

**ANTICONVULSANT AND SUB-CHRONIC TOXICITY STUDIES OF THE
METHANOL LEAF EXTRACT OF *DIOSPYROS MESPILIFORMIS* HOCHST
(EBENACEAE) IN LABORATORY ANIMALS**

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NIGERIA**

FEBRUARY 2014

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FEBRUARY, 2014

DECLARATION

I declare that the work in this thesis entitled “**Anticonvulsant and Sub-chronic toxicity Studies of the methanol leaf extract of *Diospyros mespiliformis* Hochst (Ebenaceae) in laboratory animals**” has been carried out by me in the Department of Pharmacology and Therapeutics. The information derived from the literature has been duly acknowledged in the text and a list of references provided. No part of this thesis was previously presented for another degree or diploma at this or any other institution.

Name of Student

Signature

Date

CERTIFICATION

This thesis entitled “**ANTICONVULSANT AND SUB-CHRONIC TOXICITY STUDIES OF THE METHANOL LEAF EXTRACT OF *DIOSPYROS MESPILIFORMIS* HOCHST (EBENACEAE) IN LABORATORY ANIMALS**” by Kasimu MUHAMMAD meets the Regulations governing the award of the Degree of Master of Science (Pharmacology) of the Ahmadu Bello University and is approved for its contribution to knowledge and literary presentation.

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DEDICATION

This thesis is dedicated to my late father Alhaji Muhammad Ibrahim who died on 5th March 2009 (9th Rabiul Awwal, 1430AH) may Allah (SWT) reward him with Aljannatul firdaus (Amin).

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ABSTRACT

Diospyros mespiliformis hochst (Ebenaceae) known in English as ebony is a plant that is found throughout West Africa. The Plant was reported to have wide ethnomedical application notably in fever, whooping cough, wounds, pneumonia, syphilis, leprosy and epilepsy among others. This study examined the anticonvulsant activity of its methanol leaf extract in mice and day old chicks against pentylenetetrazole, maximal electroshock, strychnine and 4 amino pyridine induced seizure tests. Valproic acid, phenytoin and phenobarbitone respectively were used as reference anticonvulsant drugs for comparison. The sub-chronic toxicity studies was also carried out in rats to determine the effect of twenty eight days p.o.(per oral) administration of the methanol leaf extract of *D.mespiliformis* on renal and hepatic function parameters followed by histopathological examination. The extract at i.p.(intraperitoneal) doses of 50, 100 and 200mg/kg protected the mice (50%, 66.67% and 66.67%) against pentylenetetrazole induced seizures respectively the mean onset of seizure was however not significantly increased when compared with the negative control at $P<0.05$. The extract at i.p doses of 250, 500 and 1000 mg/kg did not produced any significant anticonvulsant activity against maximal electroshock induced seizure, similarly, at i.p doses of 50, 100 and 200 mg/kg, the extract also did not show any significant activity against 4-amino pyridine induced seizure even though it showed only 16.67% protection with the 200mg/kg dose. Also no significant activity against strychnine induced seizure even though it significantly ($P<0.05$) prolonged the onset of seizure induced by strychnine but failed to protect the animals against strychnine induced lethality. The methanol leaf extract of *D.mespiliformis* causes significant elevation of alkaline phosphatase (ALP) at p.o. doses of 50 mg/kg ($P<0.05$), 500mg/kg ($P<0.05$) and 1000 mg/kg ($P<0.001$), the values of Alanine aminotransferase (ALT) and Aspartate aminotransferase (AST), Total bilirubin were however not significantly elevated at p.o doses of 50, 250, 500 and 1000 mg/kg. In the renal function tests, the result showed significantly elevated potassium levels at p.o dose of 250 mg/kg $P<0.05$ and also significant decrease in creatinine values at p.o. doses of 250, 500 and 1000 mg/kg at $P<0.01$ respectively. No significant changes in the values of sodium, total calcium, glucose, total cholesterol, urea, total protein and albumin at $P<0.05$. Histopathological studies on the other hand revealed moderate (with 500 mg/kg p.o dose) to severe (with 1000 mg/kg p.o. dose) degeneration of hepatocytes, hepatocellular necrosis and lose of hepatic architecture with the liver and moderate (with 500 mg/kg p.o. dose) to severe (with 1000 mg/kg p.o dose) loss of renal tubular architecture and degeneration of renal tubules but with intact glomerali with the kidney. The median lethal dose (LD_{50}) values of *D.mespiliformis* methanol leaf extract were found to be 774.6mg/kg i.p. and >5000 mg/kg p.o. in both rats and mice. While in chicks, the value was found to be >5000 mg/kg intraperitoneally. The preliminary phytochemical screening revealed the presence of alkaloids, saponins, flavonoids, tannins, carbohydrates, cardiac glycosides, and combined anthracene type of anthraquinones. These results suggests that *D.mespiliformis* leaf extract posses biologically active phytoconstituents that have anticonvulsant activity and are hepatotoxic and nephrotoxic at higher doses.

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LIST OF ABBREVIATION

ABC:	ATP-Binding Cassette
AEDs:	Antiepileptic Drugs
ALP:	Alkaline phosphatase
ALT:	Alanine Aminotransferase
AMPA:	α -amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid
ANOVA:	Analysis of Variance
AST:	Aspartate Aminotransferase
ATP:	Adenosine Triphosphate
BCG:	Bromocresol Green
BCRP:	Breast Cancer Resistant Protein
Ca ⁺⁺ :	Calcium ions
Cl ⁻ :	Chloride ions
CNS:	Central Nervous System
CRH:	Corticotrophin Releasing Hormone
CSF:	Cerebrospinal fluid
DBS:	Deep Brain Stimulation
DM:	<i>Diospyros mespiliformis</i>
EEG:	Electro Encephalograph
EHC:	Environmental Health Criteria
GABA:	Gamma Amino Butyric Acid
GAT:	GABA transporters
GFR:	Glomerular Filtration Rate
i.p.:	intraperitoneal
ILAE:	International League against epilepsy
K ⁺ :	Potassium ions
KA:	Kainate
LD ₅₀ :	Lethal dose
MES:	Maximal Electroshock
MRP-1:	Multi Resistant Proteins
Na ⁺ :	Sodium ions

NMDA:	N-Methyl-D-Aspartate
nRT:	nucleus Reticularis Thalami
OECD:	Organization for Economic Cooperation and Development
P.O.:	Per oral
PDS:	Paroxysmal Depolarizing Shift
PHB:	Phenobarbitone
PHT:	Phenytoin
PTZ:	Pentylenetetrazole
RNS:	Responsive Neurostimulator System
ScPTZ:	Subcutaneous Pentylenetetrazole
TB:	Total Bilirubin
TCN:	Thalamocortical Neurons
UK:	United Kingdom
USA:	United States of America
VA:	Valproic Acid
VNS:	Vagus Nerve Stimulation
WHO:	World Health Organization

CHAPTER ONE

1.0 INTRODUCTION

The word epilepsy is derived from ancient Greek word '*epilepsia*' literally meaning '*to take hold of*' or '*to seize*' is one of the most common of the serious neurological disorders (Hirtz *et al.*, 2007). It is however, thought to be a common and diverse set of chronic neurological disorders characterized by seizures. There are various definitions of epilepsy but most definitions require that the seizure be recurrent and unprovoked (Chang and Lowenstein, 2003), while others require only a single seizure combined with brain alterations which increases the chances of future seizures (Fisher *et al.*, 2005).

Epileptic seizures therefore can be said to result from abnormal excessive or hypersynchronous neuronal activity in the brain (Fisher *et al.*, 2005). It is estimated that about 50 million people worldwide suffer from epilepsy, including about 2.7 million in the United States (Glauser and Sankar, 2008) and that around 40 million of those affected lives in developing countries (WHO, 2001).

The prevalence of epilepsy in Nigeria based on specifically defined communities, was found to vary from 15 to 37 per 1000 (Reynolds, 1988) which is high compared with the prevalence rates of between 4 and 6 per 1000 which was reported among Caucasians (Hauser *et al.*, 1991). One of the earliest publications on the prevalence of epilepsy in Nigeria reported a prevalence rate of between 8 to 13 per 1000 inhabitants of Lagos (Dada, 1970). However, recently it has been shown that the estimated proportion of the general population with active epilepsy requiring treatment at any given time is between 4 to 10 per 1000 people (WHO,

2012). Studies have shown that epilepsy is more common in the lower socio-economic groups (Danesi *et al.*, 1980) and even in the United States of America, Shomansky and Glaser, 1979 found a higher incidence of epilepsy among blacks who are economically poor. Up to 30% of all seizures are said to be provoked by central nervous system (CNS) disorders or insults (e.g. meningitis, trauma, tumors and exposure to toxins (McAuley and Lott, 2008), studies have shown that epilepsy becomes more common as people age (Holmes and Browne, 2008; Brodie *et al.*, 2009) and that onset of new cases occurs most frequently in infants and the elderly; in other words, the incidence rates are highest in childhood, plateaus from the age of 15 to 65 years and rise again among the elderly (Shovon, 1990; Hauser *et al.*, 1991).

Epilepsy as with other neurological disorders is usually managed but not cured with medications (Antiepileptic drugs AEDs) and that it should be noted that over 30% of people with epilepsy do not have seizure control even with the best available medication hence surgery may be considered in difficult and refractory cases (Cascino, 1994; Engel, 1996). It is also worth noting that currently available antiepileptic drugs (AEDs) are usually associated with serious adverse effects on cognition and behaviour (Samren *et al.*, 1997) and these becomes a problem since the management of epilepsy is usually done on a long term basis. Since a lot of people in developing countries where epilepsy is more prevalent still depend on traditional healing practices and medicinal plants for their daily healthcare needs (Ojewole, 2004), quite a number of such plants with such activities abound that are yet to be scientifically evaluated.

1.1

Statement of Research Problem

Epilepsy, as a chronic neurological disorder is said to affect about fifty million people worldwide and that forty million of those affected are in developing countries Nigeria inclusive (WHO.2008). Major causes includes meningitis, tumours and trauma especially motor traffic accidents where Nigeria and East African countries presents the highest rate of automobile accidents in the world with attendant increases in post traumatic epilepsy (Ogunrin, 2006).

There are quite a number of agents used in the management of epilepsy but inspite of the currently available therapeutic arsenal of old and new anti epileptic drugs, almost one third of epileptic patients continue to present with seizures that appear to be refractory to all pharmacological regimen (Bialer, 2006;Perucca *et al.*, 2007).Also since epilepsy is a chronic disorder requiring long term management,the side effect also becomes a course for concern, 88% of patients in a European study reported at least one anticonvulsant related side effects or the other (Baker, *et al.*,1997).

In recent years, considerable number of reports has been published on hepatotoxicity associated with herbal products (Herera and Bruguera, 2008). Hence the liver and the kidney should be evaluated in any long term toxicity studies.

1.2

Justification of the Study

Anticonvulsants are the main stay drugs in the management of epilepsy. The use of anticonvulsants usually, will be life long and can have major impact on the quality of life

ranging from serious side effects and teratogenicity among others (Samren *et al.*, 1997). This makes the search for newer agents especially from natural sources with fewer side effects, greater efficacy and lower cost imperative.

There are many local medicinal plants that are employed either alone or in combination for the management of epilepsy but with limited scientific evidence from studies done to evaluate their safety and effectiveness (WHO, 2008). This necessitates the need to evaluate the anticonvulsant and toxicity profile of *Diospyros mespiliformis* which will validate its use in the long term management of epilepsy in traditional medicine

1.3 Theoretical Framework

Animal models used to study convulsions experimentally, falls into two main categories; acute seizure models (non-epileptic animals induced to have seizure by an electrical or chemical stimulus) and chronic seizure models (non-epileptic animals induced to have enhanced seizure susceptibility or spontaneous seizures (Rogawski, 2006), for practical purposes, the acute seizure model is the one most commonly employed in experimental research to screen potential new antiepileptic drugs (Engel and Schwartzkroin, 2006; White *et al.*, 2006).

Maximal electroshock (MES) developed in 1946 by Toman and his co-workers (Toman *et al.*, 1946) is an acute seizure model that is electrically induced and is about the best-validated model that predicts drugs effective against generalized tonic-clonic (grand-mal) seizures (Loscher and Schmidt, 1988; White, 2003; Mares and Kubova, 2006) and over the years, it

has remained as the gold standard in the preliminary stages of testing (Rogawski, 2006). Here electrical stimulus of sufficient intensity is applied to mice or rats to induce maximal seizures of their hind limbs with tonic extension as the endpoint of the test (Borowicz *et al.*, 2007), however, day old chicks could also be used.

Secondly, neurotransmitters in the central nervous system can be broadly classified into inhibitory or excitatory. Consequently, either blockade of inhibitory synaptic activity or potentiation of excitatory synaptic activity might trigger a seizure. The neurotransmitters mediating the bulk of synaptic transmission in the mammalian brain are amino acids, with GABA and glutamate being the principal inhibitory and excitatory neurotransmitters respectively. Pharmacological studies showed that antagonists of GABA_A receptors or agonists of different glutamate receptor subtypes (NMDA, AMPA, KA) triggers seizures in experimental animal models of epilepsy, conversely agents that enhance GABA mediated synaptic inhibition or glutamate-receptor antagonists are potential antiepileptic agents. Likewise agents that blocks other inhibitory neurotransmitters e.g. glycine, potentiates other excitatory neurotransmitters e.g. acetylcholine or block potassium or chloride channels can also trigger seizure in experimental animal models. GABA antagonists includes; pentylenetetrazole (Loscher *et al.*, 1991). Bicuculine and picrotoxin (De-Dyne *et al.*, 1992). While Isoniazid inhibits GABA synthesis (Costa *et al.*, 1975). Strychnine antagonizes glycine (an inhibitory neurotransmitter in the central nervous system) (Vogel and Vogel, 2002). Pilocarpine is an agonist of muscarinic cholinceptors (De-Dyne *et al.*, 1992). While 4-aminopyridine blocks k⁺ channel (Seagal *et al.*, 1999).

1.4

Objectives of the Study

The major objective of this study is to scientifically evaluate the methanol leaf extract of *Diospyros mespiliformis* for possible anticonvulsant activity as claimed by traditional medicine practitioners. Furthermore, since convulsive disorders require life long management, to scientifically evaluate the effect of sub-chronic administration of the extract on some vital organs (kidney and liver) of laboratory animals with a view to determine its safety during chronic use which will validate its use in the long term management of epilepsy.

The specific objectives therefore can be summarized thus:

- a. To conduct acute toxicity studies in mice, rats and chicks using oral and intraperitoneal routes in case of mice and rats, and intraperitoneal route in case of chicks
- b. To establish phytochemical constituents of the methanol leaf extract of the Plant
- c. To evaluate the extract for anticonvulsant activity using four models namely;
 - * Pentylenetetrazole induce convulsion in mice
 - * Strychnine induce convulsion in mice
 - * Maximal electroshock induce convulsion in chicks and
 - * 4-aminopyridine induce convulsion in mice
- d. To carry out subchronic toxicity studies on rat's kidney and liver.

1.5

Research Hypothesis

The methanol leaf extract of *Diospyros mespiliformis* has anticonvulsant activity without significant toxicity.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Traditional Medicine

Traditional medicine is the sum total of knowledge, skills and practices based on the theories, beliefs and experiences indigenous to different cultures that are used to maintain health, as well as to prevent, diagnose, improve or treat physical and mental illnesses (WHO, 2008). The clinical successes of quinine and quinidine isolated from the bark of the plant, *Cinchona officinalis* and recently artemisine from *Artemisia annua* in the treatment of malaria have rekindled interest in medicinal plants as potential sources of novel drugs (Igoli *et al.*, 2005), in the same vein, dwindling financial fortunes brought about by global economic recessions coupled with rising cost of orthodox medicines especially in developing countries have caused a shift in emphasis to traditional herbal medicines where effectiveness, minimal side effect profile and relatively low cost are the reasons behind their various applications (Valiathan, 1998).

In some Asian and African countries, up to 80% of the population depends on traditional medicine for primary healthcare (WHO, 2008). It is estimated that the global market for herbal medicines stands at over US \$60 billion annually and is still growing steadily (WHO, 2003). Despite age long, widespread use of plants to treat various ailments, limited efforts have been made to scientifically evaluate potentials of such plants for their use in modern medicine which will therefore justify their use in traditional medicine (Yaro *et al.*, 2007).

2.2

Diospyros mespiliformis

2.2.1 Plant description

Diospyros mespiliformis belongs to the family Ebenaceae; it is a tall upright tree that can reach a height of 30m with over 2m in girth found in moist places of the Guinean and Sudanian woodlands, throughout the West African region and generally widespread in such localities across Africa except in the Congo Basin (Burkill, 1985). It has a dense evergreen canopy; the bark is black to grey, with a rough texture. The inner skin of the bark is reddish; leaves are simple, alternate, leathery and dark green. The flowers are cream coloured and bell shaped and lastly it is said to be suitable specie for re-afforestation (Dalziel, 1937).

2.2.2 Plant taxonomy

Kingdom;	Plantae
Division;	Magnoliophyta
Class;	Magnoliopsida
Order;	Eriocales
Family;	Ebenaceae
Genus;	Diospyros
Specie;	<i>Diospyros mespiliformis</i>

2.2.3 Common and local names:

English:	Ebony
Hausa:	Kanya
Yoruba:	Igi dudu
Igbo:	Akawayi
Nupe:	Buswachi
Fulani:	Balchi
Kanuri:	Bergen 2

2.2.4 Ethnomedical uses

Diospyros mespiliformis has been reported to have wide applications in traditional medicine which include the use of leaf decoction as a remedy for fever, whooping cough and for wounds (Dalziel, 1937; Watt and Brandwijk R, 1962). Bark and roots are used for serious infections such as malaria, pneumonia, syphilis, leprosy and dermatomycoses, as an antihelmintic and to facilitate delivery (Irvine, 1961). In Nigeria, a leaf infusion is taken as a mild laxative and as a vermifuge, for fever, dysentery and is applied to wounds as a haemostatic. The Hausas chew the leaf and fruit or apply an infusion for gingivitis and toothache (Burkill, 1985), and locally in Dembo village, Zaria Kaduna State northern Nigeria, the plant is claimed to be useful in combination with the leaves of *Annona senegalensis* in the management of convulsive disorders (Personal Communication, 17th January 2011).

2.2.5 Non medical uses

The tree is often kept unfelled when land is cleared for farming for its shade and fruits are valued (Burkill, 1985). Ripe fresh fruits are relished especially by children. It is said to be a suitable species for reafforestation (Dalziel, 1937). The juvenile twigs are sometimes used as toothbrushes and the wood is durable and is used to make spoons and canoes. The bark contains a dark coloured gum which is used in Ghana to mend broken pottery (Irvine, 1961). The plant bark is used locally for tool handles, gun-stocks, ploughs, hut-posts and rafters, combs, stools, walking sticks, cudgels, carving and fancy goods and for charcoal for cooking and for smoking fish (Dalziel, 1937; Irvine, 1961).



Plate 2.1: *Diospyros mespiliformis* (Ebenaceae) in its natural habitat, showing the trunk and the leaves

2.3

Epilepsy

2.3.1 History

In ancient times, epilepsy was believed to be a ‘sacred disease (as was described in a 5th century BC treatise by Hippocrates) because people believe that epileptic seizures were as a result of attack by the gods. The believe then was that only gods can deprive a healthy man of his senses, throw him to the ground, convulse him and then rapidly restore him to his former self again (Reynolds, 1988). This perception of epilepsy has continued to this day in some part of the world thereby making epilepsy a dreaded disease with resultant ostracization and stigmatization of patients with epilepsy because the disease is misunderstood (Ogunrin, 2006). For example in Madagascar, patients with epilepsy are refused burial in the family grave yard (Osuntokun, 1978), likewise, in Tanzania to this day, as with other parts of Africa, epilepsy is thought to be associated with possession by evil spirits, witchcraft or poisoning and is believed by many to be contagious (Jilek-Aall, 1999). The stigmatization of epilepsy which exists to this day is decreasing with time, especially in the developed world. The issue remarkably raised by Hippocrates in his famous treatise on the sacred disease was his belief that epilepsy was not sacred, that the brain was the seat of the disease (Ogunrin, 2006). Hippocrates finally remarked, that epilepsy would cease to be considered divine the day it was understood.

2.3.2 Etiology

The most prevalent form of epilepsy in all age groups is the idiopathic epilepsy even though the frequency of symptomatic epilepsy rises progressively with age (Osuntokun, 1978). About 30% of patients with epilepsy in other parts of the world have identifiable neurological or systemic disorder and the remainders have either idiopathic or cryptogenic epilepsy (Chadwick, 1990). In Nigeria, idiopathic epilepsy is said to constitute about 55-60% of all reported cases (Osuntokun, 1978).

Infections; Central nervous system infections are the major cause of symptomatic epilepsy which comprises mainly bacterial and viral meningitis, encephalitis, neurosyphilis, brain abscess and tuberculosis (Ogunrin, 2006). These are infections that are endemic in tropical African regions and accounted for about 10 to 20% of reported cases of epilepsy in Africa (Osuntokun, 1978) The advent of Human immunodeficiency virus (HIV) has worsen the case especially in Africa because it has been shown that HIV, may lead to epilepsy by direct invasion of the brain as in HIV encephalopathy or as a consequence of opportunistic infections such as toxoplasmosis. Another infection is neurocysticercosis which is rarely reported in Nigeria (Ogunrin, 2006) but was said to be responsible for significant proportions of epilepsy in Cameroon and Togo (Zoli *et al.*, 2003). One of the clinical presentations of cerebral malaria secondary to infection by *Plasmodium falcifarum* especially in children is seizure, and a study has shown that 22% of patients with temporal lobe epilepsy had febrile convulsions in childhood from malaria (Dada, 1976).

Trauma; One of the causes of secondary or symptomatic epilepsy is brain damage from pre-natal or peri-natal injuries i.e. loss of oxygen, trauma during birth or low birth weight (WHO, 2012). Trauma and hypoxia are said to be among the commonest causes of epilepsy in Nigeria (Ogunrin, 2006). Poor obstetric care during perinatal period, acts of violence and traffic accidents are said to be contributory factors. Trauma at birth can cause epilepsy secondary to excessive moulding of the scalp and asphyxia with subsequent damaging effects on the hippocampus and amygdala leading to incisural sclerosis (Ogunrin, 2006). It accounts for 1-2% of symptomatic epilepsy in Africa (Osuntokun, 1978).

Nigeria and the East African countries have the highest rate of automobile accidents in the world and consequently post traumatic epilepsy is becoming increasingly common (Ogunrin, 2006). It accounts for between 8% and 12% of symptomatic epilepsies in Nigeria (Obembe and Ahmed, 1988).

Genetics; A number of studies carried out recently indicate that genetically inherited forms of epilepsy make up to about 20% of all patients with epilepsy, especially in children (Gardiner, 1994). It has been known that epilepsy is a component of the phenotype in over 100 Mendelian disorders but the most common genetic epilepsies display a complex non-mendelian pattern of inheritance (Ogunrin, 2006). Examples of these include several well defined syndromes such as juvenile myoclonic epilepsy, absence epilepsy and benign childhood epilepsy with centrotemporal spikes (Ogunrin, 2006). It was also discovered that many of the recessively inherited disorders of lipid and amino acid metabolism may be associated with seizures (Jennings and Bird, 1981). One study showed that a positive family

history for febrile seizures can be elicited in 25% to 40% of patients with afebrile seizure (Hauser *et al.*, 1991) signifying a strong impact that genetics plays in the etiology of epilepsy.

Tumours; Cerebral tumours are other causes of epilepsy in the general population (WHO, 2012) they account for about 3-10% of all symptomatic epilepsies in Africa (Osuntokun, 1978).

Metabolic disorders: Metabolic abnormalities such as pyridoxine deficiencies which are associated with increased glutamic acid and reduced gamma aminobutyric acid (GABA) levels in the brain may lead to seizures (Ogunrin, 2006). Other metabolic causes of epilepsy include amino aciduria, hypoglycemia, hypocalcemia, hypomagnesemia, uremia, alkalosis and water intoxication (ILAE, 1981).

Vascular disorders; Stroke is a vascular disorder that starves brain of oxygen and can be another cause of epilepsy (WHO, 2012). Vascular lesions is responsible for about 6-20% of epilepsy in Africa (Osuntokun, 1978) but lower incidence rate of 3.3% (Obembe and Ahmed 1988) and 1.3% (Danesi, 1985) were obtained among Nigerians living with epilepsy. Seizure occurs in 15% of patients with cerebral infarction and becomes chronic in less than 5%. Also inflammatory vasculitis (e.g Polyarteritis nodosa, lupus erythematosus), subdural hematoma, arteriovenous malformation and intracerebral hemorrhage can presents as seizure (Ogunrin, 2006).

Drugs; There have been reports of generalized seizures in non-epileptic Nigerian subjects with normal EEGs after toxic doses of chloroquine (ILAE, 1994) and more rarely with therapeutic doses (Adamolekun, 1992) and even during prophylactic treatment (Ogunrin, 2006). The exact mechanism of this effect is not clear, however, chloroquine is known to inhibit glutamate dehydrogenase (Ogunrin, 2006).

2.3.3 Pathophysiology of epilepsy

Despite extensive research that results in recent break throughs in the understanding of the mechanisms involved in the pathophysiology of epilepsy, the specific causes of several types of epilepsy are still unknown (Engelborgs *et al.*, 2000).

