PROTECTIVE EFFECTS OF VITAMIN C AND METHANOL LEAF EXTRACT OF *Moringa oleifera* ON CHRONIC CARBAMAZEPINE-INDUCED REPRODUCTIVE TOXICITY IN MALE WISTAR RATS

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A DISSERTATION SUBMITTED TO THE SCHOOL OF POSTGRADUATE STUDIES, AHMADU BELLO UNIVERSITY ZARIA, IN PARTIAL FULFILMENT OF THE REQUIREMENT FOR THE AWARD OF THE DEGREE OF MASTERS OF SCIENCE IN VETERINARY TOXICOLOGY

DEPARTMENT OF VETERINARY PHARMACOLOGY AND TOXICOLOGY, AHMADU BELLO UNIVERSITY, ZARIA, KADUNA STATE, NIGERIA

JUNE, 2018

DECLARATION

I declare that the work in this thesis, entitled 'PROTECTIVE EFFECT OF VITAMIN C AND METHANOL LEAF EXTRACT OF *MORINGA Oleifera* ON CHRONIC CARBAMAZEPINE-INDUCED REPRODUCTIVE TOXICITY IN MALE WISTAR RATS' has been performed by me in the Department of Pharmacology and Toxicology, Ahmadu Bello University, Zaria under the supervision of Prof S.F. Ambali and Dr H.G. Mikai'l. The information derived from the literature has been duly acknowledged in the text and list of references provided. No part of this thesis was previously presented for another degree or diploma at any university.

Ganiu Jimoh AKOREDE	_____	_____
Name of student	Signature	Date

CERTIFICATION

This thesis entitled "PROTECTIVE EFFECT OF VITAMIN C AND METHANOL LEAF EXTRACT OF *MORINGA oleifera* ON CHRONIC CARBAMAZEPINE-INDUCED REPRODUCTIVE TOXICITY IN MALE WISTAR RATS" by GANIU JIMOH AKOREDE meets the regulations governing the award of the degree of Master of Science of Toxicology of Ahmadu Bello University, Zaria, and is approved for its contribution to knowledge and literary presentation.

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DEDICATION

This thesis is dedicated to almighty Allah (S.W.T) for his assistance and guidance throughout the course of this project work and also to my late father, Alhaji Babatunde Jimoh Awoye. May almighty Allah make Aljanat firdaus his final destination, amin.

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ABSTRACT

Carbamazepine (CBZ) is one of the most widely used drugs in the management of epilepsy. However, chronic usage has resulted in male reproductive disorders, which may be partly due to oxidative stress evoked by the drug. The present study was aimed at evaluating the effect of vitamin C and methanol extract of *Moringa oleifera* (MO) leaf in ameliorating adverse reproductive effect, induced by chronic CBZ administration in male Wistar rats. Seventy adult male Wistar rats were divided at random into seven groups of 10 rats each. Group I (DW) was given distilled water (2 ml/kg) daily; Group II (Vit-C) was administered vitamin C (100 mg/kg); Group III (MO) was administered *Moringa oleifera* (250 mg/kg~ $1/20^{th}$ of LD_{50}), while Group IV (CBZ) was exposed to carbamazepine (20 mg/kg) only. Group V (Vit-C+CBZ) was pretreated with vitamin C (100 mg/kg) before exposure to CBZ (20 mg/kg), 30 minutes later. Group VI (MO+CBZ) was pretreated with MO (250 mg/kg) followed by CBZ (20 mg/kg), 30 minutes later, while group VII (Vit-C+MO+CBZ) was pretreated with a combination of vitamin C (100 mg/kg) and MO (250 mg/kg), followed by CBZ, 30 minutes later. The regimens were administered orally by gavage once daily for a period of 15 weeks. Thereafter, the rats were sacrificed by jugular venesection; the pituitary glands and testicular tissues were assayed for the levels of malondialdehyde (MDA), superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx). The sera samples were assayed for the concentrations of follicle stimulating hormone (FSH), luteinizing hormone (LH) and testosterone, while the epididymal sperm count and sperm motility were also determined from semen collected. The pituitary glands, seminal vesicles and testes were examined for gross and histopathologic changes. The result showed a significant ($P < 0.05$) increase in concentrations of pituitary and testicular MDA and a decrease in the activities of pituitary and testicular SOD, CAT and GPx in the group exposed to CBZ. These

changes were ameliorated in Vit-C+MO+CBZ, MO+CBZ and Vit-C+CBZ groups. The significant decrease in testosterone concentration in the CBZ group was ameliorated in the Vit-C+MO+CBZ and Vit-C+CBZ groups. The absolute decreased values in the FSH and LH concentrations in CBZ group were ameliorated in the Vit-C+MO+CBZ, MO+CBZ and Vit-C+CBZ groups. A significant decrease in epididymal sperm count in CBZ group was mitigated in the MO+Vit-C +CBZ group, while the relative decrease in sperm motility in the CBZ group was ameliorated in the MO+Vit-C+CBZ, MO+CBZ and Vit-C+CBZ groups. A significant increase in percentage sperm mid-piece abnormality in the CBZ group was ameliorated in the MO+Vit-C+CBZ group. Degeneration of spermatogonia cells, severe neutrophils depletion and loss of cellular projection of the pituicytes and severe haemorrhages observed in the seminal vesicle of rats in the CBZ group were not observed in the MO+Vit-C+CBZ, MO+CBZ and Vit-C+CBZ groups. The study showed that pretreatment with vitamin C and/or MO ameliorated the alterations in sex hormone, semen characteristics, oxidative stress parameters and pituitary gland and testicular pathological changes induced by chronic CBZ administration, partly due to their antioxidant potentials.

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LIST OF ABBREVIATIONS/SYMBOLS

$^1O_2$ – Oxygen singlet

AEDs - Antiepileptic drugs

ALP – Alkaline phosphate

ANOVA – Analysis of variance

ATP – Adenosine triphosphate

BPI – Bureau of plant industry

CAT- Catalase

CBZ- Carbamazepine

Cu – Copper

CYP- Cytochrome P

DHEA – Dehydroepiandrosterone

DNA – Deoxyribonucleic acid

DW – Distilled water

E- Epithelial lining

ELISA – Enzyme-linked immunosorbent assay

FSH – Follicle-stimulating hormone

GABA - Gamma amino butyric acid

GBP - Gabapentin

GnRH – Gonadotropin-releasing hormone

GPx – Glutathione peroxidase

GSH – Glutathione

GSR – Glutathione reductase

GSSH – Glutathione disulphide

GSTs – Glutathione S-transferases

H_2O – Water

H_2O_2 - Hydrogen peroxide

HA – Head abnormality

HO^- - Hydroxyl radical

IC – Interstitial cells

IS – Interstitial space

L – Lumen

LD_{50} – Median lethal dose

LDL – Low density lipoprotein

LH – Luteinizing hormone

LPO – Lipid peroxidation

MDA – Malondialdehyde

Mn – Manganese

MO- *Moringa oleifera*

N_2 – Nitrogen atom

Na^+ - Sodium ion

NADP - Nicotinamide adenine dinucleotide phosphate

NADPH – Nicotinamide adenine dinucleotide phosphate hydrogen

NMDA- *N*-methyl-D-aspartate

NO_2^- - Nitrogen dioxide radical

O_2 – Oxygen

O_2^-- Superoxide anion radical

OH^- - Hydroxyl radical

Os – Oxidative stress

P – Pituicyte

PCOS – Polycystic ovarian syndrome

PHE - Phenytoin

PUFAs – Polyunsaturated fatty acids

QAE – Quercetin equivalent

RNA – Ribonucleic acid

RNS – Reactive nitrogen species

NS – Reactive species

ROS- Reactive oxygen species

SG- Spermatogenic cells

SHBG- Sex hormone-binding globulin

SM – Smooth muscle

SOD – Superoxide dismutase

ST – Seminiferous tubule

TA – Tail abnormality

TBA – Thiobarbituric acid

TBARS – Thiobarbituric reactive substances

TCA – Trichloroacetic acid

USA – United State of America

UV – Ultraviolet

V_d – Apparent Volume of distribution

Vit-C – Vitamin C

WBC – White blood cell

Zn – Zinc

CHAPTER ONE

1.0 INTRODUCTION

Carbamazepine (5H-dibenz [b, f] azepine-5-carboxamide; $C_{15}H_{12}N_{10}$) is one of the most widely used medications for the treatment of partial-onset seizures (Chogtu *et al*., 2005). Carbamazepine is a sodium channel blocker used as an anticonvulsant to treat bipolar disorder and to inhibit epileptic seizures (Hough *et al*., 1996). Carbamazepine is widely used in Nigeria for the treatment of seizure disorders and trigeminal neuralgia (Salawu and Danburam, 2007). It is reported to have psychotropic activity in depressed epileptic patients. Carbamazepine stabilizes the inactivated state of voltage-gated sodium channels, making fewer of these channels available to subsequently open. This leaves the affected cells less excitable until the drug dissociates. Carbamazepine has also been shown to potentiate GABA receptors made up of $alpha_1$, $beta_2$, and $gamma_2$ subunits (Granger, 1995). Carbamazepine and its active metabolite, 10, 11-epoxycarbamazepine, limit repetitive firing of action potentials evoked by sustained depolarization (Shetty *et al.,* 2007). Thus, carbamazepine exerts therapeutic effects via inhibition of brain neuronal activity. The drug is absorbed slowly and erratically after oral administration and spreads rapidly into all tissues. Carbamazepine has a very high potential for drug interactions. Carbamazepine as a CYP_{450} inducer may increase clearance of many drugs and decreasing their blood levels (Wikipedia, 2010). The most common dose-related adverse effects of carbamazepine are diplopia and ataxia in humans. Other side effects include mild digestive upsets and, at much higher doses drowsiness, hyponatraemia and water intoxication have occasionally occurred (Porter and Meldrum, 2007). It is metabolized into active and inactive metabolites and excreted in urine (Isojarvi *et al*., 2005). Long time use of Carbamazepine may reduce sperm motility, induce sperm abnormalities and

decrease testicular volume (Isojarvi *et al*., 2004), with adverse effect on fertility in men (Roste *et al*., 2005).

Oxidative stress is commonly observed when pro-oxidants, such as free radicals, reactive oxygen and reactive nitrogen species that is, superoxide anion, hydroxyl radical, hydrogen peroxide and nitric oxides that overwhelm cellular antioxidant systems and induce cellular damage (Saulsbury *et al*., 2009). Cellular injury is primarily due to the inability of the antioxidants to neutralize the effects of the oxygen radicals (Saulsbury *et al*., 2009). Reactive oxygen species are highly reactive molecules and can damage cellular molecules such as carbohydrates, nucleic acids, lipids, and proteins and alter their functions (Birben *et al*., 2012). Under normal circumstances, the body is equipped with agents (antioxidants) that combat the menace of oxidative stress. However, under increased oxidative challenges, the innate antioxidants systems are overwhelmed, giving rise to its deficit in relation to the oxidants, hence oxidative stress (Misra *et al*., 2009).

Antioxidants are agents which play an important role in inhibiting and scavenging free radicals, thus providing protection to tissues against damaging effects of free radicals (Beam *et al*., 2014). Antioxidants are substances that nullify the effect of free radical by either inhibiting its initial production or inhibiting the preparative phase of free radicals (Sujatha *et al*., 2011). Two major groups of antioxidants are recognised, namely enzymatic and non-enzymatic antioxidants. The enzymatic antioxidants include the primary enzymes (superoxide dismutase, catalase and glutathione peroxidase) and the secondary enzymes (glutathione reductase and glucose-6-phosphate dehydrogenase, among others) (Ratnam *et al*., 2006). Non-enzymatic antioxidants, are either water-soluble (vitamin C and phenolic compounds) or lipid-soluble (vitamin E and carotenoids) biomolecules (Podsędek, 2007).

The antioxidant vitamin C is a highly potent reducing agent (Padayatty *et al*., 2003), which is found naturally and abundantly in vegetables and fruits (Haytowitz, 2005). It is a low-molecular weight antioxidant that protects the cellular compartment against hydrophilic oxygen-nitrogen radicals; a property that makes it an efficacious antioxidant of the hydrophilic phase (Obara and Harasawa, 2008; Ogutcu *et al*., 2008).

Moringa oleifera Linn (*M. oleifera*) (Moringaceae), commonly known as Drumstick is a highly valued small or medium sized tree of about 10 m high. The plant, *M. oleifera* is a multipurpose tree considered to have its origin in the northwest region of India, south of the Himalayan Mountain with high nutritional values. It is distributed in many countries of the tropics and sub-tropics (Anwar *et al*., 2007; Razis *et al*., 2014). It is cultivated for use as a vegetable for spice, cooking and cosmetic oil, and as a medicinal plant (Ajayi *et al*., 2014). Different parts of this plant contain profiles of important minerals, and are good source of protein, vitamins, ß-carotene, amino acids and various phenolics (Anwar *et al*., 2007). It contains various chemical constituents such as alkaloids, tannins, flavonoids, carbohydrates, amino acids and glycosides (Belay and Sisay, 2014). Leaf extracts of *M. oleifera* were reported have antioxidant properties, the free radical scavenging effect of which is comparable with that of many classical antioxidants (Sreelatha and Padma, 2009; Ratshilivha *et al*., 2014).

1.1 Statement of Research Problems

Drug is the mainstay therapy in epilepsy. Epilepsy is one of the most common neurological disorders in animals and humans (Berendt, 2005) that is characterized by spontaneous recurrence of seizures originating in the central nervous system (Potschka *et al*., 2009). However, the long time use of certain antiepileptic drugs may reduce sperm motility, induce sperm abnormalities and decrease testicular volume (Isojarvi *et al*., 2004), with adverse effect on fertility in men (Roste *et*

al., 2005). Carbamazepine (CBZ) has been shown to increase serum sex hormone-binding globulin (SHBG) concentration in both men and women with epilepsy (Verrotti *et al*., 2011). Over time, the increase in serum SHBG level leads to diminished bioactivity of testosterone and estradiol, which may result in diminished potency in men and menstrual disorders in some women, and thus reduced fertility (Isojarvi *et al*., 2005). In men, sexual dysfunction has also been described after long-term CBZ treatment and was thought to be related to low bioactive testosterone levels (Isojarvi *et al*., 2005). Furthermore, treatment with CBZ was associated with changes in semen quality as indicated by reduced sperm motility as indicated by and increased frequency of morphologically abnormal sperm cells (Isojarvi *et al*., 2004), and hormonal alterations and sexual dysfunction (Roste *et al*., 2005). The wide and long-term use of the CBZ is a common schedule in children and adolescents and has been shown to alter the testosterone levels in adult animals and humans (Oliva *et al*., 2012). Oral administration of carbamazepine caused decreased testicular weight, sperm cell concentration, plasma testosterone, follicle stimulating hormone, and luteinizing hormone (Soliman *et al*., 1999). Prolonged administration of carbamazepine also increased plasma prolactin levels, which eventually causes testicular degeneration (Soliman *et al*., 1999).

Oxidant injuries to cells result from production of reactive oxygen species (ROS) and nitrogen oxides (NO). Reactive oxygen species such as superoxide (O^{2-}) and hydrogen peroxide (H_2O_2) cause respiratory burst and damage to cells (Halliwell and Whiteman, 2004). Carbamazepines (CBZ) are capable of increasing oxidative challenge and may have been involved in the reproductive disorders (Agarwal and Bedaiwy, 2003). It is therefore imperative to explore remedies to reproductive disorders induced by chronic CBZ administration.

1.2 Justification of the Study

Epilepsy causes substantial deleterious effects on human health and quality of life and places a heavy burden on society (Farghaly *et al*., 2013). Apart from the epilepsy itself, carbamazepine is known to impair optimal reproductive performance (Isojarvi *et al*., 2005). Carbamazepine and its metabolites are known to induce oxidative stress (Santos *et al*., 2008). Since the male reproductive tract is highly vulnerable to oxidative stress (Rahmanian *et al*., 2014), it is probable that oxidative stress may be partly involved in the adverse reproductive toxicity induced by chronic CBZ administration. It is therefore possible that antioxidants such as those obtained from leaf extract of *M. oleifera* and vitamin C may mitigate oxidative changes in the reproductive tract evoked by chronic CBZ administration, thereby mitigating its adverse reproductive consequence.

Different parts of *M. oleifera* contain a profile of important minerals, and are good source of protein, vitamins, ß-carotene, amino acids and various phenolics (Anwar *et al.*, 2007). Phenolic compounds in *M. oleifera* act as free radical acceptors and chain breakers. They interfere with the oxidation of lipids and other molecules by rapid donation of a hydrogen atom to radicals (Dai and Mumper, 2010). *Moringa oleifera* leaves have been used for its hepatoprotective effect by decreasing hepatic lipid peroxides (Faizi *et al.*, 1998). Methanolic extract of *M. oleifera* has been shown to enhance seminiferous tubules, testicular and epididymal weight and seminal vesicles in rats (Sudha *et al.*, 2010).

Vitamin C (ascorbic acid) is a commonly available compound that acts effectively as an antioxidant and reducing agent at physiological pH, thus neutralizing the effect of reactive oxygen species (ROS) in the body (Noordin *et al*., 2015). Water-soluble vitamin C provides intracellular and extracellular aqueous-phase antioxidant capacity primarily by scavenging oxygen free radicals. It also converts vitamin E free radicals back to vitamin E (Birben *et al.*, 2012). As a non-

enzymatic antioxidant defence system, ascorbic acid plays a crucial role in the regulation of hydrogen peroxide levels in oxidative stress. It has been shown to work concurrently with glutathione and glutathione transferase, thus its presence may boost cellular glutathione levels (Padayatty *et al*., 2003). In the male reproductive system, vitamin C is known to protect spermatogenesis and it plays a major role in semen integrity and fertility in man and animals, increases testosterone levels (Sonmez *et al*., 2005) and prevents sperm agglutination (Agarwal *et al*., 2005). It is an important chain-breaking antioxidant, contributing up to 65 percent of the total antioxidant capacity of seminal plasma found intracellulary and extracellulary (Fernandes *et al*., 2011).

1.3 Aim of the Study

The aim of the present study was to evaluate the effect of vitamin C and methanol leaf extract of *Moringa oleifera* on chronic carbamazepine-induced reproductive toxicity in male Wistar rats.

1.4 Objectives of the Study

The objectives of the study were to evaluate the:

i. phytochemical constituents of the *Moringa oleifera* leaf
ii. effect of chronic carbamazepine administration on serum sex hormones' concentrations and sperm characteristics in adult male Wistar rats
iii. effect of chronic carbamazepine administration on pituitary gland and testicular histology in adult male Wistar rats
iv. role of oxidative stress in chronic carbamazepine-induced alterations in sex hormones' oncentrations, sperm characteristics, and pituitary gland and testicular histology

v. protective effect of vitamin C and methanol leaf extract of *Moringa oleifera* on sex hormones' concentrations, sperm characteristics, pituitary gland and testicular biochemical and histological changes induced by chronic carbamazepine administration in adult male Wistar rats.

1.5 Null Hypothesis

Vitamin C and methanol leaves extract of *Moringa oleifera* do not protect sperm characteristic, pituitary gland and testicular biochemical and histopathological changes induced by chronic carbamazepine administration in adult male Wistar rats.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Carbamazepine

Carbamazepine (CBZ) is an anticonvulsant and mood-stabilizing drug used primarily in the treatment of epilepsy and bipolar disorder, as well as trigeminal neuralgia (Afshar *et al*., 2010). This drug is highly effective, well tolerated, and, compared with other antiepileptic drugs (AEDs), considered relatively safe. Chemically, CBZ is a neutral, lipid-soluble compound that can easily pass the blood–brain barrier and other membranes in the body (Matalon *et al*., 2002). CBZ is one of the most common and effective of the older AEDs and is chosen for monotherapy due to its high effectiveness and low incidence of side-effects (Zarghi *et al*., 2007). In recent comparative clinical trials, it has been demonstrated that CBZ is equally efficacious with, but is less toxic than phenobarbital, phenytoin, primidone, or valproic acid (Fritz *et al*., 1976).

2.1.1 Historical perspectives

Carbamazepine was discovered by a chemist, Walter Schindler at J.R. Geigy A.G. (now part of Novartis) in Basel, Switzerland in 1953 (Schindler and Hafliger, 1954; Scott, 1993). It was first marketed as a drug to treat epilepsy in Switzerland in 1963 under the brand name "Tegretol"; its use for trigeminal neuralgia (formerly known as tic douloureux) was introduced at the same time (Scott, 1993). It has been used as an anticonvulsant and antiepileptic in the United Kingdom since 1965 and has been approved in the United State since 1968 (ASHSP, 2015). In 1971, carbamazepine was first used to control mania in Japanese patient's refractory to antipsychotics (Okuma and Kishimoto, 1998). Carbamazepine was studied for bipolar disorder throughout the 1970s (Okuma and Kishimoto, 1998).

2.1.2 Chemistry

Carbamazepine is an iminostilbene, a dibenzepine derivative that is chemically and pharmacologically related to tricyclic antidepressant agents (Bazil and Pedley, 2003). Carbamazepine corresponds chemically to 5H-dibenz (b, f) azepine-5-carboxamide with an empirical formula of C_{15} H_{12} N_2 O, a molecular weight of 236.27 and its structural formula is shown in Figure 2.8

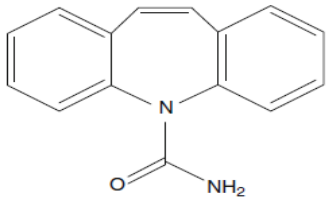

Figure 2.1: Structure of carbamazepine **(**Grzesiak *et al*., 2003).

2.1.3 Medical use

Carbamazepine is a medication used primarily in the treatment of epilepsy and neuropathic pain (Bialer, 2012). For seizures, it is effective as phenytoin and valproate. It is not as effective as valproate for absence seizures or myoclonic seizures, where valproate is much effective and the drug of choice. It may be used in augmentation for inadequately responding schizophrenia along with other medications and as a second line agent in bipolar disorder (Nolan *et al*., 2013; Powell *et al*., 2014). It is the usual drug of choice for patients with newly diagnosed partial onset seizure (Gamble *et al.*, 2009).

2.1.4 Mechanism of action

Carbamazepine is a well-known antiepileptic drug which has multiple mechanisms of action including stabilization of the inactivated stage of the voltage-gated sodium channels, potentiating GABA receptors as a GABA agonist, as well as the serotonin releasing effect (Kawata *et al*., 2001).

Carbamazepine stabilizes the inactivated stage of voltage-gated sodium channels, making fewer of these channels available to subsequent opening. This leaves the affected cells less excitable and relief the pain in trigeminal neuralgia（Wiffen *et al*., 2011）. By doing this, CBZ, like vaproate decreases influx of sodium ion and increases efflux of potassium ion (Ambrosio *et al*., 2002; Sadock *et al*., 2009).

Gamma-aminobutyric acid (GABA) is an inhibitory neurotransmitter that plays an important role in regulating dopamine and glutamate neurotransmission. It was found that patients with bipolar disorder had lower GABA levels, which results in excitotoxicity and can cause apoptosis (Granger, 1995). It is also of interest that chronic but not acute administration of carbamazepine are associated with up regulation of $GABA_B$ receptors in the hippocampus, yielding this action as a potential convergent mechanism for mood stabilization (Granger, 1995). CBZ is a GABA receptor agonist, as it has also been shown to potentiate GABA receptors made up of α_1, β_2, and γ_2 subunits (Granger, 1995; Ambrosio *et al*., 2002).

Glutamate is the universal excitatory neurotransmitter. CBZs are able to weakly block calcium influx through glutamate receptors of the N-methyl-D-aspartic acid (NMDA) subtype. CBZ could exert anti-glutamatergic effects both by decreasing the release of glutamate as well as by relative decrease in glutamate's postsynaptic efficacy through inhibition of calcium influx. Antidepressant and mood-stabilizing effects of carbamazepine are linked to its glutamate antagonism (Ambrosio *et al*., 2002; Sadock *et al*., 2009; Stahl, 2009).

Serotonin is another major neurotransmitter involved in the pathophysiologic mechanism of bipolar disorders. Carbamazepine is a serotonin releasing agent and possibly even a serotonin reuptake inhibitor (Schatzberg *et al*., 2010; Taylor *et al.*, 2011).

2.1.5 Pharmacokinetics

Carbamazepine (CBZ) is well absorbed after oral administration. The rate of absorption after oral administration varies widely among patients, although almost complete absorption apparently occurs in all (Porter and Meldrum, 2007). Its plasma half-life is about 26-30 h when it is given as single dose, but it is a strong inducer of hepatic enzymes and the plasma half-life shortens to about 15 h (range 10-25 h) when it is given repeatedly (Sadock *et al*., 2009; Schatzberg *et al*., 2010). The degree of CBZ auto induction is somewhat dose dependent but is usually complete after 3-5 weeks of treatment (Schatzberg and Nemeroff, 2010; Schatzberg *et al*., 2010). It is 80%–85% protein bound with a moderately large volume of distribution (V_d = 1.0–2.0 L/kg). However, with increasing concentrations, more free CBZ molecules and its metabolites appear in the blood (Brahmi *et al*., 2006). Bioavailability is 80% and its peak plasma levels are reached 2-8 h after a single dose. The average half-life after a single dose is 26 h, with a range of 18-54 h.

Carbamazepine is metabolized in the liver, primarily by CYP450 3A4. The active metabolite is carbamazepine-10, 11-epoxide, which also retains anti-epileptic and toxic property (Mittag *et al*., 2016). Initial half-life is 26–65 h (35-40 h) for extended release formulation); half-life 12-17 h with repeated doses. Half-life of active metabolite is approximately 34 h. It is not only a substrate for CYP450 3A4, but also an inducer of CYP450 3A4 (Guneysel *et al*., 2008). Thus, CBZ induces its own metabolism, often requiring an upward dosage adjustment (Sadock *et al*., 2009). It is excreted through the kidney via urine (Bauer, 2008). Because only 1% of CBZ is eliminated unchanged in urine, accumulation of parent drug or the epoxide metabolite is unlikely (Tolou-Ghamari, *et al*., 2013).

2.1.6 Interaction

Carbamazepine (CBZ) is a potent inducer of cytochrome P450 oxidase enzymes, particularly of the 3A4 category so that it reduces in the levels of a variety of other compounds concurrently used with it (Defendi and Tucker, 2008). CBZ concentrations are known to be increased with concurrent use of antidepressants (such as fluoxetine, fluovoxamine, nefazodone), omeperazole, efavirnaz, ratinovir, valproate, carthromycin, cimetidine, erythromycin (Schlatter *et al*., 1999), propoxyphene and calcium-channel blockers (such as verapamil) (Lacy *et al*., 2009). CBZ induce hepatic microsomal enzymes and enhance the metabolism (decrease serum levels and decrease the effectiveness) of drugs such as acetaminophen, clozapine haloperidol, benzodiazepines (especially alprazolam, triazolam), oral contraceptives, corticosteroids, cyclosporine, doxycycline, mebendazole, methadone, theophylline, thyroid supplements, valproate and warfarin (Ayano, 2016). CBZ also increases the metabolism of the hormones in birth control pills and can reduce their effectiveness leading to unexpected pregnancies (Lacy *et al*., 2009). Rifampin and Cisplatin cause cytochrome P450 enzyme induction and decreased CBZ levels. Here are common drug interactions of carbamazepine (Defendi and Tucker, 2008).

1. Decrease the levels of phenytoin when given at the same time.

2. Phenobarbital will lower CBZ levels because of microsomal enzyme induction.

3. Decreased ethosuximide levels because of cytochromeP450 enzyme induction.

4. Felbamate can increase the active metabolite of CBZ resulting in toxicity but decrease CBZ levels.

5. Lamotrigine and valproate can increase levels of the active metabolite. Patients may have signs of toxicity with normal CBZ levels. CBZ will cause decreased valproate levels (Sadock *et al*., 2009).

2.1.7 Adverse effects

The most common side effects of carbamazepine (CBZ) are skin reactions, headache, drowsiness, blurred vision, nausea, vomiting, constipation, diarrhoea, loss of appetite, sedation, ataxia and confusion (Besi *et al*., 2015). These can be prevented or significantly reduced by increasing the daily dosage slowly (Sadock *et al*., 2009).

Carbamazepine may cause life-threatening thrombocytopenia, agranulocytosis, and aplastic anaemia in 0.005% of human patients. CBZ should be discontinued if the WBC declines to less than 3000 mm, absolute neutrophil count <1500 mm or platelet count decreases to <100,000 cells per mm (Post and Leverich, 2008; Sadock *et al*., 2009). CBZ administration may cause increased lymphocytes; this may arise due to formation of epoxides by the activity of cytochrome P450. The epoxides bind covalently with macromolecules and act as hapten to stimulate immunologic actions, hence lymphocytosis (Gerson *et al*., 1983; Spielberg, 1986). In the early phase of treatment, transient and minor decreases in blood cell indices may occur and do not warrant discontinuation of CBZ therapy (Sadock *et al*., 2009).

Ashrafi *et al*. (2010) reported a decrease in immunoglobulins A and G following CBZ administration, and this may cause decreased globulin concentration. Increased alkaline phosphatase (ALP) activity following CBZ therapy was thought to be associated with its effect on bone formation, possibly related to increased bone turn-over (Merete *et al*., 2005). CBZ has been associated with rare reports of severe hepatitis and cholestatic jaundice associated with hepatic enzyme elevations, which may warrant discontinuation of therapy (Sadock *et al*., 2009).

Rashes and urticaria are relatively common. Other dermatological side effects include exfoliative dermatitis and toxic epidermal necrolysis (Stevens-Johnson syndrome), requiring immediate discontinuation of the drug (Leckband *et al*., 2013; Amstutz *et al*., 2014). Long-term treatment

with carbamazepine is associated with small degrees of weight gain, which are significantly less than those observed on valproate (Sadock *et al*., 2009).

2.2 Effects of Antiepileptic Drugs on Reproductive/Endocrine Functions

Antiepileptic drugs (AEDs) affect reproductive endocrine function in both men and women. Changes in reproductive hormone levels, decreased potency, and diminished sexual interest in men and menstrual disorders, polycystic ovarian syndrome (PCOS) and decreased fertility in women can be manifestations of the reproductive endocrine effects of AEDs. These disorders are frequently seen in association with certain AEDs (Löfgren, 2007).

The aetiology of reproductive and sexual dysfunction in men with epilepsy has been attributed to a number of possible aetiologies; including psychosocial stress, AEDs, and epilepsy itself (Herzog, 2008). Antiepileptic drugs may also influence mood and modulate sexual behaviour in women and men. Enzyme-inducing AEDs (e.g. Phenobarbital (PB), phenytoin (PHT) and carbamazepine [CBZ]) elevate serum sex hormone binding globulin (SHBG) inducing a decrease of free gonadal steroids (Yerby, 2000) in men and women. It has been speculated that antiepileptic drugs (AEDs) can induce various hormonal abnormalities; in particular, the use of the liver enzyme-inducing AEDs, such as phenytoin and CBZ, which increases serum sex hormone binding globulin concentrations. This increase leads to diminished bioactivity of testosterone, which may result in diminished potency and thus reduced fertility (Verrotti *et al*., 2011).

2.2.1 Effect of carbamazepine on testosterone concentration

Epilepsy itself and AEDs have been implicated in the pathophysiology of reproductive endocrine disorders in patients (Abdel-Reheim, 2009). The effects of carbamazepine (CBZ) on reproductive functions could be attributed to their central effects on the hypothalamic-pituitary-gonadal axis (Verrotti *et al*., 2011). The use of CBZ is related to a progressive increase in circulating levels of

sex hormone binding globulin as a result of the stimulation of the aromatase and cytochrome P450 enzyme system, which in turn accelerates the metabolism of sex steroids (Lossius *et al*., 2007). This effect decreases the proportion of free, bioactive testosterone and increases the inactive bound form which may result in sexual dysfunction in some men with epilepsy after long term CBZ treatment (Lossius *et al*., 2007; Duncan *et al*., 2009). Because sexual hormones are very important in supporting sexual drive and sexual function in man and in women it is thought to be contributing to the sexual dysfunction observed in patients with epilepsy who are on chronic treatments (Duncan *et al*., 2009). CBZ acts directly on the testis and inhibit testosterone synthesis in men (Pylvanen *et al*., 2003). In addition, CBZ affect testosterone feedback which is important for LH secretion (Pylvanen *et al*., 2003). Many studies have demonstrated the important role of dehydroepiandrosterone (DHEA) in sexual dysfunction. In fact, DHEA influences the production of endothelial nitric oxide, which lead to an improvement in erectile function but this may be altered after long term CBZ treatment (Ruige *et al*., 2013).

2.3 Oxidative Stress

Oxidative stress is characterized by an imbalance between oxidants and antioxidants, due to the excessive production of reactive oxygen species (ROS) and the reduction in the rate of its removal by the antioxidant defence system. This metabolic disturbance favours the oxidation of biomolecules, contributing to the oxidative damage in the cells and tissues and consequently to the development of several chronic diseases such as obesity, diabetes and cancer (Feng and Ruiz, 2013; Butterfield and Domenico, 2014; Pisoschi and Pop, 2015). Exposure to reactive oxygen intermediates or free radicals cause oxidative stress (Sangishetti *et al*., 2017). A free radical considered as a fragment of a molecule (possessing an unpaired electron), such as superoxide anion (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radical (HO^-) that can damage proteins, nucleic

acids and cell membranes. Increasing evidence suggests that the cumulative damage caused by reactive oxygen species contribute to numerous diseases (Lobo *et al*., 2010). An alteration between oxidants and antioxidants in favour of the oxidants, potentially leading to damage is termed oxidative stress (Betteridge, 2000). Oxidants are formed as an accustomed counting of aerobic metabolism but can be produced at elevated rates under pathological conditions (Elisa *et al*., 2012).

