of the extract, the effects of a selective benzodiazepine receptor antagonist, flumazenil (FLU) on the anticonvulsant activity of CPE was studied. Six groups of seven mice each were selected. The first four groups received CPE (100 mg kg^{-1}, *p.o.*), diazepam (0.3 mg kg^{-1}, i.p.), flumazenil (2 mg kg^{-1}) and normal saline 30 min before the administration of 3 mg kg^{-1} picrotoxin intraperitoneally. The last two groups were given flumazenil (2 mg kg^{-1}, i.p.) 5 min before the administration of CPE (100 mg kg^{-1}, *p.o.*) or diazepam (0.3 mg kg^{-1}, i.p.) and 65 min or 35 min before the injection of picrotoxin (3 mg kg^{-1}, i.p.) respectively. The latency to, the frequency and duration of clonic convulsions were tracked for each mouse as described in section 6.2.4.

6.2.7 **Data analysis**

All results are presented as mean ± SEM. Except otherwise stated, data was analysed as a one-way analysis of variance (ANOVA). When ANOVA was significant, multiple comparisons between treatments were performed using Dunnet's or Tukey *post hoc* test. GraphPad Prism for Windows Version 7 (GraphPad Software, San Diego, USA) was used for all statistical analyses.

6.3 RESULTS

6.3.1 **Strychnine – induced seizures**

Figure 6-1 indicates the effects of CPE (30-300 mg kg^{-1}, p.o.) and diazepam (0.1-1 mg kg^{-1}, i.p.) on the duration of clonic convulsions induced by strychnine in mice. One-way ANOVA revealed that the extract (100 – 300 mg/kg) exhibited a dose-dependent anticonvulsant effect against strychnine-induced clonic seizures by significantly reducing the duration of convulsions ($F_{6,20}$ = 4.196, P=0.0068). Diazepam (1 mg kg^{-1}) also significantly reduced the duration of the strychnine-induced clonic seizures. The extract (100-300 mg/kg) significantly ($F_{6,21}$ = 5.438, P=0.0016) reduced the frequency of the clonic convulsions induced by strychnine (Fig 6-2a). However, CPE, at the doses given (30 – 300 mg/kg) was unable to increase the latency for the strychnine-induced clonic convulsions.

Diazepam (0.3-1 mg kg^{-1}) significantly ($F_{6,21}$=3.308, *P*=0.0188) delayed the onset of convulsions (Figure 6-2b) and all doses administered significantly reduced the frequency of convulsions (Figure 6-2a). Administration of the extract was able to reduce the duration of convulsions as well as reducing the frequency of the clonic seizures. However, the extract showed a relatively inadequate ability to delay the onset of these seizures induced by strychnine.

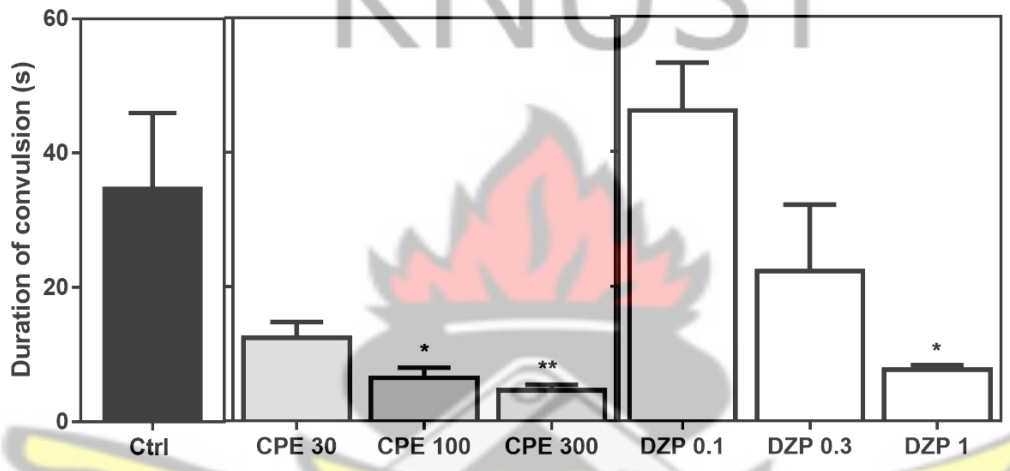

Figure 6-1 Effect of CPE (30-300 mg kg^{-1} *p.o.*) and diazepam (0.1-1.0 mg kg^{-1} i.p.) on the duration of strychnine-induced clonic seizures in mice. Data are expressed as mean ± SEM (n=7). ***P < 0.01, *P < 0.05* (One-way ANOVA followed by Tukey's *post hoc* test).

