PROTECTIVE ROLE OF KOLAVIRON, A BIFLAVONOID FROM *Garcinia kola* HECKEL SEEDS ON THE PROSTATE OF DIABETES MELLITUS RATS

BY

BOSEDE OLUWASEUN, POPOOLA
Matric No: 140863

B.Sc. Biochemistry (Ilorin), M.Sc. Biochemistry (Ibadan)

A Dissertation in the Department of Biochemistry,
Submitted to the Faculty of Basic Medical Sciences
in partial fulfillment of the requirements for the Degree of

MASTER OF PHILOSOPHY

of the

UNIVERSITY OF IBADAN

JUNE, 2019

CERTIFICATION

I certify that this research work was carried out by BOSEDE OLUWASEUN, POPOOLA with matriculation number 140863, under my supervision in the Molecular Drug Metabolism and Toxicological Unit, Department of Biochemistry, Faculty of Basic Medical Sciences, College of Medicine, University of Ibadan, Nigeria.

…….…….....

Supervisor

O. A. Adaramoye, Ph.D (Ibadan)

Professor of Biochemistry and Molecular Toxicology

Department of Biochemistry

University of Ibadan

Ibadan, Nigeria.

DEDICATION

This project is dedicated to the all wise God who is my Creator, Anchor, Strength, Sustainer, Comfort and Help.

To God be the glory.

ACKNOWLEDGEMENT

Great is thy Faithfulness Oh Lord! In whom there is no shadow of turning or variableness. I am grateful to God for making me somebody out of my nothingness to attain this level of academic pursuit.

My sincere appreciation goes to my supervisor, Prof. A.O. Adaramoye for your constructive criticism and unflinching contributions to make this research work a reality. Thank you sir for imparting me.

I am also grateful to Prof. E.O. Farombi, who is the Head of Department and also, the Unit Head for his support and encouragement during this programme. I remain grateful sir.

I am also indebted to all my teachers, Prof. O.O. Olorunsogo, Prof. A.O.Odunola and Dr. Gbadegesin for their immense encouragement and contributions. I specially thank Dr. Mrs. Adesanoye for your motherly counsel. You are blessed ma.

I thank all the members of staff of Biochemistry department, Dr. C.O. Olaiya, Dr. I. Adedara, Dr. A. Abolaji, Dr. Mrs. Nwoso, Dr. Mrs. Salami, Dr. Owumi, Dr. Olanlokun, Dr. Adegoke, Dr. Juwon, Dr. Oyebode, Mr. Olowofolahan, Mr. Esan, Mrs. Kate Nwokocha, Mr. Okewuyi, Mr. Ige, Mr. Ajiboye and others for their contributions and concerns.

My heartfelt obligation goes to my better half, my hubby; Mr. C.A. Fabiyi whose love, patience, prayers and unwavering support has been the horse I am riding on, all through the programme. You are an epitome of excellence and example to emulate. Thanks in million times and God bless you! Dearie.

To my children, I want to say a BIG THANK YOU for your cooperation. I love you always.

I deeply appreciate my parents; Mr. and Mrs. S.A. Popoola for their love, support and prayers always. I really appreciate your parental care and you will live to reap the fruits of your labour in sound mind and health.

To my siblings; Mrs. Onadeko Oluwanike, Ms Oluwafunke, Oluwabunmi and Boluwatife. God's love binds us together and thanks for being there.

My sense of gratitude to my Colleagues and friends; Sis Akanni Olubukola, Sis Sola, Mr. Olayinka, Mrs Adawa Adedoyin, and Mrs Akingbile Idowu for your love, encouragement and support. The science world awaits your manifestation.

To all Lab 101 friends and loved ones; Taiwo Oladipupo, Mr. Omage Bright, Mr. Arowoogun Jerry, Mr. Tunji Sanni, Mr. Idu, Mrs. Itunu Ajayi, Ms. Adeuga Promise, Ibi Anna, Jayesinmi Taiwo, Feyisetan Sukurah and Madu Judith. The sky is the starting point, I love you all!

To my God fathers and mothers; Mummy Adeyeye, Pastor and Mummy Aderoju, Pastor Oladunjoye, Pastor Awolola and Mummy Anjorin for your prayers and encouragement. God will reward your labour of love.

I also acknowledge all my Fellowship Leaders and members (Deeper Life Campus Fellowship U.I (Abadina centre). God will not forget your labour of love.

Finally, I return all glory to God, who knows the end from the beginning.

TABLE OF CONTENTS

LIST OF TABLES AND PLATES

LIST OF FIGURES

LIST OF ABBREVIATIONS

DM	-	Diabetes mellitus
ACE	-	Angiotensin converting enzyme
DHT	-	Dihydrotestosterone
STZ	-	Streptozotocin
KV	-	Kolaviron
MET	-	Metformin
PSA	-	Prostate specific antigen
DNA	-	Deoxyribonucleic Acid
NO	-	Nitric oxide
cGMP	-	cyclase Guanyl mono phosphate
ROS	-	Reactive Oxygen Species
IGT	-	Impaired glucose tolerance
NADPH	-	Nicotinamide adenine dinucleotide phosphate (Reduced)
NAD^+	-	Nicotinamide adenine dinucleotide (Oxidized)
$NADP^+$	-	Nicotinamide adenine dinucleotide phosphate (Oxidized)
NADH	-	Nicotinamide adenine dinucleotide (Reduced)
AGE	-	Advanced glycation end-products
HbA1C	-	Glycated haemoglobin A1c
DAG	-	Diacylglycerol
PKC	-	Protein kinase C
DHAP	-	Dihydroxyacetone phosphate
VEGF	-	Vascular endothelial growth factor
eNOS	-	Endothelial nitric oxide synthase
TGF-β	-	Transforming growth factor β
PAI-1	-	Plasminogen activator inhibitor 1

GFAT	-	Glutamine: fructose-6 phosphate amidotransferase
UDP	-	Uridine diphosphate
TCA	-	Tricarboxylic Acid
$FADH_2$	-	Flavin adenine dinucleotide (Reduced)
ATP	-	Adenine triphosphate
UCP	-	Uncoupling protein
GAPDH	-	Glyceraldehyde phosphate dehydrogenase
UDP-GLCNAC-		UDP–*N*-acetylglucosamine
PARP	-	Poly (ADP-ribose) polymerase
NFκB	-	Nuclear factor kappa B
BPH	-	Benign prostate hyperplasia
LUTS	-	Lower urinary tract symptoms
UTI	-	Urinary tract infection
AUR	-	Acute urinary retention
ZN	-	Zinc
M	-	Mitotic
AMPK	-	Adenosine monophosphate-activated protein kinase
IGF-1	-	Insulin growth factor 1
IGF-1R	-	Insulin growth factor 1 receptor
ALT	-	Alanine aminotransferase
AST	-	Aspartate aminotransferase
ALP	-	Alkaline phosphatase
GST	-	Glutathione-S-Transferase
GPx	-	Glutathione Peroxidase
GSH	-	reduced Glutathione
GSSG	-	oxidized Glutathione

LPO	-	Lipid Peroxidation
TCA	-	Trichloroacetic acid
EDTA	-	Ethylene diamine tetra acetic acid
DTNB	-	5, 5-dithio-bis-2-nitrobenzoic acid
CDNB	-	1-chloro-2,4-dinitrobenzene
MDA	-	Trichloroacetic acid
MNPCE	-	Micronucleated Polychromatic Erythrocytes
DNTB	-	5, 5-dithio-bis-2-nitrobenzoic acid
NAOH	-	Sodium hydroxide
KCl	-	Potassium chloride
FSH	-	Follicle Stimulating Hormone
LH	-	Luteinizing Hormone
ELISA	-	Enzyme-linked immunosorbent assay
BSA	-	Bovine Serum Albumin
BCG	-	Bromocresol green
ACP	-	Acid phosphatase
LDH	-	Lactate dehydrogenase
TBARS	-	Thiobarbituric acid reactive substances
TBA	-	2-thiobarbituric acid
EDTA	-	Ethylene diamine tetra acetic acid
AAS	-	Atomic Absorption Spectrophotometer
H & E	-	Hematoxylin and Eosin
ABC	-	Avidin Biotin Complex
PBS	-	Phosphate buffer saline
HRP	-	Horseradish peroxidase
FBS	-	Fasting blood sugar

ABSTRACT

Diabetes mellitus (DM) and Benign prostatic hyperplasia (BPH) is a comorbidity of elderly men and is associated with complications such as infertility. Male infertility caused by this comorbidity has also been linked to poor semen quality. The role of prostatic fluid in the maintenance of semen quality is crucial. Kolaviron (KV), a biflavonoid from *Garcinia kola* (GK) seeds, has been reported to promote fertility in male DM rats. However, the effect of KV on comorbidity-induced prostate impairment is unknown. Therefore, this study was designed to investigate biochemical and structural changes in prostate of DM and BPH rats, and the protective role of KV.

Garcinia kola seeds were obtained commercially, authenticated at Department of Botany Herbarium, University of Ibadan (UI-00138/01). Powdered GK seeds was extracted in soxhlet using methanol. The methanol extract was partitioned with chloroform and water to obtain a golden colour fraction called Kolaviron. Twenty-six male Wistar rats were assigned into three groups: Control (n=10), 3 months DM (3MDM, n=8) and 5 months DM (5MDM, n=8). Diabetes mellitus was induced by a single administration of Streptozotocin intraperitoneally (35 mg/kg). Rats were sacrificed at 3 and 5 months after DM and, blood and prostate were obtained for analysis. Another twenty rats were assigned into four groups (n=5): BPH control, BPH+DM, BPH+DM+KV (200 mg/kg) and BPH+DM+MET (Metformin, 200 mg/kg). The BPH was induced by daily administration of testosterone propionate (TP) intraperitoneally (3 mg/kg) to the castrated rats for 28 days. Biochemical assays were determined, spectrophotometrically. Luteinizing and Follicle stimulating hormones (LH and FSH) and testosterone were estimated using ELISA, while zinc and bicarbonate were estimated using Atomic Absorption Spectrophotometry. Micronucleated polychromatic erythrocytes (mnPCEs) frequencies, histology and expressions of B-cell lymphoma (Bcl-2) and ki67 were determined using standard methods. Data were analysed using ANOVA at $\alpha_{0.05.}$

The FBS, HbA1c and mnPCE were increased in 3MDM by 4.0, 2.0 and 2.0 folds, and in 5MDM by 6.0, 3.0 and 3.0 folds, respectively. Activities of ALT and AST were increased with concomitant increase in urea, creatinine and total bilirubin in 3 and 5MDM. Total ACP decreased in 3MDM and 5MDM by 32.4% and 72.2%, respectively, while prostatic ACP decreased by 47.6% in 5MDM. Prostatic Zinc and bicarbonate increased in 3MDM by 1.7 and 1.9 folds, and in 5MDM by 5.8 and 1.7 folds, respectively. In 3MDM and 5MDM, levels of LH, FSH, testosterone and activities of antioxidant enzymes decreased, while LPO level increased relative to control. In 3MDM and 5MDM, mild expression of Bcl-2 and Ki67 were observed. In BPH+DM, mnPCE increased relative to BPH control. Treatment with KV and MET

decreased mnPCE relative to BPH+DM. Serum urea, total bilirubin and total ACP increased in BPH+DM by 252.1%, 48.7% and 57.2%, respectively, while total bilirubin and total ACP decreased by 69.3% and 54.4%, respectively in BPH+DM+MET. In BPH+DM, serum ALT, AST and prostatic ALP increased, relative to BPH control, while treatment with KV and MET decreased these biochemical parameters. Histopathology of prostate revealed hyperplasia and intra-luminal budding of epithelia in BPH+DM which were attenuated by KV.

The biochemical and structural changes observed in the prostate of diabetes mellitus rats and in comorbidity with benign prostate hyperplasia were attenuated by kolaviron.

Keywords: Benign prostatic hyperplasia, Diabetes mellitus, Kolaviron.

Word count: 500

CHAPTER ONE

INTRODUCTION

Diabetes mellitus (DM), which is an indication of hyperglycemia, can be described as a long-standing metabolic and endocrine derangement that is linked with deficiencies in either insulin secretion or sensitivity or both. Oxidation of mitochondrial glucose in hyperglycemic condition (a feature of DM) enhances oxidative stress (Chang and Chuang, 2010) which causes micro- and macro-vascular complications (Giacco and Brownlee, 2010). Infertility, which is one of the complications of DM, affects 35% of people suffering from DM and reduced sperm quality is known to have positive correlation with sterility or infertility. On a regular basis, there exists an association between oxidative stress and spermatozoa dysfunction in DM (Gomez *et al.,* 1996). Also, Alves *et al.,* (2013) described disorders in endocrine control of spermatogenesis in DM. For instance, Infertility in male diabetic subject is associated with aberrations in testosterone synthesis as a result of structural and molecular variations observed in the Leydig cells (Dinulovic and Radonjic, 1990). Similarly, endocrine disorders originating from DM also affect pituitary-gonadal system. (Bhasin *et al*., 2007). Decrease in the volume of secretions from prostate gland during prostate gland pathology may contribute to male diabetic infertility (Weidner *et al.,* 1999). Also, increase in the viscosity of semen may adversely affect the sperm motility and it was suggested that people suffering from infertility with prostate gland pathology may have increased semen viscosity. Bansal *et al.,* (2013) described an antithetical correlation between diabetes and prostate pathologies, owing to modifications in the levels of sex hormone and metabolic anomalies observed in diabetes sufferer (Bonovas *et al.,* 2004). In addition, reduction in insulin ooze or its sensitivity during prostate gland pathology, may result in decline in the volume of secretions from prostate gland (Marconi *et al.,* 2009). Thus, all these causative factors contribute to the functional integrity of prostate gland, which ranges from actions of the enzymes to the motility of spermatozoa. In the meantime, BPH depicts a benignant growth and unrestrained proliferation of the prostate gland. This ailment (BPH) is the fourth utmost predominant syndrome occurring within men population that advance in age 50 and above. Amidst the male population worldwide, Diabetes mellitus and Benign Prostate Hyperplasia are dual fundamental ailments, which are extensively predominant with increasing age. Amongst other pathologies, Diabetes mellitus and BPH are the 5th utmost prevailing disorders. Although, reports from our research laboratory revealed an antithetical relationship between diabetes and prostate gland size however, derangements in DM has been implicated in the enlargement of the prostate i.e. there is correlation between diabetes mellitus and

BPH (Zongwei and Aria, 2011). One of the risk factors in the development of BPH is hyperinsulinemia linked with reduced sensitivity of insulin. It was also reported by Hammarsten *et al.,* (1998) that BPH may be characterized by the same metabolic aberrations observed in defective glucose homeostasis.

Insulin and other oral hypoglycemic compounds, which are orthodox drugs such as sulfonamides related drugs and metformin, obstruct glucose synthesis in the liver and promote uptake of peripheral glucose (Kahn *et al.,* 2005), decrease the possibility of diabetic patients being inflicted with cancer (DeCensi *et al.,* 2010) and possess antineoplastic activity towards various tumors as well as the prostate (Colquhoun *et al.,* 2012). The anti-adrenoceptor (tamsulosin, alfuzosin, doxazosin) and agents that suppress 5 alpha- reductases activity (finasteride and dutasteride) are utilized in treating BPH. Nevertheless, a number of adverse effects emanate from these drugs (Kalu *et al.,* 2016) and quest for unconventional treatments or managements of this disease are on the increase. Hence, chemopreventive agents originating from plant origin could serve as medicinal intervention in treating DM and BPH comorbidity.

A dicotyledonous plant, called *Garcinia kola* is commonly propagated all over Central and West Africa and the germs from *Garcinia kola* possess bitter astringent taste; thus, it is usually termed "bitter kola" in Nigeria. It is meant for specific occasions among the Ibo tribes while it serves major function in Yoruba people's life and beliefs (Denen *et al.,* 2015). Kolaviron, main biologically active flavonoid (bioflavonoid) compound isolated from *Garcinia kola* (GK) germs (Farombi *et al.,* 2009), demonstrates numerous therapeutic activities such as anti-hepatotoxic, anti-inflammatory, anti-diabetic, and antioxidant properties (Abarikwu, 2014). Moreover, kolaviron improves fertility in male rats with DM. However, the impact of DM and BPH on the structural and functional integrity of prostate as well as the capability of KV to improve comorbidity-induced prostate impairment is unknown.

Rationale and Objectives of the study

Diabetes mellitus (DM) is associated with complications such as nephropathy and infertility. Male infertility caused by DM has been linked to poor semen quality. The role of prostatic fluid in the maintenance of semen quality is crucial. Moreover, antidiabetic effect of kolaviron on the functional and histological changes in kidney of DM rats has been described as well as improving fertility in male rats with DM. Therefore, this study was designed to evaluate biochemical and structural modifications in prostate of DM and BPH rats, and protective role of KV.

The specific objectives of this study were therefore:

a) to examine biochemical, histological and hormonal modifications in prostate of rats after lasting streptozotocin-induced DM on:

- Prostate weight and antioxidant status of DM rats
- Fasting blood glucose, glycated haemoglobin and hormonal profile in DM rats
- Acid and alkaline phosphatase activities in DM rats
- Some trace elements in DM rats.
- Apoptotic and Proliferation markers in DM rats
- Histological examination of the prostate of DM rats.

b) to evaluate the protective role of kolaviron on the prostate of Diabetes mellitus and Benign prostate hyperplasia rats (Comorbidity) on:

- Weight of selected tissues in BPH +DM rats.
- Lactate dehydrogenase, acid and alkaline phosphatase activities in BPH +DM rats.
- Liver and kidney function test parameters in BPH +DM rats.
- Frequency of micronucleated polychromatic erythrocytes in BPH +DM rats
- Histological examination of selected tissues of BPH +DM rats.

CHAPTER TWO

LITERATURE REVIEW

2.1 DIABETES MELLITUS

Diabetes mellitus is described as a set of syndrome in metabolism in which an individual has an elevated blood glucose, either due to deficiencies in insulin ooze or sensitivity or both (American Diabetes association, 2009). People with high blood sugar usually suffer from common urination (polyuria), excessive thirst (polydipsia) and hunger (polyphagia). Diabetes, upon a lengthy duration, is an ailment that instigate high blood sugar levels. According to Williams textbook of endocrinology, 382 million people and above were projected in 2013 to have diabetes all over the world.

There are three major types of diabetes and they have something in common. According to WHO, (1999), they are:

2.1.1 Type 1 Diabetes

Diabetes mellitus (Type 1) originates from pancreatic islet beta cells damage which is facilitated by cellular autoimmune response that leads to loss of insulin production. Approximately 10% of all cases of diabetes are type 1. It was formerly alluded to as "juvenile diabetes" or "insulin-dependent diabetes mellitus" (IDDM). Its occurrence is among every age but commonly starts off in infancy and the cause is unknown.

2.1.2 Type 2 Diabetes

Diabetes mellitus (Type 2) is the most widespread diabetes that affect beyond 90% of the people suffering from diabetes globally. It is characterized by high blood glucose and insulin resistance where the body's cells does not properly respond to insulin. Low insulin sensitivity is also known as insulin resistance. It was formerly alluded to as "adult-onset diabetes" or "Non insulin-dependent diabetes mellitus" (NIDDM).

2.1.3 Gestational Diabetes

This is pregnancy related diabetes among females. This arises from high blood glucose level in women (pregnant) that have no prior record of diabetes.

2.1.4 Symptoms of diabetes mellitus

- Polydipsia
- Polyuria
- Polyphagia
- Near-sightedness
- Fatigue
- Trembling
- Dizziness
- Numbness in the feet, legs or hands (Riaz, 2009).

2.1.5 Diabetes mellitus occurrence

The global occurrence of DM heightens (Wild *et al*., 2004). Diabetes mellitus was predicted to increase to 366 million in 2030, but, the prevalence in Nigeria is between 27% (Oyegbade *et al*., 2007). Urbanization with the adoption of western lifestyles has been blamed for the increasing prevalence. The prevalence of DM in Africa relatively on the increase among young to middle-aged people while the elderly are the majority affected in the West (Akpan and Ekaidem, 2015). In developing countries, it largely affects those between 35-64years (WHO, 2004). This may have lasting unpleasant consequence on the health and economy of nations (Akpan and Ekaidem, 2015). The problem of this increase is on the countries with low and middle-income and Africa will considerably have a share in the upsurge (WHO, 2013).

2.1.6 The Complications of Diabetes Mellitus (DM)

Diabetes mellitus causes acute and chronic complications (Varim *et al*., 2014) and thus, it is connected with long-standing impairment and malfunction of several structures and organs of the

body mainly the blood vessels, nerves, eyes, kidney and heart (Gavin *et al*., 2003). Chronic hyperglycaemia is known to damage almost all cell types in the body. The deleterious consequence of hyperglycaemia are divided into microvascular (neuropathy, retinopathy, nephropathy), macrovascular (hyperlipidemia, hypertension, peripheral and cerebral vascular disease) and cancers. (WHO, 2013).

Cardiovascular disease: This is the foremost ill health leading to death in both prediabetes and type 2 DM patient. It causes oxidative stress which has significant role on the formation of abnormal fatty deposit in an artery (atherogenesis) thus, contributing to oxidation of low-density lipoprotein (LDL) (Chaturvedi, 2007).

Diabetic neuropathy: This may be linked with non-curative skin injury, foot ulcers and sexual impairment (Sanghera and Blackett, 2012). Numbness in the feet, ulceration, gangrene and other injury leading to skin infection (Vigersky, 2011) are consequences of neuropathy. Oxidative stress in cavernous tissues may cause sexual dysfunction in diabetic patients.

Diabetic nephropathy: This resultant effect from microvascular complication, is the presence of minute amounts of urinary protein (microalbumin) that are only discovered by specific testing analysis. The progression of nephropathy can be stopped, if this is identified earlier (Vigersky, 2011).

Diabetic retinopathy: Microvascular damage, which results from chronic hyperglycaemia, affects the retinal vessels, causing oedema and/or haemorrhage in the retina or the vitreous humor because of vascular permeability (Fong *et al*., 2004).

Cancers: The risk of cancer may be increased as a result of diabetes mellitus. Examples of such cancers are kidney cancer, colorectal cancer, breast cancer, liver cancer and bladder cancer. This cancer differs based on specific cancers sites.

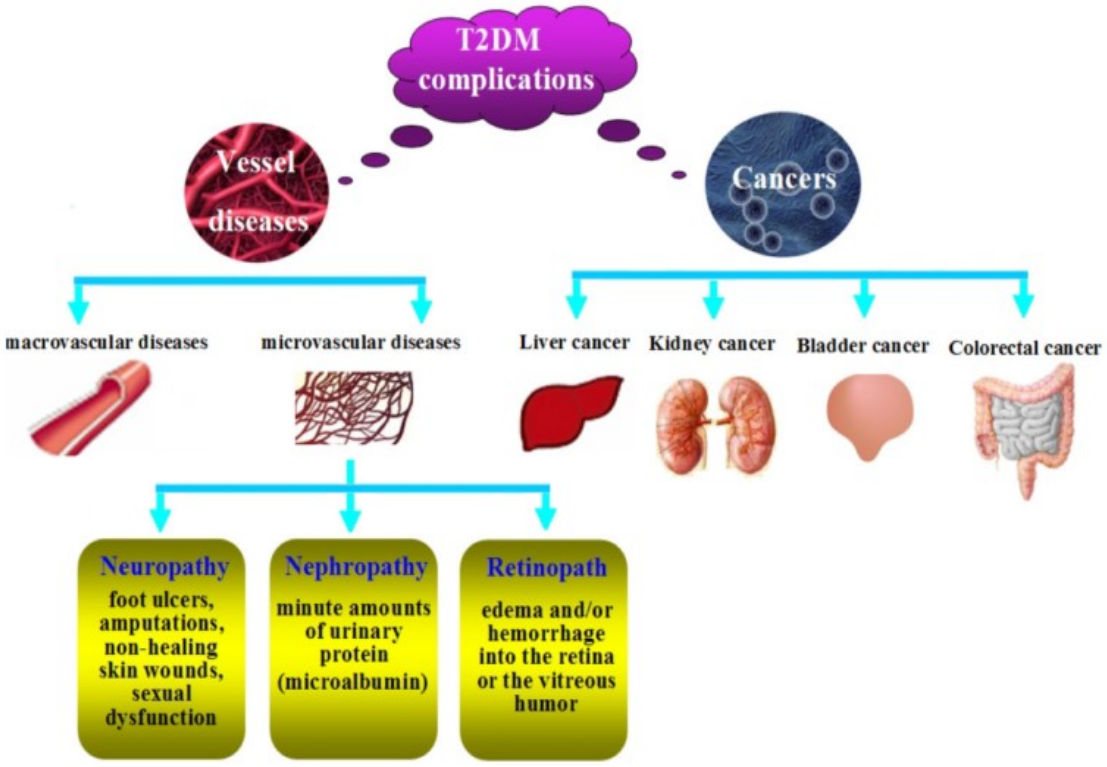


Figure 1.0: DM complications (Wu *et al.*, 2014).

2.2 Prostate gland

This is the largest virile accessory genital gland. The gland (prostate) is about the size of a walnut weighing about 20 g. The gland (prostate) is positioned at the base of the bladder and encloses a portion (proximal) of the urethra. The epithelial and stromal cells constitute the prostate gland. The secretory function is performed by epithelial cells however stromal cells make up the prostate connective tissue cells. Prostate gland secretes a milky, alkaline secretion, which is about 14–30% constituents of semen. Sperm comprises fibrinolysin and acid phosphatase, which help in promoting maximum motility of the sperm cells. At the prostatic urethra (i.e. the posterior surface of the prostate), both ejaculatory tubes open within the urethra.

2.2.1 Prostate secretions

The volume of semen from normal human being varies between 1.5 to 5 ml. The smallest quantity from the whole volume of semen represent mature spermatozoa. Secretions from the accessory of sex tissues such as epididymis, seminal vesicles and prostate make up the highest constituents of semen volume. Close to 30% of the semen volume are emissions from the prostate gland (Owen and Katz, 2005).

The vas deferens and prostate capsule contract at the same time to secrete a homogeneous, serous fluid that is a little acidic (pH 6.8) during release. Elevated quantity of zinc, phosphate, citric acid, calcium and other secretions from prostate which are vital for sperm function are constituents of the fluid.

The prostate gland has the maximum zinc level in the human body (Caldamone *et al.,* 1979). The level of zinc heightens in men at adolescence and attains the highest between ages 34 to 40 (Bedwal and Bahuguna, 1994). The stability of sperm cell chromatin in human is dependent on zinc during emission (Bjorndahl and Kvist, 2011). Thus, the absence of zinc alters the stabilization of sperm cell chromatin which exposes DNA to factors that will cause its disintegration. Zinc, as an antimicrobial agent, contribute in male propagation (Fair *et al.*, 1976). The growth of spermatozoa is related to angiotensin converting enzyme (ACE), hence, ACE damage reduction in testosterone level as well as spermatogenesis are consequences of loss in zinc (Bedwal and Bahuguna, 1994).

The epithelial cells of the prostate secrete Citric acid. Its concentration is high in prostate secretions (Kavanagh, 1994). Essential roles played by citric acid are maintenance of seminal pH, prevention of blood coagulation and prostate function.

Prostate gland secretes prostate specific antigen (PSA), which is part of human kallikrein gene family, at high concentrations into the seminal fluid. PSA enhances the motility of sperm cells by liquefying the semen. Several prostate diseases namely inflammation, benignant prostatic hyperplasia and malignant prostate increases PSA.

Enzymes, sodium, potassium, and calcium are also constituents of prostatic fluid (Zaneveld and Tauber, 1981). Lysis of the semen coagulum by profibrinolysin in the prostate increases the motility of spermatozoa (Huggins and Neal, 1942), thus, this enhances insemination and pregnancy by allowing sperm cells to move on within the female reproductive system.

5α-reductase, a type 2 enzyme, catalyses the transformation of unbound testosterone to dihydrotestosterone. Dihydrotestosterone, a strong male sex hormone (androgen), is 2–10 times active beyond testosterone. The growth of external genitals of male is associated with dihydrotestosterone.

2.3 STREPTOZOTOCIN

Intracellular actions of streptozotocin (STZ) brings about DNA fragmentation in pancreatic β-cells (Morgan *et al.,* 1994). Researchers have proposed that β-cell death via STZ induction is caused by DNA alkylation (Elsner *et al.,* 2000). The position O^6 of guanine in nitrosourea is associated to the STZ alkylation activity. Nitric oxide (NO), donated by STZ, participate in pancreatic β-cell damage (Kroncke *et al.,* 1995). Many experiments established that NO contribute to toxic effect of STZ to the cells (Kroncke *et al.,* 1995). Apart from NO, reactive oxygen species (ROS) can as well be generated from STZ. These elicit cytotoxic effects and also participate in fragmentation of DNA (Bedoya *et al.,* 1996). Consumption of oxygen by mitochondria is significantly reduced by STZ (Nukatsuka *et al.,* 1990). Conversely, fragmentation of DNA and damage as a result of STZ intoxication could be from the mutual activity of both NO and ROS. Hence, destruction of pancreatic β-cell can be prevented by deterring the production of intracellular NO or ROS.

2.4 Diabetes Mellitus and Oxidative stress

Complications from DM could be as a result of oxidative stress (Cade, 2008). Oxidative stress is as a consequence of excessive production and inadequate ejection of reactive nitrogen species (RNS) and reactive oxygen species (ROS). Mitochondrial superoxide is overproduced in diabetes mellitus (Cade, 2008) and this is the principal facilitator of diabetes mellitus complication which triggers the 5 pathways which are responsible for the causes and development of complications and inactivity of 2 antiatherosclerotic enzymes i.e. endothelial nitric oxide synthase (eNOS) and prostacyclin synthase. Presently, oxidative stress is proposed as the causal means in diabetes and its complications. Oxidative stress is a consequence of disparity between the production and scavenging mechanisms of radicals i.e. free radical buildup or reduced action of antioxidant defenses or both (Ahmed *et al*., 2014). It was discovered that oxidative stress is elevated in STZ-induced diabetic rats (Szkudelski, 2001). Diabetes mellitus interferes in the metabolism of glutathione, triggers production of lipid peroxides and derangements in antioxidant enzymes (McLennan *et al*., 1991).

2.5 Mechanisms Underlying the Association between Hyperglycaemia and Oxidative Stress

Various mechanisms have been discovered which elucidate the way hyperglycaemia triggers vascular complications. Hyperglycaemia triggers numerous pathways which are mutually beneficial to each other. Activation of these pathways is suggested to be consequence of overproduction of ROS triggered by hyperglycemia in mitochondria.

2.5.1 Aldose Reductase (Sorbitol/ Polyol) Pathway

In the cells, a reduction process of noxious aldehydes to inert alcohols is catalysed by Aldose reductase; however, it is reduced to sorbitol in the presence of excess glucose. Oxidation of sorbitol catalysed by sorbitol dehydrogenase to fructose afterwards, sorbitol is the polyol. Simultaneously, the process of oxidizing NADPH to NADP+ and reducing NAD+ to NADH (co-factors) heightens and lessens respectively; the bioavailability of NO (Dagher *et al*., 2004) results into oxidation-reduction disparity which looks like hypoxia in tissue and it is known as hyperglycaemic pseudohypoxia. The production of methylglyoxal and AGEs, also increases. Oxidative stress is triggered by all these processes (Gabbay, 1975). Reduced glutathione (an intracellular antioxidant) is restored via NADPH (an essential cofactor). Consummation of the cofactor NADPH decreases the quantity of reduced glutathione, and consequently, the exposure to oxidative stress within the

cell heightens via the polyol pathway. As sorbitol builds up, the osmotic stress elevates as well as other osmolytes (i.e. taurine and myo-inositol).

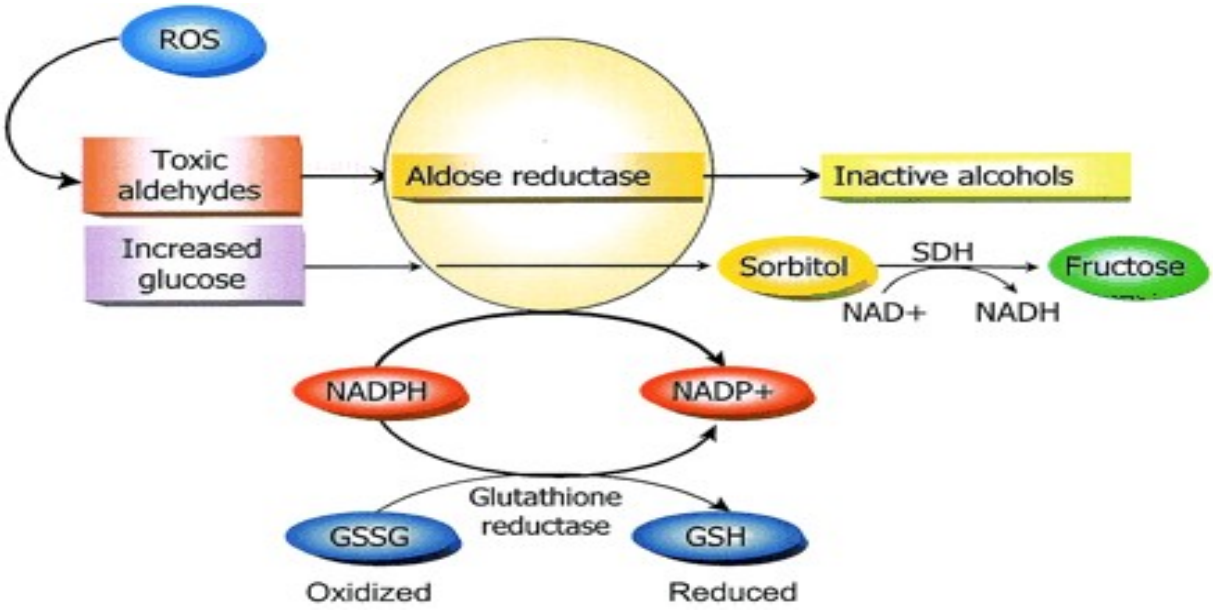


Figure 1.1: The polyol pathway (Brownlee, 2001).

ROS – Reactive Oxygen Species

SDH – Sorbitol Dehydrogenase

NADPH/NADP+ - Nicotinamide Adenine Dinucleotide Phosphate (Reduced / Oxidized)

GSSG – Oxidized Glutathione

GSH – Reduced Glutathione

NADH /NAD+ - Nicotinamide Adenine Dinucleotide (Reduced / Oxidized)

2.5.2 Non-Enzymatic Glycation End Products (AGE)

In DM and kidney damage, complex and heterogeneous compounds known as non-enzymatic glycation products buildup in the plasma and tissues. It was suggested that AGE is linked with causes and development of diabetes related chronic complications (Singh *et al.,* 2014). It was shown from earlier studies that the buildup of AGE modified proteins is linked to the gravity of microvascular complications in both diabetic humans and animals (Vlassara *et al.,* 1994).

It was suggested, as regards AGE, that the cross linking and chemical changes in lipids, proteins and DNA of tissues leads to stepwise degeneration of roles of tissues and pathogenesis of diabetes related complications as a result of disruption in their structure, function and turnover. The carbonyl group of free glucose condenses in a reaction with N-terminus of amino groups of reactive amino acids of protein such as lysine or arginine to form non-enzymatic glycation end products of proteins. This reaction produces Schiff-base intermediates which later form stable protein glucose adducts (i.e. glycated haemoglobin (HbA1c) and fructosamine (fructoselysine) after undergoing Amadori reshuffle. The polyol pathway fragmented products, decomposition of Amadori products and reaction of glycoxidative products with amino groups of protein could result in the formation of AGEs. Autooxidation of glucose generates reactive carbonyl compounds such as methylglyoxal-3-deoxyglucosone and glyoxal, which later results in glycoxidation products when these compounds react with protein. Reactive oxygen species (ROS) can as well be generated from AGEs. AGEs can decrease arterial elasticity and endothelial permeability increases via damage caused by AGEs when heparin sulphate binds to extracellular matrix and there is impaired anionic sites. AGEs are very reactive in blood circulation and several enzymes frequently render it harmless. AGE peptides are redistributed, if not removed by the kidneys, to form new AGEs which react with plasma or tissue components; thus, cell functions are impaired as a result of increased damage caused by glycation process via three mechanisms. Changes in intracellular proteins that control the transcription of gene is the first mechanism. Secondly, the diffusion of precursors of AGE outside

the cell to modify the neighboring extracellular matrix molecules, leads to cell impairment as a result of modifications in the cell/matrix signaling (Charonis *et al*., 1990). Thirdly, altered blood circulating proteins (albumin) bind and trigger the receptors of AGE which later generate inflammatory growth factors and cytokines leading to vascular pathology (Li *et al*., 1996).