2.3.1 Mechanism of oxidative damage

Reactive species (RS), are the main culprits of oxidative damage within the cells. As a byproduct of normal oxidative phosphorylation, errant electrons form free-radicals such as O^{2-}, OH^{-} or H_2O_2, which can attack DNA, proteins, and lipids, causing impairment of function and ultimately cell death, if the damage cannot be repaired. At low levels, RS are essential in gene regulation, cell signaling, and apoptosis (Dowling and Simmons, 2009) but at high levels, RS production can potentially overwhelm the antioxidant capacity of the cell and exert oxidative stress changing gene expression, and causing structural damage (Monaghan *et al*., 2009). Cells inherently contain molecules to combat damage from RS production, broadly termed the antioxidant system. These molecules function by catalyzing the oxidation of less biologically insulting molecules. Other antioxidant molecules such as vitamin E and C, act as chain-breaking antioxidants, which can scavenge for RS, remove them once they are formed, and further halt propagation of peroxidation (Nemec *et al*., 2000). Despite having a thwarting antioxidant system, oxidative damage accrues during times of stress. Two of the most insidious types of oxidative damage include damage to lipids in the form of lipid peroxidation (LPO), where an errant electron propagates across membranes, altering lipid composition and decreasing cellular function (Hulbert *et al*., 2007). The other type of damage is directly to DNA molecules, accumulating mutations that can lead to diseases like cancer (Hasty *et al*., 2003).

Biological effects of single-base damage by radiation or oxidation, such as 8-oxoguanine and thymine glycol, have been extensively studied. Tandem DNA lesions are formed at substantial frequency by ionizing radiation and metal-catalyzed H_2O_2 reactions. Under anoxic conditions, the predominant double-base lesion is a species in which C8 of guanine is linked to the 5-methyl group of an adjacent 3'-thymine (G[8,5- Me]T) (Colis *et al*., 2008). Most of these oxygen-derived species are produced by normal aerobic metabolism. Repair of oxidative damages to DNA is frequent and ongoing, largely keeping up with newly induced damages (Helbock *et al*., 1998). However, there is a steady state level of oxidative damages, as well, in the DNA of a cell (Helbock *et al*., 1998). Any damage to cells is constantly repaired. However, under the severe levels of oxidative stress that cause necrosis, the damage causes ATP depletion, preventing controlled apoptotic death and causing the cell to simply fall apart (Lelli *et al*., 1998; Lee and Shacter, 1999).

Polyunsaturated fatty acids (PUSAs), particularly arachidonic acid and linoleic acid, are primary targets for free radical and singlet oxygen oxidations (Riahi *et al*., 2010). For example, in tissues and cells, the free radical oxidation of linoleic acid produces racemic mixtures of 13-hydroxy-9*Z*,11*E*-octadecadienoic acid, 13-hydroxy-9*E*,11*E*-octadecadienoic acid, 9-hydroxy-10*E*,12-*E*-octadecadienoic acid (9-EE-HODE), and 11-hydroxy-9*Z*,12-*Z*-octadecadienoic acid as well as 4-Hydroxynonenal while singlet oxygen attacks linoleic acid to produce (presumed but not yet proven to be racemic mixtures) 13-hydroxy-9*Z*,11*E*-octadecadienoic acid, 9-hydroxy-10*E*,12-*Z*-octadecadienoic acid, 10-hydroxy-8*E*,12*Z*-octadecadienoic acid, and 12-hydroxy-9*Z*-13-*E*-octadecadienoic (see 13-Hydroxyoctadecadienoic acid and 9-Hydroxyoctadecadienoic acid) (Akazawa-Ogawa *et al*., 2015; Riahi *et al*., 2010). Similar attacks on arachidonic acid produce a far larger set of products including various isoprostanes, hydroperoxy- and hydroxy-eicosatetraenoates, and 4-hydroxyalkenals (Riahi *et al*., 2010; Vigor *et al*., 2014). While many of

these products are used as markers of oxidative stress, the products derived from linoleic acid appear far more predominant than arachidonic acid products and therefore easier to identify and quantify in, for example, atheromatous plaques (Riahi *et al*., 2010). Certain linoleic acid products have also been proposed to be markers for specific types of oxidative stress. For example, the presence of racemic 9-HODE and 9-EE-HODE mixtures reflects free radical oxidation of linoleic acid whereas the presence of racemic 10-hydroxy-8*E*,12*Z*-octadecadienoic acid and 12-hydroxy-9*Z*-13-*E*-octadecadienoic acid reflects singlet oxygen attack on linoleic acid (Cohen *et al*., 2013). In addition to serving as markers, the linoleic and arachidonic acid products can contribute to tissue and/or DNA damage but also act as signals to stimulate pathways which function to combat oxidative stress. (Kyung-Jin *et al*., 2011; Galano *et al*., 2013).

2.4 Effect of Oxidative Stress on Male Reproductive Function

Stress disturbs the testicular physiology and endocrinology (Akpinar *et al*., 2008). Prolong stress in male rats cause lower degree of sexual initiation and delayed ejaculation (Yoon *et al*., 2005). Acute stress (mental and physical) could activate catecholamine levels in brain; this could affect sexual behaviour (Yoon *et al*., 2005). Lipid peroxidation (LPO) inactivates cell constituents by oxidation causes oxidative stress (OS) by undergoing radical chain reaction, ultimately leading to loss of membrane integrity. However, the higher membrane lipid content of testes makes them more vulnerable to oxidative stress (Ahmed *et al*., 2010). ROS-induced damage leads to LPO due to high polyunsaturated fatty acids (PUFAs) content in sperm plasma membrane which results in low membrane fluidity and loss of sperm oocyte interaction. Stress increases the production of thiobarbituric reactive substances (TBARS) which is maximum under stress conditions (Tomono *et al*., 2011). There is increased catecholamine's level, thereby causing vascular contraction, which

is responsible for reduced blood flow and leads to sexual dysfunction during stress period (Yoon *et al*., 2005). In male, testosterone modulates several behaviour and response for sexual stimulation. In a stress condition, there is decreased testosterone concentration level (Yoon *et al*., 2005; Manalisha *et al*., 2011). Testicular mitochondrial membranes contain rich polyunsaturated lipids, which are highly susceptible to oxidation injury, resulting in gonadal dysfunction (Tomono *et al*., 2011).

2.4.1 Effect of stress on sperm function

Testicular membranes are rich in polyunsaturated fatty acids and thus susceptible to peroxidation injury (Elizabeth *et al*., 2008). Increased lipid peroxidation in the testis contributes to the suggested vulnerability of this organ to oxidative stress (Boguslaw, 2012). Small amount of ROS is produced by the mature spermatozoa, which are needed for capacitation acrosome reaction and fertilization. However, under stress conditions excessive amount of ROS are produced by leukocytes and immature spermatozoa, which cause damage to the normal spermatozoa by inducing lipid peroxidation and DNA damage (Elizabeth *et al*., 2008). Oxidative stress plays a major role in sperm malfunction via induction of lipid peroxidase to biomembranes (Amrit and Gurmail, 2009). Stress conditions decrease spermatogenesis and fertilizing ability (Naseem *et al*., 2007; Amrit and Gurmail, 2009). It enhances the incidence of abnormal sperm production (Bedwal *et al*., 2009; Jahangir *et al*., 2009), decreases sperm membrane fluidity (Naseem *et al*., 2007), total sperm count, quality, concentration (Naseem *et al*., 2007; Amrit and Gurmail, 2009), viability of sperm (Naseem *et al*., 2007), as well as loss of motility and sperm function (Motahareh *et al*., 2014).

2.5 Antioxidant Defence System

An antioxidant is any substance that when present at low concentrations compared to those of an oxidizable substrate significantly delays or prevents oxidation of that substrate (Fukasawa *et al*., 2009). The term oxidizable substrate includes almost everything found in living cells, including proteins, carbohydrates, lipids and DNA. Antioxidants are compounds capable of providing free radicals with the electrons they are missing while remaining stable themselves, thus preventing a cascade of interactions that could create even more free radicals (Cotgreave *et al*., 1998).

To counteract deleterious effects of oxidative stress, nature has endowed each cell with adequate protective antioxidant defences which can be broadly categorized into enzymatic or nonenzymatic antioxidants (Faraci and Didion, 2004). Antioxidants act at different levels in the oxidative process by scavenging initiator of free radicals, binding metal ions, scavenging peroxyl radicals and removing oxidatively damaged biochemicals (Ashok and Lucky, 2010). Enzymatic antioxidants include superoxide dismutase (SOD) which catalyzes the dismutation of O_2^- into H_2O_2 and O_2. SOD exists in three isoforms in mammalian system, i.e., copper/zinc SOD (SOD1), mitochondrial SOD (Mn SOD, SOD2), and extracellular SOD (ecSOD, SOD3) (Datta *et al*., 2000; Faraci and Didion, 2004). Glutathione peroxidase reduces H_2O_2 and lipid peroxides to water and lipid alcohols and in turn oxidizes glutathione to glutathione disulfide. Catalase catalyzes the conversion of H_2O_2 to water and molecular oxygen and protects the cells from harmful effects of H_2O_2 produced within the cell. These enzymes are highly effective during augmented oxidative stress, as reduced levels of glutathione or glutathione peroxidase are available. Reduced glutathione plays a major role in the regulation of the intracellular redox state of the cells as it is a major source of reducing equivalents (Schafer and Buettner, 2001). Thioredoxin reductase is responsible for thiol-dependent reductive processes in the cell (Arrigo, 1999). Glutathione S-transferase and H_2O_2 can form

spontaneously or can be formed by dismutation of O_2•− catalyzed by SOD: $2O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$. Thioredoxins are low molecular weight proteins that contain a conserved dithiol motif which is responsible for a variety of biological functions. Sulfur switches are shown as sensors in redox signaling pathways which control and integrate metabolic pathways. Three major redox controls responsible for regulation of these switches are thioredoxins, GSH/GSsL, and Cys/Cyss (Trachootham *et al*., 2008).

The nonenzymatic category of antioxidant defences includes low molecular weight molecules, e.g., glutathione, uric acid, vitamin A (retinoids), carotenoids particularly beta carotene with a high-antioxidant activity as it quenches free radicals, and α-tocopherol (vitamin E), a fat-soluble and free radical chain breaking antioxidant which, due to the presence of hydroxyl (–OH) group in its structure, is an effective hydrogen donor (Fatmah *et al*., 2012). Ascorbic acid (vitamin C) acts as a hydrogen donor and reverses oxidation and can act both as an antioxidant and as a prooxidant. Fruits and vegetables are main source of vitamin C and other nonenzymatic antioxidants, e.g., flavonoids and related polyphenols. The concentration of these antioxidants is low and varies depending on their location (Naziroglu, 2003). Bilirubin, lipoic acid, albumin, ferritin, ceruloplasmin, and transferrin also show antioxidant properties and can indirectly reduce or inhibit generation of reactive species (Ashok and Lucky, 2010).

2.5.1 Enzymatic antioxidants

Enzymatic removal of ROSs requires the coordinated responses of several antioxidant enzyme systems; individual pathways alone are insufficient (Donald *et al*., 2012). Key systems include: (1) superoxide dismutase, which converts superoxide anion to hydrogen peroxide; (2) catalase and glutathione peroxidase, which convert hydrogen peroxide to water and quench the generation of

lipid peroxides; and (3) glutathione reductase and glutathione transferase, the enzymes that regenerate reduced glutathione and conjugate it to reactive compounds (Galaris and Evangelou, 2002).

2.5.1.1 Superoxide dismutase

Superoxide dismutase (SOD) is a ubiquitous antioxidant enzyme that protects organisms from oxidative stress by catalytically converting superoxide radical ($O_2^{\cdot -}$) to hydrogen peroxide (H_2O_2) (Fridovich, 1995; Zelko *et al*., 2002). SOD competes with nitric oxide (NO) for superoxide anions, which react with NO to form peroxynitrite (Halliwell and Gutterigde, 1995). There are three classes of SODs, with different genes, unique protein structures, and distinct subcellular localizations (Zelko *et al*., 2002). CuZnSOD (SOD1) is a cytosolic enzyme, MnSOD (SOD2) is mitochondrial, and extracellular SOD (SOD3) is secreted from cells. SOD1 (and the structurally homologous SOD3) contains copper and zinc cofactors. It is distinct from manganese-containing SOD2. Expression of SOD1 is relatively constant among cell types but can be induced by redox-active metals, superoxide, and xenobiotics. SOD1 knockout mice show increased incidence of liver tumors, likely due to increased ROS (Elchuri *et al*., 2005). SOD2 is the most inducible form; its levels can increase 10-fold in response to certain drugs and cytokines. Elevated mitochondrial SOD2, and concomitant resistance to drug-induced oxidative damage, has been proposed as a mechanism of drug resistance in some lung cancers (Kinnula *et al*., 1996). Lack of SOD2 also results in increased oxidative damage in liver (Williams *et al*., 1998), while overexpression protects against this (Wheeler *et al*., 2001). SOD3 does not play a significant role in superoxide detoxification in hepatocytes.

2.5.1.2 Catalase

Catalase (CAT) is usually located in a cellular organelle called the peroxisome (Alberts *et al*., 2002). Peroxisomes in plant cells are involved in photorespiration (the use of oxygen and production of carbon dioxide) and symbiotic nitrogen fixation (the breaking apart of diatomic nitrogen (N_2) to reactive nitrogen atoms) (Alberts *et al*., 2002). Catalase (CAT) is a common enzyme found in nearly all living organisms exposed to oxygen. It catalyzes the decomposition of hydrogen peroxide to water and oxygen (Chelikani *et al*., 2004). CAT is a very important enzyme in protecting the cell from oxidative damage by reactive oxygen species (ROS) (Chelikani *et al*., 2004). Likewise, of all enzymes CAT has the highest turnover; one catalase molecule can convert millions of molecules of hydrogen peroxide to water and oxygen each second (Goodsell, 2004).

$$2H_2O_2 \rightarrow 2H_2O + O_2$$

The presence of catalase in a microbial or tissue sample can be demonstrated by adding hydrogen peroxide and observing the reaction. The production of oxygen can be seen by the formation of bubbles. This easy test, which can be seen with the naked eye, without the aid of instruments, is possible because catalase has a very high specific activity, which produces a detectable response, as well as the fact that one of the products is a gas (Goodsell, 2004).

Hydrogen peroxide is a harmful byproduct of many normal metabolic processes. Therefore, to prevent damage to cells and tissues, it must be quickly converted into other less dangerous substances. To this end, CAT is frequently used by cells to rapidly catalyze the decomposition of hydrogen peroxide into less-reactive gaseous oxygen and water molecules (Gaetani *et al*., 1996).

2.5.1.3 Glutathione Peroxidase

Glutathione peroxidase (GPx) is an important antioxidant enzyme present in virtually all tissues. It is a selenium-containing cytosolic enzyme that catalyses the reduction of H_2O_2 to water and oxygen as well as catalising the reduction of peroxidise radicals to alcohol and oxygen, and inturn oxidizes glutathione to glutathione disulfide (Tahbet and Touyz, 2007; Fanucchi, 2014).

$H_2O_2 + 2\ GSH \longrightarrow 2H_2O + GSSH$

The enzyme limits the generation of lipid peroxides and utilizes glutathione as its co-factor to convert lipid peroxide into relatively harmless hydroxylated fatty acids, water and glutathione disulfide (Dolapo and Adeniran, 2014). Glutathione peroxidase (GPx) also neutralizes superoxide, converting reduced glutathione and superoxide to oxidized glutathione and water. It is more effective than catalase for low levels of superoxide. Glutathione peroxidase also detoxifies other peroxides and phase-I electrophiles (Farrell, 2002). Glutathione peroxidases are substantially more efficient on a molar basis than other enzymes (Michiels *et al*., 1994; Gomathi *et al*., 2012). Glutathione is the most significant component which directly quenches ROS such as lipid peroxides and plays major role in xenobiotic metabolism. When an individual is exposed to high levels of xenobiotics, more glutathione is utilised for conjugation making it less available to serve as an antioxidant. It also maintains ascorbate (vitamin C) and alpha-tocopherol (vitamin E), in their reduced form, which also exert an antioxidant effect by quenching free radicals. Glutathione peroxidase requires the presence of reduced glutathione for its action. Glutathione reductase (GSR) reduces glutathione disulfide (GSSG) to the sulfhydryl form GSH an NADPH-dependent reaction, while GPx catalyses the reduction of H_2O_2 and lipid peroxides at the expense of GSH (Uner *et al*., 2005: Traber and Atkinson, 2007). GSH and GSH-related enzymes play a major role in cellular

metabolism and free radical scavenging (Liu *et al*., 2008). GSH is the major nonenzymatic antioxidant which quenches oxyradicals through the –SH group.

2.5.1.4 Glutathione S-transferases

The Glutathione S-transferases (GSTs) are important phase II biotransformation enzymes which play a key role in cellular detoxification, protecting macromolecules from attack by reactive electrophiles, environmental carcinogens, reactive oxygen species and chemotherapeutic agents (Strange, 2000). GSTs are widely distributed in nature and are present in both prokaryotes and eukaryotes as the principal phase II detoxifying enzymes (Hayes, 2005). They constitute a superfamily of ubiquitous, multifunctional enzymes which catalyze the nucleophilic addition of the tripeptide glutathione (GSH; g-Glu-Cys-Gly) to several hazardous xenobiotics, including phase I electrophilic and carcinogenic metabolites (Hayes and Strange, 2000; Nebert and Vasiliou, 2004; Senhaji, 2015) thereby, neutralizing their electrophilic sites and rendering the products more water-soluble and facilitating their elimination from the cell by phase III enzymes (Coleman, 1997). In addition, GSTs can serve as peroxidases, isomerases and thiol transferases (Board, 2000). They can also play role in non-catalytic functions like modulation of signaling processes and non-substrate ligand binding (Axarli, 2004).

2.5.2 Non-enzymatic antioxidants

The non-enzymatic antioxidant defense system includes ascorbic acid (vitamin C), α- tocopherol (vitamin E), glutathione (GSH), β-Carotene, and vitamin A. There is a balance between both the activities and intracellular levels of these antioxidants that are essential for the survival of organisms and their health (Matés *et al*., 1999; Meydani, 2001; Berr *et al*., 2004)

2.5.2.1 Vitamin C as non-enzymatic antioxidant

Vitamin C is a major water-soluble antioxidant which acts by scavenging free radicals and also by maintaining the thiols of proteins (reducing properties), which built up antioxidant compounds (Himmelfarb *et al*., 2000; Deicher and Horl, 2003). Vitamin C represents one of the most powerful antioxidants both in plasma and intracellularly, strongly inhibiting lipid peroxidation, reducing endothelial dysfunction and regenerates vitamin E in lipoproteins and membranes (Gebretsadik *et al*., 2015).

2.6 Ascorbic Acid (Vitamin C)

Vitamin C, also known as ascorbic acid and ascorbate, is a basic compound that belongs to the group of water-soluble vitamins. It has L- and D-enantiomer, but D-enantiomer is not available in nature and has no physiological significance; therefore, its always refered to as L-enantiomer of ascorbic acid and ascorbate (Figure 2.14) (Aboul-Enein *et al*., 1999). Ascorbic acid, or vitamin C, is a monosaccharide oxidation-reduction (redox) catalyst found in both animals and plants. Vitamin C can be produced by most animals and plants from D-glucose and D-galactose, whereas it is not produced in humans due to the lack of L-gulonolactone oxidase; and therefore, it should be taken externally (Naidu, 2003). Most other animals are able to produce this compound in their bodies and do not require it in their diets. Fruits, vegetable and organ meats (e.g. liver and kidney) are generally the best sources of ascorbic acid, muscle meats and most seeds do not contain significant amount of ascorbic acid (Combs, 1992).

Ascorbic acid is required for the conversion of the procollagen to collagen by oxidizing proline residues to hydroxyproline. In other cells, it is maintained in its reduced form by reaction with glutathione, which can be catalysed by protein disulfide isomerase and glutaredoxins (Smirnoff and Wheeler, 2000). Ascorbic acid is a redox catalyst which can reduced, and thereby neutralize,

ROS such as hydrogen peroxide. Ascorbic acid is present at high levels in all parts of plants and can reach concentrations of 20 millimolar in chloroplasts (Shigeoka *et al*., 2002). The World Health Organization (WHO) recommends a daily intake of 45 mg per day for healthy adults (WHO, 2004). Figure 2.14 shows structure of Ascorbic acid.

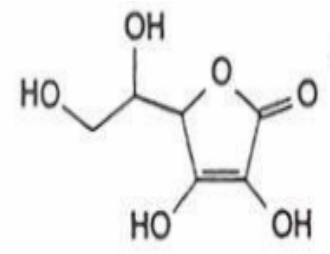

Figure 2.2: Structure of Ascorbic acid (Hacişevkđ, 2009).

2.6.1 Sources of ascorbic acid

Ascorbic acid is widely distributed in fresh fruits and vegetables. It is present in fruits like orange, lemons, grapefruit, watermelon, papaya, strawberries, cantaloupe, mango, pineapple, raspberries and cherries. It is also found in green leafy vegetables, tomatoes, broccoli, green and red peppers, cauliflower and cabbage. Most of the plants and animals synthesize ascorbic acid from D-glucose or D-galactose (Naidu, 2003). Majority of animals produce relatively high levels of ascorbic acid from glucose in liver. However, guinea pigs, fruit eating bats, apes and humans cannot synthesize ascorbic acid due to the absence of the enzyme L-gulonolactone oxidase. Hence, in human ascorbic acid has to be supplemented through food and or as tablets. Ascorbic acid is a labile molecule; it may be lost from foods during cooking/processing even though it has the ability to preserve foods by virtue of its reducing property. Synthetic ascorbic acid is available in a wide variety of supplements viz., tablets, capsules, chewable tablets, crystalline powder, effervescent tablets and liquid form. Buffered ascorbic acid and esterfied form of ascorbic acid as ascorbyl palmitate is also available commercially (Naidu, 2003). Both natural and synthetic ascorbic acid are chemically

identical and there are no known differences in their biological activities or bioavailability (Naidu, 2003).

2.6.2 Biosynthesis

The biosynthesis of ascorbic acid in animals is included in the glucuronic acid metabolic pathway. The metabolic pathway is involved in the metabolism of sugars under normal and disease conditions, and in regulation of physiological functions. It is an important pathway for major detoxification processes. The activities of the synthesizing enzymes vary from species to species (Chatterjee *et al*., 1975). Most animals can convert D-glucose into L-ascorbic acid. Humans and other primates, guinea pigs, Indian fruit bats, some fish and birds, and insects are unable to produce ascorbic acid endogenously. Most of research on ascorbic acid synthesis in animals has been carried out using rats. D-glucose is converted into L-ascorbic acid via D-glucuronic acid, L-gulonic acid, L-gulonolactone and 2-keto-L-gulonolactone as intermediates. Studies with radioactive labelling technique have indicated that, in the synthetic pathway, inversion of C-1 and C-6 takes place between D-glucuronic acid and L-gulonic acid, while the D-glucose chain remains intact (F igure 2.15). Animals that cannot synthesize ascorbic acid endogenously lack the oxidizing enzyme Lgulono-γ-lactone oxidase. This enzyme is required in the last step of the conversion of L-gulono-γ- lactone to 2-oxo-L-gulono-γ-lactone, which is a tautomer of L-ascorbic acid and, transforms spontaneously into vitamin C (Nishikim and Yagi, 1991; Nishikimi *et al*., 1994). Certain microorganisms are able to synthesize ascorbic acid or one of its isomers. A bacterial-origin enzyme, L-gulono-γ-lactone dehydrogenase, which catalyzes the oxidation reaction of the synthesis of ascorbic acid, has been isolated and characterized (Tolbert *et al*., 1975; Packer and Fuchs, 1997). The physical and chemical properties of this enzyme are entirely different from those of eucaryotic organisms.

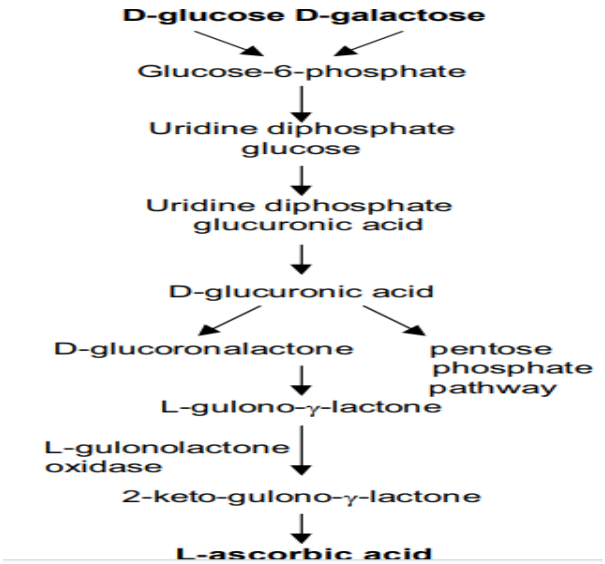

Figure 2.3: Biosynthesis of L-Ascorbic acid (Naidu, 2003).

2.6.3 Function of vitamin C

Vitamin C (ascorbic acid) is an essential water soluble nutrient that primarily exerts its effect on host defence mechanisms and immune homeostasis (Hartel *et al*., 2004). It is one of the most widely available and affordable non-enzymatic antioxidant molecules that have been used to mitigate oxidative damage (Naidu, 2003). Under physiological conditions, it functions as a potent reducing agent that efficiently quenches potentially damaging reactive oxygen species (ROS) produced by normal metabolic respiration (Arrigoni and De Tullio, 2002). Because it has a low redox potential of 280 mV, it has the potential to react with almost all other oxidized free radicals. Therefore, it is used as an antioxidant (Naidu, 2003). Vitamin C enhances antioxidant defence of T cells, T cells' responsiveness to antigens, and inhibits T cell apoptosis-signalling pathways, indicating a significant role in regulating the immune function (Campbell *et al*., 1999; Wu *et al*., 2000). Vitamin C is also a compound that plays an important role in collagen synthesis (Naidu, 2003). Vitamin C is also responsible for the synthesis of carnitine and various neurotransmitters as well as tyrosine and microsomal metabolism (Gropper *et al*., 2005).

It has been reported that vitamin C ameliorates organophosphate pesticide-induced haematological and biochemical alterations in humans and animals (Ambali and Ayo, 2011; Karmmon *et al*., 2011), and also sensorimotor and cognitive changes in animals (Ambali *et al*., 2010a; Ambali and Ayo, 2011).

Ascorbic acid regulates and participates in enzymatic reactions and transport for neurotransmitters and in hormone biosynthesis (Diliberto and Daniels, 1991). In the biosynthesis of a variety of neurochemicals, ascorbic acid is involved in many of the hydroxylation and decarboxylation reactions. Tyrosine is normally catabolized by vitamin C to fumaric and acetoacetic acid via homogenistic acid. Animals deficient in ascorbic acid metabolize tyrosine incompletely. In another metabolic pathway, tyrosine is metabolized in the presence of ascorbic acid to catecholamines by hydroxylation and decarboxylation reactions forming dopamine, norepinephrine, epinephrine, and adrenocrome. Ascorbic acid is directly involved in the dopamine-β-hydroxylase reaction to produce norepinephrine. The catecholamine biosynthesis occurs in the adrenal glands and the brain, both with relatively large amounts of ascorbic acid. Ascorbic acid protects catecholamines by direct chemical interactions and by elimination of adrenocrome, a toxic product of catecholamine oxidation, which has been linked to certain mental diseases (Bruick and McKnight, 2001; Arrigoni and De Tulio, 2002).

2.6.4 Pharmacokinetics

Vitamin C can be administered orally or intravenously (Padayatty *et al*., 2004). It is well absorbed efficiently in the small intestine via a saturable active transport mechanism. Absorption efficiency of low oral doses of vitamin C (4-64 mg) may be as high as 98% but decreases with increasing doses of vitamin C. Vitamin C is widely distributed in all tissues of the body, with higher levels found in the adrenal glands, pituitary and retina (Wandzilak *et al*., 1994). Vitamin C is oxidized to

dehydroascorbic acid, which is hydrolysed to d-ketogulonic acid and then oxidized to oxalic and threonic acid (Wandzilak *et al*., 1994). Oxidation of carbon dioxide occurs at high doses of vitamin C. Unmetabolized vitamin C and vitamin C metabolites, such as oxalate are largely excreted in the urine. Approximately 3% of the oral dose is excreted in faeces (Levine *et al*., 1999). More of the vitamin is excreted unchanged at higher level of vitamin C intake (Levine *et al*., 1999). Available data suggested that vitamin C is not associated with significant adverse effects and there are no obvious specific key toxic endpoints for vitamin C dose given orally to healthy subject. But high oral doses of vitamin C were reported to be associated with gastrointestinal effects like abdominal distension, flatulence, diarrhoea and transient colic (Cameron and Campbell, 1974).

2.6.5 Mechanism of action

Vitamin C is hydrophilic and is an important free radical scavenger in extracellular fluids, trapping radicals and protecting biomembranes from peroxide damage. Vitamin C effectively scavenges singlet oxygen, superoxide, hydroxyl, water soluble peroxyl radical and hypochlorous acid (Smirnoff and Wheeler, 2000). It is also an excellent source of electrons and therefore can donate electrons to free radicals such as hydroxyl and superoxide radicals and quench their activity (Bendich, 1990). Vitamin C is an essential co-factor involved in many biochemical functions and acts as an electron donor or reducing agent (Sies *et al*., 1992).

Vitamin C acts as a potential antioxidant due to its plethora of mechanisms like free radical and reactive oxygen species scavenging action, redox potential and prevention of oxidative damage of lipids, proteins and nuclear material (Chakraborthy *et al*., 2014). The antioxidant property of ascorbic acid is attributed to its ability to reduce potentially damaging ROS, forming, instead, resonance-stabilized and relatively stable ascorbate free radicals (Buettner, 1993). This mechanism is manifest in a number of cytoprotective functions under physiological conditions, including

prevention of DNA mutation induced by oxidation (Pflaum *et al*., 1998; Lutsenko *et al*., 2002), protection of lipids against peroxidative damage (Kimura *et al*., 1992; Barja *et al*., 1994), and repair of oxidized amino acid residues to maintain protein integrity (Cadenas *et al*., 1998). As DNA mutation is likely a major contributor to the age-related development of cancer (Deng *et al*., 1998; Halliwell, 2000), attenuation of oxidation induced mutations by vitamin C constitutes a potential anticancer mechanism. Plasma vitamin C at normal to high physiological concentrations (60-100 mmol/L) decreases oxidative stress-induced DNA damage by neutralizing potentially mutagenic ROS (Lutsenko *et al*., 2002). Consumption of vitamin C-rich foods is inversely related to the level of oxidative DNA damage *in vivo* (Rehman *et al*., 1999; Thompson *et al*., 1999). However, vitamin C switch over role as a pro-oxidant is paradoxical, stating the influence of iron and copper by the virtue of Fenton chemistry in pathological conditions (Chakraborthy *et al*., 2014), promoting oxidative damage to DNA (Stich *et al*., 1976). This occurs in the presence of free transition metals, such as copper and iron, which are reduced by ascorbate and, in turn, react with hydrogen peroxide, leading to the formation of highly reactive and damaging hydroxyl radicals (Stich *et al*., 1976). However, the relevance of this under normal physiological conditions *in vivo* has been questioned, as most transition metals exist in inactive, protein bound form *in vivo* (Halliwell and Gutteridge, 1986). However, when used at pharmacological concentrations (0.3-20 mmol/L), ascorbic acid displays transition metal-independent pro-oxidant activity, which is more profound in cancer cells and causes cell death (Chen *et al*., 2005). This tumor cell-killing response is dependent upon ascorbate incubation time and extracellular ascorbate concentration (Chen *et al*., 2005).

2.7 Phenolic Compounds in Plants

There are approximately 300,000 documented species of higher plants on the planet, which synthesize an enormous number of chemicals of diverse structure and class (more than 200,000 isolated and identified individual chemical entities). These compounds can be further divided into primary and secondary metabolites. The primary metabolites include metabolites such as sugars, fatty acids, amino, and nucleic acids, as well as chemicals considered ubiquitous to all plants for growth and development (Fiehn, 2002; Wu and Chappell, 2008). All plants produce an amazing diversity of secondary metabolites. Secondary metabolites apparently act as defence (against herbivores, microbes, viruses, or competing plants) and signal compounds (to attract pollinating or seed dispersing animals), as well as protecting the plant from ultraviolet radiation and oxidants (Lattanzio *et al*., 2008). One of the most important groups of these metabolites is phenolic compounds. Plant phenolic compounds (plant polyphenolics) are the compounds holding one or more phenolic rings and are derived from the secondary metabolism of plants (Parr and Bolwell 2000). Phenolic compounds exhibit a wide range of physiological properties, such as anti-allergenic, anti-artherogenic, anti-inflammatory, anti-microbial, antioxidant, anti-thrombotic, cardioprotective and vasodilatory effects (Puupponen-Pimia *et al*., 2001; Manach *et al*., 2005). Phenolic compounds are present in several foods and drinks of plant origin, for example, vegetables, fruits, coffee (Clifford 1999), tea (Lakenbrink *et al*., 2000), beer, wine, and chocolate (Hollman and Katan 1999). In addition to being present in many foods naturally, phenolic compounds can be extracted from their respective sources and thereafter can be added to some foods for their colouring properties and for their antioxidant effects (Maqsood *et al*., 2013).

2.7.1 Classification of plant phenolics

Phenolic compounds can be broadly divided into 2 categories; flavonoids and non flavonoid polyphenols. Non flavonoids and phenolic acids are abundant in foods. Flavonoids, the target class of polyphenols, may be divided into different subclasses according to the degree of oxidation of the heterocyclic ring: anthocyanins, flavonols, flavans, flavanol, flavones, and isoflavones (Figure 2.14) (Scalbert and Williamson 2000). Most of them are known to possess antioxidative activities. The mode of their antioxidative action may vary, depending on the structural and compositional characteristics of the individual phenolic compound. Phenolic compounds with varying antioxidative activities from diverse natural sources have been reported (Maqsood *et al*., 2014).

2.7.2 Antioxidant effect of plant phenolics

Among phenolic compounds found in plants, flavonoids are the most widely studied class with respect to their antioxidant and biological activities. They have powerful antioxidant activities *in vitro*, being able to scavenge a wide range of (ROS) and reactive nitrogen species RNS and chlorine species, such as superoxide, hydroxyl, and peroxyl radicals, and peroxynitrous acid and hypochlorous acid (Hernandez *et al*., 2009). Thus, rapid donation of a hydrogen atom to lipid radicals by these phenolic antioxidants could be one of the ways to impede lipid oxidation. In this respect, phenolic antioxidants are exceptional hydrogen or electron donors. Moreover, their radical intermediates are comparatively stable because of resonance delocalization and absence of appropriate sites for attack by molecular oxygen (Nawar, 1996). Therefore, they can terminate the chain radical reaction and thus prevent lipid oxidation in different lipid substrates. The antioxidative potential of these phenolic compounds plays an essential role in preventing lipid

oxidation and quality retention in different food products, especially those containing high amounts of PUFAs such as seafoods (Maqsood *et al*., 2013).

