THE EFFECTS OF AQUEOUS EXTRACT OF *Garcinia kola* SEEDS ON TESTICULAR HISTOLOGY AND SPERM PARAMETERS IN ADULT MALE WISTAR RATS

BY

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BMS1501738

A PROJECT WORK SUBMITTED TO THE DEPARTMENT OF ANATOMY, SCHOOL OF BASIC MEDICAL SCIENCES COLLEGE OF MEDICAL SCIENCES UNIVERSITY OF BENIN, BENIN CITY, NIGERIA.

IN PARTIAL FULFILMENT OF THE REQUIREMENT FOR THE AWARD OF BACHELOR OF SCIENCE DEGREE (B.Sc) IN ANATOMY.

DECEMBER, 2019.

CERTIFICATION

This is to certify that this research was carried out by BROWN, IGEMU (Mat. No. BMS1501738) in the Department of Anatomy, School of Basic Medical Sciences, University of Benin, Benin City, Nigeria. In partial fulfillment of the requirement for the award of Bachelor of Science Degree (B.Sc) in Anatomy.

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DEDICATION

This project work is dedicated to God Almighty for his guidance and protection throughout this project. And also to my parents Mr. and Mrs. Owhofasa Igemu for their unconditional love, support and provision throughout the period of my educational journeys.

ACKNOWLEDGEMENT

I am sincerely grateful to Almighty God for his guidance in my life as a whole and his unending protection. I deeply appreciate my supervisor Dr. Mrs. Vannessa Oigboche for her unwavering interest in my research project and her relentless support and understanding while working with her. I would not fail to acknowledge my parents and my family members Marho, Vwede, Isaiah and Karen for their consistent support and love and Odiri for her immeasurable contributions to this work. I also want to thank the Head of Department Dr. D. O Odiase and all the lecturers and staff of the department for his services to the department. I also want to thank my course mates and friends like Victory Yerumoh, Victory Sede, Aaron, Anthony, Umoh, Ugo, Friday, Olise, Peter, Faith, Andrea and all the others too numerous to mention for their support throughout my stay in UNIBEN.

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ABSTRACT

For centuries natural products have played a very crucial role in health care and prevention of diseases.Extracts of *G. kola* have been demonstrated to possess antimicrobial effects and has also been shown to possess hepatoprotective activities. The seed is used to prevent and relieve colic, chest colds and cough and can as well be used to treat headache.

The nature of the spermatozoa (their vitality, motility and morphology) and the composition of seminal fluid are important for sperm function. Basic sperm analysis measures the number of spermatozoa (per unit volume and per ejaculate), motility and morphology.

This study was carried out on twenty healthy adult male Wistar rats weighing between 200g to 250g. Group A was the control group. Group B was low dose administration group (250mg/kg body weight).Group C was intermediate dose administration group (500mg/kg body weight). Group D was high dose administration group (1000mg/kg body weight)

The result of the statistical analysis showed that Garcinia kola caused significant increases ($P < 0.05$) serum FSH, testosterone and percentage of abnormal sperm cells which in conjuction with the vascular congestion noticed. There was no significant difference in total sperm count, body weight and testes weight. There was significant decrease in sperm motility and morphology.

CHAPTER ONE

INTRODUCTION

1.0 BACKGROUND OF STUDY

For centuries natural products have played a very crucial role in health care and prevention of diseases. The ancient civilization of Chinese, Indians and North Africans provide written proof for the use of natural resources for curing various diseases. For example mandrake was prescribed for pain relief, turmeric possesses blood clotting properties and raw garlic was prescribed for circulatory disorders. These are still being used in several countries as alternative medicine (Kakare, 2007). *Garcinia kola* nut is chewed extensively in Southern Nigeria for nervous alertness and induction of insomnia. It is readily served to visitors as a sign of acceptance and peace. The roots of the plant is also used by some as bitter chew-stick. The stem bark is used as a purgative among some in Eastern Nigeria and the latex is applied to fresh wounds to prevent sepsis, thereby assisting healing (Uko et al, 2001).

