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ANTICONVULSANT AND SEDATIVE EFFECTS OF THE HYDRO-ETHANOLIC WHOLE PLANT EXTRACT OF *CLEOME RUTIDOSPERMA (DC.) (CLEOMECEAE)* IN MICE.

BY

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DECLARATION

I, Tabariyeng Naa Justice, do hereby declare that the research work described in this thesis was carried out at the Department of Animal Experimentation, Noguchi Memorial Institute for Medical Research, College of Health Sciences, University of Ghana by me and the results obtained are a true reflection of the work done under the supervision of Dr. Patrick Amoateng (Department of Pharmacology and Toxicology, School of Pharmacy) and Dr. Kennedy Kwami Edem Kukuia (Department of Medical Pharmacology, Medical School), University of Ghana, Legon. This work has not been submitted for any other degree.

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ABSTRACT

BACKGROUND: *Cleome rutidosperma* (DC.) (Family*: Cleomeceae*) is a low-growing herb used for the treatment of epilepsy in African traditional medicine. This study presents the anticonvulsant, sedative effects and safety profile of the hydro-ethanolic extract of the whole plant of *Cleome rutidosperma* (CRE) in murine experimental models.

METHODS: Preliminary phytochemical screening of the extract was conducted using standard colorimetric assays. The general effect of the extract, CRE (10, 30, 100, 300, 1000 and 3000 mg kg^{-1} *p.o.*) on the central nervous system of mice was assessed using the Irwin test. The anticonvulsant screening of CRE (100, 300 and 1000 mg kg^{-1} *p.o.*) utilized four murine models of experimental epilepsy; pentylenetetrazole (PTZ)-, picrotoxin (PIC)-, maximal electroshock (MES)- induced seizures and pentylenetetrazole-induced kindling. Phenobarbitone and carbamazepine were used as reference anticonvulsant agents in the anticonvulsant screening experiments. The duration, frequency and latency to seizures were measured for each treated animal. The extract's ability to induce sedation was also investigated using the thiopental sleeping time test. Acute and subacute toxicity studies were conducted to determine the safety of the extract in mice.

RESULTS: Preliminary phytochemical screening indicated the presence of flavonoids, alkaloids, glycosides, phytosterols and saponins. In the Irwin test, the extract produced sedation as its major effect. In the acute seizure models, the extract caused significant increase in the latency to seizure ($P< 0.0001$) and a significant reduction in the duration of tonic-clonic convulsions ($P< 0.0001$) in PTZ- induced seizure model. Also, the extract significantly decreased duration of seizures ($P< 0.0001$) and increased the latency to seizure in the PIC- induced seizure model ($P< 0.0001$). In the

MES test, the extract did not significantly reduce the duration of hind limb tonic extension (P= 0.6446) and it did not increase the latency to hind limb tonic extension (P= 0.3257). In PTZ-induced kindling test, the extract significantly (P=0.0002) reduced the percentage severity of seizures in the mice and prevented the animals from being fully kindled. The extract (30, 100, 300 and 1000 mg kg^{-1} *p.o.*) dose-dependently and significantly decreased the latency to sleep (P< 0.0001) and increased significantly the duration of sleep (P< 0.0001) induced by thiopental sodium. There were no deaths recorded in the acute toxicity studies even at a dose of 3000 mg kg^{-1} thus the LD_{50} was estimated to be more than 3000 mg kg^{-1}. The relative organ to body weight ratio for both control and treatment groups were not significantly different in both acute (P= 0.9987) and subacute (P= 0.9887) toxicity studies. Histopathological examination of the brain, and heart showed no prominent abnormalities in the extract-treated group. However, the histopathological examination of the kidney and liver showed tubular dilatation with erythrocyte infiltration and hepatocellular oedema respectively.

CONCLUSIONS: The hydro-ethanolic extract of the whole plant of *Cleome rutidosperma* produces anticonvulsant and sedative effects in mice. The extract was relatively safe in the acute and sub-acute toxicity investigations in mice, with an LD_{50} greater than 3000 mg kg^{-1}.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABBREVIATIONS

AED	Antiepileptic Drugs
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionoc acid
ATM	African Traditional Medicine
CNS	Central Nervous System
CRE	*Cleome rutidosperma* Extract
CPMR	Centre for Plant Medicine Research
ED_{50}	Dose of drug that elicits 50 % of maximum response
GABA	Gamma-aminobutyric acid
GABA-T	Gamma amino butyric acid transaminase
HLTE	Hind Limb Tonic Extension
IBE	International Bureau for Epilepsy
ILEA	International League Against Epilepsy
i.p.	Intraperitoneal
LD_{50}	Dose that causes deaths in 50 % of a population
LMIC	Low- and Middle-Income Countries
mhGAP	Mental Health Gap Action Programme
MES	Maximal Electroshock

NMDA	N-methyl-D-Aspartate
NMIMR	Noguchi Memorial Institute for Medical Research
PIC	Picrotoxin
p.o.	Per os
PTZ	Pentylenetetrazole
s.c.	Subcutaneous
WHO	World Health Organization

Chapter 1

INTRODUCTION

1.1 GENERAL INTRODUCTION

Epilepsy, a chronic non-communicable disease of the brain, affects people of all ages (Martindale, 2009; WHO, 2018). In the ancient days, epilepsy was labelled as a disease of lightening and was typified by asynchronous, dysrhythmic electrical discharges in the brain (Tripathi, 2013). Epilepsy is one of the oldest neurological diseases that has been attributed to metaphysical forces, feared, stigmatized, discriminated against and misunderstood for centuries. However, in 2005, the International League Against Epilepsy (ILEA) in conjunction with the International Bureau for Epilepsy (IBE) defined epilepsy as a disorder of the brain that is characterized by an enduring predisposition to generate epileptic seizures (Fisher *et al.*, 2005). Thus, the definition of epilepsy required the occurrence of at least one epileptic seizure. An epileptic seizure is a transient occurrence of signs and/ or symptoms due to abnormal excessive or synchronous neuronal activity in the brain (Fisher *et al.*, 2005). These definitions of epilepsy have since been reviewed by a task force and their recommendations accepted by the ILAE. The task force proposed the consideration of epilepsy as a disease of the brain defined by any of the following conditions: (1) occurrence of at least two unprovoked seizures, more than 24 hours apart; (2) one unprovoked (or reflex) seizure and a probability of further seizures similar to the general recurrence risk (at least 60 %) after two unprovoked seizures, occurring over the next 10 years; (3) diagnosis of an epilepsy syndrome (Fisher *et al.*, 2014).

Epilepsy is a key public health problem (Fisher *et al.*, 2005; England, 2014) affecting over 50 million people worldwide (1-2 % of the world's population) out of which 40 million are found in

low- and middle- income countries (LMIC) which include Ghana (WHO, 2004; Newton & Garcia, 2012). In Ghana, approximately 250,000 people are living with epilepsy and this disease is considered one of the top five burdensome medical problems (WHO, 2015). In developed countries where there are well-resourced medical care and public health policies, it is estimated that 30 to 50 per 100 000 new cases are diagnosed annually. These figures could be doubled in low- to middle- income countries (Chan, 2009; Al asmi, 2013). This can be attributed to the higher risk of endemic diseases like malaria or meningitis, pre- and perinatal related injuries, inadequate and inaccessible or poorly resourced medical infrastructure, the higher incidence of road traffic accidents and the unavailability of preventive health programmes (WHO, 2004; Ngugi *et al.*, 2013).

Epilepsy as a public health problem, has serious physical, social, economic and psychological consequences, such as premature death, discrimination, stigmatization, traumatic injury and mental health disorders for the concerned persons and their families (WHO, 2004; Fisher *et al.*, 2005; De Boer *et al.*, 2008). Notwithstanding the remarkable advances in modern medicine, the stigma and discrimination against epileptic patients and sometimes, their families persist in many countries. This stigmatization leads to a hidden burden that discourages patients from seeking the conventional diagnosis and care they need and deserve (WHO, 2004), leading them to resort to herbal remedies in order to conceal the disease. Out of stigmatization stems discrimination against people living with epilepsy. In most cultures in some African countries including Ghana, patients with epilepsy can have difficulties in acquiring a driver's license, in marrying, in securing a life insurance and in entering other occupations, among other limitations (De Boer *et al.*, 2008). They may not be permitted to participate in traditional ceremonies such as celebrating the passage to adulthood (WHO, 2004; De Boer *et al.*, 2008). Some studies have shown that children with

epilepsy are often discriminated against at school resulting in low self-esteem, under-achievement at school and high rate of social withdrawal (De Boer *et al.*, 2008).

Majority of people with epilepsy who live in countries with low and middle incomes do not receive appropriate treatment, a situation known as treatment gap (Ngugi, 2010; Lund *et al.*, 2012). The epilepsy treatment gap is defined as the number of people with active epilepsy who have not accessed biomedical services (i.e. epilepsy not diagnosed) or who are not on treatment or are on inadequate treatment, expressed as a percentage of the total number with active epilepsy (Meinardi, 2001; Mbuba *et al.*, 2012). This may be attributable to poor infrastructure, insufficient availability of medicines and scarcity of trained health personnel, the level of health care development, cultural beliefs, the erratic supply of and nonadherence to antiepileptic drugs (AEDs), and the lack of prioritization in national health policies (WHO, 2004; Mbuba, 2008). The WHO is working through a concerted effort to bridge this treatment gap and alleviate the stigma and discrimination associated with epilepsy. The WHO programme on Reducing the Epilepsy Treatment Gap and the Mental Health Gap Action Programme (mhGAP) in Ghana, Mozambique, Myanmar and Vietnam (Megiddo, 2016) are examples of such efforts.

Many Africans depend on herbal medicines for treating several infirmities such as asthma, malaria, stomach-ache, diabetes, skin diseases, psychiatric and neurological disorders including epilepsy and seizures. In Ghana, it is estimated that 65 to 70% of the population, especially the rural folks still depend on herbal medicine as a first line for primary health care (Tabi, 2006; Graz, 2011; Gyasi *et al.*, 2015). Globally, it is estimated that 80% of the population in developing countries rely on herbal medicine of folkloric origin (Spinella, 2001; Kamatenesi-Mugisha, 2005; Gunjan *et al.*, 2015). However, research into the scientific properties of many of these folkloric plants used in African Traditional Medicine seem to be deficient and literature on their medicinal uses are

scanty (Meeran, 2013; Vitry, 2013). One of such plants with numerous medicinal uses that still requires research into its medicinal properties is *Cleome rutidosperma*. This study seeks to evaluate the anticonvulsant and sedative properties of the hydro-ethanolic whole plant extract of *Cleome rutidosperma* in murine models of epilepsy.

1.2 PROBLEM STATEMENT

A lot of the crude plant medicines used in African Traditional Medicine (ATM) for the treatment of various diseases are not thoroughly researched. In other words, there is lack of scientific information on these plants in literature (Muazu & Kaita, 2008). More so, about 30% of people living with epilepsy who do not respond to any of the current AEDs live mostly in the low- and middle-income countries (Brodie, 2001; Sillanpää & Schmidt, 2006; Brodie *et al.*, 2012). The quest to satisfy the needs of this group of patients emphasizes the need to search for newer AEDs.

Moreover, none of the current AEDs has been proven to prevent epileptogenesis in patients prior to the first seizure (Temkin, 2009; Schmidt, 2012) which further strengthens the need for the search for newer AEDs which are more effective, low-cost and safe with minimal side effects to manage the disorder. Plants (herbs) have often served as effective sources of acquiring lead compounds from which newer and effective AEDs can be developed (Amoateng *et al.*, 2012).

One of the main contributory factors to the large epilepsy treatment gap is the non-availability of, and inaccessibility to antiepileptic drugs (Chin, 2012) due to lack of incentives to pharmaceutical companies because drug distribution is not lucrative (Ilangaratne, 2012). This has led to a low availability of the antiepileptic drugs in the public health care facilities suggesting that poor people especially in the rural and peri-urban communities are at a disadvantage in terms of access to the

drugs. Though oral AEDs are more likely to be available in the private sector than the public sector, availability is still a challenge (Cameron, 2012) and the high cost of newer and more effective AEDs, have compelled a greater number of Ghanaians as well as other people living in LMICs to resort to the use of herbal remedies (Amoateng *et al.*, 2012).

Cleome rutidosperma is a plant herb and its leaves are eaten as cooked vegetable or added to soup (Burkill, 1985; Edeoga, 2005 ; Khuntia *et al.*, 2013). It is occasionally taken as a pot herb (Abbiw, 1990). In Ghana, Gabon and DR Congo, the leaf sap is applied to cure ear-inflammation, ear-ache and deafness (Chakraborty, 2010). In Ghana and South Nigeria, the leafy extract is used to treat irritated skin, prickly heat and convulsion (Kirtikir & Basu, 1935; Ghosh *et al.*, 2019). However, it seems there is limited empirical evidence supporting its use in the African traditional medicine. *Cleome rutidosperma* could possess novel anticonvulsant constituents or may be injurious to health.

1.2 JUSTIFICATION FOR THE STUDY

In low-to-middle-income countries, the predominant intervention for epilepsy is far from ideal. The current treatment regimen involves the use of drugs. The conventional AEDs such as phenobarbitone, phenytoin and carbamazepine are only given for symptomatic relief (Temkin, 2009; Amoateng *et al.*, 2012; Schmidt, 2012) from epilepsy and are plagued with significant side effects such as retarded growth rate, rashes, vitamin and folate deficiencies, loss of libido, bone marrow hypoplasia and foetal abnormalities like cleft palate, cleft lip, congenital heart disease and mental deficiency. These effects deter people from the use of conventional antiepileptic drugs (Amoateng *et al.*, 2012).

Cleome rutidosperma has many traditional medicinal uses including epilepsy and therefore could be a haven for important anticonvulsant constituents. This can be scientifically exploited to broaden the limits of the current treatment outcomes of epilepsy and uncover any harmful effects of the plant.

Preliminary studies have been carried out on the anticonvulsant activity of the plant in strychnine-induced tonic convulsion in mice (Jena *et al.*, 2009). In the said research, only the strychnine-induced seizure model was used which is not one of the conventional models used in the anticonvulsant drug discovery and development. The current study is an add-on to what has been done already by Jena and colleagues. This study will investigate the anticonvulsant activity of *Cleome rutidosperma* extract in mice using both acute (PTZ and MES) - and chronic (PTZ kindling) - seizure models. The possible mechanism of action of the extract will be explored using the PIC-induced seizure model and the toxicity of the extract will be studied. The sedative effects of the extract will be assessed.

1.3 RESEARCH QUESTIONS

This study intends to give answers to the following research questions on the medicinal plant *Cleome rutidosperma.*

1. What type of seizures are possibly suppressed by the plant in murine models of epilepsy?
2. What is the possible mechanism of action of the extract?
3. Is *Cleome rutidosperma* safe as an anticonvulsant in the animal models used?

1.4 PURPOSE OF THE STUDY

This study evaluated the anticonvulsant and sedative properties of hydro-ethanolic whole plant extract of *Cleome rutidosperma*.

1.5 SPECIFIC OBJECTIVES

The specific objectives of the study include:

1. To conduct a hydro-ethanolic whole plant extraction of *Cleome rutidosperma*
2. To perform a phytochemical screening of secondary metabolites of the extract
3. To assess the central nervous system effect of the extract by using:
    1. Irwin Test
    2. Thiopental sodium sleeping time test
4. To evaluate the anticonvulsant effects of the extract in mice model of epilepsy using:
    1. Acute seizure models:
        1. Pentylenetetrazole (PTZ)- induced seizure test
        2. Picrotoxin (PIC)- induced seizure test and
        3. Maximal electroshock-induced seizure test and
    2. Chronic seizure model:
        1. Pentylenetetrazole (PTZ) kindling
5. To investigate the toxicity of the extract by:
    1. Conducting acute toxicity studies
    2. Conducting sub-acute toxicity studies

Chapter 2

LITERATURE REVIEW

2.1 EPILEPTIC SEIZURES

An epileptic seizure is defined theoretically as a transient occurrence of signs or symptoms due to abnormal excessive or synchronous neuronal activity in the brain (Fisher *et al.*, 2005). Epilepsy exists when there is spontaneous cerebral neural impulse discharges and is characterized by recurrent periodic and unpredictable seizures (Berg *et al.*, 2010). Thus, the brain must demonstrate a pathologic and enduring tendency to have recurrent seizures (Fisher *et al.*, 2014). The most commonly diagnosed type of epilepsy is termed idiopathic epilepsy, meaning there is no identifiable cause. Epilepsy that results from an identifiable medical cause is termed symptomatic or secondary epilepsy. As reported by Helmers *et al.*, (2015), the causes of secondary or symptomatic epilepsy include:

1. brain damage due to prenatal or perinatal injuries (loss of oxygen or trauma during birth, low birth weight),
2. congenital abnormalities or genetic disorders with associated brain malformations,
3. a cerebrovascular accident that results in the brain cells infarction,
4. head and spinal cord injuries from road traffic accidents,
5. infections of the brain such as meningitis, encephalitis, neurocysticercosis and
6. brain tumours.