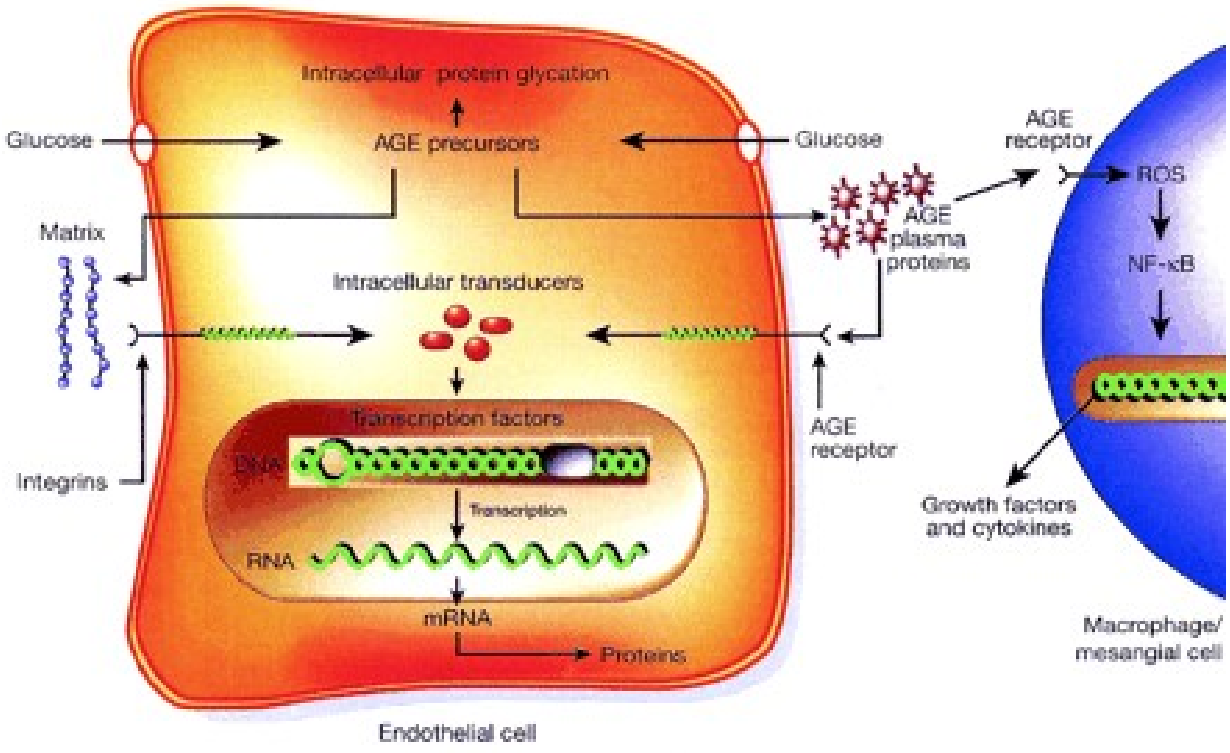


Figure 1.2: Generation of precursors of Advanced Glycation End product (Brownlee, 2001).

2.5.3 The DAG/PKC Pathway

Hyperglycaemia triggers diacylglycerol - protein kinase C (DAG - PKC) pathway which can result in vascular dysfunction. In hyperglycaemic condition, the levels of DAG are increased due to increased glycolytic intermediates dihydroxyacetone phosphate (DHAP) and glycerylaldehyde-3-phosphate, which conjugated with fatty acids and thus, de novo synthesis of DAG is elevated (Craven *et al.,* 1990). This pathway can be triggered by oxidants (e.g. H_2O_2). Induction of vascular endothelial growth factor (VEGF) alters the vascular permeability in smooth muscle cells as a result of PKC activation and this is a pathological effect (Williams *et al.,* 1997). Another effect is derangements in blood flow which reduce vasodilator that generate endothelial nitric oxide (NO) synthase, while vasoconstrictor endothelin-1 is increased. Basement membrane thickening through endothelial nitric oxide (NO) synthase facilitated by Transforming growth factor-β elevates generation of type IV collagen and fibronectin (Pugliese *et al.,* 1994). Also, it elevated PAI1 expression which lead to loss of fibrinolysis (Feener *et al.,* 1996) and triggering of superoxide generating enzymes (i.e. NADPH) with elevation of expression of abnormal uncoupled endothelial NOS, thereby causing oxidative stress increase. Transforming growth factor-β and plasminogen activator inhibitor-1, are also increased (Feener *et al.*, 1996).

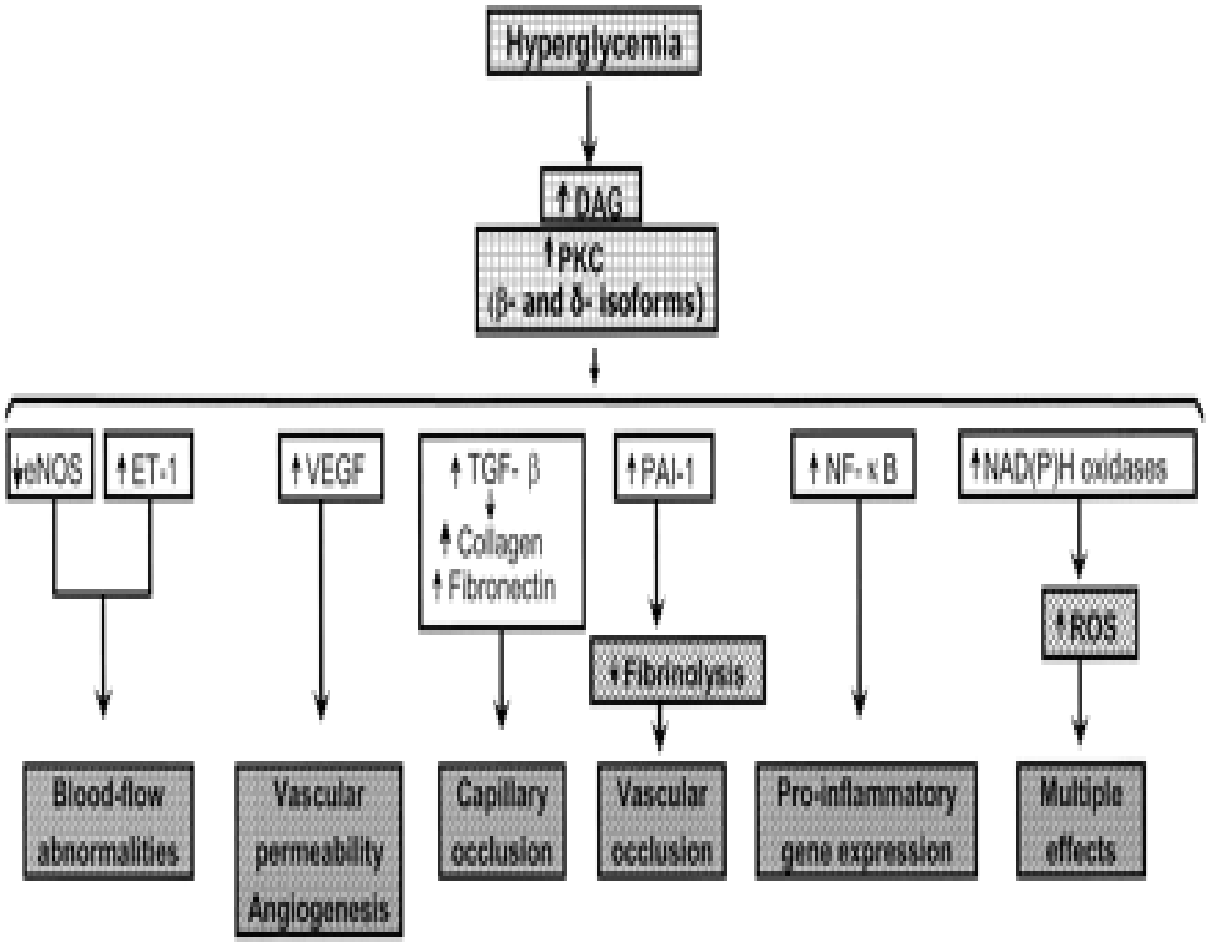


Figure 1.3: Protein Kinase C pathway activity (Brownlee, 2001).

DAG - Diacylglycerol

PKC - Protein kinase C

eNOS - Endothelial nitric oxide synthase

ET-1 - Endothelin-1

VEGF - Vascular endothelial growth factor

TGF- β - Transforming growth factor-β

PAI-1 - Plasminogen activator inhibitor-1

NF-κB - Nuclear factor κB

2.5.4 Hexosamine pathway activity

Diverting Fructose-6-phosphate (glycolysis) to a signalling route where glutamine: fructose-6 phosphate amidotransferase (enzyme) designated as GFAT catalyses the conversion of fructose-6 phosphate to glucosamine-6 phosphate and lastly to UDP (uridine diphosphate) *N*-Acetyl glucosamine. In hyperglycaemic condition, rise in fructose-6 phosphate surge into hexosamine pathway participates in the pathogenesis of diabetes related problems. Elevation in the transforming growth factor-β1 and plasminogen activator inhibitor-1 manifestation, which are equally injurious to blood vessels , are consequences of increase in altered transcription factor Sp1 triggered by hyperglycemia (Du *et al.,* 2000). This participates in the causes and development of diabetes related complications. Alterations in proteins of the endothelial cell caused by hexosamine route are markedly increased in carotid artery plaques of diabetic patients (type 2) (Federici *et al.,* 2002).

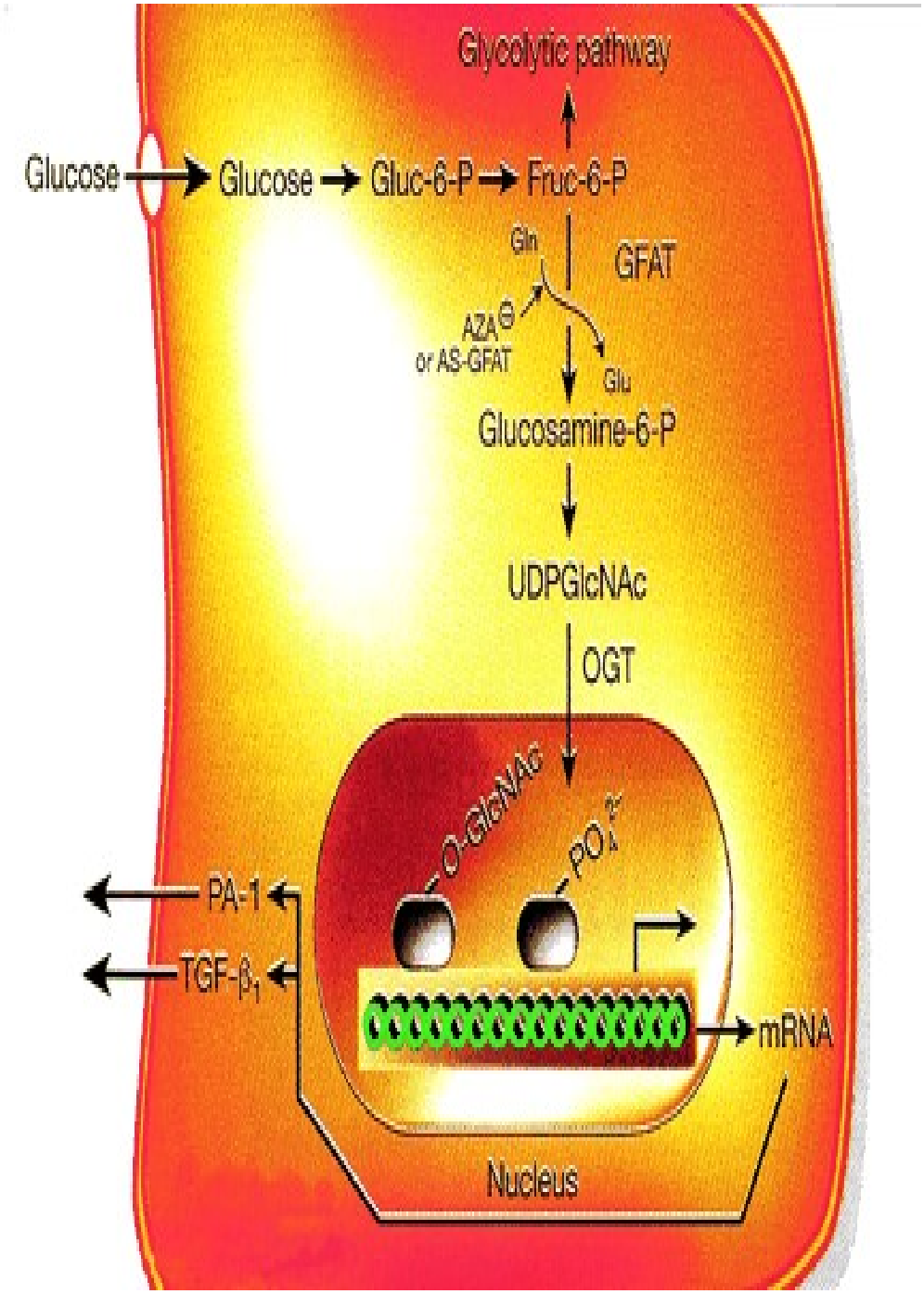


Figure 1.4: Hexosamine pathway. (Brownlee, 2001).

TGF- β - Transforming growth factor-β

PAI-1 - Plasminogen activator inhibitor-1

GFAT - Glutamine: fructose-6 phosphate amidotransferase

UDPGlcNAc - Uridine diphosphate *N*-Acetyl glucosamine

2.5.5 Mitochondrial superoxide anion overproduction: A single hyperglycemia-induced pathogenic process

Superoxide, generated from mitochondria, is transformed to another species (more reactive) that are injurious, in several ways, to cells (Wallace, 1992). In normal cells, Complexes I, III and IV transfer electron by forcing protons out into the intermembrane space, which produce a proton gradient that propels Complex V (ATP synthase) as protons get back into matrix via the inner membrane. However, in cells that are diabetic, oxidation of more pyruvate in the TCA cycle, which elevates the NADH and $FADH_2$ (electron donors) surge into the electron transport chain, thereby increasing the voltage gradient that leads to a critical threshold across the membrane of mitochondria and thus, blocks the transfer of electron inside complex III (Trumpower, 1990). This leads the electrons going backward to coenzyme Q, and gives electrons to molecular oxygen at once which generate superoxide anion.

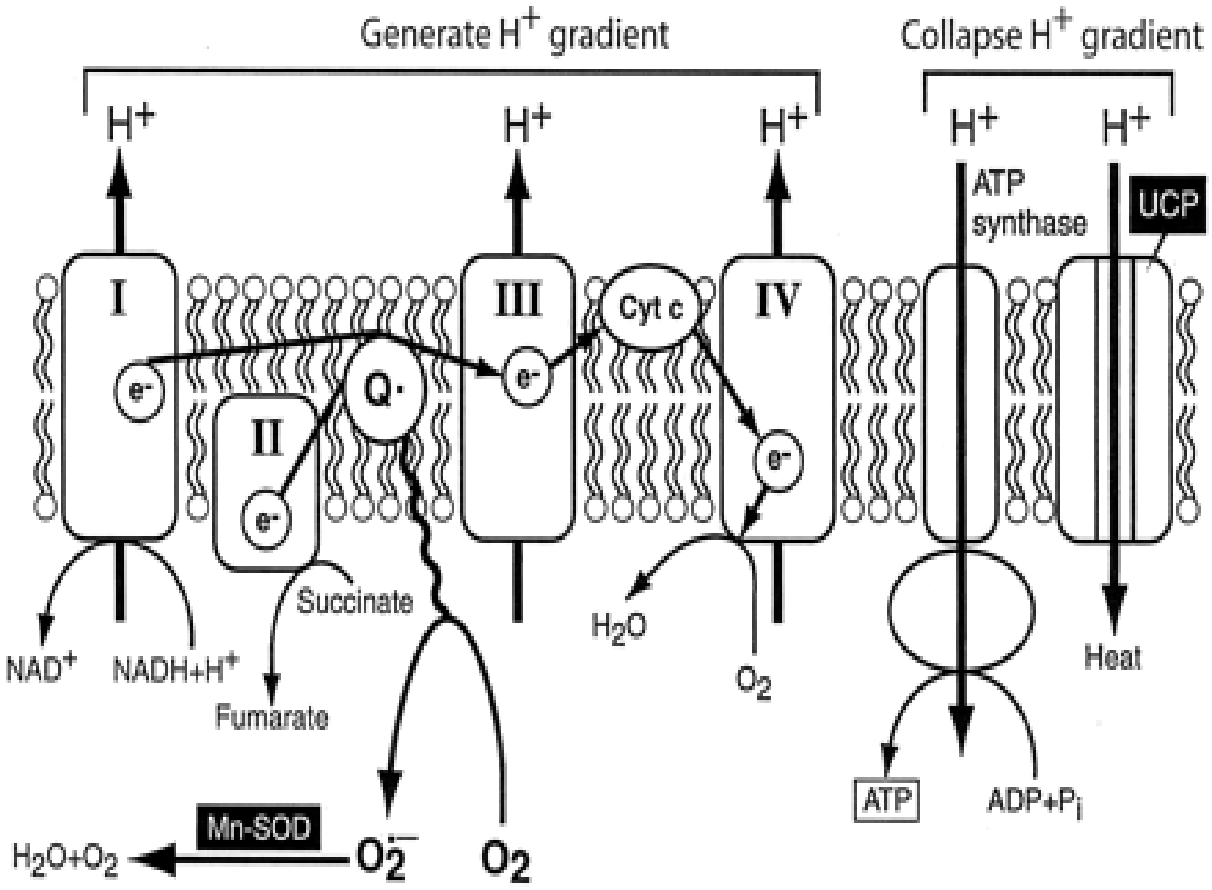


Figure 1.5: Generation of superoxide via electron transport chain in mitochondria. (Brownlee, 2001).

2.5.6 Generation of superoxide anion in mitochondria triggers deleterious pathways by inhibiting GAPDH

In hyperglycemic cells, there is reduction in glyceraldehyde 3-phosphate dehydrogenase action (GAPDH; basic glycolytic enzyme). Activity of GAPDH is not inhibited in hyperglycemia when excessive superoxide generation in mitochondria is hindered by either MnSOD or UCP-1. The level of intermediates of glucose that are upstream in glycolytic pathway increase whenever activity of GAPDH is inhibited, thereby leading to elevation of the flux into damaging pathways explained above. Increase in the levels of upstream glyceraldehyde 3-phosphate triggers two main pathways. Firstly, methylglyoxal (precursor of AGE) is produced in a non-enzymatic process from glyceraldehyde 3-phosphate and thus, triggers the AGE pathway. Recently, formation of methylglyoxal has been revealed to elevate RAGE expression (Mohammad *et al.,* 2017).

PKC pathway is also triggered by increase in the level of glyceraldehyde 3-phosphate, since diacylglycerol (a physiologic PKC activator), is produced from glyceraldehyde 3-phosphate. This promotes increased fructose 6-phosphate concentration and in turn heightens the flux via the hexosamine pathway, where GFAT enzyme catalyses the conversion of fructose 6-phosphate to UDP–*N*-Acetylglucosamine (UDP-GlcNAc). Lastly, glucose level is increased via inhibition of GAPDH and thus, the flux via the polyol pathway increases where NADPH is consumed as aldose reductase reduces glyceraldehyde 3-phosphate content (Du *et al.,* 2003).

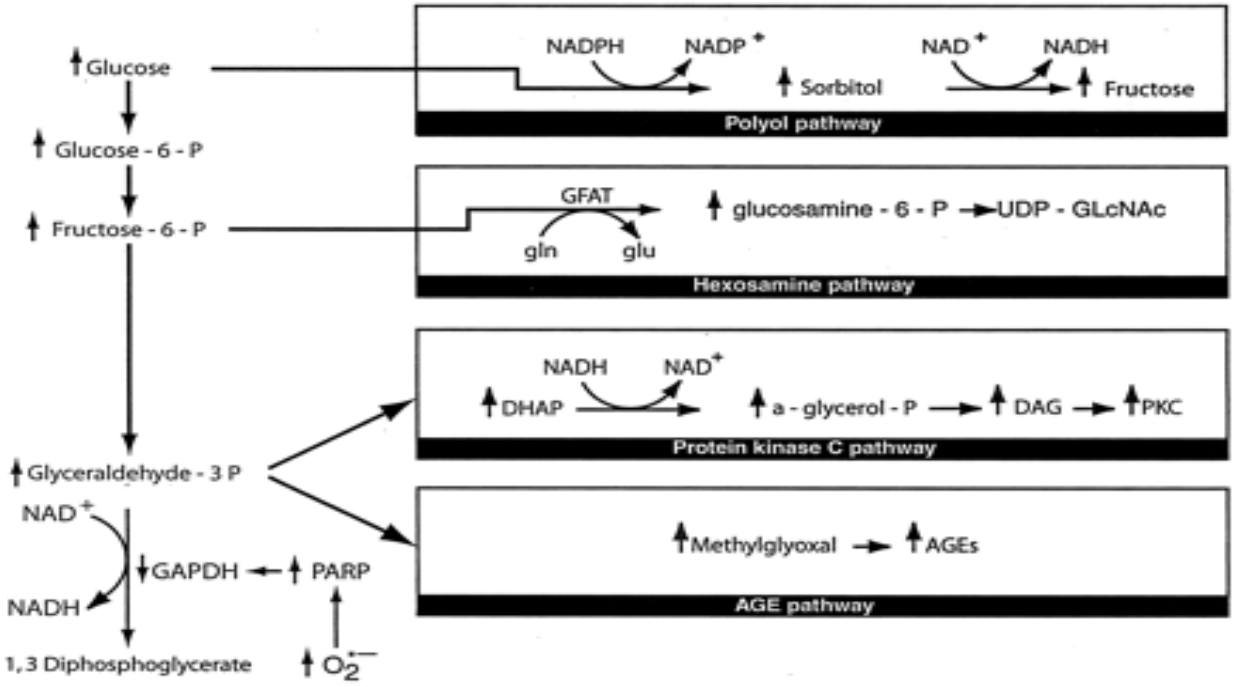


Figure 1.6: Activation of damaging pathways via Glyceraldehyde-3-Phosphate Dehydrogenase inhibition (Brownlee, 2001).

PARP – Poly (ADP-Ribose) Polymerase

GAPDH - Glyceraldehyde-3-Phosphate Dehydrogenase

GFAT - Glutamine: fructose-6 phosphate amidotransferase

UDPGlcNAc - Uridine diphosphate *N*-Acetyl glucosamine

NADPH/NADP+ - Nicotinamide Adenine Dinucleotide Phosphate (Reduced / Oxidized)

NADH /NAD+ - Nicotinamide Adenine Dinucleotide (Reduced / Oxidized)

DAG – Diacylglycerol

PKC - Protein kinase C

AGEs – Advanced Glycation End products

2.5.7 Activation of poly (ADP-ribose) polymerase via inhibition of GAPDH

Normally, PARP (DNA repair enzyme) is sited in an inert form in the nucleus expecting to be activated via DNA impairment. Increased generation of ROS in mitochondria as a result of hyperglycemia induces breakage of DNA strand which activates PARP. Upon PARP activation, the molecule (NAD^+) breaks into ADP-ribose and nicotinic acid. Then, PARP polymerizes ADP-ribose, so as to buildup on GAPDH and other nuclear proteins. In the repair of DNA, GAPDH moves in and out of the nucleus in order to serve a crucial function (Schmidt, 2001). Reduction in GAPDH action and its alteration caused by poly (ADP-ribose) can be avoided by inhibiting the generation of superoxide anion either UCP-1 or MnSOD in mitochondria.

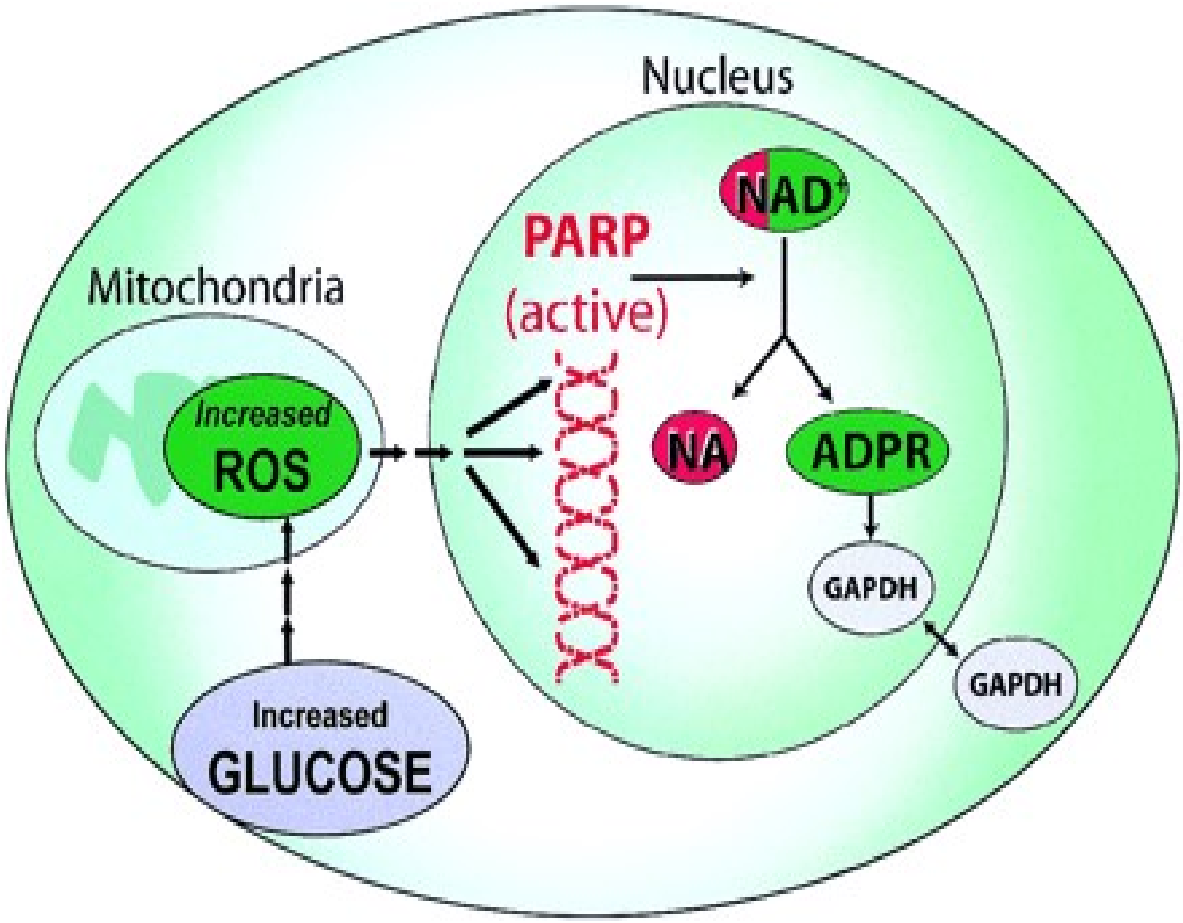


Figure 1.7: Activation of Poly (ADP-Ribose) Polymerase and modification of Glyceraldehyde Phosphate Dehydrogenase via ROS-induced DNA damage. (Brownlee, 2001).

ROS – Reactive Oxygen Species

ADPR – ADP-Ribose

GAPDH - Glyceraldehyde Phosphate Dehydrogenase

NA – Nicotinic Acid

2.5.8 The combined mechanism of cellular damage induced by hyperglycemia

As hyperglycemia grows, in target cells, with diabetes related problems, generation of free radicals (ROS) elevates inside mitochondria which results into breakage of DNA strand in the nucleus leading to activation of PARP. Activated PARP alters GAPDH, thus, decreasing its activity. Lastly, reduction in the activity of GAPDH activity triggers the polyol pathway, elevates the formation of AGE within the cell, triggers PKC pathway and consequently NFκB, and triggers the flux of hexosamine pathway. Protein Kinase C (PKC), in endothelial cells, triggers a transcription factor known as NFκB (nuclear factor κB) which simultaneously triggers several genes, causing inflammation in the vessels of blood.

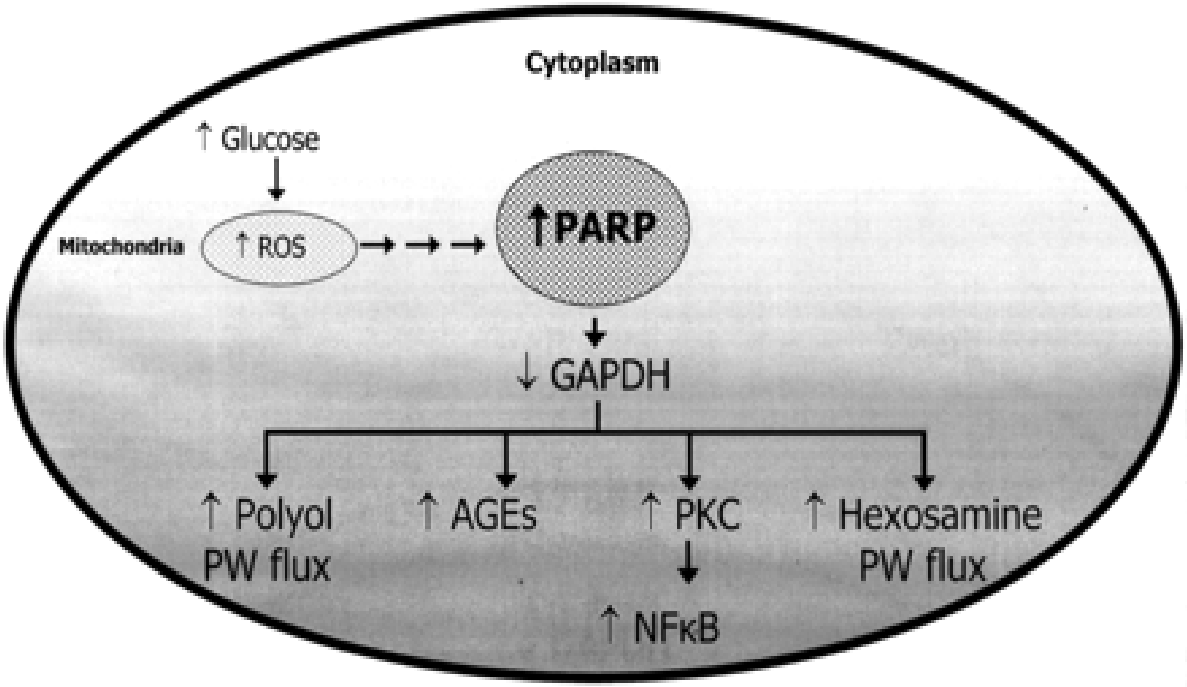


Figure 1.8: The combined mechanism of cellular damage induced by hyperglycemia (Brownlee, 2001).

ROS – Reactive Oxygen Species

GAPDH - Glyceraldehyde Phosphate Dehydrogenase

PARP – Poly (ADP-Ribose) Polymerase

DAG – Diacylglycerol

PKC - Protein kinase C

AGEs – Advanced Glycation End products

PW – Pathway

NF-κB - Nuclear factor κB

2.6 Benign Prostate Hyperplasia

The prostate gland is predisposed to disease, enlargement, and benign or cancerous growth. Generally, enlargement of the prostate begins in male population at around age 40 to 45 years. Benign prostatic hyperplasia (BPH) is a benignant enlargement of the prostate gland that arises from dysregulated propagation of the glandular epithelial cells and stroma (Dhingra and Bhagwat, 2011). It is also regarded as a discrepancy in overexpression of androgen and estrogen of epithelial and stromal growth factors, steroid hormones and cytokines. In addition, BPH is described as hyperplastic development of the epithelia and stroma cells that are existing as many microscopic and macroscopic nodes in the prostate gland (Ho and Habib, 2011). Persistent obstructions may ultimately result in blood in the urine, bladder calculi, periodic urinary tract infection (UTI) and kidney inefficiency. BPH may lead to permanent bladder impairment, sepsis, kidney failure in critical situations and death in severe cases (Roehrborn and McConnell, 2002). Age–linked hormonal changes and inflammation are also implicated in BPH. Androgens (testosterone and dihydrotestosterone (DHT)) are involved in the development, function and maintenance of intact prostate gland as well as promote BPH assault (Lowe *et al.,* 2003). An enzyme called 5α-reductase (5αR) catalyses the conversion of testosterone to DHT (Marks, 2004). The activity of DHT in the prostate, which is a potent androgen, is mediated by binding to the androgen receptor (AR) and thus, triggers the synthesis of protein and aberrant prostate growth (Maclean *et al.,* 1997). Androgens are factors implicated in the development of prostate and pathogenesis of BPH. Furthermore, peptide growth factors namely epithelial growth factor, growth hormone, transforming growth factors, insulin like growth factor and insulin are implicated in the growth of prostate.

2.6.1 Prevalence of BPH

The incidence of BPH is approximately 50-60% in males 40-60 years old and greater than 90% in men over 80 years (Ziada *et al.,* 1999).The most current report on the prevalence of BPH in Nigeria is as high as 25–35% (Ezeanyika *et al.,* 2006). BPH is the 4th most prevalent disease.

2.7 Prostate and Zinc

In normal prostate, Zn is present in peak concentrations and it is essential in male fertility. Mawson and Fischer, in the 1950s, first described prostate, as tissue that encloses more Zn than some other

soft tissues. Furthermore, high level of Zn is secreted by the prostate into the semen, which contribute to the release and motility of sperm cells (Yoshida *et al.,* 2008). Also, Zn has proven in antimicrobial action in prostate (Cho *et al.,* 2002) and its fluid (Fair *et al*., 1976). Remarkably, in contrast to most cells, Zn is isolated into vesicles and organelles and its most important role, is to mediate citrate secretion. In the prostate cytoplasm, Zn constitutes about 35% of the whole content of Zn within the cell. Zinc is reported to be biologically active because it is lightly bound to citrate (a small molecular weight molecule) (Costello and Franklin, 1998). High concentration of mitochondrial Zn inhibit the activity of m-aconitase in normal tissues, which prevent oxidation of citrate in tricarboxylic acid (TCA) cycle, which makes it accessible to be secreted into prostatic fluid (Franklin and Costello, 2007). For high Zn content and secretion to be maintained in the cell, the homeostasis of Zn must be controlled in the prostate.

2.7.1 Prostate diseases and Zinc

Benign prostatic hyperplasia and prostate carcinoma are the main disorders of the prostate that affect aged male population. Several researches have described that the content of Zn in the prostate and its secretions are decreased (Zaichick *et al.,* 1997) in male population having disorders in their prostate. Feng *et al.,* (2008) proposed that prostate carcinoma could be the consequence of low concentration of Zinc but it is not completely implicit. Excess oxidation of citrate and production of ATP from low content of Zn in the prostate heightens mitochondrial oxidative stress that aggravate damage of DNA. Malignancy could be as a result of elevated generation of ATP with probability of impaired DNA. There exists several links between low concentration of Zn in the prostate and disorders of prostate; thus, it has been reported that uncontrolled Zn level can be implicated in malignant transformation of prostate cells (Franklin and Costello, 2007). Induction of apoptosis by Zn also reveals its antitumorigenic effects. Also, induced expression of Bax by Zn, triggers apoptosis in mitochondria by initiating the caspase cascade via cytochrome c release (Feng *et al.,* 2008).