Normal Neurologic Function; During normal synaptic transmission, neurotransmitters are released into the synaptic cleft in a Ca^{2+} dependent manner and bind to their corresponding receptors. Consequently, synaptic transmission is regulated via neurotransmitter turnover, which invariably occurs via re-uptake into the vesicles and enzymatic degradation. Neuronal axons have a resting membrane potential of about -70mV inside versus outside. Action potentials however, occur due to net positive inward ion fluxes, resulting in local changes in the membrane potential. Membrane potentials vary with the activation of either ligand-or voltage-gated ion channels, which are affected by changes in either the membrane potential or intracellular ion concentrations (Lodish *et al.*, 2000).

Gamma Amino Butyric Acid (GABA);GABA is the principal and predominant inhibitory neurotransmitter in the mammalian brain (Meldrum and Rogawski, 2007) where it is released

at upto 40% of all synapses (Olsen and Avoli, 1997) impairment of GABA function is widely recognized to provoke seizures, whereas potentiation has anticonvulsant effects (Loscher, 1999).

GABA is synthesized from glutamate, exclusively in the GABAergic neurons, by action of the enzyme glutamic acid decarboxylase (GAD) (Loscher, 1999). Upon synaptic release; GABA binds postsynaptically to the ionotropic receptor GABA_A and pre-synaptically to the metabotropic receptor GABA_B (Meldrum and Rogawski, 2007). GABA receptors are distinguished by their pharmacology and function (Johnston, 1996). The GABA_A receptor belongs to the ligand-gated ion channel superfamily, and responds to GABA binding by increasing Cl⁻ conductance resulting in neuronal hyperpolarisation (Rabow *et al.*, 1995) while GABA_B receptors are G-protein linked, activation of which leads to an increase in K⁺ conductance (Olsen and Avoli, 1997).

Following receptor activation, GABA is removed from the synaptic cleft into localized nerve terminals and glial cells, by specific membrane bound transport molecules. Currently, four active transport systems, GABA Transporter (GAT)-1, GAT-2, GAT-3, and Betaine GAT (BGT)-1 have been described (Borden *et al.*, 1992). GABA has a variable affinity for these transporters, and only GAT-1, predominantly located in the cerebral cortex and hippocampus has GABA as its principal substrate (Guastella, 1990). After removal from the synapse, GABA is either recycled to the readily releasable transmitter pool (GABAergic nerve terminals only) or metabolized (neurones and glial cells) to the inactive molecule, succinic acid semialdehyde by the action of mitochondrial enzyme, GABA-transaminase (GABA-T) (Meldrum, 1995).

Many antiepileptic drugs exert their effects, at least in part by actions on the GABAergic System (Kwan *et al.*, 2001). Increased GABA synthesis, increased release, allosteric receptor facilitation and reduced inactivation have all been implicated in the mechanism of action of commonly used antiepileptic agents (Sills *et al.*, 1999).

Glutamate; *Glutamate* is the principal excitatory neurotransmitter in the mammalian brain (Meldrum, 2000). Studies has shown that focal injection of glutamate induces seizures in animals, and over-activation of glutamatergic transmission or abnormal glutamate receptor properties are observed in certain experimental seizure models and human epilepsy syndromes (Meldrum, 1995). Inhibitions of the neuronal release of glutamate and blockade of its receptors have received considerable attention in the search for novel antiepileptic drugs (Meldrum, 2000).

Glutamate is synthesized from glutamine by the action of the enzyme glutaminase in glutamatergic neurones (Daikhin and Yudkoff, 2000). Following synaptic release, glutamate binds to both ionotropic and metabotropic types of receptors (Kwan *et al.*, 2001). Glutamate acts on three classes of ionotropic receptors, namely N-Methyl-D-Aspartate (NMDA), α -amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid (AMPA) and kainate (KA). (Kwan *et al.*, 2001). AMPA receptors are the most abundant followed by NMDA and KA receptors (Meldrum, 2000; Rogawski, 2011).

These receptors contain subunits whose structure affects the biophysical properties of the receptors. AMPA receptors have lower glutamate affinity than NMDA receptors but their

faster kinetic account for the fast initial component of the excitatory postsynaptic potential (Meldrum, 2000). The metabotropic family of glutamate receptors, also classified into three distinct subtypes (Groups I, II and III), are G-protein linked and predominantly presynaptic, possibly controlling neurotransmitter release (Meldrum, 2000). Glutamate is removed from synaptic cleft into nerve terminals and glial cells by the action of several specific transporters (Meldrum *et al.*, 1999). Glial glutamate uptake is of principal importance (Kwan *et al.*, 2001). Glial cells convert glutamate into glutamine by the action of the enzyme glutamine synthetase, the resulting glutamine is then subsequently transferred into glutamatergic neurons completing the cycle (Daikhin and Yudkoff, 2000).

Although, none of the commonly used AEDs exerts their pharmacological effect solely by an action on the glutamate system (Kwan *et al.*, 2001) blockade of ionotropic glutamate receptors is believed to contribute to the antiepileptic activity of several compounds (Meldrum, 1996; White, 1999). Furthermore quite a number of AEDs have been reported to reduce glutamate release (Kwan *et al.*, 2001) although this effect may be more indicative of their action on neuronal Ca^{2+} channels than a direct effect on the glutamate system (Stefani *et al.*, 1997).

Voltage-gated ion channels

a. *Voltage gated sodium channels*; In the nervous system, voltage gated ion channels control the flow of cations across surface and internal cell membranes (Barchi, 1998), of these, the Na^+ channels is arguably of principal importance (Kwan *et al.*, 2001). Voltage dependent Na^+ channels are responsible for the upstroke of the neuronal action potential, and ultimately control the intrinsic excitability of the nervous system (Porter and Rogawski, 1992). The

neuronal Na⁺ channel has a multi-subunit structure that forms a Na⁺ selective, voltage-gated pore through the plasma membrane (Kwan *et al.*, 2001). The protein structure undergoes conformational alterations in response to changes in membrane potential, regulating conductance through the intrinsic pore (Ragsdale and Avoli, 1998). The main structural component of the neuronal Na⁺ channels is the α -subunit, which forms the ion conducting pore and confers voltage dependency (Catterall, 1992). At normal membrane potentials, most Na⁺ channels exist in a closed resting state. Upon depolarization, the channels becomes activated, facilitating ion influx. Thereafter, the Na⁺ channel enters an inactivated state, from which it is not readily reactivated (Kwan *et al.*, 2001). Repolarization of the neuronal membrane rapidly converts the channel back to a resting state, from which it can respond to subsequent depolarizations (Ragsdale and Avoli, 1998). Neuronal Na⁺ channels can cycle through the functional state within a few milliseconds (Kwan *et al.*, 2001). This characteristic is essential for sustaining the rapid bursts of action potentials necessary for some normal brain functions, and is implicated in the production of epileptic discharges (Kwan *et al.*, 2001). The neuronal Na⁺ channel represents one of the most important targets for antiepileptic drugs action (Meldrum, 1996; White, 1999).

b. Voltage gated calcium channels; Voltage gated Ca²⁺ channels share key structural elements and sequence homology with their Na⁺ channel counterparts (Barchi, 1998). The α -subunit of the Ca²⁺ channel is the homologue of the α -subunit of the Na⁺ channel (Kwan *et al.*, 2001). It forms the Ca²⁺ sensitive channel pore and confers voltage dependency (Catterall, 1995).

Voltage-gated Ca^{2+} channels can be broadly grouped into low voltage activated and high voltage activated, according to the membrane potential at which they are activated (Hoffman *et al.*, 1994). The low voltage, T-type Ca^{2+} channel is expressed predominantly in thalamocortical relay neurons, where it is believed to be instrumental in the generation of low threshold spikes that in turn trigger burst firing mediated by Na^+ channels, this burst firing is associated with the synchronicity observed in the thalamus as observed in generalized absence seizures (Coulter *et al.*, 1989).

High voltage activated Ca^{2+} channels are sub-classified by their pharmacological properties into L-, N-, P-, Q-, and R-types (Hofman *et al.*, 1994; Catterall, 1995). These channels are distributed throughout the nervous system on dendrites, cell bodies, and nerve terminals (Kwan *et al.*, 2001), the N-, P-, and Q- type channels in particular, controls presynaptic release of neurotransmitter glutamate (Stefani *et al.*, 1997). Thus blockade of voltage gated Ca^{2+} channels is an important target for antiepileptic drugs (Perez-Reyes, 2003; Meldrum and Rogawski, 2007), also some antiepileptic drugs that inhibit Na^+ channels also block T-type Ca^{2+} channels (Rogawski and Loscher, 2004).

c. Voltage gated potassium channels; Neuronal K^+ channels are large protein complexes that form tetrametric structures, the monomers of which are structurally and genetically related to the α - and α_1 - subunits of the Na^+ and Ca^{2+} channels, respectively (Barchi, 1998). The association of four subunits (monomers) in the neuronal membrane is required for the formation of a K^+ sensitive pore and therefore, channel function (Pongs 1999). More than 40 distinct K^+ channel subunits have been identified, together with several auxiliary subunits (Kwan *et al.*, 2001). Given heterologous arrangement, it is possible that countless populations

of K⁺ channels, with individual functions and distributions, are expressed in the mammalian brain (Pongs, 1999)

At the neuronal level, K⁺ channels are intimately involved in excitability (Kwan *et al.*, 2001) and they are responsible for the action potential downstroke or, more specifically, repolarization of the plasma membrane in the aftermath of Na⁺ channel activation (Pongs, 1999). Direct activation of voltage gated K⁺ Channels hyperpolarizes the neuronal membrane and limits action potential firing (Porter and Rogawski, 1992). Consequently, K⁺ channel activators have anticonvulsant effects in some experimental seizure models (Rostock *et al.*, 1996) whereas, K⁺ channel blockers precipitate seizures (Yamaguchi and Rogawski, 1992).

Potentiation of voltage gated K⁺ channel currents has proven to be an important target for antiepileptic drugs development (Kwan *et al.*, 2001). The recently developed antiepileptic agent, retigabine is believed to exert its effects, at least in part by activation of KCNQ2/KCNQ3 K⁺ channels (Rundfeldt and Netzer, 2000). Mutations in the KCNQ2/KCNQ3 channels have been reported in benign neonatal familial convulsions, a generalized epilepsy syndrome (Rogawski, 2000).

Non-receptor regulatory mechanism in epilepsy

i. GABA transporters; GABA transporters may be reduced in epilepsy as observed in human epileptogenic foci from mesial temporal lobe epilepsy, hence studies have shown that K⁺ induced release of GABA is increased while glutamate induced Ca²⁺ = independent release of GABA is decreased in the epileptogenic hippocampus (Avoli *et al.*, 2005).

ii. Gap junction; During epileptic seizures gap junctions may play an important role in the synchronization of neuronal networks under pathophysiological conditions (Nakase and Naus,

2004). Gap junctions contains protein known as connexins (Hormuzdi *et al.*, 2004) and they allow flow of electrical signals and smaller molecules including dyes between cells, hence promoting neuronal synchrony and anything that enhances the function of or blocks gap junction will increase or decrease epileptiform neuronal synchronization respectively (Carlen *et al.*, 2000).

iii. Neuromodulators; These consist basically of neuropeptide Y and corticotrophin-release hormone

a. Neuropeptide Y; It is a neuromodulatory substance that was found to be of importance in the regulation of neuronal excitability, particularly tuning interneuron discharge propensity (Baraban and Tallent, 2004). Neuropeptide Y can cause a decrease in synaptic transmission by reducing presynaptic calcium influx and consequently, suppress epileptiform activity via Y2 receptor activity (Avoli-Massimo, 2005) also tonically released endogenous neuropeptide may decrease excitability in recurrent mossy fibre projections in a limbic epilepsy model (Tu *et al.*, 2005).

b. *Neuropeptide corticotrophin releasing hormone (CRH);* The most potent epileptogenic peptide is the excitatory neuropeptide CRH, and may play a critical role in the triggering of seizures (Wasterlin and Mazarati, 1997).

2.3.4 *Primary physiologic mechanisms involved in epilepsy*

Epilepsy could be differentiated from seizure by the fact that while epilepsy is a progressive neurologic disorder of the brain, seizures are distinct, transient occurrences caused by abnormal excessive or hypersynchronous neuronal activity in the brain (Fisher *et al.*, 2005). Thus, the pathophysiology underlying the epileptic process includes mechanism involved in the initiation of seizures (ictogenesis), as well as those involved in transforming the normal brain into a seizure-prone brain (epileptogenesis) (Fisher *et al.*, 2005).

Mechanism of ictogenesis; Hyperexcitation is the key factor underlying ictogenesis. Excessive excitation may originate from individual neurons, the neuronal environment or neuronal networks (Engelborgs *et al.*, 2000).

i. Individual neurons; Excitability from individual neurons may arise from structural or functional changes in the postsynaptic membrane; alterations in the type, number and distribution of voltage and ligand-gated ion channels; or biochemical modification of receptors that increase permeability to Ca^{2+} , favouring development of prolonged depolarization that precedes seizures (AES, 2010).

ii. Neuronal environment; Excitability arising from neuronal environment may result from both physiologic and structural changes. Physiologic changes include alterations in concentrations of ions, metabolic alterations, and in neurotransmitter levels. Structural changes affect both neurons and glia. Seizure associated astrocytes reportedly are complex, arborized, highly branched processes with a stellate appearance and with a ratio of Na^+ to K^+ conductance that is 3-4 fold higher than that observed in normal astrocytes. Consequently,

glial K^+ buffering may be affected and may lead to epileptic activity (Bordey and Sontheimer, 1998; Engelborghs *et al.*, 2000). Extracellular Ca^{2+} concentration decreases by over 85% during seizure, preceding the changes in K^+ concentration by milliseconds. However, Ca^{2+} levels returns to normal faster than K^+ levels.

iii. Neuronal networks; Alternations in the neuronal network may facilitate excitability through sprouting of the axons of the granule cells of the dentate gyrus or mossy fibers; or changes in neuronal firing properties due to channelopathies (Engelborghs *et al.*, 2000)

Mechanism of ictal-Interictal Transition and epileptogenesis ;Non synaptic and synaptic mechanisms both affect synchronicity, signal amplification and spread of seizures thereby playing a role during ictal-interictal transition, hence promoting epileptogenesis (Engelborghs *et al.*, 2000).

i. Non synaptic mechanisms; According to Engelborghs *et al.*, (2000) changes in ionic concentrations observed during hyperexcitation i.e. increased extracellular K^+ or decreased extracellular Ca^{2+} , for example- may be caused by decreases in extracellular size or volume, likewise, failure of $Na^+ - K^+$ pumps due to hypoxia or ischaemia is known to promote epileptogenesis in animal models and interference with $Cl^- K^+$ transport, which controls intracellular Cl and regulates GABA-activated inhibitory Cl currents, may lead to enhanced excitation. And that excitability of synaptic terminals depends on the extent of depolarization and the amount of neurotransmitter released. Synchronization following abnormal burst of spikes in the axonal branching of thalamocortical relay cells plays a key role in epileptogenesis. Ephaptic interactions that occur between neighbouring neurons separated by

small extracellular spaces also contribute to increased synchronization. Ephaptic (a Greek verb meaning “to touch”) is the passage of neural impulse from one nerve fibre, axon or dendrite to another through the membrane. This can be a factor in epileptic seizures. (Engelborghs *et al.*, 2000)

ii. Synaptic mechanism; Synaptic pathophysiological process involved in the epileptogenesis of epilepsy and epileptic disorders primarily involves reduced GABAergic inhibition or enhanced glutamatergic excitation (Engelborghs *et al.*, 2000).

a. Reduced GABAergic inhibition; The levels of GABA have been found to be reduced in the cerebrospinal fluid (CSF) of patients with certain kinds of epilepsy, such as infantile spasms and untreated generalized tonic-clonic seizures and also in excised epileptic tissue from patients with drug resistant epilepsy, suggesting that these patients have decreased inhibition (Loscher and Siemes, 1985), similarly, dogs with epilepsy have been shown to have low CSF level of GABA, and mice genetically susceptible to audiogenic seizures have a lower number of GABA receptors than non seizure prone animals. Reduced [³H] – GABA binding to GABA receptors has been reported in human brain tissue, and low glutamic acid decarboxylase levels have been shown in kindled rats and in excised human epileptic tissue, suggestive of decreased GABAergic inhibition (Engelborghs *et al.*, 2000).

b. Enhanced glutamatergic excitation; Hippocampal recordings from conscious human brains have shown sustained increases in the levels of extracellular glutamate levels during and preceding seizures, GABA levels remain low in the epileptogenic hippocampus, but during seizures, GABA concentrations increase, although mostly in the non-epileptogenic

hippocampus. This leads to a toxic increase in extracellular glutamate due to reduce inhibition in the epileptogenic areas (During and Spencer, 1993).

In human hippocampal epilepsy, densities of glutamate AMPA receptor subunits correlated with the locations of the densest aberrant mossy fibers, increases in AMPA receptors in a KA model of epileptic rats preceded mossy fiber ingrowth and demonstrated a greater increases than the increase in pre-synaptic mossy fiber inputs (Babb *et al.*, 1996), KA receptors have also been shown to be involved in ongoing glutamatergic transmission in granule cells of chronic epileptic animals. Thus while the role of NMDA receptors in epilepsy has been known for some time, there is now growing evidence of the role of AMPA and KA receptors in epilepsy (Epsztein *et al.*, 2005).

c. Thalamocortical network excitation ;Epilepsies of the generalized types are characterized by abnormally synchronized activity in large neuronal networks. In absence seizures, the 3-4 Hz spike- and-wave patterns are thought to be the result of high frequency thalamocortical oscillations (Blumenfeld, 2003). Thalamocortical oscillations are generated by the synaptic interplay of 3 structures viz, nucleus reticularis thalami (nRT), thalamocortical neurons (TCNs) and cortical pyramidal neurons. Both nRT and TCNs have an intrinsic ability to fire in bursts when their cell membrane is hyperpolarized, a process that is dependent on extracellular Ca^{2+} and the transient or T-type Ca^{2+} channels. TCNs fire action potentials in high frequency and short duration bursts hence, they play a key role in the pathophysiology of epilepsy (Acharya, 2002).

d. Role of glial cells in excitation ;Despite the fact that substantial interest have been focused on neurones in the pathogenesis of epilepsy, recent studies have shown that glial cells play a key role in buffering functions that maintain the uptake of K^+ and glutamate; disrupting these functions may cause hyperexcitability, also recent evidence suggests that glial cells releases glutamate which can generate paroxysmal depolarizing shift (PDS), the prolonged depolarization reflected in EEG recordings of interictal discharges. Even in the absence of synaptic interactions, astrocytic release of glutamate can trigger PDS-like events (Rogawski, 2005).

2.3.5 Pathophysiology of specific epileptic disorders

The pathogenesis of some epileptic disorders is not fully understood (Engelborghs *et al.*, 2000). Insults to the brain, such as traumatic brain injury, neonatal and adult hypoxia-ischaemia, encephalitis and some degenerative disorders have all been associated with epilepsy and specific mechanisms underlying each of these conditions are unclear, they may serve as triggers to events that leads to structural and functional changes in the brain that can initiate ictogenesis and epileptogenesis (Bialer and White, 2010).

Monogenic mutations; Several familial epilepsies have complex modes of inheritance resulting from interaction of several genetic loci with environmental factors. However, some epileptic disorders, been in only 1% of patients, (Engelborghs *et al.*, 2000), are associated with single gene mutations, many of which have been found in ion channel proteins.

Autoimmune pathogenesis; Rasmussen's encephalitis is a progressive degenerative disease affecting children; patients have seizures that are typically resistant to antiepileptic drugs. It

has been shown that progressive hemiparesis with dementia is characteristic of this rare disease (Acharya, 2002). Subsequently, discovery of anti-GluR3 antibodies suggests that this disease may be the result of autoimmune pathogenesis (Engelborghs *et al.*, 2000).

Epilepsy-associated neuronal migration; Several developmental disorders of neuronal migration, with underlying genetic or intrauterine causes are associated with epilepsy, Agyria or lack of gyri and sulci, and pachygyria (thick convolutions) are commonly associated with abnormalities in neuronal migration. Such cortical malformations including microgyric cortices have been associated with increases in postsynaptic glutamate receptors and decreases in GABA receptors, a condition that can promote epileptogenesis (Engelborghs *et al.*, 2000). Tuberous sclerosis, x-linked lissencephaly, and double cortex syndrome are other examples of developmental disorders associated with epilepsy and disordered neuronal migration (Engelborghs *et al.*, 2000).

2.3.6 Seizure types.

According to Rogers and Cavazos, (2008), seizures, based on EEG recording and clinical symptomatology, are divided into two main pathophysiologic groups; partial and generalized seizures.

Partial epilepsy; Partial (focal) seizures begin in one cerebral hemisphere of the brain resulting in asymmetric motor manifestation (Rogers and Cavazos, 2008), here, clinical manifestation is determined by the cortical area involved (Ogunrin, 2006). For example, seizure arising from the occipital region presents with visual phenomena, those from the precentral gyrus with motor phenomena and those from the post central gyrus with sensory symptoms (Ogunrin, 2006). If the seizure is without loss of consciousness, it is referred to as

simple partial (focal motor or sensory) seizure (McAuley and Lott, 2008), on the other hand, complex partial (psychomotor or temporal lobe) seizure results from the spread of focal discharges to involve a larger area. Consciousness is impaired and patients may exhibit complex but inappropriate behaviour (automatism) such as lip smacking, picking at clothing or aimless wandering (McAuley and Lott, 2008). It has been demonstrated that ninety-five percent of adult patients with complex partial seizures would have automatisms (Ogunrin, 2006).

Studies carried out in Nigeria have shown that simple partial seizures are less common than complex partial seizures (Ahmed and Obembe, 1991 Obembe and Ahmed, 1998) and of the simple partial seizures, those with motor symptomatology are the most common (Ogunrin, 2006). It is however to be noted that the higher proportion of complex partial seizures might probably be due to higher incidence of birth injuries, central nervous system infections and more importantly, recurrent childhood febrile convulsions (Danesi, 1985).

Generalized epilepsy: Generalized seizures have clinical manifestations that indicate involvement of both hemispheres (Rogers and Cavazos, 2008), here, motor manifestations are bilateral with clear loss of consciousness. There are two basic types of generalized tonic-clonic seizures, primary generalized tonic-clonic seizures with bilaterally symmetrical involvement without focal features at onset and secondary generalized tonic clonic seizures which begin focally and then becomes generalized. Identification of secondarily generalized seizure is important because some antiepileptic drugs are more effective in controlling

primary generalized seizure since partial seizures are often more difficult to control (Ogunrin, 2006).

Patient might describe somatosensory symptoms as a ‘warning’ prior to the development of a generalized tonic clonic seizure known as aura (Rogers and Cavazos, 2008) and this aura is strongly indicative of a focal onset (Ogunrin, 2006). Several studies have shown that generalized tonic-clonic seizures constitute the largest subgroup of generalized epilepsies in Nigerian children (Obembe and Ahmed, 1988; Ahmed And Obembe, 1991). It accounted for between 25 and 65 percent of the generalized epilepsies in several series published in Nigeria (Danesi, 1985; Ahmed and Obembe, 1991), though community based studies showed a higher prevalence of partial seizures over generalized, the latter is more prevalent in hospital admissions data (Osuntokun, 1978).

Absence (Petit Mal) seizures occur primarily in children and often remit during puberty (McAuley and Lott, 2008). Its hallmark is suppression of mental functions, usually to the point of complete abolition of awareness, responsiveness and memory (Ogunrin, 2006), its duration is usually less than 10 seconds and rarely more than 45 seconds (Katzung, 2004). Studies have shown that alteration in the circuitry between the thalamus and the cerebral cortex may underlie the mechanism that generates absence seizure (Kostopoulos, 2001).

2.3.7 Epilepsy syndromes

Epilepsy syndromes can be defined on the basis of seizure type as well as cause (if known), precipitating factors, age of onset, characteristic EEG patterns, severity, chronicity family history and prognosis (McAuley and Lott, 2008).

Benign childhood epilepsy with centrotemporal spikes; It is one of the most common epileptic syndromes in childhood and is benign as seizures are infrequent but often responsive to treatment and typically subside in adolescence. It has an optimistic prognosis, though neuropsychiatric testing indicates that cognitive difficulties can exist in areas such as language and memory (Monjauze *et al.*, 2005).

Lennox-Gastaut syndrome; It is less frequent but severe and can consist of multiple types of seizures, developmental delay and a high prevalence of status epilepticus with poor prognosis especially in those with earlier onset (Chevrie and Aicardi, 1972; Roger *et al.*, 1987).

Primary reading epilepsy; It is reflex epilepsy first described by Bickford and co (Bickford *et al.*, 1957). Here reading in susceptible individuals' triggers characteristic seizures (Koutroumanidis *et al.*, 1998).

Dravet syndrome; Previously known as severe myoclonic epilepsy of infancy, is a neurodevelopmental disorder that begins in infancy, characterized by severe epilepsy that is refractory to treatment as first described by French Psychiatrist Charlotte Dravet in 1978 (Dravet, 1978).

Landau-Kleffners Syndrome; Very rare form of epilepsy syndrome with only about 200 reported cases since 1957 (Beaumanoir, 1995). There is abrupt onset of seizures and regression of language skills.

Temporal lobe epilepsy ;This is one of the most common forms of epilepsy that comprises simple partial seizures, complex partial seizure with automatism and secondary generalized seizures occurring in about 50% of patients (McAuley and Lott, 2008). In most cases, the epileptogenic region is found in the midline (mesial) temporal structures (e.g. the hippocampus, amygdala and parahippocampal gyrus).

Rasmussen's encephalitis; This occurs as a result of an immunological attack on glutamate receptors a common neurotransmitter in the brain (Rogers *et al.*, 1994). It affects children and start as simple or complex partial seizures progressing to *epilepsia partialis continua* (a rare form of brain disorder characterized by continuous focal jerking of a body part).