Epileptic seizures can vary in their expressions depending on the site, extent and mode of propagation of the paroxysmal discharge. The behavioural expression of a seizure is indicative of

the area of the brain involved. For instance, the involvement of the motor cortex results in convulsions, the reticular formation involvement leads to loss of consciousness and when the hypothalamus is affected, it results in peripheral autonomic discharges (psychomotor seizures) (Kwan *et al.*, 2010). Epileptic seizures are categorized into focal seizures, with a local cortical origin and generalized seizures that involve both hemispheres of the brain from the inception, which could be convulsive or nonconvulsive. Clonic convulsions are characterized by prolonged and rapid repetition of involuntary muscle contraction whereas tonic convulsions is expressed as abduction, extension of the limbs and rigid stretching of the body (Etholm *et al.*, 2013). The ILAE operational classification of seizures include: focal, generalized and seizure of unknown onset (Fisher *et al.*, 2017).

2.1.1 Focal Seizures

Focal seizures can be classified as simple or complex. Simple partial seizures usually begin at a definite locus in the brain and convulsions are limited to a single limb or a group of muscles. It is often secondary and without loss of consciousness. Complex partial seizures, however, lead to bizarre and confused behaviour and purposeless movements, emotional changes lasting several minutes with impaired consciousness. Simple or complex partial seizures secondarily generalized begins as a partial seizure first then evolves into generalized tonic-clonic seizures with loss of consciousness (Tripathi, 2013).

2.1.2 Generalized Seizures

Generalized seizures usually involve both cerebral hemisphere from the onset. It has sudden onset with immediate loss of consciousness. Generalized seizures results in tonic, clonic (grand mal) and myoclonic seizures with stiffness of extremities and immense clonic jerking (Quintans Júnior *et al.*, 2008). Absence seizures (petit mal), another type of generalized seizures, are prevalent in

children. There is momentary loss of awareness with a stare in one direction. No muscular component or little bilateral jerking is observed in absence seizures. Atonic seizures lead to unconsciousness with relaxation of all muscles due to excessive inhibitory discharges (Tripathi, 2013). The ILAE defines *status epilepticus* as a medical emergency which occurs when generalized tonic-clonic seizures persist for 30 minutes or more. The seizures could take the form of prolonged seizures or repetitive seizures without recovery in between (Berg *et al.*, 2010).

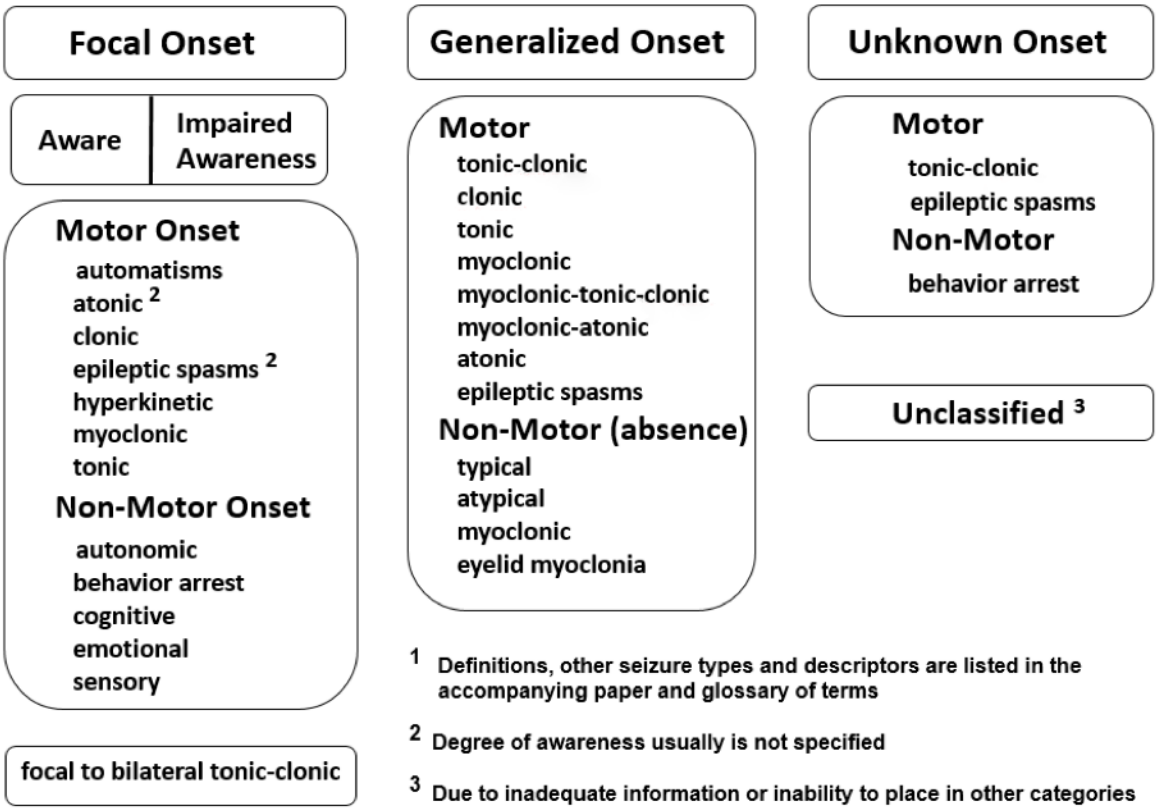

Figure 2- 1: Classification of convulsions, (ILAE, 2017)

2.2 EPILEPTOGENESIS

Normal brain neurons transition into epileptic neurons through a gradual process in which neurons become hyper-excitable and tend to instigate impulses in a hypersynchronous manner. This process is referred to as epileptogenesis (Löscher & Brandt, 2010). The proposed causes of epileptogenesis can be grouped into structural (tumours and brain traumas), metabolic (oxidative stress) and idiopathic changes in the central nervous system. Some regions of the brain that are prone to injury and can be a focus for epileptogenesis include: hippocampus, amygdala, temporal lobe and the piriform cortex (Dubey *et al.*, 2015; Pitkänen *et al.*, 2015). A lot of neurons in the brain are excitatory with glutamate as the main excitatory neurotransmitter. N-methyl-D-aspartate (NMDA) receptors are the most prevalent glutamate receptors (Mani, 2011). Gamma-aminobutyric acid (GABA) is the main inhibitory neurotransmitter which minimizes repeated neuronal excitatory activations by intact inhibitory GABAergic feedback (March, 1998; Patterson, 2013). The disparity between excitation and inhibition is thought to be pathophysiology of epilepsy (Chandler, 2006; Wu *et al.*, 2015). The balance between excitatory and inhibitory neurotransmission is also substantially affected by voltage-gated ion channels (Patterson, 2013). Epileptogenesis involves three stages: (1) latency phase, (2) spontaneous seizure activity phase which is also termed ictogenesis and (3) the development of refractory seizures phase.

2.2.1 Latency Phase

There is an increased threat of developing epilepsy following a variety of injuries to the brain. Such injuries include trauma, ischemia, infection and neurodegeneration (Dichter, 2009). The repair of the brain after an injury results in cell loss, axonal spreading, circuit reorganization, and altered glial function. After a brain injury, there is an overall glutamatergic enhancement and

GABA disruption, electrocephalographic changes including interictal spikes and high-frequency oscillations (Mani, 2011). There are micro seizures which do not progress into obvious seizures in an epileptic focus during the latent period. However, the hyper-excitability of the area around the micro seizures is increased by three processes which ultimately result in overt seizure activity. These processes are (1) stronger stimulation of repeated excitatory circuits resulting from the firing of excitatory circuits, (2) a synaptic strength decline, and (3) replacement of inhibitory interneurons with excitatory interneurons (Dichter, 2009). Genetic mutations of voltage- or ligand-gated ion channels have been implicated as a cause of epilepsy in humans (Steinlein, 2008).

2.2.2 Recurrent Seizure Period

An influx of calcium through voltage-gated calcium channels and influx of sodium through NMDA and non-NMDA receptor-gated channels collectively results in paroxysmal depolarization shift (PDS) in epileptic foci. Paroxysmal depolarization shift precedes a post-depolarization spike hyperpolarization phase caused by calcium- and voltage-dependent outward potassium currents and feedback inhibition by GABA (Engelborghs *et al.*, 2000). Amplified glutamate receptor sensitivity and GABA neuron loss contributes to epilepsy (Engelborghs *et al.*, 2000). The loss of inhibitory response to hyper-excitability is attributable to the changes in the expression and function of $GABA_A$ receptors involved in the pathogenesis of both focal and generalized epilepsy (Galanopoulou, 2010).

2.2.3 Refractory Epilepsy

A series of intracellular, calcium-mediated events and neuronal “excitotoxicity” in the amygdala, hippocampus, thalamus and neocortex results from the incessant and excessive glutamate release during seizures (Engelborghs *et al.*, 2000). The excitotoxicity considerably results in further neurodegeneration, axonal sprouting, neurogenesis, glial cell activation, angiogenesis, invasion of

inflammatory cells, and alteration of ligand- and receptor-gated ion channels (González & Brooks-Kayal, 2011).

2.3 PHARMACOLOGICAL MANAGEMENT OF EPILEPSY

In order to improve the quality of life of the epileptic patient, medical intervention preferably pharmacotherapy is required. With early and appropriate care, seizure control can be achieved in 70-80% of cases (WHO, 2004). Pharmacological advances in the past decades have made the treatment of epilepsy possible with inexpensive daily medication that costs as little as US$ 5.00 (GH ₵ 30) per year (Helmers *et al.*, 2015). The contemporary remedies for convulsions are aimed at controlling the convulsions without resolving the underlying pathophysiological causes of epilepsy (Patterson, 2013). About 70% of children and adults with epilepsy living in the low-to-middle-income countries can have their seizures completely suppressed with antiepileptic drugs. After 2 to 5 years of successful treatment and being seizure free, 70% of children and 60% of adults can be weaned off the AEDs without subsequent relapse (WHO, 2018).

2.4 DRUGS IN CLINICAL USE

Anticonvulsants are categorized into conventional antiepileptic drugs (AEDs) (those discovered before 1990) and newer generation drugs (AEDs discovered after 1990) (Glauser *et al.*, 2013). The newer generation antiepileptic drugs have broadened the therapeutic options over the past two decades; however, there is no difference in efficacy between these newer antiepileptic drugs and the conventional AEDs for treatment of new-onset epilepsy (Löscher, 2011). The new generation AEDs were primarily used as adjuncts to the classical drugs. Conversely, monotherapy with the

new generation AEDs has gained wide usage and seems to have better therapeutic outcomes and lesser side effects profile and improved tolerability (Löscher, 2011). The conventional AEDs include: phenytoin, phenobarbital, ethosuximide, valproate, carbamazepine and various benzodiazepines such as diazepam, clonazepam, and clobazam. The newer generation AEDs in current use include: vigabatrin, gabapentin, pregabalin, lamotrigine, felbamate, tiagabine, topiramate, levetiracetam, oxcarbazepine, stiripentol, rufinamide and zonisamide. Some of these AEDs are also used in other conditions such as migraine, bipolar disorders, anxiety disorders and neuropathic pain (Goodman *et al.*, 2011).

Table 2- 1: Seizure types and some recommended drug treatments

SEIZURE TYPE	FIRST LINE TREATMENT	ALTERNATIVES
Generalized seizure		
Tonic-clonic	Valproate, Lamotrigine	Topiramate, phenytoin and zonisamide
	levetiracetam	
Absence	Ethosuximide, valproate	Lamotrigine, clonazepam
		Zonisamide and levetiracetam
Atypical absence, atonic, myoclonic	Valproate, Lamotrigine	Topiramate, zonisamide,
	levetiracetam	Clonazepam and felbamte
Status epilepticus	Diazepam(iv), phenytoin(iv) phenobarbital (iv)	benzodiazepines
Focal seizures		
Simple	Carbamazepine	Gabapentin, topiramate,
Complex	Levetiracetam	Valproate, tiagabine, zonisamide lacosamide, pregabalin, felbamate, phenytoin and retiagabine.
Secondarily generalized	Lamotrigine and oxcarbazepine	

2.5 MECHANISMS OF ANTICONVULSANT ACTION

The conventional and some of the new generation AEDs exert their anticonvulsant effects by; elongation of sodium channel inactivation, enhancement of $GABA_A$-mediated chloride action, inhibition of T-type calcium current and inhibition of glutamate release or a modulation of its receptors (Goodman *et al.*, 2011).

2.5.1 Blockade of Voltage-Dependent Sodium Channels

The primary action of phenytoin, carbamazepine, oxcarbazepine, lamotrigine, topiramate, zonisamide and felbamate is the blockade of voltage-dependent sodium channels (Meldrum, 1996; Brodie & Sills, 2011). The implication of this blockage is that, high-frequency repetitive neuronal firing is selectively prevented thereby inhibiting the spread of seizure activity without interfering with normal physiologic neurotransmission. Even though these sodium channel blockers, at therapeutic concentrations, inhibit action potential firing without directly affecting synaptic responses, blockade of neuronal firing eventually prevents depolarization of the nerve terminal and the consequent release of neurotransmitters principally glutamate. It is evident that drugs sharing this property are effective against partial and secondarily generalized tonic-clonic seizures and possibly also against primarily generalized tonic-clonic seizures (Perucca, 2001). Drugs with this mechanism of action are not useful against absence and myoclonic seizures and may even aggravate these seizures (Perucca *et al.*, 1998).

2.5.2 Potentiation of GABAergic Inhibition and Other Mode of Actions

Gamma aminobutyric acid is the main inhibitory neurotransmitter in the mammalian brain. It is therefore not amazing that many of the AEDs suppress epileptic firing by potentiating GABAergic inhibition (Meldrum, 1999; Brodie & Sills, 2011). The barbiturates such as phenobarbital and the benzodiazepines bind to various allosteric sites on the $GABA_A$ receptor to facilitate GABA-mediated influx of chloride and subsequent hyperpolarization. Vigabatrin exerts its effect by inhibiting GABA transaminase (GABA-T) leading to increased pool of GABA that can be released from presynaptic nerve terminals. Tiagabine on the other hand, increases GABAergic transmission by blocking the reuptake of synaptically released GABA by GABA Transporter 1 (GAT 1) (Perucca *et al.*, 1998; Brodie & Sills, 2011; Rang HP, 2014).

Valproate, ethosuximide and clonazepam inhibit T-type low-voltage-activated calcium channels. The T-type calcium channel blockade in thalamic neurons is a primary mechanism for antiabsence effect of the drugs mentioned above (Kito *et al.*, 1994; Meldrum, 1996; Weiergräber *et al.*, 2010) whereas the modulation of excitatory transmission release through blockade of N-type and P/Q-type calcium channels appears to mediate the efficacy of gabapentin and pregabalin in epilepsy, neuropathic pain and other indications (Fink *et al.*, 2000). Newer generation AEDs such as topiramate and felbamate are postulated to act on postsynaptic NMDA and AMPA receptors, whereas lamotrigine may modulate serotoninergic transmission. In the case of levetiracetam, studies suggests that its primary action is related to modulation of SV2A, a synaptic vesicle protein involved in vesicle exocytosis (Lynch *et al.*, 2004; Löscher *et al.*, 2016) thus, blocking glutamate release.

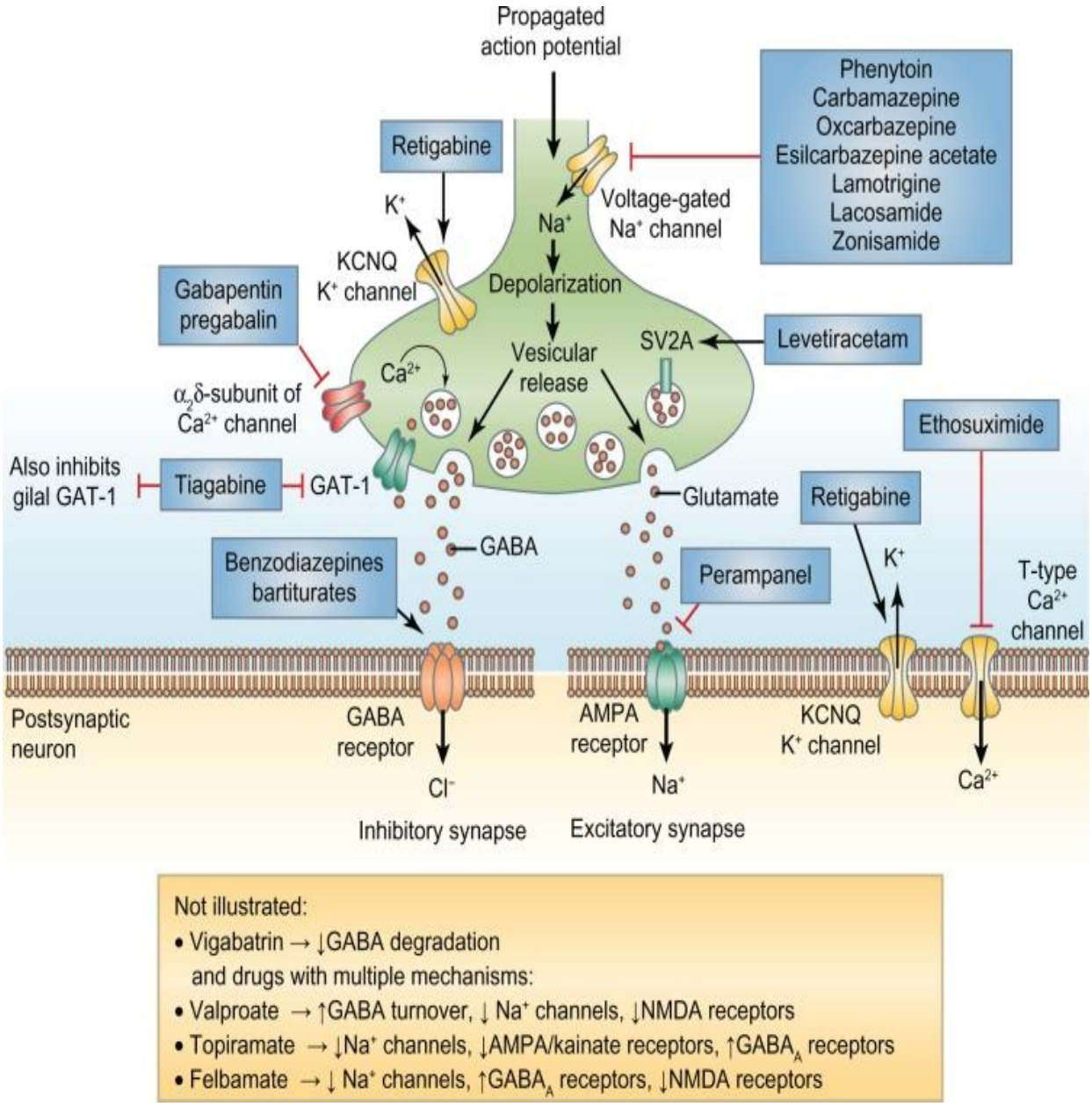

Figure 2- 2: Mechanism of action of antiepileptic drugs (Fong, Wong & Zuo, 2014)

2.6 ANIMAL MODELS OF EPILEPSY

The development of various new antiepileptic drugs in the past few decades and the search for new therapies with better efficacy, tolerability and minimal side effects still remain an essential aim (Bialer & White, 2010). The discovery and development of new AEDs relies largely on the preclinical use of animal models to establish the efficacy and safety of the test drug prior to first trials in humans (White *et al.*, 2006). Variety animal models have been created for both acute and chronic seizure models using various agents, either by chemical or electronic methods. Anticonvulsant activities of a test drug or compound can be studied using variations of two basic test methods: blockade of electroshock-induced convulsive seizures or chemical-induced convulsive seizures (Dhir, 2012).