Antioxidant action of phenolic compounds is due to their high tendency to chelate metals. Phenolics possess hydroxyl and carboxyl groups, able to bind particularly iron and copper (Jung *et al*., 2003). The roots of many plants exposed to heavy metals exude high levels of phenolics (Winkel-Shilley, 2002). They may inactivate iron ions by chelating and additionally suppressing the superoxide-driven Fenton reaction, which is believed to be the most important source of ROS (Rice-Evans *et al*., 1997; Arora *et al*., 1998). Tannin-rich plants such as tea, which are tolerant to Mn excess, are protected by the direct chelation of Mn (Lavid *et al*., 2001). According to Morgan *et al*. (1997) this general chelating ability of phenolic compounds is probably related to the high nucleophilic character of the aromatic rings rather than to specific chelating groups within the molecule. Phenolic antioxidants inhibit lipid peroxidation by trapping the lipid alkoxyl radical. This activity depends on the structure of the molecules, and the number and position of the hydroxyl group in the molecules (Millic *et al*., 1998). Among the tropical plant that is rich in phenolic compound is *Moringa oleifera*. Figure 2.16 shows generic structure of major classes of flavonoids.

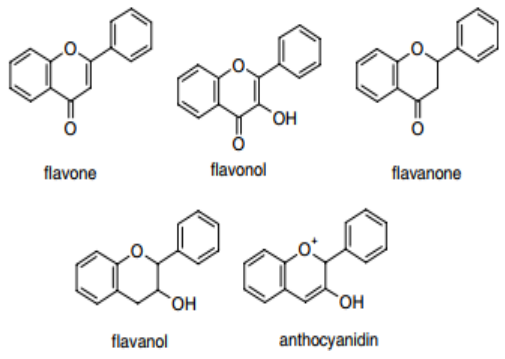

Figure 2.4: Generic structure of major classes of flavonoids (Hernandez *et al*., 2009).

2.8 *Moringa oleifera*

Moringa oleifera is the most widely cultivated species of a monogeneric family, the Moringaceae, which is native to the sub-Himalayan tracts of India, Pakistan, Bangladesh, Afghanistan, Thailand and Philippines (Fuglie, 2005). This rapidly-growing tree (also known as the horseradish tree, drumstick tree, or Ben oil tree), was utilized by the ancient Romans, Greeks and Egyptians; it is now widely cultivated and has become naturalized in many locations in the tropics (Figure 2.17) (Fahey, 2005). In Nigeria it is mostly grown in the northern part and locally known as *Zogeli* among the Hausa speaking people. The Yoruba of South-West Nigeria call it *ewe ile* or *igi iyaanu* (because of its many medicinal uses) (Saalu *et al*., 2012). Moringa grows naturally at elevations of up to 1,000 m above sea level. It also grows well on hillsides but is more frequently found growing on pastureland or in river basins. It is a fast growing tree and has been found to grow to 6 to 7 m in one year in areas receiving less than 400 mm mean annual rainfall (Odee, 1998). *M. oleifera* is a fast-growing, deciduous tree that can reach a height of 10-12 m (32-40 ft) and trunk diameter of 45 cm (1.5 ft) (Parotta, 1993).

The bark has a whitish-grey colour and is surrounded by thick cork. Young shoots have purplish or greenish-white, hairy bark. The tree has an open crown of drooping, fragile branches and the leaves build up feathery foliage of tripinnate leaves (Rashid *et al*., 2008). The flowers are fragrant and bisexual, surrounded by five unequals thinly veined, yellowish-white petals. The flowers are about 1.0-1.5 cm (1/2") long and 2.0 cm (3/4") broad. They grow on slender, hairy stalks in spreading or drooping flower clusters which have a length of 10-25 cm (Parotta, 1993). Flowering begins within the first six months after planting. In seasonally cool regions, flowering only occurs once a year between April and June. In more constant seasonal temperatures and with constant rainfall, flowering can happen twice or even all year-round (Parotta, 1993). The fruit is a hanging,

three-sided brown capsule of 20-45 cm size which holds dark brown, globular seeds with a diameter around 1 cm. The seeds have three whitish papery wings and are dispersed by wind and water (Parotta, 1993). In cultivation, it is often cut back annually to 1-2 m (3-6 ft) and allowed to regrow so the pods and leaves remain within arm's reach (Parotta, 1993).

Moringa oleifera is an important tropical crop that is used in human nutrition, medicine and in oil production (Anwar *et al*., 2005; Soliva *et al*., 2005; Hamza, 2010). All parts of this plant are applied in traditional medicine for the treatment of human diseases such as asthma, spasm, enlarged liver and spleen, infection and nervous debility, ulcer, inflammation and for wound healing (Promkum *et al*., 2010; Mishra *et al*., 2011). The biological activities of this plant as hepatoprotective (Pari and Kumar, 2002), hypocholesterolemic (Mehta *et al*., 2003), antifungal (Chuang *et al*., 2007) and antitumor (Bharali *et al*., 2003) agents have been documented.

Figure 2.5: *Moringa oleifera* leaves (Odee, 1998).

2.8.1 Antioxidant activity

M. oleifera is one of such medicinal plants reported to have high antioxidant activities due to its high contents of phenolics compound (Siddhuraju and Becker, 2003; Iqbal and Bhanger, 2006). Administration of *M. oleifera* extract enhances the SOD and catalase profiles, dose-dependently, by acting as a strong free radical quencher, hence protecting the cells (Molina *et al*., 2003; Parimoo

et al., 2014). The antioxidant potential of *M. oleifera* leaves extract may be attributed to the presence of total phenolics and flavonoids in the extract and/or active constituents like β-sitosterol, quercetin and kaempferol which have hydroxyl group(s) (Singh *et al*., 2014). The hydroxyl group(s), because of its resonance property, easily donates electron (e^-) to free radicals and effectively neutralizes the free radicals. Also, the presence of a hydroxyl group(s) increases its antioxidant potential through intermolecular hydrogen bonding involving the -SH group of non-protein thiols and enzymes resulting in the restoration of the antioxidant system against oxidative damage in mammalian tissue (Singh *et al*., 2014). The active antioxidant properties of *M. oleifera* known to contain specific plant pigments include carotenoids- lutein, chlorophyll, xanthins, alpha carotene and beta carotene. Other isolated phytochemicals in *M. oleifera* with antioxidant potentials are quercetin, rutin, kaempferol and caffeoyqumic acids (Ndong *et al*., 2007). Potent antioxidant vitamins- A, C, E (Siddhuraju and Becker, 2003; Aslam *et al*., 2005) and essential micronutrients with antioxidant ability such as selenium and zinc have been reported in the plant (Fuglie, 1999).

2.8.2 Profertility effect

Administration of *M. oleifera* L. powder shown significantly higher testicular and epididymal weight gain in male mice (Priyadarshani *et al*., 2014). It may be due to the fact that the leaves are excellent source of vitamin B, calcium, protein and potassium, Beta-carotene and other phytochemicals with known powerful antioxidant ability like kaempferol, quercetin, rutin and caffeoylquinic acids; powerful antioxidant vitamins - C, E, and A, and essential micronutrients with antioxidant activity - selenium and zinc (Fuglie, 1999; Jaiswal *et al*., 2009; Vongsak *et al*., 2013). D'cruz and Mathur *et al*. (2005) proved that the sperm cytoplasm contained very low concentrations of scavenging enzymes therefore an increase in the antioxidant enzyme system

levels by *Moringa* treatment can favour the reproductive process and also enhances spermatogenesis. A work by the Bureau of Plant Industry (BPI) showed that a steady diet of *Moringa* fruit boosts the sperm count of men thus, improves their chances of fertilizing an egg (Cabacungan, 2008). Serrano (2008) reported an increase in the sperm count in male mice when 1% concentration of *Moringa* ethanolic leaf extract was administered subcutaneously for two weeks.

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Plant Materials

3.1.1 Plant collection, identification and preparation

Fresh leaves of *Moringa oleifera* were collected from the main campus of University of Ilorin, Ilorin, Nigeria. The samples of the leaves were sent to the herbarium of the Department of Plant Biology of the University for identification. A specimen was deposited at the herbarium for reference purpose, while voucher number UILH/001/1011 was given. The leaves were air-dried to constant weight in the laboratory at room temperature and pounded to powdered form using mortar and pestle. The powdered samples were kept in polythene bag until use.

3.1.2 Plant extraction

Two kilograms (2 kg) of the powdered *Moringa oleifera* leaves was extracted with methanol (10L) using a cold extraction method in a glass funnel for 48 hours, with periodic shaking, to enhance the extraction process. Thereafter, the solution was filtered using Whatman No 1 filter paper and the filtrate concentrated *in-vacuo*, using the rotary evaporator coupled to a thermocirculator. The residue was further air-dried to a constant weight and thereafter preserved in a refrigerator at 4°C until required for use. Four (4) gram of the extract was dissolved in 100 ml of distilled water to make 4% (w/v) stock solution.

3.1.3 Phytochemical screening of leaf extract

Phytochemical screening of the leaf extract of *M. oleifera* was carried out to determine the chemical constituents, using standard methods as described by Trease and Evans (1978). Alkaloids were detected using Mayer's test; phenols using ferric chloride test; glycosides using modified

Salkowski's test; saponins using Foam test; tannins using gelatin test; flavonoids using alkaline reagent test and steroids using Salkowski's test (Appendix VIII).

3.1.4 Quantitative analysis of methanolic extract of *M. oleifera* leaves for flavonoids and total phenolics

Quantitative analysis of the total phenolic contents of the extract of *M. oleifera* was performed using high-performance liquid chromatophgraphy method as described by Makkar *et al.* (1997). Total flavonoid content of the extract was measured by the aluminium chloride colorimetric assay (Zhishen *et al*., 1999) (Appendix VIII).

3.2 Ethical Approval

Ethical approval was sought from Ahmadu Bello University Committees on Animal Use and Care (ABUCAUC) with the approval no: **ABUCAUC/2016/040.**

3.3 Drug Acquisition and Preparation

Commercial grade Carbamazepine (200 mg) marketed as Tegretol® (Novartis Farma, Italy) and vitamin C tablet (100 mg) (Biopharma, Nigeria) tablets were obtained from Aromokeye pharmaceutical stores in Ilorin, Kwara State. They were dissolved in distilled water to make solution prior to use.

3.4 Chemical Acquisition

All the chemicals used for this study were of analytical grade and were obtained from Science Research Laboratory Stores in Ilorin, Kwara State.

3.5 Experimental Animals

Apparently healthy male adult Wistar rats were obtained from the Animal House of the Department of Veterinary Pharmacology and Toxicology, University of Ilorin, Ilorin. The animals were conditioned to the laboratory environment for two weeks for acclimatization prior to the commencement of the study. All animals were housed in plastic cages in the animal holding facility of the department and were fed with commercial grower's feed (Top Feed, Nigeria). Rats were given access to feed and water *ad libitum*.

3.6 Determination of Median Lethal Dose (LD_{50}) of *Moringa oleifera* Extract

The median lethal dose (LD_{50}) of the methanol extract of *M. oleifera* was determined using a standard two phase approach as described by Lorke (1983). In the first phase of the trial, nine rats were divided at random into three groups of three rats each. The animals were deprived of feed and water for 12 hours. Group I, II and III were treated with *M. oleifera* extract orally at 10 mg/kg, 100 mg/kg and 1000 mg/kg body weight, respectively. Rats were observed for 48 hours for any sign of toxicity or mortality. In the second phase of the experiment, three animals were assigned into three groups (IV, V and VI) of one animal each. Animals in groups IV, V and VI were administered with 1600 mg/kg, 2900mg/kg and 5000mg/kg respectively. They were observed for death over a period of 48 hours. The LD_{50} was determined based on dose-response as described by Lorke (1983).

3.7. Chronic Reproductive Toxicity Study

Chronic reproductive toxicity was aimed at evaluating pituitary and testicular lipoperoxidation, superoxide dismutase, catalase and glutathione peroxidase activity, follicle stimulating hormone, luteinizing hormone and testosterone concentrations, as well as evaluating sperm characteristics.

3.7.1 Animal groupings and dosing protocol

A total of seventy (70) adult male Wistar rats (weighing 150-250 g) were used for this study. The rats were divided at random into seven groups of ten rats each. Group I was administered distilled water only at 2 ml/kg, while group II was given vitamin C at the dose of 100 mg/kg (Ambali *et al*., 2010b). Group III was administered with methanol extract of *M. oleifera* (250 mg/kg ~ $1/20^{th}$ of the LD_{50}), while group IV was administered carbamazepine at 20 mg/kg (Rajesh *et al*., 1991) only. Group V was pretreated with vitamin C (100 mg/kg) then followed by carbamazepine (20 mg/kg) 30 minutes later, group VI was pretreated with *M. oleifera* extract (250 mg/kg) followed by carbamazepine (20 mg/kg) 30 minutes later (Idoga, 2015). Group VII was pretreated with *M. oleifera* extract (250 mg/kg) and vitamin C (100 mg/kg) then followed by carbamazepine (20 mg/kg) 30 minutes later (Ambali *et al*., 2012). The regimens were administered orally, by gavage once daily for a period of 15 weeks. During this period, the rats were observed for signs of toxicity and death. At the end of the treatment period, the animals that survived were sacrificed by jugular venesection after light chloroform anaesthesia and blood, sera and tissue samples were collected for analysis. Post-mortem, gross and histopathological examinations were conducted on the euthanized animals and those that died in the course of the experiment.

3.7.2 Effect of treatments on pituitary gland and testicular tissue lipoperoxidation

Pituitary and testicular lipoperoxidation were determined by measuring the malondialdehyde (MDA) concentration using the method of Draper and Hadley (1990). The principle was based on measurement of the colour developed as a result of the reaction of thiobarbaturic acid (TBA) with MDA. Briefly, pituitary gland samples from animals in all the groups were individually weighed and homogenized in a known volume of ice-cold phosphate buffer to obtain a 10% homogenate, which were centrifuged at 2000 × *g* for 10 minutes to obtain a supernatant. The supernatant was

mixed with 1 mL of 10% TCA (trichloroacetic acid) and 1 mL of 0.67% TBA. The mixture was then heated in a boiling water bath for 15 minutes, and butan-2-ol was added to the solution. The mixture was centrifuged at 800 × *g* for 5 minutes. The thiobarbituric acid reactive substances (TBARS), MDA was determined from the absorbance at 532 nm using UV spectrophotometer (Model UV/VIS 752, Pec Medical, USA). The concentration of MDA in the samples were calculated by absorbance coefficient of MDA-TBA complex 1.56×10^5/cm/M and expressed in nmol/mg of protein for pituitary gland and testis.

3.7.3 Evaluation of pituitary and testicular superoxide dismutase activity

Superoxide dismutase activity was analysed in the pituitary gland and testes using SOD Assay Kit-WST (SOD-19160 water-soluble tetrazolium salt, Sigma-Aldrich, USA). The principle of the test was based on monitoring of the auto-oxidation rate of haematoxylin as described by Martin *et al*. (1987). Briefly, in the presence of SOD enzyme at specific pH of 7.4, the rate of auto-oxidation of haematoxylin was inhibited and the percentage of inhibition was linearly proportional to the amount of SOD present within a specific range. Sample SOD activity was determined by measuring ratio of auto-oxidation rates in the presence and absence of the sample and expressed as McCord-Fredovich "cytochrome c" units.

3.7.4 Evaluation of pituitary and testicular catalase activity

Catalase activity was analysed in the pituitary gland and testes using the catalase activity assay kit (ab83464, UK). The principle was based on monitoring and measuring the consumption of hydrogen peroxide (H_2O_2) substrate at 240 nm as described by Beers and Sizer (1952) with the following modifications to increase robustness and convenience. Catalase calibrator of known activity was used in order to prevent the need for tedious H_2O_2 substrate calibrations. Reagents

were formulated to provide better stability of diluted H_2O_2 substrate and diluted CAT standards, allowing for more convenience. The rate of peroxide removal by catalase is exponential. It is difficult to saturate catalase because of the large rate constants of compound I and II (1.7 and 2.6 $\times 10^{-7}$ M^{-1} s^{-1}, respectively). Catalase will begin to be inactivated by H_2O_2 at levels >0.1 M, when compound I is converted to compound II or III. By the end of the assay, H_2O_2 is consumed and catalase is inactivated. The catalase-positive control activity is defined in international unit equals (1 unit) as the amount of catalase necessary to decompose 1.0 µM of H2 O2 per minute at pH 7.0 at 25 °C, whereas H_2O_2 concentration falls from ≈10.3 to 9.2 mM. The concentration of H_2O_2 can be calculated from absorbance using the following expression:

[H_2O_2 mM]=(Absorbance 240nm 1,000)/39.4mol^{-1}cm where 39.4 mol^{-1} cm^{-1} is the molar extinction coefficient for H_2O_2.

3.7.5 Evaluation of pituitary and testicular glutathione peroxidase activity

Glutathione peroxidase (GPx) activity in the pituitary gland and testes was evaluated using the Fortress Diagnostic glutathione peroxidase assay kits protocol BXC0551A (Antrim, UK), adapted from the method described by Paglia and Valentine (1967). Briefly, GPx catalysed the reduction of hydrogen peroxide and oxidized the reduced glutathione to form oxidized glutathione (GSSG). GSSG was then reduced by glutathione reductase and β-nicotinamide adenine dinucleotide phosphate (NADPH) forming NADP. This resulted in decrease absorbance at 340 nm and the recycling of GSH. The decrease in absorbance at 340 nm was directly proportional to the GPx concentration. The assay can be carried out with cumene hydroperoxide or tert-butyl hydroperoxide as the substrate instead of H_2O_2 to measure total GPx. Tert-butyl and cumene hydroperoxides are both hydroperoxides and both glutathione S-transferases (non-selenium containing peroxidase or selenium independent) and GPx (contains a selenium in the active site)

can utilize hydroperoxides to determine total peroxidase activity. On the other hand, glutathione S-transferases will not detoxify H_2O_2. Thus, the assay using cumene hydroperoxide or tert-butyl hydroperoxide measures selenium-dependent GPx and activity from glutathione S-transferases (selenium-independent GPx). This assay is an indirect, coupled assay for glutathione peroxidase. This assay takes advantage of glutathione disulfide (GSSG) formed by the enzymatic action of GPx and is regenerated by excess glutathione reductase (GR) in the assay. The action of GR is monitored by following the disappearance of the co-substrate NADPH at 340 nm. The assay recording of NADPH loss measures H_2O_2 reduction by GPx to an alcohol.

3.7.6 Evaluation of concentrations of serum follicle-stimulating hormone and luteinizing hormone

The serum follicle stimulating hormone and luteinizing hormone concentrations were assayed using the Follicle Stimulating Hormone Elisa Kit (FS232F, Calbiotech Inc. USA) and Luteinizing Hormone Elisa Kit (LH231F, Calbiotech Inc. USA), respectively according to the instruction of the manufacturer. It is an enzyme immunoassay procedure. The principle of determination was based on the "Sandwich". Briefly, two separate antibodies directed at distinct antigenic determinants of FSH and LH molecules were utilized in the assay. Two hormones present in the test sample react simultaneously with one antibody immobilized on the microwell surface and with another antibody conjugated to horseradish peroxidase enzyme, leading to the formation of an Ag-Ab-Enzyme complex on the microwell surface. Then, the unbound conjugate was removed by washing, and the colour development reagents (substrate) were added. Upon exposure to the enzyme, a colour change occurred. The intensity of the colour which reflects the amount of bound anti-FSH, and anti-LH enzyme conjugates was determined and was expected to be proportional to the concentration of FSH or LH in the specimen within a dynamic range of assay. After stopping the reaction, the resulting colour was measured using a spectrophotometer (UV/VIS 752, Pec

Medical, USA) at 450 nm. A set of standards is used to plot a standard curve from which the amount of FSH and LH in the samples and controls can be directly read (Wilkle and Utley, 1987).

3.7.7 Evaluation of serum testosterone concentration

Sera samples collected were assayed for testosterone concentration using Enzyme-Linked Immunosorbent Assay (ELISA) method based on competitive binding principle. Testosterone present in the sample competes with enzyme-labelled testosterone for binding with anti-testosterone antibody immobilized on the microwell surface. The amount of conjugate that bind to the microwell surface was expected to decrease in proportion to the concentration of testosterone in the sample. The unbound sample and conjugate were removed by washing and the colour development reagents (substrates) were added. Upon exposure to the bound enzyme, a colour change occurred. The intensity of colour reflects the amount of bound enzyme-testosterone conjugate and was inversely proportional to the concentration of testosterone in the sample within dynamic range of the assay. After stopping the reaction, the resulting colour was measured using a spectrophotometer (UV/VIS 752, Pec Medical, USA) at 450 nm. The testosterone concentration in the sample and the control was determined from a standard curve, using the method of Wilkle and Utley (1987).

3.7.8 Evaluation of epididymal sperm count

One epididymis from each of the rats in each group was carefully removed, dried with blotting paper and immediately homogenized in 1 mL of 0.5% formol saline. One mililitre of the aliquot was diluted to 1:200 using erythrocyte diluting pipette. Counting was done using the improved Neubauer counting chamber, and cells were counted using a light microscope at magnification of × 20 as described by Yarube *et al*. (2009).

3.7.9 Evaluation of sperm motility

Sperm motility was evaluated using the method described by Sonmez *et al*. (2005). Briefly, caudal epididymis from each rat was cut into small pieces and transferred into the Petri dishes containing prewarmed 0.5 mL Tris buffer solution. The sperm cells were then allowed to swim out within five minutes, at 37°C. An aliquot of this solution was then observed under light microscope using magnification of 400 fold. The percentage sperm motility was calculated using the number of live sperm cells over the total number of sperm cells, both motile and immotile. The sperm cells that were not moving were considered to be immotile, while the rest, which displays some movement were considered motile.

3.7.10 Evaluation of sperm morphology

For sperm morphology evaluation, the suspension was smeared, dried and fixed with fixative (three volume of absolute methanol and one volume of glacial acetic acid), stained with haematoxylin for 15 minutes and then washed, followed by staining with 1 % eosin for 10 minutes, washed and left to dry at room temperature and observed using a light microscope at magnification of x100 (Wyrobek, 1979).

3.7.11 Evaluation of gross and histopathological changes

The pituitary glands, seminal vesicles and testicles of each rat were examined for any gross lesion. Samples of the tissues were prepared using the modified method described by Luna (1960) for histological examination. Briefly, the tissue samples were fixed in Bouin's solution for one week, the tissues were dehydrated in alcohol, cleared in xylene, infiltrated with molten paraffin wax at 60°C and blocked in paraffin according to standard procedures and labeled. Sections were cut at nominal thickness of 3-4 µm using Semi-automatic microtome (Model MR2258S, KEDEE,

Thailand). The sections were mounted on glass, deparafinized, stained with haematoxylin and eosin stain using DPX mountant (Trust Chemical Co., USA) as mounting medium. Slides prepared were viewed under microscope at × 400 magnification. Lesions were observed and recorded.

3.8 Statistical Analysis

Data obtained were expressed as mean ± standard error of mean (mean ± SEM) using column statistics and subjected to statistical analysis using one-way analysis of variance (ANOVA), followed by Tukey's *post-hoc* multiple comparison test. Graphpad prism version 5.03 for windows (Graphpad Software, San Diego, California, USA) was used to analyse all the data. Values of $P < 0.05$ were considered significant.

CHAPTER FOUR

4.0 RESULTS

4.1 Determination of Median Lethal Dose (LD_{50}) for *Moringa oleifera* Leaf Extract

All the three rats in each of the groups administered with *M. oleifera* at 10 mg/kg, 100 mg/kg and 1000 mg/kg, respectively during phase I of the LD_{50} determination survived 48 hours after administration. In the same vein, all the three rats administered with 1600 kg/kg, 2900 mg/kg and 5000 mg/kg in phase II survived 24 hours after administration without visible clinical signs of toxicity and death. The rats administered with the extract in phase II were sacrificed 48 hours after administration, and no gross lesion was noticed at post-mortem. Therefore, using Lorke's method (1983), the LD_{50} of *M. oleifera* extract was determined to be greater 5000 mg/kg, and the extract was considered relatively safe.

4.1.1 Phytochemical components of methanolic extracts of *Moringa oleifera* leaves

Phytochemical screening of the extract was positive for alkaloids, flavonoids, glycosides, phenols, tannins, steroids, lipids and amino acids, while saponin was absent. The results are summarized in Table 4.1.

Table 4.1: Summary of results of phytochemical screening of the methanol extract of *Moringa oleifera*

Components	Bioassay
Alkanoids	+
Phenols	+
Tannins	+
Flavonoids	+
Saponins	-
Steroids	+
Glycosides	+
Lipids	+
Amino acids	+

+ = positive (present)
- = negative (absent)

4.1.2 Flavonoid and phenolics contents of *Moringa oleifera* leaf extract

The results of the quantitative analysis of the flavonoid content of the extract showed that the extract contained 22.23% quercetin equivalent (QAE) of flavonoids. The results of UV visible analysis of the extract also showed that the extract contained phenols (7.8%), tannin (0.54%) and glycoside (0.004%). A summary of the results is shown in Table 4.2.

Table 4.2: Summary of the concentration of phytochemical constituents of the methanol extract of *Moringa oleifera*

Components	Concentration (% w/v)
Flavonoid	22.23
Phenol	7.8
Tannin	0.54
Glycoside	0.004

4.2 Chronic Toxicity Study

4.2.1 Clinical signs

There was no clinical sign of toxicity in animals treated with methanolic extract of *M. oleifera* (250 mg/kg). Animals in CBZ group (20 mg/kg) showed signs of dizziness and depression. The groups pretreated with *M. oleifera* (250 mg/kg) before exposure to CBZ, did not show any apparent sign of toxicity, likewise the group pretreated with vitamin C (100 mg/kg) before exposure to CBZ. Also, there was no apparent sign of toxicity in the group pretreated with *M. oleifera* (250 mg/kg) and vitamin C (100 mg/kg) before exposure to CBZ (20 mg/kg). Similarly, the DW, Vit-C and *M. oleifera* groups showed no signs of toxicity.

4.3 Effect of Treatment on Activity of Pituitary and Testicular Oxidative Stress Parameter

4.3.1 Effect of treatment on pituitary malondialdehyde concentration

Figure 4.1 shows the pituitary mean MDA concentrations in the different treatment groups. The pituitary mean MDA concentration in CBZ group was significantly ($P < 0.05$) higher when compared to those in Vit-C + MO + CBZ, MO + CBZ and DW groups. Also, the pituitary mean MDA concentration of DW group was significantly ($P < 0.05$) lower when compared with those in Vit-C + CBZ, Vit-C and MO groups.

4.3.2 Effect of treatment on testicular malondialdehyde concentration

Figure 4.2 shows the testicular mean malondialdehyde concentrations in the different treatment groups. The testicular mean MDA concentration in the CBZ group was significantly ($P < 0.05$) higher when compared to those in Vit-C + MO + CBZ, MO + CBZ, Vit-C + CBZ, MO, Vit-C and

DW groups. Also, the testicular mean MDA concentrations in Vit-C + MO + CBZ and MO + CBZ groups were significantly ($P < 0.05$) lower when compared to those in MO, Vit-C and DW groups.

4.3.3 Effect of treatment on pituitary superoxide dismutase activity

Figure 4.3 shows pituitary mean superoxide dismutase activity in the different treatment groups. The pituitary mean SOD activity in CBZ group was significantly ($P < 0.05$) lower when compared with the enzyme's activity in Vit-C + MO + CBZ, MO + CBZ, Vit-C + CBZ, MO and Vit-C groups. Also, the pituitary mean SOD activity in Vit-C + MO + CBZ group was significantly ($P < 0.05$) higher when compared to that in MO + CBZ, Vit-C + CBZ, MO, Vit-C and DW groups.

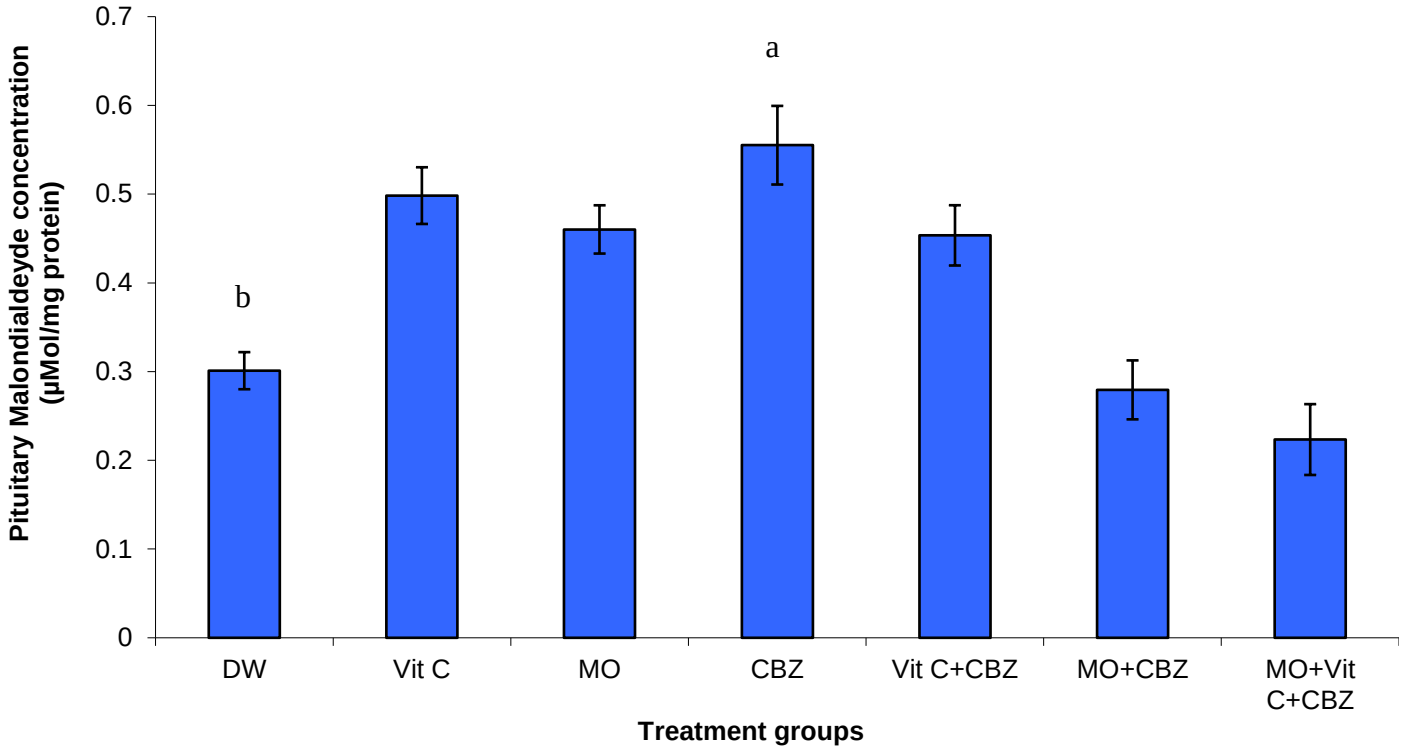

Figure 4.1: Effect of Vitamin C and Methanol leaf extract of *M. oleifera* on pituitary Malondialdehyde concentration of Adult Male Wistar Rats exposed to chronic Carbamazepine (n=5).

a= ($P < 0.05$) higher Vs Vit-C + MO + CBZ, MO + CBZ and DW groups.

b= ($P < 0.05$) lower Vs Vit-C + CBZ, Vit-C and MO groups.

Key:

DW= Distilled water

MO= *Moringa oleifera* leaf extract

Vit-C= vitamin C

CBZ= carbamazepine

Vit-C + CBZ= vitamin C and carbamazepine

MO + CBZ= *Moringa oleifera* leaf extract and carbamazepine

Vit-C + MO + CBZ= vitamin C, *Moringa oleifera* leaf extract and carbamazepine.

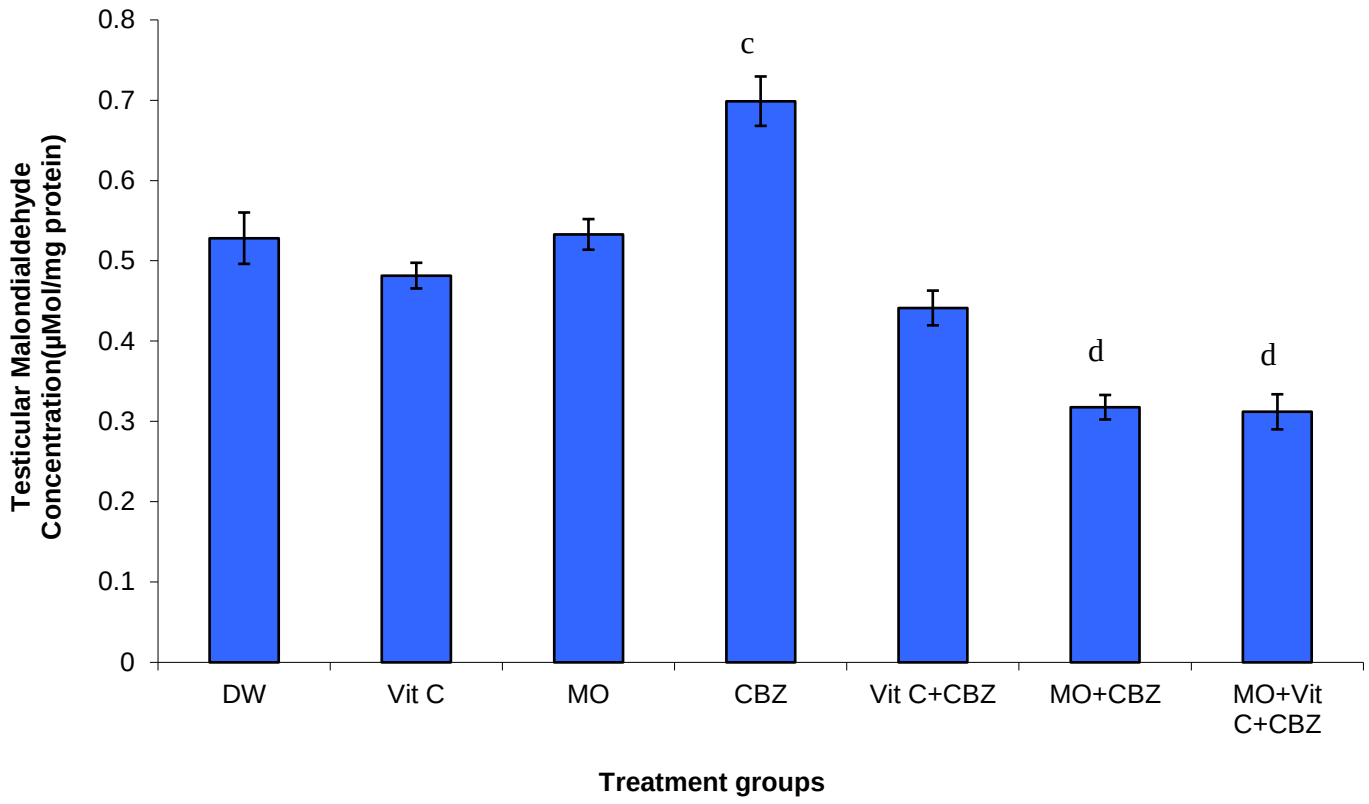

Figure 4.2: Effect of Vitamin C and Methanol leaf extract of *M. oleifera* on testicular Malondialdehyde concentration of Adult Male Wistar Rats exposed to chronic Carbamazepine (n=5).

c= ($P < 0.05$) higher Vs Vit-C + MO + CBZ, Vit-C + CBZ, MO + CBZ, MO, Vit-C and DW groups.

d= ($P < 0.05$) lower Vs MO, Vit-C and DW groups.