2.3.8 Management of epilepsy

Management of epilepsy usually requires the use of antiepileptic drugs; however, in cases of refractory epilepsy non pharmacological methods can be employed. These non pharmacological methods include surgery, ketogenic diet and implantation of medical devices like those employed for vagus nerve stimulation (VNS).

Antiepileptic drugs (AEDs);Antiepileptic drugs (AEDs) are agents used to manage various types of epilepsy disorders and most AEDs chiefly targets the basic mechanism underlying ictogenesis that is hyperexcitation. The mechanism of action of AEDs may conveniently be grouped into 3 major categories: modulation of voltage-gated ion channels (Na^+ , K^+ , Ca^{2+}), enhancement of GABA-mediated synaptic inhibition and inhibition of (particularly glutamate mediated) synaptic excitation (Rogawski and Loscher, 2004). However, there are some AEDs that act via complex complementary mechanism involving more than one of the above.

i. *Modulation of voltage-gated ion channels* ; Ion channels, Na⁺, K⁺, Ca²⁺ shapes the sub-threshold electrical activity of the neuron, regulate response to synaptic activity and thus contribute to the paroxysmal depolarizing shift (PDS) involved in seizure generation, hence agents that modulates voltage-gated ion channels are potential antiepileptic agents (Rogawski and Loscher, 2004; Meldrum and Rogawski, 2007).

ii. *Enhancement of GABA-mediated synaptic inhibition* ; Several AEDs available today aim to enhance GABAergic inhibition by interacting with fast ionotropic GABA_A receptors or by modifying the activity of enzymes and transporters involved in GABA synthesis or re-uptake (Meldrum and Rogawski, 2007).

iii. *Suppression of (particularly glutamate-mediated) synaptic excitation*; Glutamatergic excitation may be influenced through action on NMDA, AMPA or KA receptors, However, AMPA receptors are the most abundant ionotropic glutamate receptors that mediate synaptic signaling (Rogawski, 2011) .

A. Examples of some antiepileptic drugs

a. *Agents that modulates ion channels*

i. *Phenytoin*; Phenytoin was discovered following a search to identify a non-sedative analogue of phenobarbitone (Merritt and Putnam, 1934). It has become a first line treatment for primary generalized and partial seizures (Brodie and Dichter, 1996; Rogers and Cavazos, 2008).

Phenytoin is believed to exert its anticonvulsant effect primarily by an action on voltage dependent sodium channels (Tunncliffe 1996; Ferraro and Buono, 2005). Phenytoin has also been reported to block high voltage activated Ca²⁺ channels (Schumacher *et al.*, 1998), to

attenuate post-ictal glutamate release (Rowley *et al.*, 1995) and paradoxically to reduce K^+ currents (Nobile and Vercellino, 1997). There is further unsubstantiated evidence suggesting that phenytoin potentiates the action of GABA at specific molecular subtypes of the $GABA_A$ receptors (Granger *et al.*, 1995).

ii. Carbamazepine: Carbamazepine is a compound that is chemically related to the tricyclic antidepressants (Kwan *et al.*, 2001). It was first introduced in 1963 and used in the treatment of partial and generalized tonic-clonic seizures (Brodie and French, 2000). Studies have shown that carbamazepine stabilizes the inactive form of the Na^+ channel in a voltage frequency, and time-dependent fashion (Courtney and Etter, 1983).

Inhibition of glutamatergic neurotransmission has also been implicated in the mechanism of action of carbamazepine (Kwan *et al.*, 2001). Evidence suggests that carbamazepine inhibits the rise in intracellular free Ca^{2+} induced by NMDA and glycine in rat cerebellar granule cells (Hough *et al.*, 1996) and blocks veratrine-induced release of endogenous glutamate (Waldmeir *et al.*, 1995). Unlike phenytoin, there is no evidence that carbamazepine directly interacts with Ca^{2+} channels or potentiates the action of GABA (Kwan *et al.*, 2001).

iii. Lamotrigine: This is a new broad spectrum antiepileptic drug with efficacy for partial, absence, myoclonic and tonic-clonic seizures (Leach and Brodie, 1995) lamotrigine, like phenytoin and carbamazepine inhibits sustained repetitive firing of action potential (Wang *et al.*, 1993) by blocking Na^+ channels in a voltage and use dependent manner (Zona and Avoli, 1997).

The broad spectrum profile of lamotrigine may suggest that its effects on Na⁺ channel may differ from those observed with phenytoin and carbamazepine (Kwan *et al.*, 2001). Unlike phenytoin, lamotrigine acts principally on the slow inactivated state of the channel (Kuo and Lu, 1997). In fact, it has been suggested that Lamotrigine may selectively target Na⁺ channels on neurons that synthesise glutamate and aspartate (Leach *et al.*, 1986).

In addition to Na⁺ channel effects, lamotrigine reduces whole cell Ca²⁺ currents in rat amygdalar neurons, possibly via the N- and P- type channels that have been implicated in neurotransmitter release (Stefani *et al.*, 1997).

iv. Oxcarbazepine ; It is closely related to carbamazepine structurally (Kwan *et al.*, 2001), the keto substitutions at the 10 and 11 positions of the dibenzazepine nucleus do not affect the therapeutic profile of the drug when compared with carbamazepine but result in altered biotransformation and better tolerability (White, 1999). The structural modifications circumvent the 10, 11-epoxide metabolite of carbamazepine that is believed to be responsible for many of its side effects and its ability to induce cytochrome P450 dependent hepatic metabolism (Tecoma, 1999). Oxcarbazepine is a prodrug that is rapidly and completely reduced in the liver to its active metabolite (10, 11-dihydro-10-hydroxycarbamazepine) (Editorial, 1989).

Oxcarbazepine exerts its effects by blockade of voltage dependent Na⁺ channels (McLean *et al.*, 1994); it also reduces presynaptic glutamate release possibly by blocking high voltage activated Ca²⁺ channels (Stefani *et al.*, 1997), whereas carbamazepine may modulate L type Ca²⁺ channels, oxcarbazepine appears to modulate N- and P-type calcium channels (Ambrosio

et al., 1999). Unlike other AEDs oxcarbazepine may in addition increase K^+ channel conductance (McLean *et al.*, 1994).

v. *Ethosuximide*; *Ethiosuximide* exerts its antiabsence seizure effects by inhibiting T-type calcium channels in thalamocortical relay neurones (Coulter *et al.*, 1989; Ferraro and Buono, 2005). These low voltage activated T-type calcium channels predominates in these neurones where it is believed to play a fundamental role in the generation of the characteristic 3-Hz spike-and-wave discharge typical of absence epilepsy (Coulter *et al.*, 1989).

vi. *Zonisamide*; Zonisamide is effective against partial and generalized seizures, and has particular efficacy in the progressive myoclonic epilepsies that are often resistant to AED treatment (Kyllerman and Ben-menachem, 1998). Zonisamide acts by modulation of voltage gated ion channels (Kwan *et al.*, 2001). Like Lamotrigine, Zonisamide enhances slow Na^+ channel inactivation (Schauf, 1987; Welty, 2006) and reduces sustained repetitive firing in spinal cord neurones (Rock *et al.*, 1989). It also blocks low voltage activated T-type calcium channels which may account for its anti absence seizure effects (Suzuki *et al.*, 1992; Welty, 2006). Zonisamide also inhibits carbonic anhydrase (Welty 2006), although this action is believed to be too weak to contribute to its antiepileptic effect (Rho and Sankar, 1999).

b. Agents that potentiates GABA

i. *Phenobarbital*; The mechanism of action of phenobarbital is by allosteric activation of the $GABA_A$ receptor, increasing the duration of chloride channel opening, without affecting the frequency of opening or channel conductance (Macdonald *et al.*, 1989). The barbiturates can also activate the $GABA_A$ receptor directly, in the absence of GABA, an effect that may

account for their sedative properties (White, 1999), additionally, barbiturates can block high voltage activated Ca^{2+} channels (Rogawski and Porter, 1990; Ferraro and Buono, 2005) and also inhibit AMPA/Kainate subtype of glutamate receptors (Davies, 1995; Ferraro and Buono, 2005).

ii. Benzodiazepines; More than 50 chemically distinct benzodiazepines are marketed worldwide (Kwan *et al.*, 2001) but among this only Diazepam, Lorazepam, clobazam and clonazepam are most commonly employed as AEDs (Dichter and Brodie, 1996). They are broad spectrum AEDs efficacious in the management of partial and idiopathic generalized epilepsies (Dichter and Brodie, 1996) and also for acute treatment of status epilepticus (Treiman *et al.*, 1998).

The benzodiazepines bind to the α subunit of the GABA_A receptor (Macdonald and Kelly, 1995) resulting in allosteric activation of the receptor, increasing the frequency of Cl^- channel opening, without affecting open duration or channel conductance (Twyman *et al.*, 1989). Unlike Barbiturates, the benzodiazepines are unable to activate GABA_A receptor in the absence of GABA (White, 1999). Augmentation of GABAergic inhibition in the thalamus can result in the de-inactivation of T-type Ca^{2+} channels, triggering a strong low threshold burst and enhancing development of the thalamocortical rhythmicity that is characteristic of absence seizures (Coulter, 1997).

iii. Vigabatrin; *Vigabatrin* inhibits GABA-T; the enzyme responsible for the catabolism of GABA (Jung *et al.*, 1977), consequently, GABA level is elevated, thereby potentiating

inhibitory neurotransmission throughout the brain (Schechter *et al.*, 1977). It is transformed by GABA-T to an active metabolite which consequently, irreversibly binds to the active site of the enzyme (Lippert *et al.*, 1977). Evidence also exists suggesting that vigabatrin may block the uptake of GABA into glial cells (Leach *et al.*, 1996).

Vigabatrin is approved as adjunctive therapy for partial seizures with or without secondary generalizations (Dichter and Brodie, 1996) there is also evidence of efficacy against infantile spasms (Appleton *et al.*, 1999).

iv. Tiagabine; It is licensed for the adjunctive management of partial seizures with or without secondary generalizations (Leach and Brodie, 1998). Tiagabine inhibits GABA uptake into synaptosomal membranes, neurons and glial cells (Kwan *et al.*, 2001) and it has a greater affinity (2.5-fold) for glial than for neuronal uptake (Braestrup *et al.*, 1990). Tiagabine has a selective action on the GAT-1 GABA transporter, with little or no activity on GAT-2, GAT-3, or BGT-1 (Borden *et al.*, 1994), hence, the pharmacological effects of Tiagabine reflects the regional distribution of GAT-1, and is mainly restricted to the cerebral cortex and hippocampus (Meldrum and Chapman, 1999).

c. Agents with multiple mechanisms of action

I. Sodium Valproate; The antiepileptic properties of valproic acid were discovered serendipitously when valproic acid was employed in animal studies as a solvent for drugs under formal investigation (Meunier *et al.*, 1963). Valproic acid has been reported to block voltage dependent Na⁺ channels (Kwan *et al.*, 2001), it reduces sustained repetitive firing of

mouse neurones in culture (McLean and Macdonald, 1986), inhibits Na⁺ channels in *Xenopus laevis* myelinated neurones (Van Dongen *et al.*, 1986) and reduces Na⁺ currents in neocortical neurones (Zona and Avoli, 1990).

Valproic acid may also block T-type Ca²⁺ channels in a manner similar to that reported for ethosuximide and such an action may account for its efficacy against generalized absence seizures (Kwan *et al.*, 2001). There is also evidence suggesting that valproic acid elevates whole brain GABA levels and potentiates GABA responses possibly by enhancing Glutamic acid decarboxylase enzyme activity and inhibiting GABA degradation (Loscher, 1999). Anecdotal reports suggest that the drug also augments GABA release (Rowley *et al.*, 1995) and blocks GABA uptake (Sills *et al.*, 1996). Other studies showed that valproic acid also decreases brain levels of the excitatory amino acid aspartate, without influencing those of glutamate or GABA (Schechter *et al.*, 1978).

Valproic acid is a broad spectrum antiepileptic efficacious against generalized epilepsies such as myoclonic, atonic, and absence seizures (Rogers and Cavazos, 2008). It can also be used as monotherapy or adjunct in partial seizure or in patient with mixed seizure disorders, also useful in other neurological disorders like migraine headache and bipolar disorders (Rogers and Cavazos, 2008).

ii. Topiramate ; Topiramate has multiple mechanism of action including inhibition of Na⁺ and Ca²⁺ channels, blockade of AMPA/Kainate subtype of glutamate receptors and facilitation of GABA effects at GABA_A receptor (Kwan *et al.*, 2001). Like Zonisamide, it also inhibits carbonic anhydrase though this effect is believed not to contribute to the antiepileptic action

(Shank *et al.*, 1994). Topiramate is active against partial onset and generalized seizures (Brodie and French, 2000).

iii. Felbamate; Felbamate is used as a monotherapy and add-on treatment for partial onset and primary generalized tonic-clonic seizures in adults and for children with Lennox-Gastaut Syndrome (Dichter and Brodie, 1996) though it is associated with aplastic anaemia and hepatotoxicity (Rogers and Cavazos, 2008). Felbamate is the first AED with a direct action on the NMDA subtype of glutamate receptor (Kwan *et al.*, 2001). It inhibits NMDA/glycine stimulated increases in intracellular Ca^{2+} (Taylor *et al.*, 1995) and blocks NMDA receptor-mediated excitatory postsynaptic potentials (Pagliese and Coradetti, 1996). In addition to inhibiting NMDA responses, felbamate also potentiates GABA_A receptor (Rho *et al.*, 1994). Felbamate also reduces voltage dependent Na^+ channels in striated neurones (Pisani *et al.*, 1995) and reduces high voltage activated Ca^{2+} channels (Stefani *et al.*, 1996).

d. Agents with unknown mechanism of action

i. Levetiracetam; *Levetiracetam* is the s-enantiomer of the ethyl-analogue of piracetam, a widely used nootropic agent in the elderly (Loscher and Honack, 1993). As the latest AED, levetiracetam appears to have a unique mechanism of action (Kwan *et al.*, 2001) it does not interact with any of the traditional targets, including Na^+ , Ca^+ and K^+ channels or GABA and glutamate neurotransmitter systems (Noyer *et al.*, 1995), nor does it show any activity in the classic models used to test antiepileptic drugs (Rogers and Cavazos, 2008). However, it is believed to bind to the synaptic vesicle protein SV2A in the brain and this is thought to be important to its activity (Lynch *et al.*, 2004). There is also limited evidence that levetiracetam

may have epileptogenic effects, meaning that it may be able to prevent the development of epilepsy under certain circumstances (Loscher *et al.*, 1998). Clinical studies suggest that levetiracetam is effective against partial seizures with or without secondary generalization (Bialer *et al.*, 1999).

B. AEDS and refractory epilepsy ;Recent studies have made it possible for researchers to propose two major hypotheses that explain the mechanism of antiepileptic drug resistance in epilepsy, these are, the target hypothesis and the transporter hypothesis (Kwan and Brodie, 2006).

i. Target hypothesis; According to the target hypothesis, alterations in cellular or molecular target of an antiepileptic drug resulting in reduced sensitivity to the drug are responsible for drug resistance. Studies has shown that GABA_A receptor subtypes have been altered in patients with uncontrolled temporal lobe epilepsy,however, it is not clear whether the altered receptor structure would itself affect the action of the AED sufficiently to cause drug resistance (Kwan and Brodie, 2006).

ii .Transporter Hypothesis; This hypothesis tries to account for the fact that drug resistance often involves intolerability to multiple drugs with varying mechanism of actions, suggesting that there must be an independent mechanism underlying drug resistance itself (Kwan and Brodie, 2006). An over expression of certain active drug transporters belonging to the ATP-binding cassette (ABC) transporter superfamily has been implicated in drug resistance (Lazarowski *et al.*, 2007). In fact, 22 of the known 48 members of this transporter family are

associated with drug resistance. Of these, the p-glycoprotein, (p-gp), the multi-resistant proteins (MRP1-7), and the breast cancer resistant protein (BCRP) occur in the blood brain barrier and cerebrospinal fluid-brain barrier and drive the flow of their substrates against their concentration gradients resulting in lowered plasma levels of the drug despite adequate administration (Lazarowski *et al.*, 2007). High levels of P-glycoprotein and multi-resistant proteins has been demonstrated in both vascular endothelial cells and brain parenchymal cells of epileptogenic brain specimens of patients with refractory epilepsy (Lazarowski *et al.*, 2007). However, it is not clear, due to lack of control, if this over expression of transporters exist before the onset of epilepsy, or if it is a consequence of the seizure or the treatment (Lazarowski *et al.*, 2007).

2.3.9 Non pharmacological management of epilepsy

Non-pharmacological methods of managing epilepsy involve surgery, ketogenic diet and implantation of medical devices.

i. Surgery; Epilepsy surgery is an option for people with local seizures that have proven to be refractory to treatment with antiepileptic drugs (Duncan *et al.*, 2006). The goal for these procedures is total control of epileptic seizures (Birbeck *et al.*, 2002) although anticonvulsant medication may still be required (Berg *et al.*, 2007). The most common epilepsy surgery is anterior temporal lobectomy for patients with temporal lobe epilepsy and is effective, durable and results in decreased healthcare cost. (Wiebe, 2001; Kelly and Theodore, 2005).

ii. Electrical Stimulation; Electrical stimulation methods of managing epilepsy are currently in use (Theodore and Fisher, 2004). A currently approved device is the vagus nerve stimulation (VNS). This is an electrical stimulator that when implanted in the neck directs intermittent pulses to the vagus nerve. The patient can activate the stimulator magnetically if they feel a seizure is about to begin in order to prevent the seizure or reduce its severity, common side effects such as alterations in voice and tingling sensations are mild to moderate and subside with time (Ben-Menachem, 2002). Approximately, 30 - 40% of patients who are so treated have a 50% reduction in seizures (Schoenenberger, 1995; Tecoma and Iraqui, 2006). Other investigational devices include responsive neurostimulator system (RNS) and deep brain stimulation (DBS).

iii. Ketogenic diet; In the early part of the 1900's, a few people noticed that their children's seizure improved during times of fasting, the ketogenic diet is designed to imitate the chemistry of the fasting state by depriving the brain of sugar (Fisher, 2010). This low carbohydrate high fat diet results in persistent ketosis which raises seizure threshold, thereby making the brain more resistant to seizure and is believed to play a major role in the therapeutic effect (McAuley and Lott, 2008). Ketogenic diet has been used occasionally in the management of refractory epilepsy following the report of Wilder in 1921 (Wilder, 1921) and it is most commonly used and seems to be most beneficial to children (Hassan, 1999; Bainbridge, 1999).

The classical diet is based on an estimated daily requirement of 75 kilocalories per body weight; 50% of calories are given as fat, the remainder as protein and carbohydrates (Ogunrin,

2006). In some centres, fats mainly used are long chain fats (Butter, Cream etc) and medium chain triglycerides (octanoic and decanoic acids). In a study from Oxford, 41% of 57 children with epilepsy showed greater than 90% reduction in seizure frequency within one month of starting the diet, 81% of the children had a greater than 50% reduction in seizure frequency and only 19% showed no benefit (Schwartz *et al.*, 1983). However, such therapeutic option was never reported among Nigerian patients (Ogunrin, 2006).

2.3.10 Plants with antiepileptic activity

Traditional medical practices have remained as a component of health care system of many societies in spite of the availability of well established alternatives (Oyeka, 1981; Ndoye, 2005). But lately focus has shifted to the use of herbal remedies in the management of epileptic seizures, probably because these measures fit into the cultures of people and are not usually as expensive and do not possess many side effects, contraindications and possible interactions with drugs used simultaneously (Hassan *et al.*, 2012). There are so many plants that have been used locally for the management of epilepsy and whose anticonvulsant's properties have been scientifically evaluated and hence their local use justified, some few are listed below:

1. *Securidaca longipedunculata* (Polygalaceae) (Muazu and Kaita, 2008, Adeyemi *et al.*, 2010)
2. *Mitrogyna inermis* (Rubiaceae) Muazu and Kaita, 2008)
- 3 *Annona Senegalensis* (Okoli *et al.*, 2010; Konate *et al.*, 2012)
4. *Chrysanthellum Indicum* (compisitae) (Yaro *et al.*, 2007)
5. *Clausena Anisata* (rutaceae) (Kenechuckwu *et al.*, 2012)

6. *Tacazzea Apiculata* (Periplocaceae) (Ahmed *et al.*, 2009)
7. *Stereospernum Kunthianum* (Bignoniaceae) (Ching *et al.*, 2009)
8. *Ocimum sanctum* (Jaggi *et al.*, 2003). Among others.

2.3.11 Previous studies done on *Diospyros mespiliformis*

Previous studies done on the phytochemistry of the stem barks or wood of *Diospyros mespiliformis* showed the presence of triterpenes, α -amyrin-baurenol, trihydroxy-triterpenoid acid, α -amyrin, β -sitosterol, lupeol, betulin and betulinic acid (Fallas and Thompson, 1968; Khan *et al.*, 1980; Zhong *et al.*, 1984; Lajubutu *et al.*, 1995) and Naphthoquinones such as diospyrin, Isodiospyrin, diosquinone and plumbagin (Fallas and Thompson, 1968; Lajubutu *et al.*, 1995). The cytotoxicity (Lajubutu *et al.*, 1995), antibacterial (Lajubutu *et al.*, 1995; Adeniyi *et al.*, 1996; Sanogo *et al.*, 1998), and antifungal (Adeniyi *et al.*, 1996) activities of the isolated compounds and crude extract of the plant have been reported. The stem bark of the plant in a neuropharmacological studies was found to prolonged onset of the phases of seizure activity induced by pentylenetetrazole (Adzu *et al.*, 2002) and recently, subchronic toxicity studies on the root of the plant showed significant elevation of serum transaminases AST and ALT (Jigam *et al.*, 2012) and lastly extract of the leaves of the plant has been shown to inhibit caffeine induced calcium release from sarcoplasmic reticulum in dose dependent manner which explains its traditional use to treat many diseases (Belemtongri *et al.*, 2006).

2.3.12 Anticonvulsant studies

In experimental anticonvulsant studies, the animal models commonly used employ the use of chemical or electrical stimulus to induce seizure in otherwise healthy, non-epileptic animals—

the so called acute seizure models. Kindling models (e.g. amygdala kindled rat) is a chronic seizure model that is not commonly used especially in early stages of anticonvulsant studies.

a. Maximal electroshock (MES) test; The maximal electroshock test, developed by Toman and collaborators in 1946 (Toman, 1946) and modified by Swinyard and Kupferberg (1985) and Browning (1992), is probably the best validated pre-clinical test that predicts drugs effective against generalized seizures of the tonic-clonic (grand mal) type (Loscher and Schmidt 1988; White, 2003; Mares and Kubova, 2006). It allows evaluation of the ability of an agent to prevent seizure spread through neural tissue of the central nervous system (Swinyard and Kupferberg, 1985). The MES test is simple and can be conducted easily with a minimal investment in equipment and technical expertise and is well standardized (Mares and Kubova, 2006). Several standard and newly developed AEDs are effective in the MES test, hence making it possible to quantify their anticonvulsant potency after both single and combined application (White, 2003, Borowicz *et al.*, 2007). Agents that acts on sodium channels e.g. carbamazepine, phenytoin, oxcarbazepine and lamotrigine are known to suppress hind limb tonic extension induced by maximal electroshock (Rho and Sankar, 1999). Thus the high correlation between suppression of hind limb tonic extension in MES and its effectiveness in human generalized tonic clonic convulsion coupled with the ease with which anticonvulsant activity can be detected in rodents, are probably the main reasons for the popularity of the MES test (Browning and Nelson, 1985).

b. Pentylentetrazole induce seizure test: Pentylentetrazole is a chemoconvulsant agent that induces seizures in experimental animals by the non-competitive inhibition of Gamma Amino Butyric Acid (GABA) receptors and is widely accepted experimental model for absence seizure (Loscher *et al.*, 1991). Pentylentetrazole, a tetrazole derivative is the prototype agent

in the class of systemic convulsants that when administered parenterally has consistent convulsant actions in mice, rats, cats and primates (De Deyn *et al.*, 1992).