2.8 Diabetes-associated mortality and morbidity

2.8.1 Diabetes mellitus and Benign Prostate Hyperplasia

Several researchers reported that hyperinsulinemia resulting from decreased response of the body's cell to insulin and the constituents of metabolic diseases are stakes for the development of BPH (Dahle *et al.,* 2002). Hammarsten and Hogstedt, (2001) were the first to assess the correlation

between metabolic diseases and BPH and it was established in their research that insulin resistance and secondary hyperinsulinemia are main risk factors that confirm the association of metabolic disease with elevation in the risk of BPH. Increase in the risk of BPH is also related to elevated level of insulin in the serum (Dahle *et al.,* 2002). Increase in the level of insulin could cause prostatic growth via several mechanisms. The functional action of the sympathetic nervous system can be increased as a result of hyperinsulinemia. The sympathetic nervous system is controlled when hyperinsulinemia elevates the entry of glucose to the hypothalamus neurons (Landsberg, 1986). Elevation of the sympathetic activity resulting from hyperinsulinemia could be connected to pathology of BPH. In contrast, doxazosin, (an alpha-blocker drug) used for the treatment of indicative BPH, is established to elevate the sensitivity of insulin and also, reduce the levels of insulin (Shieh *et al.,* 1992). Another pathway is insulin-like growth factor axis (IGF-1), a potent mitogen, which elevates the proliferation of cell and triggers the induction of apoptosis in several tissues including the epithelium and stroma of prostate (Peehl *et al.,* 1996). Torring *et al.,* (1997) reported that the weight of prostate was increased as a result of systemic use of IGF-1 in rats for a short period. Growth hormone controls IGF-1 and IGF-binding protein-3 while insulin controls IGF-1 bioactivity (Ketelslegers *et al.,* 1996). People suffering from acromegaly as well as increased growth hormone and IGF-1 levels, have enlarged prostates despite that they have low levels of testosterone (Colao *et al.,* 1999).

2.8.2 Diabetes mellitus and prostate cancer

From animal studies, the level of testosterone in the serum and the weight of prostate gland can be reduced owing to the induction of diabetes mellitus (Ikeda *et al.,* 2000). On the other hand, such alterations can be managed by insulin (Jackson and Hutson, 1984). The male population suffering from DM are liable to reduced level of testosterone that leads to decreased libido and impotence (Hakim and Goldstein, 1996). Elevated level of testosterone contribute to prostate malignancy (Shaneyfelt *et al.,* 2000), thus, it was postulated that subjects with diabetes record may be linked with reduced risk of prostate cancer because of decreased testosterone levels. This relationship can be attributed to alteration in the level of insulin and testosterone in the body. At the onset, several men with Type 2 diabetes are hyperinsulinemic; however, insulin level may reduce as the syndrome advances (Kasper and Giovannucci, 2006). Low level of insulin may inhibit the growth of both healthy and prostate melanoma since high levels of insulin have been reported to be positively correlated with the development of both cells (Calton *et al.,* 2007). Insulin plays an important role

in the growth of prostate epithelium *in vitro* (Peehl and Stamey, 1986), so as to induce rat prostate cancer cell line growth *in vitro* (Polychronakos *et al.,* 1991). This is correlated with higher occurence and recurring prostate melanoma (Lehrer *et al.,* 2002). Thus, decline in the level of circulating insulin could cause reduction in prostate melanoma risk in people suffering from long-standing diabetes. This corresponds to the findings of some researchers (Hense *et al.,* 2011) that described low stakes of prostate melanoma with long-standing Type 2 diabetes.

2.9 Bcl-2 Protein and Diabetes mellitus

The Bcl-2 protein, one of the Bcl-2 family protein members, controls apoptosis and plays a role in the promotion or suppression of cell death. It was suggested that the relative expression of the different Bcl-2 families of proteins controls the sensitivity of cells to apoptotic stimuli (Reed, 1994). The overexpression of Bcl-2 enhances cell survival by suppressing apoptosis in a number of cells that are subjected to a wide range of apoptosis-inducing stimuli, including nerve growth factor withdrawal, radiation and chemotherapeutic agent. Hasnan *et al.,* (2010) reported that there were low expressions of Bc1-2 in diabetic condition. Sgarbosa *et al.*, (2006) who studied the role of apoptosis in the maintenance of placental homeostasis, also reported that a higher apoptosis index and lower Bc1-2 expression were observed in the placentas of diabetic women. However, some studies have noted an upregulation of Bc1-2 in the microenvironment of hyperglycaemia. Abu-El-Asrar *et al.,* (2004) found that the glial cells of the retinas of diabetics showed an upregulation of Bc1-2 when compared to non - diabetic subjects (Abu-El-Asrar *et al.,* 2004). Galkowska *et al.,* (2003), also demonstrated that there was a high expression of Bc1-2 in the edge of diabetic ulcers compared to normal venous ulcers. However, Hasnan *et al.,* (2010) suggested that the discrepancy of results could be as a result of the immunohistochemistry techniques, sample sizes, types of tissues and antibodies used.

Therefore, prolonged hyperglycaemia induces apoptosis by downregulating the expression of Bc1-2 protein in the endothelial cells of diabetic ulcers. In addition, apoptosis induced by high glucose has been shown in endothelial cells *in vitro* (Ho *et al.,* 2000) and was supported by Sekiguchi *et al.,* (2004) which verified the apoptotic phenomenon in human aortic endothelial cell culture with a high glucose concentration.

2.10 Ki67

Ki67, a non histone nuclear protein, is labile and firmly connected to the cell cycle. It is produced all through the stage of mid G_1 in proliferating cells , rises in concentration throughout S and G_2 stage and reaches to the highest concentration at the M stage of the cell cycle (Gerdes *et al.,* 1984). As the M stage winds up, Ki67 is quickly broken down and unnoticeable in quiescent (G_0 and early G_1) cells (Fitzgibbons *et al.,* 2000). Through the process of repair of DNA, Ki67 is unnoticeable, hence, it is considered as a marker of cell proliferation.

CHAPTER THREE

MATERIALS AND METHODS

3.1 REAGENTS AND CHEMICALS

Streptozotocin was purchased from (Sigma Chemical Co., Saint Louis, MO USA). Citric acid, Epinephrine, Glutathione, hydrogen peroxide, 5, 5-dithio-bis-2-nitrobenzoic acid (DNTB), Hydrochloric acid, Sodium citrate and Sodium hydroxide were purchased from (Sigma Chemical Co., Saint Louis, MO USA). Thiobarbituric acid (TBA) and Trichloroacetic acid (TCA) were obtained from British Drug House (BDH) Chemical Ltd., Poole, UK.

3.2 DIABETES MELLITUS INDUCTION

Diabetes mellitus induction was carried out in male wistar rats by a single administration of injection of 0.1 M streptozotocin (STZ) solution intraperitoneally at 35 mg/kg body weight.

Detailed preparations of the reagents are presented in Appendix 1.

3.3 PREPARATION OF EXTRACTS.

Garcinia kola (GK) seeds were purchased from Ojoo, a local market in Ibadan, Oyo state, Nigeria and authenticated at Department of Botany Herbarium, University of Ibadan (UI-00138/01). It was washed and air-dried. They were later sliced into small pieces and processed to powder. Extract of Kolaviron was isolated from powdered *Garcinia kola* (GK) seeds as stated by Iwu *et al.*, (1990) and revised by Farombi *et al*., (2000). Concisely, powdered GK seeds were distillated with light petroleum ether (b.pt 40-60^{0}C) for 24 hrs in a soxhlet. The parched marc, which its fat has been removed with hexane, was repacked and distillated with acetone. The extract was distilled and watered down twice its volume with water and distillated with ethyl acetate. The segment of ethyl acetate concentrate yielded a solid coloured yellow called Kolaviron

3.4 ANIMALS AND TREATMENT

Animals weighing 150 g – 175 g were purchased from the Animal house of the Department of Physiology, University of Ibadan, Nigeria. The animals were acclimatized for 2 weeks. All animals were maintained under standard laboratory conditions of room temperature (25^{o} C) and 12-hour light/dark cycles throughout the periods of both experiments. Rats were provided with laboratory chow (Ladokun Feeds Nig. Limited, Ibadan, Nigeria) and water *ad libitum*

3.4.1 EXPERIMENTAL DESIGN 1

At the end of acclimatization period, Twenty six adult male wistar rats were assigned to 3 groups:

Group I - Control (rats that received citrate buffer only, n=10)

Group II - 3 Months Diabetes Mellitus (rats that received STZ only, n=8)

Group III - 5 Months Diabetes Mellitus (rats that received STZ only, n=8)

Diabetes mellitus was induced by giving the rats a single intraperitoneal administration of 0.1 M Streptozotocin at 35 mg/kg body weight after an overnight fast. The weight and the blood glucose level of wistar rats prior and after induction of diabetes were determined. Blood samples were obtained from dorsal vein of the tail of conscious rats. Accu-Check Glucometer (Roche) and compatible glucometer strips were used for the determination of blood glucose levels in STZ - induced wistar rats 72 hours after induction of diabetes. Diabetes was allowed to stabilize for 14 days and only rats with glucose level greater than 250 mg/dl were considered diabetic in this study. Evaluation of the animals in each group were done on a fortnight basis for signs of weight loss, weakness, hair loss, cataract, coma and death.

Sample Collection

Half of the animals were sacrificed at the end of the experiments (3 months) by cervical dislocation after an overnight fast (~16 hours) while the remaining half were sacrificed after 5 months. Samples (Blood) were stored into Ethylene diamine tetra acetic acid (EDTA) bottles to determine the percentage of Glycated haemoglobin (HbA1c) and also into non-heparinized tubes. The samples (blood), in non-heparinized tubes, were set aside to erect for few minutes in order to coagulate and centrifuged for 10 minutes at 3000 rpm. The supernatants were decanted into already labelled tubes and kept in storage for biochemical analysis**.**

3.4.2 EXPERIMENTAL DESIGN 2

Before the beginning of the experiment, rats were acclimatized for a period of two weeks. Twenty rats were assigned to four groups of five animals each.

Group I - BPH Control (Benign Prostate Hyperplasia) (castrated rats that received Testosterone Propionate)

Group II - BPH + DM (called Diabetic BPH rats) (gelded rats that received TP and STZ)

Group III - BPH + DM + KoV (Diabetic BPH rats that received kolaviron, 200 mg/kg)

Group IV - BPH + DM + Met (Diabetic BPH rats that received metformin, 200 mg/kg).

Benign Prostate Hyperplasia was induced by daily administration of testosterone propionate (TP) intraperitoneally (3 mg/kg) to the castrated rats for 28 days. After which Diabetes mellitus was induced by a single administration of Streptozotocin intraperitoneally (35 mg/kg).

Kolaviron and Metformin solutions were prepared by dissolving in oil (corn) and water (distilled) respectively and administered orally to the animals daily.

Sample Collection

The rats were fasted overnight (~16 hours) and sacrificed at the end of the experiments (14 days after DM) by cervical dislocation. Samples (Blood) were collected via retro orbital plexus of the rats into non-heparinized tubes which were set aside to erect for few minutes in order to coagulate and centrifuged for 10 minutes at 3000 rpm. The supernatants were decanted into already labelled tubes and kept in storage for biochemical analysis**.**

3.4.3 PREPARATION OF TISSUES FOR BIOCHEMICAL ANALYSIS, IMMUNOHISTOCHEMICAL AND HISTOPATHOLOGICAL OBSERVATION

The tissue (prostate) of the animals collected after sacrifice were rinsed in 1.15% KCl (ice – cold), wiped dry and weighed. Tissue (prostate) were homogenized in 6 volumes of the homogenizing buffer (Phosphate buffer; 1mM, pH 7.4) with homogenizer (Raider Hamburg Germany, RDID0955C model). Centrifugation of the homogenate was done at 10,000 g, for 15 minutes at 4^0C. The post mitochondrial fraction PMF (supernatant) was decanted into already labelled tubes and kept in storage for biochemical estimations. Also, a part of the tissues was homogenized with Tris-EDTA buffer, centrifuged at 27, 000g for 20 minutes at 4^0C. The pellet and the supernatant were stored for DNA fragmentation assay. The tissue (prostate) of the animals were collected after the sacrifice for biochemical analysis. A section of the tissues (prostate) were also obtained and kept in formalin for histological and immunohistochemical assessment.

Detailed preparations of the reagents are presented in Appendix 2.

3.5 ESTIMATION OF PERCENTAGE GLYCATED HAEMOGLOBIN

The glycated haemoglobin in percentage unit (% HbA1c) in whole blood was assayed using the CLOVER A1C® Self test system, an absolutely computerized boronate affinity test.

Principle:

The test cartridge of the system is made up of a cartridge and a reagent pack which has the reagents needed for haemoglobin (Hb)A1C estimation, with a sample collecting area for the collection of specimen (blood). The reagent pack is prefilled with reaction solution and washing solution. The reaction solution comprises agents that break down red blood cell and precisely, bind hemoglobin together with a boronate resin that binds cis-diols of glycated haemoglobin.

Procedure:

The specimen (blood) (4µL) was collected at the sample collecting area of the reagent pack, inserted into the cartridge where the blood was instantly lysed so as to release haemoglobin and the boronate resin that binded the HbA1c. The reagent pack containing the blood sample was inserted in CLOVER A1C® Self Analyzer (in which the cartridge has been placed). Automatically, the cartridge was rotated in order to position the specimen (blood) in the measuring zone. The haemoglobin (total) was photometrically measured by the diffused reflectance of the optical sensor made up of both a PD (Photo Diode) and a LED (Light Emitting Diode).

Then assembled cartridge was spinned and the rinsing solution rinsed non-glycated Hb from the blood specimen, which allowed the photometric estimation of HbA1c to be taken.

The ratio of HbA1c and total Hb was calculated:

$$\% \text{ HbA1c} = A \times \left[\frac{\text{HbA1c}}{\text{Haemoglobin (Total)}} \times 100 \right] + B$$

'HbA1c' and 'haemoglobin (Total)' are figures got from the CLOVER A1C® Self system. 'A' and 'B' are the gradient and intercept factor to correct the value for the calibration standard of National Glycohaemoglobin Standardization Program.

3.6 HORMONAL ASSAY

The assay for the serum levels of the reproductive hormones were performed at the medical laboratory of the University College Hospital in Ibadan, Oyo State, Nigeria. The kit used for the hormonal assay was supplied by Fortress Diagnostics Limited, United Kingdom. The hormonal assay for Testosterone, FSH (follicle-stimulating) and LH (luteinizing hormone) was carried out, using second antibody (sandwich) enzyme-linked immunosorbent assay (ELISA).

Principle:

This is based on the affinity and specificity of monoclonal antibody targeted towards a definite antigenic determinant on the whole hormone molecule. The levels of the hormone are directly proportionate to the intensity of the color formed from the sample.

Procedure:

The microplate wells for each sample, calibrator and control serum were formatted to carry out the assays in replica. Single or double wells were added for blank (Tetramethyl benzidine). To each calibrator, samples and control serum (25 µL) were then pipetted into suitable wells after adding the conjugate solution (100 µL) to each well except blank. The microplate was whirled slightly between twenty–thirty seconds and then covered. The mixture was then incubated at 25°C for sixty minutes. 300 µL of washing solution was added and then decanted. Addition of washing solution and decantation were done five times. TMB-substrate (100 µL) was added into all wells at scheduled time (shaking of the plate was avoided after adding TMB-substrate). The plate was incubated again at 25°C in the dark for twenty–thirty minutes followed by the addition of stopping reagent (150 µL) to all wells as before. The incubation mixtures were slightly mixed for five-ten seconds and the absorbance taken at 450 nm after twenty minutes using microplate reader.

3.7 ESTIMATION OF ALANINE AMINOTRANSFERASE ACTIVITY

The activity of ALT was estimated based on the method described by Reitman and Frankel, (1957).

Principle:

α-oxoglutarate + L-alanine $\xrightarrow{GPT}$ pyruvate + L - glutamate

Alanine aminotransferase (ALT) activity was assessed by observing the content of pyruvate hydrazone formed with 2, 4-dinitrophenylhydrazine.

Procedure:

Diluted sample (0.1 mL) was interspersed with Reagent 1 (phosphate buffer, α-oxoglutarate, L-Alanine) and for just half an hour, the solution was incubated at 37^0C. Reagent 2 which contain 2, 4-dinitrophenylhydrazine (2 mmol/L) (0.5 ml) was put into the reaction mixture and was let stay at 25^0C for just 20 minutes. Afterward, NaOH (5.0 mL) was appended and the absorbance was taken against the reagent blank at 546 nm after 5 minutes.

Standard curve (see Appendix 3)

Detailed preparations of the reagents are presented in Appendix 3.

3.8 ESTIMATION OF ASPARTATE AMINOTRANSFERASE ACTIVITY

The activity of ALT was estimated based on the method described by Reitman and Frankel, (1957).

Principle:

α-oxoglutarate + L-aspartate $\xrightarrow{GOT}$ oxaloacetate + L - glutamate

Aspartate aminotransferase activity was assessed by observing the content of oxaloacetate hydrazone formed with 2, 4-dinitrophenylhydrazine.

Procedure:

Diluted sample (0.1 mL) was interspersed with Reagent 1 (L-aspartate, α-oxoglutarate and phosphate buffer) and for just half an hour, the solution was incubated at 37^0C. Reagent 2 which contains 2, 4-dinitrophenylhydrazine (0.5 ml) was put into the reaction mixture and was allowed to stand at 25^0C for just 20 minutes. Afterward, NaOH (5.0 mL) was appended and the absorbance was taken against the reagent blank at 546 nm after 5 minutes.

Standard curve (see Appendix 4)

Detailed preparations of the reagents are presented in Appendix 4.

3.9 ESTIMATION OF SERUM CREATININE CONCENTRATION

Creatinine (serum) concentration was estimated based on the procedure of Bartels *et al*., (1972).

Principle:

Creatinine present in alkaline liquid reacts with picric acid to produce a compound (coloured). The quantity of the compound produced is wholly relative to the content of the creatinine.

Method:

Diluted sample (0.1 mL) was interspersed with 1.0 mL of solution of the same amounts of picric acid (35 mmol/L) and sodium hydroxide (0.32 mol/L). The absorbance A_1 of the standard and sample was taken after 30 seconds at 520 nm and the absorbance A_2 was taken at subsequent 2 mins. Interspersed and allowed to stand for 20 min at 25^0C. The absorbance of sample (A_{sample}) and standard ($A_{standard}$) against blank was taken.

Calculation:

Content of creatinine in the sample.

$$\frac{A_{sample}}{A_{standard}} \times \frac{177}{1} = \mu mol/L$$

Detailed preparations of the reagents are presented in Appendix 5.

3.10 DETERMINATION OF UREA (SERUM) CONCENTRATION

Urea (serum) concentration was estimated based on the procedure of Fawcett *et al*., (1962).

Principle:

Urea (serum) undergoes hydrolysis by the activity of urease to produce ammonia. Photometrically, ammonia is calculated by Barthelot's reaction.

$$Urea + H_2O \xrightarrow{urease} 2NH_3 + CO_2$$

$$NH_3 + Hypochlorite + Phenol \longrightarrow Indophenol\ (Blue\ Compound)$$

Procedure:

Sample (10 µL) was added to 100 µL of 116 mmol/L EDTA (6 mmol/L of sodium nitroprusside and 1 g/L urease), interspersed and for 10 minutes, incubated at 37^0 C. 2.50 mL of diluted phenol (120 mmol/L) was added, then, 2.50 mL of diluted sodium hypochlorite (27 mmol/L) was also added. Instantly interspersed and for 15 minutes, incubated at 37^0C. The absorbance of the sample (A_{sample}) and standard ($A_{standard}$) against the blank was read at 546 nm. For no less than 8 hours, the reaction colour was constant.

Calculation:

$$\text{Content of Urea} = \frac{A_{sample}}{A_{standard}} \times \frac{13.3}{1} \text{ (mmol/L)}$$

Detailed preparations of the reagents are presented in Appendix 6.

3.11 ESTIMATION OF TOTAL BILIRUBIN CONCENTRATION

This was estimated based on the colorimetric method described by Jendrassik and Grof, (1938).

Principle:

Reaction of total bilirubin with diazotized sulphanilic acid liberates albumin bound bilirubin in the presence of caffeine.

Procedure

Reagent 1 (0.20 mL) was pipetted into cuvette (1 cm light path) and 1 drop (0.05 mL) of Reagent 2 was added, followed by 1.0 mL of Reagent 3 and 0.20 mL of the samples was appended. Interspersed and let stay at 20 – 25^0C for 10 mins and finally 1.0 mL of Reagent 4 (0.93 mol/Ltartrate in 1.9 N sodium hydroxide) was added. Mixed and allowed to stand at 20 – 25^0C for 5 – 30 mins and the absorbance of the sample against the sample blank (A_{TB}) at 578 nm was taken.

Calculation:

$$\text{Total Bilirubin concentration (µmol/L)} = 185 \times A_{TB}\,(578 \text{ nm})$$

Detailed preparations of the reagents are presented in Appendix 7.

3.12 ESTIMATION OF DIRECT BILIRUBIN CONCENTRATION

This was estimated by colorimetric method described by Jendrassik and Grof, (1938).

Principle:

Reaction of conjugated or direct bilirubin with diazotized sulphanilic acid produces blue coloured compound in alkaline medium.

Procedure

Reagent 1 (0.20 mL) was pipetted into cuvette (1 cm light path) and 1 drop (0.05 mL) of Reagent 2 was added, then mixed with 2 mL of sodium chloride (9 g/L) and 0.20 mL of the samples was finally added. Mixed and allowed to stand for exactly 10 min at 20 – 25^{o}C. The absorbance of the sample against the sample blank (A_{DB}) at 546 nm was taken.

Calculation: Direct Bilirubin concentration (µmol/L) = 246 x A_{DB} (546 nm).

Detailed preparations of the reagents are presented in Appendix 7.

3.13 ESTIMATION OF INDIRECT BILIRUBIN CONCENTRATION

Calculation:

Indirect Bilirubin concentration= Total Bilirubin concentration – Direct Bilirubin concentration

3.14 ASSESSMENT OF ALKALINE PHOSPHATASE ACTIVITY

Alkaline phosphatase activity was assessed based on the procedure described by Englehardt, (1970)

Principle

p-nitrophenylphosphate + H_2O $\xrightarrow{ALP}$ Phosphate + *p*-nitrophenol

Method

Reagent for the assay was prepared by reconstituting one vial of substrate Reagent 1b (10 mmol/L p-nitrophenylphosphate) with 3.0 mL of Reagent 1a. 1.0 mL of mixed reagent was appended to

sample (0.02 mL), interspersed, read initial absorbance at 405 nm (25^0C) and timer was started simultaneously. Reading was taken at another 1, 2 and 3 minutes against blank.

Calculation:

U/L = 2760 x ΔA

Detailed preparations of the reagents are presented in Appendix 8.

3.15 DETERMINATION OF ACID PHOSPHATASE ACTIVITY

Acid phosphatase activity was determined based on the procedure explained by Fishman and Lerhner (1953).

Principle

1-naphthyl phosphate + H_2O $\xrightarrow{\text{Acid Phosphatase}}$ Phosphate + 1-naphthol

1-naphthol + Fast Red TR salt $\longrightarrow$ Azo dye

Fast Red TR salt = 4-chloro-2-methylphenyldiazonium salt

Procedure

100 µL of sample was added to reagent solution a (10 mL of buffer + 1 vial of substrate (1-naphthyl phosphate and Fast Red TR salt) or b (reagent solution a + substrate (sodium tartarate). The mixture was thoroughly interspersed and incubated at 37^OC for 5 minutes. The initial absorbance was read against air at 405 nm while others were read at 60 minutes interval up to 3 minutes.

Calculation

Activity of Total Acid Phosphatase (U/L) = ΔAbs of solution a/min x 743

Activity of Prostatic Acid Phosphatase (U/L) = (ΔAbs of solution a/min – ΔAbs of solution b/min 743)

3.16 ESTIMATION OF LACTATE DEHYDROGENASE ACTIVITY

This was estimated based on the method of Weisshar, (1975)

Principle

$$\text{Pyruvate} + \text{NADH} + H^{+} \xrightarrow{\text{LDH}} \text{Lactate} + NAD^{+}$$

Procedure

1.0 mL of the mixture of substrate and the buffer was pipetted into the cuvette, and 0.02 mL of the sample was added. The mixture was mixed and incubated is at 37^{o} C. The initial absorbance was read and other readings were taken after 1 min, 2 mins and 3 mins at 340 nm.

Calculation

LDH activity (U/L) = 4127 X ΔA340 nm/min.

Detailed preparations of the reagents are presented in Appendix 9.

3.17 DETERMINATION OF PROTEIN CONCENTRATION

Protein concentrations of the several samples were estimated based on Biuret procedure as explained by Gornal *et al*., (1949). Addition of potassium iodide inhibited Cu^{2+} ions from precipitating as cuprous oxide.

Principle

A coloured compound is formed when protein, in alkaline medium, reacts with cupric ions as demonstrated by the reagent (Biuret) comprising sodium potassium tartrate, $CuSO_4$ and KI. Standard BSA curve is commonly used to standardize this method.

Procedure:

The 5 times dilution of the supernatant (post mitochondrial fractions) with water (distilled) was prepared and 3 mL of Biuret reagent was appended to it. Incubation of the mixture was done at 25^{o}C for half an hour and afterward, the absorbance (540 nm) was read with distilled H_2O as blank. From the standard curve, the protein content in the samples was deduced and multiplied by 5 to get the real quantity in the fraction.

Standard curve (see Appendix 10)

Detailed preparations of the reagents are presented in Appendix 10.

3.18 ESTIMATION OF MALONDIALDEHYDE CONCENTRATION

Malondialdehyde concentration was estimated by evaluating the TBARS (thiobarbituric acid reactive substance) produced during lipid peroxidation as described by Rice-Evans *et al*., (1993).

Principle

The reaction involving malondialdehyde (end product of lipid peroxidation) with 2-thiobarbituric acid (TBA) is the basis of this method. Upon heating in acidic pH, pink chromophore product with a maximum absorption at 532 nm is formed. This product can be extracted by organic solvents such as butanol. This test can be calibrated using MDA and results are stated as the quantity of unbound MDA formed.

Procedure

A fraction of the specimen (0.4 mL) was interspersed with Tris-KCl buffer (1.6 mL) and 30% TCA (0.5 mL) was appended. Then, 0.75% TBA (0.5 mL) was appended and positioned in a water bath (at 80^0C) for 45 minutes. This was cooled in ice and for 15 minutes, centrifuged at 3000 rpm. The absorbance of the resulting clear pink solution formed was evaluated at 532 nm against distilled water as reference blank. Based on the procedure explained by Adam-Vizi and Sergi (1982), the content of MDA was evaluated. Malondialdehyde concentration (gram tissue or units /mg protein) was calculated with a molar extinction coefficient of $1.56 \times 10^5\ M^{-1}Cm^{-1}$.

Calculation:

$$\text{MDA (units/mg protein)} = \frac{\text{Absorbance x Volume of mixture}}{E_{532\ nm} \text{ x Volume of Sample x mg Protein}}$$

Detailed preparations of the reagents are presented in Appendix 11.

3.19 ESTIMATION OF REDUCED GLUTATHIONE CONCENTRATION

The concentration of reduced glutathione (GSH) was determined based on the procedure explained by Beutler *et al*., (1963)

Principle:

In most cases, Glutathione (reduced) constitutes the major part of the non-protein sulfhydryl groups in the cell. This procedure depends on the formation of yellow colour that is slightly stable upon addition of 5, 5^1-dithiobis-(2-nitrobenzoic acid) (DTNB) or Ellman's reagent to sulfhydryl complex. 2-nitro-5-thiobenzoic acid (resultant chromophore), formed from the reaction of the glutathione (reduced) with Ellman's reagent, has a molar absorption at 412 nm. The concentration of Glutathione (reduced) in the specimen (test) is proportional to the absorbance (412 nm) of this compound formed.

Procedure

Test sample (0.1 mL) was watered down with 0.9 mL of water (distilled) to give 1 in 10 dilutions. 4% sulphosalicyclic acid solution (precipitating solution) (3 mL) was appended to the diluted specimen (test) to deproteinize it. Centrifuge the mixture for 10 minutes at 3,000 g. Thereafter, the supernatant (0.5 mL) was appended to 4 mL of phosphate buffer (0.1 M) and lastly, Ellman's Reagent (4.5 mL) was appended. The preparation of the blank was done [reaction mixture of 4 mL of phosphate buffer (0.1 M), diluted precipitating solution (0.5 mL) [i.e. addition of precipitating solution (3 mL) and 2 mL of water (distilled) and Ellman's Reagent (4.5 mL)]. The absorbance of the mixture were read at 412 nm within 5 minutes. The content of glutathione (reduced), GSH is compared to the absorbance read at 412 nm.

Detailed preparations of the reagents are presented in Appendix 12.

3.20 ESTIMATION OF CATALASE ACTIVITY

The activity of catalase was determined based on the procedure explained by Sinha, (1971).

Principle

Reduction of dichromate in acetic acid to chromic acetate with the production of perchromic acid (an unstable intermediate) when heated in the presence of hydrogen peroxide is the basis of this procedure. The chromic acetate produced is determined at 570 – 610 nm. Since dichromate has no absorbance in this region, the presence of the compound in the assay mixture does not interfere at all with the colorimetric determination of chromic acetate. At different time phases, catalase in the test sample splits hydrogen peroxide. Adding dichromate/acetic acid mixture halts the reaction at a specific time (60 seconds interval) and after heating the reaction mixture at 80^oC, the hydrogen peroxide that is left over is evaluated based on the colorimetric measurement of chromic acetate.

Estimation of Catalase activity in specimen

Sample (1 mL) was interspersed with 49 mL of water (distilled) to make 1 in 50 dilution of the specimen. 4 mL of H_2O_2 solution (800 µmoles) and 5 mL of Phosphate buffer in a 10mL flat bottom flask made up the assay mixture. The well, diluted sample (1 mL) was quickly interspersed with the assay mixture by a light whirling movement at room temperature. An aliquot from the assay mixture was appended to dichromate acetic acid reagent (2 mL) at the intervals of 1 min. The contents of H_2O_2 of the samples inhibited were estimated by the above mentioned procedure. The mononuclear velocity constant k for catalase to decompose H_2O_2 was estimated based on a first - order reaction.

$K = 1/t \log S_0/S$

Where S_0 is the initial concentration of the hydrogen peroxide and S is the concentration of the peroxide at time t minute. The values of the k were plotted against time in minutes and the velocity constant of catalase k_0 at 0 minute was estimated by inference. The catalase contents of the sample were stated as katalase feiahigkeit or "kat f".

Kat f = K_0/mg protein (mL).

$k_0 = H_2O_2$ Consumed

H_2O_2 remained = $\frac{\text{Change in absorbance/min.}}{0.171}$

H_2O_2 Consumed = 800 - H_2O_2 remained

Standard curve (see Appendix 13)

Detailed preparations of the reagents are presented in Appendix 13.

3.21 ESTIMATION OF SUPEROXIDE DISMUTASE (SOD) ACTIVITY

The activity of SOD was determined based on the procedure explained by Mistra and Fridovich, (1972).

Principle

The inhibition of the autoxidation of epinephrine by SOD at pH 10.2 is the basis for this assay. Reactions of xanthine oxidase produce superoxide radical which oxidizes epinephrine so as to produce adrenochrome, then, adrenochrome yield, from every superoxide introduced, elevates with rising pH (Valerino and Cormack, 1971) and rising epinephrine (concentration).

Procedure

Specimen (1 mL) was watered down in 9 mL of water (distilled) to make 1 in 10 dilutions. A portion of the diluted specimen (0.2 mL) was appended to 2.5 mL of carbonate buffer (0.05 M, pH 10.2) so as to equilibrate in the spectrophotometer. The reaction started by the addition of 0.3 mL of 0.3 mM adrenaline (that was freshly prepared) to the mixture and was rapidly interspersed by inversion. The reference cuvette contained water (0.2 mL), buffer (2.5 mL) and 0.3 mL of substrate (adrenaline). The absorbance at 480 nm was taken every 30 seconds for 150 seconds.

Calculation

$$Absorbance\ increase\ per\ minute = \frac{A\,3 - A\,0}{2.5}$$

Where $A\,0$ represents absorbance after 0 seconds

$A\,3$ represents absorbance after 150 seconds

$$\%\ Inhibition = \frac{Increase\ in\ absorbance\ of\ substrate}{Increase\ in\ absorbance\ of\ blank}\ X\ 100$$

The quantity of SOD required to bring about 50% inhibition of the oxidation of adrenaline represents 1 unit of SOD.

Detailed preparations of the reagents are presented in Appendix 14.

3.22 ESTIMATION OF GLUTATHIONE-S-TRANSFERASE ACTIVITY

The activity of Glutathione-S-transferase (GST) was determined based on the procedure explained by Habig *et al*., (1974).

Principle:

Activity of GST utilizing the substrate, 1-chloro-2, 4, - dinitrobenzene is the basis for this assay. It is evident that glutathione-S-transferase demonstrates a relatively high activity with 1-chloro-2, 4-dinitrobenzene. The conjugation of this substance with reduced glutathione shifts the wavelength

longer at maximum absorption. An exact measurement of the conjugate concentration is provided as the absorption increases at the new wavelength of 340 nm.

Principle

Addition of 30 µL of reduced glutathione (0.1 M) to 150 µL of 20 mM of 1-chloro- 2, 4-dinitrobenzene (CDNB), then 2.82 mL of 0.1 M Phosphate buffer (pH 6.5) was added and mixed with Cytosol/Microsomes (30 µL). The absorbance at 340 nm was taken every 60 seconds for 180 seconds against the blank. The blank was prepared as the above but 30 µL of distilled was used to replace the sample. Was used to compute the compute the enzyme activity.

Calculations:

Extinction coefficient of CDNB = 9.6 mm^{-1} Cm^{-1}

$$\text{Activity of GSH-S-transferase} = \frac{\text{OD/min}}{9.6} \times \frac{1}{0.03 \text{ ml mg protein}}$$

= µmole/min/mg protein.

Detailed preparations of the reagents are presented in Appendix 15.

3.23 ESTIMATION OF GLUTATHIONE PEROXIDASE ACTIVITY

The activity of Glutathione peroxidase was determined based on the procedure explained by Rotruck *et al*., (1973).

Procedure

Phosphate buffer (0.5 mL) was appended to NaN_3 (0.1 mL), GSH (0.2 mL), H_2O_2 (0.1 mL), 0.5 mL of sample and 0.6 mL distilled water. The whole mixture was incubated for 3 minutes at 37^0C, then, TCA (0.5 mL) was appended and thereafter, centrifuged for 5 minutes at 3000 rpm. To each supernatant (i.e. 1 mL), K_2HPO_4 (2 mL) and DTNB (1 mL) were appended and the absorbance (412 nm) was taken against the blank.

Calculation:

Glutathione peroxidase (GPx) activity was evaluated by plotting a standard curve. The content of the GSH leftover was deduced from the curve.

GSH consumed = 245.84 – GSH leftover

Glutathione peroxidase activity = $\frac{\text{GSH consumed}}{\text{mg protein}}$

Detailed preparations of the reagents are presented in Appendix 16.

3.24 THE MICRONUCLEI ASSAY

The micronuclei assay was determined based on the method described by Schmid, 1975.

Principle

The staining ability of polychromatic erythrocyte (PCE) cells differs from normocyte (normal mature erythrocyte) and this is the basis for this assay. Ribosomal RNA occurring 24 hours before the cells are formed is attributed to the staining ability of PCE. PCEs lose their ribosomal RNA and staining ability as it grows into mature erythrocytes. Erythrocytes that are matured drive their nuclei out, 8-12 hours following the final mitosis prior to the development of an erythrocyte. But micronuclei are not totally driven out due to a few reasons. Micronuclei are not commonly present in blood (circulating erythrocytes) as spleen filters out micronuclei.

Procedure

The method of Schmid, 1975 was adopted in the preparation of bone marrow smears. The femur of each rat was removed after sacrificing the rat and stripped clean of muscle. The iliac section of the femur was opened with a pair of scissors. A small pin was pushed inside the canal of the marrow at the epiphyseal end, then, marrow was exuded via the hole at the iliac end on to a slide.

A drop of fetal bovine serum was appended to the smear by means of a Pasteur pipette. The whole content was mixed to become homogeneous by using a clean edge of another slide. The homogeneous mixture was then spread on the slide as a smear and allowed to dry.

Procedure for fixing and staining of slides

The slides were fixed in methanol for 5 minutes and allowed to air dry to remove methanol. After which they were stained with 0.4% May Grunwald stain 1 and subsequently, May Grunwald stain 2 (1:1 of stain 1 and distilled water) and air dried. It was also stained in 5% Giemsa for a minimum of

half an hour, rinsed for nearly 30 seconds in phosphate buffer (pH=6.8) and in water (distilled) and air dried. The slides were lastly fixed for 20 minutes in xylene and air dried.