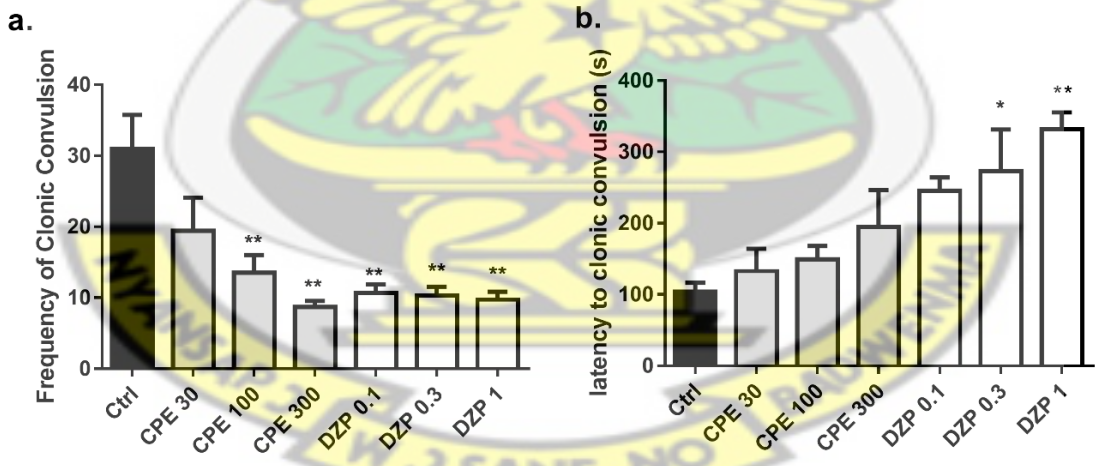

Figure 6-2 Effect of CPE (30-300 mg kg^{-1}*p.o.*) and diazepam (0.1-1.0 mg kg^{-1}i.p.) on frequency (a), latency (b) of strychnine-induced clonic seizures in mice. Data are expressed as mean ± SEM (n=7). ***P < 0.01, *P < 0.05* (One-way ANOVA followed by Dunnet's *post hoc* test).

6.3.2 **Picrotoxin – induced seizures**

The extract treated groups exhibited a significant anticonvulsant effect in this model. CPE (100 – 300 mg kg^{-1}) caused a profound dose-dependent delay in the onset of clonic convulsions ($F_{6, 25}$ = 17.43, P<0.0001) (Fig. 6-3a) and tonic convulsions in mice ($F_{6,20}$ = 43.45, P<0.0001) (Fig. 6-3b). The extract also decreased the duration of convulsions significantly ($F_{6,19}$ = 41.71, P<0.0001) (Fig. 6-4). Diazepam (0.1 – 1.0 mg kg^{-1}), the reference anticonvulsant showed similar results as the extract by increasing the latencies to clonic and tonic convulsions and the duration of the convulsions.