Pentylentetrazole was introduced as a screening test for anticonvulsant in part because the antiabsence seizure drug ethosuximide, which is effective against pentylentetrazole induced seizures, fails to alter maximal electroshock (MES) thresholds. In contrast, some drugs effective against MES seizures such as phenytoin and carbamazepine are ineffective against pentylentetrazole induce seizures (De Deyn *et al.*, 1992).

c. *Strychnine- induce seizure test;* Strychnine and related alkaloids such as brucine and thebain induces generalized convulsions by blocking postsynaptic neurotransmission mediated by glycine. Glycine is an important inhibitory neurotransmitter to motor neurones and interneurons in the spinal cord and strychnine acts as a selective, competitive antagonist to block the inhibitory effects of glycine at all glycine receptors (Vogel and Vogel, 2002) and this might explain their epileptogenic nature. Seizures induced by strychnine are different from those induced by GABA antagonists since they are mainly extensor tonic with little cortical EEG activity and are not fully relieved by acceptable doses of any of the classical anticonvulsants including benzodiazepines (De Deyn *et al.*, 1992).

d. *4-Aminopyridine induce seizure test;* 4-aminopyridine owe its convulsant activity by virtue of its voltage-gated, fast potassium channel blockade capable of improving axonal conduction by facilitating the propagation of action potential in de-myelinated nerve fibres (Segal *et al.*,

1999). The use of 4-aminopyridine-induced model was first described by Yamaguchi and Rogawski, 1992. It is a highly lethal agent that induces tonic clonic convulsions.

e. *Picrotoxin induce seizure model*; Picrotoxin is known to be a GABA_A antagonist exerting its effect by binding to the picrotoxin binding site which is closely related to the chloride ionophore in the GABA_A receptor complex. When given to laboratory animals systemically, picrotoxin induces minimal and maximal seizures in a dose dependent manner. In rat doses of 8mg/kg produce hyperactivity, body tremor and forelimb clonus followed by tonic extension of the hindlimbs and generalized tonic clonic seizures (De-Deyn *et al.*, 1992). Thus, picrotoxin induced convulsions are used to further evaluate CNS active compounds (Vogel and Vogel, 2002).

f. *bicuculine induce seizure model*; Bicuculine is believed to exert its epileptogenic effect through blockade of GABAergic neurotransmission by competing with GABA for its binding site. It has been applied focally and systemically. It has been used to induce acute simple focal epilepsy after topical application in the sensorimotor cortex in rats (De Deyn *et al.*, 1992).

g. *isoniazid induced seizure model* ;Isoniazid can precipitate convulsions in patients with seizure disorders (Vogel and Vogel, 2002). It is believed to act by inhibiting GABA synthesis (Costa *et al.*, 1975). Clonic tonic seizures are elicited in mice which are antagonised by anxiolytic drugs (Vogel and Vogel, 2002).

h. *pilocarpine induce seizure model* ;Acetylcholine or its analogues acetylcholinesterase inhibitors and acetylcholine precursors when administered into the brains of experimental

animals results in pronounced seizure activity, intra-amygdaloid, intrahippocampal or systemic. Injections of large doses of muscarinic cholinergic agonists in rats produce electroencephalographic and behavioural limbic seizures; however, while antimuscarinic drugs are effective against epilepsy induced by cholinergic agents or inhibitors of acetylcholinesterase, they do not possess significant anticonvulsant properties in any spontaneous or rather acute model of epilepsy (De Deyn *et al.*, 1992).

2.3.13 Toxicity Studies

Convulsive disorders are usually managed on a long term basis hence the safety of any herbal medicine used for such chronic management becomes a cause for concern. It is known that the liver and the kidney are usually the main organs of drug metabolism and excretion and are easily affected by potentially toxic agents; hence the functions of the liver and kidney should be monitored in any long term toxicity studies (WHO, 2000). Moreso, in the last few years, considerable number of reports have been published on hepatotoxicity associated with herbal products (Herera and Bruguera, 2008) thereby making toxicological studies on herbal products essential.

The rat are the usual species selected for subchronic and chronic toxicity studies because of their availability and the large amount of background information available on them (EHC, 1978) and the route of administration is usually that through which the plant extract is being administered for the claimed antiepileptic effect.

Liver and liver toxicity; The basic structure of the liver consists of rows of hepatic cells (hepatocytes) perforated by specialized blood capillaries called sinusoids and also in the liver three main functions occur; storage, metabolism and biosynthesis (Hodgson and Levi, 2004a).

The liver is an extremely resilient organ that is well equipped to resist toxic insults because of its unique cellular attributes e.g. cell cooperation, acute phase response, synthesis of hepatoprotective substances. Despite this resiliency, the liver is vulnerable to injury because it is frequently exposed to agents in their most reactive, thus toxic forms. Because the liver is located between the absorptive lining of the gastrointestinal tract and drug targets within the body, orally administered drugs enter the portal circulation and undergo “first pass metabolism” leading to significant exposure of the drug or its metabolites to the hepatocytes (Holt, 2008). Several factors are known to promote the close contact between hepatocytes, blood and drugs, these factors includes:

1. The structure of hepatic sinusoids
2. The fenestrated hepatic endothelium and
3. The enhanced overall surface area of the hepatocytes (Kaplowitz, 2007). The liver may also be rapidly exposed to intravenously administered drugs because it receives approximately 25% of the cardiac output (Holt, 2008).

Pathophysiology of drug induced hepatic injury

Hepatic uptake of drugs is thought to occur through passive diffusion, carrier mediated uptake, facilitated transport or active transport (Holt, 2008). After drugs have entered the hepatocytes, back diffusion out of the cells may be minimized through cytosolic transfer proteins, such as glutathione s-transferase, fatty acid binding proteins and 3-hydroxysteroid dehydrogenase (LeBlanc, 1994). Drugs are thereby transferred to either the endoplasmic reticulum, where they are metabolised, or to the canalicular membrane, where transporters

actively secrete endogenous or exogenous substances into the bile (Holt, 2008). Because most drugs are lipophilic, they are not readily excreted in the urine or bile and must be transformed to a more excretable hydrophilic form. The liver is responsible for the biotransformation either through phase I mediated cytochrome P₄₅₀ oxidative metabolism and formation of potentially hepatotoxic metabolites (toxification) or through phase II conjugation (detoxification) (Schwabe and Brenner, 2006).

In drugs undergoing oxidative metabolism, an activated oxygen molecule is integrated into lipophilic substrates resulting in the formation of reactive electrophiles, free radicals and reduced oxygen compounds (Schwabe and Brenner, 2006). Reactive electrophiles can bind to cellular membranes, disrupt their function and subsequently result in hepatocellular necrosis (Kaplowitz, 2007). Examples of agents that cause this type of liver injury are isoniazid and acetaminophen, free radical formation from agents, such as carbon tetrachloride (CCl₄) may lead to peroxidative injury of membrane lipids and necrosis (Losser and Payen, 1996).

Because cellular function is overwhelmingly disrupted through these cytochrome P₄₅₀ mediated mechanisms, cellular death is a common result (Holt, 2008) and this process, according to Schwabe *et al.*, 2006 occurs through the following mechanisms:

- i. Plasma membrane alteration and disruption of the cytoskeleton (e.g. loss of ionic gradients);
- ii. Mitochondrial dysfunction (e.g. decline in Adenosine triphosphate levels and disrupting fatty acid oxidation)
- iii. Loss of intracellular homeostasis; and fatty acid oxidation
- iv. Activation of degradative enzymes

A second means of drug metabolism is by phase II (detoxification) conjugation reactions. Phase II reactions occur through binding of drug metabolites to glutathione, glucuronate, or sulfate, which leads to the formation of non-toxic readily excretable hydrophilic products (Schwabe and Brenner, 2006). Subsequently, these compounds are excreted from the body in bile or urine through excretory transporters in the hepatocyte canalicular and sinusoidal membrane (phase III elimination). However, inadequate detoxification due to reduced binding substance concentrations (e.g. inadequate glutathione stores) may lead to a greater concentration of a reactive metabolite). An example of a phase II induced liver injury may arise from the inability to detoxify the antiepileptic agent, phenytoin metabolites. Also, the combination of phase I and phase II-induced liver injury may occur in situations resulting in both formation of toxic metabolites and inadequate detoxification e.g. Acetaminophen (Holt, 2008).

Overall, the pathogenesis of hepatotoxic drug reactions is likely a result of “multi-hit” process (Lee, 2003) and several genetic P₄₅₀ isoenzyme variants that result in toxic metabolites, inadequate detoxification pathways and individual genetic predisposition may all contribute to drug induced liver injury. Histopathologically, there are no absolute definitive features of drug induced liver injury (Holt, 2008). However, certain patterns suggest a drug etiology; these include (a) zonal necrosis or fatty changes that are associated with mitochondrial injury and (b) mixed histologic manifestations of necrosis and cholestasis. The presence of

neutrophils and/or eosinophils within destructive bile duct lesions is suggestive of drug induced liver injury (Lee, 2003).

Classification of drug-induced liver injury; Drug induced liver injury can be classified into intrinsic hepatotoxicity, which is generally dose dependent, and has a short and consistent latency period between drug exposure and liver injury and idiosyncratic hepatotoxicity which occur without warning, are unrelated to dose and may have a latency period from a few days to 12 months (Holt, 2008).

i. Intrinsic hepatotoxicity; This can be subdivided into direct and indirect toxins (Holt, 2008). Direct intrinsic hepatotoxins (e.g. carbon tetrachloride) destroy hepatocytes by a physicochemical attack, mostly through the toxic effects of their metabolites and there are no known direct hepatotoxins that are used as therapeutic agents (Lee, 2003; Andrade *et al.*, 2006) while indirect hepatotoxins (e.g. antimetabolites) induce structural changes in the hepatocytes by competitive inhibition of essential metabolites or by interference with selective metabolic or secretory processes of the hepatocytes. These changes can be due to either cytotoxic or cholestatic mechanism (Holt, 2008). Indirect hepatotoxins that produce cytotoxic changes include tetracycline, mechlorethamine, alcohol, acetaminophen, and mercaptopurine (Larrey, 2000). Indirect intrinsic cholestatic hepatotoxins produce jaundice and hepatic dysfunction by interfering with mechanisms for the excretion of bile from the liver e.g. C-17 alkylated anabolic steroids (Schwabe *et al.*, 2006).

ii. *Idiosyncrasy*

This may be cytotoxic, cholestatic or mixed (Andrade *et al.*, 2006). Most idiosyncratic drug reactions cause damage to hepatocytes throughout the hepatic lobule with various degrees of necrosis and apoptosis. Hepatic injury due to host idiosyncrasy can be caused by hypersensitivity reactions or by other mechanisms (e.g. an aberrant metabolic pathway for the drug in susceptible patient) (Holt, 2008). Examples of drugs causing allergic hepatic dysfunctions are methyldopa, phenytoin, paraaminosalicylic acid, chlorpromazine, erythromycin estolate, rarely clarithromycin and sulfonamides (Lee, 2003; Zimmermann, 2000).

Liver function tests: Routine liver function tests includes test for alkaline phosphatase, bilirubin, aspartate amino transferase, (AST), alanine amino transferase (ALT), Gamma-glutamyl transpeptidase (GGT).

i. *Aminotransferases;* ***The*** aminotransferases, AST and ALT are enzymes located in the cytoplasm of hepatocytes. The degree of elevation and rate of rise in aminotransferase levels is helpful in suggesting possible etiologies (Sease *et al.*, 2008). Studies have shown that the highest levels of aminotransferases are typically seen in acute viral, ischemic or toxic liver injury (Giannini *et al.*, 2005).

ii. *Alkaline phosphatase and gamma glutamyl transpeptidase*

Elevated serum levels of alkaline phosphate and gamma glutamyl transpeptidase occur as a result of the bile flow obstruction that accompanies conditions such as primary biliary cirrhosis, primary sclerosing cholangitis, drug induced cholestasis, gallstone disease and

autoimmune cholestatic liver disease (Sease *et al.*, 2008) hence they measure hepatobiliary function.

iii. Bilirubin; Bilirubin is a by product of haemoglobin derived from senescent red blood cells. Elevations of the serum bilirubin level are common in end stage liver disease and obstruction of the common bile duct caused by gallstone or malignancy (Sease *et al.*, 2008).

Kidney and kidney toxicity; The kidney has 3 major anatomical areas; the cortex, the medulla and the papilla. The renal cortex is the outermost region of the kidney and contains glomeruli, proximal and distal tubules and peritubular capillaries. Since the cortex receives almost 90% of the renal blood flow, cortical functions will be preferentially affected by blood borne toxicants. The fact that the kidney receives 25% of cardiac output, coupled with its ability to concentrate tubular fluid and its role in the biotransformation of chemicals to reactive and thus potentially toxic metabolites underlie the kidney susceptibility to chemical or drug toxicity (Hodgson and Levi, 2004b)

Under normal condition, each of the 2 million nephrons of the kidneys work in an organized fashion to filter, reabsorb and excrete various solutes and water (Nolin and Himmelfarb, 2008). The kidney is a primary regulator of sodium and water balance as well as of acid-base homeostasis (Melanie *et al.*, 2008). The kidney also produces hormones necessary for red blood cell synthesis and calcium homeostasis (Nolin and Himmelfarb, 2008).

Drug induced nephrotoxicity: Drug induced nephrotoxicity is a relatively common complication of several therapeutic agents (Nolin and Himmelfarb, 2008). It is manifested by acid-base abnormalities, electrolyte imbalances, urine sediment abnormalities, proteinuria, pyuria and/or hematuria (Choudhury and Ahmed, 2006). However, the most common manifestation of drug-induced nephrotoxicity is a decline in the glomerular filtration rate

(GFR) which results in a rise in the serum creatinine and blood urea nitrogen (Nolin and Himmelfarb, 2008).

Drug induced nephrotoxicity is often reversible on discontinuation of the offending agent but may also lead to acute kidney injury and/or end stage renal disease. Currently, many different mechanism are responsible for the pathogenesis of drug induce nephrotoxicity and the introduction of new drugs with novel mechanisms of action provides the potential for the identification of new presentation of acute kidney injury and chronic kidney disease (Nolin and Himmelfarb, 2008).

CHAPTER THREE

3.0 MATERIALS AND METHOD

3.1 Materials, Equipments, Chemicals and Animals

3.1.1 Materials and equipments

- Beaker
- Sample bottles
- Electroconvulsive machine (ugo basile, model no. 7801)
- Evaporation disc
- *Diospyros mespiliformis* leaves
- Filter paper
- Funnel
- Metler balance (P 162 Gallenkamp, UK)
- Mortar and pestle
- Plastic animal cages
- Spatula
- Stop watch
- Syringe and needles (1, 2, 5 and 10ml)
- Water bath
- Weighing balance (Ohio, New York USA)
- RT-9200 semi-auto chemistry analyser
- AC 9900 Automatic Electrolyte Analyser

3.1.2 Chemicals/drugs

- Methanol (BDH chemicals limited Poole England)
- Chloroform (Sigma Chemical CO., USA)
- Pentylenetetrazole (Sigma Chemical Company, Louis, MO., USA)
- 4-Aminopyridine (Sigma Chemical Company MO, USA)
- Strychnine (Sigma Chemical Company Louis MO, USA)
- Phenytoin sodium (Mancare pharmaceuticals Pvt Ltd India)
- Sodium valproate (Sanofi Synthelabo Ltd UK)
- Phenobarbitone (Sterop, Belgium)
- Sodium Chloride (Fisher Scientific Co. USA)

3.1.3 Animals

- Adult Swiss albino mice (males and females)
- Adult Wistar rats (males and females)
- Day old cockerels

3.2 Preparation of Plant Extract

3.2.1 Collection and identification of plant materials

The plant sample of *Diospyros Mespiliformis* was collected in February, 2011 from Dembo village in Zaria Local Government area of Kaduna State, Northern Nigeria. The plant was taken to the Herbarium section of the Department of Biological Sciences, Ahmadu Bello University, Zaria for taxonomic identification and authentication. This was done by the Botanist, Mallam Umar Gallah of the herbarium section by comparison with already deposited specimen with reference number 1611 for the purpose of future reference.

3.2.2 Preparation of plant extract

The plant material of *Diospyros mespiliformis* leaves were cleaned, air dried in a shade for 7 days and then crushed into fine powder with a pestle and mortar. It weighed 1326g; the whole mass was macerated with 7000ml of methanol (98%) for 72 hours with occasional mixing. The mixture was filtered using a filter paper and the filtrate was evaporated using water bath at 60°C to constant weight.

3.3 Study Animals

Three species of animals were employed in these studies; these species are rats, mice and day old chicks. Young adult Swiss albino mice of both sexes weighing between 25-34g and wistar rats with body weight range of between 156 – 247g obtained from the Animal House, Department of Pharmacology and Therapeutics, Ahmadu Bello University, Zaria were used for the study, Also day old chicks weighing between 24-33g obtained from the National Animal Production Research Institute (NAPRI) hatchery, Ahmadu Bello University, Shika Zaria were used for the study.

3.3.1 Experimental groupings

In grouping the animals, body weight and sex (in case of rats and mice) were taken into consideration. This was done to achieve approximately uniform condition among groups and hence minimize biological variations.

3.4 Preliminary Studies

3.4.1 Phytochemical screening

The methanol leaf extract of *Diospyros mespiliformis* was subjected to preliminary phytochemical screening to test the presence or absence of alkaloids, saponins, flavonoids, tannins, anthraquinones, cardiac glycosides and carbohydrates according to standard procedures as outlined by Sofowora, (1993); Evans (1996).

Test for alkaloids: 0.5g of the extract was mixed with 10ml of 1% aqueous hydrochloric acid on a water bath. The resulting filtrate was used for the following tests:

i. Meyer's test: To 1ml of the above sample of the extract was added few drops of Meyer's reagent in a test-tube. A cream coloured precipitates is indicative of the presence of alkaloids.

ii. Dragendorff's test: Few drop of Dragendorff's reagent (potassium bismuth iodide solution) was added to 1ml of the extract sample solution in a test tube. A rose red precipitate is indicative of the presence of alkaloid.

iii. Picric acid test ; Few drops of picric acid solution were added to about 1ml of the sample extract solution in a test tube. Formation of a yellow coloured precipitate is indicative of the presence of alkaloid.

Test for saponins

Frothing test; Small quantity of the extract was dissolved in 10ml of distilled water. The solution was then vigorously shaken for about 30 seconds and was allowed to stand for 30 minutes. A honey comb froth formed for more than 15 minutes is indicative of the presence of saponins.

Test for flavonoids

Sodium hydroxide test ;0.5g of the extract was dissolved in 10ml of water,then to a few drops of aqueous sodium hydroxide was added 5ml of extract solution, a yellow colouration is indicative of the presence of flavonoids.

Test for tannins

Ferric Chloride Test; 0.5ml of extract was dissolved in 10ml of distilled water and then filtered. Few drops of ferric chloride solution were added to about 4ml of the filtrate. The formation of a blue black precipitate indicates the presence of hydrolysable tannins, while the formation of green precipitate is indicative of the presence of condensed tannins.

Test for carbohydrates

Molisch test ;To a little quantity of the extract solution in a test tube was added few drops of molisch reagent and then a small quantity of concentrated sulphuric acid (H_2SO_4) was allowed to run down the side of the test tube. Formation of a lower purple to violet colour at the interface is indicative of the presence of carbohydrates.

Test for cardiac glycosides

Kella-Killiani test; 0.5g of the extract was dissolved in glacial acetic acid containing ferric chloride solution. The test tube was held at an angle of 45 degrees. 1ml of concentrated sulphuric acid was added down the side of test tube. Formation of purple ring colour at the interface indicates the presence of cardiac glycosides (Trease and Evans, 1983).

Test for anthraquinones

i. Borntrager's Test; 3g of extract was shaken with 10ml of benzene and filtered. 5ml of 10% ammonia solution was added to the filtrate and shaken. The production of a pink-red or violet colour indicates the presence of free anthraquinones.

ii. Test for combined anthracene (Modified Borntrager's Test): 5ml of sample of extract was added to 5ml of 10% hydrochloric acid and boiled for 3 minutes. This is to hydrolyse the glycosides to yield aglycone which is soluble in hot water only. The solution was filtered hot, the filtrate was cooled and extracted with 5ml of benzene. The benzene layer was filtered off and shaken gently with half its volume of 10% ammonia solution. A rose-pink or cherry red colour indicates combined anthracene (Trease and Evans, 1983).

3.4.2 Preparation and administration of drugs

To guarantee the efficacy and potency of drug and extract preparations, only freshly prepared drug and extract solutions were used. Calculations of doses to be administered were done based on stock concentration of each solution and the body weight of experimental animals to be employed. The time interval between drug administrations and the route of administration employed are as prescribed by each individual method.

3.4.3 Acute toxicity studies.

The method of Lorke (1983) was employed using thirteen Wistar rats, Swiss albino mice and day old cockerel chicks. For the rats and mice, both oral and intraperitoneal (i.p.) routes of drug administration were employed while for the day old cockerel chicks only the i.p. route was employed.

The method was divided into phase I and II. In phase I three groups of three animals each were administered 10, 100 and 1000 mg/kg of the extract respectively to ascertain the extent of toxicity of the extract. The animals were observed for any sign of toxicity or death within 24 hour period. In phase II which depends on the outcome of the first phase, other specifically graded doses were administered to four different sets of rats, mice and chicks using both oral and i.p routes as the case apply and observed for sign of toxicity or death within another 24 hour period. From the outcome of phase II, LD₅₀ value was determined by calculating the geometric mean of lowest dose that causes death and the highest dose for which the animals survived, as shown by the formula below:

$$LD_{50} = G.M. = i$$

LD₅₀ = Median Lethal Dose

G.M. = Geometric Mean

X = lowest lethal dose (1/1)

Y = Highest non-lethal dose (0/1)

3.5 Anticonvulsant Studies

3.5.1 General study design

The studies were carried out using five groups each containing ten or six animals as the case may be. Groups 1 and 5 served as negative and positive controls using normal saline and a standard drug respectively while groups 2, 3 and 4 received graded doses of the extract. Results obtained for each experiment were recorded accordingly

3.5.2 Pentylenetetrazole induced seizure model

The method of Swinyard *et al.*, (1989) was employed. Thirty mice of either sex were divided into five groups of six mice each. Mice in group 1 were treated with equivolume of distilled water per kg body weight intraperitoneally, the second, third and fourth group were treated with 50, 100 and 200 mg of the extract per kg body weight i.p., the fifth group were treated with 200 mg valproic acid per kg body weight i.p. Thirty minutes later, mice in all the groups were treated with 90 mg/kg of freshly prepared pentylenetetrazole subcutaneously. The mice were observed for presence or absence of clonic spasm of at least 5 seconds duration, hind limb extension or death.

3.5.3 Maximal electroshock test

The method of Toman (1946) as modified by Swinyard and Kupferberg (1985) and Browning (1992) was employed. Fifty day old chicks were divided randomly into five groups of 10 chicks per group. The first group was treated with distilled water (10ml/kg) i.p., second, third and fourth groups were treated with 250, 500 and 1000mg of the extract per kg i.p. respectively and the fifth group was treated with 20mg phenytoin per kg i.p. as positive control. Thirty minutes later, maximal electroshock was administered to induce seizure in the

chicks using ugo Basile electroconvulsive machine (Model 7801) with corneal electrodes placed on the upper eyelids of the chicks. The current, shock duration, frequency and pulse width used were maintained at 90 mA, 0.8s, 100 pulse per second and 0.8ms respectively. The chicks were observed for hind limb tonic extension which was considered as convulsion while abolition of hind limb tonic extension was considered as protection against electrically induced convulsion.

3.5.4 Strychnine induced seizure model

The method of Porter *et al.*, (1984) was employed. Thirty mice of either sex were divided into five groups of six mice each. Mice in group 1 were treated with 10 ml/kg of distilled water per kg body weight i.p., the second, third, and fourth group were treated with 50, 100 and 200 mg of the extract per kg body weight i.p., and the fifth group was treated with 30 mg /kg phenobarbitone i.p. as positive control. Thirty minutes later, mice in all the groups were treated with 2.5 mg of freshly prepared strychnine per kg s.c. The mice were observed for tonic extensor jerks of the hind limb which was considered as convulsion and abolition of such was considered as protection.

3.5.5 4-Aminopyridine induced seizure model

The method of Yamaguchi and Rogawski, (1992) was employed. Thirty mice of either sex were divided into five groups of six mice each. Mice in the first group were treated with 10ml normal saline per kg body weight i.p. The second, third and fourth group were treated with 50, 100 and 200 mg of the extract per kg body weight i.p. respectively. The fifth group was treated with 30 mg phenobarbitone per kg body weight i.p. Thirty minutes later, mice in all

the groups were treated with 14 mg of freshly prepared 4-aminopyridine per kg s.c.. The mice were observed for presence or absence of hind limb tonic extension, onset of episodes of convulsion and possibly death, abolition of any of these was considered as protection.

3.6 Subchronic Toxicity Studies

Five groups of six adult Wistar rats consisting of three males and three females' rats per group were used for the study. Rats in group 1 were given distilled water only orally this is the control group while rats in groups 2, 3, 4 and 5 were administered with 50, 250, 500 and 1000 mg. per kg body weight orally of the methanol leaf extract of *Diospyros mespiliformis* respectively on daily basis for 28 days. At the end of the study period, the animals were anaesthetized in light chloroform and then sacrificed, blood samples were collected by cardiac puncture into clean dry centrifuge tubes. They are processed individually and allowed to stand for about 10 minutes at room temperature and then centrifuged at 1000 rpm for 15 minutes on laboratory centrifuge (SM 800B, surgifrend medicals, England), the supernatant (serum) was removed carefully with pastor pipette and stored in a refrigerator.

3.6.1 Measurement of liver function

For hepatic/liver toxicity studies, Organisation for Economic Cooperation and Development (OECD), 2008 recommended at least two appropriate tests for hepatocellular and two for hepatobiliary–evaluation (OECD, 2008) and for hepatocellular function, we tested for alanine aminotransferase (ALT) and aspartate aminotransferase (AST) while for hepatobiliary function, we tested for alkaline phosphatase (ALP) and total bilirubin (TB).

3.6.2 Measurement of kidney function

According to Weingand *et al.*, (1996), the following list of parameters were investigated to evaluate kidney function; glucose, urea (urea, nitrogen), creatinine, total protein, calcium, sodium, potassium and total cholesterol.

3.6.3 Clinical chemistry methods

Different clinical chemistry methods were employed to determine both hepatic and renal function parameters, serum alanine aminotransferase (AST) and aspartate aminotransferase (ALT) were assayed by the method of Reitman and Frankel, (1957). While the activities of alkaline phosphatase (ALP) was assayed using the nitrophenol method of Bassey-lowry-Brock, 1946. Total bilirubin, however was assayed using the method of Jendrassik and Grof, (1938).