2.6.1 Acute Epileptic Seizure Models

Acute seizures can be induced chemically using such chemoconvulsants as pentylenetetrazole (PTZ), picrotoxin (PIC), strychnine (STC) lithium-pilocarpine (Li-Pc), 4-aminopyridine (4-AP) and electrically as in the electroconvulsive maximal electroshock (MES) test. Acute seizure models are mainly used to explore the anticonvulsant effects of first-hand chemicals. The maximal electroshock threshold test and the PTZ-induced seizure test are primary screening tests for identifying compounds that have the ability to prevent the spread of seizures and raise the seizure threshold, respectively. Compounds that can limit the spread of a seizure from its focus, also inhibit hind-limb extension in the MES test and compounds that block clonic convulsions in the PTZ-induced seizure test are able to raise the seizure threshold. PTZ and PIC are used to test for agents effective against myoclonic or generalized absence seizures, whereas MES test identifies chemicals that are effective against generalized tonic-clonic seizures (Dhir, 2012). This study

made use of the three major conventional anticonvulsant screening tests to evaluate the anticonvulsant properties of *Cleome rutidosperma*.

2.6.2 Chronic Epileptic Seizure Models

Kindling is a chronic model of epilepsy where repetitive and intermittent administration of sub-convulsive electrical or chemical stimuli can lead to progressive amplification of seizures, culminating in generalized seizure activity (McNamara, 1984; Ahmadi *et al.*, 2017). Chronic kindling model permits the study of long-term effects of drugs or epileptogenesis. These models are often modified forms of the acute models: chronic administration of low dose of a convulsant, direct application of a convulsant to a specific region of the brain, sudden withdrawal of neuronal suppressing agents, genetic alteration of the animal specimen and chronic application of low intensity current in electrophysiological methods. For example, electrical stimulation of the limbic system can induce chronic spontaneous epilepsy similar to temporal lobe epilepsy several weeks after treatment, also PTZ at doses of (20 to 40 mg kg^{-1} *i.p.*) can induce chronic myoclonic convulsions (Hellier & Dudek, 2005; Löscher, 2011). In this study, the anticonvulsant properties of *Cleome rutidosperma* was evaluated using test models as described (Dhir, 2012) with some minor modifications.

2.7 *CLEOME RUTIDOSPERMA*

Cleome rutidosperma (DC) *(Cleomeceae)* is a low-growing herb, up to 70 cm tall, found in waste grounds, humid or grassy places with trifoliate leaves and small, violet-blue flowers, which turn pink as they age. The elongated capsules display asymmetrical, dull black seeds (Khuntia *et al.*, 2013). The fruit is cylindrical and curved, about 5 cm long. *Cleome rutidosperma* is an annual species reproducing mainly by seed. Flowering and fruiting plants of *C. rutidosperma* can be found

throughout the year, although most abundantly in the rainy season (Bosch, 2014). It belongs to the family of *Cleomeceae* and native to West Africa, from Guinea to Nigeria. It can also be found in Democratic Republic of Congo and Angola (Widespread, 1972; Waterhouse, 1998) and has been reported to be among the 50 species of *Cleome* occurring in Africa (Jansen, 2004). It is commonly known as Fringed Spider Flower (Khuntia *et al.*, 2013).

Figure 2- 3: *Cleome rutidosperma (Cleomaceae*). Centre for Plant Medicine Research, Mampong-Akuapem, Ghana.

2.7.1 Traditional Medicinal Uses of *Cleome rutidosperma*

The leaves, roots and seeds preparations of *C. rutidosperma* have been used by various traditional medicinal practitioners for anti-convulsant, anti-inflammatory, anti-stimulant, anti-scorbutic, rubefacient, anti-diarrheal, vesicant and carminative purposes. The leaves are eaten as cooked vegetable or added to soup (Burkill, 1985; Edeoga, 2005 ; Khuntia *et al.*, 2013). It is occasionally taken as a pot herb (Abbiw, 1990). In Ghana, Gabon and DR Congo, the leaf sap is applied to cure ear-inflammation, ear-ache and deafness (Chakraborty, 2010). In Ghana and South Nigeria, the leafy extract is used to treat irritated skin, prickly heat and convulsions (Kirtikir & Basu, 1935; Ghosh *et al.*, 2019).

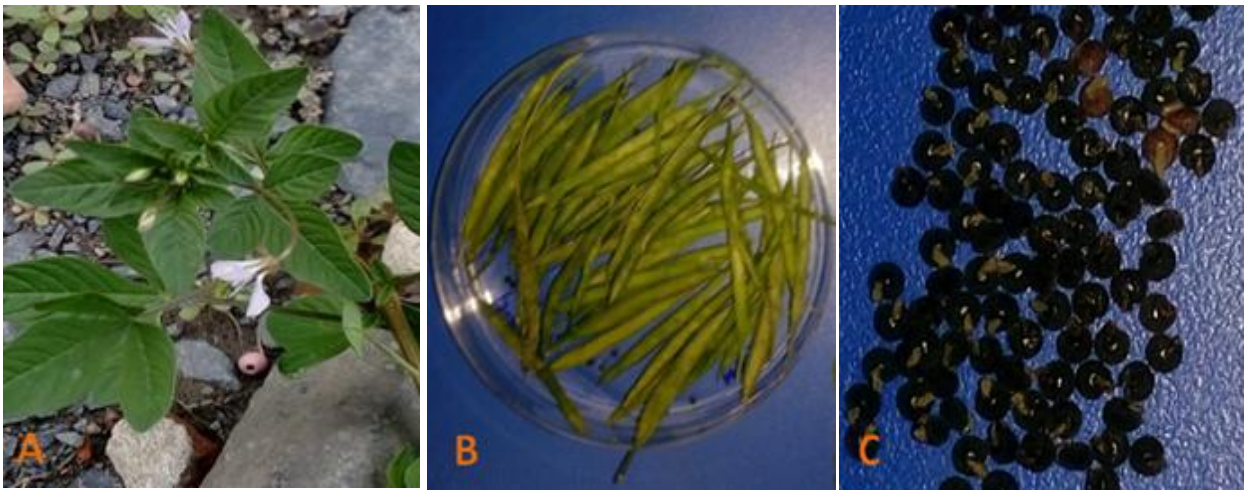

Figure 2- 4: A) Leaves and flowers; B) Pods; C) Seeds of *Cleome rutidosperma* (Ghosh, Chatterjee, Das, Karmakar & Mahapatra, 2019)

2.8 TOXICITY REPORTS FROM TADITIONAL USES

In the LMIC where herbal remedies are the mainstay for primary health care, these plants are indiscriminately recommended without standardized safety information to guide their usage. Folk medicines are used as a single or combination remedy which may contain many active elements with numerous physiological and pharmacological effects. The presence of numerous phytochemicals including alkaloids, glycosides, flavonoids, steroids, saponins and many other secondary metabolites are thought to be responsible for the pharmacological activity of these plants (Kagbo & Ejebe, 2010). *Cleome rutidosperma*, aside from the multiple folkloric applications, seems to lack any reports of toxicity from their traditional uses.

2.9 PREVIOUS WORKS ON *CLEOME RUTIDOSPERMA*

Earlier investigations have centred on preliminary phytochemical screening of the leaves, stem and roots of *C. rutidosperma:* glycosides, saponins, alkaloids, flavonoids, tannins, steroids and carbohydrates (Okonwu *et al.*, 2017; Ghosh *et al.*, 2019). Ethanolic extract of the leaves and stem have shown significant anti-inflammatory and anti-pyretic activity. The methanolic extracts of the roots of the plant showed analgesic, anti-pyretic and anti-inflammatory activity (Bose, 2007). The analgesic and locomotive effect of methanol, chloroform and petroleum ether extracts of *Cleome rutidosperma* have also been studied (Bose *et al.*, 2004). Study of the crude aqueous extract of the plant showed laxative, diuretic effects and antimicrobial properties against different gram-positive and gram-negative bacterial strains (Bose *et al.*, 2005; Bose, 2006; Bose *et al.*, 2007). The anti-oxidant activity (Bose *et al.*, 2008; Chakraborty, 2010), wound healing property (Mondal & Suresh, 2012) and the anti-diabetic activity (Okoro *et al.*, 2014) have been investigated. The anticonvulsant activity of *Cleome rutidosperma* extracts using ethanol, petroleum ether, ethyl

acetate and n-butanol as solvents for extraction have been investigated (Jena *et al.*, 2009). The anti-neuro-inflammatory activity of CR using LPS-stimulated microglial cell line BV2 (Ding, 2016), the anti-nociceptive property (Ansari *et al.*, 2016), anti-plasmodial activity (Bidla, 2004), anti-cancer property (Prabha, 2017), and anti-depressant activity (Archi, 2016) have been explored.

Chapter 3

MATERIALS AND METHODS

3.1 PLANT COLLECTION AND EXTRACTION

3.1.1 Plant Collection

Samples of the whole plant of *Cleome rutidosperma* were collected at the arboretum of the Centre for Plant Medicine Research (CPMR), Mampong-Akuapem in the Eastern region of Ghana (latitude 5.9107950 and longitude 0.1342191) in July 2017. The plant sample was authenticated at the CPMR, Mampong-Akuapem and voucher specimen labelled (CPMR 208/17) was archived in the herbarium.

3.1.2 Extraction Process

The collected fresh plant material of *C. rutidosperma* was washed under running tap water to remove dirt, shade-dried for 7 days, chopped and pulverized in a mechanical blender to obtain a fine powder. The powdered plant (2.75 kg) was extracted using 90% $^{V}/_{V}$ ethanol over a 48-h period using a Soxhlet apparatus at a temperature of 60-80ºC. The resultant extract was concentrated under reduced pressure at 40-60ºC to a syrupy mass in a rotary evaporator. The syrupy mass was further air-dried in a water bath and kept in a desiccator. The yield was 6.4% $^{w}/_{w}$ with respect to dry starting material. The dried extract which had characteristic odour and greasy consistency is subsequently referred to as *Cleome rutidosperma* ethanolic extract (CRE) or extract in this study.

3.2 DRUGS AND CHEMICALS

The ensuing drugs and chemicals were used: Pentylenetetrazole (PTZ) and Picrotoxin (PIC) were obtained from Sigma-Aldrich Inc., St. Louis, MO, USA, while phenobarbitone (PHB) and diazepam (DZP) was purchased from Ernest Chemists Ltd, Ghana, and carbamazepine (CBZ) was obtained from Pharm-Inter, Brussels, Belgium.

3.3 ANIMALS

Female ICR mice (25±5 g) were purchased from the Noguchi Memorial Institute for Medical Research, College of Health Sciences, University of Ghana, and kept at the breeding centre of the Department of Animal Experimentation of the Institute. The animals were housed in groups of 5 in stainless steel cages (34 cm x 47 cm x 18 cm) with soft wood shavings for bedding and maintained under laboratory conditions (temperature 24-26°C, relative humidity 60-70 % and 12 h light-dark cycle). Animals were allowed water *ad libitum* and fed with standard commercial pellets. The mice were brought into the test environment for at least five days to allow for acclimatization to experimental conditions. All procedures and techniques used in this study were in accordance with principles regarding the protection of animals used for experimental purposes. All protocols used were duly approved by the Scientific and Technical Committee (STC), NMIMR with protocol number NIACUC-2018-02-2V.

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3.4 PHYTOCHEMISTRY OF EXTRACT (*CLEOME RUTIDOSPERMA*)

The preliminary phytochemical screening of CRE was conducted using various methods to determine the phytoconstituents of the extract (Kala, 2014 ; Trease & Evans, 2016).

3.4.1 Flavonoids (Ammonia Test)

A volume of 5 ml of dilute NH_3 was added to a 1 ml aqueous filtrate of the extract and 5 ml conc H_2SO_4 was added. A yellow coloration that disappeared on standing indicated the presence of flavonoids.

3.4.2 Saponins (Frothing Test)

A volume of 4 ml of H_2O was added to 0.2 g of CRE and agitated vigorously to froth. The mixture was observed for about 5 min to determine if the froth persisted.

3.4.3 Alkaloids (Dragendorff's Test)

A volume of 5 ml of HCl was added to 0.2 g CRE and brought to a boil for 5 min and later cooled and filtered. Dragendorff's reagent was added to 1 ml of the filtrate and visually observed for a reddish-orange precipitate.

3.4.4 General Test for Glycoside (Reducing Sugars)

Dilute H_2SO_4 5 ml and CRE 0.2 g mixture was brought to boil for 2 min in a water bath, cooled and 5 drops of NaOH (20%) was added. A volume 1 ml of Fehling's A solution and 1 ml Fehling's B were dissolved in it. The content was heated and monitored for a precipitate of brick-red of cuprous oxide.

3.4.5 Phytosterols (Liebermann-Burchard's Test)

CRE 0.2 g dissolved in a 4 ml $CHCl_3$ and 2 ml $C_4H_6O_3$ was mixed in a test tube. Conc. H_2SO_4 was carefully streamed down along the inner walls of the test tube and observed for a blue coloration at the interface.

3.4.6 Tannins (Ferric Chloride Test)

About 0.2 g of CRE was boiled in 5ml of H_2O in a test tube. It was then cooled under running water and filtered. A volume of 1 ml of the filtrate was added to 2 ml H_2O and 5 drops of 1% $FeCl_3$ and monitored for blue-black or olive-green precipitates.

3.5 PRELIMINARY TESTS

3.5.1 Irwin Test

This test was conducted as described by (Irwin, 1968; Amoateng *et al.*, 2012) with slight modifications. The test was conducted with the aim of evaluating the effects of CRE on the behaviour and physiological functions of the mice. Female ICR mice (25±5 g) were randomized into seven groups of five mice (n=5) and maintained in optimum experimental conditions for three days for acclimatization. The animals were fasted overnight but allowed access to water *ad libitum* and then treated with oral doses of CRE (10, 30, 100, 300, 1000 and 3000 mg kg^{-1}) of body weight by gavage. A control group was administered 10ml kg^{-1} of distilled water per oral route. The mice were monitored for 48 h for manifestations of alterations in physiological function at 0, 15, 30, 60, 120, 180 min and 24 and 48 h after treatment with CRE. Demonstration of neurotoxicity effects (convulsions, tremor) and CNS stimulant effects (excitation, straub tail, jumping, hypersensitivity, stereotypies, and aggression) were monitored. CNS depressant (sedation, rolling gait, loss of balance, motor incoordination, hyposensitivity to external stimuli, decreased muscle tone, akinesia

and catalepsy) and autonomic effects (urination, salivation, and defecation) were also monitored. The symptoms of toxicity and the rate of mortality were noted for each mouse for 48 h (Kagbo & Ejebe, 2010).

3.5.2 Acute Toxicity Test

After the administration of the extract / distilled water, mice in each group were monitored 8 hourly for clinical signs of toxicity such as piloerection, abnormal gait, salivation, respiration, frequency of stool and mortality within 48 h. Death(s) within 24 and 48 h after administration of extract or vehicle were recorded and lethal dose (LD_{50}) determined. After 48 h of the study, the animals were euthanized in a chloroform chamber and were autopsied without delay. Organs (brain, heart, lungs, liver, kidneys and spleen) were macroscopically inspected, harvested, weighed and stored in formalin for histopathological investigations. Thorough post-mortem examination was performed.