FIG. 1.0 Garcinia kola tree (courtesy of ijpjournal.com)

Nutritionally, *G. kola* has been observed to contain high proportions of Carbohydrates, little amounts of Crude Fibre, Proteins, Ash and Crude Fats. It was also shown to contain Anti-Nutrients such as Oxalate (0.423 g/100 g), Phytate (0.57 g/100 g) and Trypsin Inhibitor (0.37 g/100). Mineral content show high level of Calcium, Potassium and Sodium (Adesuyi et al, 2012). Phytochemical assay showed that Tannin (0.347%), Sapoium (0.680%), Phytic acid (0.550%), Phenol (0.163%), Sterols (0.093%), Flavonoids (2.130%), Alkaloids (0.433%), Caffeine (0.607%) and Hydrogen cyanide (1.347 mg/kg) were present in significant amounts (Mazi et al 2013). The dominant fatty acids in the seed and hull are palmitic, oleic and linoleic acids. Glutamic acid is the dominant non-essential amino acid in the seed and hull while lysine and valine are the dominant essential amino acids (Eleyinmi et al, 2006).

Extracts of *G. kola* have been demonstrated to possess antimicrobial effects (Arekemase et al, 2012, Amalu et al, 2014). It has also been shown to possess hepatoprotective activities (Adaramoye et al, 2008). Iranloye and Owokunle (2008) demonstrated on female rats that it may be used as contraceptive with a possible advantage of reversibility. It has also portrayed has potential antidiabetic, antilipidemic and antiatherogenic agent (Udenze et al, 2012). The seed is used to prevent and relieve colic, chest colds and cough and can as well be used to treat headache (Ayensu, 1978).

The testes are the two sperm-producing organs in males. They also produce androgens, the male hormones primarily testosterone. The nature of the spermatozoa (their vitality, motility and morphology) and the composition of seminal fluid are important for sperm function. Basic sperm analysis measures the number of spermatozoa (per unit volume and per ejaculate), motility and morphology (WHO, 2010). Hence, sperm analysis has an important role in the routine evaluation of idiopathic male infertility, usually manifested as low sperm counts, impaired sperm motility, or absence of sperm, and remains the most common single diagnostic tool (Omu, 2013).

1.1 STATEMENT OF RESEARCH PROBLEM

Reports show increasing rise in cases of male infertility around the world, Africa and Nigeria in particular (WHO, 1991). Male factor infertility accounts for up to 50% of all cases. Despite this high prevalence in male infertility, no significant efforts have been made in tackling the problem. (Uadia and Emokpae, 2015, Ugwuja et al, 2008, Larsen, 1995).

This clearly show the need for a research work like this on the possible toxicity or benefits of *G. kola* on the fertility parameters of Nigerian men. The scarcity of research work on the effects of *Garcinia kola* on reproductive health in general further augments the issue.

1.2 JUSTIFICATION OF STUDY

Garcinia kola seed is popularly consumed by many Nigerians and other Africans of varying ages. The evaluation of its effects on testicular histology and sperm properties will demonstrate the benefits and/or dangers of *Garcinia kola* on the general male population of Africa.

1.3 AIM AND OBJECTIVES

The aim of this research is to study the effects of extracts of *Garcinia kola* seeds on spermatogenesis in adult male wistar rats. This will demonstrate possible corresponding effects on humans (Clause, 1993).

The specific objectives are the following fertility parameters to be analyzed at the end of this research project:

(1) The sperm count of the semen samples.

(2) The sperm motility morphology.

(4) The histological structure of the testicles and epididymis.

(5) The hormonal profile of blood samples obtained. (WHO, 1999).

CHAPTER TWO

LITERATURE REVIEW

2.1.0 PLANT OF STUDY

Garcinia *kola* is a medium-size tree, but sometimes grows up to 12 m tall and 1.5 m wide. It is a spreading tree with a dense and heavy crown; the trunk is straight; the bark is greenish brown, thick, and smooth. It has broad leaves, 5–10 cm long, elongated elliptic to broadly elliptic, acute or shortly acuminate, cuneate, leathery, with very distinct resinous canals. It has 10 pairs of lateral veins that run parallel to the margin but not forming a marginal nerve; the midrib is prominent at the underside; the stalk is stout, finely hairy in young leaves, and about 8 mm long. It bears male and female flowers separately, usually December–March and May–August. Female flowers are yellow and fleshy, globose, 1.5 cm wide; male flowers are smaller but with more prominent stamens (4 bundles), 4 sepals, and 4 greenish-white petals. It produces characteristic large fruits (6 cm in diameter), with the size and color of an orange, containing 2–4 brown seeds embedded in an orange-color pulp.