Scoring of the slides

The fixed cells on the slides were viewed under light microscope to detect the presence of micronucleated polychromatic erythrocytes (PCEs). Tally counter was used to make scoring easier. The slides were first screened at medium magnification to get suitable regions for scoring. PCEs and micronuclei stained blue while normal mature erythrocytes stained red, with this one can distinguish between micronuclei and normocytes.

Detailed preparations of the reagents are presented in Appendix 17.

3.25 DETERMINATION OF PERCENTAGE DNA FRAGMENTATION

The percentage DNA fragmentation was determined based on the procedure explained by Wu *et al*., (2005)

Procedure

The prostates were homogenized in 10 volumes of Tris-EDTA buffer (TE, pH 8.0). Homogenates were centrifuged for 20mins at 27,000g to set apart the intact chromatin (pellet named A) from the fragmented ones (supernatant named B). The pellet (A) was suspended in Tris-EDTA buffer ($TE^{!}$) of pH 8.0. Then, 0.5ml aliquot of each sample (pellet and supernatant) was placed in separate test tubes and 1.5ml of freshly prepared diphenylamine solution was appended to each test tubes. The reaction mixture was incubated for 20hours at 37^{0}C. Absorbance of mixture was then measured at 620nm.

Calculation

Estimation of fragmented DNA Quantity by via the formula:

$$\% \text{ fragmented DNA} = \frac{B}{(A+B)} \times 100$$

Where A is the Absorbance of pellet and B is the Absorbance of supernatant

Detailed preparations of the reagents are presented in Appendix 18.

3.26 DETERMINATION OF CONCENTRATION OF MINERALS (ZINC)

The level of Zn in the prostate was determined based on the method described by Ammerman *et al.,* (1974).

Procedure

Prostate PMF (1ml) was deproteinised with 9ml of 10% TCA in 0.1% Lanthanum solution and centrifuged at 3000g for 10 minutes. The supernatant was diluted with 0.1% Lanthanum depending on concentration of individual element (Selenium) and aspirated to the AAS. Another portion of the supernatant was diluted in ratio 1:4 with water (for Zinc) and read in AAS. Standards and blanks were prepared by diluting with 5% glycerin.

Calculation A mg/L = ppm= R x D

Where:

A= Zn/Se

R= AAS reading

D=Dilution factor

Detailed preparations of the reagents are presented in Appendix 19.

3.27 DETERMINATION OF BICARBONATE CONCENTRATION

Procedure

A few drops of Phenolphthalein was added to 50ml of water until a pink colour was produced. The pink solution was titrated with sulphuric acid by adding a drop of sulphuric acid every 2 or 3 seconds until the pink colour disappears. Titration continued until the methyl orange end point was reached and the total reading was noted. Blank was determined by running the reagents with CO_2 –free distilled water.

Detailed preparations of the reagents are presented in Appendix 20.

3.28 HISTOPATHOLOGY

Prostate samples were fixed in 10% formaldehyde. After dehydration procedures, the samples were blocked in paraffin. Ultra-thin (4-5µm) sections of the organ were cut using a microtome knife and

stained with Hematoxylin and Eosin (H & E) solution. The sections were examined under a light microscope and the micrographs of the organs were taken.

3.29 IMMUNOHISTOCHEMISTRY

The method of Chakravarthi *et al.*, 2010 was used for Immunohistochemistry analysis.

Principle

Immunohistochemical (IHC) staining techniques allow for the visualization of antigens via the sequential application of a specific antibody to the antigen (primary antibody), a secondary antibody to the primary antibody and an enzyme complex with a chromogenic substrate with interposed washing steps. The enzymatic activation of the chromogen results in a visible reaction product at the antigen site. The specimen may then be counterstained and cover slipped. The use of a light microscope helps in the interpretation of results and aid in the differential diagnosis of pathophysiological processes, which may or may not be associated with a particular antigen.

Procedure

The method of immunohistochemistry used in this study is Avidin Biotin Complex (ABC) method, also known as Avidin Immunoperoxidase method. All chemicals used were manufactured by Novocastra (LEICA). The antibody dilution factor used was 1: 100 dilutions for all the antigen markers. Prostate tissue was sectioned at 2µ on the rotary microtome and fixed on slides followed by placing the slides on the hot plate at 70ºC for about 1 hour. The sections were passed into water, after being passed through two changes of Xylene and three changes of descending grades of alcohol. Antigen retrieval was performed on the sections by heating them on citric acid solution (pH 6.0) using microwave for 15 minutes. The sections were equilibrated gradually with cold water to displace the hot citric acid for about 5 minutes to allow cooling of the sections. Peroxidase blocking was carried out on the sections by covering the sections with 3% hydrogen peroxide for 15minutes. Prostate sections were washed with PBS and protein blocking was done using avidin for 15 minutes. Prostate sections were washed with PBS and endogenous biotin in the prostate was blocked for 15 minutes. Prostate sections were incubated with 5µg/ml each of anti-Bcl-2 and anti-ki67 antibodies (1: 100 dilutions) for 60 minutes. Phosphate buffer saline (PBS) (pH 7.4) was used to wash excess antibody followed by application of a secondary antibody (LINK) on the sections for 15 minutes. The sections were washed and Horseradish peroxidase (HRP) label was applied on

the sections for15 minutes. The sections were washed with PBS for 5 minutes to remove HRP and treated with DAB solution (1 drop of DAB chromogen + 1ml of DAB substrate). Excess DAB solution and precipitate were washed off with water. The tissue sections were counterstained with Haematoxylin solution for 2 minutes. The slides were dehydrated in alcohol, cleaned in xylene, mounted in DPX mountant and observed under microscope.

Scoring of Slides

Slides were observed under a Binocular microscope. Cells with specific brown colors in the cytoplasm, cell membrane or nuclei depending on the antigenic sites were considered positive. The cells were scored based on haematoxylin staining. Zero or no staining if there is less than 5% staining, 1 or weak staining if there is 6-24% staining (light yellow), 2 or moderate staining if there is 25-49% staining (yellow brown), 3 or strong staining if there is 50-74% staining (brown) and 4 or very strong if there is 75-100% staining.

3.30 STATISTICAL ANALYSIS.

All values were expressed as the mean ± SD (Standard deviation). Data were analysed using One-Way Analysis Of Variance (ANOVA) followed by the post-hoc Duncan multiple range test to identify significantly different groups using SPSS (24.0) and student's t-test for comparing the treated with the control using Graph Pad prism (6.0). Values were considered statistically significant at $p \leq 0.05$. Graphs and Tables were used to represent the data pictorially.

CHAPTER FOUR

RESULTS

4.1 EXPERIMENT ONE: BIOCHEMICAL, HISTOLOGICAL AND HORMONAL MODIFICATIONS IN THE PROSTATE OF RATS AFTER LASTING STREPTOZOTOCIN-INDUCED DIABETES

4.1.1 INTRODUCTION

Diabetes mellitus (DM), which is an indication of hyperglycemia, can be described as a long-standing metabolic and endocrine derangement that is linked with deficiencies in either insulin secretion or sensitivity or both. Oxidation of mitochondrial glucose in hyperglycemic condition (a feature of DM) enhances oxidative stress (Chang and Chuang, 2010) which causes micro- and macro-vascular complications (Giacco and Brownlee, 2010). Infertility, which is one of the complications of DM, affects 35% of people suffering from DM and reduced sperm quality is connected to infertility. On a regular basis, oxidative stress is interrelated with spermatozoa dysfunction in DM (Gomez *et al.,* 1996). Also, Alves *et al.,* (2013) described disorders in endocrine control of spermatogenesis in DM. For instance, infertility in male diabetic subject is associated with abberrations in testosterone synthesis as a result of structural and molecular variations observed in the Leydig cells (Dinulovic and Radonjic, 1990). Similarly, endocrine disorders originating from DM also affect pituitary-gonadal system (Bhasin *et al*., 2007). Decrease in the volume of secretions from prostate gland during prostate gland pathology may contribute to male diabetic infertility (Weidner *et al.,* 1999). Also, increase in the viscosity of semen may adversely

affect the sperm cell motility and it was suggested that infertile people with prostate gland pathology may have increased semen viscosity. Bansal *et al.,* (2013) described an antithetical correlation between diabetes and prostate pathologies, owing to modifications in the levels of sex hormone and metabolic anomalies observed in diabetes patients (Bonovas *et al.,* 2004). In addition, reduced secretion or sensitivity to insulin (Meyer *et al.,* 2000) during prostate gland pathology, may result in decline in the volume of secretions from prostate gland (Marconi *et al.,* 2009). Thus, all these causative factors contribute to the functional integrity of prostate gland, which ranges from actions of the enzymes to the motility of spermatozoa. Hence, the structural and functional integrity of the prostate as regards the biochemical, histological and hormonal modifications in DM needs to be fully elucidated so as to lessen the risks of infertility in male diabetic subjects. Hence, the aim of this study was to examine the biochemical, histological and hormonal modifications in the prostate of male rats (wistar) after long-standing streptozotocin-induced diabetes mellitus.

PROCEDURE

A total of twenty six wistar rats were assigned at random into 3 groups. Group I-Control (rats that received citrate buffer only, n=10), Group II-3 Months Diabetes Mellitus (rats that received STZ only, n=8) and Group III-5 Months Diabetes Mellitus (rats that received STZ only, n=8). Diabetes mellitus was induced by a single administration of 0.1 M streptozotocin (STZ) solution to male rats (wistar) at 35mg/kg body weight intraperitoneally (Adaramoye *et al*., 2012). Following 72 hours of STZ dose, determination of concentration of blood glucose in the rats were done using Accu-Check Glucometer (Roche) with suitable glucometer strips. The concentration of blood glucose that exceeded 250 mg/dL in animals were regarded as diabetic and recruited for the study. Evaluation of the animals in each group were done on a fortnight basis for signs of weight loss, weakness, hair loss, cataract, coma and death.

RESULTS

Diabetes mellitus caused a marked ($p<0.05$) reduction in prostate weight of 3- and 5-MDM rats by 33% and 59%, respectively relative to the control. Similarly, in 3- and 5-MDM, the body weight of rats was reduced ($p<0.05$) by 41% and 97%, respectively, relative to controls as presented in Table 1. Table 2 revealed marked ($p<0.05$) increase in FBS (fasting blood glucose) and glycated haemoglobin relative to controls at both 3- and 5-MDM. Diabetes mellitus triggered a marked ($p<0.05$) elevation in the levels of serum creatinine and urea in 3-MDM rats by 57% and 56%, and

in 5-MDM rats by 45% and 46%, respectively, relative to the control. Moreover, in Table 2, the total bilirubin level, in 3- and 5-MDM rats, was elevated ($p<0.05$) by 45% and 31%, respectively, relative to controls. The FSH, LH and testosterone contents in the serum were markedly ($p<0.05$) reduced in 3-MDM rats by 2.3, 1.9 and 3.1 folds, and in 5-MDM rats by 2.1, 1.6, and 4.7 folds, respectively, compared to control, as presented in Table 3. However, Table 3 showed considerable elevation ($p<0.05$) in the contents of prostatic zinc and bicarbonate in 3- and 5- MDM rats, relative to control. The GSH concentration and GPx activity in both 3- and 5- MDM of STZ diabetic rats prostate considerably ($p<0.05$) reduced relative to control as shown in Table 3. Similarly, Table 3 revealed that CAT, SOD and GST activities in the prostate of 3- and 5- MDM rats were markedly ($p<0.05$) reduced relative to control. Activities of AST and ALT in Figure 1.9, were markedly ($p<0.05$) heightened in 3-MDM rats relative to control. Nevertheless, in 5-MDM rats, no marked difference ($p>0.05$) was observed in AST and ALT activities relative to control. The total acid phosphatase (tACP) activities in the prostate of diabetic rat was markedly ($p<0.05$) reduced by 56% and 71% in 3- and 5-MDM, respectively, relative to control as presented in Figure 2.0. Similarly, marked ($p<0.05$) reduction (48%) in the prostatic ACP activities was discovered in 5-MDM rats relative to the control. Furthermore, alkaline phosphatase (ALP) activity in 3-MDM rats was considerably ($p<0.05$) heightened by 28% relative to the control. However, marked reduction ($p<0.05$) in the activity of ALP was discovered in 5-MDM rats (Figure 2.1). Figure 2.2 revealed considerable ($p<0.05$) elevation in MDA concentration by 1.2 and 1.4 folds in the prostate of both 3- and 5-MDM rats, respectively, relative to control. The micronucleated polychromatic erythrocytes (mnPCEs) frequency in bone marrow of 3- and 5- MDM rats was heightened by 112% and 159% respectively (figure 2.3). No considerable change ($p>0.05$) was observed in the fragmented DNA percentage in 3- and 5-MDM rats,relative to the control as shown in Figure 2.4. Analysis of the histopathology of prostate showed distortion, hyperplasia with intraluminal budding of epithelia cells in 3- and 5-MDM rats (Plate 1B and C) relative to control (Plate 1A). Moreover, findings from immunohistochemistry showed marked reduction in Bcl-2 protein expression (Plate II) and mild expression of Ki67 (Plate III) in the prostate of 3- and 5-MDM rats, relative to controls.

Table 1.0: Animal weight, organ and Organosomatic weight changes in Streptozotocin-induced diabetic rats

Treatment	3-MDM					5-MDM				
	Animal weight (g)			Prostate		Animal weight (g)			Prostate	
	Initial	Final	Weight Gain	Weight (g)	Organosomatic weight[1]	Initial	Final	Weight Gain (g)	Weight (g)	Organosomatic weight[1]
Control	264.0±19.7	314.2±8.0	50.2±11.8	0.47±0.03	0.15±0.01	248.9±8.6	295.4±8.6	46.46±0.01	0.39±0.03	0.13±0.01
Diabetic	193.2±8.9	222.6±13.3	29.4±4.4*	0.25±0.01*	0.11±0.01	181.9±1.7	183.3±4.9	1.40±0.65*	0.16±0.01*	0.09±0.01*

Results are stated as Mean ± STD (n=5)

* depicts marked difference ($p<0.05$) relative to control

1 represents % body weight

3-MDM represents 3 months diabetes mellitus

5-MDM represents 5 months diabetes mellitus

Table 2.0: Modifications in the level of biochemical indices in the serum of Streptozotocin-induced diabetic rats

Parameters	3 – MDM		5 - MDM	
	Control	Diabetic	Control	Diabetic
Glycated Hb (%)	3.51±0.02	7.00±0.52*	3.59±0.1	9.31±0.03*
Blood glucose[x]	131.60±2.90	496.0±14.77*	93.70±3.50	536.71±18.20*
Urea[x]	77.41±1.14	120.67±2.28*	50.50±2.18	73.89±1.25*
Creatinine[x]	5.41±0.55	8.57±1.19*	4.11±0.01	7.48±0.01*
Protein[y]	22.64±1.04	24.76±0.38	35.98±1.93	28.93±0.32
Bilirubin (Total)[x]	1.10±0.03	1.99±0.11*	1.04±0.08	1.48±0.16*
Bilirubin (Direct)[x]	0.78±0.07	1.72±0.13*	0.54±0.17	1.23±0.31
Bilirubin (Indirect)[x]	0.32±0.01	0.29±0.05	0.51±0.14	0.68±0.09

Results are stated as Mean ± STD (n=5)

* depicts marked difference ($p<0.05$) relative to control

[x] = mg/dL ; [y] = mg/g.

3-MDM represents 3 months diabetes mellitus

5-MDM represents 5 months diabetes mellitus

Table 3.0: Changes in the hormone concentration, zinc level and antioxidative enzyme activities in the serum of Streptozotocin-induced diabetic rats

Parameters	3 – MDM		5 - MDM	
	Control	Diabetic	Control	Diabetic
FSH[a]	12.01±2.23	5.20±1.16*	4.51±0.89	2.06±0.50*
LH[a]	15.59±1.76	8.03±0.92*	7.01±1.03	4.29±0.70*
Testosterone[a]	1.98±0.36	0.63±0.05*	1.63±0.03	0.34±0.05*
GSH[b]	39.54±5.12	21.04±3.46*	37.70±0.46	15.41±0.38*
GPx[b]	174.49±8.16	93.20±7.04*	219.62±7.34	103.26±5.34*
CAT[c]	24.66±1.27	15.86±0.15*	159.58±5.76	101.19±9.19*
SOD[c]	0.17±0.01	0.07±0.01*	0.21±0.01	0.03±0.004*
GST[e]	3.58±0.06	3.07±0.06*	2.94±0.09	2.24±0.16*
Zn[d]	1.01±0.03	1.67±0.67*	0.04±0.02	0.24±0.01*
Bicarbonate[d]	1031.67±23.20	1920.00±16.10*	1225.00±35.00	2135.00±31.00*

Results are stated as Mean ± STD (n=5)

* represents marked difference ($p<0.05$) relative to control

[a] = IU/L; [b] = mg/g; [c] = U/mg protein; [d] = µg/dL; [e] = µmol/min/mg Protein

3-MDM represents 3 months diabetes mellitus

5-MDM represents 5 months diabetes mellitus

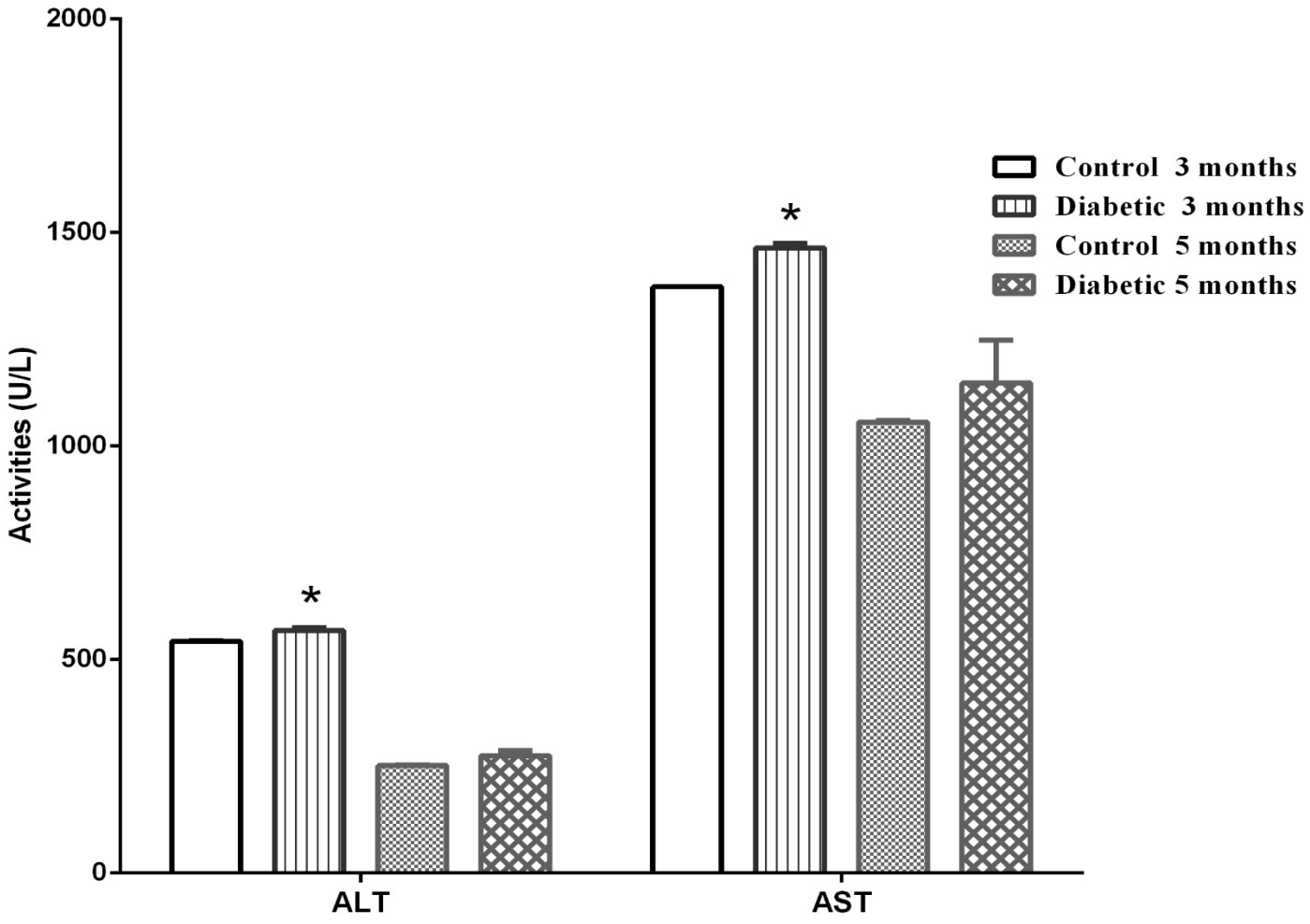


Figure 1.9: Serum Alanine Aminotransferase and Aspartate Aminotransferase activities in Streptozotocin-induced diabetic rats.

Results are stated as Mean ± STD (n=5)

* depicts marked difference ($p<0.05$) relative to control

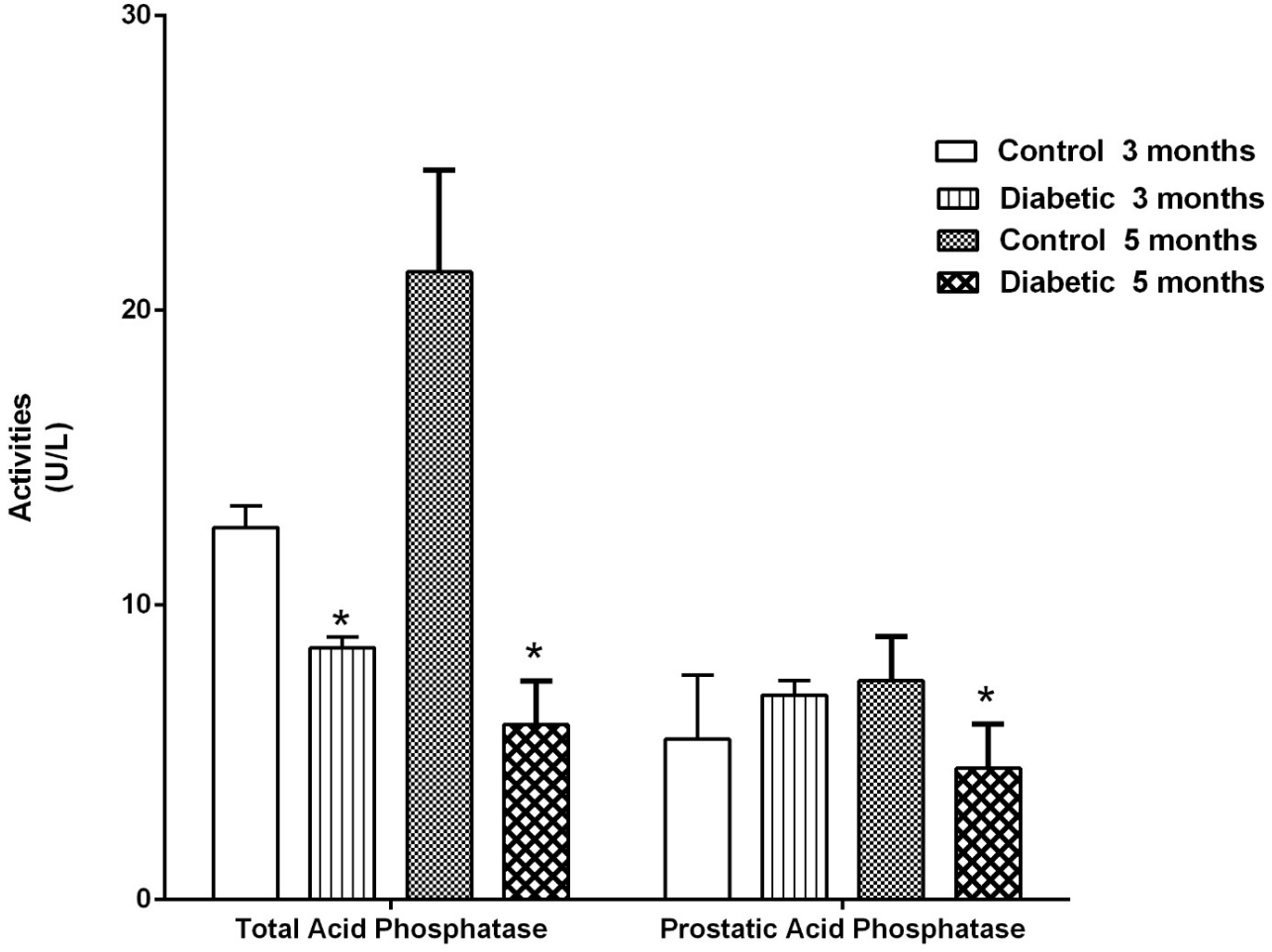


Figure 2.0: Acid Phosphatase activities in the prostate of Streptozotocin-induced diabetic rats.

Results are stated as Mean ± STD (n=5)

* depicts marked difference ($p<0.05$) relative to control

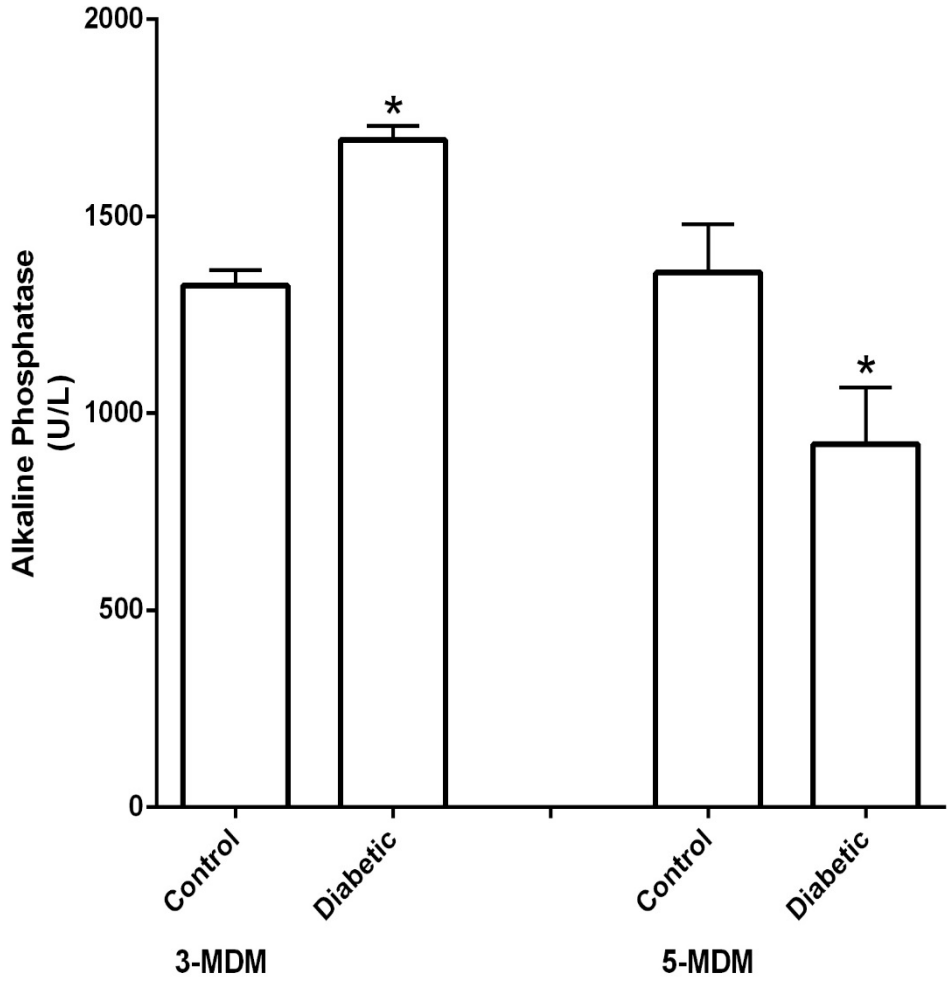


Figure 2.1: Alkaline Phosphatase activities in the prostate of Streptozotocin-induced diabetic rats.

Results are stated as Mean ± STD (n=5)

* depicts marked difference ($p<0.05$) relative to control

3-MDM represents 3 months diabetes mellitus

5-MDM represents 5 months diabetes mellitus

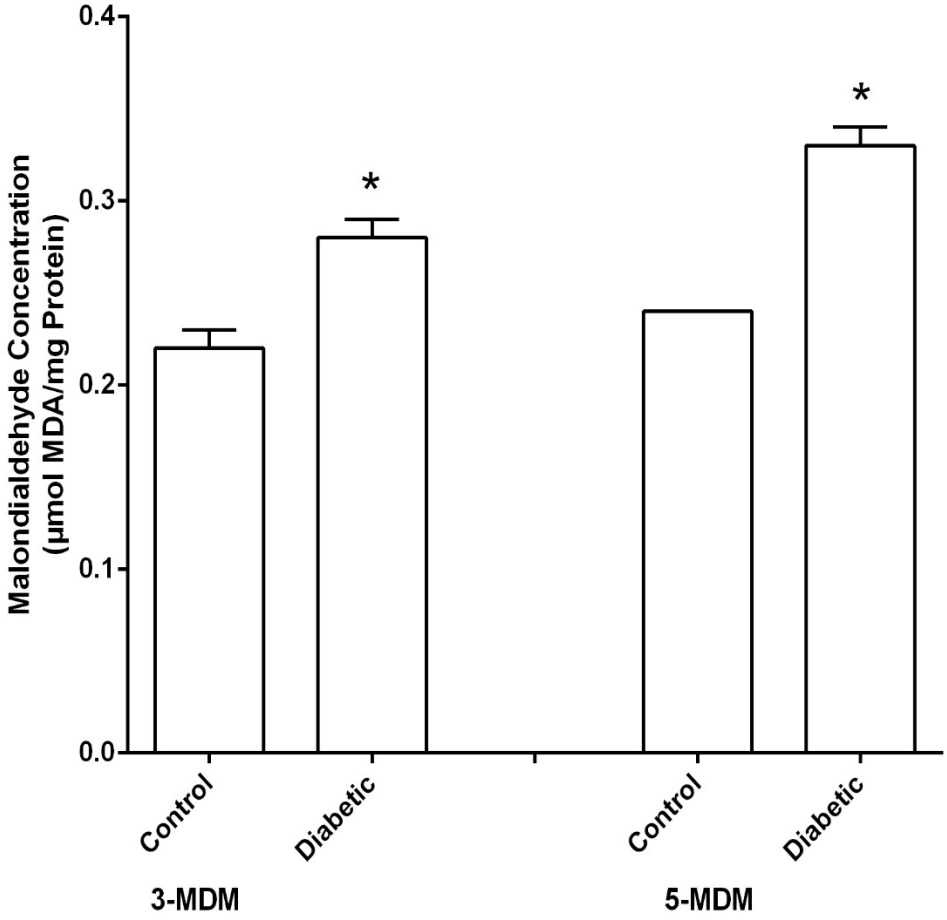


Figure 2.2: The prostatic Malondialdehyde concentration in Streptozotocin-induced diabetic rats.

Results are stated as Mean ± STD (n=5)

* depicts marked difference ($p<0.05$) relative to control

3-MDM represents 3 months diabetes mellitus

5-MDM represents 5 months diabetes mellitus

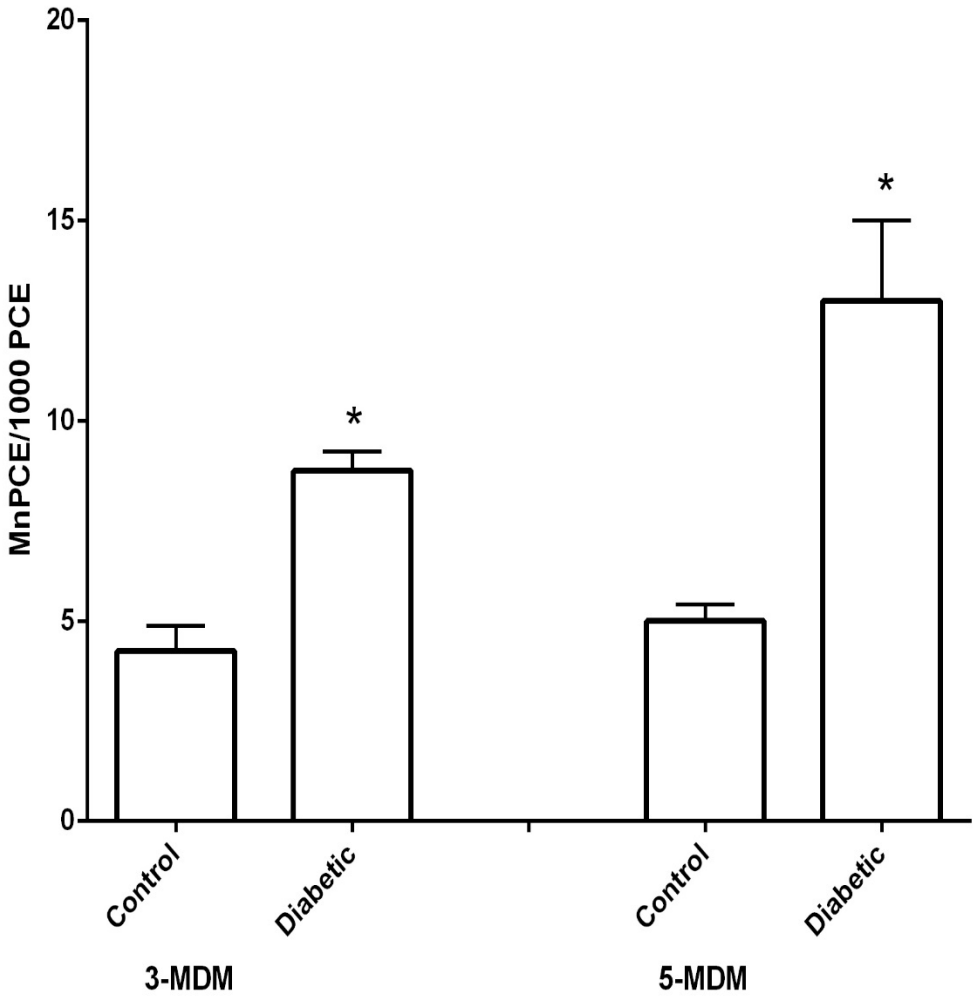


Figure 2.3: The frequency of Micronucleated Polychromatic Erythrocytes (MnPCEs) in the bone marrow of Streptozotocin-induced diabetic rats.

Results are stated as Mean ± STD (n=5)

* depicts marked difference ($p<0.05$) relative to control

3-MDM represents 3 months diabetes mellitus

5-MDM represents 5 months diabetes mellitus

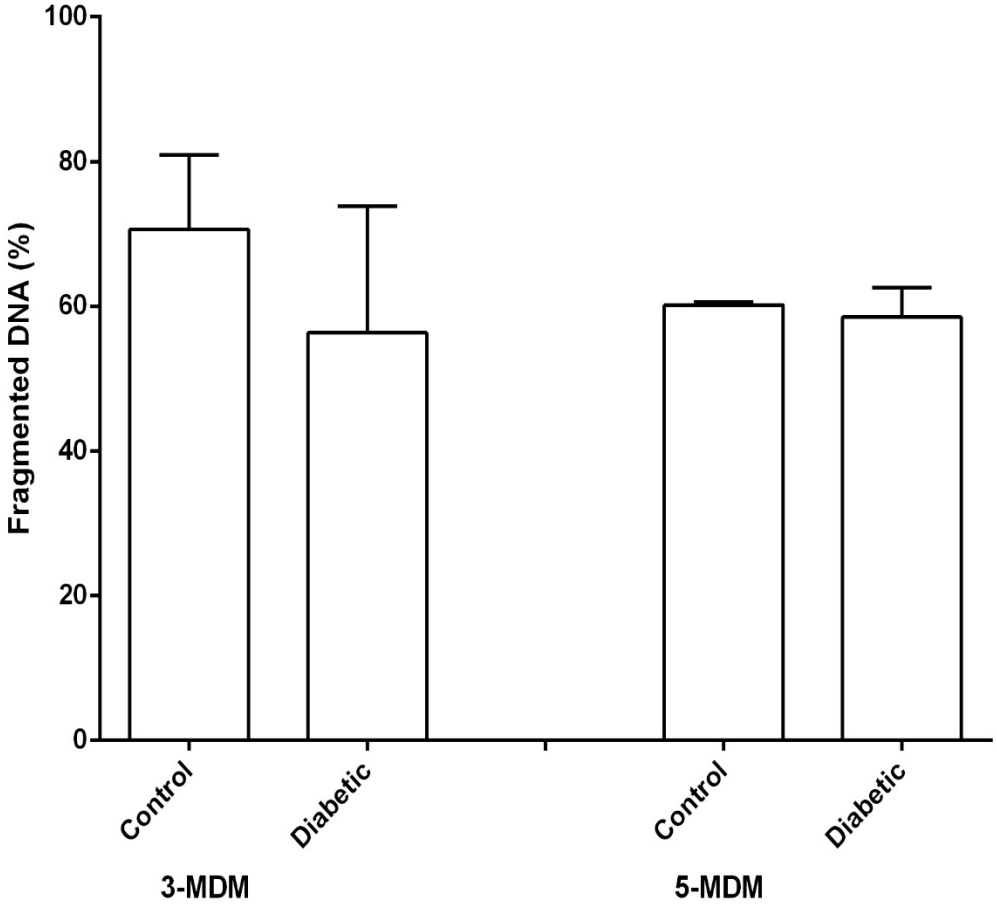


Figure 2.4: The Fragmented DNA in the prostate of Streptozotocin-induced diabetic rats.