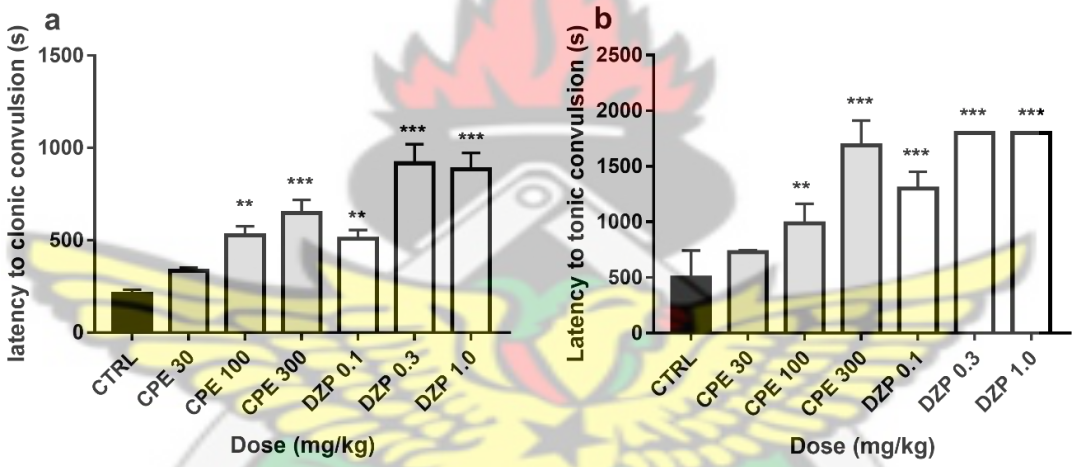

Figure 6-3 Effect of CPE (30 - 300 mg kg^{-1}, *p.o.*) and diazepam (0.1 - 1 mg kg^{-1}, i.p.) on the latency to (a) clonic convulsions and (b) tonic convulsions, in the picrotoxin-induced seizure test in mice. Data are presented as mean ± S.E.M. (n = 7); ****P<0.001;* ** *P<0.01*; compared to the vehicle-treated group (One-way ANOVA followed by Dunnet's *post hoc* test).

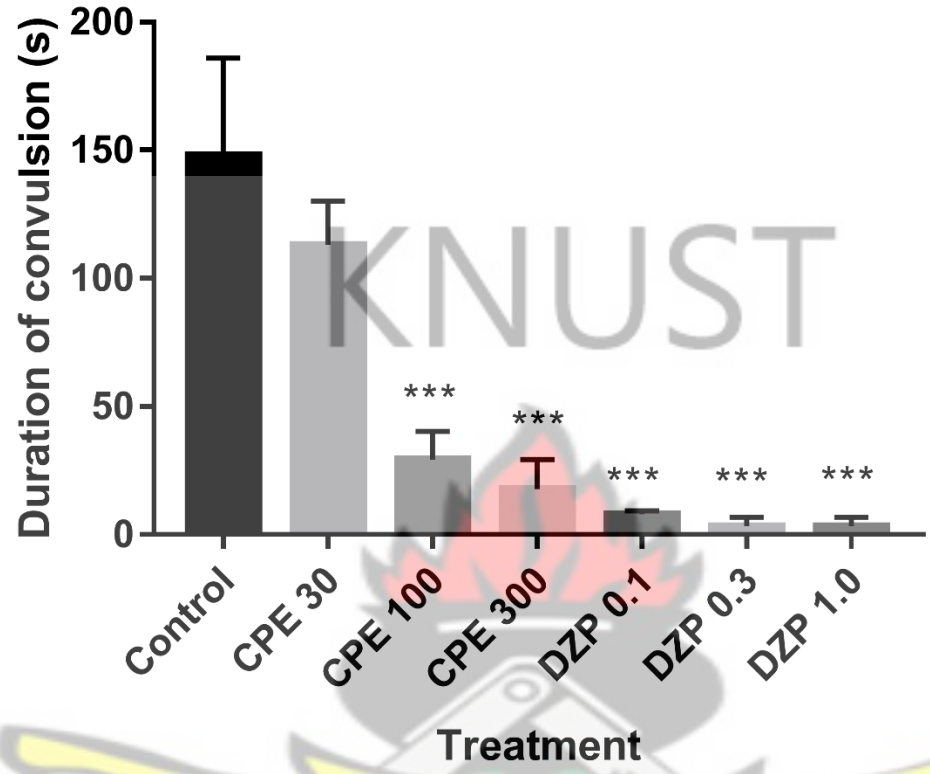

Figure 6-4 Effect of CPE (30 - 300 mg kg^{-1} *p.o.*) and diazepam (0.1 - 1 mg kg^{-1}, i.p.) on the duration of convulsions, in the picrotoxin-induced seizure test in mice. Data are presented as mean ± S.E.M. (n = 7); ****P<0.001;* ** *P<0.01*; compared to the vehicle-treated group (One-way ANOVA followed by Dunnet's *post hoc* test).