Key:

DW= Distilled water

MO= *Moringa oleifera* leaf extract

Vit-C= vitamin C

CBZ= carbamazepine

Vit-C + CBZ= vitamin C and carbamazepine

MO + CBZ= *Moringa oleifera* leaf extract and carbamazepine

Vit-C + MO + CBZ= vitamin C, *Moringa oleifera* leaf extract and carbamazepine.

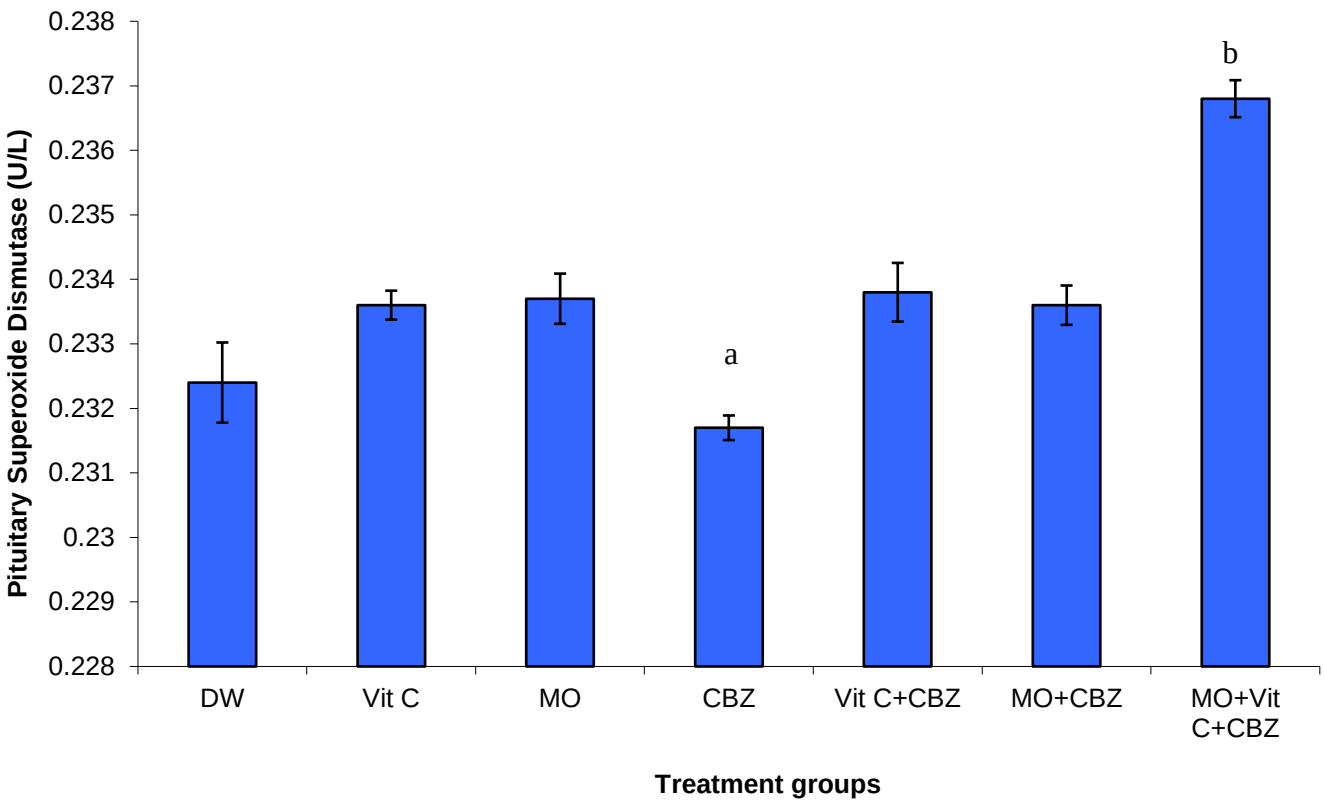

Figure 4.3: Effect of Vitamin C and Methanol leaf extract of *M. oleifera* on pituitary SOD activity of Adult Male Wistar Rats exposed to chronic Carbamazepine (n=5).

a= ($P < 0.05$) lower Vs Vit-C + MO + CBZ, Vit-C + CBZ, MO + CBZ, MO and Vit-C groups.

b= ($P < 0.05$) higher Vs MO + CBZ, Vit-C + CBZ, MO, Vit-C and DW groups.

Key:

DW= Distilled water

MO= *Moringa oleifera* leaf extract

Vit-C= vitamin C

CBZ= carbamazepine

Vit-C + CBZ= vitamin C and carbamazepine

MO + CBZ= *Moringa oleifera* leaf extract and carbamazepine

Vit-C + MO + CBZ= vitamin C, *Moringa oleifera* leaf extract and carbamazepine.

4.3.4 Effect of treatment on testicular superoxide dismutase

Figure 4.4 shows testicular mean superoxide dismutase activity concentrations in the different treatment groups. The testicular mean SOD activity in CBZ group was significantly ($P < 0.05$) lower when compared with the enzyme's activity in Vit-C + MO + CBZ, MO + CBZ and Vit-C + CBZ, MO and Vit-C groups. Also, the testicular mean SOD activity in Vit-C + MO + CBZ and MO + CBZ groups were significantly ($P < 0.05$) higher when compared with the enzyme's activity in MO, Vit-C and DW groups.

4.3.5 Effect of treatment on pituitary catalase

Figure 4.5 shows the pituitary mean catalase activity in the different treatment groups. The pituitary mean CAT activity in CBZ group was significantly ($P < 0.05$) lower when compared with that of Vit-C + MO + CBZ group. The pituitary mean CAT activity in CBZ group shows no significant ($P > 0.05$) change but increased in absolute values were recorded when compared to MO + CBZ (17.4%), Vit-C + CBZ, MO (19.8%), Vit-C (11%) and DW groups.

4.3.6 Effect of treatment on testicular catalase

Figure 4.6 shows the testicular mean catalase activity in the different treatment groups. The testicular mean CAT activity in CBZ group was significantly ($P < 0.05$) lower when compared to that of Vit-C + MO + CBZ, MO + CBZ, Vit-C + CBZ, Vit-C, MO and DW groups. The testicular mean CAT activity in MO group was significantly ($P < 0.05$) higher when compared to that of MO + CBZ, Vit-C + CBZ and DW groups (Figure 4.6)

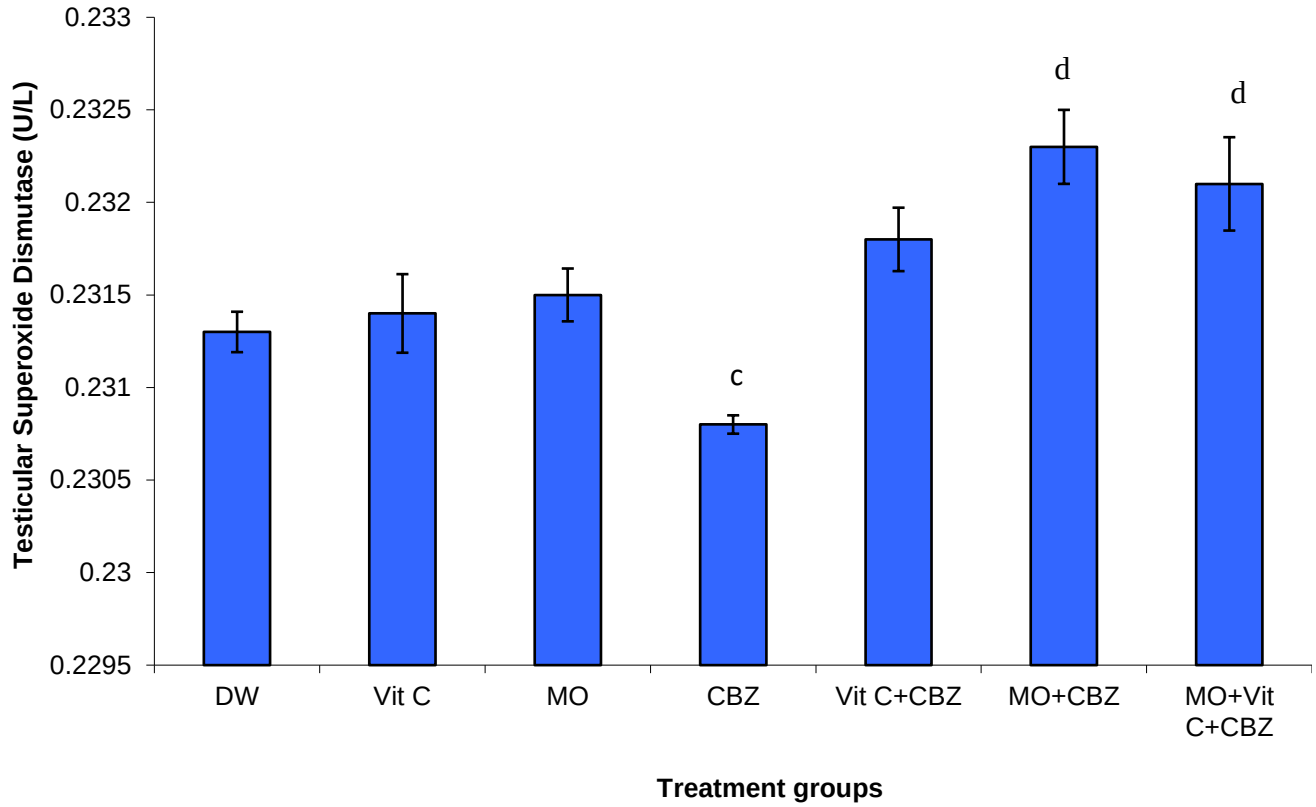

Figure 4.4: Effect of Vitamin C and Methanol leaf extract of *M. oleifera* on testicular SOD activity of Adult Male Wistar Rats exposed to chronic Carbamazepine (n=5).

c= ($P < 0.05$) lower Vs Vit-C + MO + CBZ, Vit-C + CBZ, MO + CBZ, MO and Vit-C groups.

d= ($P < 0.05$) higherVs MO, Vit-C and DW groups.

Key:

DW= Distilled water

MO= *Moringa oleifera* leaf extract

Vit-C= vitamin C

CBZ= carbamazepine

Vit-C + CBZ= vitamin C and carbamazepine

MO + CBZ= *Moringa oleifera* leaf extract and carbamazepine

Vit-C + MO + CBZ= vitamin C, *Moringa oleifera* leaf extract and carbamazepine.

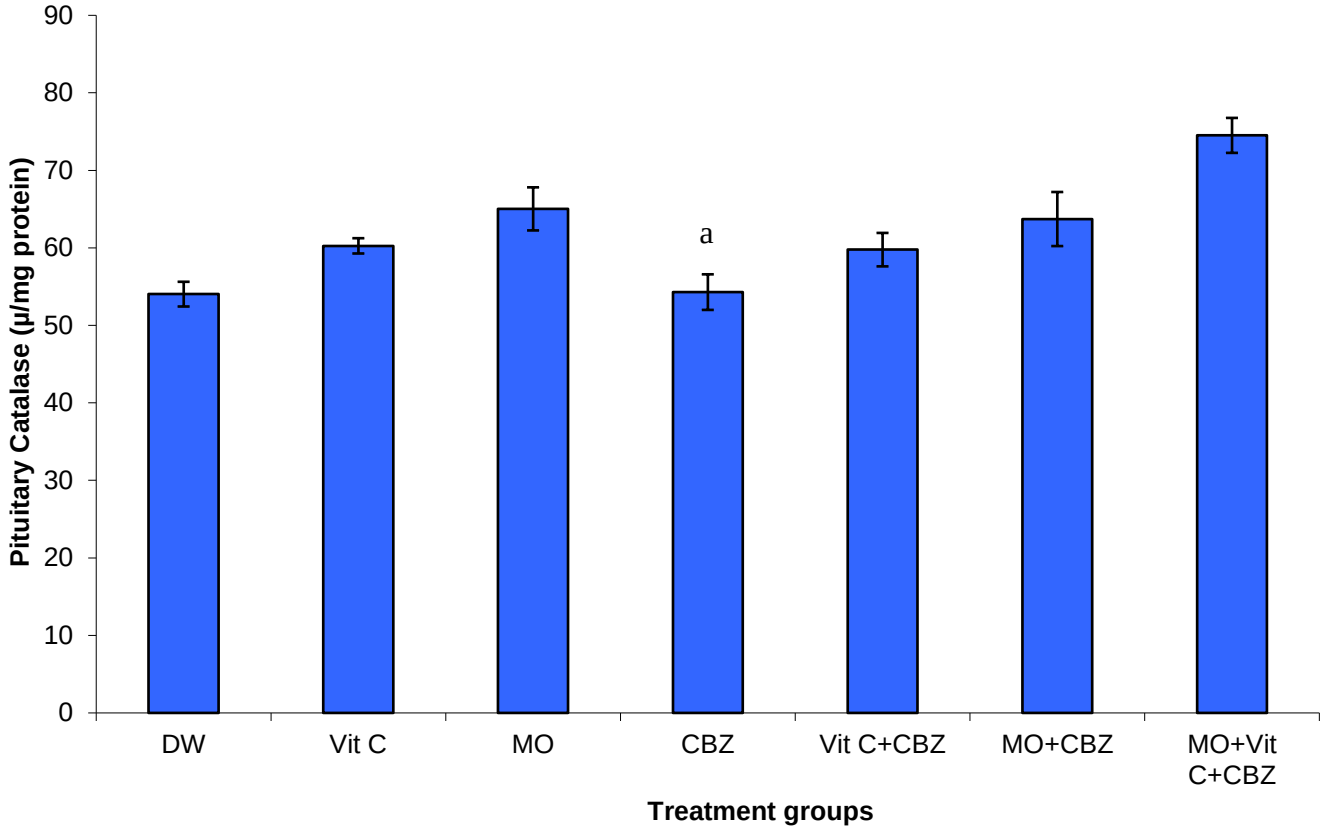

Figure 4.5: Effect of Vitamin C and Methanol leaf extract of *M. oleifera* on pituitary CAT activity of Adult Male Wistar Rats exposed to chronic Carbamazepine (n=5).

a= ($P < 0.05$) lower Vs Vit-C + MO + CBZ group.

Key:

DW= Distilled water

MO= *Moringa oleifera* leaf extract

Vit-C= vitamin C

CBZ= carbamazepine

Vit-C + MO + CBZ= vitamin C, *Moringa oleifera* leaf extract and carbamazepine.

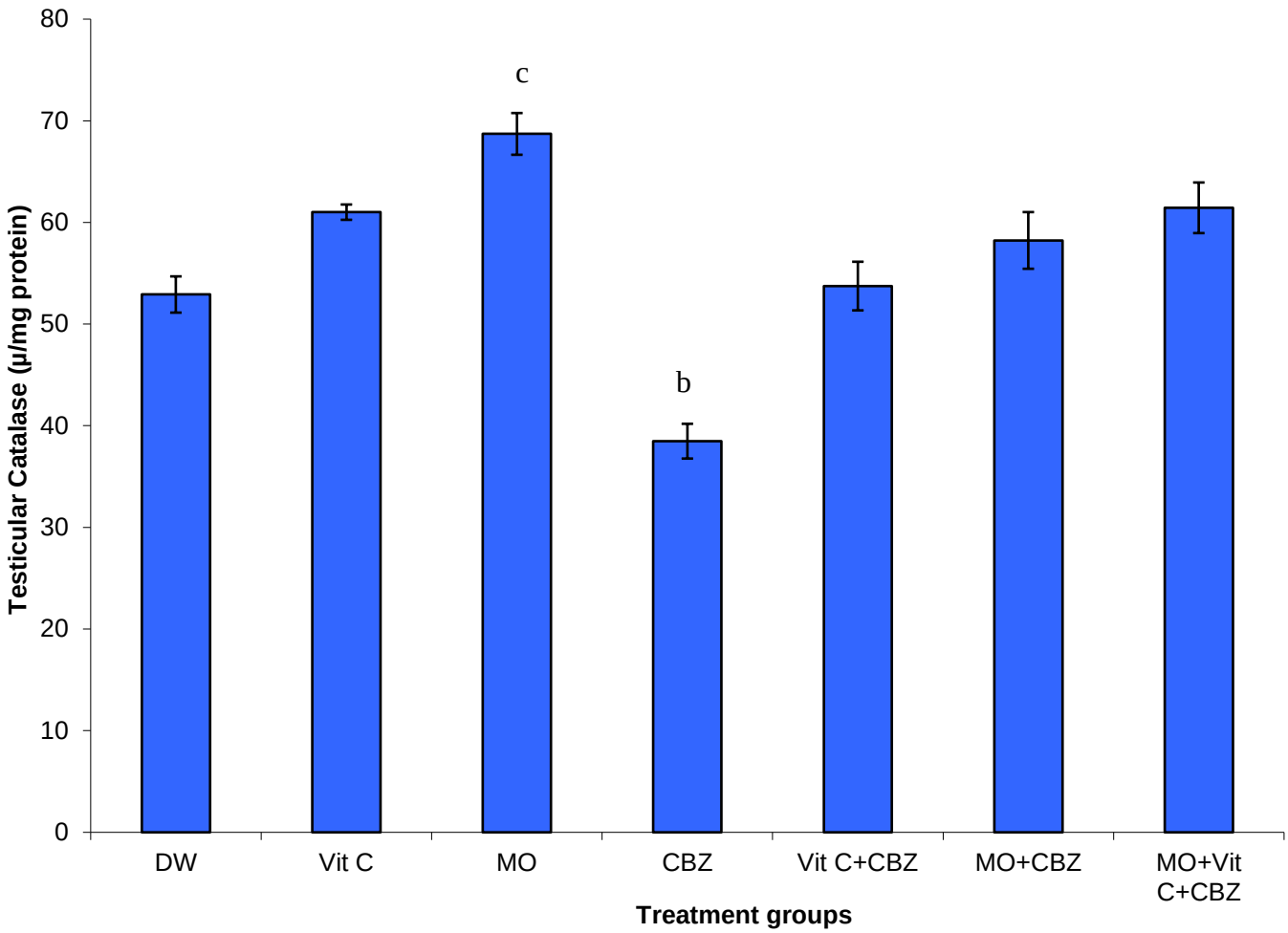

Figure 4.6: Effect of Vitamin C and Methanol leaf extract of *M. oleifera* on testicular CAT activity of Adult Male Wistar Rats exposed to chronic Carbamazepine (n=5).

b= ($P < 0.05$) lower Vs Vit-C + MO + CBZ, MO + CBZ, Vit-C + CBZ, MO, Vit-C and DW groups.

c= ($P < 0.05$) higher Vs MO + CBZ, Vit-C + CBZ and DW groups.

Key:

DW= Distilled water

MO= *Moringa oleifera* leaf extract

Vit-C= vitamin C

CBZ= carbamazepine

Vit-C + CBZ= vitamin C and carbamazepine

MO + CBZ= *Moringa oleifera* leaf extract and carbamazepine

Vit-C + MO + CBZ= vitamin C, *Moringa oleifera* leaf extract and carbamazepine.

4.3.7 Effect of treatment on pituitary glutathione peroxidise activity

Figure 4.7 shows the pituitary mean glutathione peroxidise activity in the different treatment groups. The pituitary mean GPx activity in CBZ group was significantly ($P < 0.05$) lower when compared to that of Vit-C + MO + CBZ, MO + CBZ, Vit-C + CBZ, MO, Vit-C and DW groups.

4.3.8 Effect of treatment on testicular glutathione peroxidise activity

Figure 4.8 shows the testicular mean glutathione peroxidise activity in the different treatment groups. The testicular mean GPx activity in CBZ group was significantly ($P < 0.05$) lower when compared with the enzyme's activity in Vit-C + CBZ, Vit-C and MO groups. There was increase in absolute values of testicular mean GPx activity in Vit-C + MO + CBZ (16.2%), MO + CBZ (12.8%) and DW (11.3%) groups when compared with CBZ group. Also, the testicular mean GPx activity in MO group was significantly ($P < 0.05$) higher when compared to Vit-C + MO + CBZ, MO + CBZ and DW groups.

4.4 Effect of Treatment on Hormonal Concentration

4.4.1 Effect of treatment on follicle stimulating hormone concentration

Figure 4.9 shows the mean follicle stimulation hormone concentrations in the different treatment groups. There was no significant ($P > 0.05$) change in the FSH concentration in all the groups. However, the absolute mean FSH concentration in the CBZ group was relatively lower when compared to that of Vit-C + MO + CBZ (17.2%), MO + CBZ (11.5%), Vit-C + CBZ, MO (18.2%), Vit-C and DW groups.

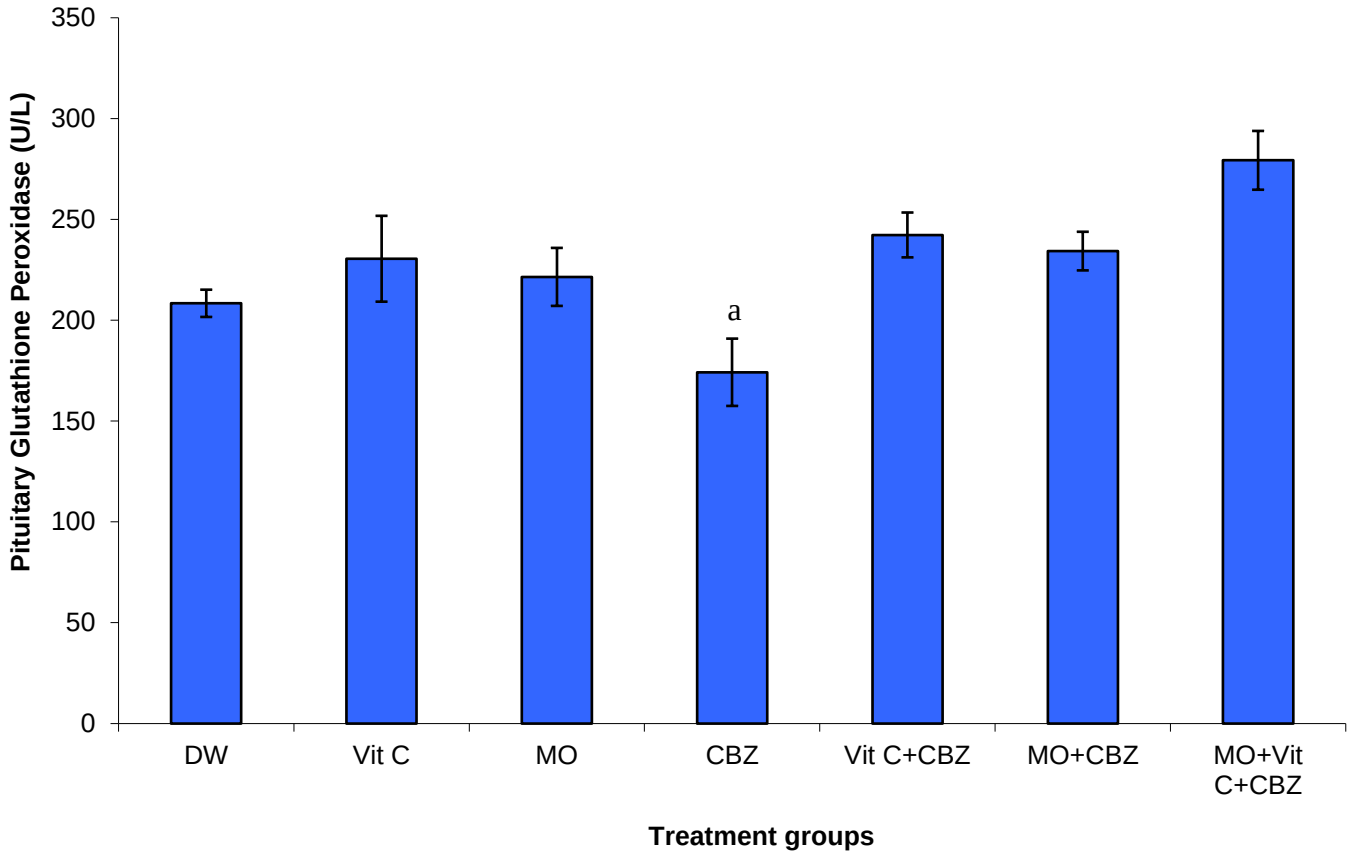

Figure 4.7: Effect of Vitamin C and Methanol leaf extract of *M. oleifera* on pituitary GPx activity of Adult Male Wistar Rats exposed to chronic Carbamazepine (n=5).

a= ($P < 0.05$) lower Vs Vit-C + MO + CBZ, MO + CBZ, Vit-C + CBZ, MO, Vit-C and DW groups.

Key:

DW= Distilled water

MO= *Moringa oleifera* leaf extract

Vit-C= vitamin C

CBZ= carbamazepine

Vit-C + CBZ= vitamin C and carbamazepine

MO + CBZ= *Moringa oleifera* leaf extract and carbamazepine

Vit-C + MO + CBZ= vitamin C, *Moringa oleifera* leaf extract and carbamazepine.

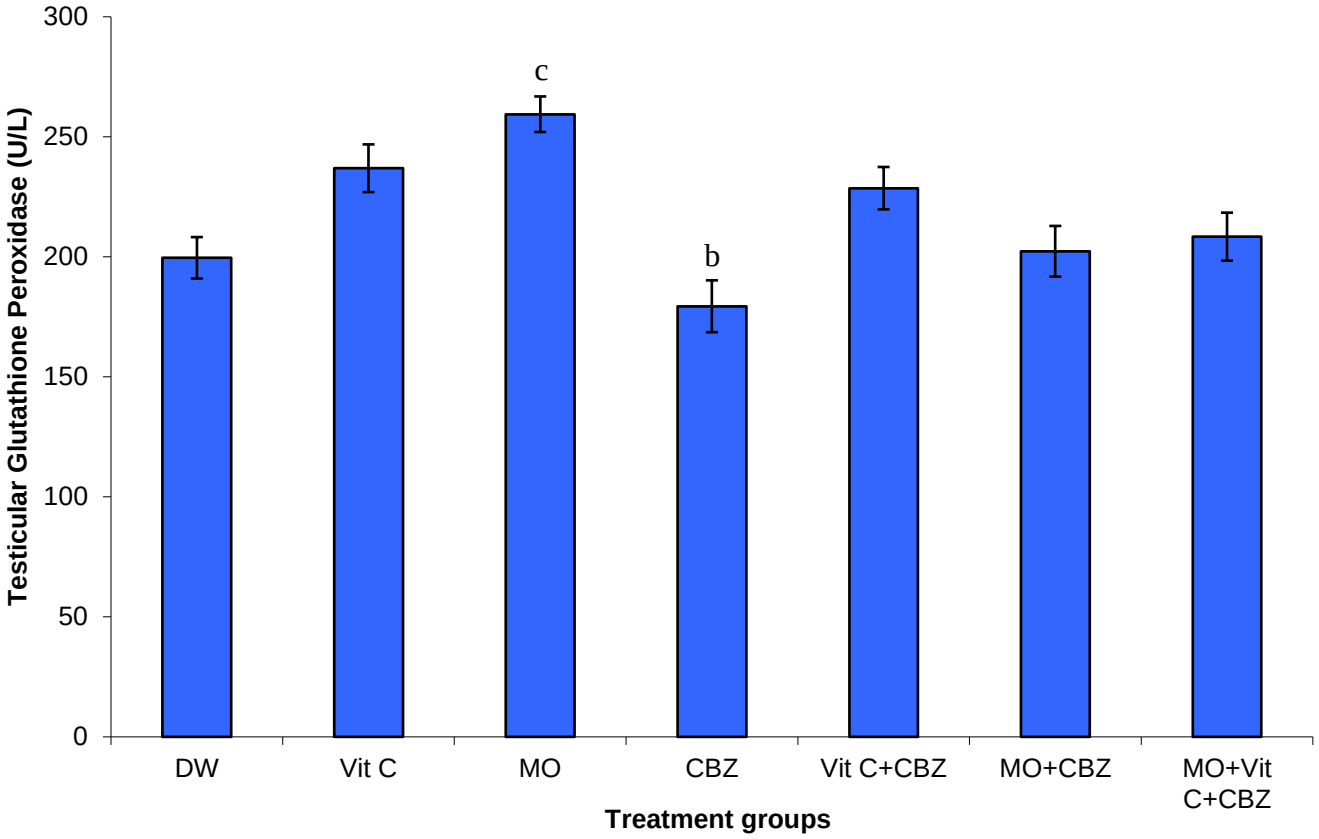

Figure 4.8: Effect of Vitamin C and Methanol leaf extract of *M. oleifera* on testicular GPx activity of Adult Male Wistar Rats exposed to chronic Carbamazepine (n=5).

b= ($P < 0.05$) lower Vs Vit-C + CBZ, MO and Vit-C groups.

c= ($P < 0.05$) higher Vs MO + CBZ + CBZ, MO + CBZ and DW groups.

Key:

DW= Distilled water

MO= *Moringa oleifera* leaf extract

Vit-C= vitamin C

CBZ= carbamazepine

Vit-C + CBZ= vitamin C and carbamazepine

MO + CBZ= *Moringa oleifera* leaf extract and carbamazepine

Vit-C + MO + CBZ= vitamin C, *Moringa oleifera* leaf extract and carbamazepine.

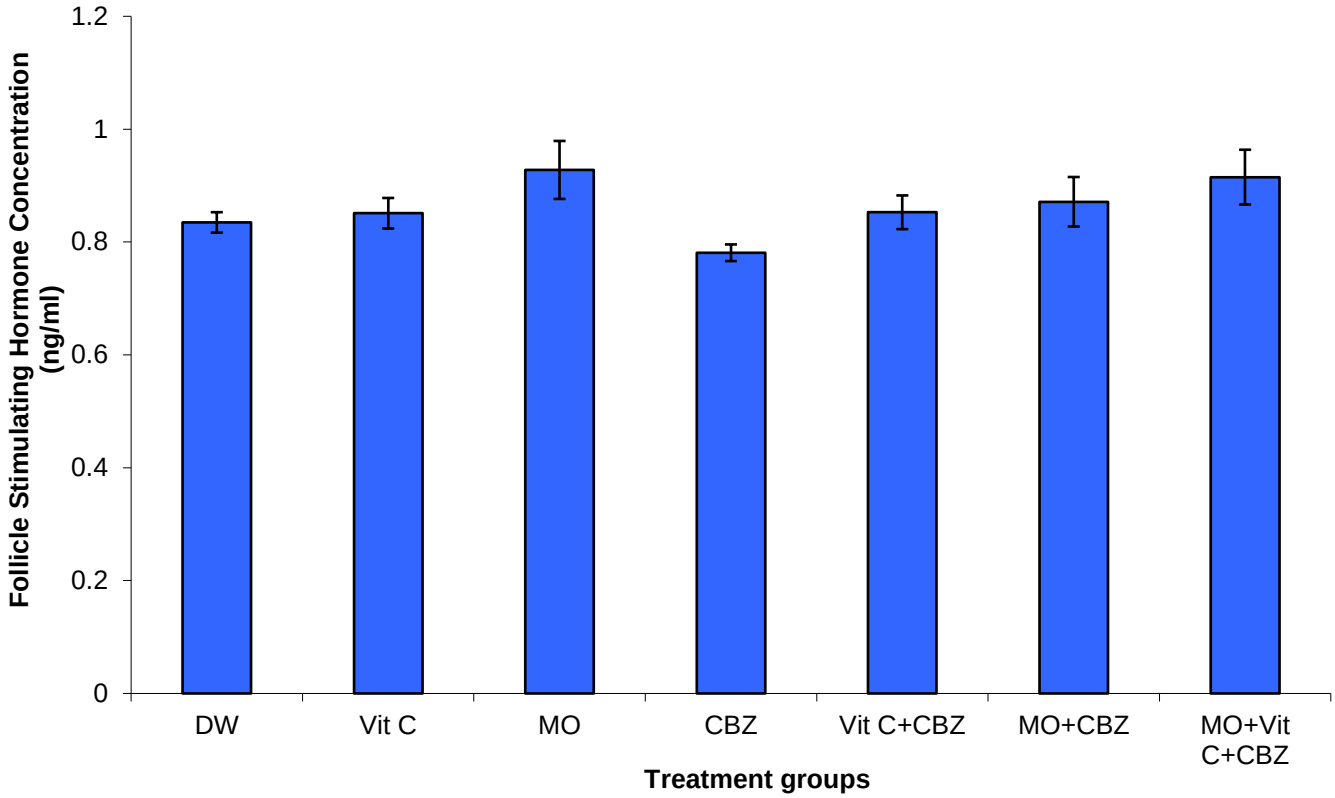

Figure 4.9: Effect of Vitamin C and Methanol leaf extract of *M. oleifera* on Follicle stimulating hormone concentration of Adult Male Wistar Rats exposed to chronic Carbamazepine (n=5).