Results are stated as Mean ± STD (n=5)

* depicts marked difference ($p<0.05$) in relative to control

3-MDM represents 3 months diabetes mellitus

5-MDM represents 5 months diabetes mellitus

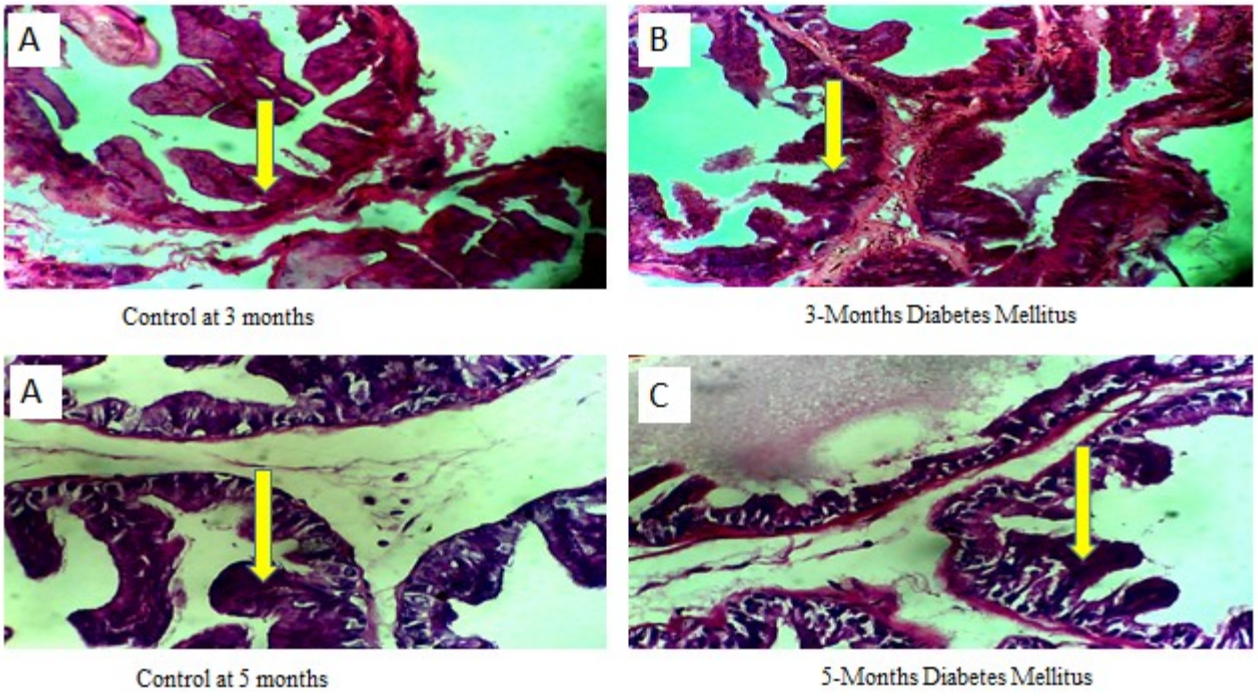


Plate I: Changes in histoarchitecture of prostate gland in Streptozotocin-induced diabetic rats. (Stain: H & E; Mag.: X400)

A = Control at 3 months – yellow arrow showing normal architecture

B = 3-Months Diabetic mellitus – yellow arrow showing distortion, profuse glandular proliferation with budding of epithelia cells

A = Control at 5 months – yellow arrow showing normal architecture

C = 5-Months Diabetic mellitus – yellow arrow showing distortion, mild hyperplasia with intraluminal budding of epithelia cells

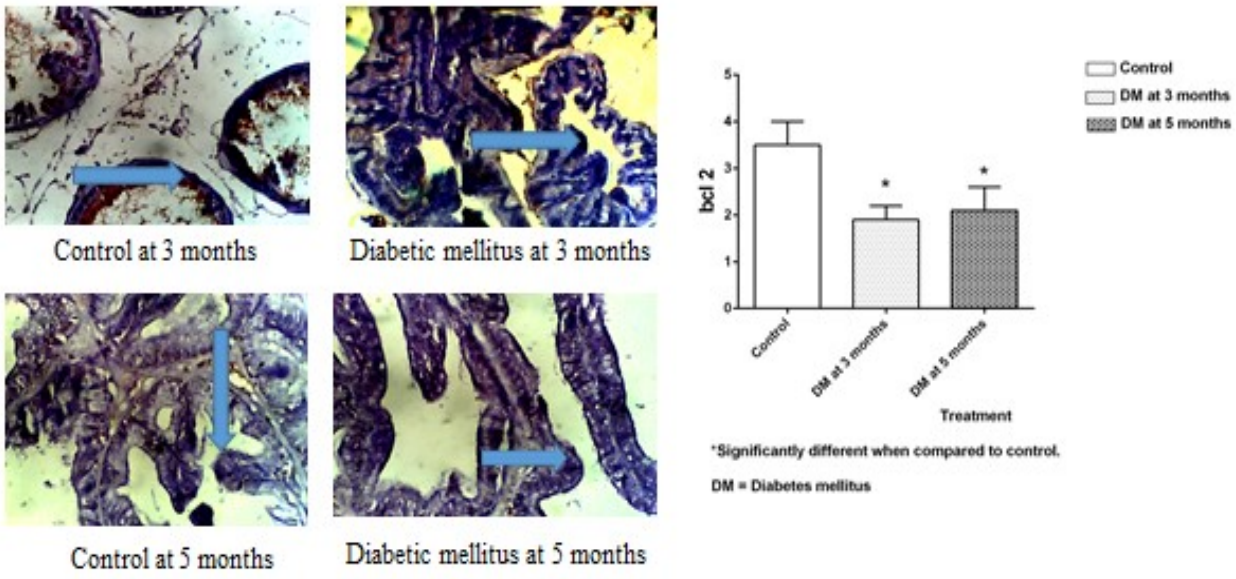


Plate II: Expression of Bcl-2 protein in prostate of Streptozotocin-induced diabetic rats after 3 and 5 months. (Mag. X400)

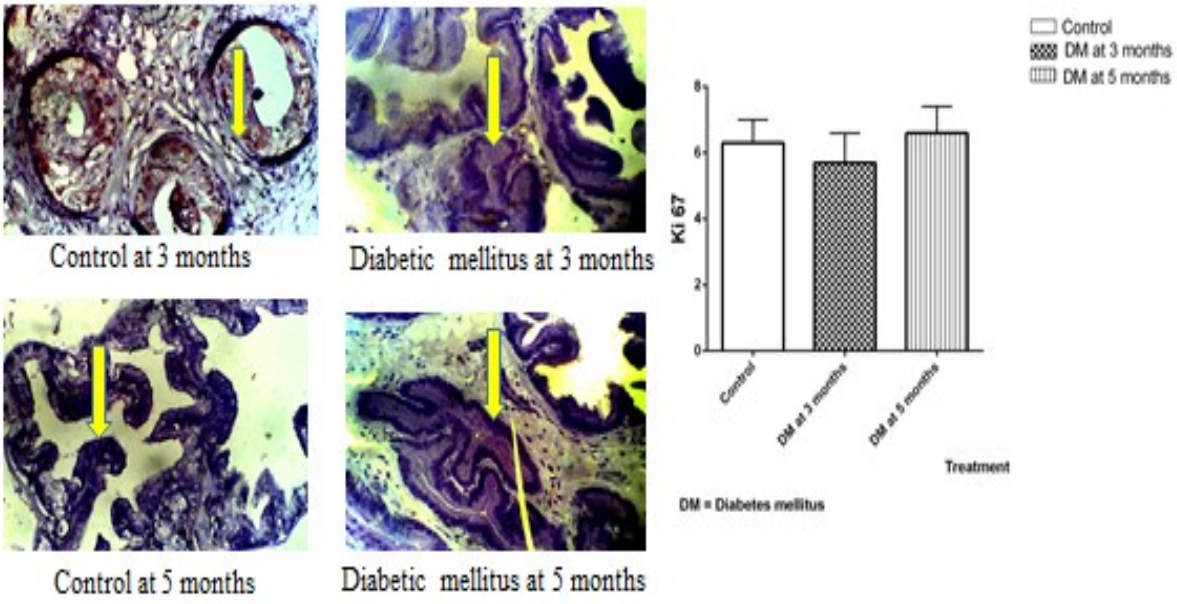


Plate III: Expression of Ki-67 protein in prostate of Streptozotocin-induced diabetic rats after 3 and 5 months. (Mag. X400)

4.2 EXPERIMENT TWO: THE PROTECTIVE ROLE OF KOLAVIRON ON THE PROSTATE OF BPH +DM RATS (COMORBIDITY)

INTRODUCTION

Benign Prostate Hyperplasia depicts a benignant enlargement and unrestrained proliferation of the prostate gland cells. This ailment (BPH) is the 4th highly predominant syndrome among the male populace that are 50 years and above. Amidst the male population worldwide, DM and BPH are dual fundamental ill-health (Jeffrey and Stacy, 2010), that are predominant to a large measure with increasing age. Both conditions were among the 5th highly pervasive syndrome (Issa and Regan, 2007). Although current research from our laboratory showed an antithetical association exists between prostate gland size and DM however, derangements in DM has been implicated in the enlargement of the prostate i.e. there is correlation between diabetes mellitus and BPH (Zongwei and Aria, 2011). One of the stakes in the development of BPH is hyperinsulinemia linked with reduced insulin sensitivity (Nandeesha *et al.,* 2006). It was described by Hammarsten *et al.,* (1998) that BPH may be characterized by the same metabolic aberrations observed in defective glucose homeostasis.

Insulin and other oral hypoglycemic compounds, which are orthodox drugs such as sulfonamides related drugs and metformin, obstruct glucose generation in the hepatocyte and promote the assimilation of peripheral glucose (Kahn *et al.,* 2005), thereby decreasing the possibility of diabetic patients being inflicted with cancer (DeCensi *et al.,* 2010) and possess antineoplastic activity towards various tumors as well as the prostate (Colquhoun *et al.,* 2012). The anti-adrenoceptor (tamsulosin, alfuzosin, doxazosin) and inhibitors of 5 alpha- reductases (finasteride and dutasteride) are utilised in BPH therapy. Nevertheless, a number of adverse effects emanate from these drugs (Kalu *et al.,* 2016) and quest for unconventional treatments or managements of this disease are on the increase. Hence, chemopreventive agents originating from plant origin could serve as medicinal intervention in treating DM and BPH comorbidity.

A dicotyledonous plant, called *Garcinia kola* is commonly propagated all over Central and West Africa and the germs from *Garcinia kola* possess bitter astringent taste; thus, it is usually termed “bitter kola” in Nigeria. It is meant for specific occasions among the Ibo tribes while it serves major function in the Yoruba people’s lives and culture (Denen *et al.,* 2015). Kolaviron, the main biologically active flavonoid (bioflavonoid) compound isolated from *Garcinia kola* (GK) germs (Farombi *et al.,* 2009), has been reported to demonstrate numerous therapeutic activities such as anti-hepatotoxic, anti-inflammatory, antioxidant, and anti-diabetic properties (Abarikwu, 2014). Moreover, kolaviron improves fertility in male rats with DM (Adaramoye and Lawal, 2013). However, the effect of DM and

BPH on the structural and functional integrity of prostate as well as the capability of KV to improve comorbidity-induced prostate impairment in castrated rat model is unknown. Thus, this study was designed to evaluate biochemical and structural modifications in prostate of DM and BPH rats, and protective role of KV in Streptozotocin (STZ) diabetic BPH rats.

PROCEDURE

Twenty rats were assigned to four groups of five animals each.

Group I represented Benign Prostate Hyperplasia [BPH] Control (castrated rats that received Testosterone Propionate (TP))

Group II as Diabetic BPH rats [BPH + DM] (castrated rats that received TP and STZ)

Group III - BPH + DM + KoV (Diabetic BPH rats that received kolaviron, 200 mg/kg)

Group IV - BPH + DM + Met (Diabetic BPH rats that received metformin, 200 mg/kg).

Benign Prostate Hyperplasia was induced by daily administration of testosterone propionate (TP) intraperitoneally (3 mg/kg) to the castrated rats for 28 days. After which Diabetes mellitus was induced by a single administration of Streptozotocin solution intraperitoneally (35 mg/kg).

Kolaviron and Metformin solutions were prepared by dissolving in oil (corn) and water (distilled) respectively and administered orally to the animals daily.

RESULTS

In Table 4, the weights (body) of BPH+DM and BPH+DM+KoV as well as BPH+DM+Met rats, were considerably ($p<0.05$) reduced by 3, 2.5 and 4 folds, respectively, relative to control (BPH) rat. Though, there were insignificant ($p>0.05$) reduction of 22% and 21% ,in the weights (prostate) of BPH+DM and BPH+DM+KoV rats, respectively, relative to control (BPH) rats, however, the prostate weights as well as organo-somatic weights were markedly decreased by 86% and 51%, in BPH+DM+Met rats, respectively, relative to BPH+DM rats. Furthermore, the kidney weights and organo-somatic weights were markedly ($p<0.05$) increased in BPH+DM, BPH+DM+KoV and BPH+DM+Met rats by 15%, 15%, 31% and 24%, 25%, 44%, respectively, relative to control (BPH) rats. Alongside, the weight of kidney in the BPH+DM+Met rats was elevated ($p<0.05$) considerably by 16% relative to BPH+DM rats. Also, no marked differences ($p>0.05$) were observed in the liver weight among the groups of rats. Table 5 shows significant ($p<0.05$) increase in the serum urea concentration in BPH+DM rats by 4 folds, relative to control (BPH) rats, however, insignificant ($p>0.05$) decrease was observed in the serum urea contents of BPH+DM+KoV and BPH+DM+Met rats, relative to BPH+DM rats. Moreover, unconjugated (indirect) and conjugated (direct) bilirubin contents were

markedly ($p<0.05$) elevated by 14 folds and 284%, respectively, in BPH+DM rats, relative to control rats; Conversely, treatments with kolaviron and metformin significantly ($p<0.05$) decreased unconjugated and conjugated bilirubin concentrations by 73%, 38% and 41%, 41%, respectively, relative to BPH+DM rats. Figure 2.5 shows that administration of STZ markedly ($p<0.05$) elevated blood glucose (fasting) level in the BPH+DM, BPH+DM+KoV and BPH+DM+Met rats by 6, 6 and 5 folds, respectively, relative to control (BPH) rats. Conversely , insignificant ($p>0.05$) reduction was observed in blood glucose (fasting) level of BPH+DM+KoV and BPH+DM+Met rats, relative to BPH+DM rats (Figure 2.5). Figures 2.6 and 2.7 show marked ($p<0.05$) elevation in the serum AST and ALT activities of BPH+DM rats by 46% and 66%, respectively, relative to control rats; however, serum AST and ALT activities considerably reduced ($p<0.05$) in the BPH+DM+KoV and BPH+DM+Met rats with percentage reduction of 31%, 44% and 23%, 42%, respectively, relative to BPH+DM rats. Figures 2.8 and 2.9, show that the serum and prostatic total acid phosphatase (TACP) activities of BPH+DM rats elevated considerably ($p<0.05$) by 56% and 14%, respectively, relative to control (BPH) rats. In contrast, the TACP activities in the serum of BPH+DM+KoV and BPH+DM+Met rats and TACP activity in the prostate of BPH+DM+Met rats were markedly ($p<0.05$) decreased, relative to BPH+DM rats. In addition, in Figure 3.0, the ALP activity in the serum was insignificantly ($p>0.05$) increased in BPH+DM rats, relative to control (BPH) rats ; however, the ALP activity in the serum of BPH+DM+Met rats was markedly ($p<0.05$) reduced by 50%, relative to BPH+DM rats. In Figures 3.1 and 3.2, the alkaline phosphatase (ALP) and lactate dehydrogenase (LDH) activities were elevated considerably ($p<0.05$) in the prostate of BPH+DM rats by 44% and 412%, respectively, relative to the control (BPH) rats. Conversely, the activities of prostatic ALP and LDH were markedly ($p<0.05$) reduced by 29% and 89% in BPH+DM+KoV and in BPH+DM+Met rats by 39% and 95%, respectively, relative to BPH+DM rats. In the bone marrow of BPH+DM rats, the micronucleated polychromatic erythrocyte (mnPCE) number was markedly ($p<0.05$) increased by 3 folds, relative to control (BPH) rats (Figure 3.3). Treatment with kolaviron and metformin markedly ($p<0.05$) reduced the number of micronucleated polychromatic erythrocyte (mnPCE) in BPH+DM+KoV and BPH+DM+Met rats by 64% and 56%, respectively, relative to BPH+DM rats. Cross sections of prostate of BPH+DM rats revealed moderate fibroblast with mild infiltration of inflammatory cells as well as mild proliferating columnar secretory cells (Plate IV[BPH+DM]). Administration of kolaviron and metformin (200 mg/kg) each, mitigated the pathological modifications in (Plate IV [BPH+DM+KoV] and IV [BPH+DM+MET]). Also, Cross sections of liver of BPH+DM rats revealed moderate congestion of the portal vein (Plate V[BPH+DM]); however, treatment with kolaviron and metformin (200 mg/kg each) mitigated the pathological alterations in (Plate V[BPH+DM+KoV] and

V[BPH+DM+Met]). Cross sections of kidney of BPH+DM rats revealed infiltration of the interstitial spaces by inflammatory cells as well as glomeruli with mild sclerosis (Plate VI[BPH+DM]); however, administration of kolaviron and metformin (200 mg/kg each) mitigated the pathological modifications (Plate VI[BPH+DM+KoV] and VI[BPH+DM+Met]).

TABLE 4: Effect of kolaviron on animal weight, organ and organo-somatic weight of selected tissues in rats with Diabetes mellitus and Benign Prostate Hyperplasia (Comorbidity)

Treatment	animal weight (g)			weight (organs) (g)			organo-somatic weight (organs) (% body wt)		
	Initial	Final	Wt diff.	Prostate	Liver	kidney	Prostate	Liver	kidney
BPH (Control)	200.00±0.00	218.30±7.63	18.33±7.63	0.35±0.03	6.03±0.49	1.20±0.00	0.16±0.02	2.76±0.15	0.55±0.22
BPH+DM	250.00±0.00	200.00±0.00	-50.00±0.00 [a]	0.28±0.08	7.45±1.76	1.35±0.07 [a]	0.14±0.04	3.73±0.88	0.68±0.04 [a]
BPH+DM+KoV (200 mg/kg)	237.50±5.00	196.25±21.36	-41.25±19.31 [a]	0.28±0.03	6.78±1.54	1.34±0.07 [a]	0.13±0.01	3.54±1.12	0.69±0.08 [a]
BPH+DM+Met (200 mg/kg)	273.33±2.89	210.00±13.23	-63.33±10.41 [a]	0.14±0.02 [a, b]	7.85±0.66	1.57±0.06 [a, b]	0.07±0.02 [a, b]	3.76±1.15	0.75±0.06 [a]

Results are stated as Mean ± STD (n=5)

[a] depicts marked difference ($p<0.05$) compared to control (BPH)

[b] depicts marked difference ($p<0.05$) compared to BPH+DM

Wt diff. = weight difference

BPH (Control) represents Benign Prostate Hyperplasia Control; BPH+DM represents Diabetic BPH rats; BPH+DM+KoV (200 mg/kg) represents Diabetic BPH rats treated with kolaviron (200 mg/kg); BPH+DM+Met (200 mg/kg) represents Diabetic BPH rats treated with metformin (200 mg/kg).

TABLE 5: Effect of kolaviron on the level of creatinine, urea and bilirubin in the serum of diabetic (STZ) BPH rats

Treatment	Creatinine Level	Urea level	Bilirubin level		
			Conjugated	Total	Unconjugated
	(mg/dL)		(mg/dL)		
BPH (control)	1.33±0.50	35.95±10.16	2.12±0.56	1.78±0.42	-0.23±1.09
BPH+DM	1.82±0.12	126.43±17.61 [a]	8.14±1.12 [a]	2.65±0.23	-5.49±0.89 [a]
BPH+DM+KoV (200 mg/kg)	2.17±0.30	123.24±5.85	2.09±0.85 [b]	1.97±0.57	0.31±0.27 [b]
BPH+DM+Met (200 mg/kg)	2.08±0.76	114.50±11.50	5.00±0.66 [b]	1.57±1.17	-3.28±0.94 [b]

Results are stated as Mean ± STD (n=5)

[a] depicts marked difference ($p<0.05$) compared to control (BPH)

[b] depicts marked difference ($p<0.05$) compared to BPH+DM

STZ - Streptozotocin

BPH (control) represents Benign Prostate Hyperplasia Control rats ; BPH+DM represents Diabetic BPH rats; BPH+DM+KoV (200 mg/kg) represents Diabetic BPH rats treated with kolaviron (200 mg/kg); BPH+DM+Met (200 mg/kg) represents Diabetic BPH rats treated with metformin (200 mg/kg).

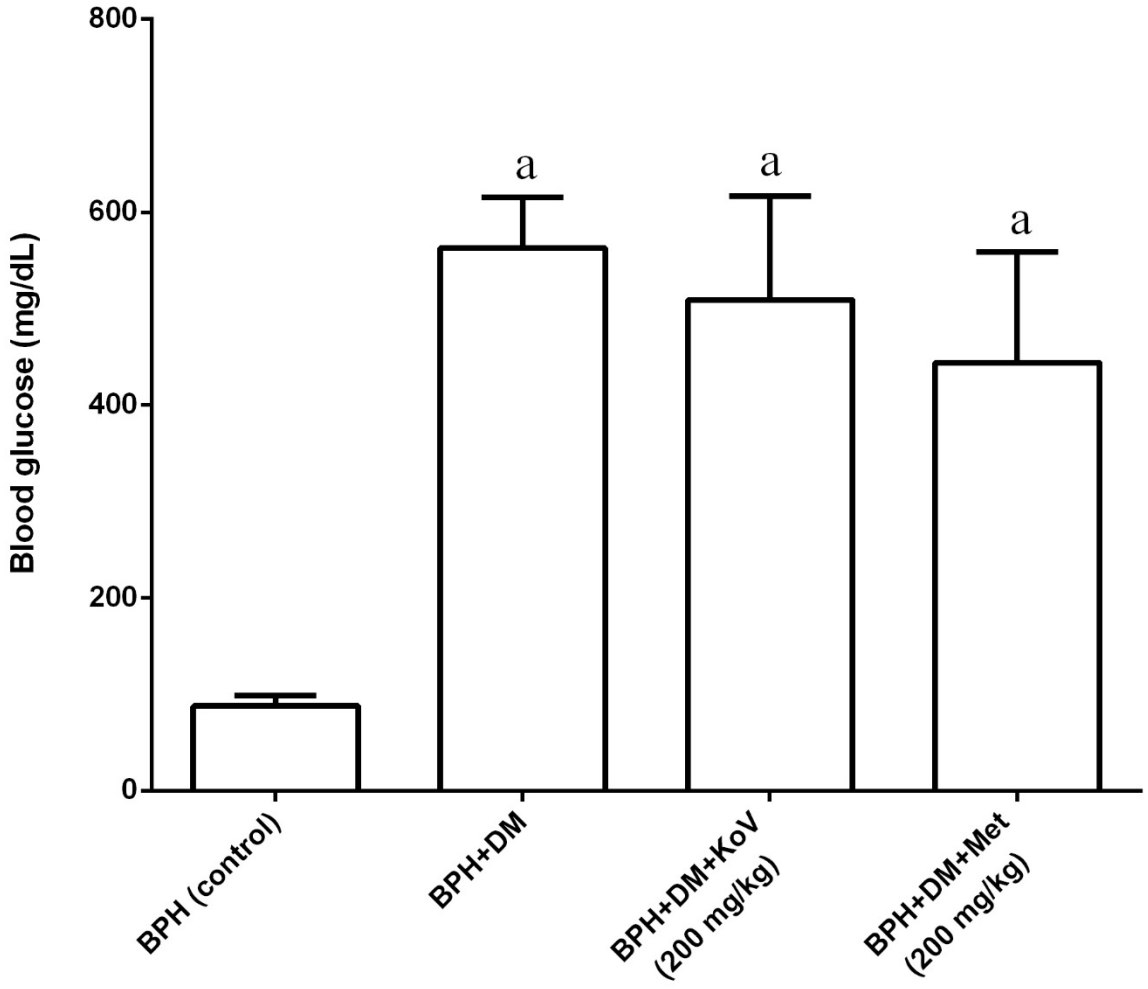


Figure 2.5: Effect of kolaviron on the blood glucose concentration in diabetic (STZ) BPH rats.

Results are stated as Mean ± STD (n=5)

[a] depicts marked difference ($p<0.05$) compared to control (BPH)

STZ - Streptozotocin

BPH (control) represents Benign Prostate Hyperplasia Control rats ; BPH+DM represents Diabetic BPH rats; BPH+DM+KoV (200 mg/kg) represents Diabetic BPH rats treated with kolaviron (200 mg/kg); BPH+DM+Met (200 mg/kg) represents Diabetic BPH rats treated with metformin (200 mg/kg).

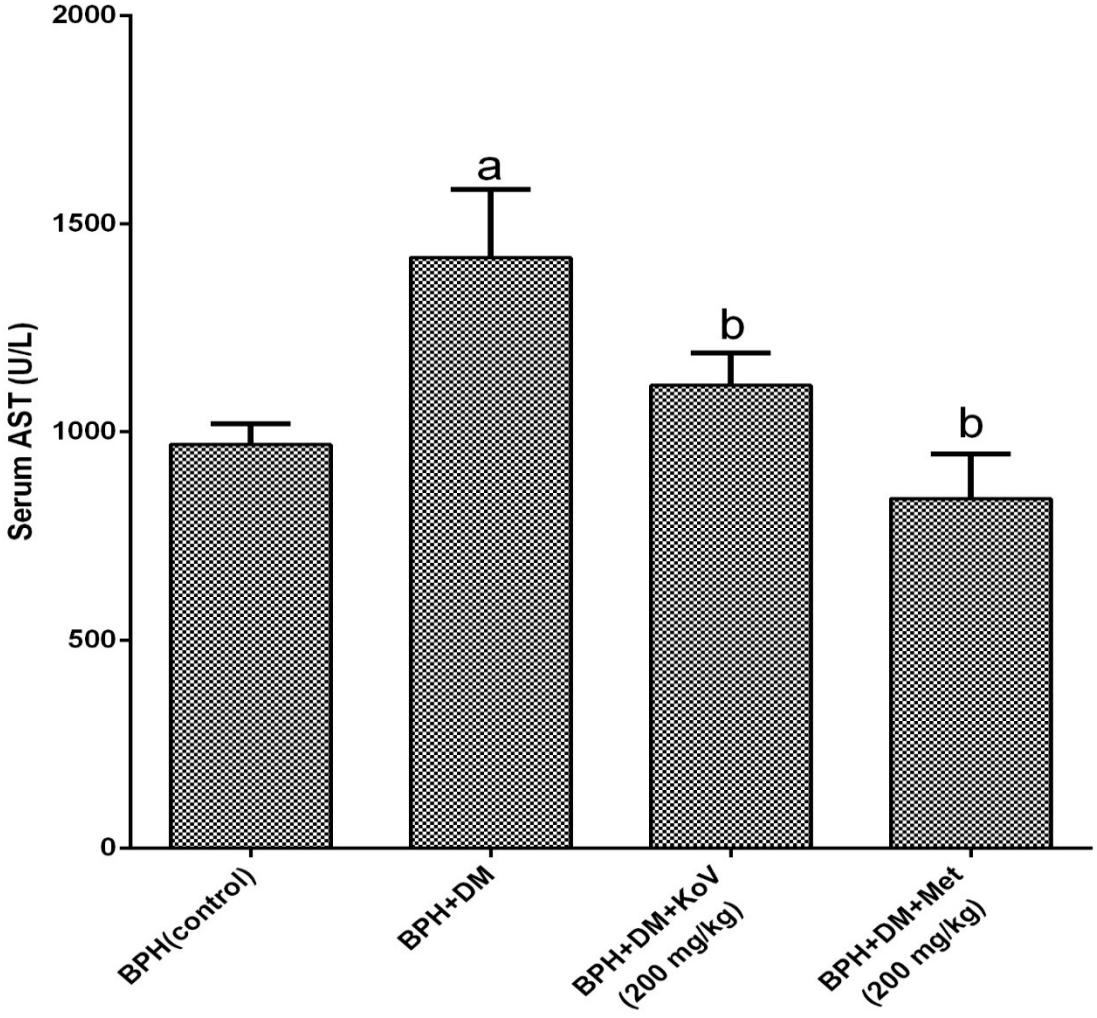


Figure 2.6: Effect of kolaviron on the activity of Serum Aspartate aminotransferase in diabetic (STZ) BPH rats.

Results are stated as Mean ± STD (n=5)

[a] depicts marked difference ($p<0.05$) compared to control (BPH)

[b] depicts marked difference ($p<0.05$) compared to BPH+DM

STZ - Streptozotocin

BPH (control) represents Benign Prostate Hyperplasia Control rats ; BPH+DM represents Diabetic BPH rats; BPH+DM+KoV (200 mg/kg) represents Diabetic BPH rats treated with kolaviron (200 mg/kg); BPH+DM+Met (200 mg/kg) represents Diabetic BPH rats treated with metformin (200 mg/kg).

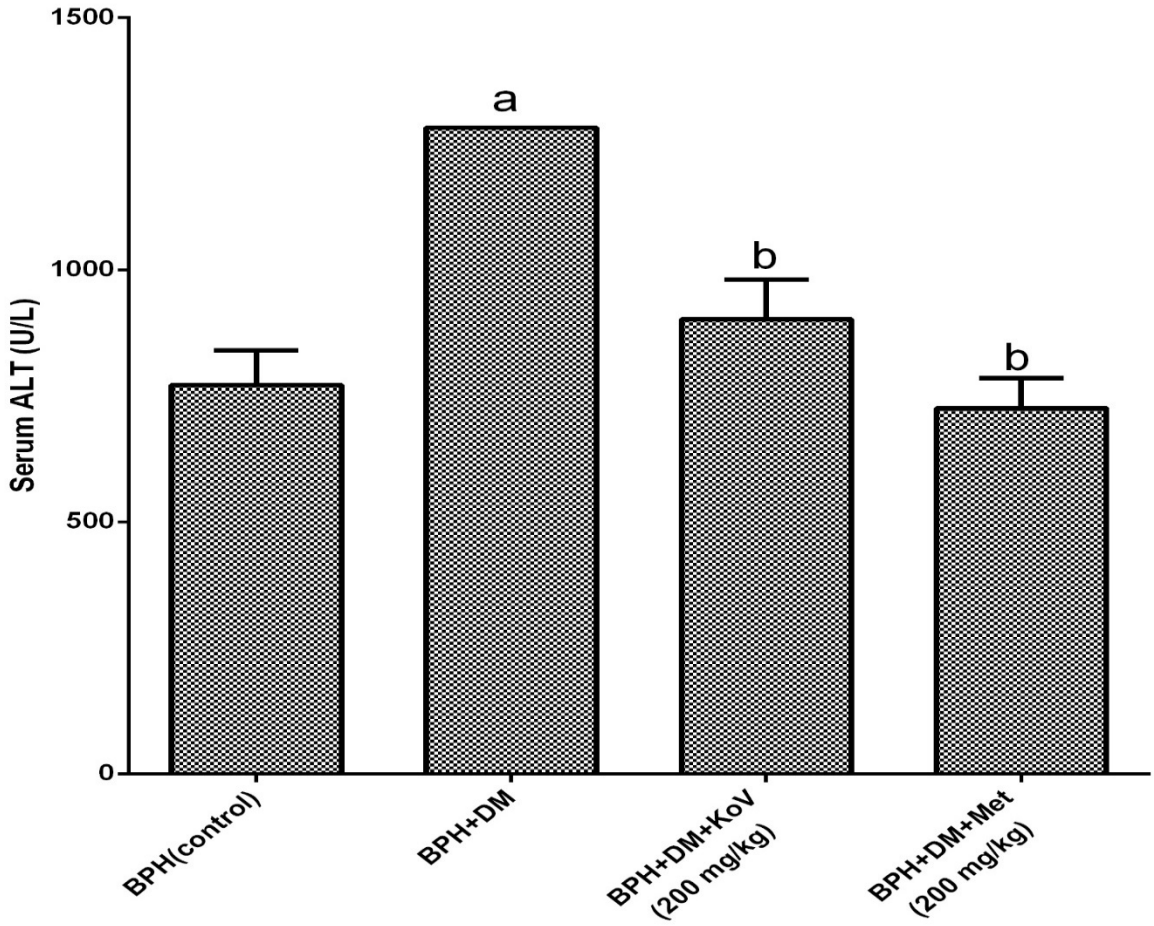


Figure 2.7: Effect of kolaviron on the activity of Serum Alanine aminotransferase in STZ diabetic BPH rats.

Results are stated as Mean ± STD (n=5)

[a] depicts marked difference ($p<0.05$) compared to control (BPH)

[b] depicts marked difference ($p<0.05$) compared to BPH+DM

STZ - Streptozotocin

BPH (control) represents Benign Prostate Hyperplasia Control rats ; BPH+DM represents Diabetic BPH rats; BPH+DM+KoV (200 mg/kg) represents Diabetic BPH rats treated with kolaviron (200 mg/kg); BPH+DM+Met (200 mg/kg) represents Diabetic BPH rats treated with metformin (200 mg/kg).

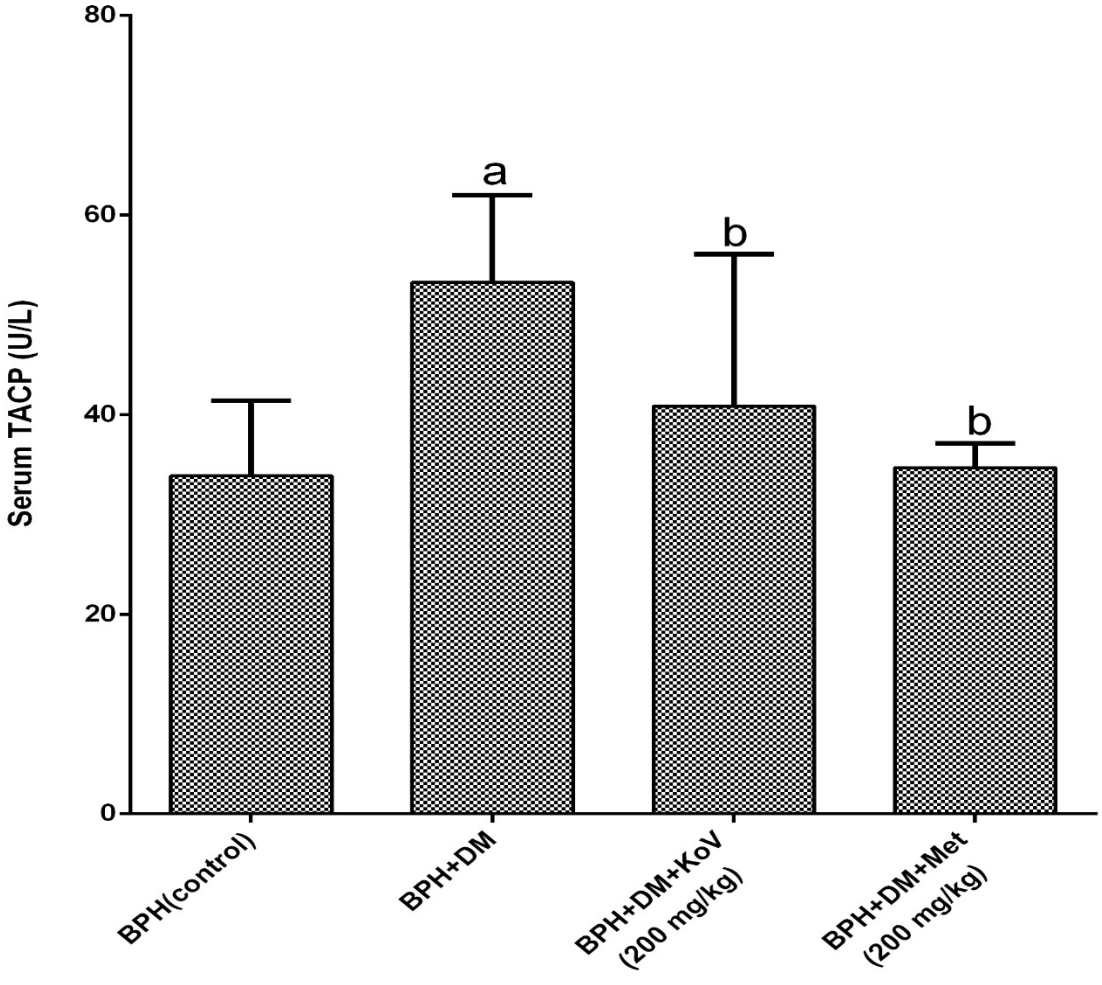


Figure 2.8: Effect of kolaviron on the Serum Total Acid Phosphatase activity in diabetic (STZ) BPH rats.