6.3.3 **Pilocarpine – induced *status epilepticus***

One-way ANOVA showed that CPE dose-dependently delayed the onset of clonic ($F_{6,22}$ = 7.995, P =0.0001) (Fig. 6-5a) and tonic convulsions ($F_{6,\ 25}$ =7.365, P=0.0001) (Fig. 6-5b). Diazepam, the reference anticonvulsant agent (0.1-1.0 mg kg^{-1}) showed similar effects as the extract by increasing the latencies to clonic and tonic convulsions. The oral dose of CPE (100-300 mg kg^{-1}), showed profound anticonvulsant effect by the protection of the test animals against death which was caused by the convulsions induced by the pilocarpine (Fig. 6-6) (Hazard Ratio = 0.1819 *P<0.0021*). ED_{50} (~ 0.1007) and E_{max} values calculated from the dose-response curves (Fig. 6-7) demonstrated that the extract was less potent than diazepam in reducing the duration of convulsions and delaying the onset of convulsions, but their efficacies were comparable

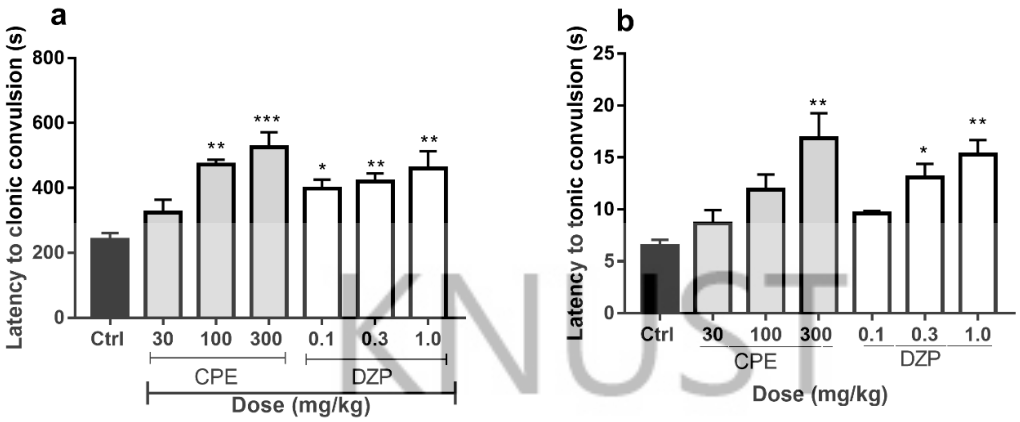

Figure 6-5 Effect of CPE (30 - 100 mg kg^{-1}, p.o.) and diazepam (0.1 - 1 mg kg^{-1}, i.p.) on the latency to (a) clonic and (b) tonic convulsions in the pilocarpine-induced status epilepticus in mice. Data is presented as mean ± S.E.M. (n = 7); ****P< 0.001;* ** *P< 0.01*, * *P< 0.05* compared to vehicle-treated group (One-way ANOVA followed by Dunnet's *post hoc* test).