Scientific classification of *Garcinia kola:*

Kingdom = Plantae

Division = Magnoliophyta;

Class = Magnoliopsida

Order = Malpighiales

Family = Guttiferae

Genus = Garcinia

Species = Garcinia kola

2.1.1 HABITAT AND DISTRIBUTION

It is found in moist forests and cultivated in homesteads. It is distributed throughout West and Central Africa and has been located in Sierra Leone, Ghana, Nigeria, Cameroon, and Congo.

FIG. 2.0 Garcinia kola fruits in tree (courtesy of Google.com)

FIG. 2.1 Garcinia kola fruit cut open to expose seeds (courtesy of kitchenbutterfly.com)

FIG. 2.2 Garcinia kola seed cut in half showing the edible content (courtesy of kitchenbutterfly.com)

2.1.2 ETHNOMEDICINAL USES

It is used extensively in traditional medicine for the treatment of various diseases. The drug is chewed in southern Nigeria and parts of West Africa as a masticatory, in spite of its very bitter taste. The stem bark is used as a purgative, and the powdered bark is used for the treatment of malignant tumors. The sap is used for parasitic skin diseases. The latex (gum) is used internally for gonorrhea treatment and applied externally to fresh wounds. The twigs of *Garcinia kola* can be used as tapers, and the roots yield the favorite bitter chew sticks sold in small bundles in local markets in West Africa. The seeds are used to prevent or relieve colic, cure head or chest colds, and relieve cough. The seeds are chewed as an aphrodisiac and the dried nuts for dysentery treatment. The seeds are also used in the treatment of diabetes, bronchitis, and throat infections. The plant has been employed in the treatment of liver disorders.

The peeled stem and twigs are cut into small pieces and placed into a bottle of local gin, which is allowed to “mature” over a couple of days for drinking as an aphrodisiac. The alcohol is replenished several times until the resultant infusion becomes colorless (Iwu, 1993).

2.1.3 CONSTITUTION AND PYTHOCHEMISTRY

Garcinia kola contains alkaloids, saponins, tannins, flavonoids, glycosides, sterols and phenols. The major constituents of the plant are kolaviron, garcinia biflavonoid (GB)-1a-glucoside (1), GB-1a (2), GB-1 (3), GB-2 (4), kolaflavonone (5), benzophenone (6), xanthone (7), coumarin (8), apigenin (9), quercetin (10), garcinoic acid (11), Garcinia in (12) (Nmaju, et al 2014 and Buba, et al 2016). Hexadecanoic acid, 9-octadecanoic acid, methyl ester, linoleic acid, heptadecane-(8)-carbonic acid, formaldehyde*, N, N*-Diethyl, n-tetradecanoic acid amide; 3,4,8-trimethyl-2-nonenal were also isolated from the seed of *Garcinia kola* (Seanego and Ndip, 2012).

The mineral composition of *Garcinia kola* seeds and hulls has been reported, potassium and phosphorus were the most abundant in the seed, while phosphorus and calcium were the most abundant in the hull. Other constituents include ash, crude protein, crude fiber, crude lipid, water-soluble oxalate, terpenoids, and fat (Aniche and Uwakwe, 1990). The chemical constituents of *Garcinia kola* seed and hull had been studied using gas-liquid chromatography and High-Performance Liquid Chromatography. The seed oil composed of fatty acid and amino acid derivatives, namely meristic, pentadecanoic, margaric, trans-palmitoleic, cis-vaccenic, cis-oleic, cis-linoleic, α-linolenic, threonine, tyrosine, methionine, serine, histidine and alanine. The hull yielded the following fatty acid and

amino acid derivatives, pentadecanoic, margaric, pentadecanoic, myristoleic, cis-palmitoleic, cis-vaccenic and eicosadienoic, methionine, tyrosine, histidine, and arginine (Eleyinmi, et al, 2006).