Results are stated as Mean ± STD (n=5)

[a] depicts marked difference ($p<0.05$) compared to control (BPH)

[b] depicts marked difference ($p<0.05$) compared to BPH+DM

STZ - Streptozotocin

BPH (control) represents Benign Prostate Hyperplasia Control rats ; BPH+DM represents Diabetic BPH rats; BPH+DM+KoV (200 mg/kg) represents Diabetic BPH rats treated with kolaviron (200 mg/kg); BPH+DM+Met (200 mg/kg) represents Diabetic BPH rats treated with metformin (200 mg/kg).

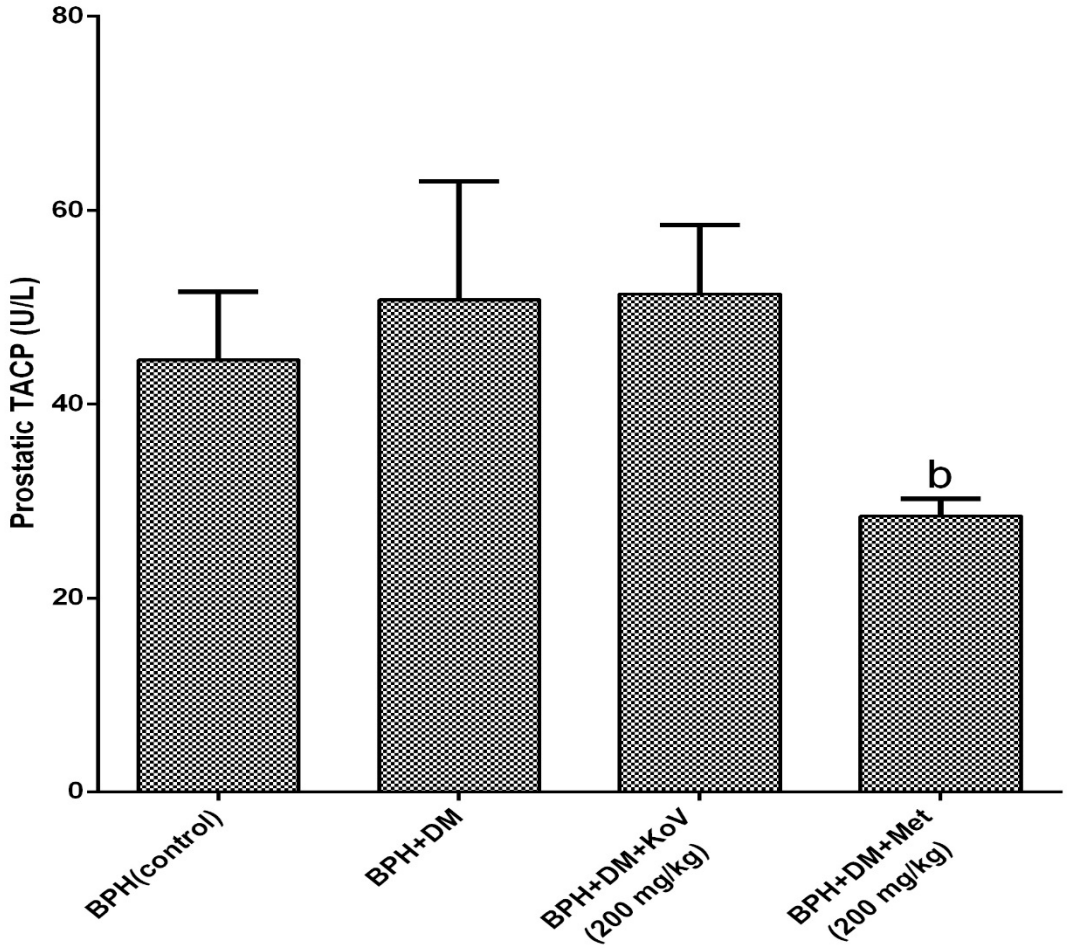


Figure 2.9: Effect of kolaviron on the Prostatic Total acid phosphatase activity in diabetic (STZ) BPH rats.

Results are stated as Mean ± STD (n=5)

[b] depicts marked difference ($p<0.05$) compared to BPH+DM

STZ - Streptozotocin

BPH (control) represents Benign Prostate Hyperplasia Control rats ; BPH+DM represents Diabetic BPH rats; BPH+DM+KoV (200 mg/kg) represents Diabetic BPH rats treated with kolaviron (200 mg/kg); BPH+DM+Met (200 mg/kg) represents Diabetic BPH rats treated with metformin (200 mg/kg).

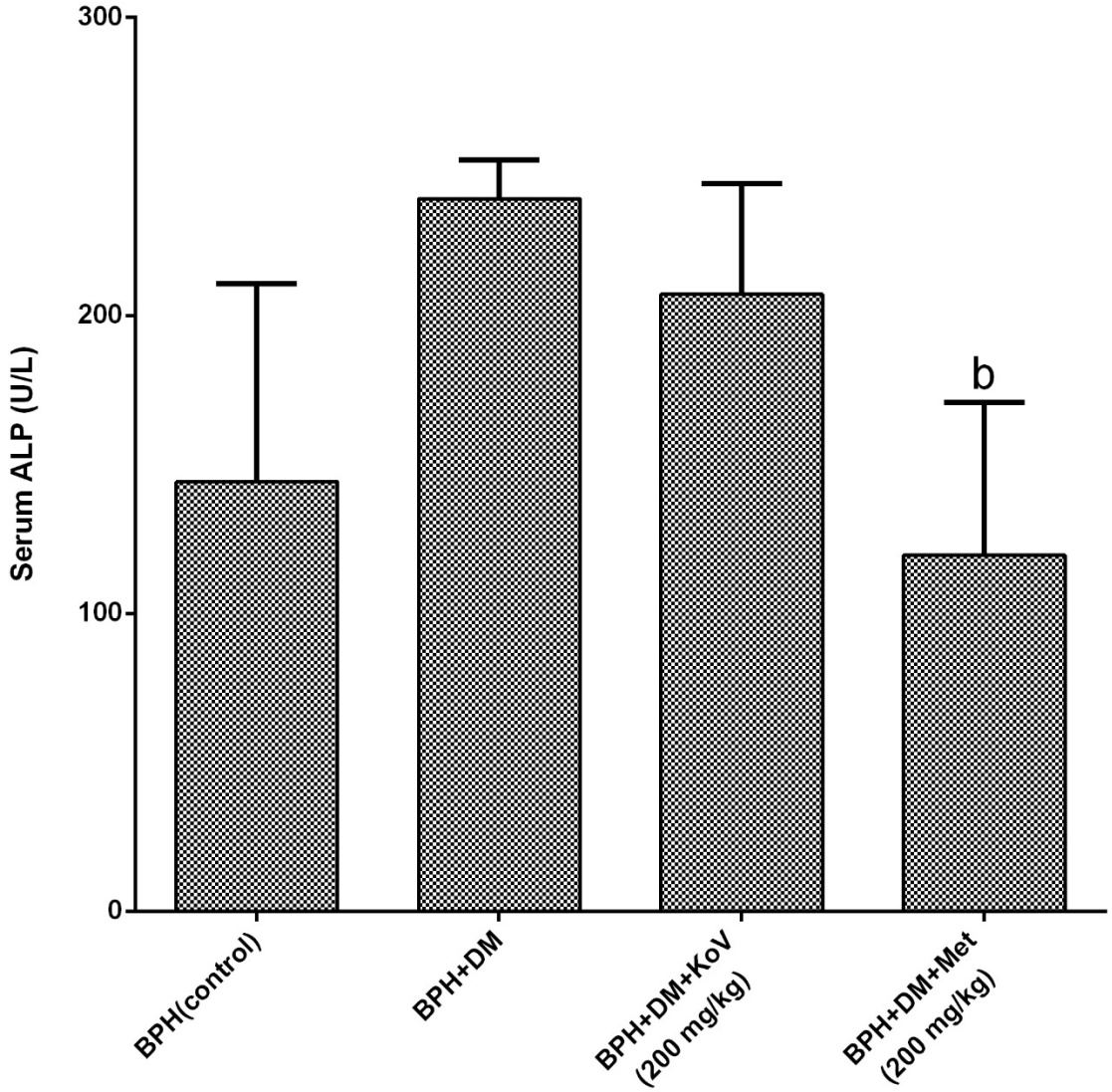


Figure 3.0: Effect of kolaviron on the Serum Alkaline phosphatase activity in diabetic (STZ) BPH rats.

Results are stated as Mean ± STD (n=5)

[b] depicts marked difference ($p<0.05$) compared to BPH+DM

STZ - Streptozotocin

BPH (control) represents Benign Prostate Hyperplasia Control rats ; BPH+DM represents Diabetic BPH rats; BPH+DM+KoV (200 mg/kg) represents Diabetic BPH rats treated with kolaviron (200 mg/kg); BPH+DM+Met (200 mg/kg) represents Diabetic BPH rats treated with metformin (200 mg/kg).

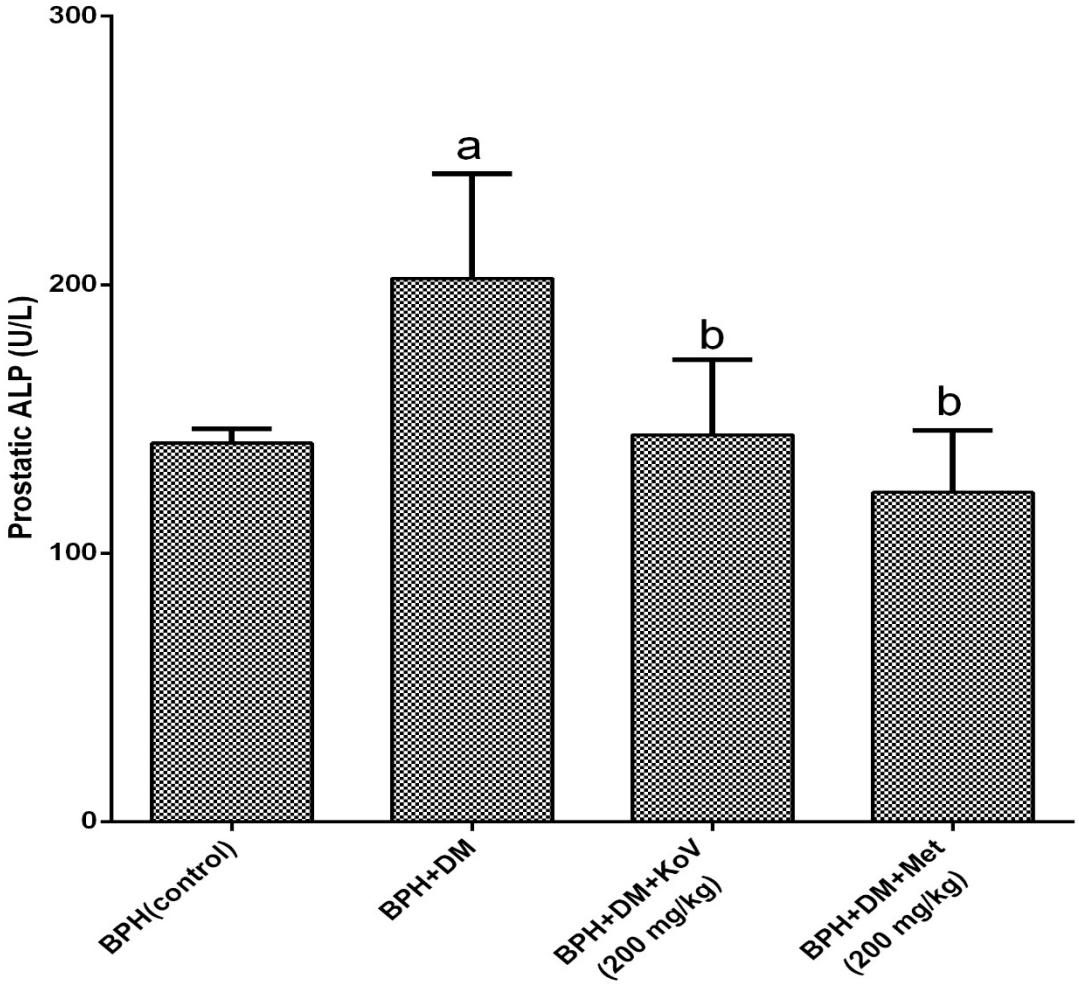


Figure 3.1: Effect of kolaviron on Prostatic Alkaline phosphatase activity in diabetic (STZ) BPH rats.

Results are stated as Mean ± STD (n=5)

[a] depicts marked difference ($p<0.05$) compared to control (BPH)

[b] depicts marked difference ($p<0.05$) compared to BPH+DM

STZ - Streptozotocin

BPH (control) represents Benign Prostate Hyperplasia Control rats ; BPH+DM represents Diabetic BPH rats; BPH+DM+KoV (200 mg/kg) represents Diabetic BPH rats treated with kolaviron (200 mg/kg); BPH+DM+Met (200 mg/kg) represents Diabetic BPH rats treated with metformin (200 mg/kg).

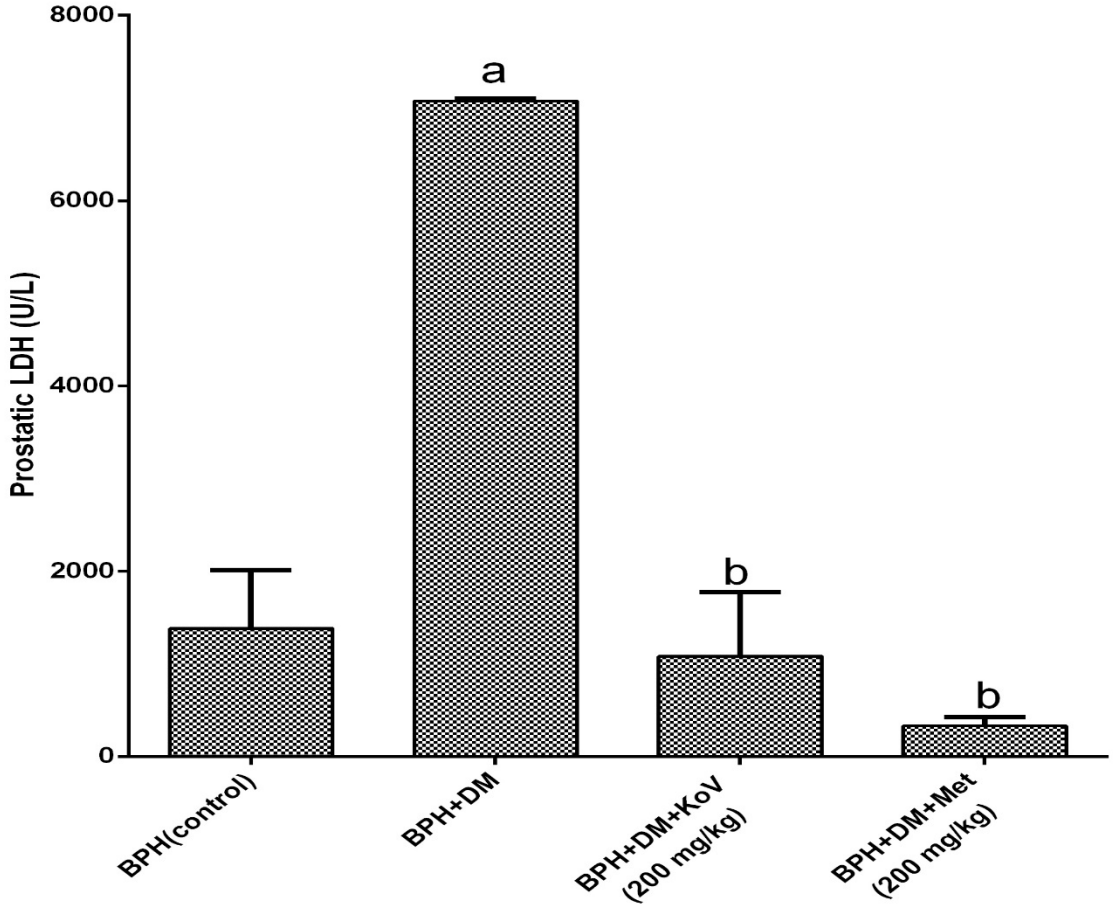


Figure 3.2: Effect of kolaviron on the activity of Prostatic Lactate dehydrogenase in diabetic (STZ) BPH rats.

Results are stated as Mean ± STD (n=5)

[a] depicts marked difference ($p<0.05$) compared to control (BPH)

[b] depicts marked difference ($p<0.05$) compared to BPH+DM

STZ - Streptozotocin

BPH (control) represents Benign Prostate Hyperplasia Control rats ; BPH+DM represents Diabetic BPH rats; BPH+DM+KoV (200 mg/kg) represents Diabetic BPH rats treated with kolaviron (200 mg/kg); BPH+DM+Met (200 mg/kg) represents Diabetic BPH rats treated with metformin (200 mg/kg).

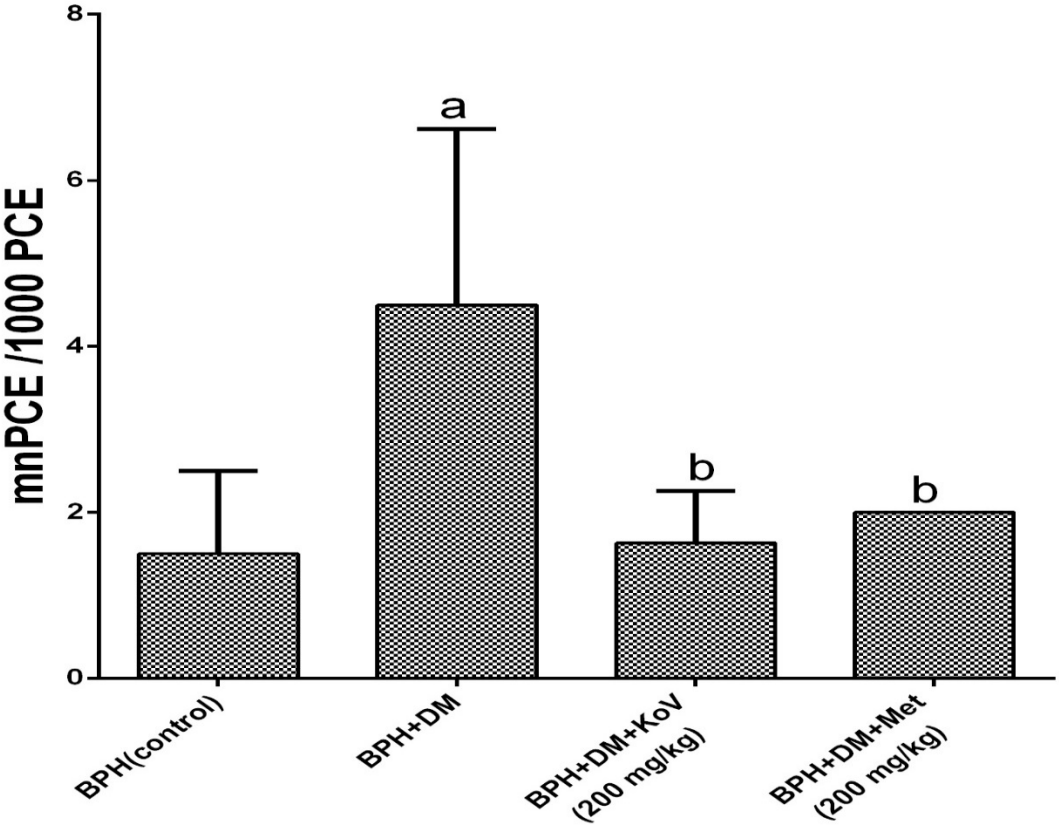


Figure 3.3: Effect of kolaviron on micronucleated polychromatic erythrocytes number in diabetic (STZ) BPH rats.

Results are stated as Mean ± STD (n=5)

[a] depicts marked difference ($p<0.05$) compared to control (BPH)

[b] depicts marked difference ($p<0.05$) compared to BPH+DM

STZ - Streptozotocin

BPH (control) represents Benign Prostate Hyperplasia Control rats ; BPH+DM represents Diabetic BPH rats; BPH+DM+KoV (200 mg/kg) represents Diabetic BPH rats treated with kolaviron (200 mg/kg); BPH+DM+Met (200 mg/kg) represents Diabetic BPH rats treated with metformin (200 mg/kg).

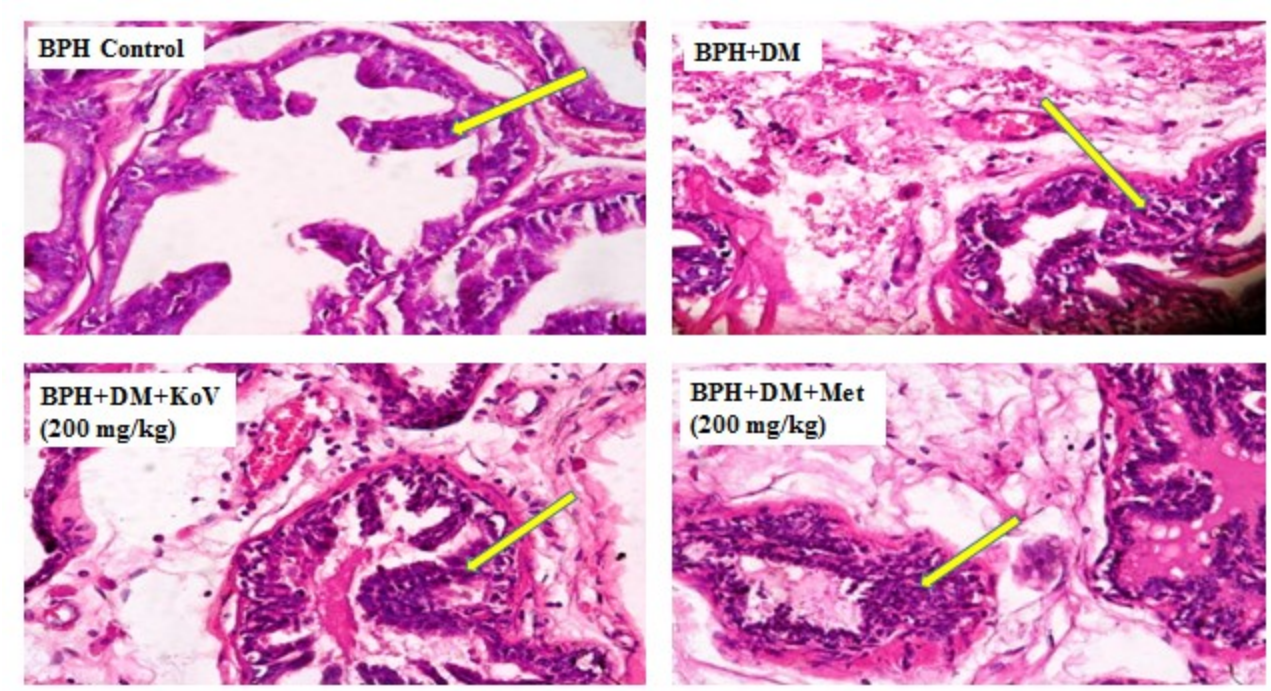


Plate IV: Effect of kolaviron on the histology of prostate sections of diabetic (STZ) BPH rats. (Stain: H&E; Mag. X400)

BPH control – yellow arrow showing normal architecture

BPH+DM – yellow arrow showing moderate fibroblast and mild infiltration of inflammatory cells

BPH+DM+KoV (200 mg/kg) – yellow arrow showing moderate to severe fibrosis and moderate infiltration of inflammatory cells

BPH+DM+Met (200 mg/kg) – yellow arrow showing severe fibrosis and moderate infiltration of inflammatory cells.

STZ - Streptozotocin

BPH (control) represents Benign Prostate Hyperplasia Control rats ; BPH+DM represents Diabetic BPH rats; BPH+DM+KoV (200 mg/kg) represents Diabetic BPH rats treated with kolaviron (200 mg/kg); BPH+DM+Met (200 mg/kg) represents Diabetic BPH rats treated with metformin (200 mg/kg).

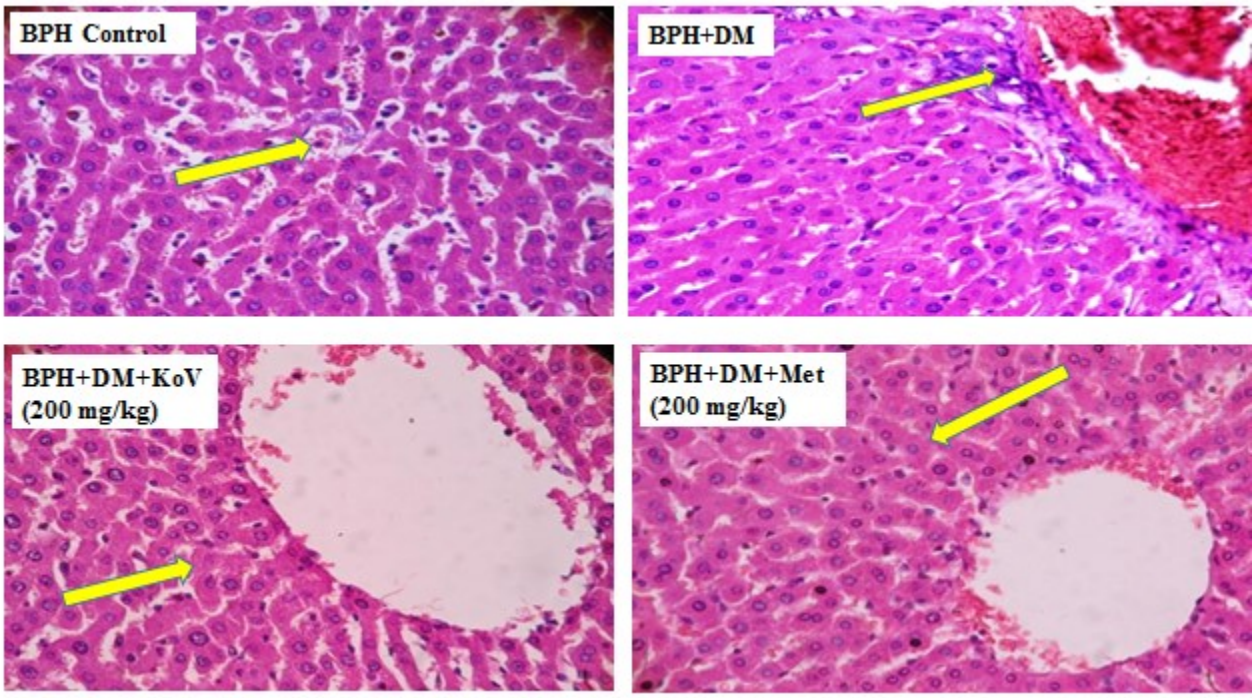


Plate V: Effect of kolaviron on the histology of Liver sections of diabetic (STZ) BPH rats.

(Stain: H&E; Mag. X400)

BPH control – yellow arrow showing mild congestion of the portal vein and scanty infiltration of inflammatory cells.

BPH +DM – yellow arrow showing moderate congestion of the portal vein.

BPH+DM+KoV (200 mg/kg) – yellow arrow showing normal and uninfiltrated sinusoids.

BPH+DM+Met (200 mg/kg) – yellow arrow showing normal and uninfiltrated sinusoids.

STZ - Streptozotocin

BPH (control) represents Benign Prostate Hyperplasia Control rats ; BPH+DM represents Diabetic BPH rats; BPH+DM+KoV (200 mg/kg) represents Diabetic BPH rats treated with kolaviron (200 mg/kg); BPH+DM+Met (200 mg/kg) represents Diabetic BPH rats treated with metformin (200 mg/kg).

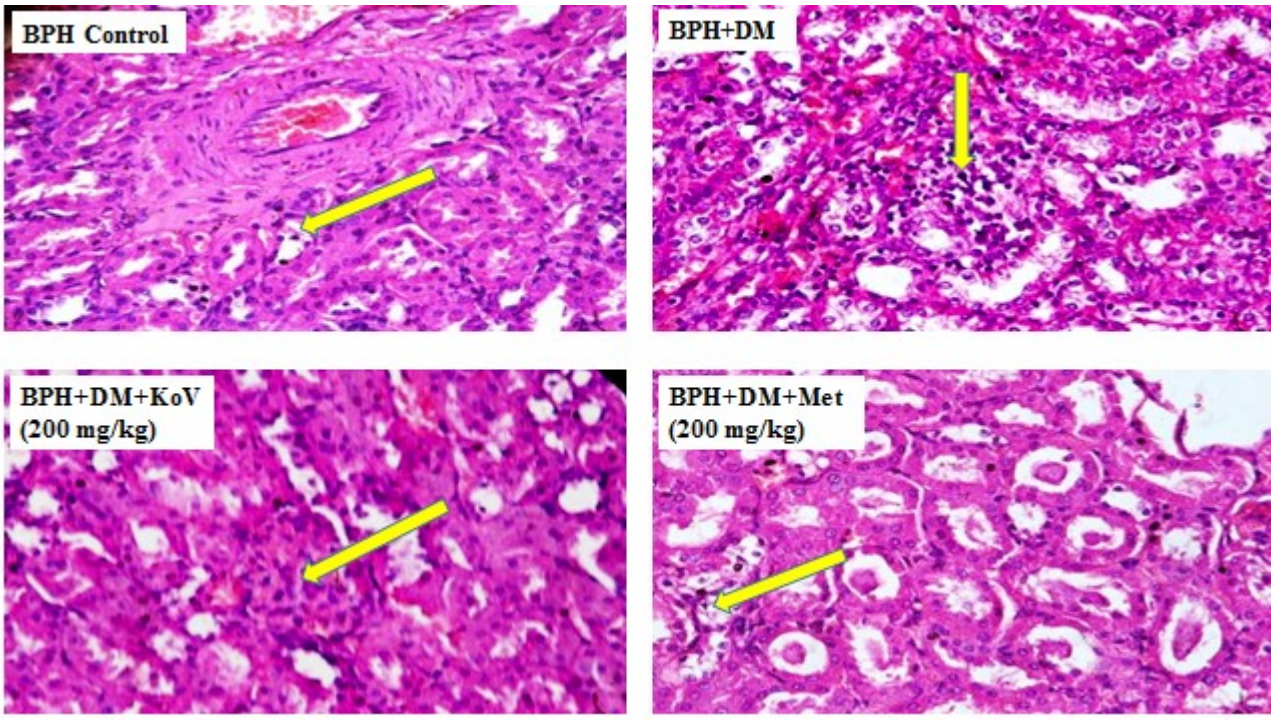


Plate VI: Effect of kolaviron on the histoarchitecture of kidney sections of diabetic (STZ) BPH rats. (Stain: H&E; Mag. X400)

BPH control – yellow arrow showing moderate vascular congestion and normal renal cortex.

BPH+DM – yellow arrow showing mild sclerosis and mild infiltration of inflammatory cell.

BPH+DM+KoV (200 mg/kg) – yellow arrow showing renal cortex exhibiting mesengial hyperplasia, some of the renal tubules are normal while some are attenuated.

BPH+DM+Met (200 mg/kg) – yellow arrow showing area of inflammation with scanty infiltration of inflammatory cells.

STZ - Streptozotocin

BPH (control) represents Benign Prostate Hyperplasia Control rats ; BPH+DM represents Diabetic BPH rats; BPH+DM+KoV (200 mg/kg) represents Diabetic BPH rats treated with kolaviron (200 mg/kg); BPH+DM+Met (200 mg/kg) represents Diabetic BPH rats treated with metformin (200 mg/kg).

CHAPTER FIVE

DISCUSSION, CONCLUSION AND CONTRIBUTION TO KNOWLEDGE

5.1 DISCUSSION

Reproductive activity is suppressed in streptozotocin-induced DM due to oxidative stress generated by hyperglycemia and impairment in the histoarchitecture of male reproductive gland (e.g. testes). Thus, hyperglycaemia, one of the indications of impairments caused by STZ, causes complications on a relatively short and long period of time (Pacher and Szabo, 2005). The resultant effect of extreme ROS (reactive oxygen species) production due to a defect in the activation of the polyol pathway and mitochondrial electron transfer is a consequence of DM-induced hyperglycaemia leading to oxidative stress (Ceriello, 2003). In this study, the level of malondialdehyde (lipid peroxidation products) in the prostate increased in both 3- and 5-months DM, this is in agreement with the account of Chen *et al.,* (2015). Diabetes mellitus related alterations in antioxidant system (both enzymic and non-enzymic) regulate the level of lipid peroxidation. Reduction in endogenous antioxidant throughput heightens DM-related oxidative stress (Pérez-Matute *et al.,* 2009). The activities of enzymic (CAT, SOD and GPx) antioxidants and the level of non-enzymic (GSH) antioxidants were decreased in this study. These observations are in line with the report of Samout *et al.,* (2016) who stated that decrease in the activities of enzymic antioxidants leads to alterations in the redox equilibrium state of cells thereby aggravating oxidative stress. Several investigations centered on the regulation of blood glucose level and general complications of DM such as diabetic neuropathy, skin ulcer, cardiomyopathy etc. (Enomoto *et al.,* 2016). In this study, the control of blood glucose level and diabetes complications were evaluated by the percentage glycated Hb, levels of fasting blood glucose, serum creatinine and urea. Consequentially, the increase observed in micronucleated polychromatic erythrocyte number in the bone marrow of diabetic rats may be due to oxidative stress generated from diabetes and its complication and this is in agreement with the observations of Toneline *et al.,* (2014). Interference in the process of apoptosis in diabetic rats is an indication pointing towards the observed insignificant change in the percentage DNA fragmentation in the prostate of diabetic rats as revealed by this study. Diabetes mellitus has injurious consequence on the fertility of male subjects and the major focus is on testis (Abdelali *et al.,* 2016). Retrograde ejaculation, erectile dysfunction, decrease in testicular hormone and alterations in the quality of semen are consequences of diabetes mellitus. Reduced levels of LH, FSH and testosterone were observed in DM rats at both 3- and 5- months. This result is in line with the abnormal changes observed in testicular and epididymal structure as a result of STZ-induced DM which may be associated with defect in the serum testosterone level as documented by Cai *et al.,* (2000). In addition, the activity of leydig cell may be affected by diabetes leading to decrease in the levels of testosterone. It has been shown that decline in LH levels of diabetic rats may be linked with reduction in

testosterone levels (Kianifard and Kianifard, 2014). In contrast, decline in the production of testosterone may obstruct the growth of male sex accessories as well as prostate gland (Gilad *et al.,* 1998). Moreover, increase in zinc level, altered pH of prostatic fluid as well as reduction in the weight of prostate were observed in this study. In the prostate, these factors imply substantial increase in apoptotic process and decrease in cell proliferation (Melloul *et al.,* 2002), thus confirming marked reduction in Bcl-2 protein expression and mild expression of Ki67 protein in the prostate of diabetic rats. The Bcl-2 protein regulates apoptosis while Ki-67 is a cell proliferation marker. In this study, the histology of prostate revealed hyperplasia as well as distortion in intraluminal budding of prostate epithelia cells.