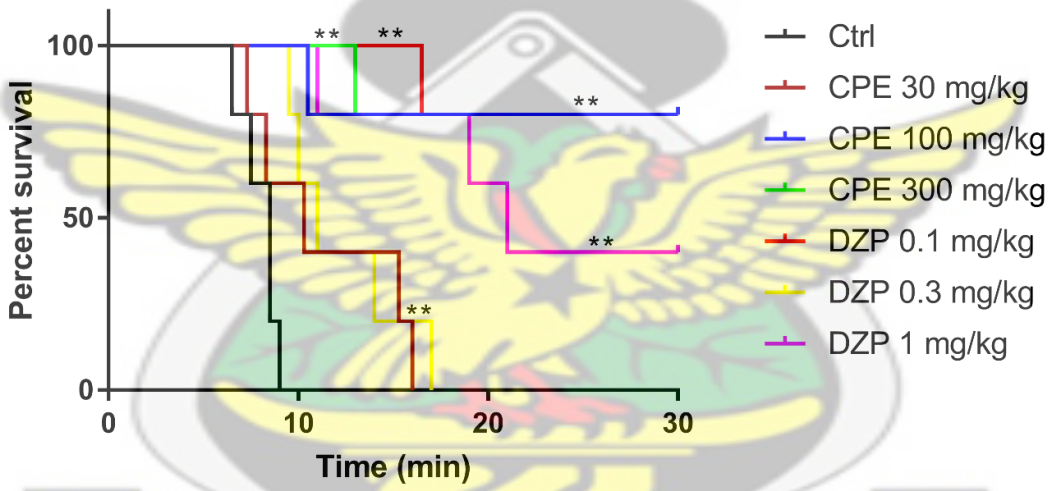

Figure 6-6 Percentage survival of mice for extract (30-300 mg kg-1) and diazepam (0.1-1 mg kg-1, i.p.). Each point is the mean ± S.E.M. of 7 animals.

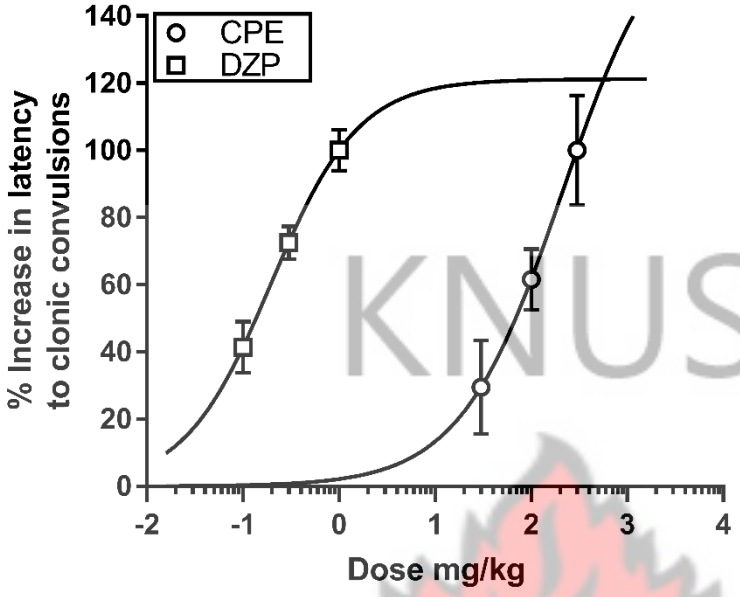

Figure 6-7 Dose-response curve for the anticonvulsant activity induced by the administration of CPE (30-100 mg kg^{-1}, *p.o.*) and diazepam (0.1-1 mg kg^{-1}, i.p.) in the pilocarpine-induced seizure test in mice.

6.3.4 **Involvement of GABAergic mechanism**

From the results obtained, CPE alone delayed the onset of convulsions and duration of convulsions just like diazepam alone (Fig. 6-8). Flumazenil alone (2 mg kg^{-1} i.p.) did not alter the onset or duration of convulsions. Pre-treatment with flumazenil, could not inhibit the anticonvulsant effect of extract but completely reversed the onset ($F_{4,\ 16}$ = 47.09, P<0.0001) and duration ($F_{4,15}$ = 24.13, *P*<0.0001) of convulsion effect of diazepam in the picrotoxin-induced seizure model.