2.1.4 THERAPEUTIC USES OF GARCINIA KOLA

2.1.4.1 ANTIMICROBIAL ACTIVITY

Antimicrobial activities of crude extract of *Garcinia kola* against some bacterial isolates comprising of both Gram-positive and Gram-negative organisms had been reported (Adegboye et al, 2008). In another study, the antimicrobial interaction between *Garcinia kola* seed and gatifloxacin, a fourth-generation fluoroquinolone, was evaluated by a modification of the checkerboard technique (Ofokansi K C, 2008). The antimicrobial activity of five different solvent extracts of *Garcinia kola* seed had been investigated against 30 clinical strains of *Helicobacter pylori* and a standard control strain, NCTC 11638, using conventional microbiological techniques (Collise et al, 2011). A study to investigate the anti-bacterial activity of bitter kola and ginger (*Zingiber officianale*) on four respiratory tract pathogens, namely *Staphylococcus aureus, Streptococcus pyogens, Streptococcus pneumonia, and Haemophilus influenza* revealed that the extracts from ginger and *Garcinia kola* exhibit anti-bacterial activities against the pathogens (Akoachere et al, 2001). The effect of aqueous extracts of *Garcinia kola* seeds on membrane stability of human erythrocytes indicated possible use of the extract for the management of sickle cell. Crude ethanol extracts of *Garcinia kola* seed demonstrated inhibitory effects on some pathogenic organisms of medical importance. The inhibitory effects shown by the ethanol extracts may be due to the presence of some phytochemical

components (Iwu, 1993). The antimicrobial properties of ethanol extracts of *Garcinia kola* seed were attributed to the presence of benzophenone. Research involving the bioassay of fractions of the seed showed mixtures of triterpenes, phenolic compounds, benzophenones, kolanone with potent antimicrobial properties (Okunji et al, 2002).

2.1.4.2 ANTIVIRAL ACTIVITY

The bioflavonoids constituents of the seeds of *Garcinia kola* have shown remarkable broad spectrum antiviral activity against a variety of viruses including *puntatoro, pichinde, sandfly fever, influenza A, Venezuelan Equine Encephalomyelitis,* HIV-1, and Ebola, with IC_{50} values of 7.2-32 µg/ml and TMC of more than 320 µg/ml (Iwu, 2002).

2.1.4.3 ANTIINFLAMMATORY ACTIVITY

The anti-inflammatory activities of flavonoids are complemented by their ability to activate NF-E2 related factor 2 (Nrf2), thus increasing anti-oxidant defenses. The analgesic and anti-inflammatory properties of kolaviron, a defatted seed extract of *Garcinia kola*, was investigated in mice and found to exhibit a weak analgesic but very strong anti-inflammatory activity when compared to a standard reference drug, acetylsalicylic acid (González-Gallego et al, 2007). Kolaviron from the seed of *Garcinia kola* had been shown to interfere with LPS signaling by reducing the activation of several inflammatory transcription factors and signaling pathways (Abarikwu, 2014).

2.1.4.4 ANTIDIABETIC ACTIVITY

Garcinia kola seed powder had also been shown to have antidiabetic, antilipidemic and anti-atherogenic properties with tremendous potential to protect against coronary heart disease (Udenze et al, 2014). Kolaviron reduced blood sugar levels in STZ-induced

diabetic rats within 4 h of oral administration and showed a favorable effect on the plasma lipid profile of diabetic animals (Adaramoye and Adeyemi, 2006). Kolaviron at 100 mg/kg significantly ameliorated hyperglycemia and liver dysfunction. It also prevented diabetes-induced increase in the hepatic levels of proinflammatory cytokines, interleukin (IL)-1beta, IL-6, tumor necrosis factor (TNF-α) and monocyte chemotactic protein (MCP-1) (Ayepola et al, 2013).

2.1.4.5 ANTIOXIDANT ACTIVIY

The leaf extract of *Garcinia kola* produced an antioxidant effect and protective response against the destructive effects of free radicals on both brain and liver (Oloyede et al, 2013). Antioxidant property of *Garcinia kola* is attributed to its very high content of ascorbic acid. Antioxidant potential of five fractions (ME1–ME5) of methanolic extract of *Garcinia kola* seeds was studied. ME4 fraction possessed the most significant activities. Fraction ME4 strongly inhibited nitric oxide production in lipopolysaccharide-activated macrophage U937 cells (Okwu, 2005). Polyphenolic compounds, flavonoids, and their derivatives are known to have antioxidant activities. Also, some anthraquinones have been reported to possess antioxidant activity (Daramola et al, 2012).