In consistence with Akanni *et al*. (2017), prostate gland impairment that is directly related to BPH was established in this study via daily intraperitoneal injection of Testosterone propionate to rats which was confirmed by the observed increase in prostate weight of control rats. Presence of hyperglycemia in BPH rats in this study confirmed the induction of experimental diabetes mellitus via intraperitoneal injection of STZ at a low dosage. Also, reduced prostate weight, body weight, and organosomatic weight as well as elevated levels of bilirubin and urea, activities of ACP, ALT and AST in the serum and activities of LDH and ALP in the prostate were demonstrated in diabetic BPH rats in this study. Moreover, histological analysis revealed distortion in the liver, kidney and prostate gland sections.

However, the demonstrated increment in the level of blood glucose in BPH+DM rats was not alleviated in kolaviron and metformin-treated rats. Therefore, the attested hypoglycemic actions of kolaviron and metformin were disproved in the management of DM and BPH comorbidity. Thus, this finding speculates that the capability of kolaviron and metformin to reduce blood glucose level may be reliant on normal prostate gland.

Furthermore, in this study, DM caused body weight loss in BPH which was not alleviated by kolaviron and metformin. One of the essential indicators of BPH is increase in the prostate cell volume (weight) (Mee-Young *et al*., 2012). Decrease in weight of prostate was demonstrated in metformin-treated rats and this finding, established the capability of metformin at the administered dose to contract the prostate tissue. This is in line with the findings of Pais, (2010). Furthermore, increase in the weight of kidney was attributed to a defect that leads to severe diabetic nephropathy (Raju *et al.,* 2001). The development of diabetic nephropathy depends on the extent of hyperglycemia (Doan *et al.,* 2013). The kidney weight in diabetic BPH rats, was heightened in this study and there was no significant change in this kidney pathology in either kolaviron or metformin treated rats compared to BPH+DM rats. The flow of urine can be impeded by the tightening of the urethra which disrupts the draining of urine from the bladder and this was reported to be the consequence of anomalies in prostate gland enlargement (Arora *et al,* 2003). Disruption in urine flow over a long time may later result in bladder calculi, periodic urinary tract

infection (UTI), renal failure, acute urinary retention (AUR) and blood in the urine (Curtis, 2006). Renal dysfunction, one of the problems that emanates from BPH, leads to elevation in the levels of serum creatinine and urea (Curtis, 2006). This dysfunction could also lead to metabolic and electrolyte derangements in rats (Daisy and Feril, 2013). Substantial increase in the level of serum urea in BPH+DM rats was revealed in this study and there was no significant change in this index in either kolaviron or metformin treated rats. Thus, insignificant change observed in the serum level of urea in kolaviron or metformin-treated rats, compared to BPH+DM rats, suggests that either kolaviron or metformin is non-toxic to the kidney. Related reports were given by kalu *et al.,* (2016).

Rise in the level of bilirubin and activities of enzymes in the liver (such as AST, ALT) are usually indicators of mild or severe injury to the hepatocytes. Thus, assessment of these biomarkers are used to diagnose the extent of impairment in the liver of diabetic rats (Hye-Jin *et al.,* 2005). Altered level of liver enzymes and derangements in the metabolism of glucose and lipid are linked to diabetes related hyperglycemia (Daisy and Feril, 2013). Reduced activities of AST and ALT in the serum were observed in kolaviron and metformin treated rats in this study. Modifications in acid phosphatase (ACP) and alkaline phosphatase (ALP) activities, which are clinically and toxicologically crucial, suggest impairment of tissue (liver and prostate) by toxic substances (Daisy and Feril, 2013). Elevation in total ACP and ALP activities, in the present study, in the serum and prostate respectively, were significantly reduced in kolaviron and metformin treated rats. This is in agreement with the observations of Daisy and Feril, (2013) who reported that significant reduction of these enzymes (tACP and ALP) may be as a result of cellular regeneration that leads to prevention of leakage of intracellular enzyme and this suggests the protective ability of kolaviron. Prostate secretes Lactate Dehydrogenase (LDH), which catalyzes the conversion of lactate to pyruvate. Increase in activity of LDH, a pointer to BPH related oxidative stress (Mohammad *et al.,* 2014), an evidence of tissue damage (Devaki *et al.,* 2010), leads to production of more lactate in enlarged prostate cells (Gisselsson and Lerche, 2008). In this study, elevation in LDH activity observed is in consonance with Mohammad *et al.,* (2014) findings. Conversely, drastic decrease in LDH activity was observed in kolaviron and metformin treated rats compared to BPH+DM rats. Furthermore, animals treated with toxic compound have been reported to exhibit elevation in the frequency of mnPCEs in the bone marrow of rats (Natalia *et al.,* 2004). In this study, similar finding was obtained, however, the rate of mnPCEs was markedly decreased in the bone marrow of kolaviron- and metformin treated rats.

Histopathological examinations of the sections of liver, prostate and kidney of diabetic BPH rats showed moderate congestion of the portal vein, mild infiltration of inflammatory cells and mild sclerosis respectively. However, the pathological changes in the kidney and liver sections were mildly improved via treatments with kolaviron and metformin.

5.2 CONCLUSION

Conclusively, significant histological and biochemical modifications demonstrated in both 3 and 5 months DM resulted in reduced reproductive hormones content, prostate weight, downregulated Bcl-2 expression and zinc buildup in prostate. In due course, these derangements may cause infertility in diabetic rats. Also, in the comorbidity of DM with benign prostate hyperplasia, alterations in the histoarchitecture (liver and kidney) and biochemical indices (i.e. reduction in body weight as well as elevation in the conjugated and unconjugated bilirubin levels, activities of total ACP, liver function test parameters in the serum and activities of LDH and ALP in the prostate of diabetic BPH rats were attenuated by kolaviron.

5.3 CONTRIBUTION TO KNOWLEDGE

The study on the protective role of kolaviron on the prostate impairment rats with diabetes mellitus and Benign Prostate Hyperplasia (Comorbidity) has shown the following:

1. Kolaviron ameliorated the biochemical alterations (TACP, LDH and MnPCEs) observed in diabetes mellitus and Benign Prostate Hyperplasia rats (Comorbidity).
2. Kolaviron modified the distortions observed in the histoarchitecture of selected tissues (liver and kidney) in diabetes mellitus and Benign Prostate Hyperplasia rats (Comorbidity).
3. The hypoglycemic property of kolaviron and metformin were disproved in this study and thus, speculated that the hypoglycemic property of kolaviron and metformin may be dependent on intact prostate.
4. Accumulation of prostatic Zn and bicarbonate ions as well as reduction in the level of hormonal profile observed altered the integrity of prostatic fluid which may contribute to infertility of the male diabetic rats.
5. Increase in the activity of prostatic ALP, reduction in TACP and PACP activities in the prostate and decreased expression of Bcl2 and ki67 proteins observed suggest compromised prostate health with no progression of abnormal prostate growth.

Recommendation

Consumption of nutraceuticals e.g. *Garcinia kola* seeds will be beneficial to male population in order to prevent comorbidity of diabetes mellitus and Benign Prostate Hyperplasia.

REFERENCES

Abarikwu S.O. (2014). Kolaviron, a natural flavonoid from the seeds of Garcinia kola, reduces LPS-induced inflammation in macrophages by combined inhibition of IL-6 secretion, and inflammatory transcription factors, ERK1/2, NF-κB, p38, Akt, p-c-JUN and JNK. *Biochim. Biophys. Acta* 1840: 2373–2381.

Abdelali A., Al-Bader M., Kilarkaje N. (2016). Effects of Trans-Resveratrol on hyperglycemia-induced abnormal spermatogenesis, DNA damage and alterations in poly (ADP-ribose) polymerase signaling in rat testis. *Toxicol. Appl. Pharmacol*., 311: 61-73.

Abu-El-Asrar A.M., Dralands L., Missotten L., Al-Jadaan I.A., Geboes K. (2004). Expression of apoptosis markers in the retinas of human subjects with diabetes. *Invest Ophthalmol Vis Sci*. 45:2760-6.

Adam-Vizi V., Seregi A. (1982). Receptor independent stimulatory effect of noradrenaline on Na,K-ATPase in rat brain homogenate. Role of lipid peroxidation. *Biochem Pharmacol*. 31(13):2231-6

Adaramoye O., Amanlou M., Habibi-Rezaei M., Pasalar P. and Moosavi-Movahedi A. (2012). Methanolic extract of African mistletoe (*Viscum album*) improves carbohydrate metabolism and hyperlipidemia in streptozotocin-induced diabetic rats. *Asian Pac. J. Trop. Med*. 5: 427-433.

Adaramoye O., Lawal S. (2013). Effect of kolaviron, a biflavonoid complex from Garcinia kola seeds, on the antioxidant, hormonal and spermatogenic indices of diabetic male rats. *Andrologia* 46 (8): 878-886

Ahmed D., Kumar V., Verma A., Gupta P.S., Kumar H., Dhingra V., Mishra V., Sharma M., (2014). Antidiabetic, renal/ hepatic/pancreas/cardiac protective and antioxidant potential of methanol/dichloromethane extract of Albizia lebbeck Benth. Stem bark (ALEx) on streptozotocin induced diabetic rats. *B.M.C. Complement. Altern. Med*. 14 (1): 214–243.

Akanni O.O., Abiola O.J., Adaramoye O.A. (2017). Methyl Jasmonate ameliorates testosterone propionate-induced prostatic hyperplasia in castrated wistar rats. *Phytother Res.* 31(4):647-656

Akpan H.D., Ekaidem I. (2015). Modulation of Immunological and Hematological Disturbances of Diabetes Mellitus by Diets Containing Combined Leaves of Vernonia amygdalina and Gongronema latifolium. *British Journal of Applied Science and Technology*, 6(5): 534-544.

Alves M.G., Martins A.D., Rato L., Moreira P.I., Socorro S., Oliveira P.F. (2013). Molecular mechanisms beyond glucose transport in diabetes-related male infertility. *Biochimica et Biophysica Acta*. 1832: 626–635.

American Diabetes Association. (2009). Diagnosis and classification of diabetes mellitus. *Diabetes care.* 32(1): 62-7.

Ammerman C.B., Loaiza J.M., Blue W.G., Gamble J.R., Martin F.G.(1974). Mineral composition of tissues from beef cattle under grazing conditions in Panama. *J Anim Sci.* 38: 158–162

Arora R.P., Nayak R.L., Malhotra V., Mohanty N.K., Kulkarni K.S. (2003). Role of herbal drugs in the management of benign prostatic hyperplasia: clinical trial to evaluate the efficacy and safety of Himplasia. *Med. Update* 11: 55–58.

Bansal D., Bhansali A., Kapil G., Undela K., Tiwari P. (2013). Type 2 diabetes and risk of prostate cancer: a meta-analysis of observational studies. *Prostate Cancer and Prostatic Disease*, 16: 151–158.

Bartels, H., Bohmer, M., (1972). *Clin. Chem*. Acta 37: 193.

Bedoya F.J., Solano F., Lucas M. (1996). N‐monomethyl‐arginine and nicotinamide prevent streptozotocin‐induced double strand DNA break formation in pancreatic rat islets. *Experientia* .52 (4):344‐7.

Bedwal R.S., Bahuguna A. (1994). Zinc, copper and selenium in reproduction. *Experientia.* 50: 626–640.

Beutler E., Duron O., Kellin B.M. (1963). Improved method for the determination of blood glutathione. *J.Lab.Clin. Medic*. 61:882-888.

Bhasin S., Enzlin P., Coviello A., Basson R. (2007). Sexual dysfunction in men and women with endocrine disorders. *Lancet*, 369: 597-611.

Bjorndahl L., Kvist U. (2011). A model for the importance of zinc in the dynamics of human sperm chromatin stabilization after ejaculation in relation to sperm DNA vulnerability. *Syst. Biol. Reprod. Med*. 57: 86–92.

Bonovas S., Filioussi1 K., Tsantes A. (2004). Diabetes mellitus and risk of prostate cancer: a meta-analysis. *Diabetologia*, 47:1071–1078.

Brownlee M. (2001). Biochemistry and molecular cell biology of diabetic complications. *Nature*, 414: 813–820.

Cade W.T. (2008). Diabetes-related microvascular and macrovascular diseases in the physical therapy setting. *Physical Therapy*, 88(11): 1322–1335.

Cai L., Chen S., Evans T., Deng D.X., Mukherjee K., Chakrabarti S. (2000). Apoptotic germ-cell death and testicular damage in experimental diabetes, prevention by endothelin antagonism. *Urol. Res.* 28: 342–347.

Caldamone A.A., Freytag M.K., Cockett A.T. (1979). Seminal zinc and male infertility. *Urology*, 13: 280–281

Calton B.A., Chang S.C., Wright M.E., Kipnis V., Lawson K., Thompson F.E., Subar A.F., Mouw T., Campbell D.S., Hurwitz P., Hollenbeck A., Schatzkin A., Leitzmann M.F. (2007). History of diabetes mellitus and subsequent prostate cancer risk in the NIH-AARP Diet and Health Study. *Cancer Causes Control*, 18: 493–503.

Ceriello A. (2003). New insights on oxidative stress and diabetic complications may lead to a "causal" antioxidant therapy. *Diabetes Care*. 26:1589-1596.

Chakravarthi S., Long AS., Hannien B., Pasupati T., Palayan K., Talib A. (2010). The expression of p53 as a reliable immunohistochemical marker of gastric adenocarcinomas. *Res. J. Med. Sci*. 4: 15-19.

Chang Y.C., Chuang L.M. (2010). The role of oxidative stress in the pathogenesis of type 2 diabetes: from molecular mechanism to clinical implication. *Am. J. Transl. Res*. 2: 316–331.

Charonis A.S., Reger L.A., Dege J.E., Kouzi-Koliakos K., Furcht L.T., Wohlhueter R.M., Tsilibary E.C. (1990) Laminin alterations after in vitro nonenzymatic glycosylation. *Diabetes*, 39: 807–814.

Chaturvedi N. (2007). The burden of diabetes and its complications: Trends and implications for intervention. *Diabetes Res. Clin. Pract.* 76(3): S3–S12.

Chen K. C., Sung S.Y., Lin Y.T., Hsieh C.L., Shen K.H., Peng C.C., Peng R.Y. (2015). Benign prostatic hyperplasia complicated with T1DM can be alleviated by treadmill exercise - evidences revealed by the rat model. *B.M.C. Urology*, 15:113. doi 10.1186/s12894-015-0104-8

Cho Y.H., Lee S.J., Lee J.Y., Kim S.W., Lee C.B., Lee W.Y., Yoon M.S. (2002). Antibacterial effect of intraprostatic zinc injection in a rat model of chronic bacterial prostatitis. *Int J. Antimicrob. Agents*. 19: 576–82

Colao A., Marzullo P., Spiezia S., Ferone D., Giaccio A., Cerbone G., Pivonello R., Di Somma C., Lombardi G. (1999). Effect of growth hormone (GH) and insulin-like growth factor I on prostate diseases: an ultrasonographic and endocrine study in acromegaly, GH deficiency, and healthy subjects. *J. Clin. Endocrinol. Metab*.84:1986–91.

Colquhoun A.J. Venier N.A., Vandersluis A.D., Besla R., Sugar L.M., Kiss A., Fleshner N.E., Pollak M., Klotz L.H., Venkateswaran V. (2012). Metformin enhances the antiproliferative and apoptotic effect of bicalutamide in prostate cancer. *Prostate Cancer Prostatic Dis*. 15: 346–352.

Costello L.C., Franklin R.B. (1998). Novel role of zinc in the regulation of prostate citrate metabolism and its implications in prostate cancer. *Prostate,* 35:285–96.

Craven P.A., Davidson C.M., DeRubertis F.R. (1990). Increase in diacylglycerol mass in isolated glomeruli by glucose from de novo synthesis of glycerolipids. *Diabetes.* 39: 667–74.

Curtis N.J. (2006). BPH: costs and treatment outcomes, *Am. J. Manag. Care,* 12: s141–148

Dagher Z., Park Y. S., Asnaghi V., Hoehn T., Gerhardinger C., Lorenzi M. (2004). "Studies of rat and human retinas predict a role for the polyol pathway in human diabetic retinopathy," *Diabetes*, 53:9: 2404–2411.

Dahle S.E., Chokkalingam A.P., Gao Y.T., Deng J., Stanczyk F.Z., Hsing A.W. (2002). Body size and serum levels of insulin and leptin in relation to the risk of benign prostatic hyperplasia. *J. Urol*. 168:599–604.

Daisy P., Feril G. (2013). Hypolipidemic and hepatoprotective effects of *cassia auriculata* linn bark extracts on streptozotocin induced diabetics in male wister albino rats. *Asian J Pharm Clin Res.* 6 (2): 43-48.

DeCensi A., Puntoni M., Goodwin P., Cazzaniga M., Gennari A., Bonanni B., Gandini S. (2010). Metformin and cancer risk in diabetic patients: a systematic review and meta-analysis. *Cancer Prev. Res. (Phila.)* 3:1451–1461.

Denen A., Samuel O.O., Egesie U.G., Joseph E.T. (2015). Effect of ethanolic extract of Garcinia kola seed on some reproductive parameters of male Wister rats. *IOSR J. Pharm*. 5:4–10.

Devaki M., Nirupama R., Yajurvedi H.N. (2010). Repeated Acute Stress Alters Activity of Serum Aminotransferases and Lactate Dehydrogenase in Rat. *JPBS*; 23(2): 1-4.

Dhingra N., Bhagwat D. (2011). Benign prostatic hyperplasia: an overview of existing treatment. *Indian J Pharmacol.* 43: 6–12.

Dinulovic D., Radonjic G. (1990). Diabetes mellitus/male infertility. *Arch. Androl.* 25:277-293.

Doan V. B., Nguyen T. D., Le T.T., Nguyen B. N., Phan V.C. (2013). Macro- and Microvascular Complications of Diabetes Induced by High-Fat Diet and Low-Dose Streptozotocin Injection in Rats Model. *International Journal of Diabetes Research.* 2(3): 50-55. doi: 10.5923/j.diabetes.20130203.03.

Du X., Matsumura T., Edelstein D., Rossetti L., Zsengeller Z., Szabo C., Brownlee M. (2003): Inhibition of GAPDH activity by poly (ADP-ribose) polymerase activates three major pathways of hyperglycemic damage in endothelial cells. *J Clin Invest*.112:1049–1057.

Du X.L., Edelstein D., Rossetti L., Fantus I.G., Goldberg H., Ziyadeh F., Wu J., Brownlee M. (2000). Hyperglycemia-induced mitochondrial superoxide overproduction activates the hexosamine pathway and induces plasminogen activator inhibitor-1 expression by increasing Sp1 glycosylation. *Proc. Natl. Acad. Sci. U. S. A.* 97: 12222–12226.

Elsner M., Guldbakke B., Tiedge M., Munday R., Lenzen S. (2000). Relative importance of transport and alkylation for pancreatic beta‐cell toxicity of streptozotocin. *Diabetologia* , 43 (12):1528‐33.

Englehardt A (1970). Measurement of alkaline phosphatase. *Aerztl Labor*. 16: 42-43.

Enomoto M., Ishizu T., Seo Y., Kameda Y., Suzuki H., Shimano H., Kawakami Y., Aonuma K. (2016). Myocardial dysfunction identified by three-dimensional speckle tracking echocardiography in type 2 diabetes patients relates to complications of microangiopathy. *J. Cardiol*. 68:282-287.

Ezeanyika L.U.S., Ejike C.E.C.C., Obidoa O., Elom S.O. (2006). Prostate disorders in an apparently normal Nigerian population: prevalence, *Biokemistri.* 18:127–132.

Fair W.R., Couch J., Wehner N. (1976): Prostatic antibacterial factor. Identity and significance. *Urology*. 7: 169–77.

Farombi E.O., Tahnteng J.G., Agboola A.O., Nwankwo J.O., Emerole G.O. (2000). Chemoprevention of 2-acetylaminofluorene-induced hepatotoxicity and lipid peroxidation in rats by kolaviron- a Garcinia kola seed extract. *Food Chem. Toxicol*. 38: 535–541.

Farombi E.O., Tahnteng J.G., Agboola A.O., Nwankwo J.O., Emerole G.O. (2000). Chemoprevention of 2-acetylaminofluorene-induced hepatotoxicity and lipid peroxidation in rats by kolaviron- a Garcinia kola seed extract. *Food Chem. Toxicol*. 38: 535–541.

Farombi E.O., Shrotriya S., Surh Y.J. (2009). Kolaviron inhibits dimethyl nitrosamine-induced liver injury by suppressing COX-2 and iNOS expression via NF-kappaB and AP-1. *Life Sci*. 84(5-6):149-55. doi: 10.1016/j.lfs.2008.11.012

Fawcett J. K., Scott J. E. (1960). A rapid and precise method for the determination of urea. *J Clin Pathol.* 13:156-9.

Federici M., Menghini R., Mauriello A., Hribal M.L., Ferrelli F., Lauro D., Sbraccia P., Spagnoli L.G., Sesti G., Lauro R. (2002). Insulin-dependent activation of endothelial nitric oxide synthase is impaired by O-linked glycosylation modification of signaling proteins in human coronary endothelial cells. *Circulation* 106: 466–472.

Feener E.P, Xia P., Inoguchi T., Shiba T., Kunisaki M., King G.L. (1996). Role of protein kinase C in glucose and angiotensin II-induced plasminogen activator inhibitor expression. *Contrib Nephrol*. 118:180–187.

Feng P., Li T., Guan Z., Franklin R.B., Costello L.C. (2008). The involvement of Bax in zinc-induced mitochondrial apoptogenesis in malignant prostate cells. *Mol. Cancer*. 7:25–30.

Fishman W.H., Lerner F.A. (1953). Method for estimating serum acid phosphatase of prostatic origin. *J Biol Chem*.200: 89–97.

Fitzgibbons P.L., Page D.L., Weaver D., Thor A.D., Allred D.C., Clark G.M., Ruby S.G., O'Malley F., Simpson J.F., Connolly J.L., Hayes D.F., Edge S.B., Lichter A., Schnitt S.J. (2000). Prognostic factors in breast cancer. College of American Pathologists Consensus Statement 1999. *Arch. Pathol. Lab. Med.* 124 (7):966–978.

Fong D.S., Aiello L., Gardner T.W., King G.L., Blankenship G., Cavallerano J.D., Ferris F.L. 3rd, Klein R. (2004). Retinopathy in diabetes. *Diabetes Care*; 27(1): S84–87.

Franklin R.B., Costello L.C. (2007). Zinc as an anti-tumor agent in prostate cancer and in other cancers. *Arch. Biochem. Biophys*. 463:211–7.

Gabbay K. H. (1975). Hyperglycemia, polyol metabolism, and complications of diabetes mellitus. *Annual Review of Medicine,* 26: 521–536.

Galkowska H., Olszewsk W.L., Wojewodzka U., Mijal J., Filipiuk E. (2003). Expression of apoptosis- and cell cycle -related proteins in epidermis of venous leg and diabetic foot ulcers. *Surgery*, 134:213-20

Gavin J.R., Alberti K.G.M.M., Davidson M.B., Defronzo R.A., Drash A., Gabbe S.G., Genuth S., Harris M.I., Kahn R., Keen H., Knowler W.C., Lebovitz H., Mclaren N.K., Palmer J.P., Raskin P., Rizza R.A.,Stern M.P. (2003). Report of the Expert Committee on the Diagnosis and Classification of Diabetes Mellitus. *Diabetes care,* 26: S5-S20.

Gerdes J., Lemke H., Baisch H., Wacker H.H., Schwab U., Stein H. (1984). Cell cycle analysis of a cell proliferation-associated human nuclear antigen defined by the monoclonal antibody Ki-67. *J. Immunol*. 133:1710–1715.

Giacco F., Brownlee M. (2010). Oxidative stress and diabetic complications. *Circ. Res.*107: 1058–1070.

Gilad E., Laudon M., Matzkin H., Zisapel N. (1998). Evidence for a local action of melatonin on the rat prostate. *J. Urol.* 159: 1069–1073.

Gisselsson A., Lerche M. (2008). Metabolic activity of diseased and healthy prostate cells investigated as lactate formation from hyperpolarized 13Cpyruvate. *Proc. Intl. Soc. Mag. Reson. Med.* 16: 251.

Gomez E., Buckingham D.W., Brindle J., Lanzafame F., Irvine D.S., Aitken R.J. (1996). Development of an image analysis system to monitor the retention of residual cytoplasm by human spermatozoa: correlation with biochemical markers of the cytoplasmic space, oxidative stress, and sperm function. *J. Androl.* 17:276–287.

Gornal, A. G., Bardawill C. J., David M. M. (1949). Determination of serum protein by means of Biuret reaction. *J Biol. Chem.,* 177: 571-576.

Habig W.H., Pabst M.J., Jakoby W.B. (1974). Glutathione-S-transferases. The first enzymatic step in mercapturic acid formation. *J. Biol. Chem.* 249: 7130-7139.

Haddle, J. A. and M. F. Salamone, (1981). The micronucleus assay 1. *In vivo* in Stich H. F. and R. H. San, editors. Topic in Environment Physiology and Medicine. *Short Term Test for Chem Carcinogens*. Heidelberg, Berlin: Springer-Verlag. Page 243-249.

Hakim L.S., Goldstein I. (1996). Diabetic sexual dysfunction. *Endocrinol. Metab. Clin. North. Am*. 25:379–400.

Hammarsten J., Hogstedt B. (2001). Hyperinsulinaemia as a risk factor for developing benign prostatic hyperplasia. *Eur Urol*; 39:151–8.

Hammarsten J., Hogstedt B., Holthuis N., Mellstrom D. (1998). Components of the metabolic syndrome—risk factors for the development of benign prostatic hyperplasia. *Prostate Cancer and Prostatic Diseases* 1:157–162

Hasnan J., Yusof M.I., Damitri T.D., Faridah A.R., Adenan A.S., Norbaini T.H. (2010). Relationship between apoptotic markers (Bax and Bcl-2) and biochemical markers in type 2 diabetes mellitus. *Singapore Med J*. 51(1): 50-55.

Hense H.W., Kajüter H., Wellmann J., Batzler W.U. (2011). Cancer incidence in type 2 diabetes patients - first results from a feasibility study of the D2C cohort. *Diabetol. Metab. Syndr*. 3: 15.

Ho C.K.M., Habib F.K. (2011). Estrogen and androgen signaling in the pathogenesis of BPH. *Nat. Rev. Urol.* 8: 29–41.

Ho F.M., Liu S.H., Liau C., Huang P.J., Lin-Shiau S.Y. (2000). High glucose -induced apoptosis in human endothelial cells is mediated by sequential activations of c-Jun NH(2)-terminal kinase and caspase-3. *Circulation*. 101:2618-24.

Huggins C., Neal W. (1942). Coagulation and liquefaction of semen: proteolytic enzymes and citrate in prostatic fluid. *J. Exp. Med*. 76: 527–541.

Hye-Jin H., Sang – W., Jong – M., Ji – H., Hyun – O., Hyun – M., Jong – W. (2005). Hypoglycemic effect of crude epoxypolysaccharides produced by a medicinal mushroom *Phellinus baumii* in Streptozotocin induced diabetic rats. *Life sci.* 76: 3069-3080.

Ikeda K., Wada Y., Foster H.E. Jr., Wang Z., Weiss R.M., Latifpour J. (2000). Experimental diabetes induced regression of the rat prostate is associated with an increased expression of transforming growth factor-beta. *J. Urol.* 164:180–185.

Issa M.M., Regan T.S. (2007). Medical therapy for benign prostatic hyperplasia–present and future impact. *Am. J. Manag. Care*.13: S4–9.

Iwu M.M., Igbok O.A., Okunji C.O., Tempesta M.S. (1990). Antidiabetic and aldose reductase activities of biflavanones of *Garcinia Kola*, *J. Pharm. Pharmacol*. 42: 290–292.

Jackson F.L., Hutson J.C. (1984). Altered responses to androgen in diabetic male rats. *Diabetes*, 33: 819–824.

Jeffrey K. Mullins & Stacy Loeb. (2010). Diabetes mellitus and prostate cancer risk. *Expert Review of Endocrinology & Metabolism*, 5(6): 787-789.

Jendrassik L., Grof P. (1938). Simplified photometric method for the determination of blood bilurubin. *Biochem. Z.* 297: 81

Kahn B.B., Alquier T., Carling D., Hardie D.G. (2005). AMP-activated protein kinase: ancient energy gauge provides clues to modern understanding of metabolism. *Cell Metab*. 1: 15–25.

Kalu W. O., Okafor P. N., Ijeh I. I., Eleazu C. (2016). Effect of kolaviron, a biflavanoid complex from *Garcinia kola* on some biochemical parameters in experimentally induced benign prostatic hyperplasic rats. *Biomedicine & Pharmacotherapy,* 83: 1436–1443.

Kasper J.S., Giovannucci E. (2006): A Meta-analysis of Diabetes Mellitus and the Risk of Prostate Cancer. *Cancer Epidemiol. Biomarkers Prev*. 15: 2056-2062.

Kavanagh J.P. (1994). Sodium, potassium, calcium, magnesium, zinc, citrate and chloride content of human prostatic and seminal fluid. *Journal of reproduction & Fertility.* 75: 35-41.

Ketelslegers J.M., Maiter D., Maes M., Underwood L.E., Thissen J.P. (1996). Nutritional regulation of the growth hormone and insulin-like growth factor-binding proteins. *Horm Res.* 45: 252–7.

Kianifard D., Kianifard L. (2014). Effects of Morus Alba Extract on the Microscopic Changes of Spermatogenesis in Experimentally Induced Diabetes Mellitus in Adult Rats. *Medicine Science* 3(3):1491-506. doi: 10.5455/medscience. 2014.03.8151

Kroncke K.D., Fehsel K., Sommer A., Rodriguez M.L., Kolb‐Bachofen V. (1995). Nitric oxide generation during cellular metabolization of the diabetogenic N‐methyl‐N‐nitroso‐urea streptozotozin contributes to islet cell DNA damage. *Biol. Chem. Hoppe Seyler*. 376 (3):179‐85.

Landsberg L. (1986). Diet, obesity and hypertension: an hypothesis involving insulin, the sympathetic nervous system and adaptive thermogenesis. *Q J Med*. 61: 1081–90

Lehrer S., Diamond E.J., Stagger S., Stone N.N., Stock R.G. (2002). Increased serum insulin associated with increased risk of prostate cancer recurrence. *Prostate*, 50: 1–3.

Li Y.M., Mitsuhashi T., Wojciechowicz D., Shimizu N., Li J., Stitt A., He C., Banerjee D., Vlassara H. (1996). Molecular identity and cellular distribution of advanced glycation endproduct receptors: relationship of p60 to OST-48 and p90 to 80K-H membrane proteins. *Proc. Natl. Acad. Sci. U. S. A.* 93: 11047–11052.

Lowe F.C., McConnell J.D., Hudson P.B., Romas N.A., Boake R., Lieber M., Elhilali M., Geller J., Imperto-McGinely J., Andriole G. L., Bruskewitz R.C., Walsh P., Bartsch G., Nacey J. N., Shah S., Pappas F., Ko A., Cook T., Stoner E., Waldstreicher J. (2003). Long-term 6-year experience with finasteride in patients with benign prostatic hyperplasia. *Urology.* 61: 791–796.

MacLean H.E., Warne G.L., Zajac J.D. (1997). Localization of functional domains in the androgen receptor. *J Steroid Biochem. Mol. Biol.* 62: 233–42.

Marconi M., Pilatz A., Wagenlehner F., Diemer T., Weidner W. (2009). Impact of infection on the secretory capacity of the male accessory glands. *Int. Braz. J. Urol*. 35: 299–308.

Marks L.S. (2004). 5alpha-reductase: History and clinical importance. *Rev Urol.* 69: S11–21.

Mawson C. A. and Fischer M. I. (1952). The Occurrence of Zinc in the Human Prostate Gland. *Can. J. Med. Sci.* 30: 336 – 339.

McLennan S.V., Heffernan S., Wright L., Rae C., Fisher E., Yue D.K., Turtle J.R., (1991). Changes in hepatic glutathione metabolism in diabetes. *Diabetes*. 40 (3): 344–348.

Mee-Young L., In-Sik S., Chang-Seob S., Nam-Hun L., Hye-Kyung H., Jong-Keun S.,Hyeun-Kyoo S.(2012). Effects of *Melandrium firmum* methanolic extract on testosterone-induced benign prostatic hyperplasia in Wistar rats. *Asian J. Androl.* 14: 320–324

Melloul D., Marshak S., Cerasi E. (2002). Regulation of insulin gene transcription. *Diabetologia.* 45: 309–326.

Meyer K., Deutscher J., Anil M., Berthold A., Bartsch G., Kiess W. (2000). Serum androgen levels in adolescents with type I diabetes: relationship to pubertal stage and metabolic control. *J. Endocrinol. Invest*. 23: 362–368.

Misra H.P., Fridovich I. (1972). The role of superoxide anion in the autooxidation of epinephrine and a simple assay for superoxide dismutase. *J Biol Chem*. 247: 3170– 5.

Mohammad A.J., Mohammad R.A., Rafal A.L. (2014). Biochemical Changes in Patients with Benign Prostate Hyperplasia. *Journal of Babylon University Pure and Applied Sciences* 22: 9

Mohammad H. B., Arif T. J., Gulam R., Khurshid A., Jalaluddin M. A., Taeyeon K., Han S. M., Yong H., L., Won-Kyung C., Jin Y. M., Eun J., L., Inho C. (2017). Methylglyoxal and Advanced Glycation End products: Insight of the regulatory machinery affecting the myogenic program and of its modulation by natural compounds. *Sci Rep*. 7: 5916. doi: 10.1038/s41598-017-06067-5

Morgan N.G., Cable H.C., Newcombe N.R., Williams G.T. (1994). Treatment of cultured pancreatic B‐cells with streptozotocin induces cell death by apoptosis. *Biosci Rep*. 14 (5):243‐50.

Nandeesha H., Koner B.C., Dorairajan L.N., Sen S.K. (2006). Hyperinsulinemia and dyslipidemia in non-diabetic benign prostatic hyperplasia. *Clin. Chim. Acta*. 370: 89–93.

Natalia K., Irena S., Jaroslav L., L'ubomír L., Marcel F., Juraj P., Gabriel K. (2004). Induction of micronuclei in rat bone marrow after subchronic inhalation exposure to mixture of benzene, cyclohexanone and cyclohexane. *Bull. Vet. Inst. Pulawy*. 48:341-345

Nukatsuka M., Yoshimura Y., Nishida M., Kawada J. (1990). Importance of the concentration of ATP in rat pancreatic beta cells in the mechanism of streptozotocin‐induced cytotoxicity. *J. Endocrinol*. 127 (1):161‐5.

Owen D.H., Katz D.F. (2005). A review of the physical and chemical properties of human semen and the formulation of a semen simulant. *J. Androl.* 26: 459–469.

Oyegbade O.O., Abioye-Kuteyi E.A., Kolawole B.A., Ezeoma I.T., Bello I.S. (2007). Screening for diabetes mellitus in a Nigerian family practice population. *SA Fam Pract.* 49: 15-19.

Pacher P., Szabó C. (2005). Role of poly (ADP-ribose) polymerase-1 activation in the pathogenesis of diabetic complications: endothelial dysfunction, as a common underlying theme. *Antioxid. Redox Signal*, 7:1568-1580.

Pais P. (2010). Potency of a novel *saw palmetto* extract, SPET-85, for inhibition of 5alpha-reductase II. *Adv.* Ther. 27:555-563.

Peehl D.M., Cohen P., Rosenfeld R.G. (1996). The role of insulin-like growth factors in prostate biology. *J Androl*.17: 2–4.

Peehl D.M., Stamey T.A. (1986). Serum-free growth of adult human prostatic epithelial cells. *In Vitro Cell Dev. Biol*. 22: 82–90.

Perez-Matute P., Zulet M.A., Martinez J.A. (2009). Reactive species and diabetes: Counteracting oxidative stress to improve health. *Curr. Opin. Pharmacol.* 9: 771-779.