The isolated organs (heart, brain, lungs, kidneys, liver and spleen) were preserved in 10% neutral buffered formalin solution for 10 days and washed thoroughly with water. Using a disposable microtome blade, individual tissues were cut into approximately 3 mm thick slices and appropriately labelled. Three slices each were obtained from each of the organs under investigation. The labelled sections were placed in a tissue processor (LEICA TP1020) for 18 to 21 h. The organs were dehydrated using ethanol and in xylene. Preparation of paraffin wax tissues blocks were made, and the tissue blocks were cast using a molten wax dispenser, plastic cassettes and mould boxes. Using a rotary microtome, tissue blocks were cut at 5 µm and mounted onto microscope slides. The cut sections were then put into a hot air oven for at least 2 h. The slides were later stained using haematoxylin and eosin (H&E) dyes. The slides were identified via codes written on the frosted parts of the slides.

3.5.3 Sub-Acute Toxicity Test

For 28 consecutive days, 10 female ICR mice (25±5 g) divided into two groups of 5 mice (n=5) were administered distilled water (10 ml kg^{-1}) or CRE (1000 mg kg^{-1}). The vehicle and CRE were administered orally by gavage to mimic folkloric administration route. All procedures and techniques used on the animals in this study were in accordance with the Noguchi Institute Animal Care and Use Committee (NIACUC) guidelines as well as the National Institute of Health Guidelines for the Care and Use of Laboratory Animals. The mice in each group were monitored every 8 h for autonomic effects of the extract (toxicity) such as piloerection, abnormal gait, salivation, respiration, frequency of stool and mortality throughout the study period. The animals were weighed on the 1^{st}, 8^{th}, 15^{th}, 22^{nd} and on the 28^{th} day. After the 28^{th} day, the mice were sacrificed in a chloroform chamber and were autopsied immediately. All visible organs (brain, heart, lungs, liver, kidneys and spleen) were macroscopically harvested, weighed, inspected and stored in formalin for thorough post-mortem examination as well as histopathological investigations as described in the acute toxicity study.

3.5.4 Thiopental Sodium Sleeping Time Test

Female ICR mice (25±5 g) were randomized into six groups of five mice (n=5) and maintained in optimum experimental conditions for two days for acclimatization. The animals were fasted overnight but allowed access to water *ad libitum* and then treated with oral doses of CRE (30, 100, 300, and 1000 mg kg^{-1}) of body weight and diazepam (10 mg kg^{-1}) of body weight per oral route. A control group was administered 10ml kg^{-1} of distilled water per oral route. After 30 min of pre-treatment with the test drugs, thiopental sodium (60 mg kg^{-1}) was administered intraperitoneally to all groups. The time from loss to regain of righting reflex was noted and the time between administrations of thiopental sodium and the loss of reflex was recorded.

3.6 ANTICONVULSANT EFFECT OF *CLEOME RUTIDOSPERMA*

3.6.1 Pentylenetetrazole-Induced Seizure Test

Female ICR mice (25±5 g) were pre-treated with CRE (100, 300, and 1000 mg kg^{-1} *p.o.*) or phenobarbitone sodium (3, 10, and 30 mg kg^{-1} *p.o.*) and after 30 min clonic seizures were induced by subcutaneous injection of 80 mg kg^{-1} PTZ into the loose skin fold on the back of the neck of the mice. The negative control group received distilled water (10ml kg^{-1} *p.o.*). After the PTZ injection, animals were placed in a testing chamber (made of Perspex of dimensions 15 cm x 15 cm x 15 cm). A mirror placed at an angle of 45˚below the floor of the chamber allowed a complete view of the convulsive events in the mice. The behaviour of the animals was captured with a camcorder, placed directly opposite the mirror, for 30 min. The animals were observed for the frequency, duration and latency to convulsions using animal behaviour tracking software Behaviour Tracker® version 1.0. The ED_{50} (a measure of anticonvulsant potency) of the extract and the reference anticonvulsant were calculated from the dose-response curves of the percent seizure inhibition by the drug/ extract to the vehicle-treated group. The ability of the extract to prevent the seizures or delay/ prolong the latency or onset of the clonic seizures, reduce the frequency and the duration of seizures was considered an indication of anticonvulsant activity.

3.6.2 Picrotoxin-Induced Seizure Test

The effects of CRE on PIC- induced seizures were examined with the use of protocols previously described. Thirty-five (35) female ICR mice were randomized into seven groups of five mice (n=5). The control group was pre-treated with distilled water (10ml kg^{-1} *p.o.*) and the other groups were pre-treated with CRE (100, 300, 1000 mg kg^{-1} *p.o.*) or phenobarbitone sodium (3, 10, 30 mg kg^{-1} *p.o.*). Thirty (30) min after pre-treatment with test drugs, picrotoxin was administered to all groups, at 3 mg kg^{-1} of body weight intraperitoneally. The animals were monitored in a transparent

plexiglass observation chambers and videotaped for clonic-tonic convulsions for 30 min. The videos were tracked for duration, frequency and latency to seizures. The ED_{50} of the extract and phenobarbitone sodium were calculated as described in the PTZ test.

3.6.3 Maximal Electroshock Threshold Test

The ability of the extract to stop the spread of a seizure from its focus was tested using the maximal electroshock (MES) threshold test. The protocol used in this model is similar to that described by (Giardina & Gasior, 2009). Five mice were used to pre-test the current required to induce hind limb tonic convulsions (60 mA, 80 Hz and 0.2 s). Then, 70 mice were divided into seven groups (n= 10). Group I received oral distilled water (10 ml kg^{-1}). The next three groups (II, III, and IV) received 100, 300 and 1000 mg kg^{-1} oral CRE respectively. Groups V, VI and VII were administered carbamazepine (3, 10, and 30 mg kg^{-1} *p.o.*). After 30 min, in the case of extract-treated or carbamazepine-treated groups, an electroconvulsive therapy (ECT) apparatus was used to induce hind limb tonic convulsions (HLTE) by passing a current of 60 mA at a frequency of 80 Hz in 0.2 s through ear electrodes. The mice were then videotaped for 30 min in transparent plexiglass observation chambers. The latency and duration of hind limb tonic convulsions were recorded.

3.6.4 PTZ-Induced Kindling

To induce kindling, a 35 mg kg^{-1} dose of PTZ was injected intraperitoneally into CRE-phenobarbitone sodium- or vehicle- treated female ICR mice (25±5 g) every 48 h for 32 days. The control group received distilled water (10 ml kg^{-1} *p.o.*) while the other groups were administered CRE (100, 300, and 1000 mg kg^{-1} *p.o.*) or phenobarbitone sodium (3, 10, and 30 mg kg^{-1} *p.o.*). After each PTZ injection, the mice were placed in the testing chamber and recorded as in the PTZ

experiment described previously. Seizure intensities were classified according to the Racine score as follows:

Stage 0: no response

Stage 1: ear and facial twitching

Stage 2: convulsive waves throughout the body

Stage 3: myoclonic jerks, rearing

Stage 4: turning over onto one side

Stage 5: turning onto the back, generalized tonic-clonic seizures.

Each mouse was considered fully kindled after showing stage 4 or 5 on two consecutive PTZ administrations. Seven days after kindling had been achieved, the mice were challenged with 35 mg kg^{-1} of PTZ, and the entire event, recorded. The ED_{50} of the extract was calculated as described in the PTZ test.

3.8 STATISTICAL ANALYSIS

Animal behaviour tracking software Behaviour Tracker® version 1.0 was used to track duration, frequency and latency to convulsions. Graphpad Prism® Version 6.0 (GraphPad Software, San Diego, CA, USA) was used to analyse data. One/two-way analysis of variance (ANOVA) followed by an appropriate *post hoc* test were applied to detect significant differences in means between treated groups. Values were reported as Mean± S.E.M. and in all cases, $P < 0.05$ was considered statistically significant.

Chapter 4

RESULTS

4.1 PRELIMINARY PHYTOCHEMICAL SCREENING

The phytochemical screening of the whole plant extract of *Cleome rutidosperma* (CRE) confirmed the presence of various phytoconstituents such as glycosides, saponins, alkaloids, sterols, flavonoids except for tannins (Table 4-1).

Table 4- 1: Preliminary phytochemical screening of CRE

Colorimetric Test	Phytoconstituent	Remark
Ferric chloride & Lead acetate Test	Tannins	-
Dragendorff's Test	Alkaloids	+
General (Fehling's) Test	Glycosides	+
Liebermann-Burchard's test	Phytosterols	+
Ammonia Test	Flavonoids	+
Frothing Test	Saponins	+

(+) – present, (-) – absent

4.2 IRWIN TEST

Grouped mice were administered with CRE doses between 10 - 3000 mg kg^{-1} which resulted in reduced activity in some mice. Sedation was observed at 30 - 3000 mg kg^{-1} dose range as the main CNS effect (Table 4-2). No mortality was recorded in the Irwin test and the LD_{50} was estimated to be above 3000 mg kg^{-1}.

Table 4- 2: Behavioural changes observed after CRE administration in Irwin Test.

Group	Dose Kg^{-1}	Mortality (x/n)	Observations (0-48 Hours)
I	Distilled water (10 ml kg^{-1})	0/5	NIL
II	CRE 10 mg kg^{-1}	0/5	Stereotypy (chewing and sniffing), scratches
III	CRE 30 mg kg^{-1}	0/5	Sedation+, Stereotypy (chewing and sniffing), Analgesia
IV	CRE 100 mg kg^{-1}	0/5	Sedation++, Stereotypy (chewing and sniffing), Analgesia, Hypothermia
V	CRE 300 mg kg^{-1}	0/5	Sedation++, Stereotypy, Analgesia, Hypothermia, Decreased reactivity to stimulus
VI	CRE 1000 mg kg^{-1}	0/5	Sedation++, Stereotypy, Analgesia, Hypothermia, Decreased reactivity to stimulus, Ptosis
VII	CRE 3000 mg kg^{-1}	0/5	Sedation++, Stereotypy, Analgesia, Hypothermia, Decreased reactivity to stimulus, Abnormal gait, Ptosis

4.3 ACUTE TOXICITY TEST

4.3.1 Autonomic Effects of the Extract

The administration of CRE (10, 30, 100, 300, 1000 and 3000 mg kg^{-1}*p.o.*) resulted in no obvious anomaly in the autonomic effects in the mice compared with the vehicle treated group within the first 48 h.

4.3.2 Median Lethal Dose-LD_{50}

Monitoring the CRE-treated mice for 48 h, no deaths were recorded. Thus, the LD_{50} of the extract can be said to be more than 3000 mg kg^{-1} when given orally.

4.3.3 Post-Mortem Observations

Morphological examination of the CRE- and vehicle-treated mice did not show any visible abnormal effects in all the organs.

4.3.4 Relative Organ-Body Ratio

The relative organ to body ratio indices measured revealed no significant difference (P=0.9987) between the group treated with the vehicle and the group treated with the extract even at a dose of CRE 3000 mg kg^{-1} (Figure 4-1).

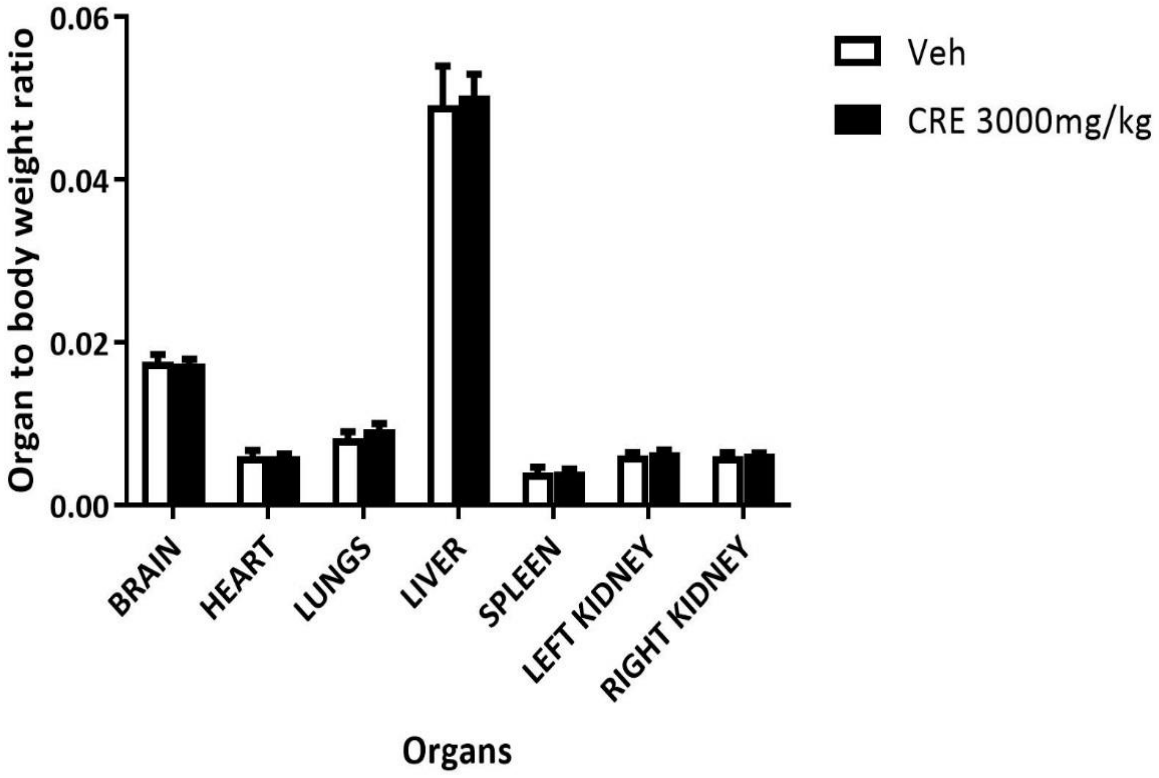

Figure 4- 1: Relative organ-body ratio of female ICR mice following the administration of CRE (3000 mg kg^{-1}) or distilled water (Veh). Data are Mean ± SEM. $P \leq 0.05$ was considered significant (One-way ANOVA followed by a Dunnett's multiple comparison test).

4.3.5 Histopathological Examination of Some Isolated Organs

The histopathological examination of the brain and heart of vehicle (distilled water ml kg^{-1})- and extract-treated mice revealed no anomaly even at the highest dose (3000 mg kg^{-1}) respectively. The kidney of the vehicle-treated mouse was normal (Figure 4-4 A) while the kidney of the extract-treated mouse showed tubular dilatation with erythrocyte infiltration (Figure 4-4 B). The liver of the vehicle- treated mouse was normal (Figure 4-5 A). However, the liver of the extract-treated mice revealed a liver microstructure with hepatocellular oedema (Figure 4-5 B).

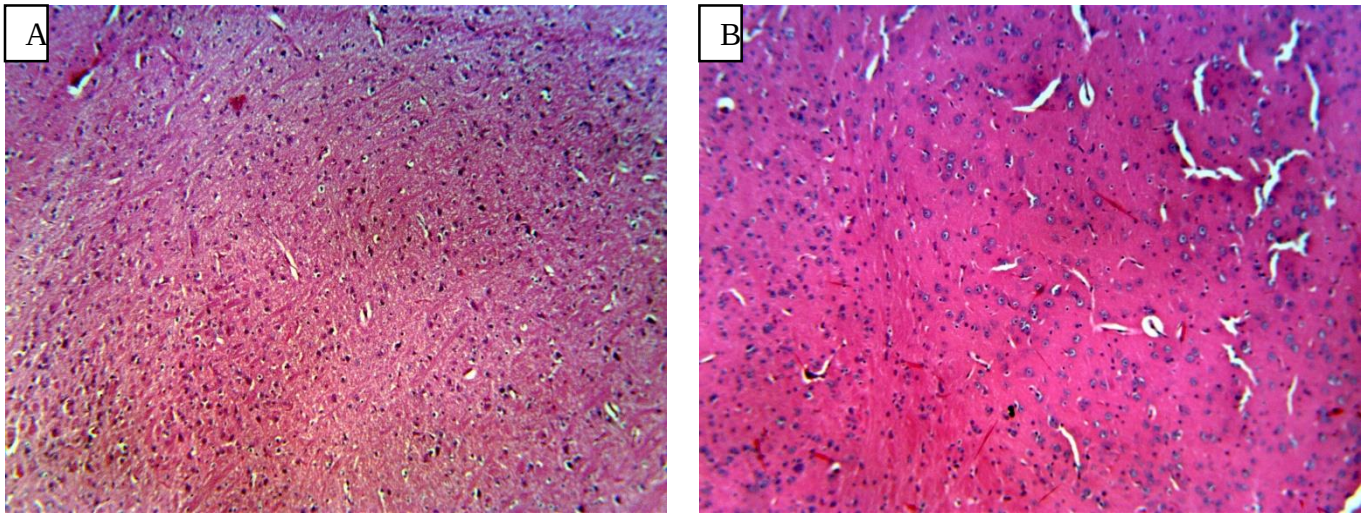

Figure 4- 2: Photomicrographs of brain from mice after single administration of (A) Vehicle (distilled water, 10ml kg^{-1}) and (B) CRE 3000 mg kg^{-1} showing normal brain microstructure with no infarct or necrosis. (H&E staining X10)

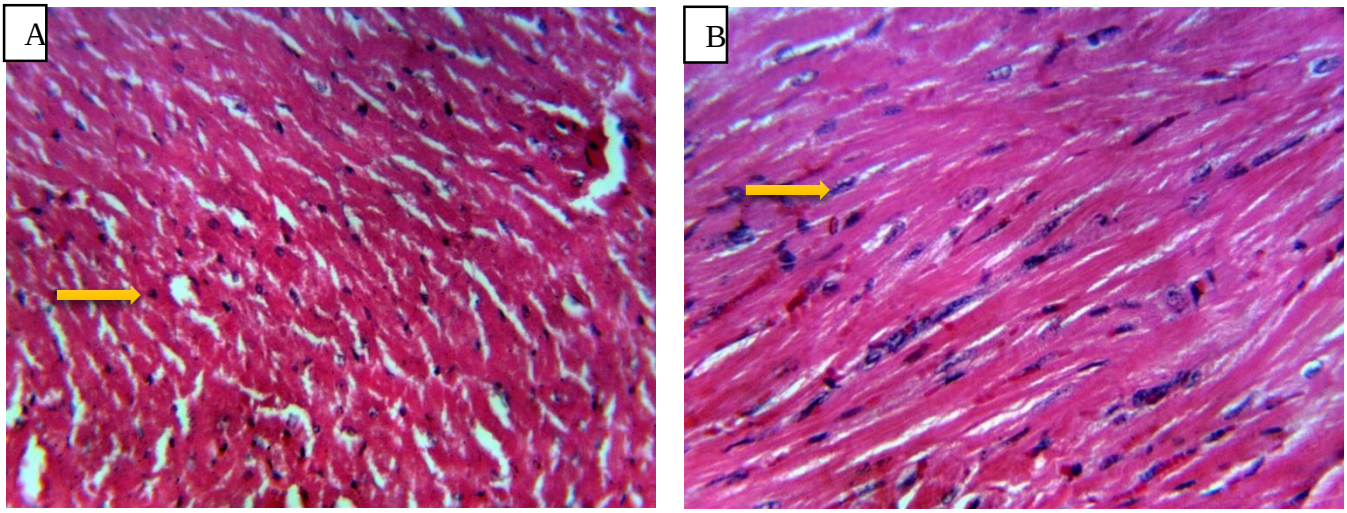

Figure 4- 3: Photomicrographs of heart from mice after single administration of (A) Vehicle (distilled water, 10ml kg^{-1}) and (B) CRE 3000 mg kg^{-1} showing normal myocardial fibre (arrow) with laterally located nuclei (H&E staining X40).