For the renal function parameters, serum creatinine was determined using the Jaffe-slot modified alkaline picrate colorimetric method (Slot, 1965) while serum albumin was determined using the bromocresol green (BCG) binding method of Spencer and Price, (1977) and Webster, (1977). Urea was assayed using the diacetyl-monoxime method of Wybenga *et al.*, (1971), and Blood glucose was assayed using the glucose oxidase enzymatic method (Trinder, 1969). Total protein was determined using the Biuret method (Domas, 1975) while Na^+ and K^+ were determined by ion selective electrode (ISE) technology using an ISE analyzer which measures (senses) the activities of sodium and potassium ions and converts the activity measurements to mmol/L (Cheesbrough, 1998) and serum calcium was however determined using the method of Baginski *et al.*, (1973) and lastly, total cholesterol was assayed using the method of Liebermann and Burchard as described by Xiong *et al.*, (2007).

3.6.4 Histopathological Studies

The liver and kidney of at least a male and a female rat from each group administered with methanol leaf extract of *Diospyros mespiliformis* including the control group administered with normal saline were removed after draining the blood and fixed in 10% Buffered neutral formalin for 48 hours and then with bovine solution for 6 hours. Paraffin sections were taken at 5mm thickness and processed in alcohol-xylene series and was stained with alum hematoxylin and eosin. The sections were examined microscopically for histopathological changes (Disbrey and Rack, 1970)

3.7 Statistical Analysis

Results were expressed as the Mean \pm Standard Error of the Mean (SEM) and Percentages. Statistical analysis of data was done using statistical package for the Social Sciences (SPSS) version 16. The data were subjected to student t-test followed by one-way analysis of variance (ANOVA) and the difference in the mean between the control and the tested groups were considered significant at $P < 0.05$). Differences between the control (distilled water) group and the group that received the standard drug were tested using Chi square test and was considered significant at $P < 0.05$. They were presented as Tables, figures and plates.

CHAPTER FOUR

4.0 RESULTS

4.1 Phytochemical constituents of *Diospyros mespiliformis*

The preliminary phytochemical screening of methanol leaf extract of *Diospyros mespiliformis* revealed the presence of alkaloids, saponins, flavonoids, tannins, carbohydrates, cardiac glycosides, and combined anthracene type of anthraquinones (table 4.1). The free type of anthraquinone was however found to be absent

Table 4.1: Phytochemical constituents of Methanol Leaf Extract of *Diospyros mespiliformis*

Chemical Constituents	Inference
Alkaloids	Present
Saponins	Present
Flavonoids	Present
Tannins	Present
Carbohydrates	Present
Cardiac Glycosides	Present
Antraquinones (combined anthracene)	Present
Antraquinones (free)	Absent

4.2 Acute Toxicity Studies

The median lethal dose (LD₅₀) of the methanol leaf extract of *Diospyros mespiliformis* plant in rats was found to be 774.6 mg/kg body weight intraperitoneally and above 5000 mg per kg body weight orally. In mice, the LD₅₀ values were found to be 774.6 mg/ kg intraperitoneally and above 5000mg/kg orally while in chicks, the LD₅₀ value was found to be above 5000mg/kg body weight via the intraperitoneal route of administration (Table 4.2).

Table 4.2: LD₅₀ Values of Methanol Leaf Extract of *Diospyros mespiliformis* via Intraperitoneal and Oral routes of administration

Species	Route of Administration	LD₅₀ Values (mg/kg)
Rats	Intraperitoneal	774.6
	Oral	>5000
Mice	Intraperitoneal	774.6
	Oral	>5000
Chicks	Intraperitoneal	>5000

4.3 Anticonvulsant Studies

4.3.1 *Pentylenetetrazole (PTZ)-induced seizure in mice*

The methanol leaf extract of *Diospyros mespiliformis* protected the animals against PTZ-induced seizure. At 50 mg/kg of the extract, the percentage protection was found to be 50% which then goes up to 66.7% protection when the dose was raised to 100 mg/kg of the extract, while increase in dose of the extract above 100 mg/kg did not confer additional protection against seizure, The percentage protection at 200mg/kg of the extract is still 66.7% same as that of 100 mg/kg. Valproic acid (200 mg/kg) produced 100% protection against seizure and mortality as well. There was no statistically significant difference in the mean onset of seizure between the control (normal saline) group and extract at doses of 50, 100 and 200 mg/kg respectively at $P < 0.05$ (Table 4.3).

Table 4.3: Effect of Methanol Leaf Extract of *Diospyros Mespiliformis* (DM) and Valproic Acid on Pentylenetetrazole (PTZ) induced seizure in mice

Treatment (mg/kg)	Mean onset of seizures (min)	Quantal Protection	Protection against seizure (%)	Mortality rate (%)
N/saline (10ml/kg)	9.00±1.00	1/6	16.67	83.33
DM 50	11.00± 3.46	3/6	50.00	50.00
DM 100	6.50 ± 1.50	4/6	66.67	33.33
DM 200	10.00 ± 4.00	4/6	66.67	33.33
VA 200	0.0	6/6	100.0	0.00

Values are presented as mean ± SEM, n=6 per group, DM=*Diospyros mespiliformis* VA=valproic acid. No significant difference in the mean onset of seizure between control (normal saline) group and treated groups at P<0.05. Significant difference between control (saline) group and standard (VA) at P<0.05 (chi-square test).

4.3.2 Maximal electroshock (MES) –induced seizure in chicks

The methanol leaf extract of *Diospros mespiliformis* did not confer protection against MES-induced seizure in chicks at all the doses of the extract tested, while phenytoin (20 mg/kg) demonstrated 90% protection against seizure. No mortality was recorded in all the groups and there was no statistically significant difference observed in the mean recovery time from seizure between the control (normal saline) group and treated groups at $P < 0.05$ (Table 4.4).

Table 4.4: Effect of Methanol Leaf Extract of *Diospyros Mespiliformis* (DM) and Phenytoin (PHT) on maximal electroshock (MES) – induced seizure in chicks

Treatment (mg/kg)	Mean Time of Recovery from seizures (min)	Quantal Protection	Protection against seizure (%)	Mortality rate (%)
N/saline (10ml/kg)	14.80±5.09	0/10	0.00	0.00
DM 250	11.90± 4.88	0/10	0.00	0.00
DM 500	9.10 ± 1.54	0/10	0.00	0.00
DM 1000	11.90 ± 3.30	0/10	0.00	0.00
PHT 20	17.0	9/10	90.00	0.00

Values are presented as mean ± SEM, n=10 per group DM= *Diospyros Mespiliformis* PHT=Phenytoin. No statistically significant difference between control (normal saline) group and the treated groups at doses of 250, 500 and 1000mg/kg at P<0.05, significant difference between control (saline) group and the standard drug (PHT) at P<0.05 (chi-square test).

4.3.3. Strychnine induced seizure in mice

The methanol leaf extract of *Diospyros mespiliformis* demonstrated statistically significant difference in the latency of death by prolonging the onset of seizure at the tested doses of 50, 100 and 200 mg/kg at $P < 0.05$. However, the extract fails to protect the animals against seizure and lethality induced by strychnine at all the tested doses, meanwhile, the standard agent employed as positive control, phenobarbitone offers 83.33% protection (Table 4.5).

Table 4.5: Effect of methanol leaf extract of *Diospyros mespiliformis* (DM) and phenobarbitone (PHB) on strychnine induced seizure in mice

Treatment (mg/kg)	Mean onset of seizures (min)	Quantal Protection	Protection against seizure (%)	Mortality rate (%)
N/saline (10ml/kg)	2.67±0.33	0/6	0.00	100.00
DM 50	5.33 ± 0.71	0/6	0.00	100.00
DM 100	6.00 ± 0.37	0/6	0.00	100.00
DM 200	5.50 ± 0.50	0/6	0.00	100.00
PHB 20	0.0	5/6	83.33	16.67

Values are presented as mean ± SEM, n=6 per group, DM= *Diospyros Mespiliformis*, PHB =phenobarbitone. There is statistically significant difference in the mean onset of seizure between the control (Normal saline) group and the treated groups given 50, 100 and 200mg/kg of the extracts at P<0.05 and also between the control group and the standard drug (PHB) at P<0.05) (chi-square test).

4.3.4. 4-aminopyridine induced seizure in mice

The methanol leaf extract of *Diospyros mespiliformis* showed 16.67% protection at the highest dose of the extract tested (200 mg/kg), however at the other doses tested i.e. 50 and 100mg/kg it could not protect the animals against 4-aminopyridine induced seizure and lethality. Also there is no statistically significant difference in the mean onset of seizure between the control (normal saline) group and the treated groups given 50, 100 and 200 mg/kg of the extract respectively at $P < 0.05$ (Table 4.6).

Table 6: Effect of methanol leaf extract of *Diospyros mespiliformis* (DM) and phenobarbitone (PHB) on 4-amino pyridine induced seizure in mice

Treatment (mg/kg)	Mean onset of seizures (min)	Quantal Protection	Protection against seizure (%)	Mortality rate (%)
N/saline (10ml/kg)	13.67±1.69	0/6	0.00	100.00
DM 50	20.67± 3.21	0/6	0.00	100.00
DM 100	15.33 ± 2.96	0/6	0.00	100.00
DM 200	16.00 ± 2.49	1/6	16.67	83.33
PHB 30	0.0	6/6	100.00	0.00

Values are presented as mean ± SEM, n=6 per group, DM=*Diospyros mespiliformis*, PHB=Phenobarbitone. There is no statistically significant difference in the mean onset of seizure between the control (normal saline) group and the groups given 50, 100 and 200mg/kg of the extract respectively at P<0.05, while there is significant difference between the control group and the standard drug (PHB) at P<0.05 (chi square test).

4.4 Results of sub-chronic toxicity studies

4.4.1 Liver function tests.

i. Hepatocellular function tests: The methanol leaf extract of *Diospyros mespiliformis* slightly raise the value of AST at 250 and 500mg/kg dose of the extract. While at 1000mg/kg AST values was found to be decreased. The increase in the AST was found not to be statistically significant when compared with the control at $P < 0.05$. Similarly, there is a slight increase in ALT values with the 500 and 1000mg/kg dose of the extract, however, the increase was also found not to be statistically significant when compared with the control at $P < 0.05$ (Fig.4.2).

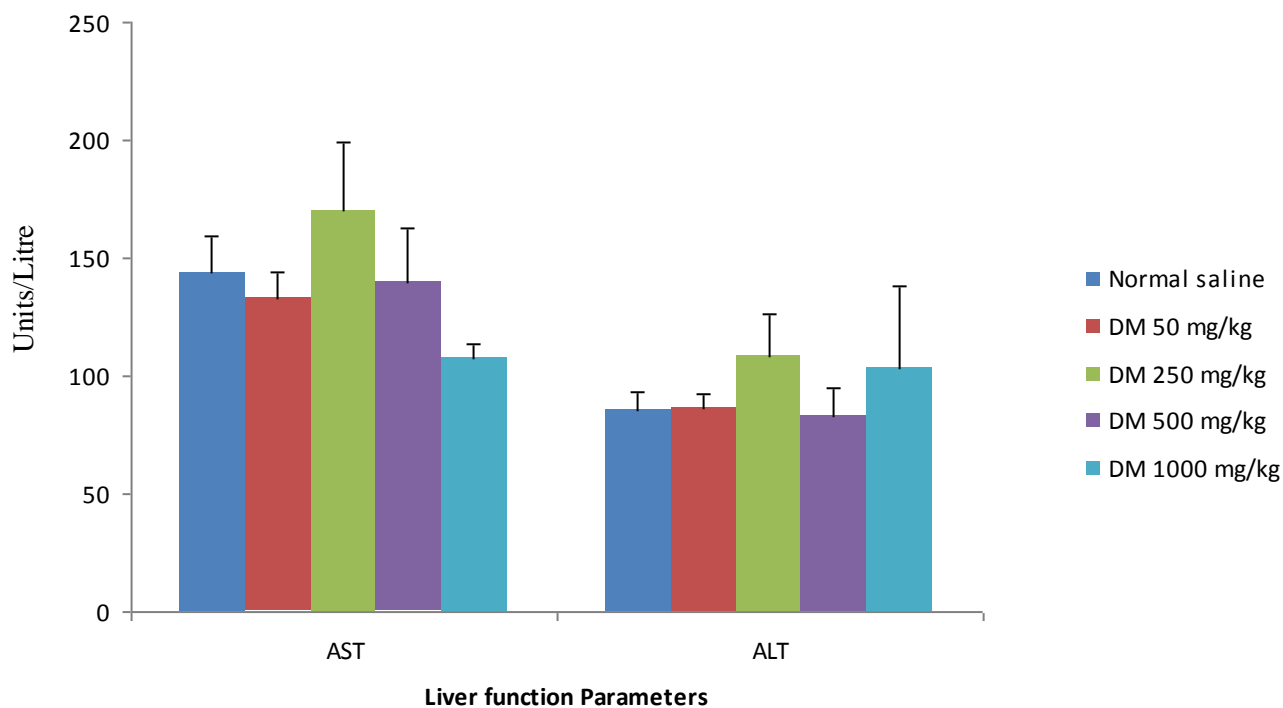


Fig.4.2: Effect of 28 days administration of DM on AST and ALT. No statistically significant increase in AST and ALT when compared with the control at $P < 0.05$

ii. **Hepatobiliary function test;** The methanol leaf extract of *Diospyros mespiliformis* causes a dose dependent increase in the values of ALP especially at 50, 500 and 1000mg/kg doses of the extract, this increase in ALP was found to be statistically significant at *P<0.05(50 mg/kg), P<0.05(500 mg/kg) and***P<0.001(1000 mg/kg) respectively, however the extract at all the tested doses causes only a slight decrease in TB even though the decrease in TB was not statistically significant at P<0.05.(Fig. 4.3 and 4.4).

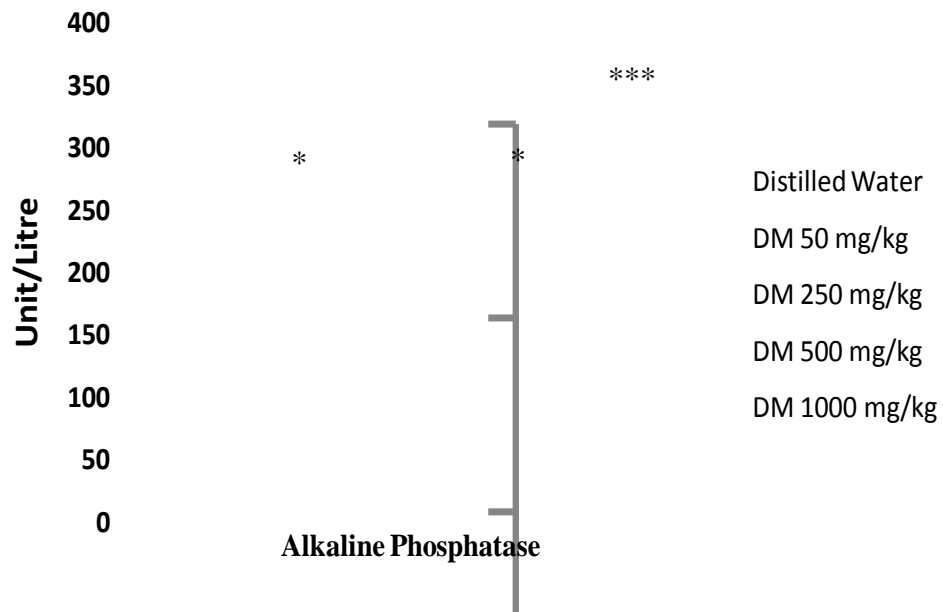


Fig. 4.3: Effect of 28 days administration of DM on ALP. There is statistically significant increase in the level of ALP when compared with the control at *P<0.05 and *P < 0.001**

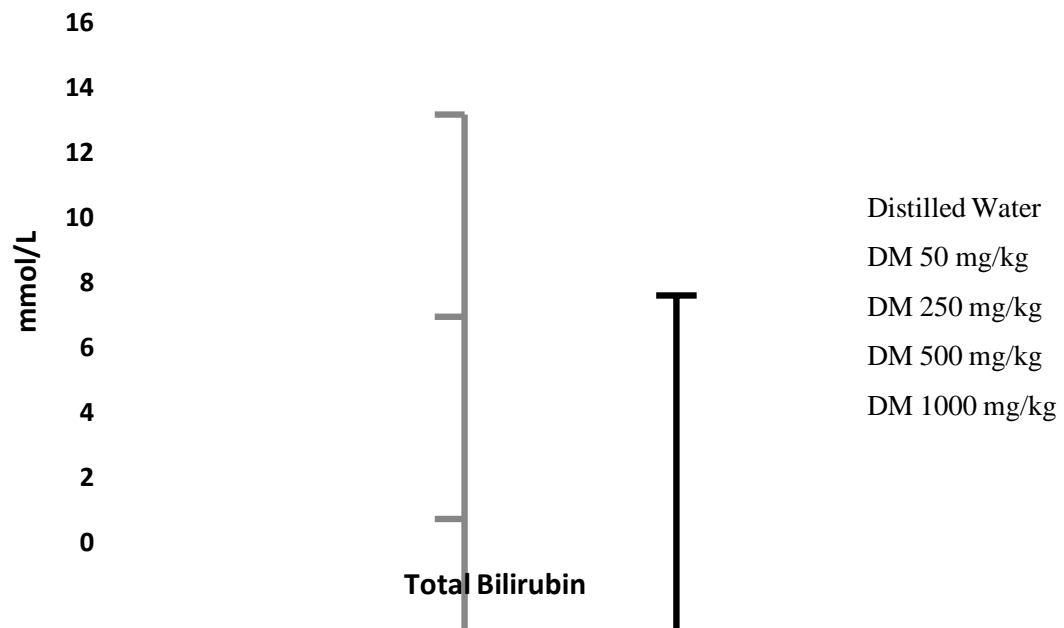


Fig. 4.4: Effect of 28 days administration of DM on TB. There is no statistically significant increase in the level of TB when compared with the control at $P < 0.05$

4.4.2. Kidney function tests

The methanol leaf extract of *Diospyros mespiliformis* when administered orally for 28 days at doses of 50, 250, 500 and 1000mg/kg causes only a slight increase in sodium, calcium and a slight decrease in glucose, urea, total protein, albumin, and total cholesterol, these increases and decreases are however, not statistically significant when compared with the control at $P < 0.05$.

However, there is statistically significant decrease in the value of creatinine at doses of 250(* $p < 0.05$), 500(* $p < 0.05$) and 1000mg/kg(** $p < 0.001$) of the extract when compared with the control at $P < 0.05$, similarly, there is increase in the value of potassium at 250mg/kg dose of the extract and this increase was found to be statistically significant at $P < 0.05$ when compared with the control group (Fig. 4.5-4.9).

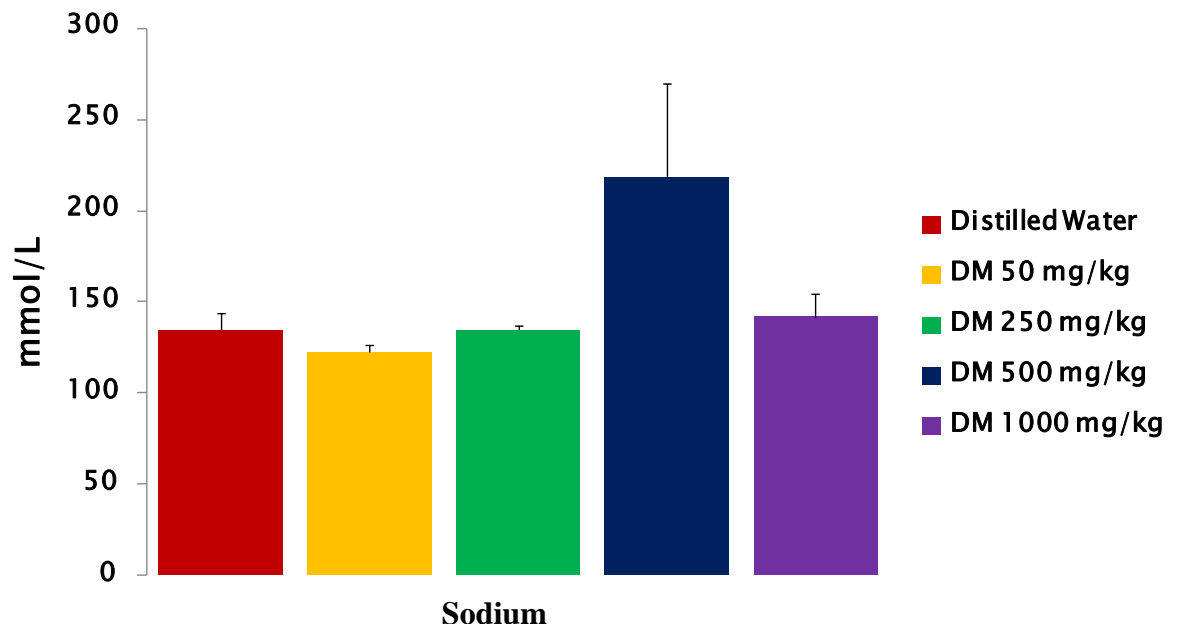


Fig 4.5: Effect of 28 days administration of DM on sodium levels .No statistically significant increase in the level of sodium when compared with control a $p < 0.05$

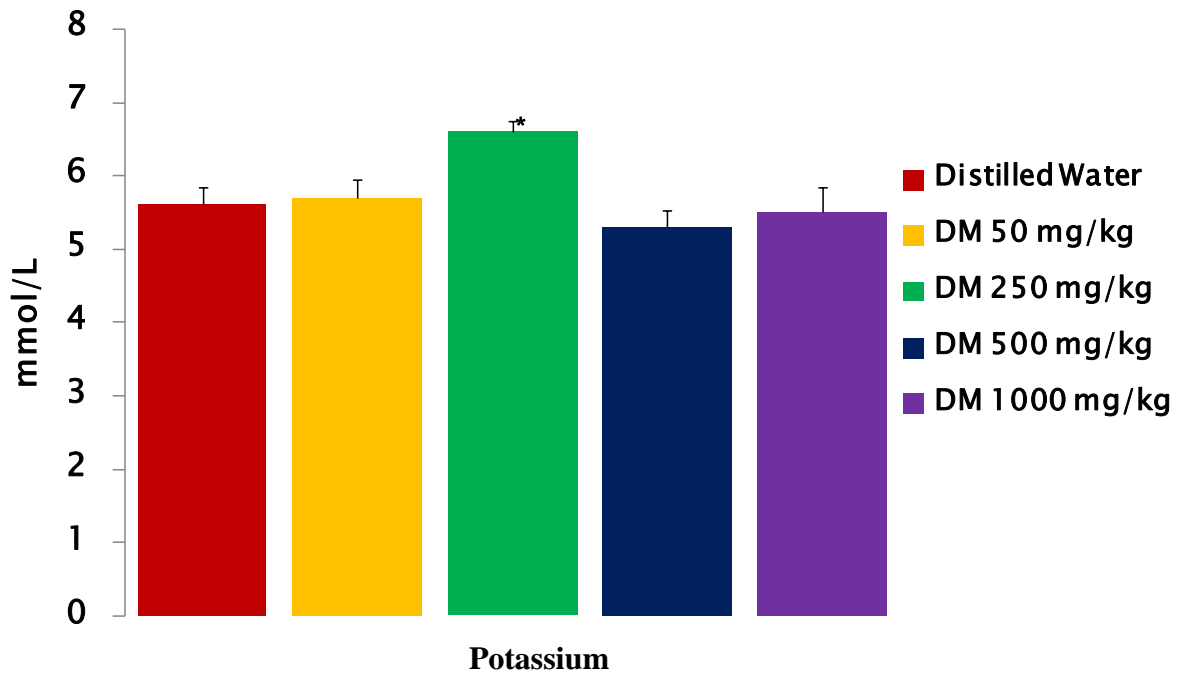


Fig.4.6; Effect of 28 days administration of DM on Potassium levels. Statistically significant increase in the level of potassium with the 250mg/kg dose when compared with control at $p < 0.05$. * $p < 0.05$

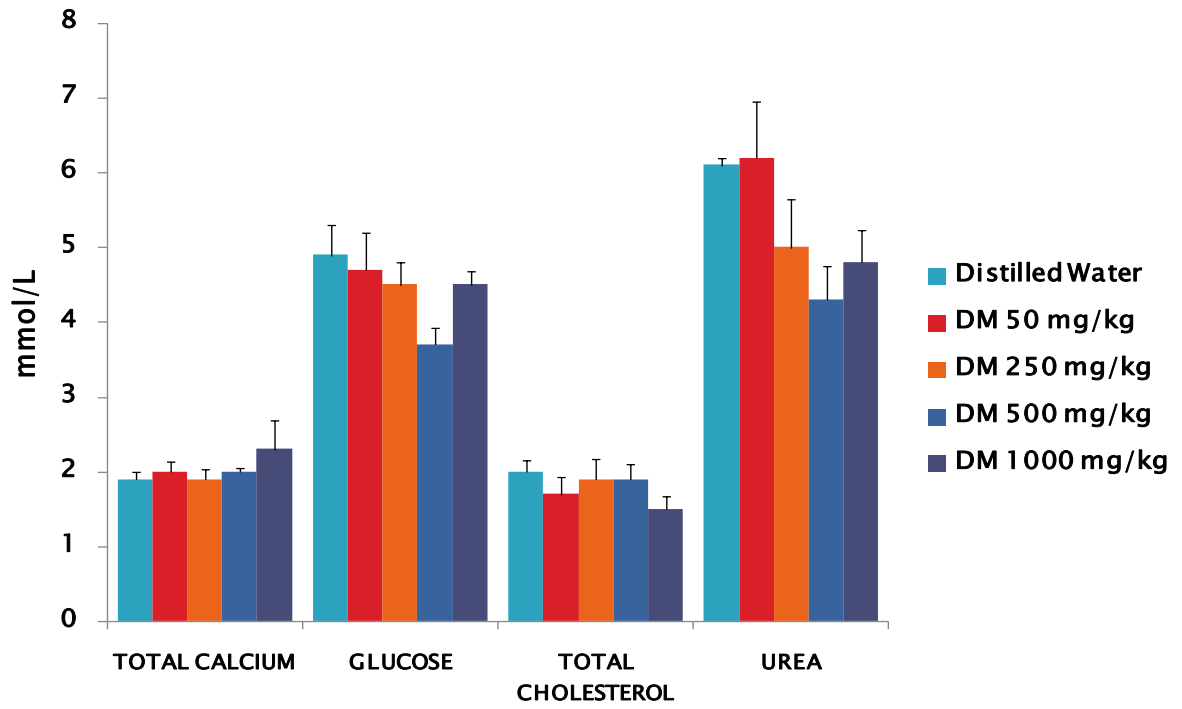


Fig.4.7: Effect of 28 days administration of DM on total calcium, glucose. Total cholesterol and urea. No statistically significant increase in the level of total calcium, glucose, total cholesterol and urea when compared with control at $p < 0.05$