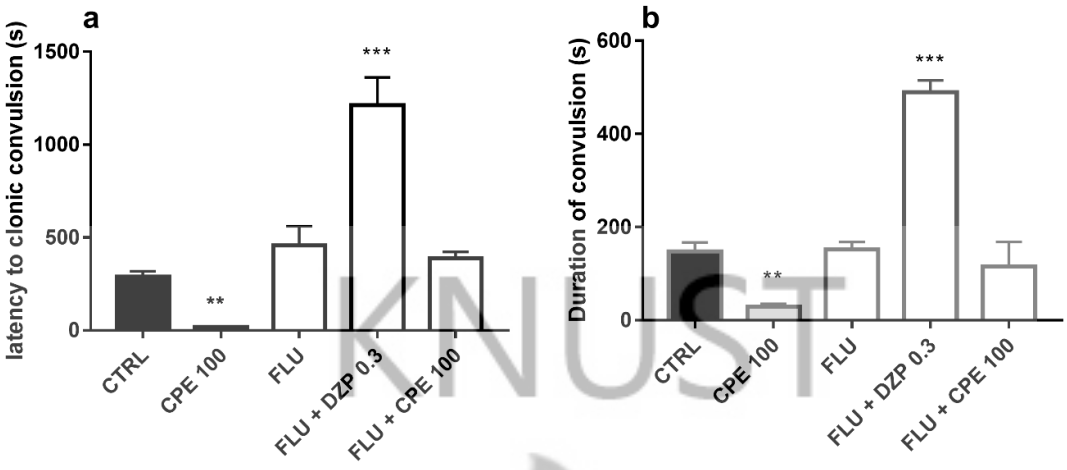

Figure 6-8 Effect of CPE (100 mg kg^{-1}, *p.o.*) and diazepam (0.1 mg kg^{-1}, i.p.) on the effect of flumazenil pre-treatment on the (a) latency and (b) duration of clonic convulsions in the picrotoxin-induced seizure test in mice. Data are presented as mean ± S.E.M. (n =7); ****P<0.001; **P<0.01*; compared to the vehicle-treated group (One-way ANOVA followed by Dunnet's *post hoc* test).

6.4 DISCUSSION

Despite the increased discovery of new antiepileptic drugs (AEDs) over the years, a good number of epilepsy patients experience recurrent seizures and many experience undesirable side effects (Kwan and Brodie, 2000; Panayiotopoulos *et al.*, 2007). The unmet needs for the treatment of epilepsy underscore the need to develop new AEDs that can reduce seizure frequency and severity as well as improve tolerability and safety (Brandt *et al*., 2006; French, 2007; Perucca *et al*., 2007; Cretin and Hirsch, 2010). Medicinal plants used traditionally in treating epilepsy can serve as means of identifying and developing alternative AEDs since they may contain bioactive molecules that can attenuate seizures (Kumar, 2006; Stafford *et al*., 2008). *Calotropis procera* leaf extract is an example of such medicinal plants (Kinda *et. al.,* 2017).

From the studies carried out, it is evident that oral administration of *Calotropis procera* leaf extract (CPE) has anticonvulsant effect in both acute generalized seizure models – pentylenetetrazole, picrotoxin, and strychnine – induced seizure tests and partial seizure model; the pilocarpine-induced *status epilepticus*.

Together with the $GABA_A$ receptors, the glycine receptor is responsible for mediating fast inhibitory neurotransmission in the mature central nervous system (Webb and Lynch, 2007) making this receptor a potential target for antiepileptic drugs (Lopez-Corcuera *et al*., 2001; Bowery and Smart, 2006;). Strychnine causes convulsions by antagonizing the activity of strychnine-sensitive glycine receptors and increasing postsynaptic excitability and ongoing activity in the brainstem and spinal cord (Wang *et al*., 2001; Wood *et al*., 2002). Since the extract reduced the frequency and duration of convulsions induced by strychnine, an interaction of the extract with glycine receptors/pathways is conceivable. It is possible the extract may contain bioactive compounds that activate glycinergic inhibitory neurotransmission.

Picrotoxin is a $GABA_A$ receptor antagonist (Olsen, 1981). Picrotoxin exerts its convulsant effect by blocking the $GABA_A$ receptor-linked chloride ion channel which normally opens to allow increased chloride ion conductance into the brain cells following the activation of $GABA_A$ receptors by GABA (Nicoll, 2001, Velíšek, 2006). GABAergic ionotropic receptors can mediate both pre- and postsynaptic inhibition. Pre-synaptic inhibition

mediated by GABA often leads to inhibition of neurotransmitter release from the excitatory arm (MacDermott *et al.*, 1999). Data from this study shows that CPE and diazepam exhibited anticonvulsant activity against picrotoxin-induced seizures by significantly and dose-dependently delaying the occurrence as well as decreasing the duration of clonic and tonic seizures. The extract, being effective in the picrotoxin-induced seizure test points to action on GABA-mediated neurotransmission.

Many barbiturates and benzodiazepines generally potentiate the inhibitory action of $GABA_A$ receptors, reducing neuronal excitability and increasing the threshold for convulsions (Loscher, 2002). Since CPE was effective against both pentylenetetrazole-induced seizures and picrotoxin-induced seizures, a possible interaction with GABAergic mechanisms was investigated using flumazenil, a benzodiazepine receptor antagonist (Przegalinski *et al*., 2000) in the picrotoxin – induced seizure test. However, there was no reversal of the anticonvulsant effect of CPE by flumazenil in this test and this indicates a potentially different or complex mechanism involved in the attenuation of convulsion by the *Calotropis procera* extract.