2.1.4.6 HEPATOPROTECTIVE ACTIVITY

Garcinia kola has a protective effect against a variety of experimental hepatotoxins. Anti-hepatoxic efficacy of this plant seed was due to its kolaviron content (Galam N Z et al, 2013). Garcinia bioflavonoids protected against hepatotoxicity induced by phalloidin, amanita, 2-acetylaminofluorene, carbon tetrachloride, paracetamol, aflatoxin, dimethylnitrosamine in rodents (Farombi and Owoeye, 2011). Even at 500 mg/kg

Garcinia kola did not cause significant degenerative or trophic changes in liver cells. Hepatic lobules which are polyhedral three dimensional in shape were preserved ((Galam et al, 2013)). *Garcinia kola* seed alleviated the hepatic degenerative changes associated with ciprofloxacin. The hepatoprotection exhibited by *Garcinia kola* seed as an adaptogen is generally ascribed to the presence of constituents with antioxidant properties (Esimone et al, 2007). *Garcinia kola* extract at 60 mg/kg significantly protected against damages caused by exposure to hepatotoxic antitubercular drug (Ogono et al, 2014).

2.1.4.7 ANTIHYPERTENSIVE ACTIVITY

Garcinia kola reduced glutathione concentration and also inhibits prostaglandin synthesis. *Garcinia kola* has a spasmolytic effect on gastrointestinal smooth muscle. It relaxes the smooth muscles of the uterus and gastrointestinal tract. It has been reported to stimulate histamine-dependent gastric acid secretion. Antithrombotic activity of *Garcinia kola* has also been reported. Aqueous extract of the plant stabilized the membranes of HbAA, HbAS and HbSS human erythrocytes and reduced blood viscosity. The effect of *Garcinia kola* on blood pressure has been traced to its ability to reduce total peripheral resistance either by direct or indirect action on the vascular smooth muscle. It has been observed that Ryanodine lowered mean arterial pressure and suppressed basal heart rate. This may be via a calcium chelating mechanism as it is known that most flavonoids are anti-nutrients, removing cholesterol, calcium, and glutathione from the blood.

Also, the removal of glutathione from the blood could help the vasodilatation of the resistant vessel as it has been observed that reduced glutathione level improved coronary endothelial vasomotor function by potentiating the vasodilator function of Nitroglycerine.

Membrane stabilization and reduction of blood viscosity is another possible way by which *Garcinia kola* may reduce blood pressure. It also contains a vasoactive ingredient, which is capable of lowering blood pressure

(Iwu et al, 1987).

2.1.5 TOXICOLOGY

Acute toxicity studies was done using the Probit Analysis method and was determined to be 6741.43mg/kg showing that it is relatively non-toxic and doses up to 900mg/kg were found to be very safe (Udenze et al, 2014). Another study indicated that that the aqueous extract of G. kola seeds at the doses of 25, 50 and 100 mg/kg body weight caused functional toxicity to the organs of the animals. The extract significantly ($P<0.05$) increased the testes-body weight ratio, activities of testicular alkaline phosphatase (ALP), heart, testes and serum gamma glutamyl transferase (GGT) activity, serum concentrations of uric acid, K^+, creatinine and PO_4^{3-}. The liver-body weight ratio, activities of kidney and serum ALP, liver, heart and serum alanine and aspartate aminotransferases (ALT and AST), serum and testicular acid phosphatase (ACP), concentrations of serum albumin, globulin, urea, Na^+ , HCO^{3-}, conjugated and total bilirubin were reduced (Yakubu, and Quadri, 2016).