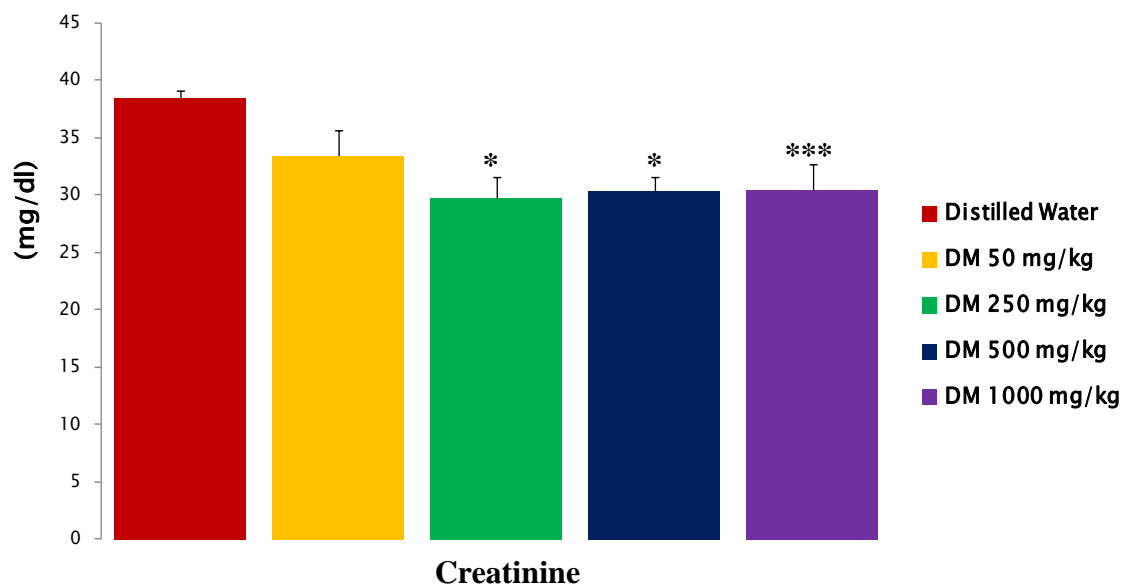


Fig 4.8: Effect of 28 days administration of DM on creatinine levels statistically significant decrease in the level of creatinine when compared with control at * $p < 0.05$, * $p < 0.001$**

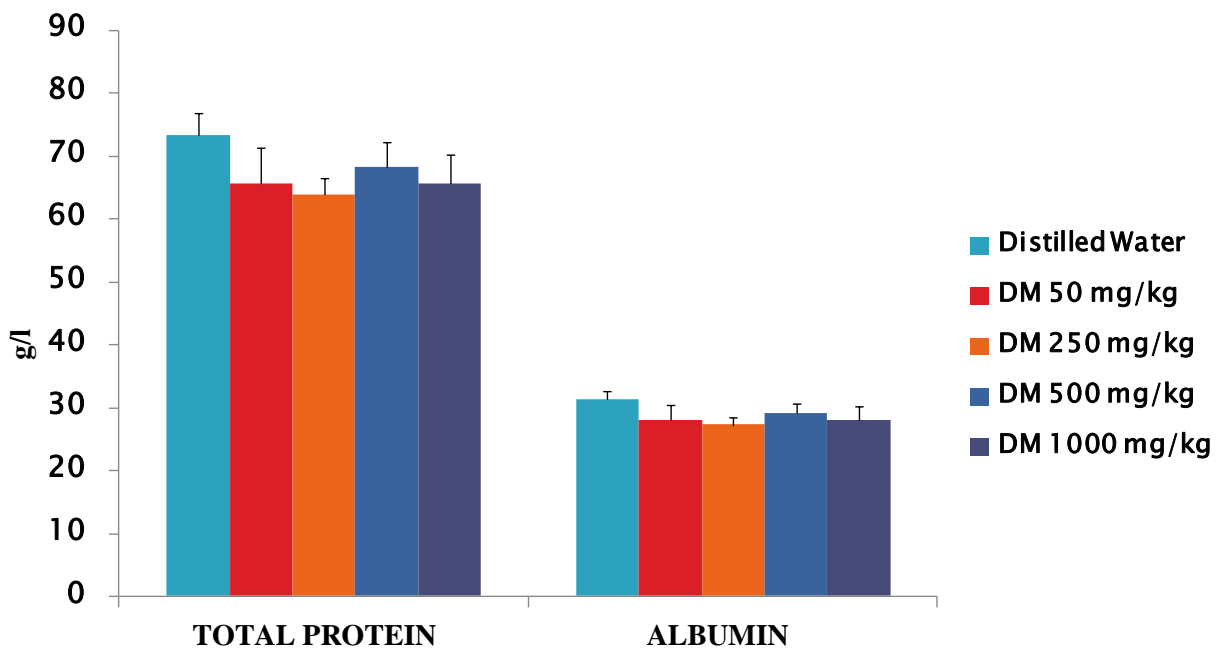


Fig.4.9; Effect of 28 days administration of DM on Total protein and Albumin. No statistically significant decrease in the level of total protein and Albumin when compared with control a $p < 0.05$

4.5. Histopathological Studies

4.5.1. Liver

The methanol leaf extracts of *Diospyros mespiliformis* when administered orally for 28 days at doses of 50 and 250mg/kg does not cause any significant histopathological changes on the liver similar to the control (A). The central vein (CV), Hepatocytes (H) and Sinusoidal Spaces (SS) are evident and intact signifying normal hepatic architecture (Plate 4.1 and 4.2). At a dose of 500mg/kg however, there was degeneration of hepatocytes, moderate hepatocellular necrosis and loss of hepatic architecture (Plate 4.3). While with a dose of 1000mg/kg there was degeneration of hepatocytes, loss of hepatic architecture and necrosis of hepatocytes (Plate 4.4)

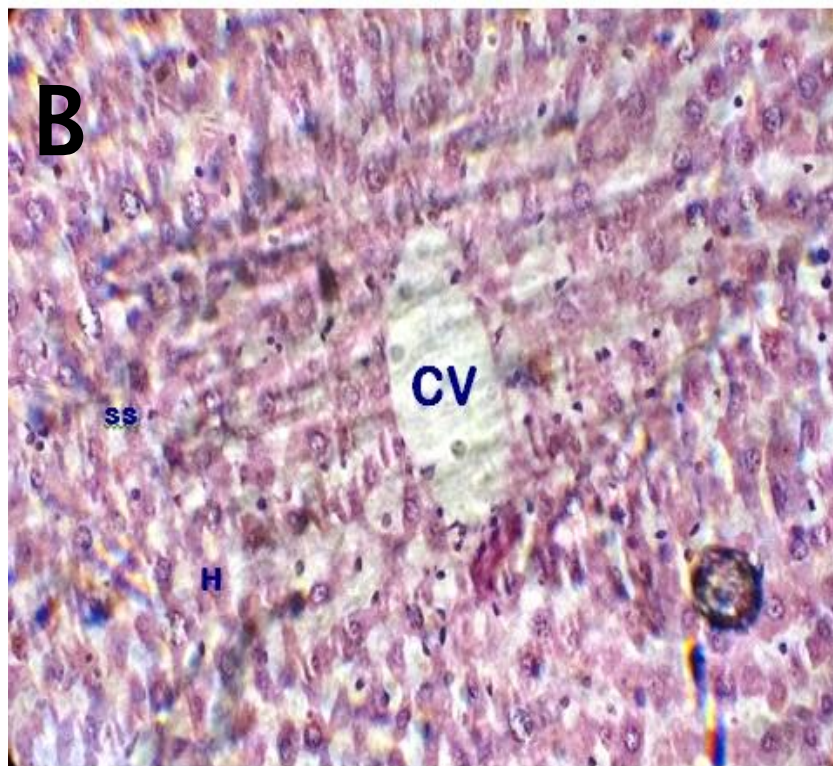
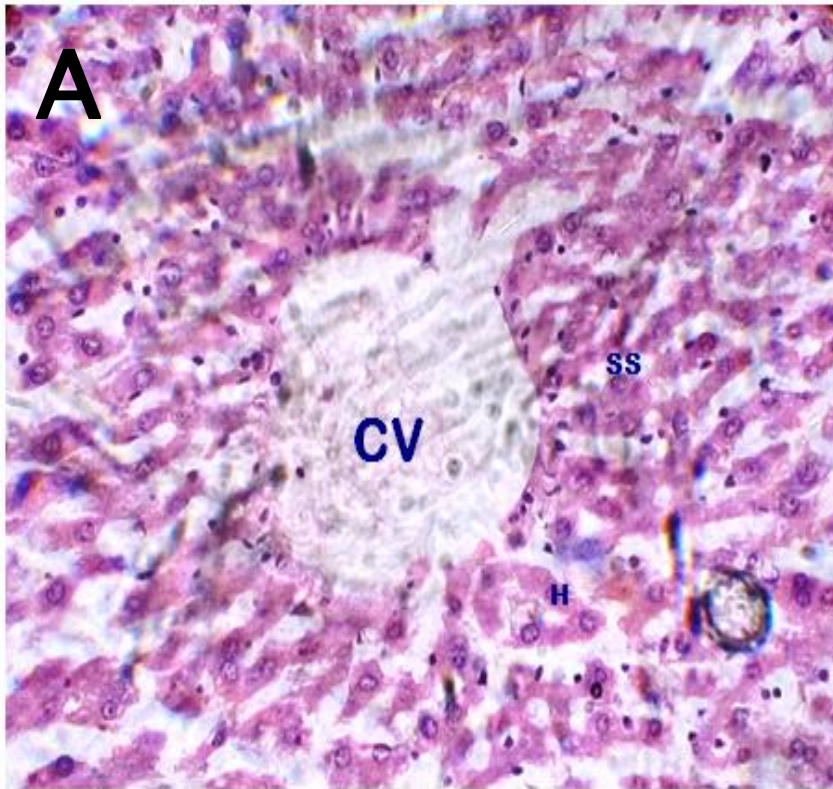


Plate 4.1: Photomicrograph of a section of the rat liver given distilled water only (control) (A) and rat liver administered with 50mg/kg of DM (B) for 28 days (magnification x 400) showing normal hepatic architecture i.e. central vein (CV), Hepatocytes (H) and Sinusoidal spaces (SS). No significant histopathological changes

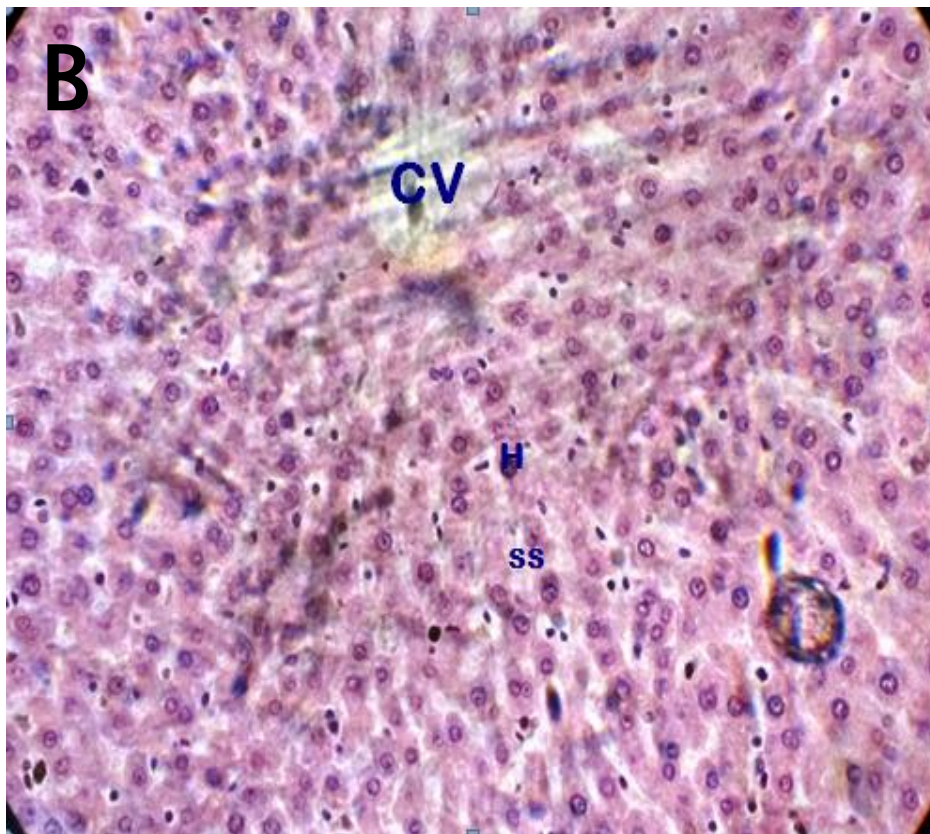
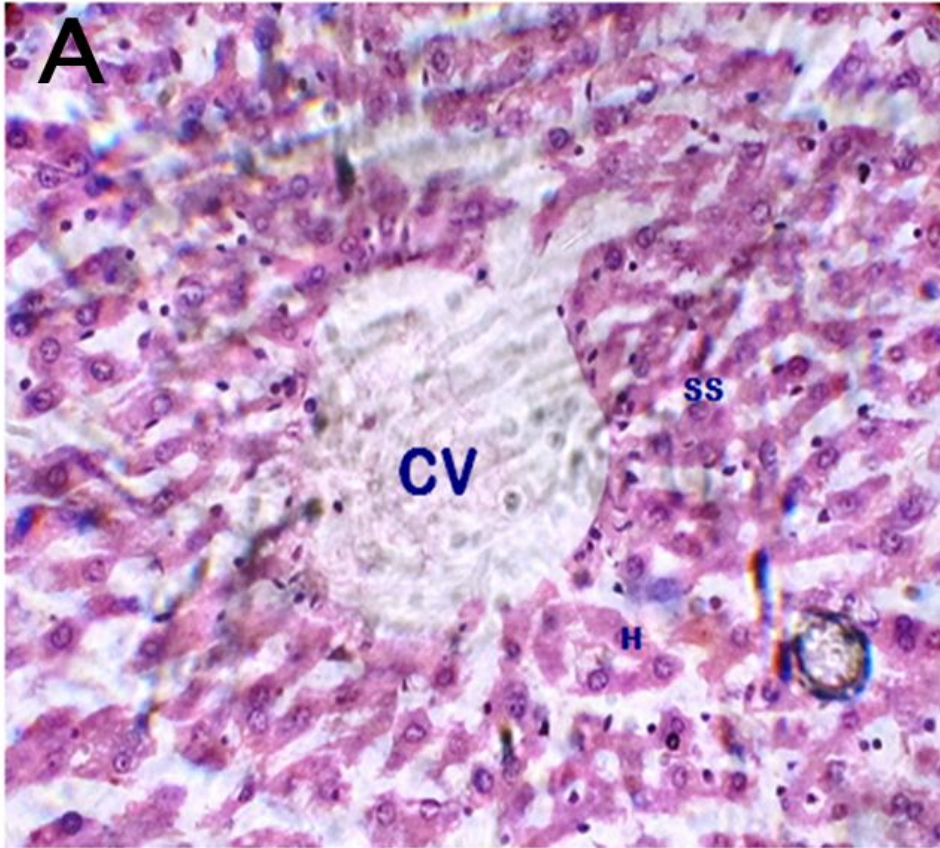


Plate 4.2: Photomicrograph of a section of the rat liver given distilled water only (control) (A) and rat liver administered with 250 mg/kg of DM (B) for 28 days (magnification x 400) showing normal hepatic architecture i.e. central vein (CV), Hepatocytes (H) and Sinusoidal spaces (SS). No significant histopathological changes

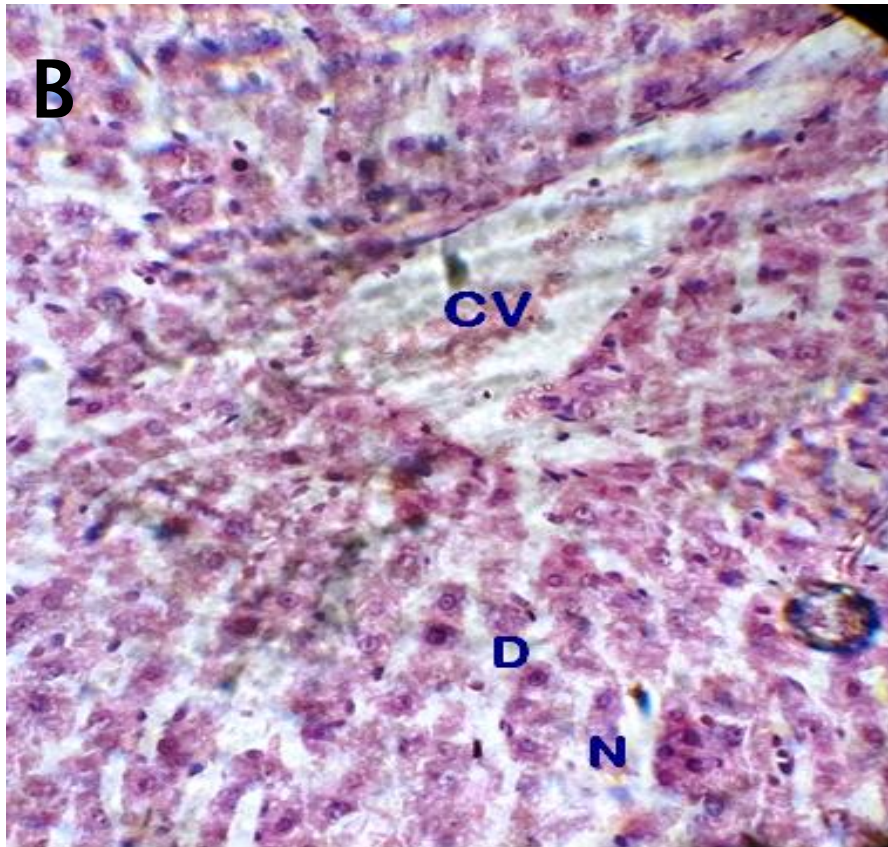
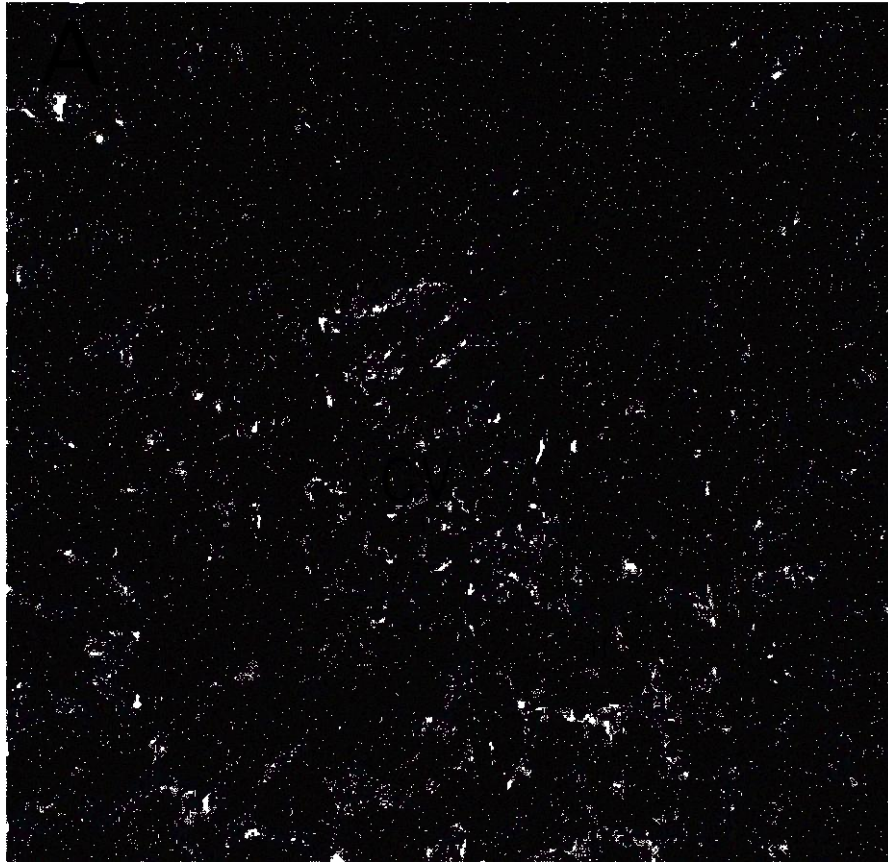


Plate 4.3: Photomicrograph of a section of the rat liver given distilled water only (control) (A) and rat liver administered with 500 mg/kg of DM (B) for 28 days (magnification x 400). Showing normal hepatic architecture i.e. central vein (CV), Hepatocytes (H) and Sinusoidal spaces (SS) in (A) and degeneration of hepatocytes (D), moderate hepatocellular necrosis, (N) and loss of hepatic architecture in (B)

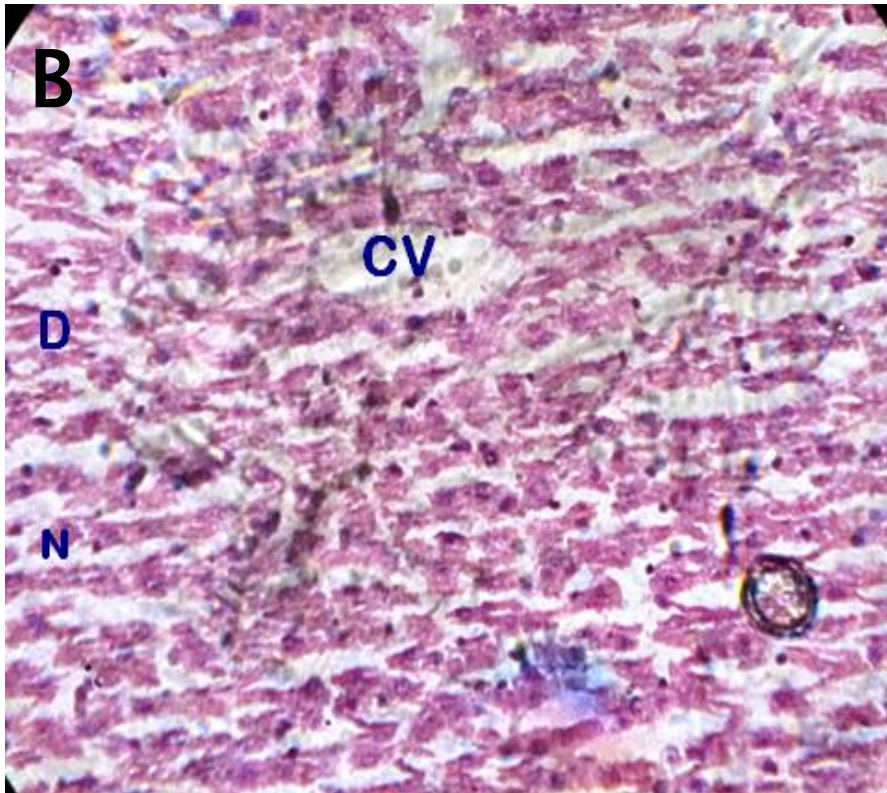
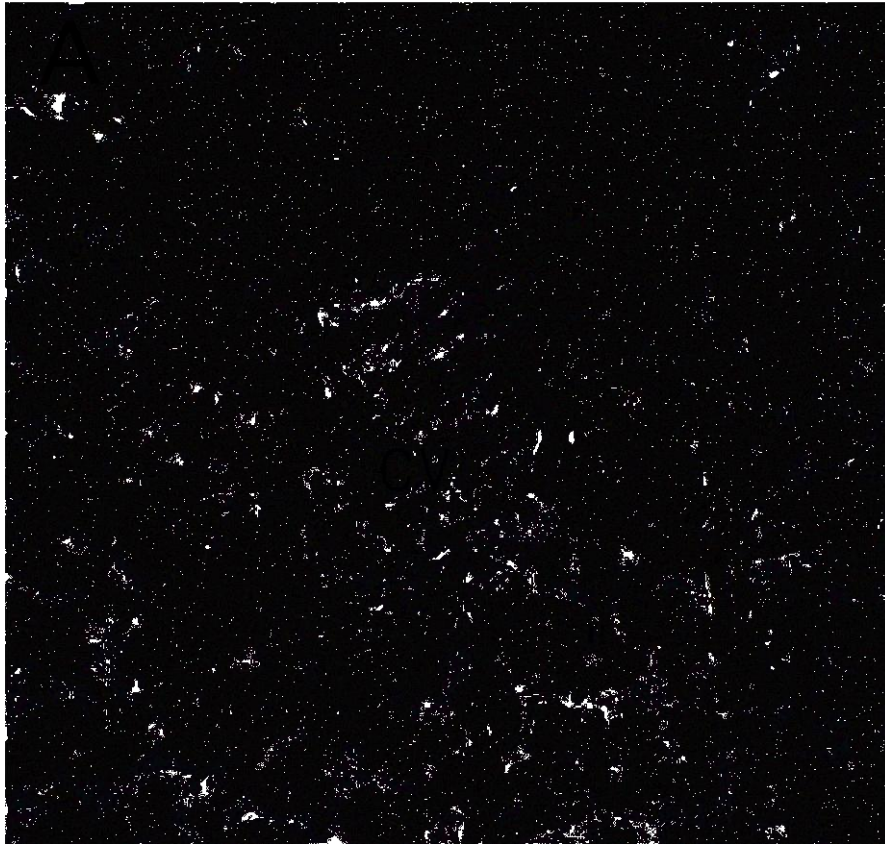