4.4.2 Effect of treatment on luteinizing hormone concentration

Figure 4.10 shows the mean luteinizing hormone concentrations in the different treatment groups. There was no significant ($P > 0.05$) change in the LH concentration in all the groups. However, the absolute mean LH concentration in CBZ group was relatively lower when compared to that of Vit-C + MO + CBZ (11.3%), MO + CBZ (32.1%), Vit-C + CBZ (16.9%), MO (27%), Vit-C (13.2%) and DW (12.6%) groups.

4.4.3 Effect of treatment on testosterone concentration

Figure 4.11 shows the mean testosterone concentrations in the different treatment groups. The mean testosterone concentration in CBZ group was significantly ($P < 0.05$) lower when compared to Vit-C + MO + CBZ and Vit-C + CBZ groups. The mean testosterone concentration in Vit-C + MO + CBZ group was significantly ($P < 0.05$) higher when compared to Vit-C and DW group.

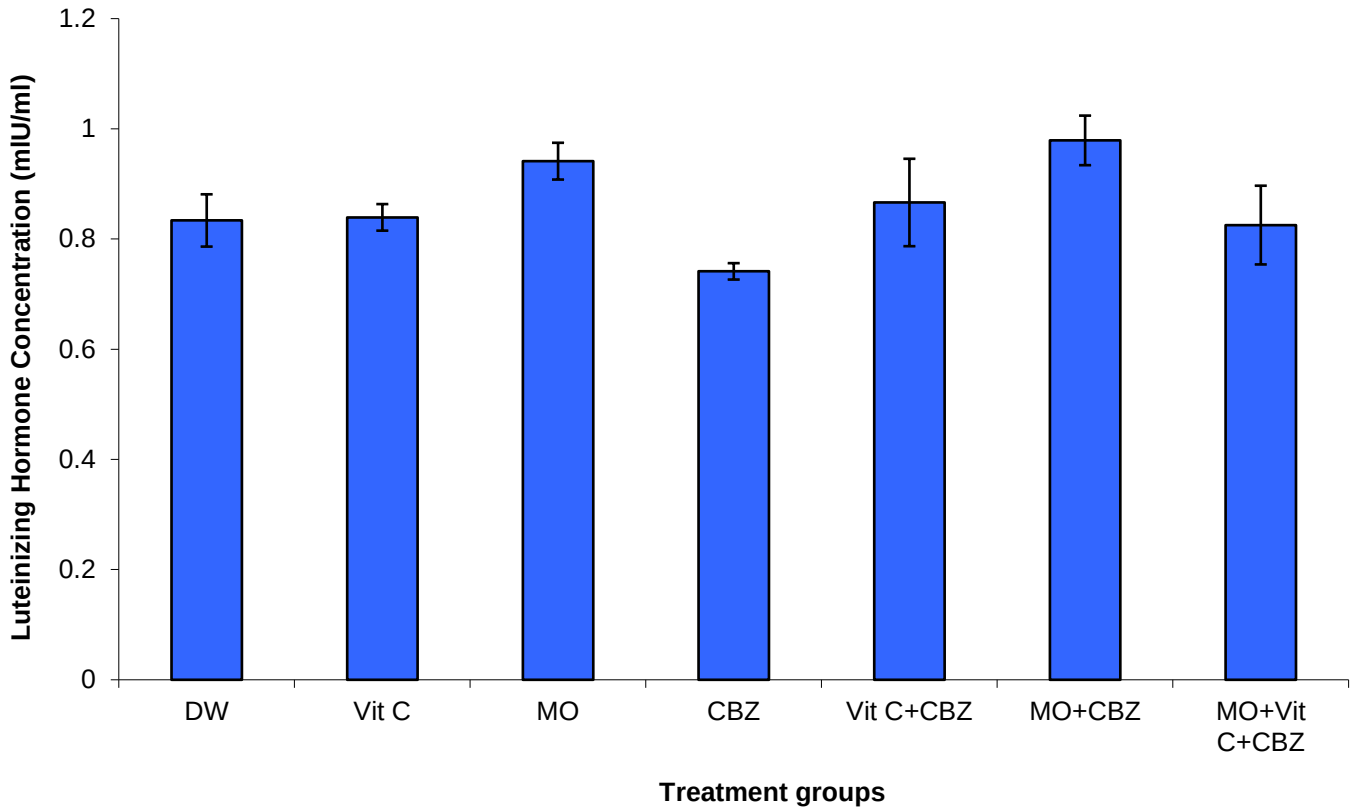

Figure 4.10: Effect of Vitamin C and Methanol leaf extract of *M. oleifera* on Luteinizing hormone concentration of Adult Male Wistar Rats exposed to chronic Carbamazepine (n=5).

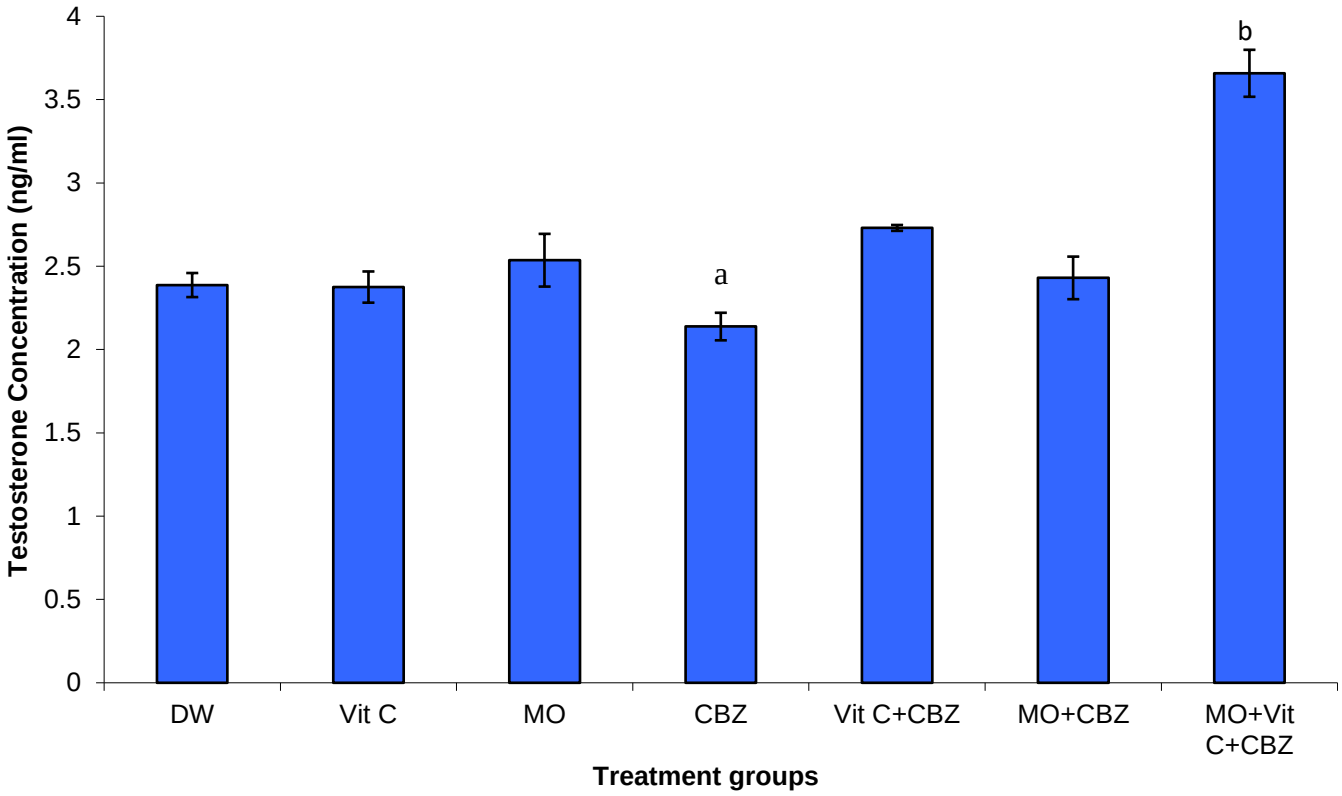

Figure 4.11: Effect of Vitamin C and Methanol leaf extract of *M. oleifera* on Testosterone concentration of Adult Male Wistar Rats exposed to chronic Carbamazepine (n=5).

a= ($P < 0.05$) lower Vs MO + Vit-C + CBZ and Vit-C + CBZ groups.

b= ($P < 0.05$) higher Vs DW and Vit-C groups.

Key:

DW= Distilled water

MO= *Moringa oleifera* leaf extract

Vit-C= vitamin C

CBZ= carbamazepine

Vit-C + CBZ= vitamin C and carbamazepine

MO + CBZ= *Moringa oleifera* leaf extract and carbamazepine

Vit-C + MO + CBZ= vitamin C, *Moringa oleifera* leaf extract and carbamazepine.

4.5 Effect of Treatment on Sperm Characteristics

4.5.1 Effect on epididymal sperm count

Figure 4.12 shows the mean epididymal sperm count in the different treatment groups. The mean epidydimal sperm count in CBZ group was significantly ($P < 0.05$) lower when compared to that of MO + Vit-C + CBZ, MO, Vit-C and DW groups. The mean epidydimal sperm count in DW group was signicantly ($P < 0.05$) higher when compared with MO, MO + CBZ, Vit-C + CBZ and MO + Vit-C + CBZ groups.

4.5.2 Effect on percentage sperm motility

Figure 4.13 shows the mean percentage sperm motility in the different groups. There was no significant ($P > 0.05$) change in mean percentage sperm motility in CBZ group but relative decreased in absolute values were recorded when compared to MO + Vit-C + CBZ (15.8%), MO + CBZ, Vit-C + CBZ, MO (15.8%) and Vit-C (21.1%) groups. The mean percentage sperm motility in CBZ group was significantly ($P < 0.05$) lower when compared with that of DW group. The mean percentage sperm motility in DW group was significantly ($P < 0.05$) when compared to MO, Vit-C, MO + CBZ, Vit-C + CBZ and MO + Vit-C + CBZ groups.

4.5.3 Effect on percentage immotile sperm

Figure 4.14 shows the mean percentage immotile sperm in the different treatment groups. The mean percentage immotile sperm in CBZ group was significantly ($P < 0.05$) higher when compared with DW group. There was no significant ($P > 0.05$) change in mean percentage immotile sperm in CBZ group but increased absolute values were recorded when compared with MO + Vit-C + CBZ (26.7%), MO + CBZ, Vit-C + CBZ, MO (26.7%) and Vit-C (35.9%) groups. The mean

percentage immotile sperm in DW group was significantly ($P < 0.05$) lower when compared with that of Vit-C, MO, Vit-C + CBZ, MO + CBZ and MO + Vit-C + CBZ groups.

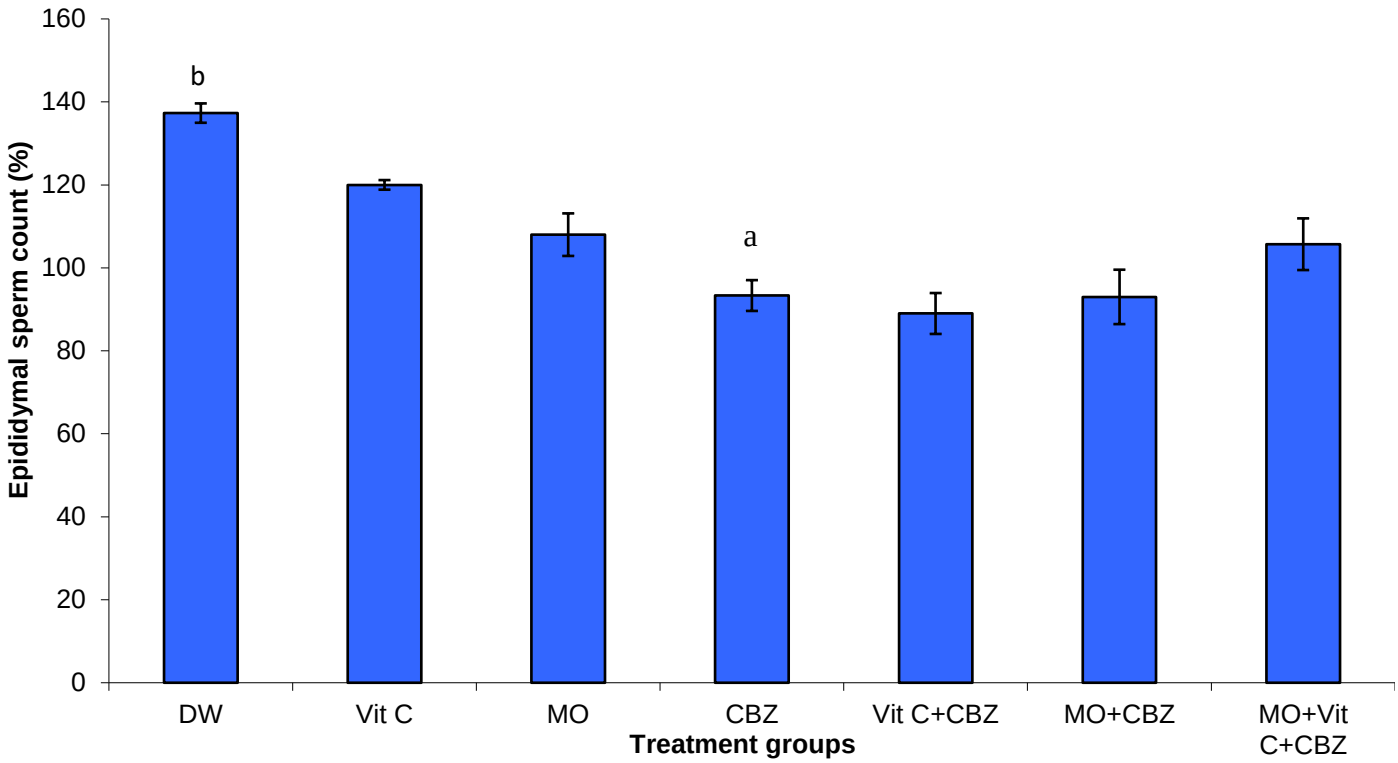

Figure 4.12: Effect of Vitamin C and Methanol leaf extract of *M. oleifera* on Epididymal sperm cell count of Adult Male Wistar Rats exposed to chronic Carbamazepine (n=5).

a= ($P < 0.05$) lower when compared to MO + Vit-C + CBZ, MO, Vit-C and DW groups.
b= ($P < 0.05$) higher when compared to MO, Vit-C + CBZ, MO + CBZ and MO + Vit-C + CBZ groups.

Key:

DW= Distilled water

MO= *Moringa oleifera* leaf extract

Vit-C= vitamin C

CBZ= carbamazepine

Vit-C + CBZ= vitamin C and carbamazepine

MO + CBZ= *Moringa oleifera* leaf extract and carbamazepine

Vit-C + MO + CBZ= vitamin C, *Moringa oleifera* leaf extract and carbamazepine.

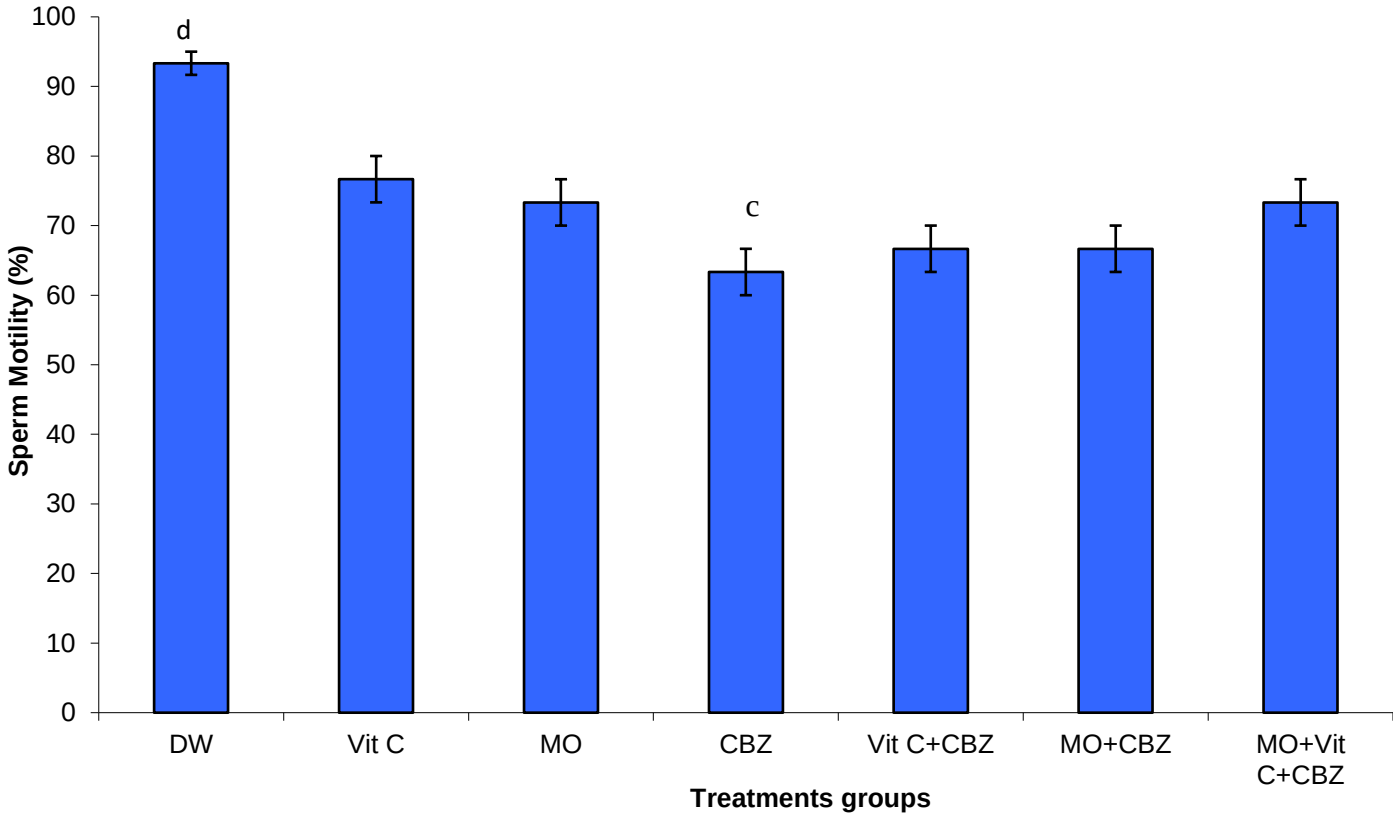

Figure 4.13: Effect of Vitamin C and Methanol leaf extract of *M. oleifera* on Sperm motility of Adult Male Wistar Rats exposed to chronic Carbamazepine (n=5).
c= ($P < 0.05$) lower Vs DW group.
d= ($P < 0.05$) higher Vs MO, Vit-C + CBZ, MO + CBZ and MO + Vit-C + CBZ groups.
Key:

DW= Distilled water

MO= *Moringa oleifera* leaf extract

Vit-C= vitamin C

CBZ= carbamazepine

Vit-C + CBZ= vitamin C and carbamazepine

MO + CBZ= *Moringa oleifera* leaf extract and carbamazepine

Vit-C + MO + CBZ= vitamin C, *Moringa oleifera* leaf extract and carbamazepine.

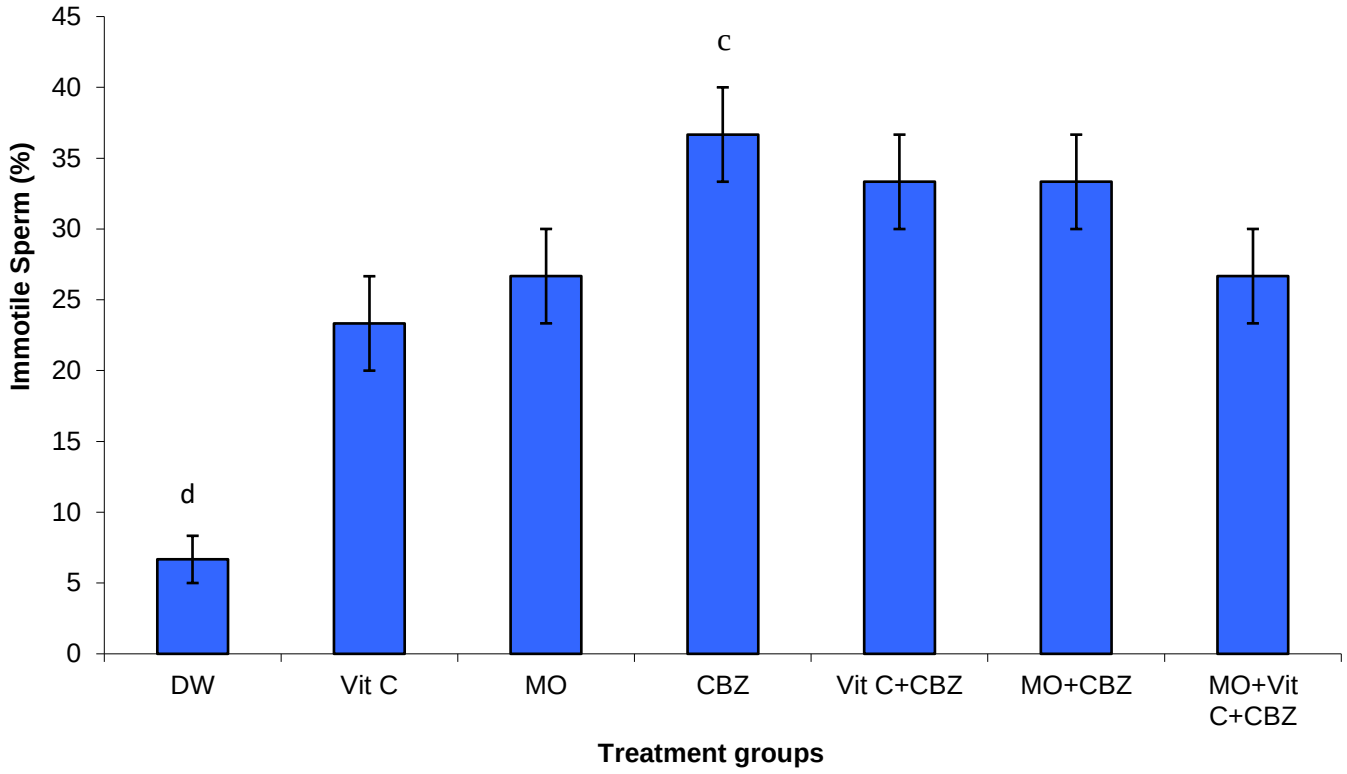

Figure 4.14: Effect of Vitamin C and Methanol leaf extract of *M. oleifera* on Immotile sperm of Adult Male Wistar Rats exposed to chronic Carbamazepine (n=5).
c= ($P < 0.05$) higher Vs DW group.

d= ($P < 0.05$) lower Vs Vit-C, MO, Vit-C + CBZ, MO + CBZ and Vit-C + MO + CBZ groups.

Key:

DW= Distilled water

MO= *Moringa oleifera* leaf extract

Vit-C= vitamin C

CBZ= carbamazepine

Vit-C + CBZ= vitamin C and carbamazepine

MO + CBZ= *Moringa oleifera* leaf extract and carbamazepine

Vit-C + MO + CBZ= vitamin C, *Moringa oleifera* leaf extract and carbamazepine.

4.5.4 Effect on percentage sperm head abnormality

Figure 4.15 shows the mean percentage sperm head abnormality in the different treatment groups. There was no significant ($P > 0.05$) change in percentage sperm head abnormality in between the groups. However, the DW group had the lowest mean percentage of sperm head abnormality when compared to Vit-C + MO + CBZ (41.9%), Vit-C + CBZ (39.5%), MO + CBZ (38.1%), CBZ (49.7%), Vit-C (31.6%) and MO (34.2%) groups (Plate XXII).

4.5.5 Effect on sperm tail abnormality

Figure 4.16 shows the mean sperm tail abnormality in the different treatment groups. The mean sperm tail abnormality in DW group was significantly ($P < 0.05$) lower when compared with CBZ, Vit-C + CBZ, MO + CBZ and Vit-C + MO + CBZ groups (Plate XXIII).

4.5.6 Effect on sperm mid-piece abnormality

Figure 4.17 shows the mean sperm mid-piece abnormality in the different treatment groups. The mean sperm mid-piece abnormality in CBZ group was significantly ($P < 0.05$) when compared with that of Vit-C + MO + CBZ and DW groups. CBZ group shows slight increase in sperm mid-piece abnormality than in Vit-C + CBZ (11.3%), Vit-C (20%), MO (17.7%) and MO + CBZ (16.1%) groups. The mean sperm mid-piece abnormality in DW group was significantly ($P < 0.05$) when compared with Vit-C + CBZ group.

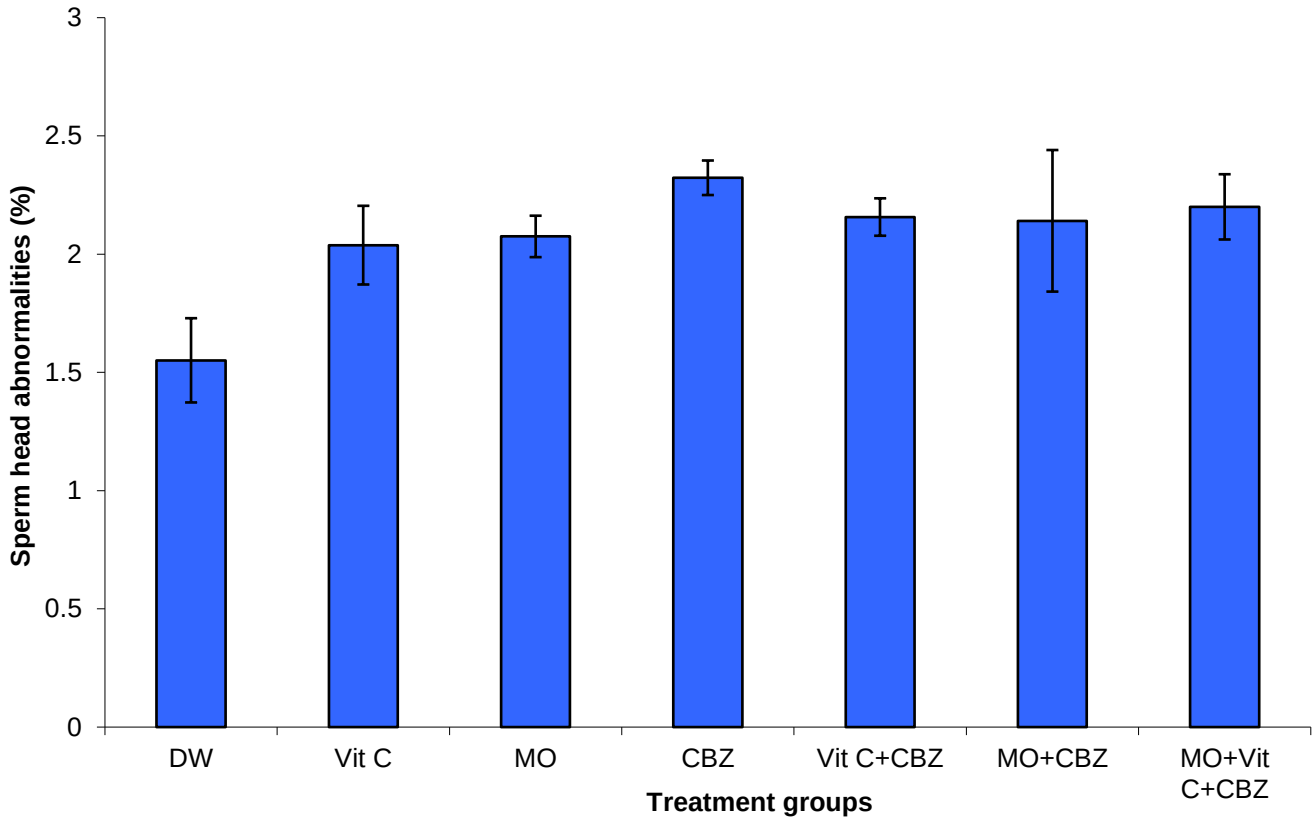

Figure 4.15: Effect of Vitamin C and Methanol leaf extract of *M. oleifera* on Sperm head abnormality of Adult Male Wistar Rats exposed to chronic Carbamazepine (n=5).

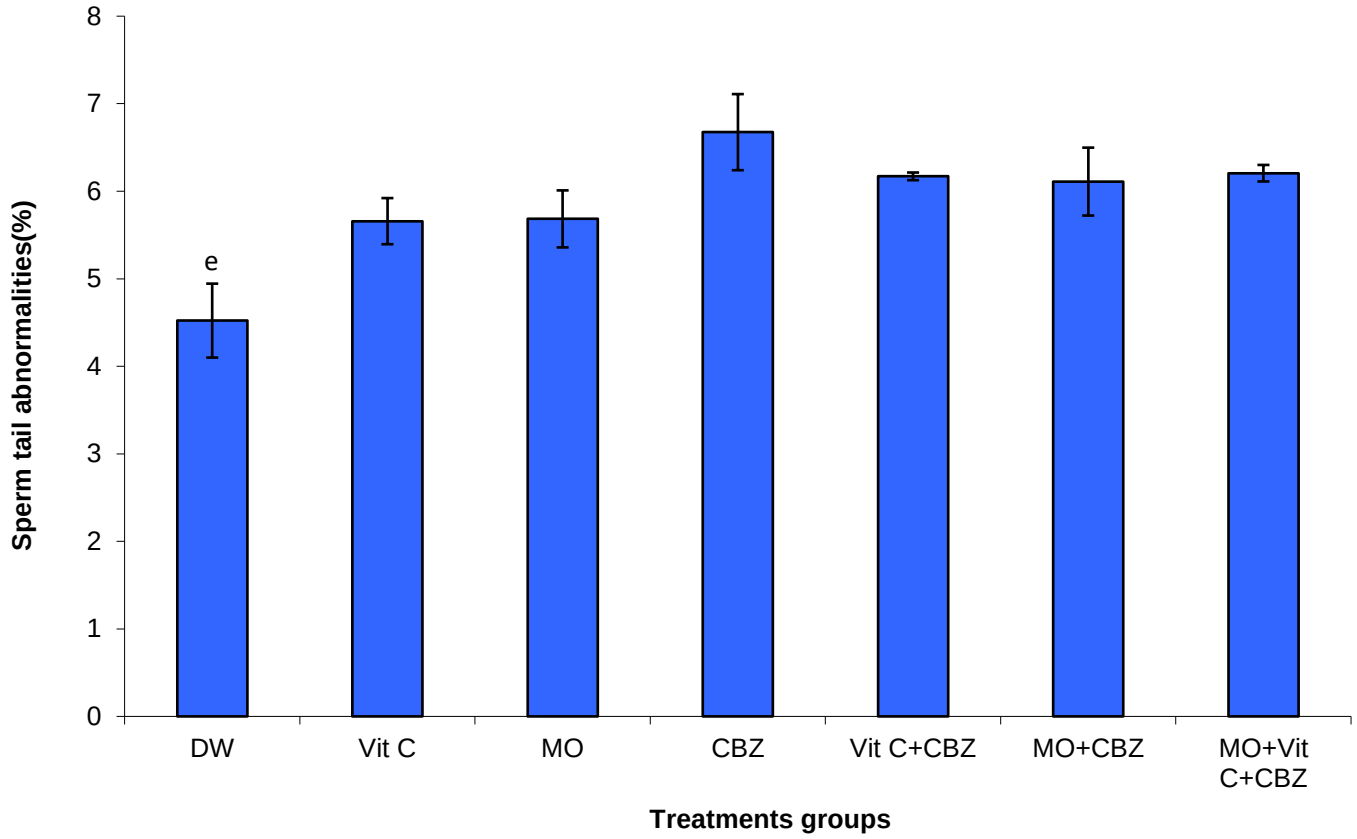

Figure 4.16: Effect of Vitamin C and Methanol leaf extract of *M. oleifera* on Sperm tail abnormality of Adult Male Wistar Rats exposed to chronic Carbamazepine (n=5).
e= ($P < 0.05$) lower Vs CBZ, Vit-C + CBZ, MO + CBZ and Vit-C + MO + CBZ and CBZ groups.

Key:

DW= Distilled water

MO= *Moringa oleifera* leaf extract

Vit-C= vitamin C

CBZ= carbamazepine

Vit-C + CBZ= vitamin C and carbamazepine

MO + CBZ= *Moringa oleifera* leaf extract and carbamazepine

Vit-C + MO + CBZ= vitamin C, *Moringa oleifera* leaf extract and carbamazepine.