Systemic administration of pilocarpine, a nonselective muscarinic agonist, is an animal model of intractable epilepsy (Turski *et al*., 1989; Eglen *et al*., 2001; Mirza *et al*., 2003; Wirtshafter, 2006). Histological studies have shown that this model has important similarities to temporal lobe epilepsy in humans thus drugs effective in this model are potential candidates for managing temporal lobe epilepsy (Liu *et al*., 1994; Wall *et al*., 2000; Szyndler *et al*., 2005; Perez-Mendes *et al*., 2011). The extract was able to protect the animals from death from the acute convulsions described as *status epilepticus* induced by pilocarpine. It is, therefore, possible that the extract may have potential value in the management of temporal lobe epilepsy and/or other partial seizures. This is evidenced in the survival curve drawn and the hazard ratio calculated. The lower the hazard ratio, the better the treatment as there is a low risk of death compared to an untreated population (Chen *et al.*, 2012).

Secondary metabolites such as the triterpenes, steroids, saponins, and alkaloids have been reported to possess anticonvulsant activity in some experimental seizure models such as PTZ (Chauhan *et al*., 1988; Kasture *et al*., 2002). The identified secondary metabolites in

the plant – alkaloids, saponins and sterols may be responsible for the observed anticonvulsant effect of the extract (Obese *et al.,* 2018).

6.5 CONCLUSION

These findings demonstrated an anticonvulsant effect of the extract. CPE activation of the GABAergic system may be absent but may act through glycinergic systems. The anticonvulsant effect exhibited makes the extract a likely therapeutic potential for both generalised and partial seizure events.

Chapter 7

GENERAL DISCUSSION

Epilepsy, pain and inflammation remain a major health menace in the world because the burden of these non-communicable diseases is on the ascendancy. There is an overwhelming percentage of the population with conditions that are refractory to the current medications; the onset of action of current medications is slow; adverse effects and toxicity are rife (Lambert and Robertson, 1999; Jackson and Turkington, 2005). These reasons accentuate the need for alternative medications that are effective against the refractory conditions, safe, have a rapid onset of action and can improve patient quality of life.

The therapeutic potential of plants, on the other hand, has been gaining credibility not only in developing nations but also the developed ones (Verma and Singh, 2008). Hence researching into the possibility of medicinal plants to treat pain, inflammation, and epilepsy is prudent. *Calotropis procera*, a common shrub used traditionally to treat pain, inflammation, and other central nervous disorders was effective as an anticonvulsant, analgesic, and anti-inflammatory in the respective models assessed.

The *Calotropis procera* extract inhibited visceral pain in the acetic acid-induced writhing test. This provided scientific evidence to the traditional use of the plant as an analgesic for the treatment of visceral pain such as colic pain. Similarly, the extract blocked thermal pain elicited in the tail-immersion test indicating the involvement of central and peripheral mechanisms in the anti-nociceptive effects of the extract. The tail-immersion response could also involve higher neural structures (Jensen and Yaksh, 1986). The activity of the extract in this model shows the extract acts, at least in part, by central mechanisms.

Results obtained from the formalin-induced nociception model showed that the extract inhibited both neurogenic and inflammatory pain. The inflammatory phase of the formalin test, like the inflammation induced by carrageenan, involves the release of proinflammatory pain mediators such as iNOS, COX-2 products, TNF- α, serotonin, histamine, and prostaglandins. These pro-inflammatory pain mediators have also been implicated in the development of musculoskeletal pain. It is therefore not surprising that the extract inhibited greatly phase 2 of the formalin test (Leung et al., 2005; Park et al., 2007). The formalin test is a very useful test because not only can it detect drugs that inhibit neurogenic and/or

inflammatory pain but can also predict drugs that may be effective in neuropathic and neurogenic painful conditions (Ellis et al., 2008; Vissers et al., 2006). The extract inhibited the release and induction of some of the inflammatory cytokines.