Plate 4.4: Photomicrograph of a section of the rat liver given distilled water only (control) (A) and rat liver administered with 1000 mg/kg of DM (B) for 28 days (magnification x 400). Showing normal hepatic architecture i.e. central vein (CV), Hepatocytes (H) and Sinusoidal spaces (SS) in (A) and degeneration of hepatocytes (D) loss of hepatic architecture and necrosis of hepatocytes (N)

4.5.2 Kidney

The methanol leaf extracts of *Diospyros mespiliformis* when administered orally for 28 days at doses of 50 and 250mg/kg does not cause any significant histopathological changes on the kidney similar to the control (A).The glomeruli, renal tubules and Bowman's spaces are clear and intact (Plate 4.5 and 4.6).At doses of 500mg/kg however, there was moderate degeneration of renal tubules with intact glomeruli (Plate 4.7). While with a dose of 1000mg/kg there was loss of renal tubular architecture and degeneration of renal tubules but with intact glomeruli (Plate 4.8)

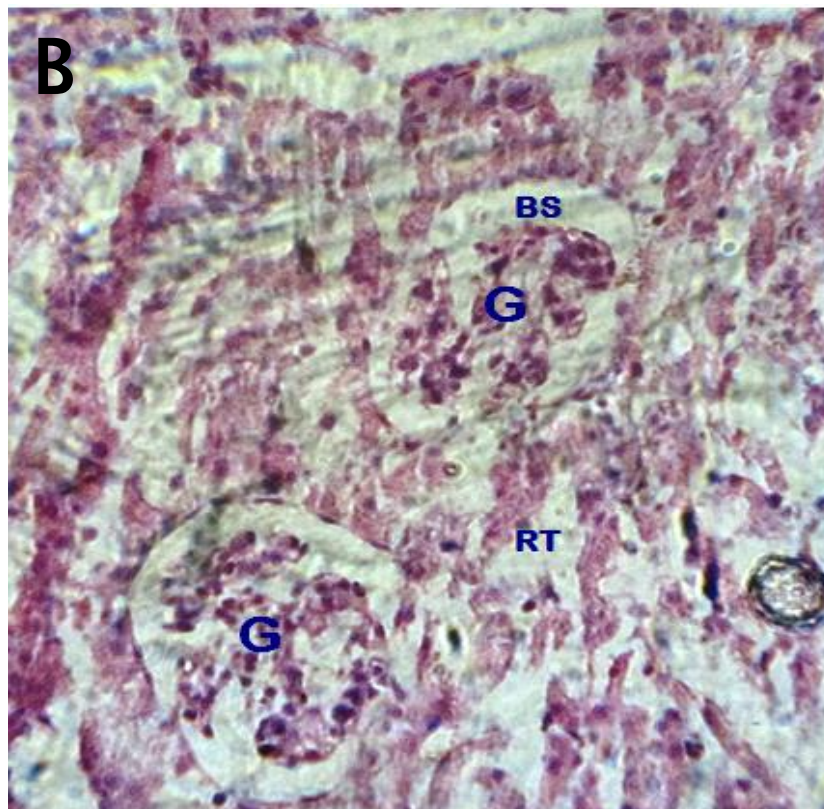
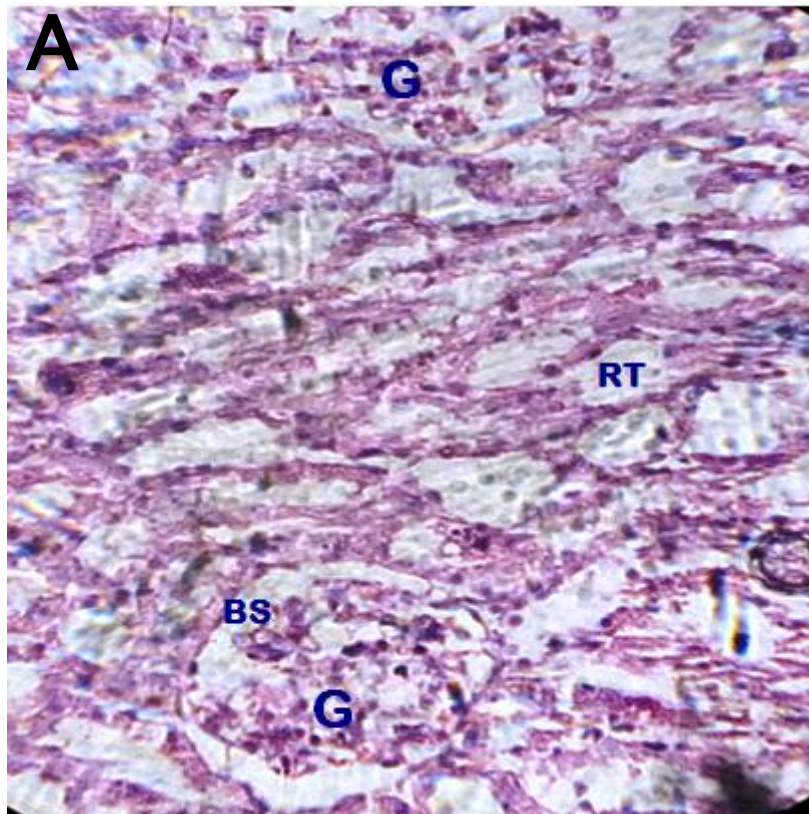


Plate 4.5: Photomicrograph of a section of the rat kidney given normal feed and distilled water only (control) (A) and 50 mg/kg DM (B) for 28 days (magnification x 400) showing normal glomeruli (G), Renal tubules (RT) and Bowman's spaces (BS). No any significant histopathological changes

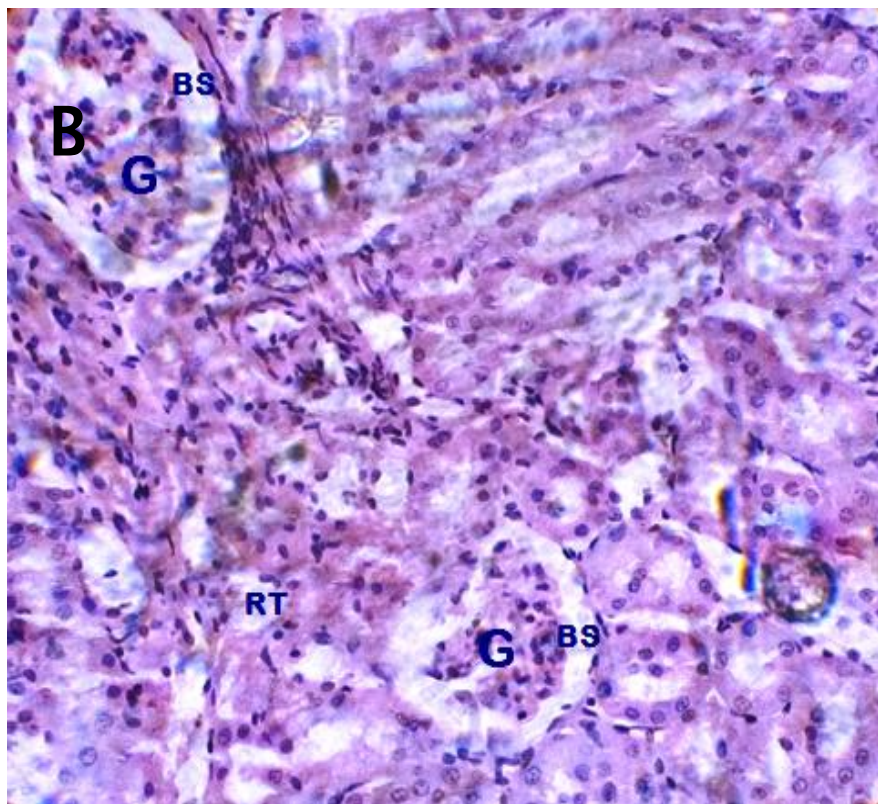
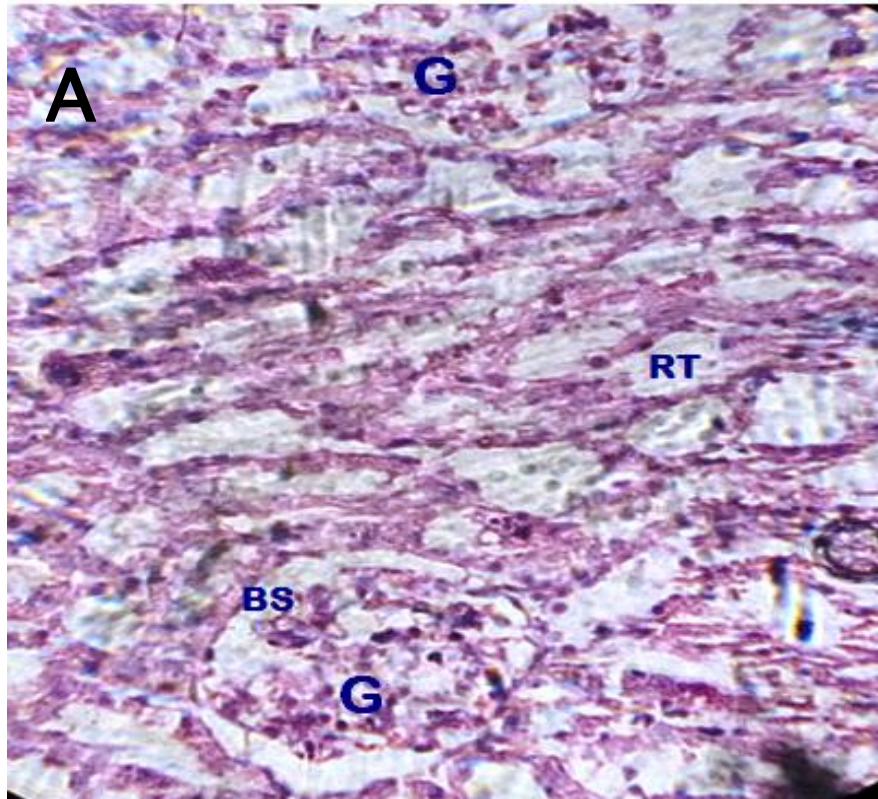


Plate 4.6: Photomicrograph of a section of the rat kidney given normal feed and distilled water only (control) (A) and 250 mg/kg DM (B) for 28 days (magnification x 400) showing normal glomeruli(G), Renal tubules (RT) and Bowman's spaces (BS). No any significant histopathological changes

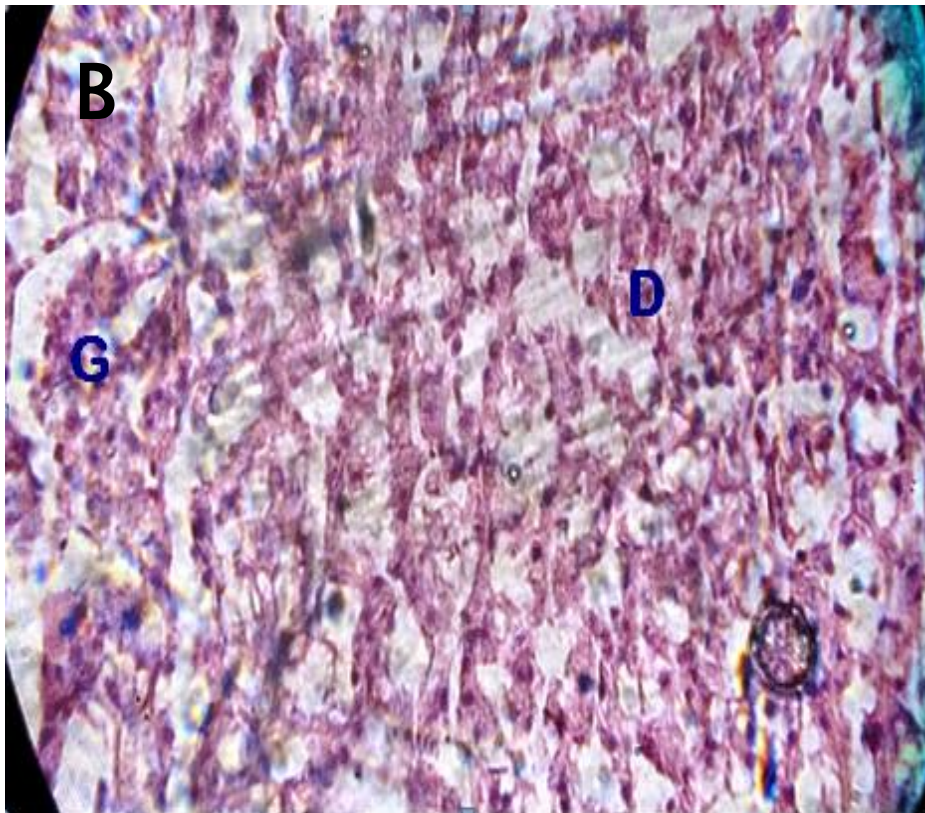
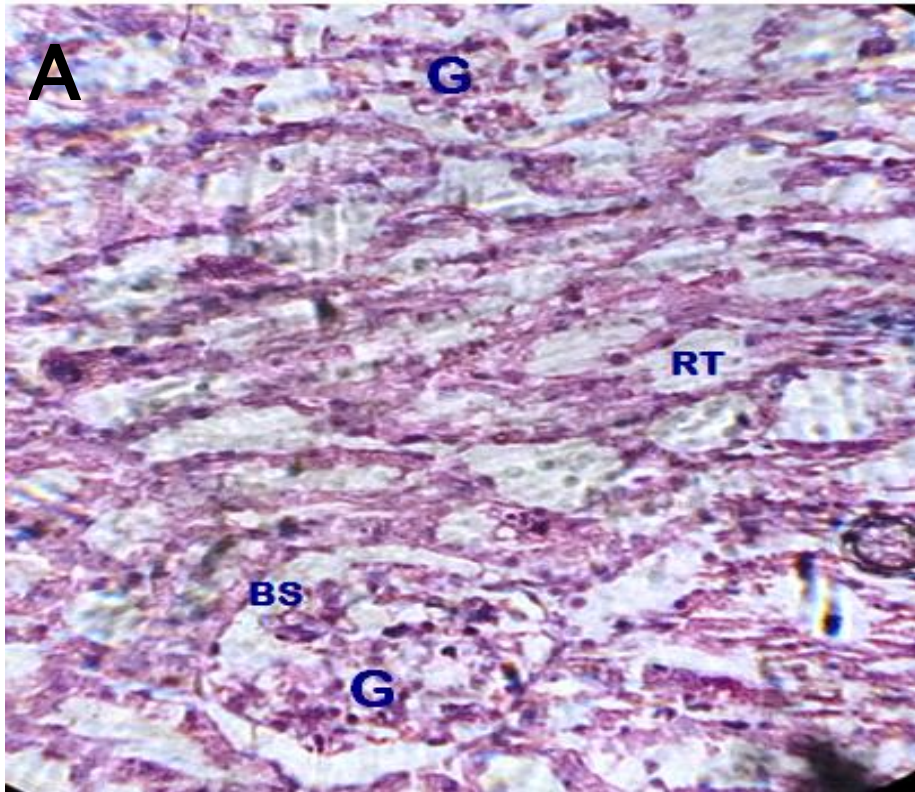


Plate 4.7: Photomicrograph of a section of the rat kidney given normal feed and distilled water only (control) (A) and 500 mg/kg DM (B) for 28 days (magnification x 400). A is showing normal glomeruli (G), Renal tubules (RT) and Bowman's spaces (BS). B is showing moderate degeneration of renal tubules (D) with intact glomeruli (G).

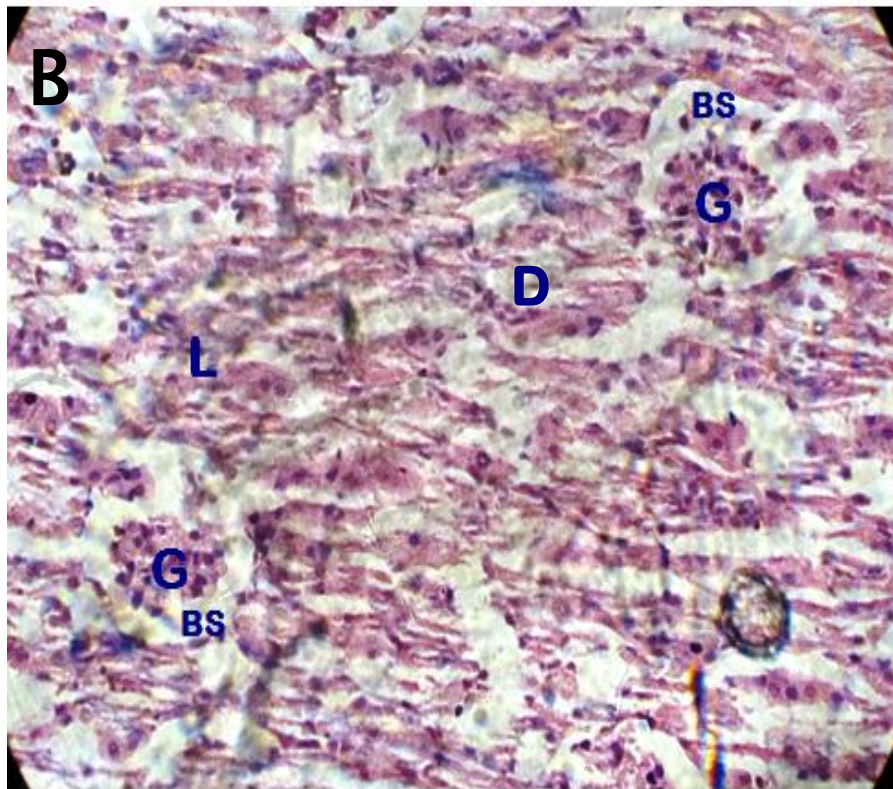
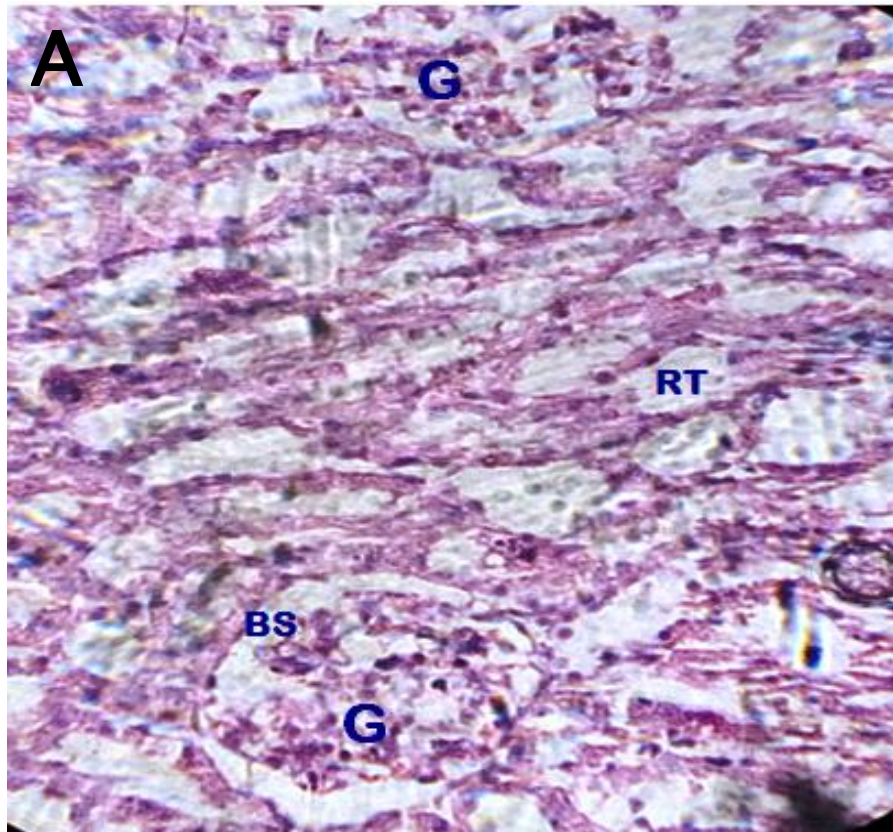


Plate 4.8: Photomicrograph of a section of the rat kidney given normal feed and distilled water only (control) (A) and 1000 mg/kg DM (B) for 28 days (magnification x 400) A is showing normal glomeruli (G), Renal tubules (RT) and Bowman's spaces (BS). B is showing loss of renal tubular architecture (L); degeneration of renal tubules (D) with intact glomeruli (G).

CHAPTER 5

5.0 DISCUSSION

The preliminary phytochemical studies of the methanol leaf extract of *Diospyros mespiliformis* showed the presence of alkaloids, saponins, flavonoids, tannins, carbohydrates, cardiac glycosides and the combined anthracene type of anthraquinones. Flavonoids, tannins and saponins are phytoconstituents that have been reported to modulate central nervous system activities (Danjuma *et al.*, 2010). For example, the saponin present in the plant, *Baccopa moniera* has been shown to possess anticonvulsant activity (Ray, 2004). Similarly, some alkaloids (nantenine derivatives) have been found to be effective in inhibiting pentylenetetrazole induced seizure and maximal electroshock-induced seizures (Ribeiro, 2003) hence, it can be inferred that the observed pharmacological activity shown by the leaf extract of *Diospyros mespiliformis* may be due to the presence of the aforementioned phytoconstituents.

The LD₅₀ values of the extract in mice and rats when administered via oral route were above 5000 mg/kg each while for the intraperitoneal route of administration, the LD₅₀ was found to be 774.6 mg/kg for both mice and rats. The result showed that the route of administration determines the extent of toxicity of extracts as the oral route is relatively safer than the intraperitoneal route. When drugs are administered intraperitoneally, the bioavailability is higher and hence the onset of action will be faster. The magnitude of action and the duration will also be higher depending on the affinity of the drug to the site of action leading to enhanced pharmacological effect. It is on the basis of this therefore that the toxicity of the extract is more pronounced when administered intraperitoneally than orally. Also the LD₅₀ in chicks was found to be above 5000 mg/kg intraperitoneally, this result showed that

species differences could play a key role in assessing toxicity of extracts as doses that were found to be toxic in mice and rats were subsequently found to be practically non-toxic when administered to chicks. This can be explained by the fact that drug metabolism to a very large extent is affected by species differences as some of the toxic effects elicited by some compounds might be due to their metabolites or the inability of the animal to detoxify and excrete the toxic compound

Studies have shown that pentylenetetrazole is a chemoconvulsant agent that induces seizure by the non-competitive blockage of the major inhibitory pathways mediated by the predominant inhibitory-neurotransmitter GABA, at all levels of the central nervous system (Desarro *et al.*, 1999) and is widely accepted experimental model for absence seizure (Loscher *et al.*, 1991). This GABA system represents the most successful target for the rational design of novel antiepileptic compounds (Loscher, 1998). It has been shown that seizures induced by pentylenetetrazole, can be blocked by drugs such as ethosuximide that reduces T-type Ca^{2+} currents (Rho and Sankar, 1999) while drugs such as phenytoin and carbamazepine that were found to be effective against maximal electroshock seizure are ineffective against pentylenetetrazole induce seizure (De Deyn *et al.*, 1992).

Activation of NMDA receptor system appears to be involved in the initiation and propagation of pentylenetetrazole-induced seizures (Velisek *et al.*, 1999). It has also been reported that agents that block glutamatergic excitation mediated by NMDA receptors such as felbamate also showed anticonvulsant activity against pentylenetetrazole-induced seizure (White, 1997).

Based on the foregoing, therefore it is possible that the observed anticonvulsant activity shown by the crude extract of *Diospyrus mespiliformis* leaves against pentylenetetrazole induced seizure might be due to either activation of GABA neurotransmission or blockade of NMDA mediated glutamatergic neurotransmission in the central nervous system. Anticonvulsant activity in the subcutaneous PTZ test identifies compounds that can raise seizure threshold in the brain (White *et al.*, 1998) and that agents such as phenorbabitone and benzodiazepines that were found to be effective in the management of generalized seizures of (absence or myoclonic) petit mal type are capable of raising seizure threshold induced by PTZ (Loscher *et al.*, 1991). The extract therefore can be said to possess some bioactive phytoconstituents that may be effective in the management of absence or myoclonic seizures. This finding agrees with the work of Adzu *et al.*, (2002) where the stem bark of the same plant *Diospyros mespiliformis* was found to significantly prolong onset of the phases of seizure activity induced by PTZ even though the stem bark extract could not protect the animals against lethality induced by PTZ (Adzu *et al.*, 2002).