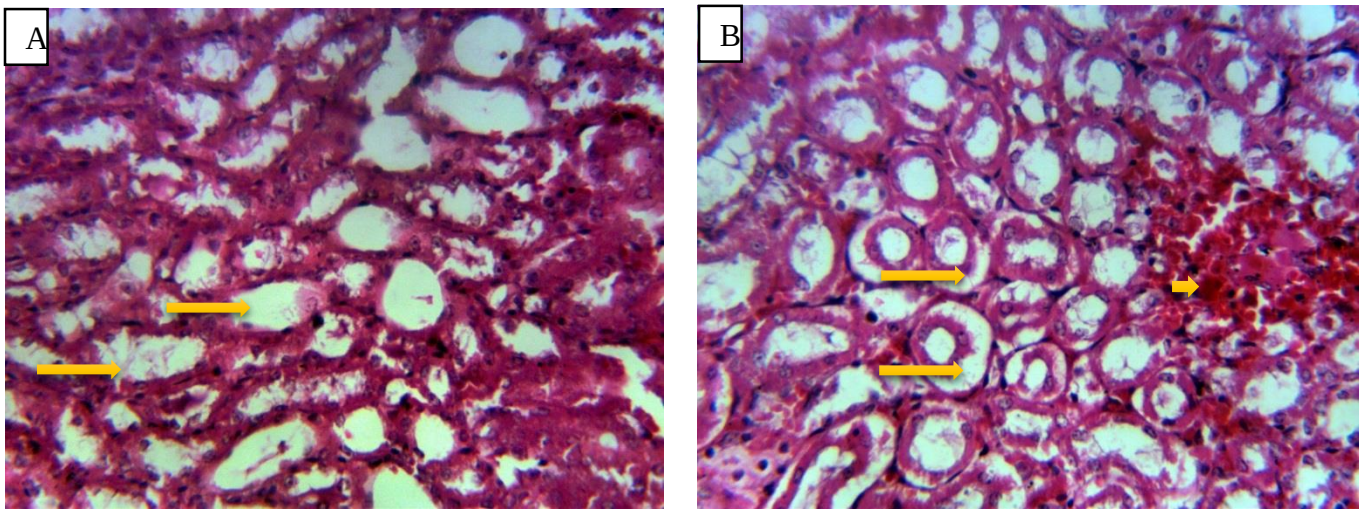

Figure 4- 4: Photomicrographs of Kidney from mice after single administration of (A) Vehicle (distilled water, 10ml kg^{-1}) and (B) CRE 3000 mg kg^{-1} showing normal tubular structures in the kidney at the medulla (arrow) (A) and tubular dilatation (arrow) with erythrocyte infiltration (arrow head) (B) (H&E staining X40)

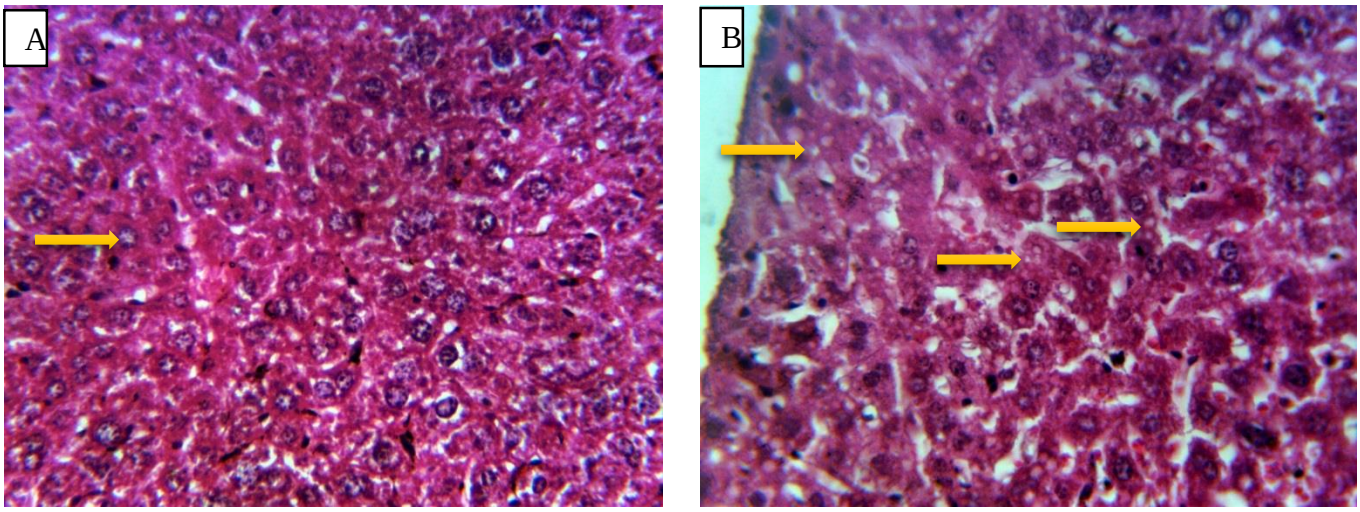

Figure 4- 5: Photomicrographs of liver from mice after single administration of (A) Vehicle (distilled water, 10ml kg^{-1}) and (B) CRE 3000 mg kg^{-1} showing normal hepatocyte with no inflammatory cells and no hepatocellular necrosis (arrow) (A) and liver microstructure with hepatocellular oedema (arrow) (B) (H & E staining X40).

4.4 SUB-ACUTE TOXICITY TEST

4.4.1 Autonomic Effects of the Extract

The administration of CRE (100, 300 and 1000 mg kg^{-1}) orally for 28 days did not yield any recognisable abnormality in the physiological function of the mice. The autonomic effects of the extract on the mice were normal compared with the vehicle-treated group in the entire period of the study.

4.4.2 Post-Mortem Examination

From the post-mortem examination of the CRE- treated group and the group treated with the vehicle, there was no visible unusual effects in the major organs that were examined.

4.4.3 Animal Weights

The weights of the mice treated with the extract were not significantly different ($P > 0.05$) from the vehicle-treated group when measured on the 1st, 8th, 15th, 22nd and 28th day of the study (Table 4-3). However, a gradual decline of weight for both treatment groups was observed throughout the study period to day 28.

Table 4- 3: Analysis of weights of female ICR mice in a 28-day oral administration of CRE (1000 mg kg_{-1}). Data are mean ± S.E.M. (n=5). $P \leq 0.05$ was considered significant when compared to the vehicle-treated group (One-way ANOVA)

Day	Veh	CRE-1000mg/kg	P-value
1	24.0000 ± 1.264911	22.40000 ± 0.40000	P > 0.05
8	15.2000 ±6.343501	12.80000 ± 5.23832	P > 0.05
15	9.6000 ± 6.177378	12.80000 ± 5.314132	P > 0.05
22	6.0000 ± 6.00000	14.00000 ± 5.727129	P > 0.05
28	6.0000 ± 6.00000	14.00000 ± 5.727129	P > 0.05

4.4.4 Relative Organ-Body Weight Ratio

There was no significant difference (P=0.9887) between the vehicle-treated group and the extract-treated group (CRE 1000 mg kg^{-1}) regarding the relative organ to body ratio indices measured (Figure 4-6)

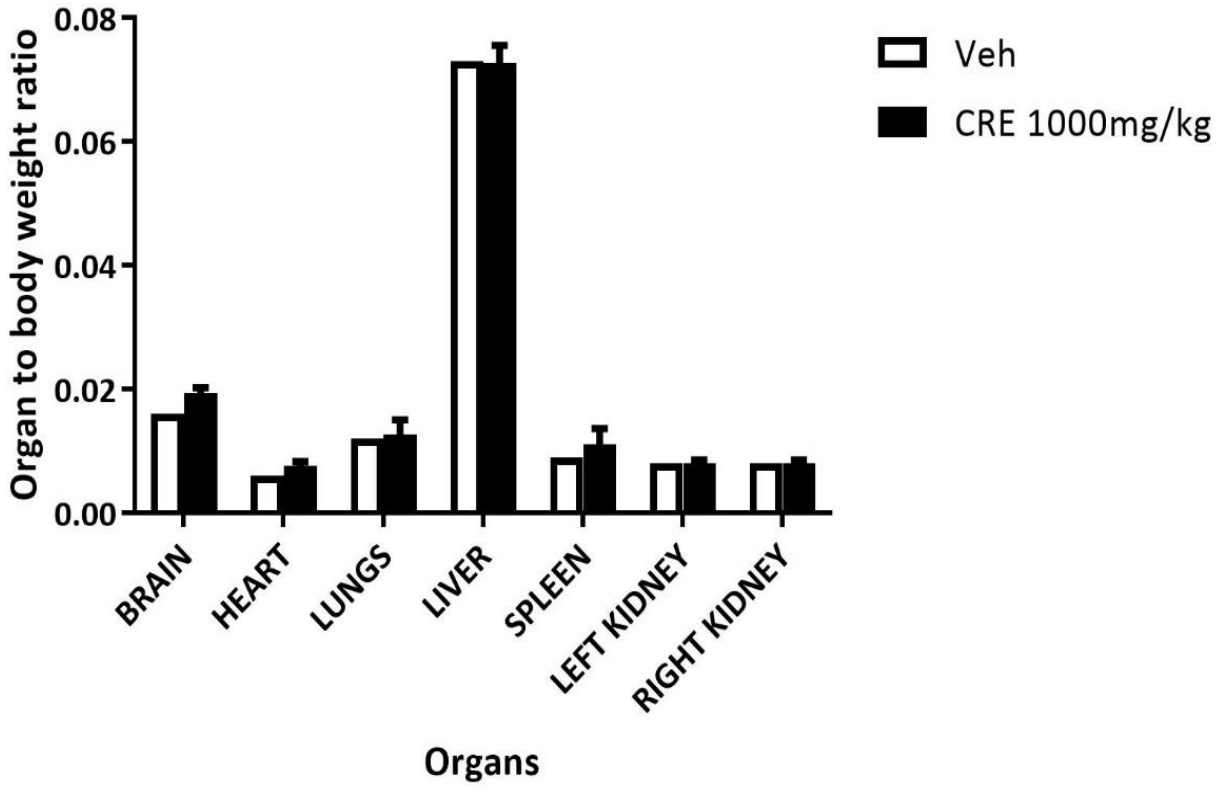

Figure 4- 6: Relative organ-body ratio of female ICR mice following the administration of CRE (1000 mg kg^{-1}) or distilled water (Veh). Data are Mean ± SEM. $P \leq 0.05$ was considered significant (One-way ANOVA followed by a Dunnett's multiple comparison test).

4.4.5 Histopathological Examination of Some Isolated Organs

The histopathological examination of the brain and heart of the vehicle-treated (Distilled water 10 mg kg^{-1}) and extract-treated mice did not reveal any abnormality even at 1000 mg kg^{-1} respectively. The brain showed normal microstructure with no infarct or inflammatory cell infiltration. The heart revealed normal myocardial fibre with no infarct or inflammatory cells present. The kidney of the extract-treated mice showed renal tubular dilatation with associated tubular epithelial sloughing (Figure 4-9). There was however, no glomerular or tubular necrosis or inflammatory cells present. The liver of the extract- and vehicle- treated mice were normal with no inflammatory cells present or hepatocellular necrosis (Figure 4-10).

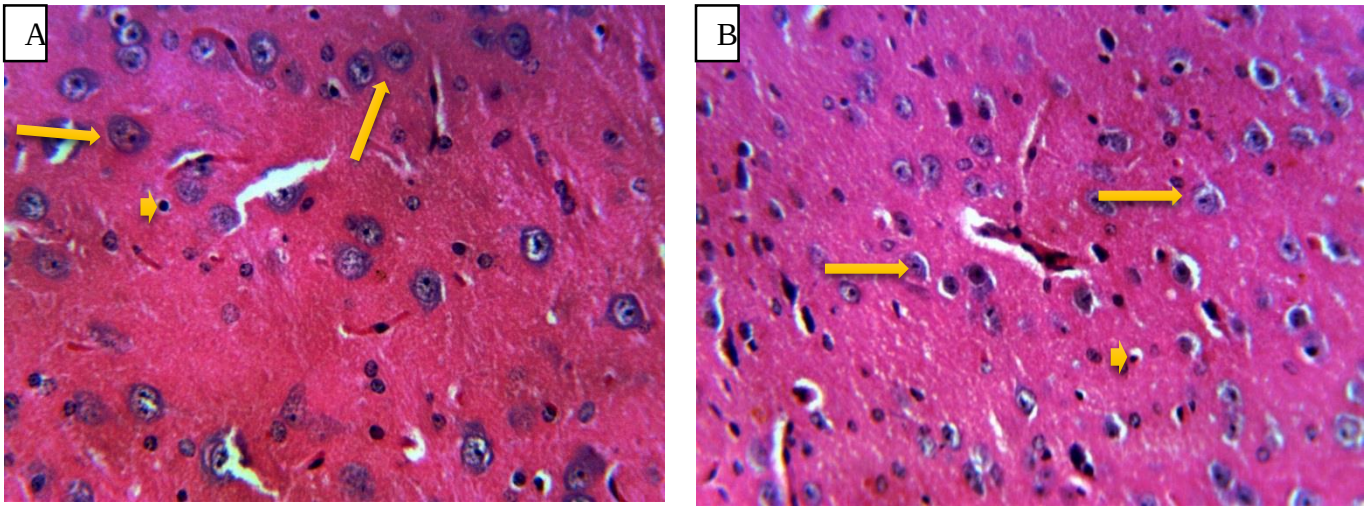

Figure 4- 7: Photomicrographs of brain from mice after a 28-day administration of (A) Vehicle (distilled water, 10ml kg^{-1}) and (B) CRE 1000 mg kg^{-1} showing normal brain neuron surrounded by glial cells (arrow head). No evidence of necrosis or inflammatory cell infiltration. (H&E staining X40).

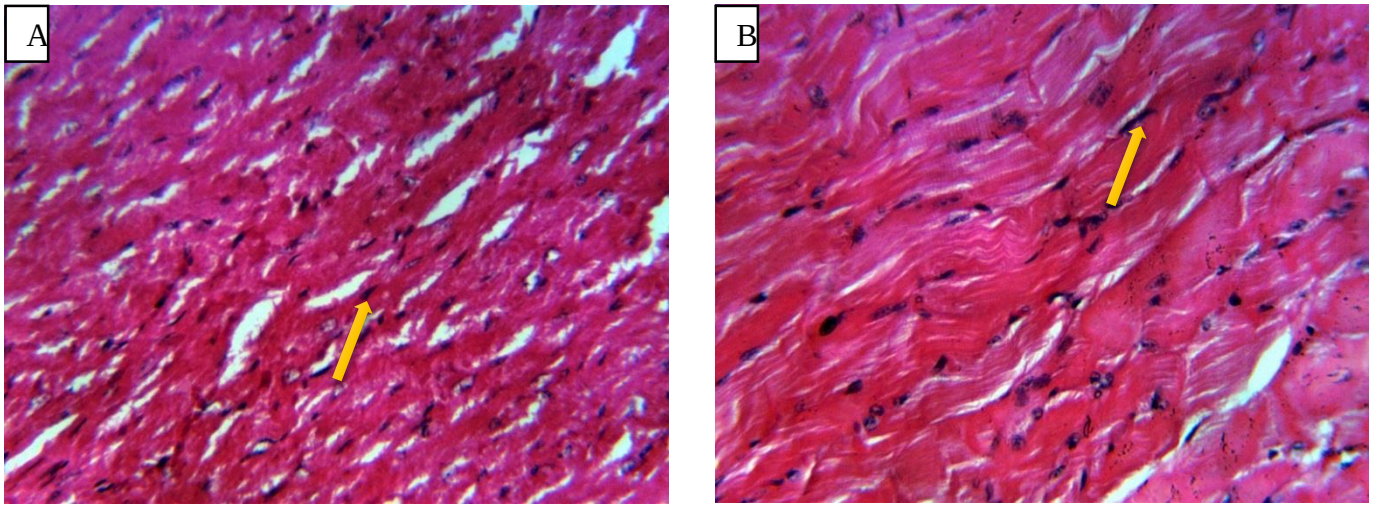

Figure 4- 8: Photomicrographs of heart from mice after a 28-day administration of (A) Vehicle (distilled water, 10 ml kg^{-1}) and (B) CRE 1000 mg kg^{-1} showing normal myocardial fibre (arrow) with no infarct or necrosis or inflammatory cells. (H&E staining X40).