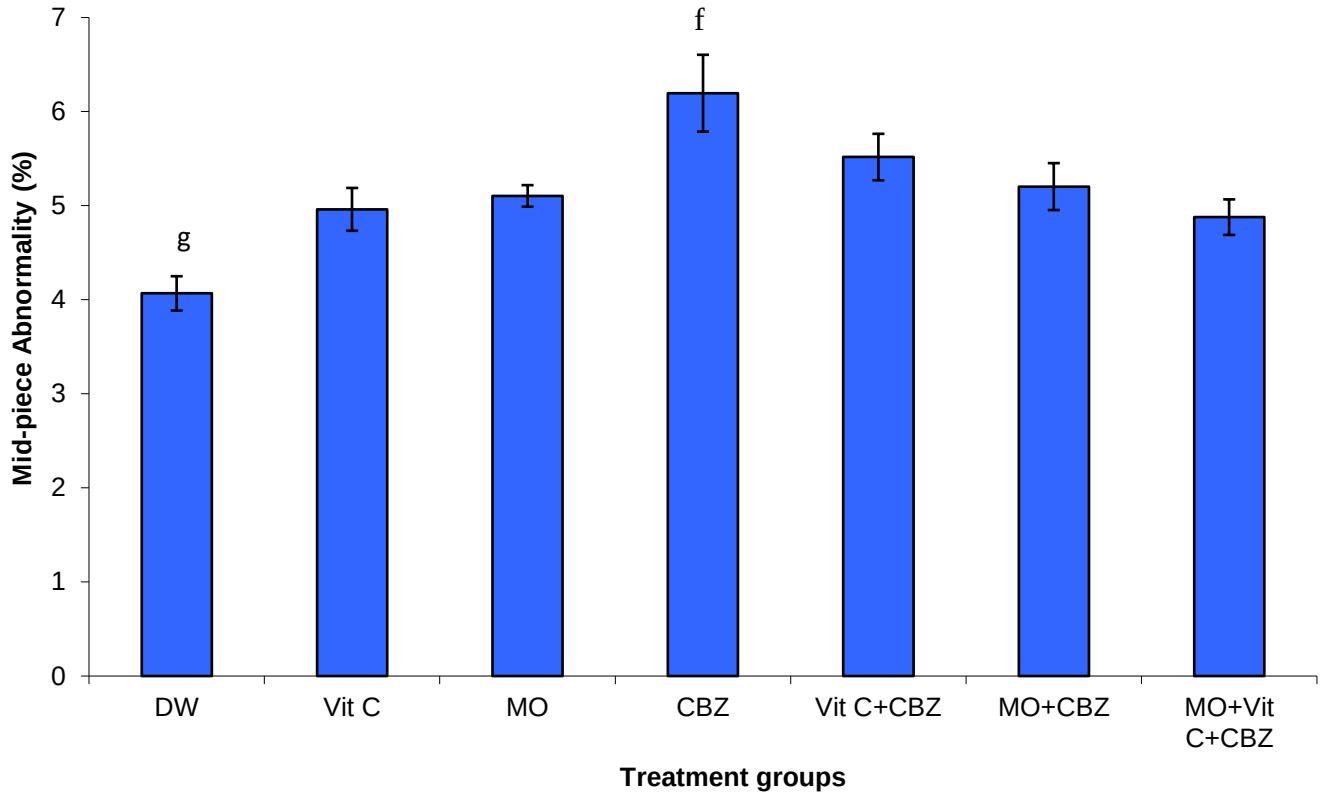

Figure 4.17: Effect of Vitamin C and Methanol leaf extract of *M. oleifera* on Sperm mid-piece abnormality of Adult Male Wistar Rats exposed to chronic Carbamazepine (n=5).
f= ($P < 0.05$) higher Vs Vit-C + MO + CBZ and DW groups.
g= ($P < 0.05$) lower Vs Vit-C + CBZ group.

Key:

DW= Distilled water

MO= *Moringa oleifera* leaf extract

Vit-C= vitamin C

CBZ= carbamazepine

Vit-C + CBZ= vitamin C and carbamazepine

MO + CBZ= *Moringa oleifera* leaf extract and carbamazepine

Vit-C + MO + CBZ= vitamin C, *Moringa oleifera* leaf extract and carbamazepine.

4.6 Effect of Treatment on Relative Organ Weights

4.6.1 Effect of treatment on relative pituitary gland weight

Figure 4.18 shows the mean relative pituitary gland weight in the different groups. The mean pituitary relative pituitary gland weight in CBZ group was significantly ($P < 0.05$) when compared to that of Vit-C + MO + CBZ and Vit-C + CBZ groups.

4.6.2 Effect of treatment on relative testicular gland weight

Figure 4.19 shows the mean relative testicular gland weight in the different groups. The mean relative testicular gland weight in CBZ group shows no significant ($P > 0.05$) change when compared to Vit-C + MO + CBZ, MO + CBZ, Vit-C + CBZ, MO, Vit-C and DW groups.

4.6.3 Effect of treatment on relative seminal vesicular weight

Figure 4.20 shows the mean relative seminal vesicular weight in the different treatment groups. The mean relative seminal vesicular weight in CBZ group was significantly ($P < 0.05$) lower when compared to that of MO + CBZ, Vit-C and DW groups. The mean relative seminal vesicular weight in CBZ group shows no significant ($P > 0.05$) change but a relative increased in absolute values in Vit-C + MO + CBZ (13.2%), Vit-C + CBZ and MO (10.5%) groups were recorded.

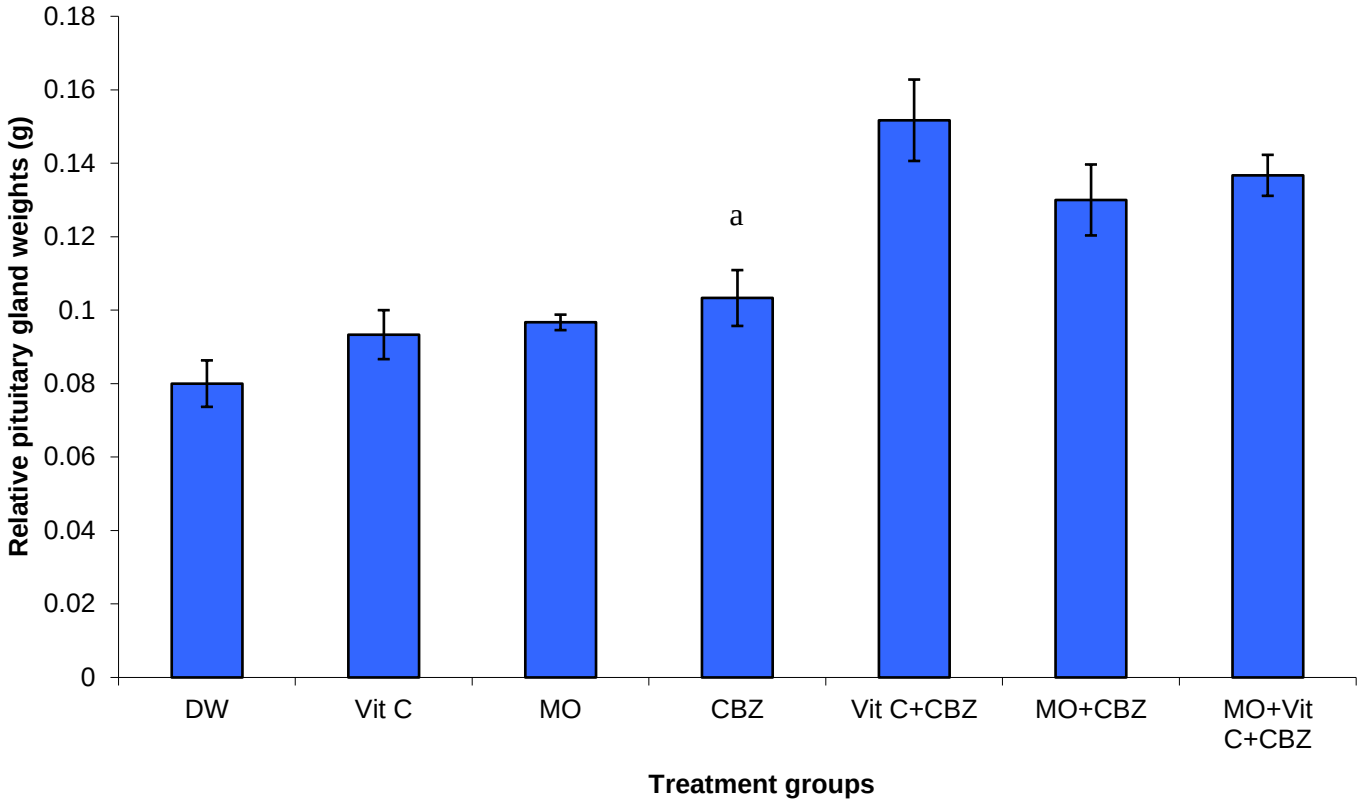

Figure 4.18: Effect of Vitamin C and Methanol leaf extract of *M. oleifera* on Relative pituitary gland weight of Adult Male Wistar Rats exposed to chronic Carbamazepine (n=5).

a= ($P < 0.05$) lower Vs MO + Vit-C + CBZ and Vit-C + CBZ groups.

Key:

DW= Distilled water

MO= *Moringa oleifera* leaf extract

Vit-C= vitamin C

CBZ= carbamazepine

Vit-C + CBZ= vitamin C and carbamazepine

MO + CBZ= *Moringa oleifera* leaf extract and carbamazepine

Vit-C + MO + CBZ= vitamin C, *Moringa oleifera* leaf extract and carbamazepine.

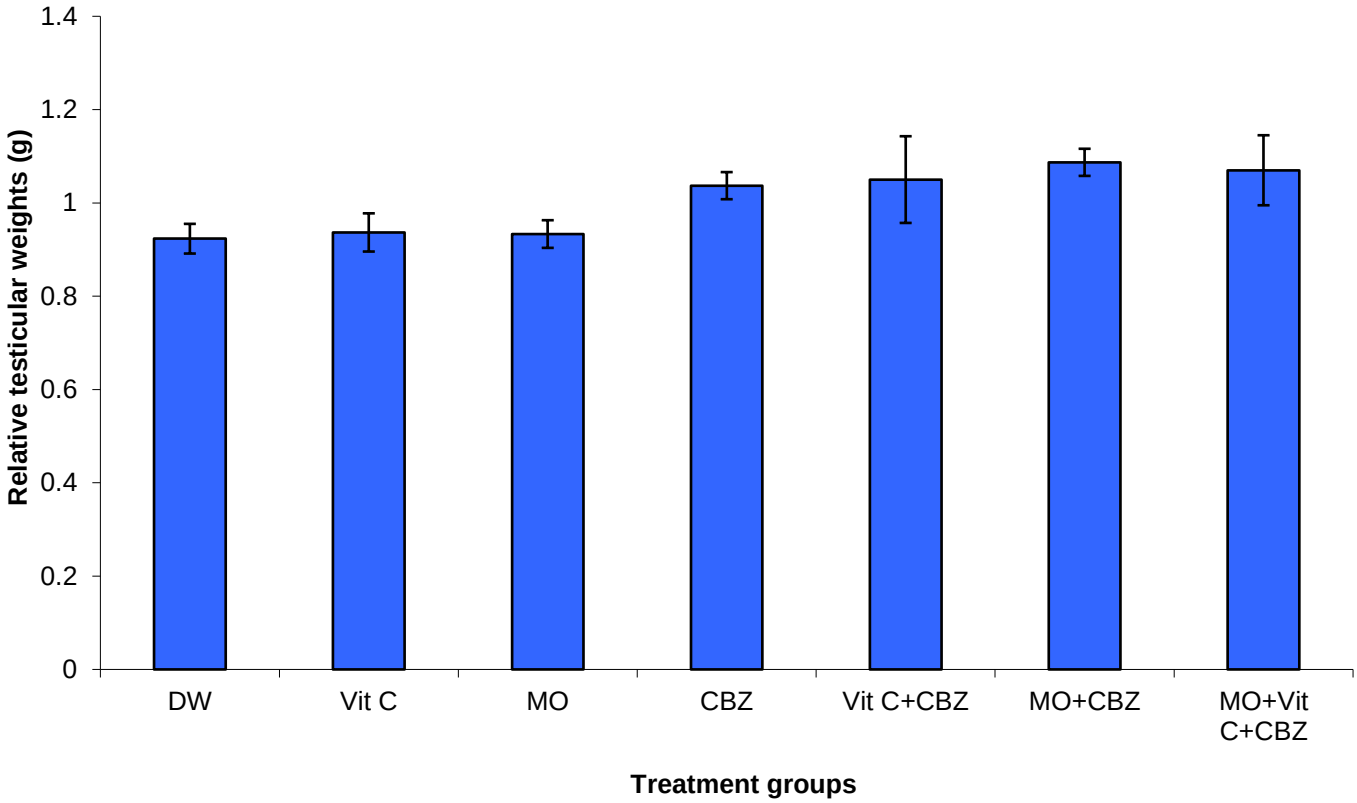

Figure 4.19: Effect of Vitamin C and Methanol leaf extract of *M. oleifera* on Relative testicular weight of Adult Male Wistar Rats exposed to chronic Carbamazepine (n=5).

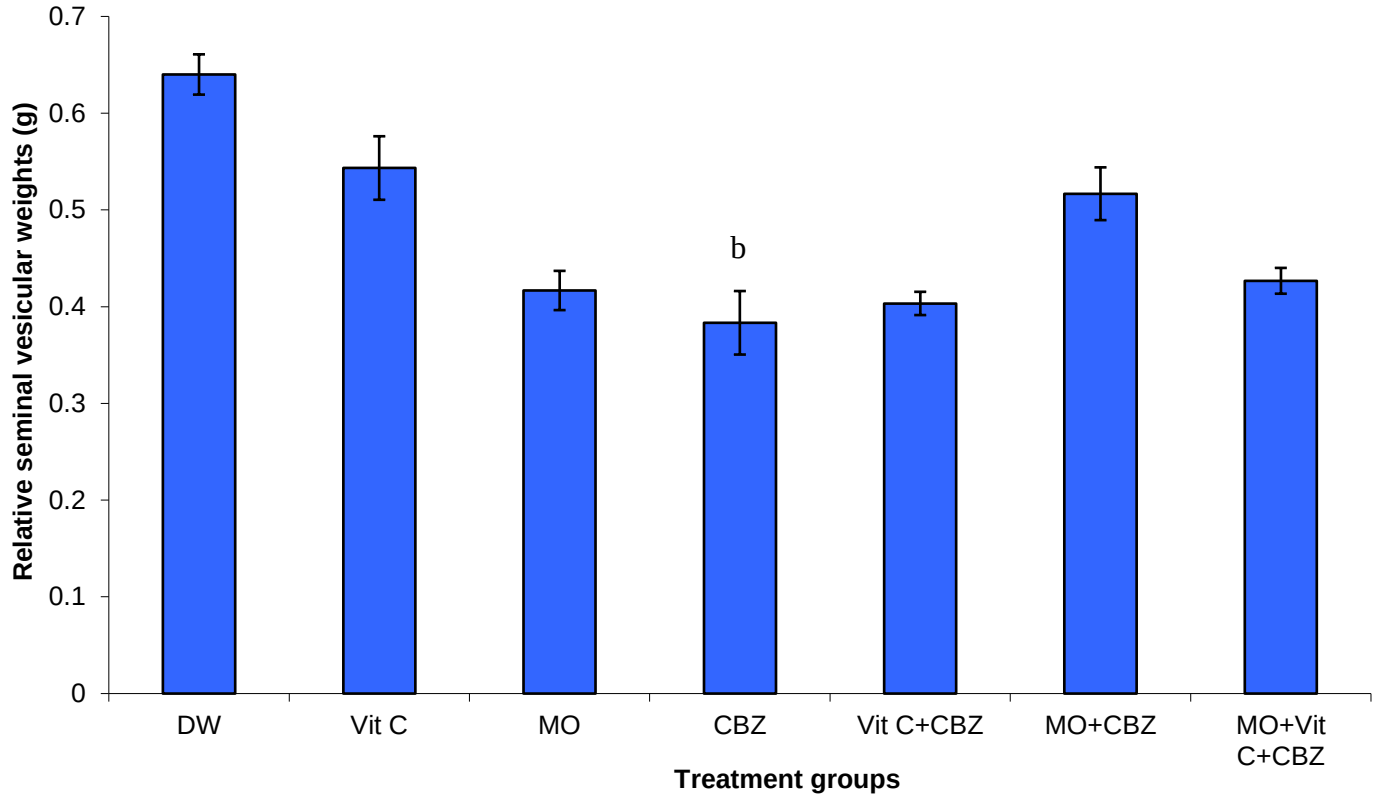

Figure 4.20: Effect of Vitamin C and Methanol leaf extract of *M. oleifera* on Relative seminal vesicular weight of Adult Male Wistar Rats exposed to chronic Carbamazepine (n=5).

b= ($P < 0.05$) lower Vs MO + CBZ, Vit-C and DW groups.

Key:

DW= Distilled water

MO= *Moringa oleifera* leaf extract

Vit-C= vitamin C

CBZ= carbamazepine

Vit-C + CBZ= vitamin C and carbamazepine

MO + CBZ= *Moringa oleifera* leaf extract and carbamazepine

Vit-C + MO + CBZ= vitamin C, *Moringa oleifera* leaf extract and carbamazepine.

4.7 Pathological Effect of Chronic Exposure to Carbamazepine, Vitamin C and Methanol Leaf Extract of *M. oleifera* on Adult Male Wistar Rats.

4.7.1 Effect of chronic exposure to carbamazepine, vitamin C and Methanol leaf extract of *M. oleifera* on gross post-mortem and pathological changes

All the groups (I-VII) showed no gross lesions. In the group treated with CBZ only, the pathological changes observed were mild congestion of the brain and testes. Rats in the other groups showed no gross lesions.

4.7.2 Effects of chronic exposure to carbamazepine, vitamin C and Methanol leaves extract of *M. oleifera* on histo-architecture of various organs

Pituitary gland: There were no apparent histopathological changes in group I (DW) (Plate I), II (Plate II), III (MO) (Plate III) and VII (MO + Vit-C + CBZ) (Plate VII). Treatments received by groups III (MO) (Plate III), IV (CBZ only), V (Vit-C + CBZ) and VI (MO + CBZ) showed induce degenerative changes in the cytoarchitectural presentation of the pituitary gland characterized by neutrophil infilterations surrounding the pituicytes, as well as loss of cellular projections as demonstrated by Haematoxylin and Eosin stain.

Seminal vesicles: Apparently normal histological presentations were seen in group I (DW) (Plate VIII), groups II (Vit-C) (PlateIX), III (MO) (Plate X), V (Vit-C + CBZ) (Plate XII), VI (MO + CBZ) (Plate XIII) and VII (MO + Vit-C + CBZ) (Plate XIV). While severe haemoharrgic changes occurring on the smooth muscles walls can be seen conspicuously in the group IV (CBZ only) (Plate XI).

Testis: There was no apparent histopathological changes in all the groups except in group II (Vit-C) which is characterized by mild degeneration of spermatogenic cells (Plate XVI) and IV (CBZ only) (Plate XVIII) which is characterized by the seminiferous tubules (ST) indicating spermatogenic arrest (incomplete maturation of sperm cells), degenerative changes occurring to

the spermatogenic cell nuclei, and increased interstitial space diameter across the micrographs as well as presence of fibrotic and h aemorrhagic cells.

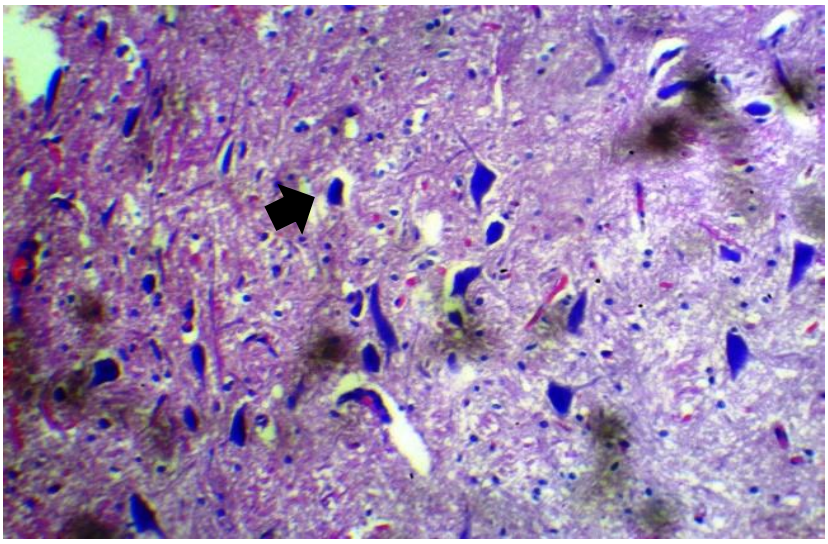

Plate I: Photomicrograph Showing Section of the Hypophysis of Adult Male Wistar Rat Exposed to Distilled Water. Note the normal pituicytes (block arrow), with no apparent histopathological lesions (H and E x 400).

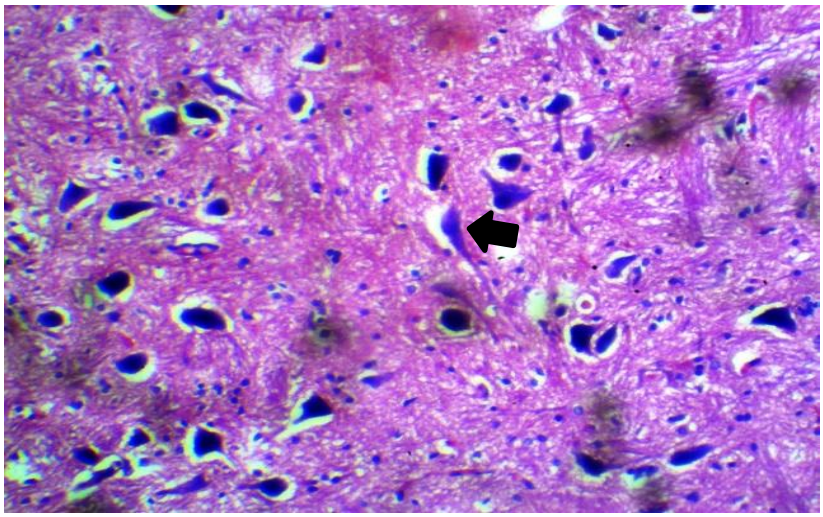

Plate II: Photomicrograph Showing Section of the Hypophysis in Adult Male Wistar Rat Exposed to Vitamin C. Note the normal pituicytes (block arrow), with no apparent histopathological lesions (H and E x 400).

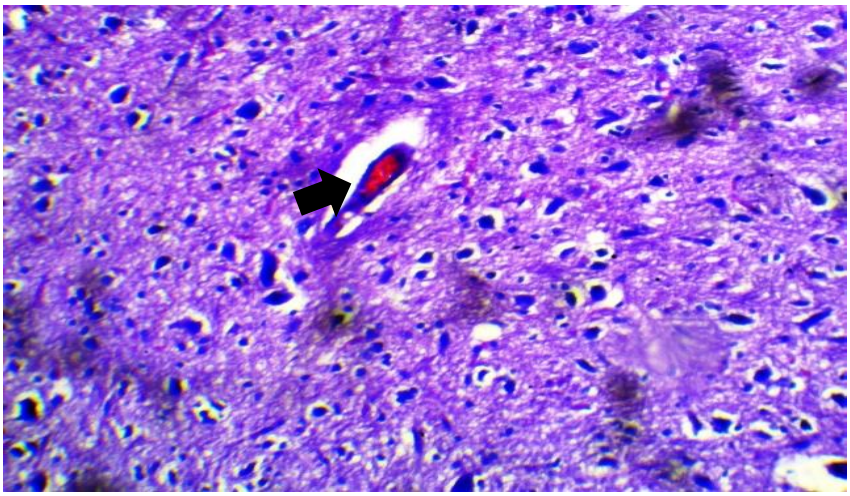

Plate III: Photomicrograph Showing Section of the Hypophysis in Adult Male Wistar Rat Exposed to Methanol leaf extract of *M. oleifera.* Note the pituicyte (block arrow) with mild loss of cellular projection (H and E x 400).

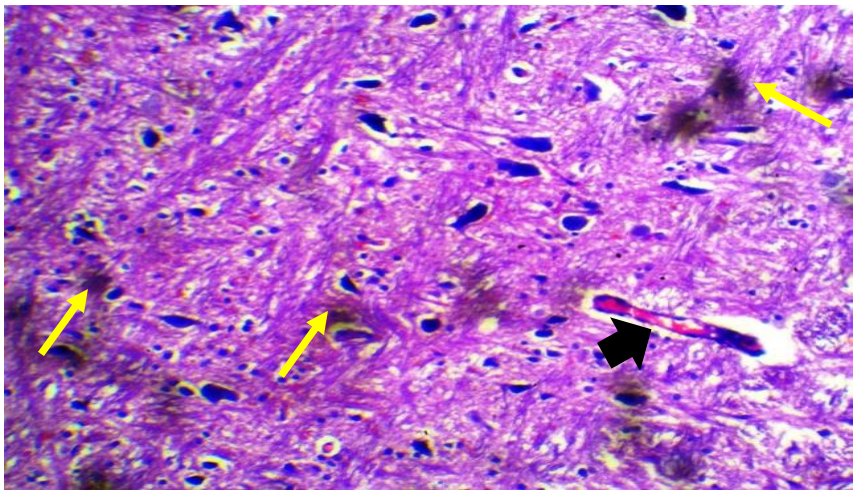

Plate IV: Photomicrograph Showing Section of the Hypophysis in Adult Male Wistar Rat Exposed to Carbamazepine. Note the neutrophil (line arrow) infilterations surrounding the pituicytes, as well as loss of cellular projections lesion (block arrow) (H and E x 400).

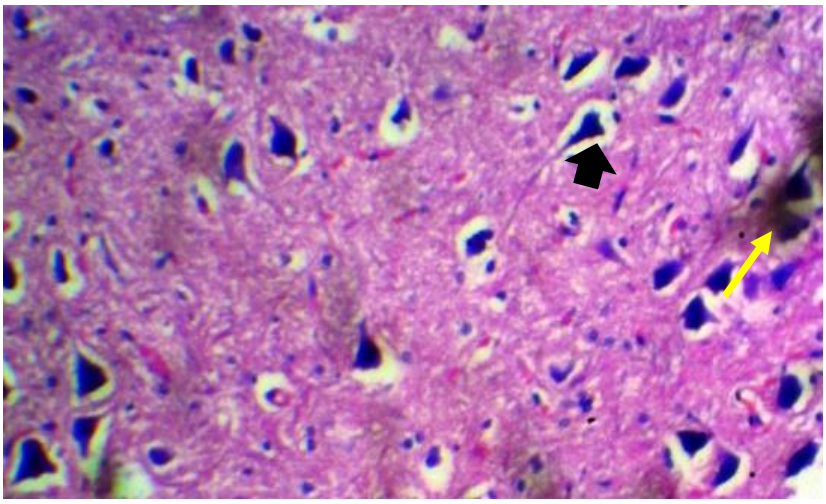

Plate V: Photomicrograph Showing Section of the Hypophysis in Adult Male Wistar Rat Exposed to Vit-C + CBZ. Note the neurophils infilteration (line arrow) surrounding the normal pituicyte (block arrow) (H and E x 400).

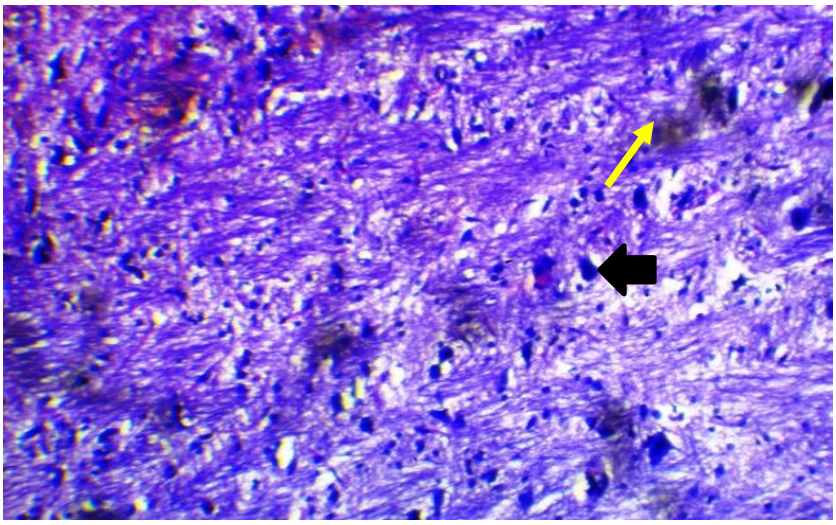

Plate VI: Photomicrograph Showing Section of the Hypophysis in Adult Male Wistar Rat Exposed to MO + CBZ. Note the neutrophil infilteration (line arrow) surrounding the pituicytes (block arrow) (H and E x 400).

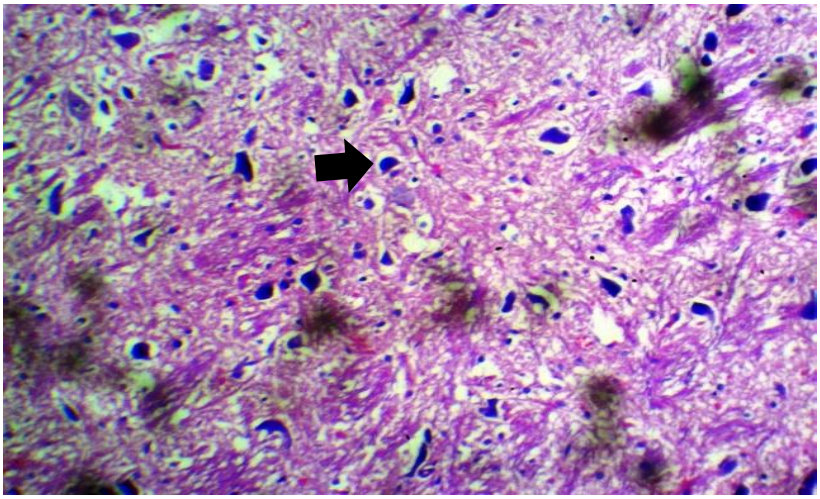

Plate VII: Photomicrograph Showing Section of the Hypophysis in Adult Male Wistar Rat Exposed to MO + Vit-C+ CBZ. Note the pituicytes (block arrow) with no apparent histopathological lesions (H and E x 400).

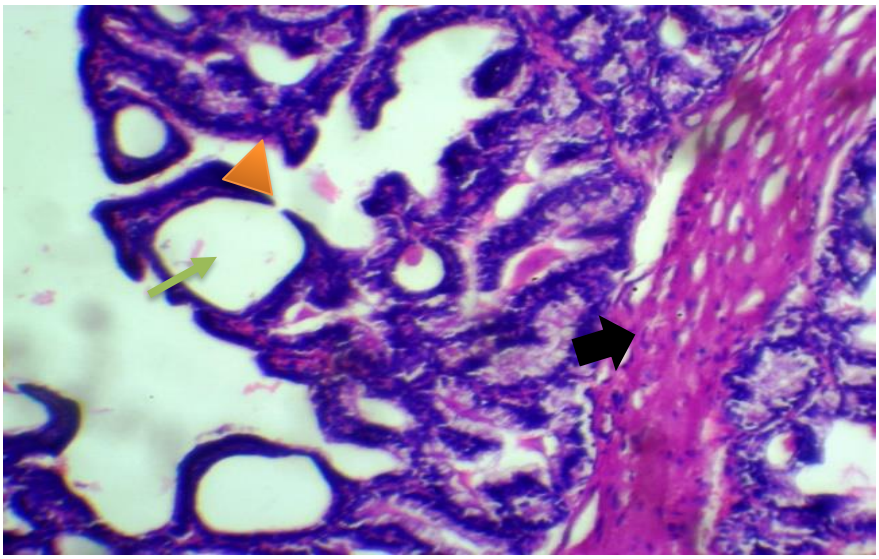

Plate VIII: Photomicrograph Showing Section of the Seminal Vesicle of Adult Male Wistar Rat Exposed to Distilled Water. Note the vesicle which consists of a highly coiled duct ranging from single to multiple fold coiled duct surrounded by two layers of smooth muscle that expel the luminal contents during ejaculation. The epithelial lining (triangle arrow), Lumen (line arrow) and Smooth musculature (block arrow) are all demonstrated (H and E x 400).

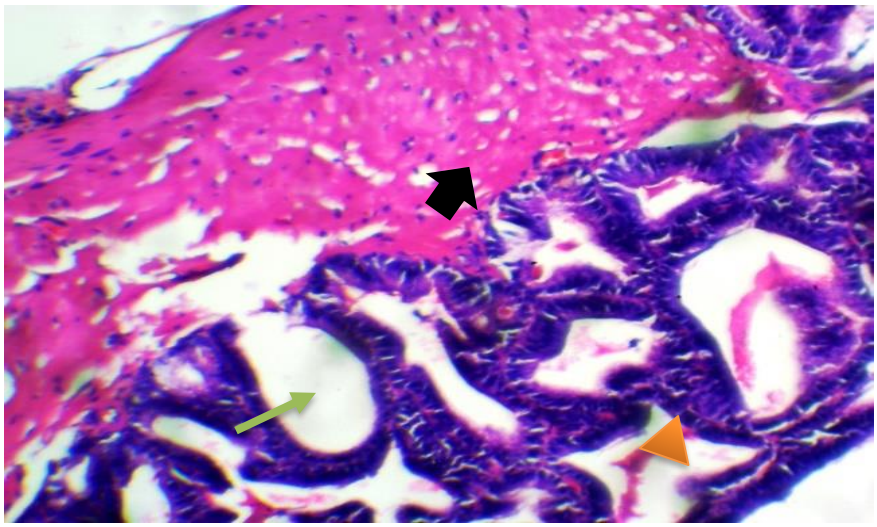

Plate IX: Photomicrograph Showing Section of the Seminal Vesicle of Adult Male Wistar Rat Exposed to Vitamin C. Note the normal vesicle which consists of a highly coiled duct ranging from single to multiple fold coiled duct surrounded by two layers of smooth muscle that expel the luminal contents during ejaculation. The epithelial lining (triangle arrow), Lumen (line arrow) and Smooth musculature (block arrow) are all demonstrated (H and E x 400).

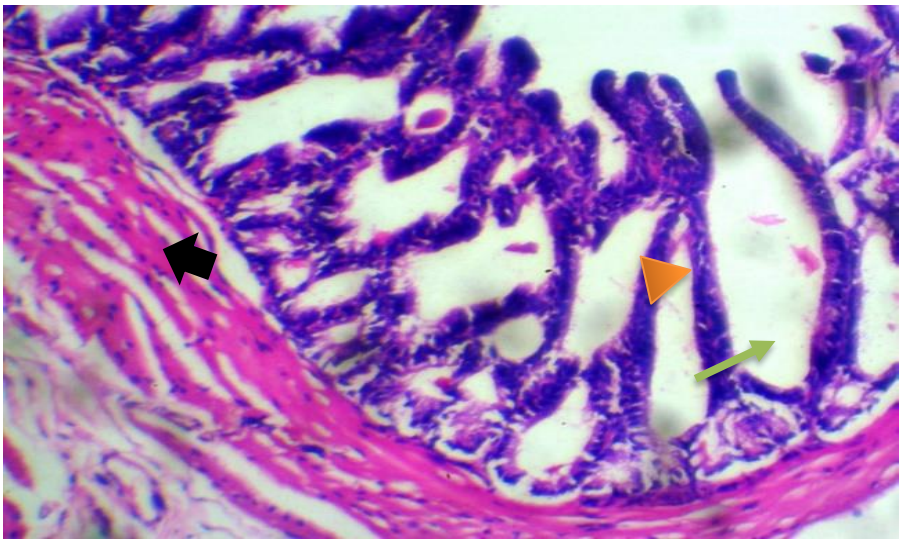

Plate X: Photomicrograph Showing Section of the Seminal Vesicle of Adult Male Wistar Rats Exposed to Methanol leaf extract of *M. oleifera*. Note the vesicle which consists of a highly coiled duct ranging from single to multiple fold coiled duct surrounded by two layers of smooth muscle that expel the luminal contents during ejaculation. The epithelial lining (triangle arrow), Lumen (line arrow) and Smooth musculature (block arrow) are all demonstrated (H and E x 400).