Polychronakos C., Janthly U., Lehoux J.G., Koutsilieris M. (1991). Mitogenic effects of insulin and insulin-like growth factors on PA-III rat prostate adenocarcinoma cells: characterization of the receptors involved. *Prostate*.19: 313–321.

Pugliese G., Pricci F., Pugliese F., Mene P., Lenti L., Andreani D., Galli G., Casini A., Bianchi S., Rotella C.M. (1994). Mechanisms of glucose-enhanced extracellular matrix accumulation in rat glomerular mesangial cells. Diabetes.43: 478–90.

Raju J., Gupta D., Rao A.R., Yadava P.K., Baquer N.Z. (2001). *Trigonella foenum-graecum* (Fenugreek) seed powder improves glucose homeostasis in alloxan diabetic rats tissues by reversing the altered glycolytic, gluconeogenic and lipogenic enzymes; *Mol. Cell. Biochem.* 224: 45-51.

Reed J.C. (1994). Bcl -2 and the regulation of programmed cell death. *J. Cell Biol*. 124: 1-6

Reitman S., Frankel S.A. (1957). Colorimetric method for the determination of serum level of glutamate-oxaloacetate and pyruvate transaminases. *Am J Clin Pathol*. 28: 56-63.

Riaz S., (2009): Diabetes Mellitus. *Sci Res Essays*. 4 (5): 367-373.

Rice-Evans C.A., Burton R. (1993). Free radical lipid interactions and their pathological consequences. *Prog Lipid Res*. 32: 72-110.

Roehrborn C.G., McConnell J. (2002). Etiology, pathophysiology, epidemiology and natural history of benign prostatic hyperplasia, in: P. Walsh, A. Retik, E. Vaughan, A. Wein (Eds.), *Campbell's Urology, 8th edn, Saunders, Philadelphia*. 1297–1336.

Rotruck J.T., Pope A.L., Ganther H.E., Swanson A.B., Hafeman D.G., Hoekstra W.G. (1973). Selenium: biochemical role as a component of glutathione peroxidase. *Science.* 179: 588-590.

Samout N., Bouzenna H., Dhibi S., Ncib S., ElFeki A., Hfaiedh N. (2016). Therapeutic effect of apple pectin in obese rats. *Biomed. Pharmacother.* 83:1233-1238.

Sanghera D.K, Blackett P.R. (2012). Type 2 diabetes genetics: beyond GWAS. *J Diabetes Metab.* 3(198): pii6948.

Schmid W. (1975).The micronucleus test. *Mutat Res*. 31(1):9-15

Schmidtz H.D. (2001). Reversible nuclear translocation of glyceraldehyde-3-phosphate dehydrogenase upon serum depletion. *Eur J Cell Biol*. 80: 419–427.

Sekiguchi N., Inoguchi T., Kobayashi K., Nawata H. (2004). Effect of erythropoietin on endothelial cell apoptosis induced by high glucose. *Diabetes Res. Clin. Pract*. 66 Suppl 1:S103-7

Sgarbosa F., Barbisan L.F., Brasil M.A., Costa E., Calderon I.M., Gonçalves C.R., Bevilacqua E., Rudge M.V.(2006). Changes in apoptosis and Bc1-2 expression in human hyperglycemic, term placental trophoblast. *Diabetes Res. Clin. Pract*. 73:143-9.

Shaneyfelt T., Husein R., Bubley G., Mantzoros C. (2000). Hormonal predictors of prostate cancer: a meta-analysis. *J .Clin. Oncol.* 18:847–53.

Shieh S.M., Sheu W.H., Shen D.C., Fuh M.M., Chen Y.D., Reaven G.M. (1992). Glucose, insulin, and lipid metabolism in doxazosintreated patients with hypertension. *Am J Hypertens.* 5:827–31.

Singh V. P., Bali A., Singh N., Jaggi A.S. (2014). Advanced glycation end products and diabetic complications. *Korean J Physiol Pharmacol*. 18:1–14.

Sinha, A.K. (1971). Colorimetric assay of catalase. *Anal Biochem*., 47: 389-394.

Szkudelski T. (2001). The mechanism of alloxan and streptozotocin action in B cells of the rat pancreas. *Physiol. Res.* 50 (6): 537–546.

Toneline M.T., Filho J.B., Rodrigueiro D.A., Novo N.F. (2014). Frequency of Micronuclei and other nuclear abnormalities in Diabetic patients. *Rev. Fac. Ciênc. Méd. Sorocaba*, 16 (2): 80 – 85.

Torring N., Vinter-Jensen L., Pedersen S.B., Sorensen F.B., Flyvbjerg A., Nexo E. (1997). Systemic administration of insulin like growth factor I (IGF-I) causes growth of the rat prostate. *J. Urol.* 158:222–7

Trumpower B.L. (1990). The protonmotive Q cycle: energy transduction by coupling of proton translocation to electron transfer by the cytochrome bc1 complex. *J Biol. Chem.* 265: 11409–11412

Valerino D.M., McCormack J.J. (1971). Xanthine oxidase-mediated oxidation of epinephrine. *Biochem Pharmacol*. 20(1):47-55

Varım C., Kaya T., Nalbant A., Ucar A., Tamer A. (2014). Effects of Glucose Control on Hematological Indices in Patients with Diabetes Mellitus. *Maced J Med Sci*. 15: 7(1), 83-88. doi .org /10.388 9/MJ MS .1857577 3.

Vigersky R.A. (2011). An overview of management issues in adult patients with type 2 diabetes mellitus. *J Diabetes Sci Technol*. 5(2): 245–250.

Vlassara H., Striker L.J., Teichberg S., Fuh H., Li Y.M., Steffes M. (1994). "Advanced glycation end products induce glomerular sclerosis and albuminuria in normal rats," *Proceedings of the National Academy of Sciences of the United States of America.* 91(24): 11704–11708.

Wallace D.C. (1992). Disease of the mitochondrial DNA. *Annu Rev Biochem.* 61:1175–212.

Weidner W., Krause W., Ludwig M. (1999). Relevance of male accessory gland infection for subsequent fertility with special focus on prostatitis. *Hum. Reprod. Update*. 5: 421–432

Weisshaar D., Gossrau E., Faderl B. (1975). Normal ranges of alpha-HBDH, LDH, AP, and LAP as measured with substrate-optimated test charges. *Med Welt.* 26 (9):387-92.

WHO. (2013). Diabetes Fact sheet N°312". Archived from the original on 26 August 2013. Retrieved 25 March (2014).

Wild S.H., Roglic G., Green A., Sicree R., King H. (2004). Global prevalence of diabetes: estimate for year 2000 and projections for 2030. *Diabetes Care* .27(5):1047-1052.

Williams B., Gallacher B., Patel H., Orme C. (1997). Glucose-induced protein kinase C activation regulates vascular permeability factor mRNA expression and peptide production by human vascular smooth muscle cells in vitro. *Diabetes.* 46:1497–503.

Williams textbook of endocrinology (12th ed.). Philadelphia: Elsevier/Saunders. pp. 1371–1435. *ISBN* 978-1-4377-0324-5

World Health Organization (WHO). (1999). Definition, Diagnosis and Classification of Diabetes Mellitus and its Complications: Report of a WHO Consultation. Part 1: Diagnosis and Classification of Diabetes Mellitus. Geneva, World Health Org.

World Health Organization/International Diabetes Federation (2004). Diabetes Action Now. An initiative of the WHO library cataloguing – in – publication data. Geneva, *WHO.* 49:149.

Wu B., Ootani A., Iwakiri R., Sakata Y., Fujise T., Amemori S., Yokoyama F., Tsunada S., Toda S., Fujimoto K. (2005). T cell deficiency leads to liver carcinogenesis in Azoxymethane-treated rats. *Exp. Biol. Med.* 231: 91-98.

Wu Y., Ding Y., Tanaka Y., Zhang W. (2014). Review: Risk Factors Contributing to Type 2 Diabetes and Recent Advances in the Treatment and Prevention. *Int. J. Med. Sci.* 11(11): 1185-1200. doi: 10.7150/ijms.10001.

Yoshida K., Kawano N., Yoshiike M., Yoshida M., Iwamoto T., Morisawa M. (2008). Physiological roles of semenogelin I and zinc in sperm motility and semen coagulation on ejaculation in humans. *Mol Hum Reprod*. 14:151–6.

Zaichick V.Y., Sviridova T.V., Zaichick S.V. (1997). Zinc in the human prostate gland: normal, hyperplastic and cancerous. *Int Urol Nephrol*. 29:565–74.

Zaneveld L.J., Tauber P.F. (1981). Contribution of prostatic fluid components to the ejaculate. *Prog. Clin. Biol. Res*. 75A: 265–277.

Ziada A., Rosenblum M., Crawford E.D. (1999). Benign prostatic hyperplasia: An overview. *Urology*. 53:1–6.

Zongwei W., Aria F.O. (2011). Diabetes, growth hormone-Insulin-like growth factor pathways and association to benign prostatic hyperplasia. *Differentiation.* 82: 261 –271.

APPENDICES

Appendix 1 – Preparation of 0.1 M Streptozotocin solution

CITRATE BUFFER (0.1 M Sodium citrate, 0.1 M Citric acid, pH 4.0)

1 0.1 M Sodium citrate

1.47 g of sodium citrate tribasic dihydrate was dissolved in water (distilled), added up to 50 mL and kept in storage at room temperature.

2 0.1 M Citric acid

1.05 g of citric acid was dissolved in water (distilled), added up to 50 mL and kept in storage at room temperature.

20 mL of 0.1 M Sodium citrate and 30 mL of 0.1 M Citric acid were interspersed to yield 0.1 M Citrate buffer. Adjustment of pH to 4.0 with 1 N NaOH were done and kept in storage in a sterilized conical tube on ice.

0.1 M STREPTOZOTOCIN (STZ)

50 mg of STZ was dissipated in 0.1 M Citrate buffer, added up to 2.5 mL and kept on ice.

Appendix 2 – Preparation of buffer

1. Rinsing Buffer (1.15% of Potassium Chloride)

11.5 g of Potassium Chloride (BDH Chemical Limited, England) was dissolved in distilled water and made up to 1000 ml and stored at 4^0C.

2. Homogenizing Buffer (0.1 M Phosphate buffer (pH 7.4)

a. 7.1628 g of $Na_2HPO_4.12H_2O$ was dissolved in 200 ml of distilled water.
b. 1.5603 g of $NaH_2PO_4.2H_2O$ was dissolved in 100 ml of distilled water.

Finally, 0.1 M phosphate buffer was prepared by adding 200 ml of (a) to 100 ml of (b) and the pH was adjusted to 7.4 with drops of HCl or NaOH as the case may be.

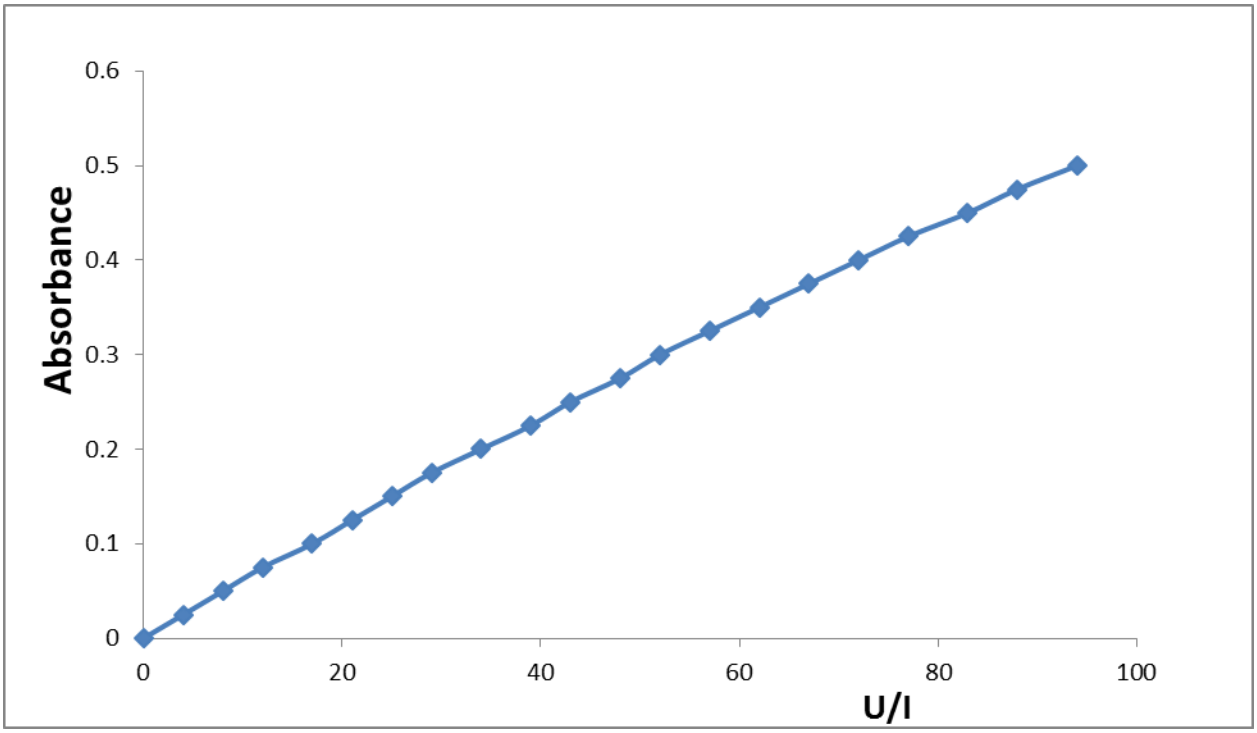
Appendix 3 – Preparation of reagents for the Estimation of ALT Activity

Reagents

Reagent 1 - Buffer (phosphate buffer – 100 mmol/L, PH 7.4, α-oxoglutarate – 2 mmol/L, L-Alanine – 100 mmol/L). Reagent 2 - 2, 4-dinitrophenylhydrazine – 2 mmol/L and Sodium hydroxide – 0.4 mol/L.

Table of Calibration for ALT standard curve

Absorbance	U/I	Absorbance	U/I
0.025	4	0.275	48
0.050	8	0.300	52
0.075	12	0.325	57
0.100	17	0.350	62
0.125	21	0.375	67
0.150	25	0.400	72
0.175	29	0.425	77
0.200	34	0.450	83
0.225	39	0.475	88
0.250	43	0.500	94



Standard curve for ALT Estimation

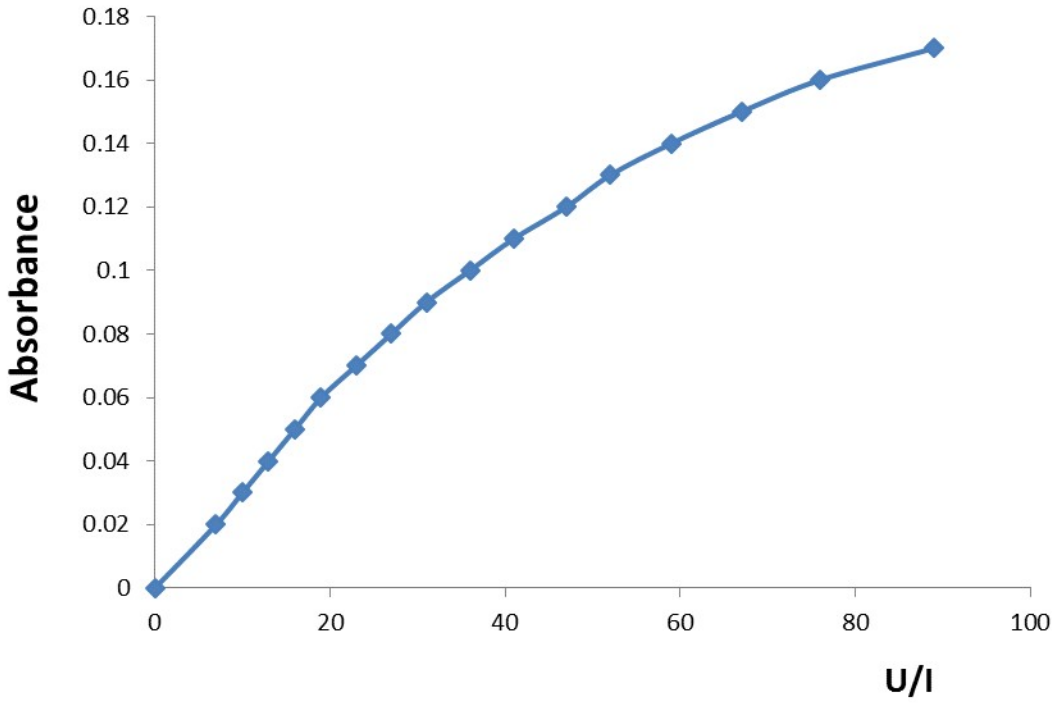
Appendix 4 – Preparation of reagents for the Estimation of AST Activity

Reagents

Reagent 1 - Buffer (L-Aspartate – 100 mmol/L, phosphate buffer – 100 mmol/L, PH 7.4, α-oxoglutarate – 2 mmol/L). Reagent 2 - 2, 4-dinitrophenylhydrazine – 2 mmol/L and Sodium hydroxide – 0.4 mol/L.

Table of Calibration for AST standard curve

Absorbance	U/I	Absorbance	U/I
0.020	7	0.100	36
0.030	10	0.110	41
0.040	13	0.120	47
0.050	16	0.130	52
0.060	19	0.140	59
0.070	23	0.150	67
0.080	27	0.160	76
0.090	31	0.170	89



Standard curve for AST Estimation

Appendix 5 – Preparation of reagents for the Estimation of serum Creatinine concentration

Reagents

Reagent 1a - (Picric acid - 35 mmol/L), Reagent 1b - (Sodium hydroxide - 0.32 mol/L), CAL, Standard - 177 µmol/L (2 mg/dL).

Appendix 6 – Preparation of reagents for the Determination of serum Urea concentration

Reagents

Reagent 1- (EDTA - 116 mmol/L, Sodium nitroprusside - 6 mmol/L, Urease - 1 g/L), Reagent 2 - (Diluted phenol - 120 mmol/L), Reagent 3 - (Diluted Sodium hypochlorite – 27 mmol/L), Sodium hydroxide - 0.14 N, CAL, Standard - 13.3 mmol/L (80 mg/dL).

Appendix 7 – Preparation of reagents for the Estimation of Bilirubin concentration

1. **Total Bilirubin**

Reagent 1 (Hydrochloric acid 0.17 N, Sulphanilic acid 29 mmol/L,), Reagent 2. (Sodium Nitrite - 38.5 mmol/L), Reagent 3. (Sodium benzoate – 0.52 mol/L, Caffeine - 0.26 mol/L), Reagent 4. (Sodium Hydroxide – 1.9 N, Tartrate 0.93 l/L).

2. Direct Bilirubin

Reagent 1 - (Hydrochloric acid 0.17 N, Sulphanilic acid 29 mmol/L), Reagent 2 - (Sodium Nitrite - 38.5 mmol/L), Reagent 3 - (Sodium benzoate – 0.52 mol/L, Caffeine - 0.26 mol/L), Reagent 4 - (Sodium Hydroxide – 0.253 N, Tartrate 0.93 l/L).

Appendix 8 – Preparation of reagents for the Estimation of Alkaline Phophatase activity

Reagents

Reagent 1a – Buffer (Diethanolamine buffer - 1 mol/L, pH 9.8, $MgCl_2$ - 0.5 mmol/L), Reagent 1b - Substrate (p-nitrophenylphosphate - 10 mmol/L).

Appendix 9 – Preparation of reagents for the Estimation of Lactate Dehydrogenase Activity

Reagents

1. 5.0 mmol/L of phosphate buffer at pH 7.5
2. 0.6 mmol/L of pyruvate
3. 0.18 mmol/L of NADH.

All are mixed together to form the substrate.

Appendix 10 – Preparation of reagents for the Estimation of Protein Concentration

Reagents

1. Sodium hydroxide (NaOH) (0.2 M)

NaOH (8 g) was dissipated in water (distilled) then, solution was topped up to a litre.

2. Biuret reagent

Sodium potassium tartarate (9 g) and Copper sulphate $CuSO_4.5H_2O$ (3 g) were dissipated in 0.2 M NaOH (500 mL). KI (5 g) was appended and the solution topped up to a litre with 0.2 M NaOH.

3. Stock Bovine Serum Albumin (standard)

BSA (7.4 g) was dissipated in 100 mL of 0.9% NaCl so as to have 7.4 mg/100 mL as the final concentration.

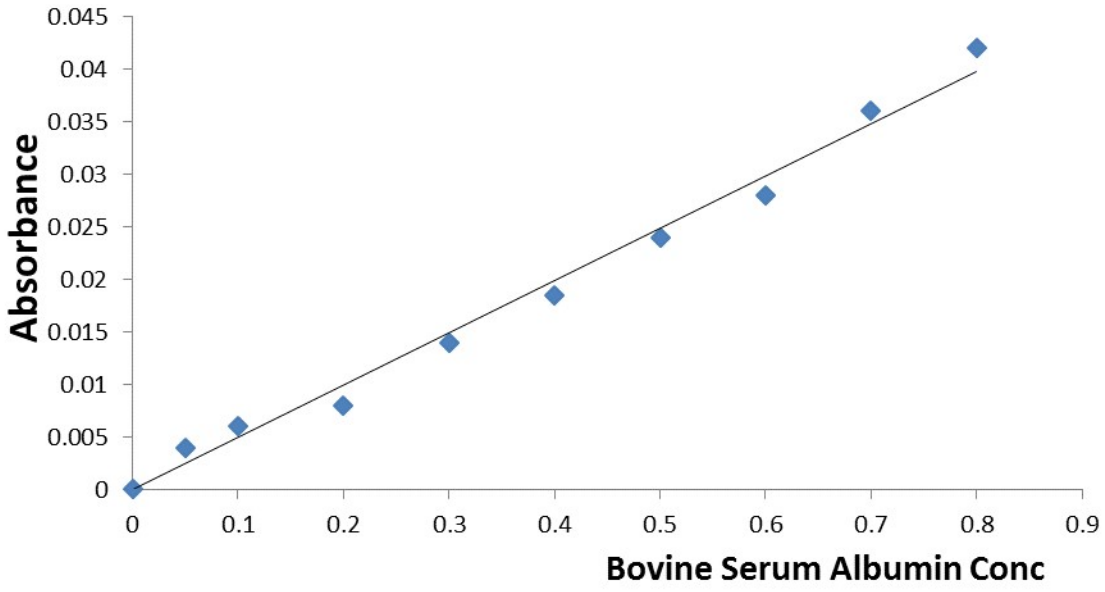
4. Curve of standard BSA via the Biuret method

Various dilutions were prepared from of the stock solution that comprises 2-10 mg protein/mL. Biuret reagent (4 mL) was appended into each protein standard solution (1 mL) in a test tube. Let the mixture stay at 25^{o}C for half an hour and the absorbance of the resultant solutions was read (540 nm). Optical density against protein concentration curve was plotted.

Table of Calibration for Protein estimation

Test tube number	1	2	3	4	5	6	7	8	9
Stock BSA(ml)	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9
Distilled water (ml)	0.9	0.8	0.7	0.6	0.5	0.4	0.3	0.2	0.1
Biuret reagent (ml)	4	4	4	4	4	4	4	4	4

BSA concentration (mg/ml)	0.05	0.10	0.20	0.30	0.40	0.50	0.60	0.70	0.80
Absorbance (540nm)	0.004	0.006	0.008	0.014	0.0185	0.024	0.028	0.042	0.042



Standard curve for protein Estimation

Appendix 11 – Preparation of reagents for the Estimation of Malondialdehyde Concentration

Reagents

1. Trichloroacetic acid (TCA) (30%)

TCA (30 g) was dissipated in 100 mL of water (distilled) and stored at 4^0C.

2. Thiobarbituric acid (TBA) (0.75%) in HCl (0.1 M)

TBA (0.075 g) was dissipated in 0.1 M HCl (10 mL). Shaking the solution in a boiling water bath helped in its dissolution. It was prepared fresh.

3. Tris- KCl buffer (0.1 M), pH 7.4

Tris base (2.36 g) and KCl (1.12 g) were dissipated singly in water (distilled) and topped up to 100 mL with water (distilled). pH = 7.4.

Appendix 12 – Preparation of reagents for the Estimation of reduced Glutathione Concentration

Reagents

1. Phosphate buffer (0.1 M, pH 7.4)

a. $NaH_2PO_4.2H_2O$ (1.5603 g, Mol.Wt.358.22) was dissipated in 100 mL of water (distilled).
b. $Na_2HPO_4.12H_2O$ (7.1628 g, Mol.Wt. 358.22) was dissipated in 200 mL of water (distilled).

Lastly, phosphate buffer (0.1 M) was made by mixing 200 mL of (b) to 100 mL of (a) and the pH was tuned to 7.4.

2. Reduced Glutathione (GSH) working standard

GSH (40 mg, Mol. Wt. 307.3 g) was dissipated in Phosphate buffer (0.1 M, pH 7.4) and topped up with idem to 100 mL and then stored at 4^0C.

3. 5, 5^1-dithiobis-(2-nitrobenzoate) (DTNB) also called Ellman's Reagent

This was made by dissipating Ellman's reagent (40 mg) in phosphate buffer (0.1 M) and topped up with idem to 100 mL. DNTB is stable for at least 3 weeks in the refrigerator.

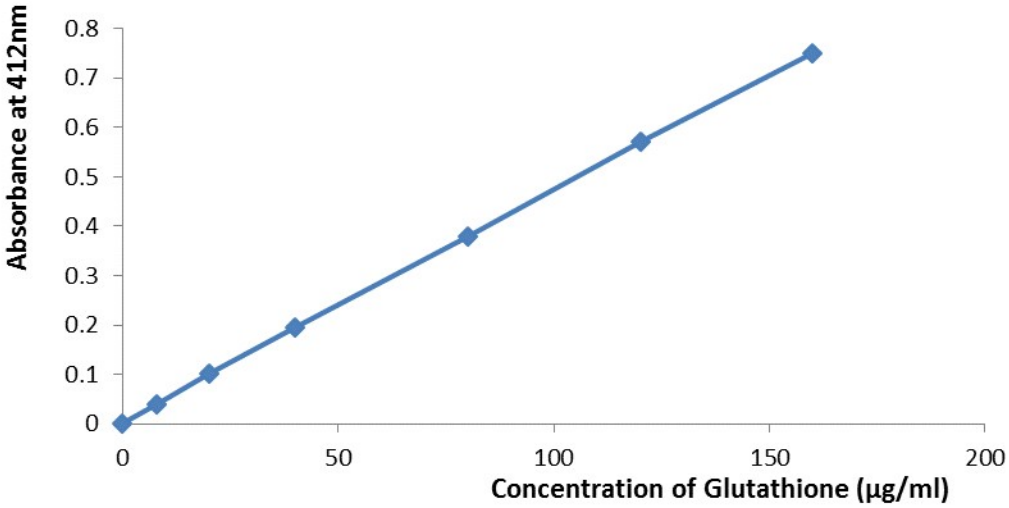
4. Sulphosalicyclic acid (Precipitating) Solution.

Sulphosalicyclic acid ($C_7H_6O_6S.2H_2O$, Mol.Wt. 254.22, 4%) was made by dissipating sulphosalicyclic acid (4 g) in little quantity of water (distilled), top up to 100 marks in a volumetric flask (standard). At 4^oC, this reagent was kept stable for just about 3 weeks.

Table of Calibration for GSH standard curve

Stock	PO_4	Ellman's Reagent	Absorbance (412nm)	GSH Conc.(ug/ml)
0.02	0.48	4.50	0.033	8

0.05	0.45	4.50	0.099	20
0.10	0.40	4.50	0.246	40
0.20	0.30	4.50	0.346	60
0.30	0.20	4.50	0.505	80
0.40	0.10	4.50	0.683	100



Calibration curve for GSH Estimation

Appendix 13 – Preparation of reagents for the Estimation of Catalase Activity

Reagents:

1. 5% $K_2Cr_2O_7$

Potassium dichromate ($K_2Cr_2O_7$) (5 g) was dissipated in 80 mL of water (distilled) and topped up to 100 mL.

2. Dichromate/acetic acid.

Mix 5% solution of $K_2Cr_2O_7$ with glacial acetic acid (1:3 by volume) to prepare the solution.

3. 0.2 M H_2O_2

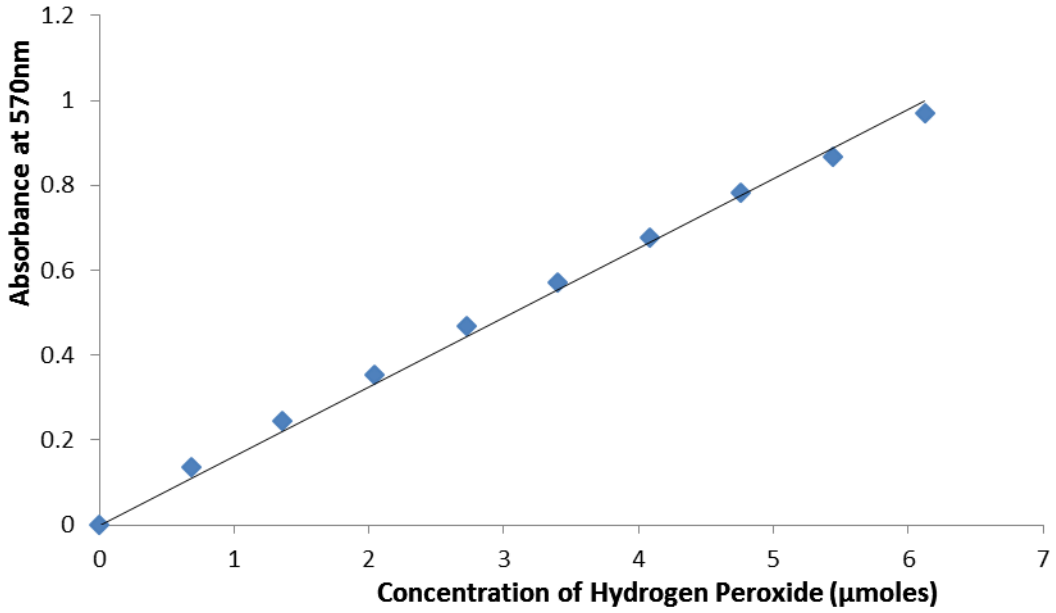
H_2O_2 (11.50 mL) was mixed with water (distilled) in a flask (volumetric) and topped up the solution to 500 mL.

3. 0.01M of Phosphate buffer (pH=7.0)

$NaH_2PO_4.2H_2O$ (1.19 g) and $Na_2HPO_4.12H_2O$ (3.581 g) was dissipated in 900 mL of water (distilled), then, topped up to 1 litre. The pH was adjusted to 7.0

Table of Protocol for the Estimation of H_2O_2

Test tube number	1	2	3	4	5	6	7	8	9
H_2O_2 (ml)	0.05	0.10	0.15	0.20	0.25	0.30	0.35	0.40	0.45
Dichromate/acetic acid	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0
Distilled water(ml)	0.95	0.90	0.85	0.80	0.75	0.70	0.65	0.60	0.55
H_2O_2conc. (µmoles)	0.68	1.36	2.04	2.72	3.40	4.08	4.76	5.44	6.12
Absorbance (570nm)	0.136	0.245	0.355	0.467	0.570	0.676	0.782	0.867	0.969



Standard curve for the Estimation of Hydrogen peroxide for catalase assay

Appendix 14 – Preparation of reagents for the Estimation of Superoxide Dismutase Activity

Reagents

1. Adrenaline (0.3 M)

Adrenaline (0.0137 g) was dissipated in 200 mL of water (distilled), then, topped up to 250 mL. It was prepared before the experiment.

2. Carbonate Buffer (0.05 M, pH 10.2)

$NaHCO_3$ (4.2 g) and $Na_2CO_3.10H_2O$ (14.3 g) were dissipated in 900 mL of water (distilled) and then topped up to 1 litre.

Appendix 15– Preparation of reagents for the Estimation of Glutathione-S-Transferase Activity

Reagents

1. 1-Chloro- 2, 4, - dinitrobenzene (20 mM)

1 chloro-2, 4-dinitrobenzene (3.37 mg) was dissipated in ethanol (1mL).

2. Reduced Glutathione (0.1 M)

Glutathione (30.73 mg) was dissipated in 1mL of phosphate buffer (0.1 M) with pH 6.5.

3. Phosphate buffer (0.1 M) with pH 6.5

Potassium dihydrogen phosphate (9.73 g) (KH_2PO_4, M.W. 136.09) and dipotassium hydrogen phosphate (4.96 g) (K_2HPO_4 M.W. = 174.18) were dissipated in water (distilled) and volume was topped up to 1000 mL and the pH was adjusted to 6.5.

Appendix 16 – Preparation of reagents for the Estimation of Glutathione peroxidase Activity

Reagents

1. Sodium azide (NaN_3, 10 mM)

NaN_3 (0.0325 g) was dissipated in 50 mL of H_2O (distilled)

2. Reduced glutathione (GSH 4 mM)

Reduced GSH (0.0123 g) was dissipated in phosphate buffer (10 mL, pH=7.4)

3. Hydrogen peroxide (H_2O_2, 2.5 mM)

H_2O_2 (0.028 mL) was dissipated in distilled water (100 mL).

4. Tricholoroacetic acid (10%)

TCA (2 g) was dissipated in distilled water (20 mL)

5. Dipotassium Hydrogen orthophosphate K_2HPO_4 (0.3 M)

K_2HP0_4 (5.23 g) was dissipated in distilled water (100 mL)

6. 5-5-dithiobis (2-dinitrobenzoic acid) [DTNB]

DTNB (0.04 g) was dissipated in phosphate buffer (100 mL, pH=7.4).

7. Phosphate buffer

KH_2PO_4 (1.946 g) and K_2HPO_4 (0.992 g) was dissipated water (distilled) (200 mL) and pH was adjusted to 7.4.

Appendix 17 – Preparation of reagents for Micronuclei assay

Reagents

Bovine serum, Absolute methanol, Xylene, 0.4% May Grunwald Stain, 5% Giemsa Stain, Phosphate buffer (0.01 M, pH 6.8).

1. Phosphate Buffer (0.01 M, pH 6.8)

Disodium hydrogen phosphate (0.71 g) and 0.68 g of potassium dihydrogen phosphate (BDH Chemicals Ltd Poole England- 29608) was dissipated in water and topped up to 1L, and then, pH was adjusted to 6.8. During washing, this was used to bathe the slides.

2. Giemsa Stain (5%)

Giemsa (5 g) was dissipated in phosphate buffer (pH 6.8) and the volume topped up with idem to 100 mL.

3. May-Grunwald Stains 1 (0.4%)

May-Grunwald stain (0.4 mL) was dissolved and made up to 100 mL with absolute methanol.

4. May-Grunwald Stains 2 (0.4%)

1:1 dilution was made using stain 1 and distilled water. They were used in staining of slides.

Appendix 18 – Preparation of reagents for the Determination of percentage DNA Fragmentation

Reagents

1. 5mMTris, 20mM EDTA (TE) buffer pH 8.0

0.61g of Tris-HCl and 7.45g of EDTA was dissolved in 900ml of distilled water. 2.0ml of Triton-X100 solution was then added and the pH (of solution) was adjusted to 8.0 and the buffer was topped up to1000ml with distilled water.

2. 5mMTris, 20mM EDTA (TE$^{!}$) buffer pH 8.0

This was prepared as above but without the addition of Triton-X100.

3. Diphenylamine (DPA) solution

1.5g of diphenylamine was dissipated in 100ml of acetic acid and 1.5ml of concentrated H_2SO_4 was appended to the solution.

Appendix 19 – Preparation of reagents for the Determination of Zn concentration

Equipments

- Atomic Absorption Spectrophotometer (AAS)
- Block heater

The Prostatic Zn were determined by direct method using Atomic Absorption Spectroscopy, Shimadzu model AA-580 fitted with a Shimadzu PR-5 graphic printer following the manufacturer's analytical techniques.

Reagents

- 10% glycerin
- 10% Trichloro acetic acid (TCA)
- 0.1% Lanthanum Chloride
- Standard solution of the respective elements to be analyzed
- 20% TCA

Appendix 20 – Preparation of reagents for the Determination of Bicarbonate concentration

Reagents

- 0.25% Phenolphthalein in 50% alcohol
- Sulphuric acid, standard 0.025M
- 0.1% Methyl orange in water