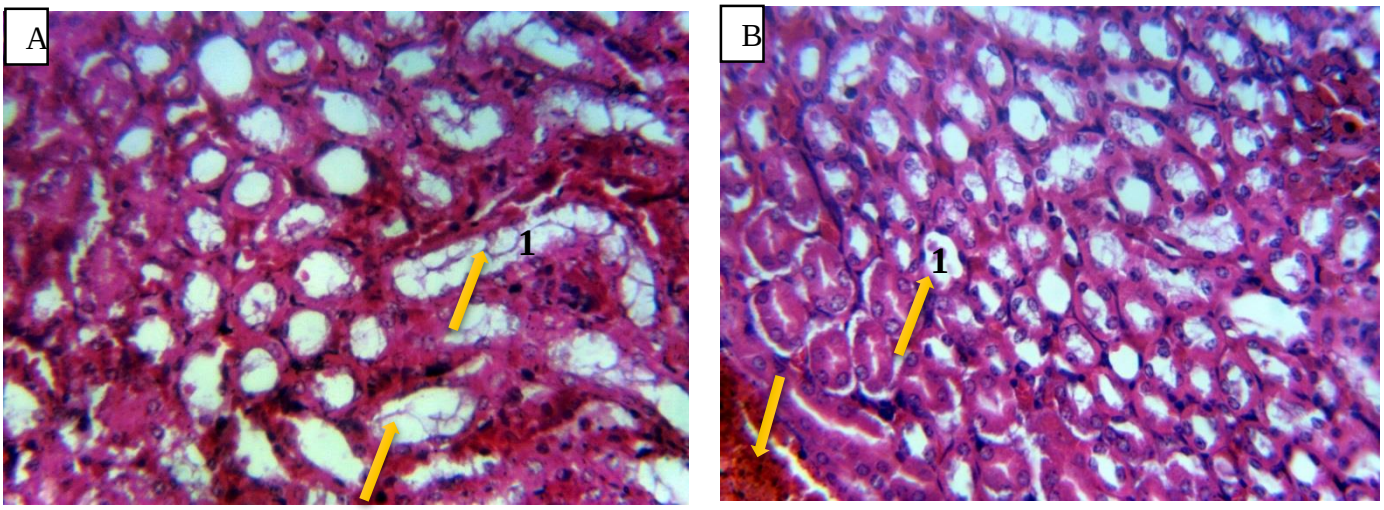

Figure 4- 9: Photomicrographs of kidney from mice after a 28-day administration of (A) Vehicle (distilled water, 10 ml kg^{-1}) and (B) CRE 1000 mg kg^{-1} showing renal tubular dilatation with associated tubular epithelial sloughing (1) (arrow) (A) and tubular alterations with epithelial cast (1) (arrow) and interstitial hemorrahges (left corner). No inflammatory cells present (B) (H & E staining X40)

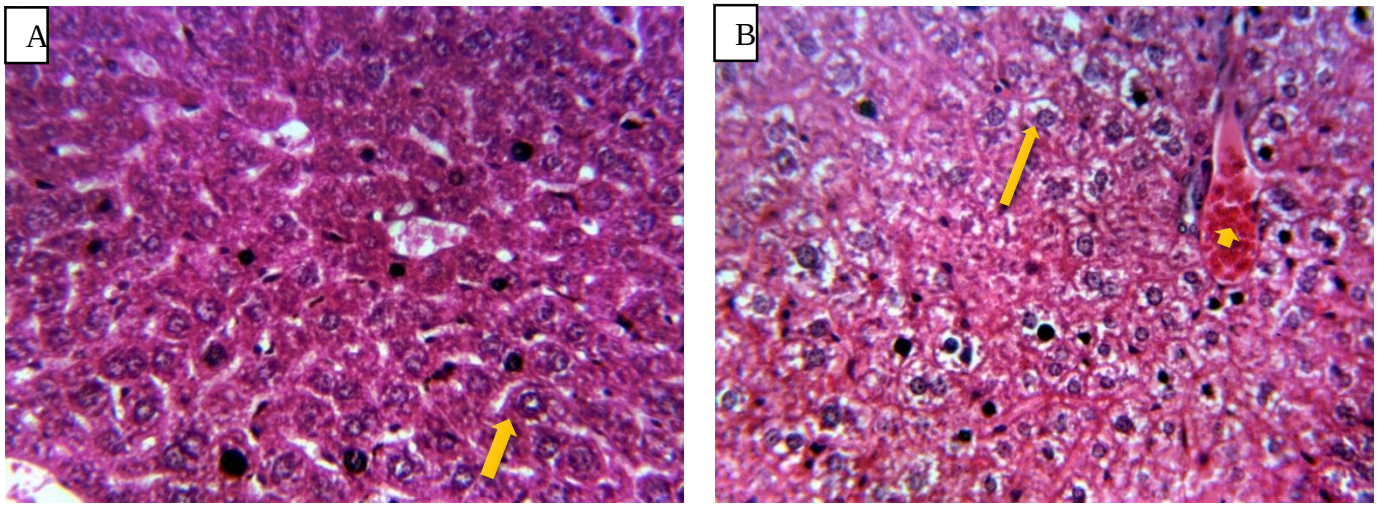

Figure 4- 10: Photomicrographs of liver from mice after a 28-day administration of (A) Vehicle (distilled water, 10 ml kg^{-1}) showing normal hepatocyte (arrow) and (B) CRE 1000 mg kg^{-1} showing normal hepatocyte (arrow) and congestion in central vein (arrow head). No inflammatory cells or hepatocellular necrosis. (H & E staining X40)

4.5 THIOPENTAL SODIUM SLEEPING TIME TEST

Thiopental sodium administered at a dose of 60 mg kg^{-1}, induced sleep in all the animals used in the study (Figure 4-11). The extract at 30, 100, 300 and 1000 mg kg^{-1} significantly ($P < 0.0001$, $F_{(5, 24)} = 111.5$) reduced the latency to sleep (Figure 4-11 A). CRE also significantly (($P < 0.0001$, $F_{(5, 24)} = 73.89$) increased the duration of sleep (Figure 4-11 B). Diazepam (positive control) administered at 10 mg kg^{-1} orally, significantly ($P < 0.0001$) reduced the latency to sleep and also increased the duration of sleep ($P < 0.0001$, Figure 4-11 A, B).

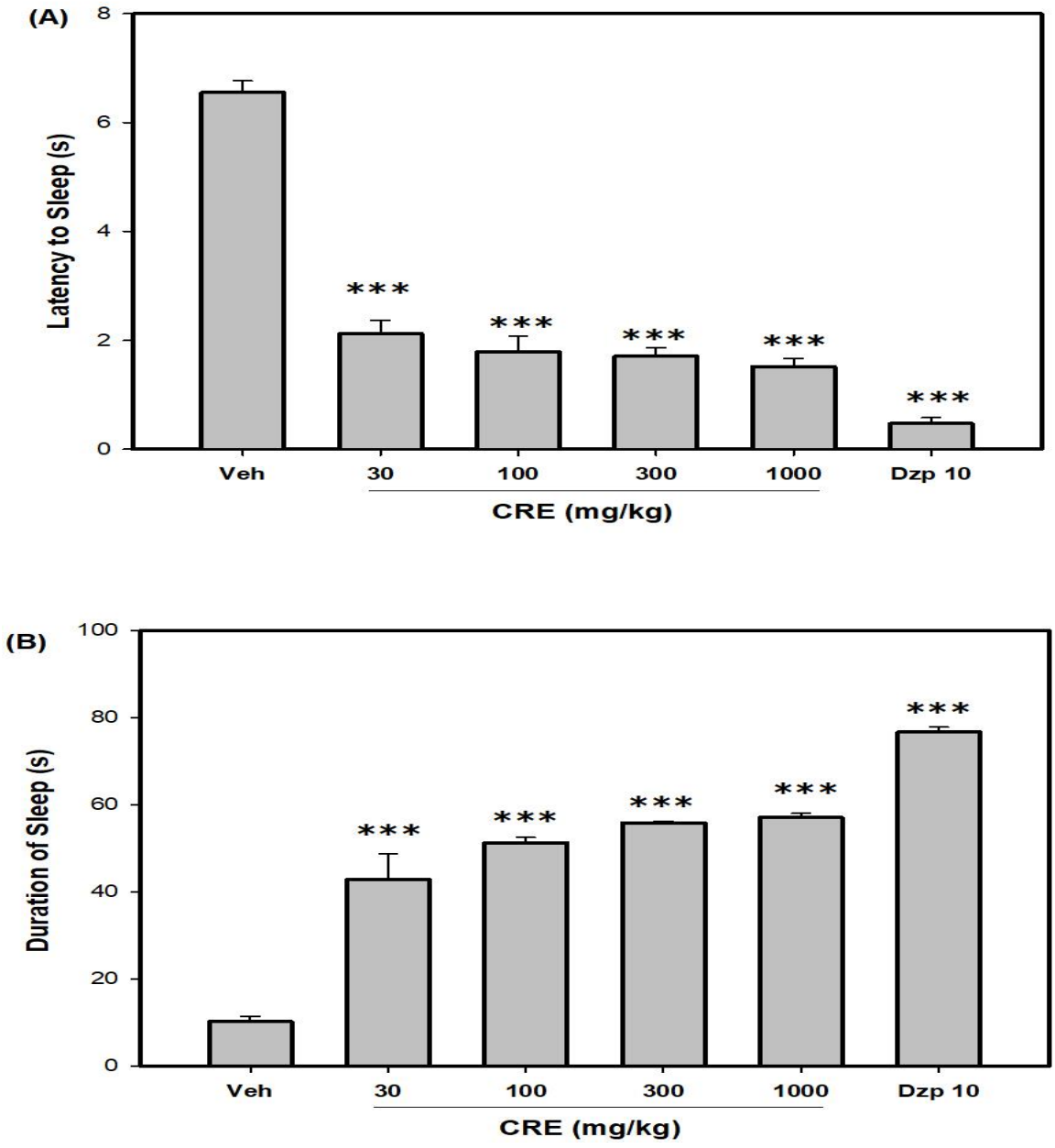

Figure 4- 11: Effects of CRE (30-1000 mg kg^{-1}) and Diazepam (10 ml kg^{-1}) on latency to sleep (A) and duration of sleep (B) in thiopental sodium sleeping time test. Data are presented as group mean ± SEM (n=5). Analysis by One-way ANOVA followed by a Dunnett's multiple comparison test. Significantly different from control. ***P< 0.001.

4.6 ANTICONVULSANT ACTIVITY STUDY

4.6.1 Pentylenetetrazole (PTZ)-Induced Seizure Test

The administration of PTZ (80 mg kg^{-1} *s.c.*) induced a sequence of events that started with myoclonic jerks, followed by an intense clonic-tonic convulsive phase. The extract, CRE, showed significant anticonvulsant effect against PTZ- induced seizures. The extract dose dependently, increased the latency to myoclonic seizures and this was significant at all doses used ($P < 0.0001$, $F_{(3, 16)} = 98.59$, Figure 4-12 a). CRE significantly ($P < 0.0001$, $F_{(3, 16)} = 72.78$, Figure 4-12 c) reduced the duration of seizures at all dose levels used. The frequency of seizures was significantly reduced at 300 mg kg^{-1} ($P=0.001$, Figure 4-12 b) and 1000 mg kg^{-1} ($P=0.0011$, Figure 4-12 b). The reference anticonvulsant used, Phenobarbitone, delayed the onset of myoclonic seizures ($P<0.0001$, $F_{(3, 16)} =127.4$, Figure 4-12 a) and reduced the duration of myoclonic seizures ($P<0.0001$, $F_{(3, 16)} = 156.90$, Figure 4-12 c). Phenobarbitone significantly ($P=0.0001$, $F_{(3, 16)} = 13.57$, Figure 4-12 b) reduced the frequency of seizures.

The anticonvulsant effects of CRE was less compared to phenobarbitone as shown in the ED_{50} values (Table 4-5).

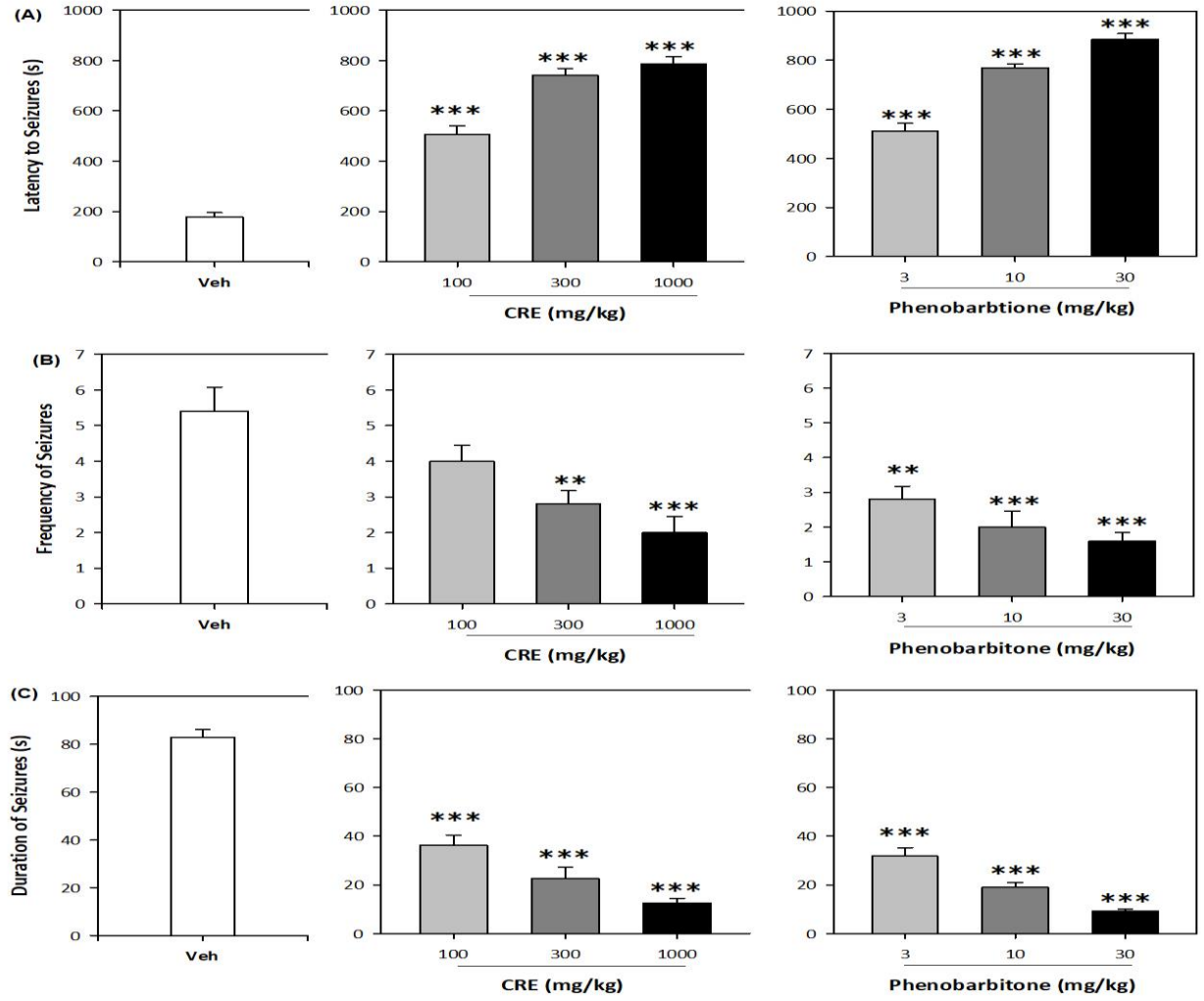

Figure 4- 12: Effects of CRE (100-1000 mg kg^{-1}) and Phenobarbitone (PHB) (3-30 mg kg^{-1}) on the latencies to myoclonic seizures (a), frequencies of seizures (b) and the duration of myoclonic seizures(c) induced by PTZ. Each column represents the mean ± S.E.M. n=5. **P<0.01, ***P< 0.001 compared to vehicle-treated group (One-way ANOVA followed by a Dunnett's multiple comparison test).