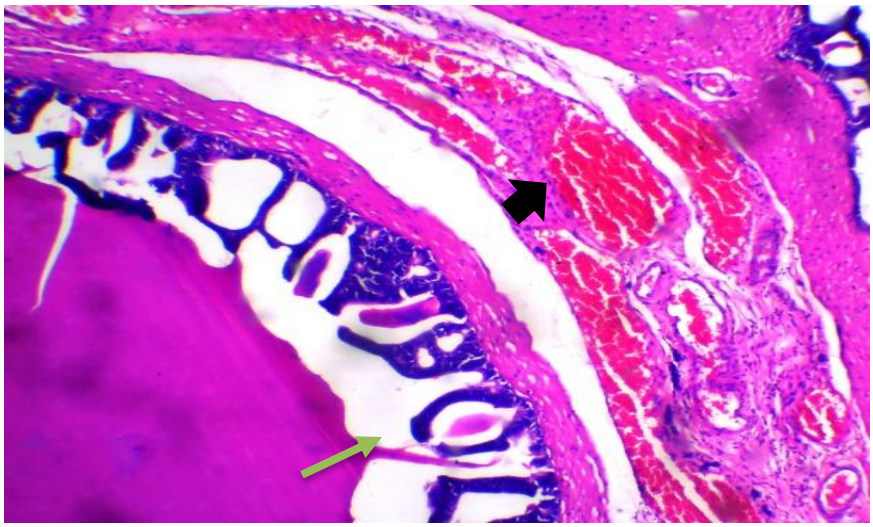

Plate XI: Photomicrograph Showing Section of Seminal the Vesicle of Adult Male Wistar Rat Exposed to Carbamazepine (CBZ). Note the severe haemoharrgic changes (block arrow) occurring on the smooth muscles walls. The lumen (line arrow) and smooth musculature are demonstrated (H and E x 400).

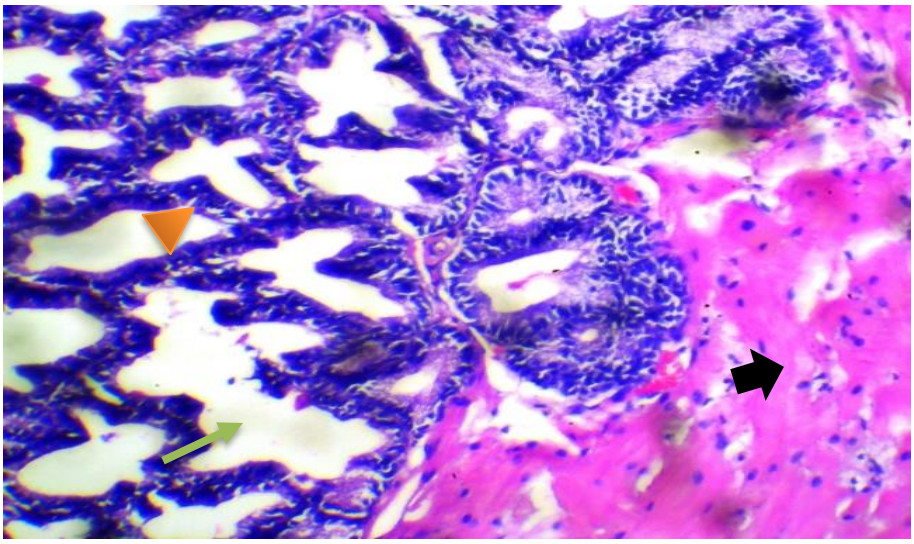

Plate XII: Photomicrograph Showing Section of the Seminal Vesicle of Adult Male Wistar Rat Exposed to Vit-C + CBZ. Note the apparently normal vesicle which consists of a highly coiled duct ranging from single to multiple fold coiled duct surrounded by two layers of smooth muscle (SM) that expel the luminal contents during ejaculation. The epithelial lining (triangle arrow), Lumen (line arrow) and Smooth musculature (block arrow) are all demonstrated (H and E x 400).

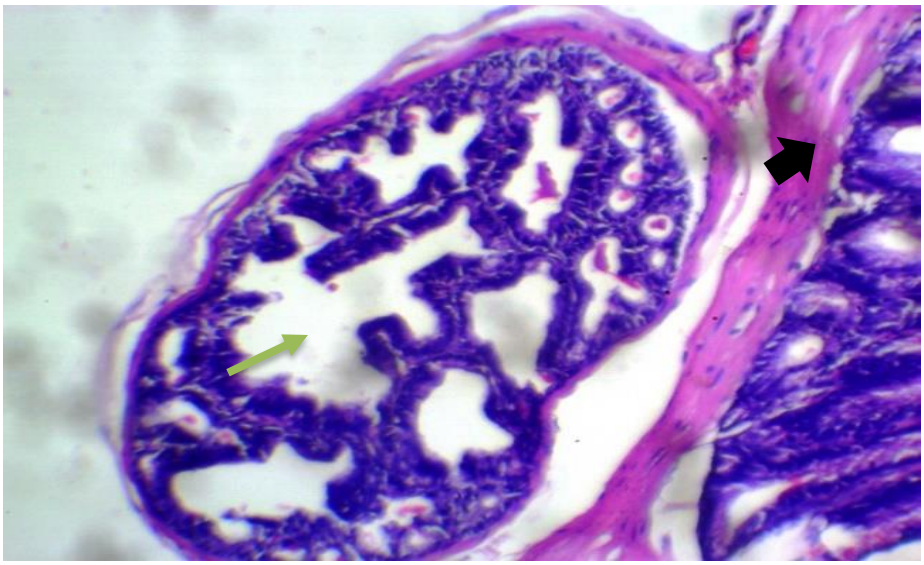

Plate XIII: Photomicrograph Showing Section of the Seminal Vesicle of Adult Male Wistar Rat Exposed to MO + CBZ. Note the apparently normal vesicle, Lumen (line arrow) and Smooth musculature (block arrow) are demonstrated (H and E x 400).

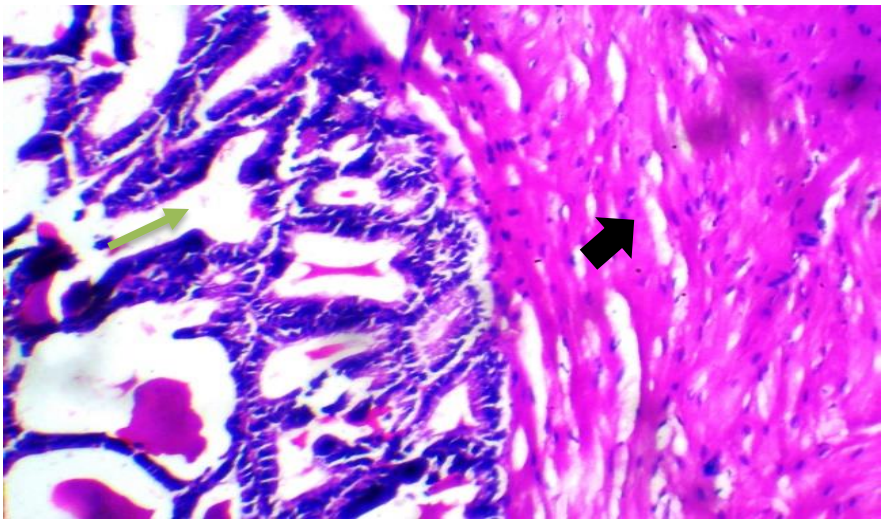

Plate XIV: Photomicrograph Showing Section of the Seminal Vesicle of Adult Male Wistar Rat Exposed to MO + Vit-C + CBZ. Note the vesicle with no apparent histopathological lesions. The Lumen (line arrow) and Smooth musculature (block arrow) are demonstrated (H and E x 400).

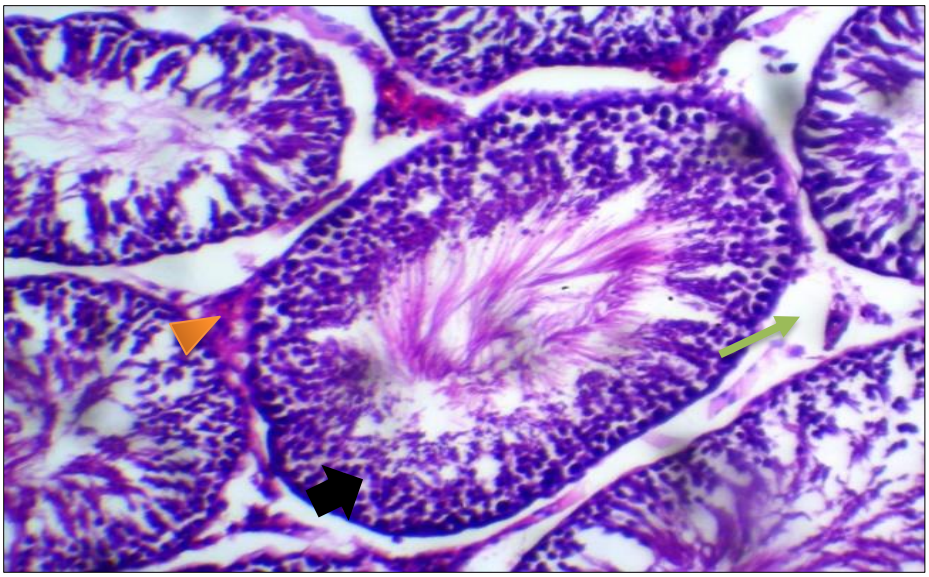

Plate XV: Photomicrograph Showing Section of Testis of Adult Male Wistar Rat Exposed to Distilled Water. Note the seminiferous tubule appears intact (block arrow), Leydig cell (triangle arrow) and interstitial space (line arrow) could be seen conspicuously (H and E x 400).

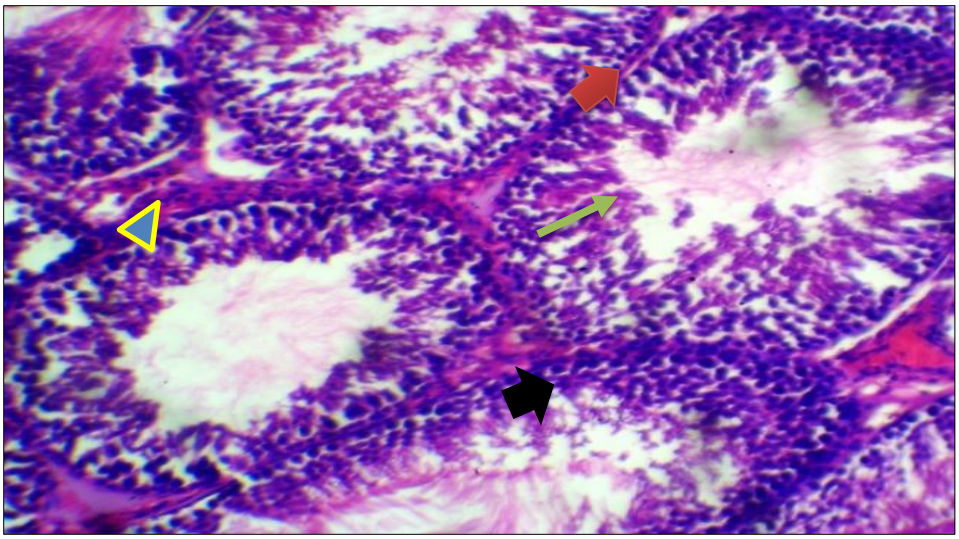

Plate XVI: Photomicrograph Showing Section of Testis of Adult Male Wistar Rat Exposed to Vitamin C. Note the seminiferous tubule appears intact (block arrow), mild degeneration of spermatogenic cells (line arrow), Leydig cell (triangle arrow) and narrow interstitial space (block arrow) could be seen conspicuously (H and E x 400).

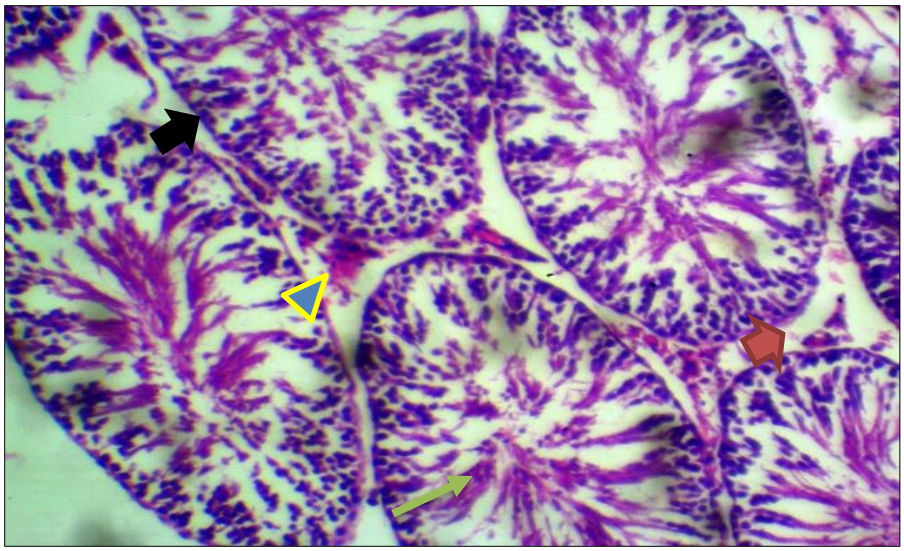

Plate XVII: Photomicrograph Showing Section of Testis of Adult Male Wistar Rat Exposed to Methanol leaf extract of *M. oleifera.* Note the seminiferous tubule appears intact (block arrow), Leydig cell (triangle arrow), spermatogenic cells (line arrow) and narrow interstitial space (block arrow) could be seen conspicuously (H and E x 400).

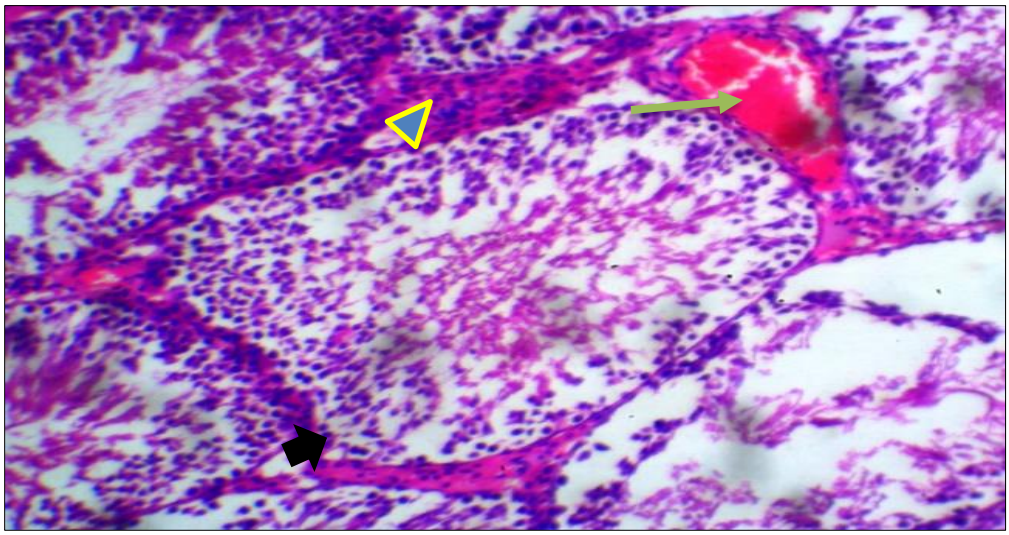

Plate XVIII: Photomicrograph Showing Section of Testis of Adult Male Wistar Rat Exposed to Carbamazepine (CBZ only). Note the seminiferous tubules (block arrow), indications of spermatogenic arrest (incomplete maturation of sperm cells), mild hemorrhage of the Leydig cell (triangle arrow), as well as presence of fibrotic and hemorrhagic cells (line arrow) (H and E x 400).

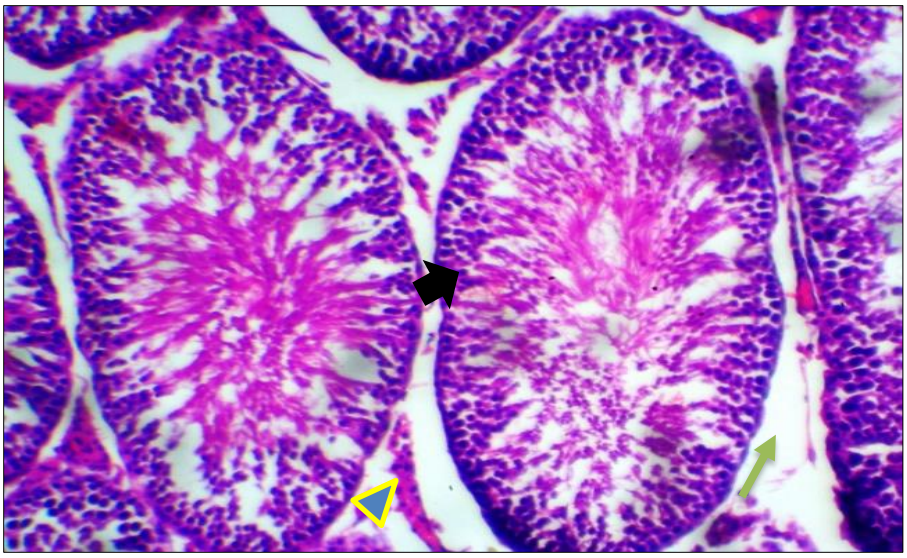

Plate XIX: Photomicrograph Showing Section of Testis of Adult Male Wistar Rat Exposed to Vit-C + CBZ. Note the seminiferous tubule appears intact (block arrow), Leydig cell (triangle arrow) and interstitial space (line arrow) could be seen conspicuously (H and E x 400).

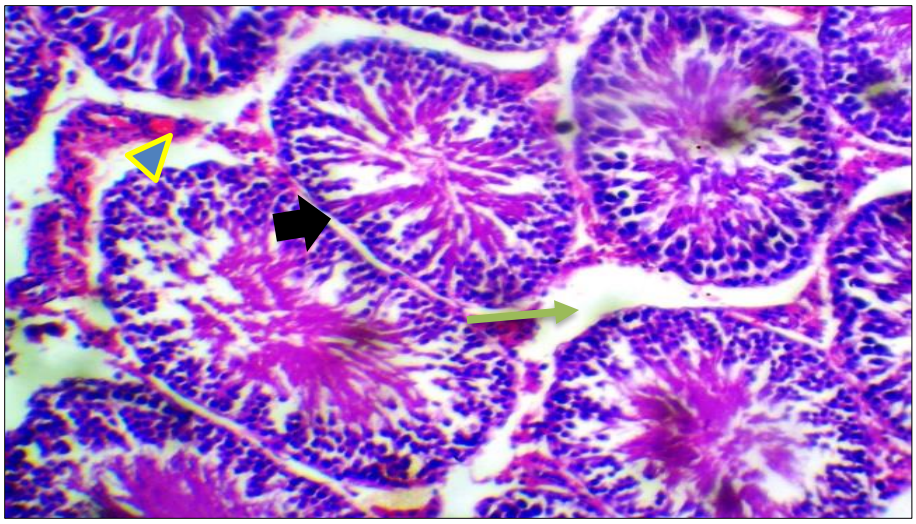

Plate XX: Photomicrograph Showing Section of Testis of Adult Male Wistar Rat Exposed to MO + CBZ. Note the seminiferous tubule appears intact (block arrow), Leydig cell (triangle arrow) and interstitial space (line arrow) could be seen conspicuously (H and E x 400).

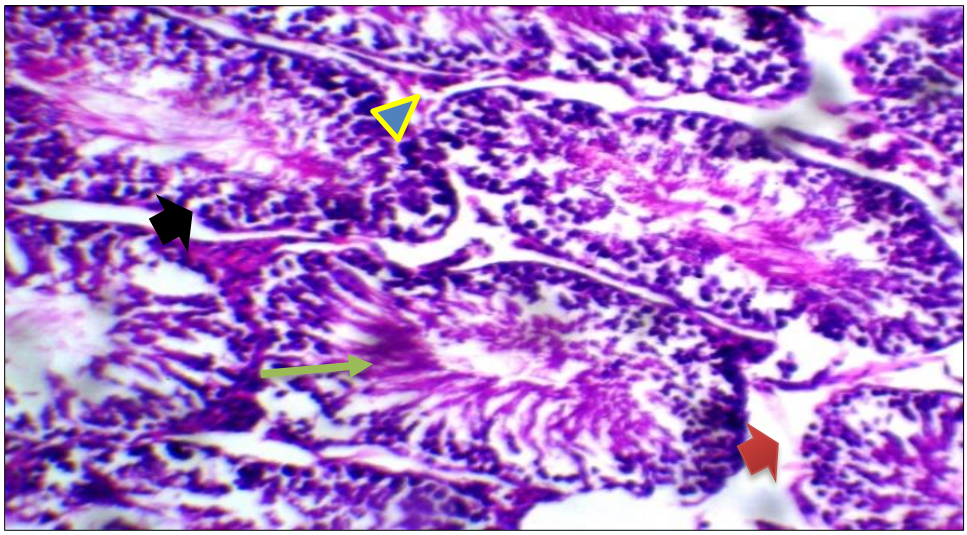

Plate XXI: Photomicrograph Showing Section of Testis of Adult Male Wistar Rat Exposed to MO + Vit-C + CBZ. Note the seminiferous tubule (block arrow) appears apparently intact, spermatogenic cells (line arrow), Leydig cell (triangle arrow) and interstitial space (blood arrow) could be seen conspicuously (H and E x 400).

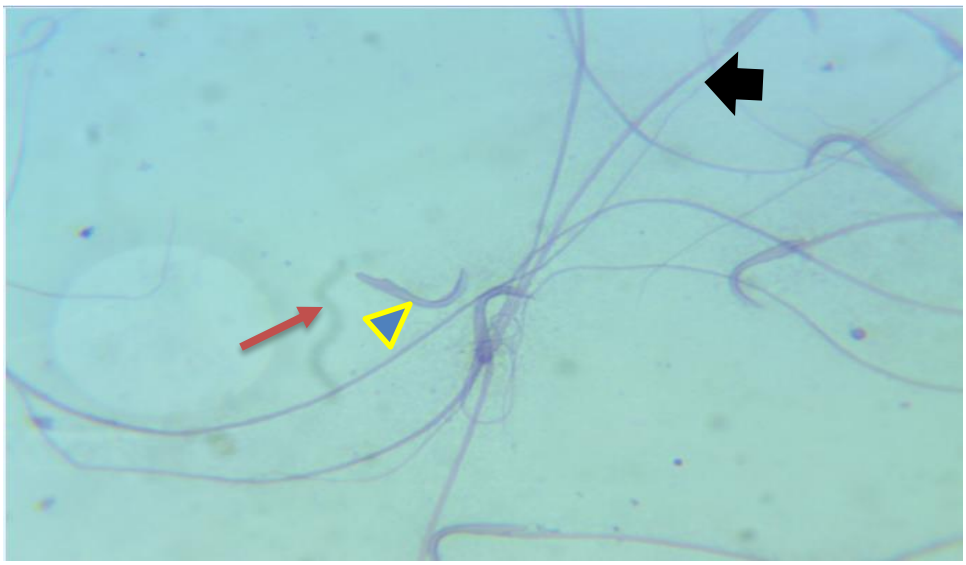

Plate XXII: Photomicrograph Showing Morphological Abnormalities of Sperm Cells using Eosin x 400. Note the Tail Abnormality (triangle arrow) — Tailess spermatozoa, Head Abnormality (line arrow) ---Headless spermatozoa, and Normal Sperm (block arrow) cells.

Plate XXIII: Photomicrograph Showing Morphological Abnormalities of Sperm Cells using Eosin x 400. Note the Tail Abnormality (TA)—Looped tail (line arrow), Curved tail (triangle arrow) and Normal Sperm (block arrow) cell.

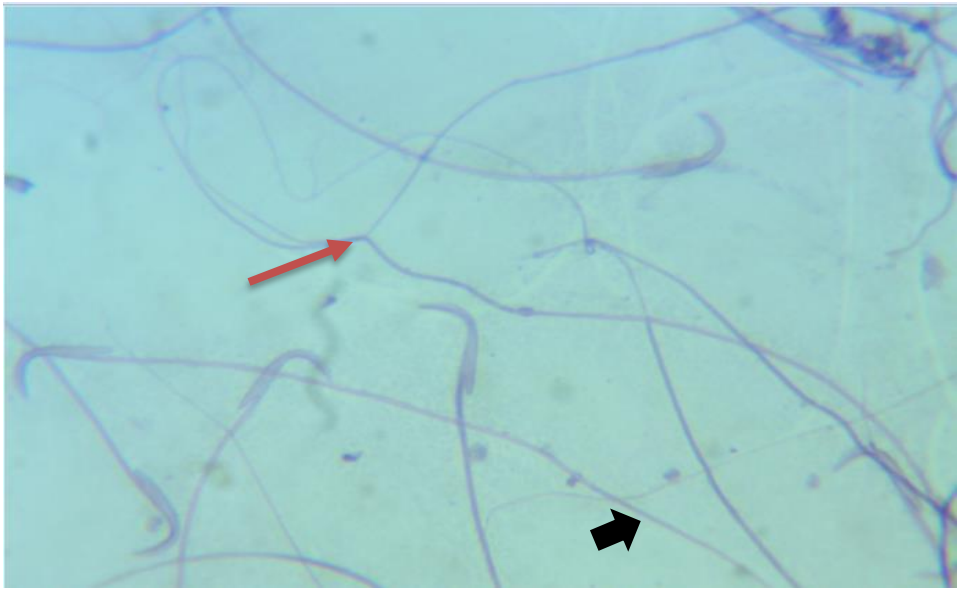

Plate XXIV: Photomicrograph Showing Morphological Abnormalities of Sperm Cells using Eosin x 400. Note the Tail Abnormality —Abaxial tail (line arrow) and Normal Sperm (block arrow) cell.

CHAPTER FIVE

5.0 DISCUSSION

The median lethal dose of methanolic extract of *Moringa oleifera* leaves in the present studies was found to be greater than 5000 mg/kg. This was within the range reported by other researchers, indicating that the plants extract is relatively safe. Akunna *et al*. (2012) reported an LD_{50} of > 3000 mg/kg for orally administered MO leaf extract in rats. Kasolo *et al*. (2012) reported an LD_{50} of 39600 mg/kg for ethanol extract, and 16100 mg/kg for aqueous extract of MO leaves, following intragastric administration in mice. This apparent non-toxicity probably explains the widespread use of MO as food and medicine without adverse effects.

Phytochemical screening of MO leaf extract revealed the presence of alkaloids, glycosides, flavonoids, phenols, tannins, steroids, lipids and amino acids while saponin is absent. This result was consistent with the reports of several authors (Okorondu *et al*., 2013; Misra *et al*., 2014). Unuigbe *et al*. (2014) also reported absence of saponins in methanolic and ethanolic leaf extract of MO respectively.

The total flavonoid content of the MO leaf extract of 22.23% was obtained from this present study, which is considered very high and by far above that reported by Sreelatha and Padma (2009) (2.7%), Oluduro (2012) (0.77%) and Muhammad *et al*. (2017) (21.76%) but lower than that reported by Moyo *et al*. (2013) (29.5% quercetin equivalent). The variation in the quantity of flavonoid content may be dependent of the soil, growing location, agricultural practices, processing techniques, preparation methods, and analytical variability. Flavonoids are secondary metabolites produced by plants in response to various environmental stresses such as climate and ultraviolet radiation (Haytowitz *et al*., 2013). Flavonoids which are part of the phytochemical

constituents of MO exhibit a wide range of biological activities, one of which are their ability to scavenge for hydroxyl radicals, and superoxide anion radicals, and thus has health-promoting action (Ferguson, 2001). Flavonoids also exhibit anti-inflammatory, antiangionic, antiallergic, analgesic and antioxidant properties (Hodek *et al*., 2002).

The total phenolic content of the MO leaf extract of 7.8% from this present study is considered very high and above that reported by Sreelatha and Padma (2009) (2.6%), Oluduro (2012) (0.29%), El-Sohaimy *et al*. (2015) (4.83%) and Ojiako (0.19%) (2014). A number of scientific reports indicate certain terpenoids, steroids and phenolic compounds protective effects due to their antioxidant properties (Chandrasekhar *et al*., 2006). Phenols are widespread secondary metabolite in plant kingdom. These diverse groups of compounds have received much attention as potential natural antioxidant in terms of ability to act as both efficient radical scavengers and metal chelator. It has been reported that the antioxidant activity of phenol is mainly due to their redox properties, hydrogen donors and singlet oxygen quenchers (Bramley and Pridham, 1995). Phenols are strong antioxidants which prevent oxidative damage to biomolecules such as DNA, lipids and proteins which plays a role in chronic diseases such as cancer and cardiovascular disease (Hollman, 2001). The tannin content of the MO leaf extract of 0.54% from this present study is considered very low when compared to values of 8.22% (Ojiako, 2014), 9.19% (Nweze and Nwafor, 2014) and 5.8% (Unuigbe *et al*., 2014) reported in previous studies.

The brain is known to be easily susceptible to foreign substances due to its inherent factors such as abundant polyunsaturated fatty acids in the plasma membrane (PUFAs), ions, etc (Eren *et al*., 2007). The consequence of CBZ induced increased lipoperoxidation in the pituitary glands and testicular tissues are biological dysfunctions, as a result of interference with their cellular integrities and alterations in their cytomorphological organizations (Shittu *et al*., 2012). MDA is

in many instances the most abundant aldehyde arising from lipid peroxidation (Verma and Srivastava, 2003) and hence is used as an index of oxidative damage in tissues. Serum and tissue MDA level is an index of free radical generation, which increases in conditions of oxidative stress (Kehrer, 1993). The present study has shown a significant higher in pituitary gland and testicular MDA concentrations in the CBZ treated group. A possible explanation for the increased MDA concentration might be partly due to the decreased formation of antioxidants and increased activity of reactive oxygen species (ROS) in CBZ treated animals. The significant higher in pituitary and testicular MDA concentrations in the CBZ group recorded in the present study was in conformity with previous findings by Suleiman *et al*. (2015) and Tarun *et al*. (2010). The MDA levels in the pituitary gland of the groups treated with Vit-C + MO + CBZ, MO + CBZ, Vit-C + CBZ, MO, Vit-C and DW groups, were significantly lower than that of the CBZ treated group. This is suggestive of a compensatory mechanism by the pituitary and testicular antioxidant enzymes, induced by MO, Vit-C and even synergic effect of MO and Vit-C to reduce the generation of free radicals; thus, ameliorating oxidative stress in the pituitary glands and testes. Tochopherols and other antioxidants in MO have a significant protective activity against oxidative damage and may have played a central role in decreasing the MDA levels (Elham *et al*., 2017).

The higher SOD activity recorded in the Vit-C + MO + CBZ and MO + CBZ groups relative to that of CBZ group suggest that MO and Vit-C caused an increase in intracellular scavenger activity against lipid peroxidation, thereby mitigating the oxidative injury. The low pituitary gland and testicular SOD activities in CBZ group may be related to the chronicity of the CBZ exposure. The persistent increase in lipoperoxidation as exemplified by increased MDA concentration in the CBZ treated group may have eventually resulted in deficit SOD activities resulting from increased usage and subsequent metabolic degradation. The significant lower in the pituitary and testicular SOD

activities in the CBZ group can also be attributed to an enhanced superoxide anion generations and utilization of SOD during reactive metabolites detoxification induced by chronic administration of CBZ. The reduction in the activity of SOD in CBZ treated group was in consistent with the report of Suleiman *et al*. (2015) and Li *et al*. (2010).

MO leaves extract diminished superoxide toxicity (Sharida *et al*., 2012). The SOD level was increased significantly in this study suggesting either the extrac itself reacts with the reactive oxygen species or boosting SOD production of the tissues. Catalytic removal of ROS by SOD is extremely important as it prevents numerous lipid peroxidation by-products, protein adduction and organ dysfunction. Based on high amounts of phenolic compounds and flavonoids concept of this plant, which are known to effectively scavenge free radicals, iujt is conceivable that these effects may be due, at least in part, to its higher antioxidant activity. The hydroxyl anion (OH^-) scavenging ability of the MO leaf extract could be attributed to the presence of polyphenols which are capable of donating hydrogen atoms to OH radicals, thus inhibiting the oxidation process (Zang *et al*., 2003). Among the antioxidant enzymes, SOD is always considered as the first line of defense against oxygen toxicity due to the inhibitory effects on oxyradical formation (Firat *et al*., 2009). It scavenges the superoxide anion to form oxygen and hydrogen peroxide and thus diminishing the toxic effect caused by the former (Afolabi *et al*., 2013). Superoxide anions and other reactive oxygen species have been shown to be a major contributor to the pathogenesis of erectile dysfunction via the initiation of lipid peroxidation (Azadzoi and Siroky, 2009). Vitamin C is an electron donor (i.e., reducing agent), and by donating its electrons, it prevents other compounds from being oxidized. Thus, one of the mechanisms of antioxidant action of vitamin C is through the scavenging or quenching of free radicals (Aprioku, 2013). The higher SOD activity in Vit-C+ MO + CBZ was a demonstration of synergistic antioxidant effect of MO and Vit-C.