From the studies carried out, it is evident that oral administration of *Calotropis procera* leaf extract (CPE) has an anticonvulsant effect by reducing the frequency, duration and onset of the seizures in both acute generalized seizure models – pentylenetetrazole, picrotoxin and strychnine – induced seizure tests and partial seizure model; the pilocarpine-induced *status epilepticus*.

Many barbiturates and benzodiazepines generally potentiate the inhibitory action of $GABA_A$ receptors, reducing neuronal excitability and increasing the threshold for convulsions (Loscher, 2002). Since CPE was effective against both pentylenetetrazole- and picrotoxin – induced seizures, a possible interaction with GABAergic mechanisms was investigated using flumazenil, a benzodiazepine receptor antagonist (Przegalinski *et al*., 2000) using the picrotoxin – induced seizure model. However, there was no reversal of the anticonvulsant effect of CPE by flumazenil in this test. This indicates a potentially different or complex mechanism involved in the attenuation of convulsion by the *Calotropis procera* extract.

The Irwin test revealed that the extract is safe as no death or other adverse effects of the extract, even at a relatively high dose, were recorded over the course of 24 h the experiment was performed. This result suggests that CPE is relatively non-toxic since substances with an LD_{50} above 3000 mg kg^{-1} by the oral route and are regarded as being safe or of low toxicity

The result of the phytochemical screening of hydro-ethanolic leaf extract of *Calotropis procera* revealed the presence of alkaloids, tannins and saponins, flavonoids, reducing sugars, sterols, amino acids, glycosides and terpenoids as has been reported by several authors, the presence of many biologically active phytochemicals such as flavonoids, triterpenes , alkaloids, steroids, tannins and glycosides in various plant extracts may be responsible for their respective pharmacological properties (Singh *et al.*, 2002; Yokosuka and Mimaki, 2009; Gomes *et al.*, 2009; Okarter and Liu, 2010, Perez-Amador *et al.*, 2010, Maganha *et al.*, 2010). For instance, plants rich in saponins have immune-boosting, anti-inflammatory, anticonvulsant, antidepressant, anti-inflammatory, anxiolytic, sedative,

cytotoxic and analgesic properties (Konoshima *et al.*, 1999; Nemmani and Ramarao, 2002; Gurib-Fakim, 2006; Wei *et al.*, 2007; Jiang *et al.*, 2007; Zhou *et al.*, 2010; Xiang *et al.*, 2011). Flavonoids, also exhibit sedative, anticonvulsant and antidepressant effects (Medina *et al.*, 1990; Wolfman *et al.*, 1998; Machado *et al.*, 2008; Yi *et al.*, 2010; Cho *et al.*, 2012). Thus, since the extract contained most of these secondary metabolites, its anticonvulsant, antinociceptive and anti-inflammatory effects could be due to the presence of such metabolites.

Chapter 8
CONCLUSIONS AND RECOMMENDATIONS

8.1 CONCLUSIONS

1. This study has provided pharmacological evidence to support the traditional use of the leaf extract of *Calotropis procera* as an analgesic, anti-inflammatory and use in managing seizures. The analgesic effect of the hydro-ethanolic extract of *C. procera* was mediated by:
    a. Stimulation of opioidergic receptors
    b. Stimulation of adenosinergic, pathways.
    c. Inhibition of prostaglandin E_2, bradykinin, IL-1β, and TNF-α pathways
2. The leaf extract of *C. procera* exhibited anticonvulsant activity and the mechanism involved may be mediated by a complex mechanism which may include glycinergic systems.
3. The leaf extract of *C. procera* was safe as observed in the Irwin test.

8.2 RECOMMENDATIONS

1. The extract should be fractionated to isolate the compounds responsible for the observed pharmacological effects especially the compound(s) responsible for the analgesic, anti-inflammatory and anticonvulsant effect seen in the experiments performed.
2. Animal models such as genetic absence epilepsy rats from Strasbourg (GAERS) or lethargic mice should be used to further confirm the possible anti-absence properties of the extract observed.
3. Acute, sub-acute and chronic toxicity should be carried out to establish the safety of the extract.
4. This work should be repeated in non-human primates in order to assess and/or confirm the scientific information gathered or discover those that were not apparent in this study.

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