4.6.2 Picrotoxin- Induced Seizure Test

The administration of picrotoxin induced tonic-clonic convulsive episodes that were preceded by myoclonic jerks. The extract exhibited significant anticonvulsant effect against the PIC- induced seizures. CRE significantly delayed the latencies to tonic-clonic seizures induced by picrotoxin ($P< 0.0001$, $F_{(3, 16)} = 16.93$, Figure 4-13 A) and also dose-dependently reduced the duration of seizures significantly ($P< 0.0001$, $F_{(3, 16)} = 19.22$, Figure 4-13 C). CRE significantly and dose-dependently reduced the frequency of seizures at 1000 mg kg^{-1} ($P<0.0001$, $F_{(3, 16)} = 18.08$ Figure 4-13 B). Phenobarbitone significantly delayed the latency to tonic-clonic seizures induced by picrotoxin at 30 mg kg^{-1} ($P=0.0081$, $F_{(3, 16)} =5.585$, Figure 4-13 A). Phenobarbitone significantly and dose-dependently reduced the frequency of tonic-clonic seizures ($P=0.0003$, $F_{(3, 16)} = 15.19$, Figure 4-13 B) and the duration of the PIC-induced seizures ($P=0.0002$, $F_{(3, 16)} = 14.70$, Figure 4-13 C). The anticonvulsant effects of CRE was less compared to phenobarbitone as shown in the ED_{50} values (Table 4-5).

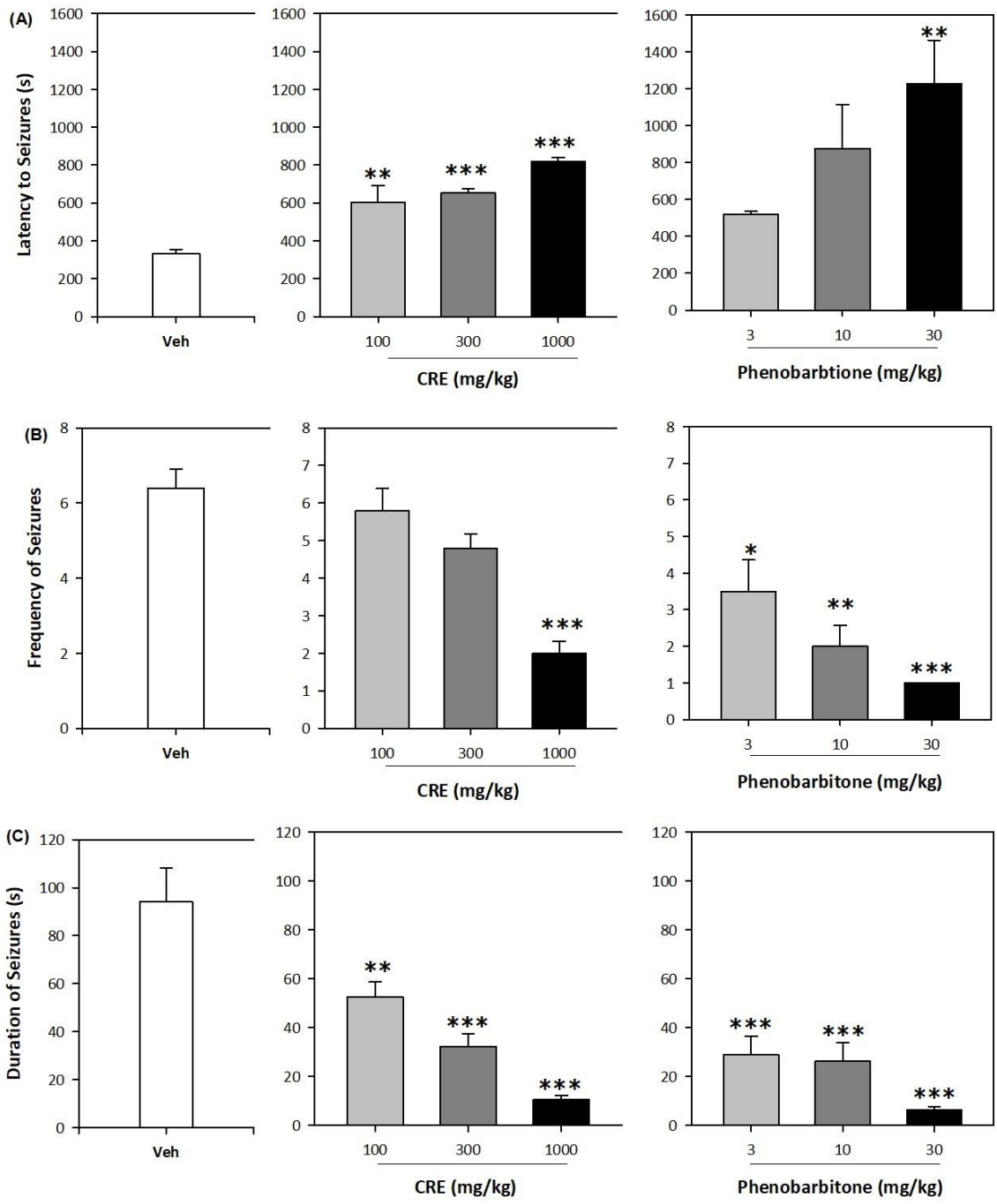

Figure 4- 13: Effects of CRE (100-1000 mg kg^{-1}) and Phenobarbitone (PHB) (3-30 mg kg^{-1}) on the (A) latencies to tonic-clonic seizures, (B) frequencies of seizures and (C) the duration of myoclonic seizures induced by PIC. Each column represents the mean ± S.E.M. n=5. *P< 0.05, **P< 0.01, ***P< 0.001 compared to vehicle-treated group (One-way ANOVA followed by a Dunnett's multiple comparison test).

4.6.3 Maximal Electroshock Seizure Test

Maximal electroshock current of 60 mA at a frequency of 80 Hz for 0.2 s induced hind limb extensions in all the vehicle-treated mice. CRE, dose-dependently delayed the latency to hind limb tonic extensions (HLTE) but this was not significant ($P= 0.3257$, $F_{(3, 35)} = 1.195$, Figure 4-14 A). The extract similarly, though reduced the duration of hind limb tonic extensions induced by the electroshock did not reach statistical significance ($P= 0.6446$, $F_{(3, 35)} = 0.5605$, Figure 4-14 B). The reference anticonvulsant, carbamazepine significantly ($P<0.0001$, $F_{(3, 19)} = 26.54$, Figure 4-14 A) caused an increase in latency to tonic hind limb convulsions and likewise, significantly ($P<0.0001$, $F_{(3, 33)} = 253.0$, Figure 4-14 B) reduced the duration of tonic convulsions at all doses.

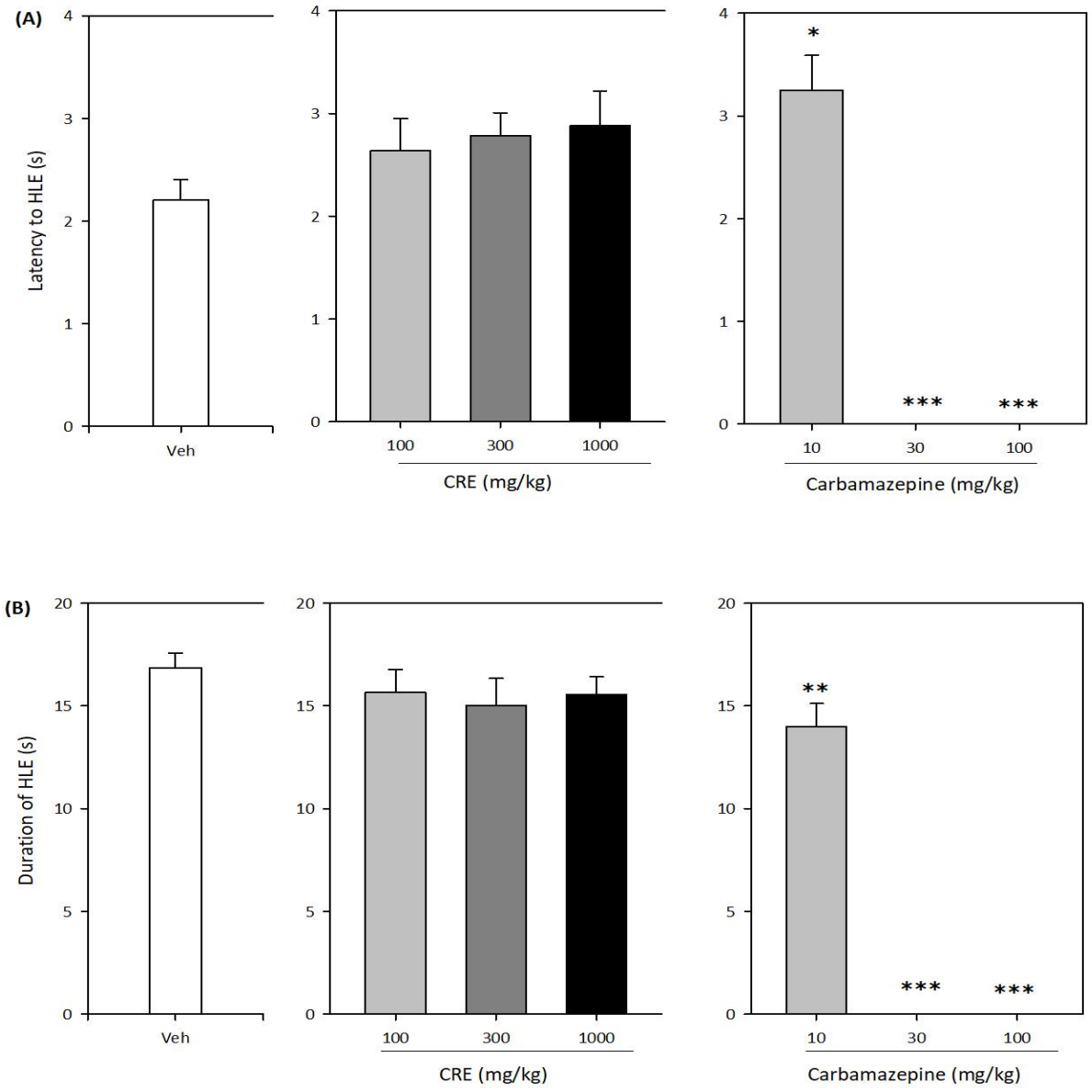

Figure 4- 14: Effects of CRE (100-1000 mg kg^{-1}) and carbamazepine (10-100 mg kg^{-1}) on (A) the latency and (B) duration of hind limb tonic convulsions in maximal electroshock- induced seizures. Data are represented as mean ± S.E.M. (n=10). *P< 0.05, **P< 0.01, ***P< 0.001 compared to vehicle-treated group (One-way ANOVA followed by a Dunnett's multiple comparison test).

Table 4- 4: Percentage presence of hind limb tonic extensions and mortality in MES- test.

	HLTE	
TREATMENT	% PRESENT	% MORTALITY
Vehicle	100	40
CRE 100mg/kg	100	30
CRE 300mg/kg	90	20
CRE 1000mg/kg	100	20
Carbamazepine 10mg/kg	70	0
Carbamazepine 30mg/kg	0	0
Carbamazepine 100mg/kg	0	0

4.7 PTZ-KINDLING SEIZURE TEST

The repeated administration of 35 mg kg^{-1} of PTZ on alternate days resulted in a gradual escalation in convulsant responses in the vehicle-treated group in the Racine score. The score had increased from 0 to 3 by the 13th day, reaching a peak severity on the Racine score of 5 by the 28th day which was sustained for the remaining period of the study. The extract significantly suppressed the kindled seizure at all dose levels used ($P=0.0002$, $F_{(3, 64)} = 7.683$, Figure 4-15 A), but this effect was statistically significant at both 300 and 1000 mg kg^{-1} as none of the animals in these treatment groups attained seizure score 5 even after 16 injections of PTZ for 32 days. The percentage severity of seizures as calculated from the area under curve (AUC), shows that CRE diminished PTZ kindling seizure activity by reducing the five scaled score from 60 % to 30 % at 100-1000 mg kg^{-1} (Figure 4-15 B). Phenobarbitone exhibited a significant dose-dependent suppression of the kindling seizure activity ($P<0.0001$, $F_{(3, 64)} = 12.47$, Figure 4-16 A) and the percentage severity of seizures was significantly reduced from 55 % for (3-30 mg kg^{-1}) to 20 % (Figure 4-16 B).

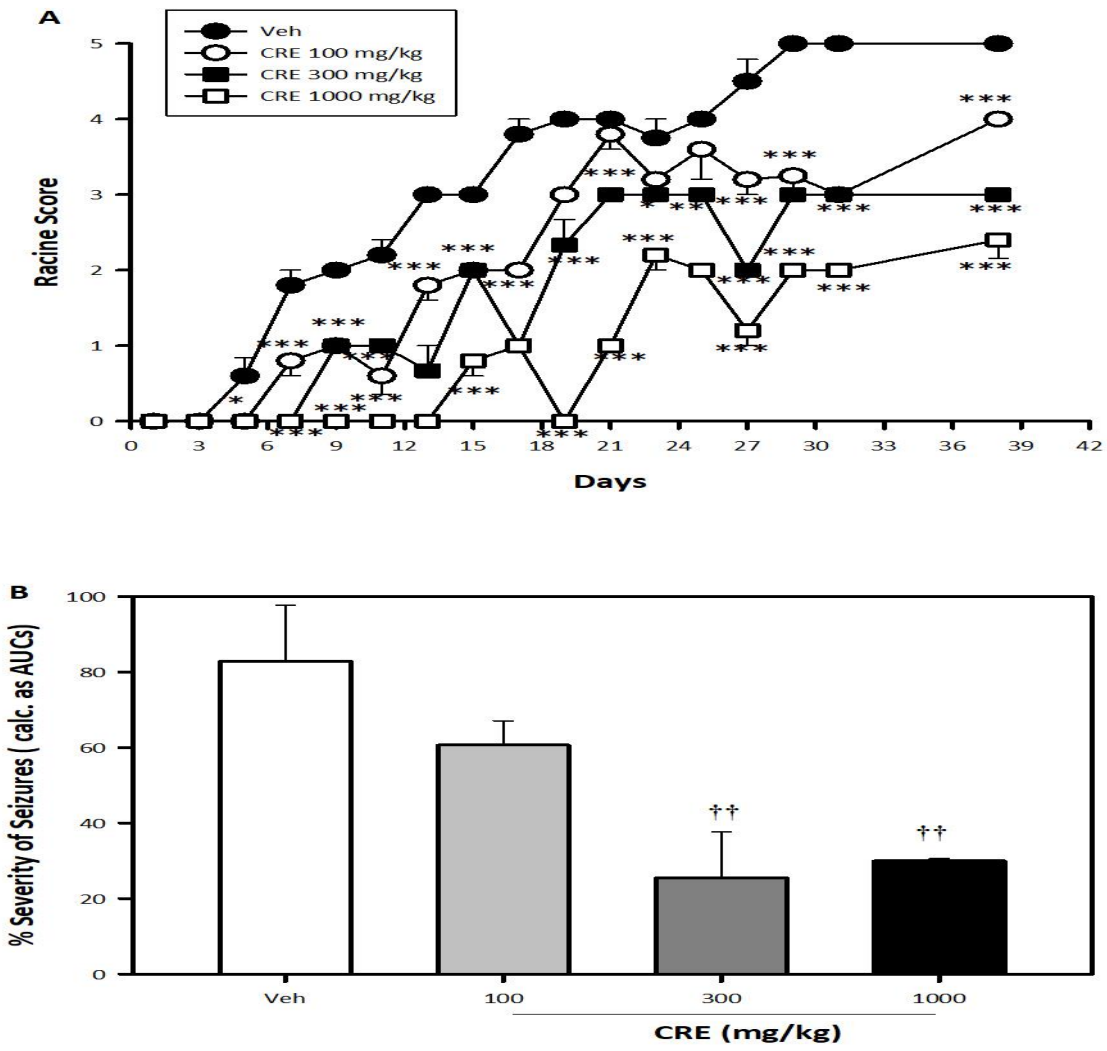

Figure 4- 15: The dose-response effects of CRE (100-1000 mg kg^{-1}) on the PTZ- induced kindling in mice. Graph (A) shows the time course effects over the 32-day period and (B) shows the percentage severity of seizure calculated from the AUCs for the duration of the test. Data are represented by Mean ± S.E.M. (n=5). *P< 0.05, **P< 0.01, ***P< 0.001 compared to vehicle-treated group (Two-way ANOVA followed by Bonferroni's *post hoc* test). †P< 0.05, ††P< 0.01, †††P< 0.001 compared to vehicle-treated group (One-way ANOVA followed by a Dunnett's multiple comparison test).