The maximal electroshock test, is probably the best validated preclinical test that predicts drugs effective against generalized seizure of the tonic clonic (grand mal) type (White, 2003; Mares and Kupova, 2006; Holmes, 2007). It permits evaluation of the ability of a substance to prevent seizure spread through neural tissue of the central nervous system (Swinyard and Kupferberg, 1985; Loscher *et al.*, 1991; Holmes, 2007). Antiepileptic agents that act via this pathway are able to limit the repetitive firing of action potentials by slowing the rate of recovery of voltage-activated sodium channels from inactivation and suppress hind limb tonic extension in maximal electroshock seizures (Rho and Sankar, 1999). In

other words, the maximal electroshock tests predict drugs acting on sodium channels e.g. carbamazepine and phenytoin (Meldrum, 1996, 2002) and these agents are ineffective against PTZ induced seizure, (De Deyn *et al.*, 1992). The findings that the extract showed activity on pentylenetetrazole induced seizure and did not show any activity against maximal electroshock-induced seizures agrees with the work of De-Deyn *et al.*, (1992) who showed that agents that are effective against pentylenetetrazole induced seizure fails to alter maximal electroshock (MES) thresholds (De Deyn *et al.*, 1992). The extract was found not to alter maximal electroshock seizure thresholds as it fails to protect the chicks against maximal electroshock seizure possibly signifying that the extract does not act on sodium channels and hence may not be effective in generalized tonic clonic and partial seizures

The ability of the extract of *Diospyros mespiliformis* to delay onset of seizure in the strychnine test showed that the extract has the ability to raise seizure threshold even though it could not protect the animals against seizure and lethality induced by strychnine. 100% mortality was recorded for the control group and all the tested doses, however, the standard drug phenobarbitone afforded 83.33% protection and only 16.67% mortality. Strychnine and related alkaloids such as brucine and thebain induces generalized convulsion by selectively and competitively blocking postsynaptic neurotransmission mediated by glycine, an important inhibitory neurotransmitter to motor neurones and interneurones in the spinal cord (Vogel and Vogel, 2002). Agents that reverse the action of strychnine (an antagonist of glycine) have been shown to have antiepileptic effects (Raza *et al.*, 2001). The ability of the extract to exert activity by delaying the onset of strychnine induced convulsion and death may be a strong indication of glycine activation in the central nervous system.

4-aminopyridine induces tonic-clonic convulsions by blocking potassium channels (Yamaguchi and Rogawski, 1992); conversely agents that activate potassium channels have anticonvulsant effects in some experimental seizure models (Rostock *et al.*, 1996). Potassium channels play a vital role in the control of neuronal excitability and seizure susceptibility, and would be of importance for the suppression of seizure initiation and spread (Wickenden, 2002). The extract at doses of 50, 100, and 200 mg/kg seems not to have any activity on 4-aminopyridine induced seizure as it failed to significantly delay the onset of seizure or significantly protect the animals against 4-aminopyridine induced lethality.

The methanol leaf extract of *Diospyros mespiliformis* when administered at doses of 50, 250, 500 and 1000 mg/kg for twenty eight days causes increases in serum AST values especially at 250 mg/kg and 500 mg/kg and then a decrease AST value at 1000mg/kg, likewise, increase in ALT values was seen at 50, 500 and 1000 mg/kg, there is however a slight decrease in ALT value at 250mg/kg when compared with the control, but all these increases in both AST and ALT values were found not to be statistically significant when compared to the control at $P < 0.05$ (ANOVA post hoc dunnet). This findings disagrees with the work of Jigam *et al* 2012 who showed significant elevation of serum AST and ALT values with the methanolic root extract of *Diospyros mespiliformis*

The enzyme, AST formerly referred to as serum glutamic oxaloacetic transaminase (SGOT) is found in the heart and liver and moderately present in skeletal muscle, kidney and the pancreas (Schwartz and Garrison, 2008) while ALT, formerly called serum glutamic

pyruvic transaminase (SGPT) is predominantly and primarily found in the liver with clinically negligible quantities found in the kidneys, heart and skeletal muscle, hence elevation in serum ALT are more specific for liver related injuries or diseases (Schwartz and Garrison, 2008). Both AST and ALT are commonly measured in (diagnostic enzymology) serum and are sensitive indicators of necrotic lesions within the liver because of their high concentrations and easy liberations from the hepatocyte cytoplasm (Sease et al, 2008) where they are located and then released into the blood from the damaged cells (Schwartz and Garrison, 2008).

ALT catalyses the transfer of amino groups between L-alanine and glutamate to meet physiological needs. AST catalyses the transfer of amino acid and keto groups between alpha-amino acids and alpha-keto acids thereby acquiring the term transferase (Tolman and Rej, 1999) although serum levels of both ALT and AST rise whenever there is hepatocellular necrosis, elevations of ALT persist longer than do those of AST activity (Johnston, 1999) and AST/ALT ratio may sometimes determine if liver damage is related to alcohol dependence (Pratt and Kaplan, 2000; Rochling, 2001). Decrease AST and ALT levels; however have been observed in end stage liver disease.

The alkaline phosphatases (ALPs) constitutes a large group of isoenzymes that play important roles in the transport of sugar and phosphates and are derived primarily from liver and bones (Schwartz and Garrison, 2008). Though ALP is present in the liver only in small quantities, the enzyme is secreted into the bile and substantial elevation of serum ALP is seen with mild intrahepatic or extrahepatic biliary obstruction (Schwartz and Garrison, 2008) and early bile duct abnormalities leads to elevated ALP even before increases in

serum bilirubin is observed, while bilirubin is a breakdown product of haemoglobin derived from senescent red blood cells (Sease *et al.*, 2008) and because some bilirubin are also excreted into the bile (Schwartz and Garrison, 2008) its level rises in hepatobiliary obstruction secondary to gallstone or malignancy (Sease *et al.*, 2008). Hepatic damage involving the bile canalicular systems is referred to as cholestatic injury and is described as disturbance of the subcellular actin filaments around the canaliculi which prevent the movement of bile through the canaliculi system (Leonard *et al.*, 1987). The inability of the liver to remove bile causes intrahepatic accumulation of toxic bile acids and excretion products (Cullen, 2005). Drug induced cholestasis can occur either as an acute disorder (e.g. cholestasis with or without hepatitis and cholestasis with bile duct injury) or as a chronic disorder (e.g. vanishing bile duct syndrome, sclerosing cholangitis and cholelithiasis, Jaeschke *et al.*, 2002). However, the most common form of drug induced cholestasis is cholestasis with hepatitis (Kirchain and Allen, 2008). Elevations in serum ALP levels are more prominent and usually precede the elevations of other liver enzymes in serum (Navarro and Senior, 2006), hence the significant elevation of serum ALP observed with the extract might be due to extract-induced intrahepatic or extrahepatic cholestasis and this may probably precedes elevations in serum total bilirubin levels (Schwartz and Garrison, 2008) or other liver enzymes such as AST and ALT in serum (Navarro and Senior, 2006). Drugs such as chlorpromazine or sulfonamide (Schwartz and Garrison, 2008) and others such as erythromycin estolate, amoxicillin/clavulanic acid and carbamazepine, (Watkins and Seeff, 2006) are known to cause cholestatic injury.

Potassium is the major intracellular monovalent cation in the body and is filtered freely at the glomerulus of the kidney, reabsorbed in the proximal tubule and secreted into the distal segment of the nephron (Schwartz and Garrison 2008). Because majority of potassium is sequestered within cells, a serum potassium concentration is not a good measure of total body potassium (Schwartz and Garrison, 2008), even though studies have shown that specimen processing, handling and transport are known to affect specimen quality (Stankovic and Smith, 2004). Delays in transport and processing, transport conditions, centrifugation and specimen storage temperature can cause haemolysis and influence the serum potassium level (Stankovic and Smith, 2004). It was reported that serum potassium level increases after 2 hours from collection (Laessig *et al.*, 1976) and that excessive agitation or mixing can also lead to increase potassium level in serum (Pragay, 1974). Also plant cells with their very high intra-cellular potassium content especially with the higher doses might possibly contribute to elevated potassium levels, However, the elevated potassium level observed with the extract might be as a result of extract induced distal tubular necrosis which leads to hyperkalaemia from impaired potassium excretion . Other manifestations of drug induced distal tubular necrosis are polyuria from failure to maximally concentrate urine and metabolic acidosis from impaired urinary acidification. Studies has shown that acute tubular necrosis is the most common presentation of drug induced nephrotoxicity and that primary agents implicated are aminoglycosides, radiocontrast media, cisplatin, amphotericin B, foscarnet and Osmotically active agents (Nolin and Himmelfarb, 2008).

Creatinine on the other hand is an amino acid that is freely filtered by the glomerulus (Stevens *et al.*, 2006), it is derived from creatine and phosphocreatine, major constituents of muscle (Schwartz and Garrison, 2008). Each day about 1-2% of muscle creatine is converted to creatinine and that men tend to have higher level of creatinine than women because they generally have a higher mass of skeletal muscle (Howard, 1989). Once creatinine is released from muscle to plasma, it is excreted almost exclusively by glomerular filtration and a decrease in glomerular filtration rate (GFR) results in an increase in serum creatinine (Schwartz and Garrison, 2008), consequently, measuring serum creatinine is a simple kidney function test and it is the most commonly used indicator of renal function, though dietary intake of creatine or eating a lot of meat can increase daily creatinine excretion (Howard, 1989) and variations in creatinine level have been noted in different age, geographic, ethnic and racial groups (Jafar *et al.*, 2003). The methanol leaf extract of *Diospyros mespiliformis* causes a significant decrease in the level of serum creatinine and this can only be seen in debilitation or decreased muscle mass like muscular dystrophy or myasthenia gravis and does not reflect drug induced decrease in glomerular filtration rate, even though studies have shown that extra renal elimination of creatinine may be increased at low levels of glomerular filtration rates (Stevens *et al.*, 2006). This increase is mainly related to the degradation of creatinine by intestinal bacteria (Stevens and Levey, 2005) hence for these reasons, the relationship between the level of serum creatinine and GFR varies substantially among persons and over time (Stevens *et al.*, 2006) and the use of a single range of serum creatinine to distinguish between a normal and abnormal GFR can be misleading (Stevens and Levey, 2005).

A slight increase in sodium is observed with the extract especially at 500 and 1000mg/kg though not significant but it might possibly be due to dehydration as decrease serum sodium is probably the result of dilutional hyponatremia of cirrhosis or depletion hyponatremia e.g. salt losing nephritis or may be tubular lesion.

There is also a slight increase in total calcium especially at 50, 500 and 1000mg/kg of the extract, though not statistically significant but might reflect hyperparathyroidism or thiazide diuretic-like effects (Schwartz and Garrison, 2008).

The extract causes a slight decrease in glucose at all the tested doses, though not significant but can be suggestive of severe liver disease or insufficient carbohydrate intake (Schwartz and Garrison, 2008).

Urea nitrogen is an end product of protein metabolism, and it is produced solely by the liver, transported in the blood and excreted by the kidneys (Schwartz and Garrison, 2008) hence measurement of serum urea nitrogen is reflective of renal function because urea nitrogen is also like creatinine, filtered completely at the glomerulus of the kidney, reabsorbed and tubularly secreted within nephrons (Schwartz and Garrison, 2008) hence it measures kidney function. The slight decrease in urea observed with the extract at doses of 250, 500 and 1000mg/kg though not significant, might be related to renal failure, significant malnourishment (decreased protein intake) or severe liver disease where the liver can no longer form urea (Schwartz and Garrison, 2008).

The extract at all the tested doses causes a slight decrease in total protein, though not significant but it can be suggestive of possible hepatic insufficiency

There is also a slight decrease in the level of albumin at all the tested doses when compared with the control, though not significant but can be reflective of impaired albumin synthesis by the liver secondary to hepatic insufficiency (Schwartz and Garrison, 2008). Albumin can also be lost directly into the urine secondary to nephrosis.

Total cholesterol levels are elevated in biliary obstruction and nephrotic syndrome and the values are decreased in severe liver insufficiency. The extract at all the tested doses causes a slight decrease in total cholesterol when compared with the control, though not significant but can be indicative of liver insufficiency.

The histopathological studies of the rat liver revealed normal hepatic architecture devoid of any significant histopathological findings with the 50 and 250mg/kg of the extract similar to the control group. The 50mg/kg dose of the extract is the lowest dose that does not produce any detectable toxic reaction (Leclair and Willard, 1970), i.e. the so called No observed Adverse effect level (NOAEL) dose and the 250mg/kg is close to 30% of the LD₅₀ signifying that the methanol leaf extract of *Diospyros mespiliformis* at LD₅₀ dose used in this study and below is relatively non toxic to the liver, i.e. not hepatotoxic at the subchronic duration, this is evident by the normal hepatic architecture, clear hepatic cord, sinusoid, central vein etc, However, at doses of 500mg/kg, there is moderate degeneration of hepatocytes, necrosis of hepatocytes and overall loss of hepatic architecture and with 1000mg/kg dose of the extract, there is significant degeneration of hepatocytes, necrosis of

hepatocytes and loss of hepatic architecture, meaning that there is dose dependent increase in hepatotoxicity with doses above 500mg/kg. The hepatocellular necrosis and degeneration observed with the extract especially at doses of 500 and 1000mg/kg might be responsible for some of the results observed with the blood chemistry for both the liver and kidney function parameters. Significant elevation of alkaline phosphatase (ALP) is suggestive of drug induced cholestasis in which case elevation of ALP usually precedes elevation of aspartate Aminotransferase and alanine aminotransferase (Navarro and Senior,2006) and since cholestasis with hepatitis was reported to be the most common form of drug induced cholestasis. This probably explains the insignificant increase in TB, ALT and AST despite hepatocellular degeneration and necrosis observed with the histopathological findings. The extract therefore can be said to probably cause cholestasis with hepatitis which is the most common form of drug induced cholestasis (Kirchain and Allen,2008).

The histopathological studies of the rat kidney also revealed intact glomeruli and renal tubules with the 50 and 250 mg/kg of the extract similar to the control group, devoid of any significant histopathological findings. The 50mg/kg is the lowest dose that doesn't cause any adverse effect while the 250mg/kg almost equals the LD₅₀ dose used in this study. This shows that the LD₅₀ dose used in the study is relatively non toxic to the kidney, However, at doses of 500mg/kg dose of the extract, there is moderate degeneration of renal tubules with intact glomeruli and with 1000mg/kg dose of the extract there is significant loss of renal tubular architecture, degeneration of renal tubules but with intact glomeruli signifying a dose dependent nephrotoxicity at doses of 500mg/kg and above. The renal tubular toxicity might be responsible for some of the results observed with the renal function parameters. It

has been shown that the primary function of the cells of the proximal tubules is to return approximately 80 percent of the glomerular filtrate into the peritubular blood and hence into the systemic circulation (Gans and Mercer, 2004), consequently, indication of proximal tubular injury might includes reduction in serum phosphate, uric acid, magnesium and potassium as a result of increased urinary losses. The fact that our extract causes renal tubular injury without affecting the glomerulus agrees with the findings that nephrotoxicity may be evidence by the alterations in renal tubular function without loss of glomerular filtration (Nolin and Himmelfarb, 2008).

CHAPTER 6

6.0 SUMMARY, CONCLUSION AND RECOMMENDATIONS

6.1 Summary

The plant *Diospyros mespiliformis* belongs to the family of Ebenaceae and is indigenous to Nigeria and other West African countries. The methanol leaf extract of *Diospyros mespiliformis* contain important phytoconstituent such as alkaloids, saponins, flavonoids, tannins, carbohydrates, cardiac glycosides and combined anthracene type of anthraquinones.

LD₅₀ values in rats and mice were found to be 774.6 mg/kg intraperitoneally and >5000 mg/kg orally while for chicks, the LD₅₀ value was found to be >5000 mg/kg intraperitoneally. The result showed that toxic effect of extracts is greatly influenced by routes of administration as well as specie variation.

The methanol leaf extract of *Diospyros mespiliformis* showed a significant anticonvulsant activity against pentylenetetrazole induced seizure in mice. From 50% protection with a dose of 50 mg/kg of extract to 66.7% protection at 100 mg/kg dose. The extract also significantly delayed the onset of seizure against strychnine induced seizure even though it could not protect the animals against lethality induced by strychnine. The extract however could not protect the animals against seizure induced by both maximal electroshock and 4-aminopyridine. However, the extract in the sub-chronic toxicity studies showed significant elevation of ALP possibly signifying drug induced cholestasis. There was, though not statistically significant elevations of AST and ALT, since cholestasis with hepatitis is the most common form of drug induced cholestasis, it can be inferred that the rise in ALP

probably precedes the rise in TB, AST and ALT hence their low values despite apparent hepatocellular necrosis from the histopathological findings.

Serum potassium was significantly elevated signifying possible extract induced renal distal tubular necrosis that leads to impaired potassium excretion with consequent hyperkalaemia. The significantly decreased creatinine level might possibly be due to debilitation or muscular dystrophy induced by the extract. Histopathological studies revealed dose dependent hepatotoxicity and nephrotoxicity of the extract. Doses of 50 to 250 mg/kg of the extracts were found to be relatively safe with no apparent hepatotoxicity or nephrotoxicity. However doses of 500 and 1000 mg/kg of the extract revealed moderate (with 500 mg/kg) to significant (with 1000 mg/kg) degeneration of hepatocytes, necrosis of hepatocytes and loss of hepatic architecture with the liver and moderate (with 500 mg/kg) to significant (with 1000 mg/kg) loss of renal tubular architecture and degeneration of renal tubules but with intact glomeruli with the kidney. This also showed that the extract might have a low therapeutic index as almost a doubling of the LD₅₀ dose might portends danger of hepatotoxicity and nephrotoxicity.

6.2

Conclusion

The data presented, analyzed and discussed suggests that the use of the leaves of *Diospyros mespiliformis* in some parts of Nigeria as a traditional remedy for convulsive disorders is to some extent scientifically justified. However, at higher doses, the plant is capable of causing drug induce cholestasis with hepatitis progressing to significant liver and kidney impairment. So the dose of the extract should be carefully measured and kept low to avoid extract induced hepatotoxicity and nephrotoxicity.

6.3

Recommendations

1. Since the plant is widely distributed in Nigeria, the leaves should be recollected from another geographical area (possibly southern Nigeria); to see if results obtained from this study can be reproduced.
2. The methanol leaf extract of *Diospyros mespiliformis* should be subjected to fractionation using a variety of solvents of different polarities such as n-Hexane, chloroform, ethyl acetate, n-Butanol etc. This might possibly lead to the isolation of the pure compound responsible for the observed pharmacological activity.
3. Since anticonvulsant agents also exhibit behavioural properties, it is good to investigate the behavioural properties of the methanol leaf extract of *Diospyros mespiliformis* as this may further validate its use in the management of convulsive disorders.

4. The extract should be subjected to chronic toxicity studies possibly at doses of 250mg/kg and less to determine the effect of long time administration on vital organs notably liver and kidney of laboratory animals so as to fully ascertain its safety profile. This is important since convulsive disorders usually require long time management.

5. There is need to carry out further studies on the methanol leaf extract of *Diospyros mespiliformis* so as to isolate and characterize the bioactive phytoconstituents responsible for the observed pharmacologic effects which can lead to the development of more active compounds through structural modification of the parent compound

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APPENDIX I

Percentage Yield

Weight of crushed leaves of *Diospyros mespiliformis* before maceration = 1326 g. Weight of extract of *Diospyros mespiliformis* after filtration and evaporation = 63g.

$$\begin{aligned}\% \text{ Yield} &= \frac{\text{Weight of extract}}{\text{Weight of crushed leaves}} \times 100 \\ &= \frac{63}{1326} \times 100 = 4.75\%\end{aligned}$$

APPENDIX II

LD₅₀ Determination in Rats, Mice and Chicks by Intraperitoneal (i.p) and Oral (p.o) Routes of Administration

I (a) RATS (ORAL)

1ST PHASE

Number of animals	Dose (mg/kg)	Mortality
3	1000	0/3 (none died)
3	100	0/3 (none died)
3	10	0/3 (none died)

2ND PHASE

Number of animals	Dose (mg/kg)	Mortality
1	5000	0/1 (no death)
1	2900	0/1 (no death)
1	1600	0/1 (no death)
1	1200	0/1 (no death)

Therefore, LD₅₀ is greater than 5000 mg/kg orally in Rats

I (b) RATS (ip)

1ST PHASE

Number of animals	Dose (mg/kg)	Mortality
3	1000	3/3 (all died)
3	100	0/3 (none died)
3	10	0/3 (none died)

2ND PHASE

Number of animals	Dose (mg/kg)	Mortality
1	600	0/1 (no death)
1	370	0/1 (no death)
1	225	0/1 (no death)
1	140	0/1 (no death)

$$LD_{50} = G.M = \sqrt{XY}$$

LD_{50} = Median lethal dose

G.M. = Geometric mean

Y = Highest non lethal dose = 600 mg/kg

X = lowest lethal dose = 1000 mg/kg

$$\text{Hence } LD_{50} = \sqrt{(600 \times 1000)}$$

$$= \sqrt{600,000}$$

$$= 774.6 \text{ mg/kg}$$

LD_{50} = 774.6 mg/kg (ip) in Rats

II (a) MICE (ORAL)

1ST PHASE

Number of animals	Dose (mg/kg)	Mortality
3	1000	0/3 (none died)
3	100	0/3 (none died)
3	10	0/3 (none died)

2ND PHASE

Number of animals	Dose (mg/kg)	Mortality
1	5000	0/1 (no death)
1	2900	0/1 (no death)
1	1600	0/1 (no death)
1	1200	0/1 (no death)

Therefore, LD₅₀ is greater than 5000 mg/kg orally in Mice

ii (b) MICE (ip)

1ST PHASE

Number of animals	Dose (mg/kg)	Mortality
3	1000	3/3 (all died)
3	100	0/3 (none died)
3	10	0/3 (none died)

2ND PHASE

Number of animals	Dose (mg/kg)	Mortality
1	600	0/1 (no death)
1	370	0/1 (no death)
1	225	0/1 (no death)
1	140	0/1 (no death)

$$LD_{50} = G.M = \sqrt{XY}$$

LD_{50} = Median lethal dose

G.M. = Geometric mean

Y = Highest non lethal dose (1/1) = 600 mg/kg

X = lowest lethal dose (0/1) = 1000 mg/kg

$$\text{Hence } LD_{50} = \sqrt{(600 \times 1000)}$$

$$= \sqrt{(600,000)}$$

$$= 774.6 \text{ mg/kg}$$

LD_{50} = 774.6 mg/kg (ip) in Mice

I11(a) CHICKS (ip)

1ST PHASE

Number of animals	Dose (mg/kg)	Mortality
3	1000	0/3 (none died)
3	100	0/3 (none died)
3	10	0/3 (none died)

2ND PHASE

Number of animals	Dose (mg/kg)	Mortality
1	5000	0/1 (no death)
1	2900	0/1 (no death)
1	1600	0/1 (no death)
1	1200	0/1 (no death)

Therefore, LD₅₀ is greater than 5000 mg/kg intraperitoneally in chicks

APPENDIX III

LIVER ENZYMES

Hepatocellular Function Parameters

Treatment	Dose mg/kg	AST	ALT
Distill water		136.6±16.10	85.5±7.57
DM	50	133±10.73	86.8±5.50
DM	250	143.3±15.8	80.7±3.48
DM	500	152.3±25.21	90.3±11.88
DM	1000	112±5.29	122±41.47

Data were analyzed using ANOVA post hoc dunnet and presented as mean ± SEM, n=6 per group DM=*Diospyros mespiliformis*. AST = *Aspartate aminotransferase*, ALT=*Alanine aminotransferase*. Values not significant at $P < 0.05$

APPENDIX IV

LIVER ENZYMES

Hepatobiliary Function Parameters

Treatment	Dose mg/kg	ALP	TB
Distill water		189.5±9.57	10.8±3.42
DM	50	225.7*±19.48	8.2±3.64
DM	250	142±9.65	6.5±3.96
DM	500	254*.4±23.15	9.8±3.99
DM	1000	320***.3±22.24	8.2±4.71

Data were analyzed using ANOVA post hoc dunnet and presented as mean ± SEM, n=6 00.per group DM=*Diospyros mespiliformis*. ALP = Alkaline Phosphatase, TB Total Bilirubin. * Statistically significant at $P < 0.05$, *** $P < 0.001$

APPENDIX V

KIDNEY FUNCTION PARAMETERS

	K	Na	Total Ca	Glucose	Total cholesterol	Urea	Creatinine	Total protein	Albumin
D.water	5.6±0.23	134.3±9.41	1.9±0.10	4.9±0.41	2.0±0.15	6.1±0.10	38.6±0.6	73.2±3.67	31.2±1.46
DM 50	5.7±0.24	122.2±4.24	2.0±0.14	4.7±0.51	1.7±0.23	6.2±0.75	33.5±2.20	65.5±5.74	28.0±2.49
DM 250	6.6*±0.15	134.5±2.38	1.9±0.13	4.5±0.30	1.9±0.28	5.0±0.65	29.8*±1.88	63.8±2.75	27.2±1.16
DM 500	5.3±0.23	217.7±51.89	2.0±0.06	3.7±0.23	1.9±0.21	4.3±0.46	30.4*±1.17	68.2±4.05	29±1.67
DM 1000	5.5±0.34	141.8±12.72	2.3±0.39	4.5±0.19	1.5±0.18	4.8±0.43	30.5***±2.25	65.5±4.72	28±2.12

Data were analyzed using ANOVA post hoc dunnet and presented as mean ± SEM, n=6 per group, DM = *Diospyros mespiliformis*, K=Potassium, Na=Sodium, Total Ca = Total Calcium. Statistically Significant at *P<0.05 and *** P<0.001