The present study showed a significant lower in pituitary GPx activity in CBZ treated group when compared to Vit-C+ MO + CBZ, MO + CBZ, Vit-C + CBZ, MO, Vit-C and DW groups. This study also showed significant decrease in testicular GPx activity in CBZ treated group when compared to Vit-C + CBZ, MO and Vit-C groups, but a relative decrease in GPx activity in CBZ treated group was observed when compared with Vit-C+ MO + CBZ, MO + CBZ and DW groups. The low level of GPx activity in CBZ treated group was in consistence with the report of Li *et al*. (2010). The GPx may have been used up in response to CBZ-provoked oxidative injury. The implication of low GPx activity as recorded in the CBZ treated group is that the hydrogen peroxides and lipid peroxides are not detoxified and may be converted to $OH^{\cdot}$ radicals and lipid peroxyl radicals respectively by transition metals (Tahbet and Touuyz, 2007).

The increased in GPx levels of Vit-C+ MO + CBZ, MO + CBZ, Vit-C + CBZ, MO, Vit-C and DW groups could boost redox reactions, thereby removing ROS, which is a steady-state cellular event in respiring cells, thereby terminating the harmful effect of chronic use of CBZ. The increase in this antioxidant enzyme will also reduce the damage of cellular macromolecules (DNA, protein, and lipids) (Droge, 2002). The higher level of GPx observed in our study in the group supplemented with the antioxidants is in agreement with the work of Dolapo and Adeniran (2014) and Ashok and Pari (2003) who observed that MO has the potential to prevent oxidative damage by inducing the synthesis of GPx in the tissues.

The present study showed significant lower in both pituitary and testicular catalase (CAT) activity in CBZ treated group when compared to Vit-C+ MO + CBZ group. This finding corroborates with the report of Suleiman *et al*. (2015) who observed in their study a marked decrease in testicular CAT activity following CBZ exposure in rats. The decrease in the CAT activity in the CBZ group may be linked to a decrease in SOD activity, which reduces the rate of conversion of O^{2-} to H_2O_2

the substrate for CAT. The lack of substrate for CAT activity eventually results in its reduced activity. Accumulation of O^{2-} through SOD activity inhibition has been shown to inhibit CAT activity (Kono and Fridovich, 1982), since O^{2-} converts feroxy state of CAT to ferryl state, which is an inactive form of the CAT (Freeman and Crapo, 1982). The decreased CAT activity may also be due to the persistent long-term increased lipoperoxidation induced by chronic administration of CBZ, which results in, increased CAT utilization and decreased synthesis due to high amounts of free radicals in the biosystem. The increased CAT activity recorded in the Vit-C+ MO + CBZ group showed that MO and Vit-C have the ability as antioxidant molecules to boost the endogenous antioxidant reserve and accelerate the removal of ROS induced by CBZ.

In the current study, there was absolute lower FSH and LH levels in the CBZ-treated group as compared to the Vit-C + MO + CBZ, MO + CBZ, Vit-C + CBZ, MO, Vit-C and DW treated groups. Our finding was in consistence with the work of Sameer *et al*. (2004) and Vijay *et al*. (2009) who reported that antiepileptic drugs act on the pituitary gland and decrease the secretion of FSH and LH responsible for spermatogenesis. Baptista *et al*. (1999) also reported that a significant reduction in plasma testosterone, FSH, and LH concentrations were found in rats treated with sodium valproate and carbamazepine. The lower serum FSH and LH concentrations in the CBZ-treated group may be linked to the inhibitory effect of CBZ on the secretion of Gonadotropic releasing hormone (GnRH) from the hypotalamus, which aid in the release of gonadotropins (FSH and LH) from the anterior pituitary. The present study showed increase in the absolute level of FSH and LH in the group treated with Vit-C + MO + CBZ, MO + CBZ, Vit-C + CBZ, MO and Vit-C when compared with CBZ-treated group.

The process of spermatogenesis and development of accessory reproductive organs are androgen dependent. Decreased androgen production will cause a decrease in the number of mature Leydig

cells and their functional status (Osuntokun *et al*., 2017). FSH regulates the growth of seminiferous tubules and maintenance of spermatogenesis in males. FSH is also critical for sperm production, as it supports the function of Sertoli cells, which in turn support many aspects of sperm cell maturation (Egba *et al*., 2014). Diminished secretion of LH or FSH can result in failure of gonadal function (hypogonadism). This condition typically manifest in males as failure in production of normal numbers of sperm (Egba *et al*., 2014). FSH binds with receptors in the sertoli cells and directly stimulates spermatogenesis (O'Donnel *et al*., 1994). LH stimulates the production of testosterone in Leydig cells, which in turn may act on the Sertoli and peritubular cells of the seminiferous tubules and indirectly stimulates spermatogenesis (O'Donnel *et al*., 1994; Singh *et al*., 1995). Testosterone is an androgen that exerts both endocrine activity and intra-testicular activity on spermatogenesis (Colao *et al*., 2004).

The current study showed a significant lower in the serum testosterone level in the CBZ-treated group when compared to Vit-C + MO + CBZ and Vit-C + CBZ groups. This report corroborates with the findings from previous studies (Suleiman *et al*., 2015; Osuntokun *et al*., 2017) who observed a significant decrease in the serum testosterone level of CBZ-treated rats. The lower serum testosterone concentration in the CBZ group may be linked to the inhibitory effect of CBZ on the secretion of pituitary gonadotropins (FSH and LH). Similarly, decrease in testosterone concentration seen in the CBZ-treated group may occur due to direct damage to Leydig cells (Oliva and Miraglia, 2009), apparently due to oxidative injury. Furthermore, the lower testosterone concentration produced by CBZ may be due to the ability of antiepilepsy to induce the production of aromatase enzyme in the liver, which converts testosterone to estradiol, a hormone known to further reduce testostosterone concentration (Vijay *et al*., 2009). Therefore, the low testosterone concentration in the CBZ-treated group as compared to the Vit-C + MO + CBZ and Vit-C + CBZ

groups may be due to deleterious effect of CBZ on both the brain and the testicular tissues due to oxidative injury. CBZ has been shown to suppress fertility in rats, not only by interfering with testicular functions, but possibly by affecting the hypothalamo-pituitary-testicular (gonadal) axis and thus reducing the amount of testosterone in circulation (Daoud *et al*., 2004). The significant improvement in testosterone concentration seen in the Vit-C + MO + CBZ and Vit-C + CBZ groups suggests an ameliorative effect of MO and Vit-C on CBZ-induced testicular and sperm defects. This result may be due to presence of flavonoids present in MO. Flavonoids are well known antioxidants that can ameliorate oxidative stress- related testicular impairments in animal tissues (Ghosh *et al*., 2002; Kujo, 2004). It also stimulates testicular androgenesis and is essential for testicular differentiation, integrity and steroidogenic functions (Luck *et al*., 1995; Salem *et al*., 2001). MO treatment can favour the reproductive process and also enhances spermatogenesis (D'cruz, 2005; Mathur *et al*., 2005). Methanol extract of MO has been shown to enhance seminiferous tubules, testicular and epididymal weight and seminal vesicles in the male rats (Sudha *et al.,* 2010). The significant higher in testosterone level in Vit-C pretreated groups may be linked to ability of Vitamin C to raise the blood testosterone levels, has it affect the hypothalamic-pituitary-testicular axis, thus elevating blood testosterone levels (Ashamu *et al*., 2010). Vitamin C has been reported to increase peripheral testosterone level, prevents sperm agglutination and also attenuates testicular toxicity (Saalu *et al*., 2007). Vitamin C has been shown to improve sperm motility and enhances semen quality and fertility of rats (Saalu *et al*., 2007). Deficiencies of vitamins C or E leads to a state of oxidative stress in the testes that disrupts both spermatogenesis and the production of testosterone (Rekha *et al*., 2009). Vitamin C has also been shown to increase total sperm output and sperm concentration (Nayanatara *et al*., 2008; Rekha *et al*., 2009). Apart from the protective effects of Vitamin C on membrane integrity that safeguarded

cellular functions, it makes available more active/functional seminiferous tubules with good cyto-architecture (Ashamu *et al*., 2010).

The present study showed a significant lower in epididymal sperm concentration in the CBZ group as compared to MO + Vit-C + CBZ, MO, Vit-C and DW groups, after 105 days of oral administration. This study agreed with the result by other researchers (Asadi-Pooya *et al*. 2015; Suleiman *et al*., 2015; Osuntokun *et al*., 2017). The lower sperm count in the CBZ group could be partly due to the induction of oxidative stress in the testes and the spermatozoa as reported by earlier researchers (Gallup *et al*., 2002; Isojarvi *et al*., 2004; Krishnamoorthy *et al*., 2007). It can also be due to the reduced testosterone concentration possibly due to low LH and oxidative damage to the Leydig cells, consistent with the finding of Sharpe *et al*. (2003).

The spermatozoa are highly susceptible to damage from ROS due to high concentration of polyunsaturated fatty acids within their plasma membrane (Krishnamoorthy *et al*., 2007), high production of free radicals resulting from mitochondrial respiration and deficient DNA repair mechanism (Zini and Gagnon, 1993). Although, spermatozoa require a short exposure to low amount of free radicals for sperm capacitation and to become hyperactive (Mariana and Mara, 2009), reactive oxygen species (ROS) must continuously be inactivated to a level that will not be injurious to the spermatozoa. Spermatozoa possess an array of antioxidant defence mechanisms such as superoxide dismutase (SOD), catalase (CAT) and glutathione (GPx) (Alverez and Storey, 1989). However, in the face of oxidative challenge such as those witnessed following chronic CBZ administration, the inate antioxidant defence systems are overwhelmed, resulting in oxidative injury to the testes. The present study has shown that treatment with the methanol extract of MO + Vit-C ameliorated the CBZ-induced low sperm count. This may be due to antioxidant activities of the extract and Vit-C apparently acting synergistically to reduce free radical load in the testes.

The antioxidant potential of MO leaves extract may be attributed to the presence of total phenolics and flavonoids in the extract and/or isolated active constituents—β-sitosterol, quercetin and kaempferol which have hydroxyl group(s) (Singh *et al*., 2014). The hydroxyl group(s), because of its resonance property, easily donates electron (e^-) to free radicals and effectively neutralizes them. Also, the presence of a hydroxyl group(s) increases its antioxidant potential through intermolecular hydrogen bonding involving the -SH group of non-protein thiols and enzymes resulting in the restoration of the antioxidant system against oxidative damage in mammalian tissue (Singh *et al*., 2014). Vitamin C is a strong antioxidant and decreases fatty acid peroxidation in the cell membrane (Dawson *et al*., 1992). Vitamin C supplementation has been shown to decrease free radical formation in human seminal plasma (Dawson *et al*., 1992) and decreased the incidence of secondary sperm abnormalities by reducing free radical formation (Sikka, 1996). Vitamin C is also involved in the synthesis of sex steroids such as testosterone, and peptide hormones; hydroxylation of steroids is especially vitamin C-dependent (Luck *et al*., 1995; Weber *et al*., 1996).

The present study showed a significant lower in the sperm motility in the group exposed to CBZ when compared to DW group, while it shows an absolute decreased volume when compared with other treated groups. This result agreed with previous findings by Suleiman *et al*. (2015) who recorded a marked decrease in the sperm motility in rat treated with CBZ. Shen and Chen (1990) reported that carbamazepine has membrane stabilizing effect and inhibits human sperm motility. Chen *et al*. (1992) observed that anti-epileptic drugs cause a reduction in sperm motility, because of their ability to interfere with sperm membrane functions. Osuntokun *et al*. (2017) also reported a marked decreased in sperm motility in rat treated with CBZ. The reduced sperm motility in CBZ treated group can also be ascribed to accumulation of proteins in the testes and epididymis, secondary to androgen deprivation, resulting in increased formation of abnormal spermatozoa

(Rao and Chinoy, 1983). Carbamazepine (CBZ) had shown negative effects on sperm analysis in both animal and human studies (Reis *et al*., 2013). CBZ has been associated with changes in semen quality and has been linked with reduced sperm motility and increased frequency of morphologically abnormal sperm cells (Isojarvi *et al*., 2004). Carbamazepine (CBZ) is capable of increasing oxidative challenge and has been involved in the reproductive disorders (Agarwal and Bedaiwy, 2003).

Spermatozoa are particularly susceptible to oxidative stress-induced damage because their plasma membranes contain large quantities of polyunsaturated fatty acids (PUFAs) and their cytoplasm contains low concentrations of scavenging enzymes (Sharma and Agarwal, 1996). Sperm DNA damage occurs secondary to oxidative stress, exacerbated by a decline in local antioxidant protection, especially during epididymal maturation. This results in variety of adverse clinical outcomes (Aitken and De Iuliss, 2004). CBZ also has direct effect on spermatogenesis, specifically its interference with acetylcholinesterase and glucose, both of which are required for the process of spermatogenesis (Nelson, 1972). This is because sperm motility, in addition to being under the control of acetylcholine, also depends on glucose availability (Nelson, 1972). The present study has shown that treatment with the methanol extract of MO + Vit-C ameliorated the CBZ-induced low sperm motility. This is so because phenolic compounds in *M. oleifera* act as free radical acceptors and chain breakers. They interfere with the oxidation of lipids and other molecules by rapid donation of a hydrogen atom to radicals (Dai and Mumper, 2010). Sperm cytoplasm contained very low concentrations of scavenging enzymes (D'cruz, 2005; Mathur *et al*., 2005) therefore an increase in the antioxidant enzyme system levels by *Moringa* treatment can favour the reproductive process and also enhances spermatogenesis. Vitamin C (ascorbic acid) is a commonly available compound that acts effectively as an antioxidant and reducing agent at

physiological pH, thus neutralizing the effect of reactive oxygen species (ROS) in the body (Noordin *et al*., 2015). Vitamin C has been shown to have beneficial effect on sperm motility from this study. Earlier studies shown that vitamin C increased sperm motility in goat (Fazeli *et al*., 2010) and ram (Sonmez and Demirci, 2003). Vitamin C also protects membranes against oxidative stress (Kujo, 2004), it has been shown to improve sperm motility and enhances semen quality and fertility of rats (Rekha *et al*., 2009).

Current study showed that CBZ mildly affects sperm morphological parameters such as head and tail abnormality, as well as mid-piece abnormality. This is contrary to the findings of Osuntokun *et al*. (2017) who reported that CBZ and gabapentin have marked adverse effect on sperm morphology.

The current study showed a significant lower in relative pituitary gland weight of CBZ-treated group when compared with Vit-C + MO + CBZ and Vit-C + CBZ groups, while a relatively lower but not significant value was observed when compared with MO + CBZ group. The decrease in pituitary gland weight is an indication of the effect of chronic CBZ treatment on the rats. This work is in line with the findings of Osuntokun *et al*. (2017) who observed a significant decrease in the relative brain weight in CBZ-treated rats when compared with gabapentin (GBP) and CBZ-GBP combination treated rats. Simmons *et al*. (2002) indicated that reduction in weight or a change in either absolute or relative organ weight after administration of a chemical is an indication of the toxic effect of such chemical or agent. The lower relative pituitary weight obtained in rats exposed to CBZ may be due to induction of lipid peroxidation thereby depleting the body mass.

Pretreatment of rats with a combination of vitamin C and *M. oleifera* showed a progressive rise in relative organ weight compared to those exposed to CBZ only, indicating the role of oxidative stress in the mechanism of CBZ-induced alteration in organ weight.

The apparent reduction in testicular weight changes in the CBZ treated group when compared to Vit-C + MO + CBZ and Vit-C + CBZ groups, suggests that chronic administration of CBZ has toxic effects on the testis. Similar to the findings in the present study, Osuntokun *et al*. (2017) noticed a reduced testicular weight following GBP, CBZ and GBP-CBZ combination treatment in rats. This may be related to oxidative damage to the testis and subsequent impairment of optimal testicular activity. Vitamin C and/or MO has been shown by the present study to mitigate the adverse relative testicular weight changes provoked by chronic CBZ administration.

Histological examination of the testes of rats treated with CBZ revealed degeneration in spermatogonia cells, lining of the seminiferous tubule, associated with incomplete spermatogenesis when compared with the control group. The degeneration of germ cells and abnormal interstitial spaces in CBZ treated group seen in this study may be due to the cytotoxic effect of CBZ. This agrees with the findings of Shetty and Naryana (2007) and Suleiman *et al*. (2015), who reported that CBZ depresses spermatogenesis, which results in the death of immature germ cells present in the seminiferous epithelium. Improvements in the testicular histoarchitecture observed in rats treated with Vit-C + MO + CBZ, MO + CBZ and Vit-C + CBZ could be ascribed to the antioxidant effects of MO and Vit-C. This is consistent with the findings of Dare *et al*. (2012), who showed that flavonoids, which are part of the phytochemical constituents of MO, exhibit a wide range of biological activities, especially their ability to scavenge for hydroxyl and superoxide anion radicals. The Vit-C + MO + CBZ group had relatively normal seminiferous tubule and germ cells, when compared to the CBZ, MO + CBZ and Vit-C + CBZ treated groups. This result suggests better ameliorative effect of Vit-C + MO on testicular cytoarchitecture as compared with MO or Vit-C alone. Therefore, there is synergistic interaction between MO and vitamin C.

Histological examination of the pituitary gland of rats treated with CBZ only revealed severe neutrophil infilterations surrounding the pituicytes, as well as loss of cellular projection when compared with the apparently normal pituicyte cytoarchitecture in the Vit-C + MO + CBZ, MO, Vit-C and DW groups. This may be partly due to oxidative injury induced by chronic CBZ administration.

Seminal vesicle secretion is important for semen coagulation and may promote sperm motility, increase stability of sperm chromatin, and suppress the immune activity in the female reproductive tract (Zhang and Jin, 2007). The abnormal function of seminal vesicles may give rise to sexual dysfunction and infertility of males (Zhang and Jin, 2007). The seminal vesicles contain 5α-reductase, which metabolizes testosterone into its much more potent metabolite, dihydrotestosterone (DHT) (Gonzales, 2001). The severe haemorrhages in the wall of seminal vesicle following chronic CBZ administration is a demonstration of the ability of antiepileptic drug to provoke damage to the seminal vesicle, due to oxidative injury. Vit-C and/or MO pretreatment was able to mitigate the oxidative damage resulting in an apparently normal seminal vesicle.

CHAPTER SIX

6.0 SUMMARY, CONCLUSION AND RECOMMENDATION

6.1 Summary

The present study has demonstrated that chronic CBZ administration to male Wistar rats for 15 weeks caused decrease in epididymal sperm count, sperm motility, as well as decrease in serum testosterone and absolute decrease in LH and FSH levels. This study also observed decrease in pituitary and testicular SOD, GPx and CAT, and an increase in pituitary and testicular MDA. The present study also demonstrated that administration of MO and vitamin C ameliorated the oxidative stress and histological alterations in the pituitary glands and testes caused by CBZ treatment. These deleterious effects by CBZ that were ameliorated by MO and Vit-C resulted from increased synergistic process, evidenced by high activities of antioxidant enzymes in groups treated with MO and vitamin C.

6.2 Conclusion

In conclusion, the results obtained from the present study has shown that:

1. Methanol leaf extract of *Moringa oleifera* contains phytochemical constituents such as flavonoids, phenols, glycosides, tannins, amino acids, lipids and alkaloids.
2. Chronic CBZ administration causes decrease in epididymal sperm count, motility and significant reduction in testosterone concentration, absolute decrease in LH and FSH concentrations in adult male Wistar rats.
3. Chronic administration of CBZ caused changes in pituitary and testicular histoarchitectures in adult male Wistar rats.

4. Oxidative stress is partly involved in the alterations of sex hormones' concentrations, sperm characteristic and pituitary and testicular histoarchitecture induced by chronic CBZ administration in adult male Wistar rats.
5. *Moringa oleifera* and/or vitamin C ameliorated chronic carbamazepine-induced alterations in sex hormones' concentrations, sperm characteristics, and pituitary and testicular histoarchitecture in adult male Wistar rats.

6.3 Recommendations

Based on the finding of this study, the followings are recommended:

1. Parameters measuring reproductive status should be constantly evaluated in individuals or animals that are on long-term CBZ therapy.
2. *Moringa oleifera* and/or vitamin C may be useful in mitigating the adverse reproductive consequence in individuals or animals that are on long-term CBZ administration.

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APPENDIX I

Effect of vitamin C and methanol leaf extract of *M. oleifera* on epididymal sperm cells count, mortility and morphology of adult male Wistar rats exposed to chronic carbamazepine

Parameters (%)	Distill Water	Vitamin C	*Moringa oleifera*	Cabamazepine (only)	Vitamin C + Cabamazepine	*Moringa oleifera* + Cabamazepine	Vitamin C + *Moringa oleifera* + Cabamazepine
Sperm count (10^6/ml)	137.00 ± 2.33^{b}	120.00 ± 1.16	108.00 ± 5.13	93.33 ± 3.71^{a}	89.00 ± 4.93	93.00 ± 6.56	105.70 ± 6.23
Motile (%)	93.33 ± 1.67^{d}	76.67 ± 3.33	73.33 ± 3.33	66.67 ± 3.33^{c}	66.67 ± 3.33	66.67 ± 3.33	73.33 ± 3.33
Immotile (%)	6.67 ± 1.67^{d}	23.33 ± 3.33	26.67 ± 3.33	36.37 ± 3.33^{c}	33.33 ± 3.33	33.33 ± 3.33	26.67 ± 3.33
Head Abnormality (%)	1.55 ± 0.18	2.04 ± 0.17	2.08 ± 0.09	2.32 ± 0.07	2.16 ± 0.08	2.14 ± 0.30	2.20 ± 0.14
Mid-piece Abnormality (%)	4.07 ± 0.18^{g}	4.94 ± 0.23	5.10 ± 0.11	6.02 ± 0.41^{f}	5.52 ± 0.25	5.20 ± 0.25	4.88 ± 0.19
Tail Abnormality (%)	4.52 ± 0.42^{e}	5.66 ± 0.26	5.68 ± 0.33	6.68 ± 0.43	6.17 ± 0.04	6.11 ± 0.39	6.21 ± 0.09

Mean ± SEM (standand error of mean)

a= ($P < 0.05$) lower Vs Vit-C+MO+CBZ, MO, Vit-C and DW groups.

b= ($P < 0.05$) higher Vs MO, MO+CBZ, Vit-C+CBZ and Vit-C+MO+CBZ groups.

c= ($P < 0.05$) lower Vs DW group.

d= ($P < 0.05$) higher Vs MO, Vit-C, Vit-C+CBZ, MO+CBZ and Vit-C+MO+CBZ groups.

e= ($P < 0.05$) lower Vs CBZ, Vit-C + CBZ, MO + CBZ and Vit-C + MO + CBZ groups.

f= ($P < 0.05$) higher Vs Vit-C + MO + CBZ and DW groups.

g= ($P < 0.05$) lower Vs Vit-C + CBZ group.

APPENDIX II

Effect of vitamin C and methanol leaf extract of *M. oleifera* on pituitary and testicular malondialdehyde (MDA) concentration of adult male Wistar rats exposed to chronic carbamazepine

Parameter	Distill Water	Vitamin C	*Moringa oleifera*	Cabamazepine (only)	Vitamin C + Cabamazepine	*Morina oleifera* + Cabamazepine	Vitamin C + *Moringa oleifera* + Cabamazepine
Pituitary MDA (µMol/mg protein)	0.301 ± 0.11^{b}	0.498 ± 0.06	0.460 ± 0.03	0.555 ± 0.12^{a}	0.454 ± 0.03	0.279 ± 0.05	0.223 ± 0.07
Testicular MDA (µMol/mg protein)	0.528 ± 0.03	0.482 ± 0.02	0.533 ± 0.02	0.700 ± 0.03^{c}	0.441 ± 0.02	0.318 ± 0.02^{d}	0.312 ± 0.02^{d}

Mean ± SEM (standand error of mean)

a= ($P < 0.05$) higher Vs Vit-C + MO + CBZ, MO + CBZ and DW groups.

b= ($P < 0.05$) lower Vs Vit-C + CBZ, Vit-C and MO groups.

c= ($P < 0.05$) higher Vs Vit-C + MO + CBZ, MO + CBZ, Vit-C + CBZ, MO, Vit-C and DW groups.

d= ($P < 0.05$) lower Vs MO, Vit-C and DW groups.

APPENDIX III

Effect of vitamin C and methanol leaf extract of *M. oleifera* on pituitary and testicular superoxide dismutase (SOD) activity of adult male Wistar rats exposed to chronic carbamazepine

Parameter	Distill Water	Vitamin C	*Moringa oleifera*	Cabamazepine (only)	Vitamin C + Cabamazepine	*Morina oleifera* + Cabamazepine	Vitamin C + *Moringa oleifera* + Cabamazepine
Pituitary SOD (U/L)	0.232 ± 0.06	0.234 ± 0.02	0.234 ± 0.03	0.232 ± 0.01[a]	0.234 ± 0.04	0.234 ± 0.03	0.237 ± 0.02[b]
Testicular SOD (U/L)	0.2313 ± 0.01	0.2314 ± 0.02	0.2315 ± 0.01	0.2308 ± 0.01[c]	0.2318 ± 0.01	0.2323 ± 0.02[d]	0.2321 ± 0.02[d]

Mean ± SEM (standand error of mean)

a= ($P < 0.05$) lower Vs Vit-C + MO + CBZ, MO + CBZ, Vit-C + CBZ, MO and Vit-C groups.

b= ($P < 0.05$) higher Vs MO + CBZ, Vit-C + CBZ, MO, Vit-C and DW groups.

c= ($P < 0.05$) lower Vs Vit-C + MO + CBZ, MO + CBZ and Vit-C + CBZ groups.

d= ($P < 0.05$) higher Vs MO, Vit-C and DW groups.

APPENDIX IV

Effect of vitamin C and methanol leaf extract of *M. oleifera* on pituitary and testicular catalase (CAT) activity of adult male Wistar rats exposed to chronic carbamazepine

Parameter	Distill Water	Vitamin C	*Moringa oleifera*	Cabamazepine (only)	Vitamin C + Cabamazepine	*Morina oleifera* + Cabamazepine	Vitamin C + *Moringa oleifera* + Cabamazepine
Pituitary CAT (µ/mg protein)	54.03 ±1.59	60.26 ±1.01	65.03 ± 2.78	54.29 ± 2.30[a]	59.77 ± 2.16	63.72 ± 3.49	74.51 ± 2.26
Testicular CAT (µ/mg protein)	52.91 ±1.78	61.01 ± 0.75	68.71 ± 2.05[c]	38.48 ± 1.71[b]	53.74 ± 2.39	58.23 ± 2.79	61.44 ± 2.49

Mean ± SEM (Standard error of mean)

a= ($P < 0.05$) lower Vs Vit-C + MO + CBZ group.

b= ($P < 0.05$) lower Vs Vit-C + MO + CBZ, MO + CBZ, Vit-C + CBZ, Vit-C, MO and DW groups.

c= ($P < 0.05$) higher Vs MO + CBZ, Vit-C + CBZ and DW groups.

APPENDIX V

Effect of vitamin C and methanol leaf extract of *M. oleifera* on pituitary and testicular glutathione peroxidase (GPx) activity of adult male Wistar rats exposed to chronic carbamazepine

Parameter	Distill Water	Vitamin C	*Moringa oleifera*	Cabamazepine (only)	Vitamin C + Cabamazepine	*Morina oleifera* + Cabamazepine	Vitamin C + *Moringa oleifera* + Cabamazepine
Pituitary GPx (U/L)	208.4 ± 6.75	230.5 ± 21.28	221.5 ± 14.38	174.2 ± 16.69[a]	242.3 ± 11.12	234.3 ± 9.57	279.3 ± 14.57
Testicular GPx (U/L)	199.6 ± 8.62	236.9 ± 9.93	259.4 ± 7.41[c]	179.4 ± 10.81[b]	228.6 ± 8.82	202.3 ± 10.56	208.4 ± 9.99

Mean ± SEM (Standard error of mean)

a= ($P < 0.05$) lower Vs Vit-C + MO + CBZ, MO + CBZ, Vit-C + CBZ, MO, Vit-C and DW groups.

b= ($P < 0.05$) lower Vs Vit-C + CBZ, Vit-C and MO groups.

c= ($P < 0.05$) higher Vs Vit-C + MO + CBZ, MO + CBZ and DW groups.

APPENDIX VI

Effect of vitamin C and methanol leaf extract of *M. oleifera* on follicle stimulating hormone, luteinizing hormone and testosterone concentrations of adult male Wistar rats exposed to chronic carbamazepine

Parameters	Distill Water	Vitamin C	*Moringa oleifera*	Cabamazepine (only)	Vitamin C + Cabamazepine	*Morina oleifera* + Cabamazepine	Vitamin C + *Moringa oleifera* + Cabamazepine
FSH (ng/mL)	0.835 ± 0.02	0.851 ± 0.03	0.928 ± 0.05	0.781 ± 0.01	0.853 ± 0.03	0.871 ± 0.04	0.915 ± 0.05
LH (mlU/mL)	0.834 ± 0.04	0.839 ± 0.02	0.941 ± 0.03	0.741 ± 0.01	0.866 ± 0.08	0.979 ± 0.05	0.825 ± 0.07
Testosterone (ng/mL)	2.387 ± 0.07	2.375 ± 0.09	2.536 ± 0.16	2.138 ± 0.08[a]	2.730 ± 0.02	2.430 ± 0.13	3.658 ± 0.14[b]

Mean ± SEM (Standard error of mean)

a= ($P < 0.05$) lower Vs Vit-C + MO + CBZ and Vit-C + CBZ groups.

b= ($P < 0.05$) higher Vs DW group.

APPENDIX VII

Effect of vitamin C and methanol leaf extract of *M. oleifera* on relative pituitary, seminal vesicular and testicular organ weights of adult male Wistar rats exposed to chronic carbamazepine

Parameters	Distilled Water	Vitamin C	*Moringa oleifera*	Cabamazepine (only)	Vitamin C + Cabamazepine	*Morina oleifera* + Cabamazepine	Vitamin C + *Moringa oleifera* + Cabamazepine
Pituitary (g)	0.080 ± 0.006	0.093 ± 0.007	0.010 ± 0.002	0.103 ± 0.01[a]	0.152 ± 0.011	0.130 ± 0.010	0.137 ± 0.006
Seminal vesicle (g)	0.640 ± 0.02	0.540 ± 0.03	0.420 ± 0.02	0.380 ± 0.03	0.400 ± 0.01	0.520 ± 0.03	0.430 ± 0.01
Testis (g)	0.923 ± 0.032	0.937 ± 0.041	0.933 ± 0.030	1.037 ± 0.029[b]	1.050 ± 0.093	1.087 ± 0.029	1.070 ± 0.075

Mean ± SEM

a= ($P < 0.05$) lower Vs Vit-C + MO + CBZ and Vit-C + CBZ groups.

b= ($P < 0.05$) lower Vs MO + CBZ, Vit-C and DW groups.

APPENDIX VIII

Phytochemical screening of methanol leaf extract *Moringa oleifera*

A stock concentration of 1% (w/v) solution of the leaf extract of *Moringa oleifera* was prepared. The leaf extract was tested for the presence of active phytochemicals viz: tannins, alkaloids, flavonoids, as described by Trease and Evans (1978) as briefly described below:

Tannins

Ferric chloride Test: A few drops of 5% ferric chloride solution was added to 2 ml of the test solution. Formation of blue coloration was observed, which indicate the presence of hydrolysable tannins.

Flavonoids

Alkaline reagent test: 5 drops of 5% sodium hydroxide was added to 1 ml of the test solution, this resulted to increased in the intensity of the yellow colour. A few drops of 2 M hydrochloric acid were added to the solution, it then turned colorless, indicative of presence of flavonoids.

Alkaloids

50 mg of extract was dissolved in 5 ml of distilled water. Further 2M hydrochloric acid was added until an acid reaction occurred and filtered. The filtrate was then tested for the presence of alkaloids using Meyer's Test.

Mayer's Test: To 1 ml of test solution or filtrate, a drop of the Mayer's reagent was added along the sides of the test tube. A white or a creamy precipitate confirmed the test as positive.

Phenol

2ml of extract was added to 2mls of ferric chloride solution ($FeCl_3$), a deep bluish green solution is formed with presence of phenols.

Glycosides

25ml of dilute sulphuric acid was added to 5ml of extract in a test tube and boiled for 15 minutes, cooled and neutralized with 10% NaOH, then 5ml of fehling solution A and B was added. A brick red precipitate of reducing sugars indicates presence of glycosides.

Steroids

Salkowski's test: In 2 ml of plant extract, 2ml of chloroform and 2 ml of concentrated H2SO4 was added and shaken well. Chloroform layer appeared red and acid layer greenish yellow fluorescent. This confirms the presence of sterols.

Total flavonoid content

The total flavonoid content (mg/mL) was determined using aluminum chloride ($AlCl_3$) method. The assay mixture consisting of 0.5 mL of the plant extract, 0.5 mL distilled water, and 0.3 mL of 5% $NaNO_2$ was incubated for 5 min at 25°C. This was followed by addition of 0.3 mL of 10% $AlCl_3$ immediately. Two milliliters of 1 M NaOH was then added to the reaction mixture, and the absorbance was

measured at 510 nm with a M8500 UV-VISIBLE spectrophotometer. Quercetin was used as a standard for a calibration curve. The flavonoid content was calculated using the following linear equation based on the calibration curve:

$A=0.01069C-0.001163$, $r=0.9998$

where A is the absorbance

C is the flavonoid content in $\mu g\ g^{-1}$.

Total phenolic content

Gallic acid monohydrate was used as the standard and represented the phenolic compound in the *Moringa oleifera* leaf. Ten (10) mg of this standard was dissolved in 100ml of methanol to give a stock solution of 100µg/ml concentration. Preparation of standard calibration curve of gallic acid Aliquots of 0.25, 0.5, 1.0, 1.5 and 2.0 from the above stock solution were taken in 5 different 10ml volumetric flasks. To each flask, 2.5ml of a tenfold dilution of Folin-Ciocalteau phenol reagent (1:10 v/v with distilled water) and 2.0 ml of a 7.5% w/v sodium carbonate (Na2CO3) solution were added. The mixture was then made up to the mark with distilled water to make concentrations ranging from 2.5-20 µg/ml. A mixture of reagents and water was used as blank. The resultant solutions were then kept on a water bath set at 45 °C for 15 minutes. Thereafter, the absorbance was read at 765nm using a Milton Roy Spectronic 21D UV-VIS Spectrophotometer (USA). A standard calibration curve of Absorbance (y) against Concentration (x) was then plotted.