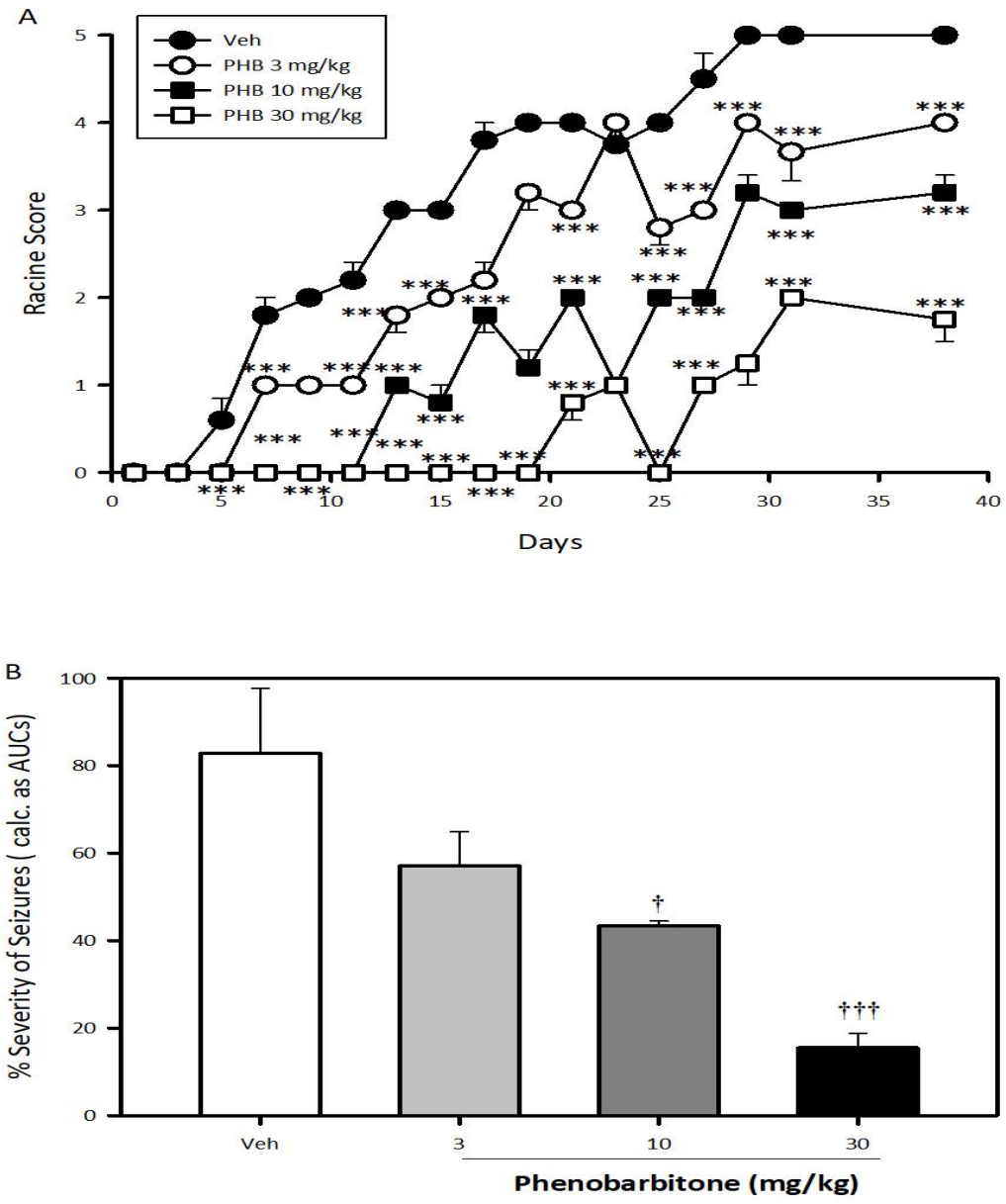

Figure 4- 16: The dose-response effects of PHB (3-30 mg kg^{-1}) on the PTZ- induced kindling in mice. Graph (A) shows the time course effects over the 32-day period and (B) shows the percentage severity of seizure calculated from the AUCs for the duration of the test. Data are represented by Mean ± S.E.M. (n=5). *P< 0.05, **P< 0.01, ***P< 0.001 compared to vehicle-treated group (Two-way ANOVA followed by Bonferroni's *post hoc* test). †P< 0.05, ††P< 0.01, †††P< 0.001 compared to vehicle-treated group (One-way ANOVA followed by a Dunnett's multiple comparison test).

Table 4- 5: ED_{50} (mg kg-^{1}) of CRE and Phenobarbitone in Pentylenetetrazole (PTZ)-, Picrotoxin (PIC) - induced seizures and PTZ-kindling in murine models of seizure.

TREATMENT	**ED_{50} (mg kg^{-1})**					
	PTZ			**PICROTOXIN**		**PTZ-KINDLING**
	Latency	Frequency	Duration	Frequency	Duration	
CRE	3605 ± 0.2232	407.2 ± 0.1285	67.57 ± 0.1437	597.9 ± 0.06652	134.2 ± 0.07432	134.5 ± 0.1918
PHENOBARBITONE	45.94 ± 0.08251	3.327 ± 0.2401	1.503 ± 0.1119	2.261 ± 0.2256	1.041 ± 0.3556	0.3151 ± 0.9021

Chapter 5

DISCUSSION AND CONCLUSION

5.1 DISCUSSION

The result of this study provides evidence that the hydro-ethanolic extract of the whole plant of *Cleome rutidosperma* has anticonvulsant and possible sedative effects in murine models of experimental epilepsy. The effectiveness of the extract in the seizure models used suggests that the herb can be used in *petit mal* type of epilepsy.

The pharmacological effects of plants are dependent on the presence of one or more of their secondary metabolites. This action of the secondary metabolites may be additive or synergistic in nature and may act on a single or multiple target sites associated with a physiological process (Briskin, 2000; Wink, 2010). Preliminary phytochemical analysis of the extract of *Cleome rutidosperma* revealed the presence of flavonoids, alkaloids, glycosides, phytosterols and saponins. The effects of these secondary metabolites may either be beneficial or deleterious to life.

The Irwin test evaluates the qualitative effects of a test substance on the behaviour and physiological function of experimental animals, from the first dose that produces observable effects up to doses that generate distinct behavioural toxicity or even death (Irwin, 1968; Lynch III *et al.*, 2011). The test also allows for the estimation of the duration of action of the test substance on the different outcome parameters (Irwin, 1968; Vogel *et al.*, 2006). In this study, the extract caused sedation, a decrease in response to external stimulus and abnormal gait indicative of possible central nervous system (CNS) depressant activity. The CNS depressant effect induced by the extract suggests GABAergic or serotonergic mechanisms (Trevor, 2012). There was no impairment of respiration. Data from the Irwin test were used to predict potential biological effects, select doses for subsequent efficacy tests, assess the risk associated with the use of the extract. The

effects of the extract in the Irwin test may suggest utility in conditions involving CNS excitation such as convulsions, epilepsy or anxiety.

The sedative effect of the extract observed in the Irwin test was substantiated by the thiopental-induced sleep time test. This test demonstrated that the extract possessed sedative properties (Marder & Paladini, 2002). Thiopental is a hypnotic agent and causes hypnosis by potentiating GABA-mediated postsynaptic inhibition through allosteric modification of GABA receptors (Hasan, 2009; Löscher & Rogawski, 2012). Substances with CNS depressant activity either decrease the onset or prolong the duration of sleep or both (Shilpi *et al.*, 2004; Nyeem *et al.*, 2006; Hasan, 2009). The most abundant inhibitory receptor system in the CNS are the GABA-benzodiazepine receptors and binding of a benzodiazepine agonist to its binding site leads to an increase in chloride ion flux (Trofimiuk *et al.*, 2005; Sieghart, 2006) culminating in hyperpolarization of the postsynaptic membrane at a lower threshold of spike generation. Diazepam, the reference drug, also induced sedation. Diazepam is a GABA-benzodiazepine receptor agonist and therefore will produce sedation in patient (Bourin, 2018; Sigel & Ernst, 2018). The secondary metabolites especially, flavonoids individually or in combination with other phytochemicals might be responsible for the sedative effect exhibited by the extract. Flavonoids have been found to be ligands for the $GABA_A$ receptors in the CNS, thus the hypothesis that they act like benzodiazepine molecules (Hanrahan *et al.*, 2011; Citraro *et al.*, 2016; Silva *et al.*, 2019). It is possible the flavonoids as detected in the extract may be responsible for its sedative effects (Trofimiuk *et al.*, 2005; Ferdousy *et al.*, 2017). However, further investigations are needed to confirm this assertion.

Cleome rutidosperma seem to be generally nontoxic and thus posed no notable health risk at doses less than 3000 mg kg^{-1}. The extract in a 48-h acute toxicity study caused no mortality in the mice,

thus LD_{50} was estimated to be more than 3000 mg kg^{-1}. This implies CRE is relatively safe with respect to the therapeutic dose range used in the acute toxicity study. All the doses of CRE (10, 30, 100, 300, 100 and 3000 mg kg^{-1}) used in the study produced no observable anomaly in the salivation, movement, mydriasis, piloerection, respiratory pattern and frequency and consistency of stool of mice compared with the vehicle treated group within the first 48 h. The absence of any statistically significant changes in the absolute weights and the relative organ to body weight ratio and the morphological changes in the harvested organs of the mice that were treated with CRE further supports the safety profile of the extract. Moreover, the leaves of the plant is eaten as food (Burkill, 1985) yet there have not been any reported or documented adverse effects, it could be said that the plant is less toxic to humans. The highest dose of CRE 3000 mg kg^{-1} administered resulted in hepatocellular oedema and renal erythrocyte infiltration respectively. Though it is unclear how this resulted, it is important for the traditional healer to exercise caution when using this plant on patients with kidney and liver diseases. While relative organ weight sometimes gives useful indication to identify a target organ, histopathology which is supposed to be the gold standard in addition, is useful for identifying a treatment related effect to an organ (Nirogi *et al.*, 2014).

Sub-acute toxicity studies are conducted to obtain information on the toxicity of a substance after repeated administration and serves to establish the doses for sub-chronic studies. The reason for gradual decline in weight for both treatment groups is not known but the fact that it happened in all treatment groups suggests that the effect is not related to the treatment but to other causes yet to be investigated. Changes in the morphological examination of the harvested organs and the relative organ to body ratio were not also significant. Organ weight is one of the most delicate indicators of an effect of a test substance, because prominent discrepancies in organ weight may

occur between treated and untreated (control) animals even in the absence of any morphological changes (Stevens, 1982; Bailey *et al.*, 2004). This was the preliminary indication of the safety of the extract. The histopathological examination revealed renal epithelial sloughing in the CRE treated mice, thus caution must be exercised by traditional healers when using this plant on patients with kidney diseases. The heart and brain were normal morphologically and histopathologically. However, the histopathology of the liver and kidneys showed hepatocellular oedema and renal erythrocyte infiltration respectively.

Pentylenetetrazole-induced seizure test is one of the conventional core battery CNS studies models that is used in anticonvulsant screening and classification of test compounds. The PTZ- induced seizure test is representative of human generalized and absence seizures (Loscher & Schmidt, 1988; Kasthuri *et al.*, 2013). PTZ, a GABA antagonist (Shimada & Yamagata, 2018), induces acute seizures in high doses (60-100 mg kg^{-1}) and chronic convulsions at low dose range (20-40 mg kg^{-1}) (Hellier & Dudek, 2005). Pentylenetetrazole initially induces myoclonic jerks which is followed by tonic- clonic seizures in doses of 60- 90 mg kg^{-1} (De Deyn *et al.*, 1992; Shimada & Yamagata, 2018). It has been reported that PTZ induces seizures by inhibiting GABA neurotransmission (De Sarro *et al.*, 2003; Shimada & Yamagata, 2018). The potentiation of GABAergic activity is known to inhibit seizures whereas the inhibition of GABAergic neurotransmission promotes and facilitates seizures (Engelborghs *et al.*, 2000; Avoli & Krnjević, 2016). Anticonvulsants such as diazepam and phenobarbitone inhibit PTZ-induced seizures by enhancing the action of $GABA_A$ receptors thus facilitating the GABA-mediated opening of chloride ion channel (Twyman *et al.*, 1989; Gale, 1992; Kasthuri *et al.*, 2013; Kobayashi *et al.*, 2019). Since CRE significantly increased the latency to seizure, reduced the frequency and duration of PTZ-induced seizures, it suggests its ability to raise seizure threshold and could be

used in the treatment of absence seizures in humans. The results from this study seem to give credence to the folkloric use of *Cleome rutidosperma* as a remedy for neurological disorders including epileptic seizures. The inhibition of PTZ induced seizures by CRE may suggest that the extract is likely to produce this effect by enhancing GABAergic activity though it could also do so by depressing glutamate mediated excitation.

Picrotoxin is a $GABA_A$ receptor antagonist (Xu *et al.*, 2018) that produces seizures by blocking the chloride ion channel linked to the $GABA_A$ receptor which prevents the entry of chloride ions into the neurons (Kasthuri *et al.*, 2013; Afrin *et al.*, 2017). This action of PIC leads to a decrease in GABA neurotransmission and activity in the brain. Therefore seizures that arise from PIC are due to a decrease in $GABA_A$ receptor mediated inhibition which tips in favour of the glutamate-mediated excitatory neurotransmission (Gale, 1992; Afrin *et al.*, 2017; Xu *et al.*, 2018). In this experiment, the administration of 3.5 mg kg^{-1} PIC to the mice produced hyperactivity, tremor, fore limb clonus, tonic extension of the hind limb and generalised tonic- clonic seizure. Phenobarbitone (the reference anticonvulsant) is known to enhance GABAergic neurotransmission by increasing chloride ion influx through the chloride ion channel of the $GABA_A$ receptors (Kasthuri *et al.*, 2013; Kobayashi *et al.*, 2019). The ability of the extract to reduce the duration and frequency of seizure and increase the latency to PIC-induced seizures significantly may suggest that CRE might have enhanced GABA related activity. Also, because CRE imitated to a larger extent the anticonvulsant actions of phenobarbitone, it could imply that CRE antagonizes PIC – induced seizures by opening the chloride ion channels associated with $GABA_A$ receptors. More so, the extract could have suppressed the glutamate mediated excitation but this has to be verified by investigating the effect of CRE on a pure glutamate- mediated excitatory postsynaptic responses in the brain. Further

studies would have to be done to determine the exact mechanism of action by which CRE attenuates seizures.

The maximal electroshock test is also one of the basic anticonvulsant screening models that suggests a substance's ability to inhibit the spread of seizure from its epileptic focus in the brain (Holmes, 2007; Sarma & Bhattacharyya, 2014). Test substances effective in the MES seizure test tend to protect against partial seizures and generalized tonic clonic seizures (GTCS) (White, 2003; Kasthuri *et al.*, 2013; Jagannatha, 2015). Examples of antiepileptic drugs that are able to limit the spread seizure from the epileptic focus include; carbamazepine, phenytoin, valproate, lamotrigine and topiramate (Kasthuri *et al.*, 2013; Tripathi, 2013; Afrin *et al.*, 2017). Carbamazepine (the reference anticonvulsant) significantly increased the latency to hind limb tonic extension (HLTE) and reduced the duration of HLTE. Carbamazepine is known to stabilize neuronal membranes by prolonging the inactivated state of voltage sensitive sodium ion channel and preventing high frequency discharges (Rogawski *et al.*, 2015; Gierbolini *et al.*, 2016; Petty *et al.*, 2016). The extract on the other hand, did not significantly affect the latency and duration of HLTE suggesting CRE may not have the ability to attenuate seizure discharge and spread from the brain stem. CRE did not show significant protection against MES induced seizures hence the extract may be ineffective in GTCS occurring in grand mal epilepsy.

The PTZ- induced kindling model is an acknowledged model for the study of chronic epilepsy and refers to the repeated administration of an initially sub convulsive dose of PTZ that results in an increase in seizure activity, culminating generalized tonic clonic seizures (Girgis, 1981; Kasthuri *et al.*, 2013; Shimada & Yamagata, 2018). The kindling models has been widely accepted as a means to study seizure mechanisms and useful experimental model for human epilepsy (Mason & Cooper, 1972; Löscher & Brandt, 2010; Shimada & Yamagata, 2018). Pentylenetetrazole-induced

kindling is associated with cognitive inconsistency, changes in emotional behaviour and neuronal loss in the hippocampus (Pavlova *et al.*, 2006). Though the exact mechanism of PTZ is not known in detail, records show that it causes modifications in GABAergic systems, glutamatergic systems and antioxidant defence systems (Erkeç & Arihan, 2015). The repeated administration of PTZ resulted in kindling that may be caused by permanent attenuation of inhibitory function of GABAergic systems in the brain (Corda *et al.*, 1991). Thus, the repetitive single dose administration ended up in a decrease in GABAergic activity. Also, PTZ is known to cause changes in the density and sensitivity of different glutamate receptor sub types (Schroeder *et al.*, 1998; Cremer *et al.*, 2009; Erkeç & Arihan, 2015) in many parts of the brain and increase in the density of glutamate neurotransmitter at the hippocampal region (Li *et al.*, 2004; Erkeç & Arihan, 2015). The increase in the glutamate mediated activity and glutamate receptor density during PTZ kindling tilts the balance in favour of excitation leading to seizures. The changes in molecular expression of glutamate transporters in the kindling process may trigger the development of epileptogenesis (Doi *et al.*, 2009). In the current study, CRE was able to reduce the maximal effects of PTZ kindling in the mice used and this might have slowed the progression of epileptogenesis by enhancing GABA related activity. Therefore, the ability of the extract to prevent the CRE-treated mice from becoming fully kindled suggests that it could prevent normal brain neurons from transitioning into epileptic neurons through a gradual process in which neurons become hyper-excitable and tend to instigate impulses in a hypersynchronous manner.

5.2 CONCLUSION

The hydro-ethanolic extract of the whole plant of *Cleome rutidosperma* possesses anticonvulsant and sedative effects in mice. The extract was also less toxic in the acute and sub-acute toxicity investigations in mice, with an LD_{50} greater than 3000 mg kg^{-1}. This study gives credence to the use of the plant as a remedy for epilepsy in traditional medicine.

RECOMMENDATIONS

The following are recommended:

1. The active constituent(s) responsible for the neuro pharmacological effects detected should be isolated and classified and studied further.
2. A herbal product to be used as an adjunct in epilepsy management should be formulated from the extract.
3. Investigate exact mechanisms of action of CRE in convulsions / epilepsy.

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