2.2 ORGAN OF STUDY

2.2.1 THE TESTES

In humans the testes occur as a pair of oval-shaped organs and are contained within the scrotal sac. In humans each testis weighs about 25 grams and is 4-5 cm long and 2-3 cm

in diameter. Each is divided by partitions of fibrous tissue from the tunica albuginea into 200 to 400 wedge-shaped sections or lobes. Within each lobes are 3 to 10 seminiferous tubules. The partitions between the lobes and the seminiferous tubules both converge in one area near the anal side of each testis to form what is called the mediastinum testis. The testes contain germ cells that differentiate into mature spermatozoa, supporting cells called Sertoli cells, and testosterone producing cells called Leydig (interstitial) cells (Rogers, 2011). The front and sides of the testes lie free in a serous space formed by the overlying tunica vaginalis, a remnant of fetal processus vaginalis. This serous membrane covers also the anterolateral part of the epididymis and a slit-like space, the sinus of the epididymis, which lies between the testis and epididymis. Testis, epididymis, and tunica vaginalis lie in the scrotum surrounded by surrounded by the membranes adherent to each other that are downward prolongations of the spermatic cord. Right and left testes are separated by the median scrotal septum. The testicular artery, from the aorta, runs in the spermatic cord, gives off a branch to the epididymis, and reaches the back of the testis, where it divides into medial and lateral branches. Venules reach the mediastinum, from which several veins pass upwards in the spermatic cord as a mass of communicating veins called the pampiniform plexus which surrounds the testicular artery. The left vein joins the left renal vein while the right vein drains directly into the inferior vena cava. The testes are supplied by the sympathetic nerves. Most of the connector cells lie in the T10 segment of the spinal cord (Sinnatamby, 1999).

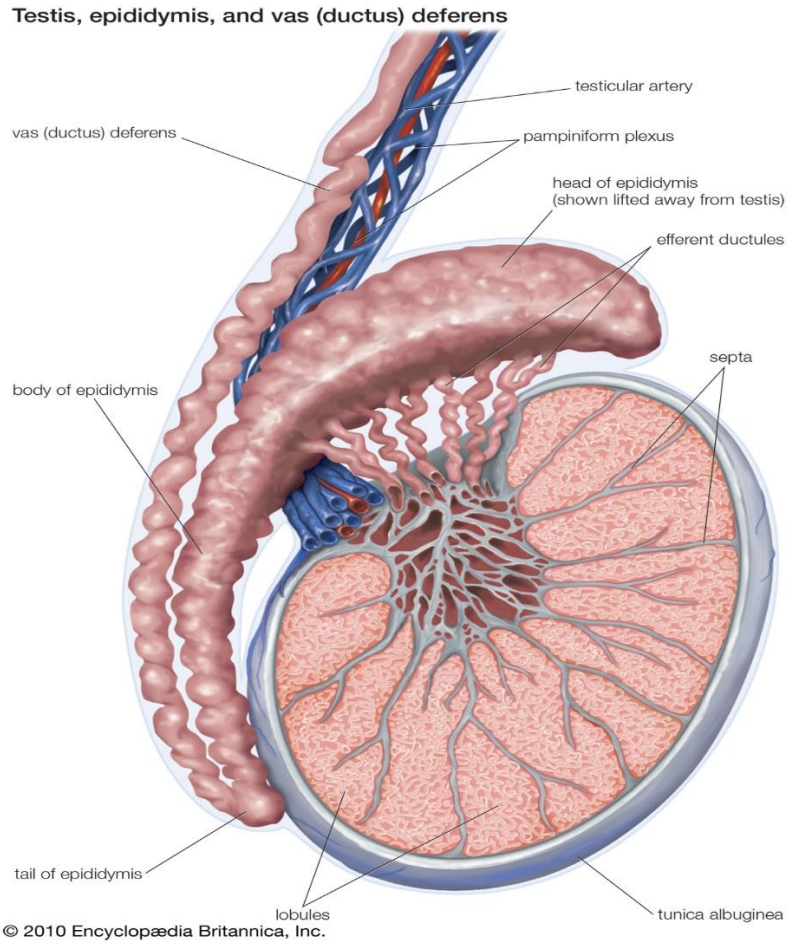

FIG 2.3 Illustration of the testis

Spermatogonial stem cells (SSCs) are the adult tissue stem cells in the testes that are at the foundation of spermatogenesis and essential for male fertility. SSCs are defined like all other cells, by their ability to balance self-renewing divisions and differentiating divisions. SSCs arise from gonocytes in the postnatal testis, which arise from primordial germ cells (PGCs) during foetal development. (Phillips et al, 2010). The process of spermatogenesis is highly conserved among many organisms and can be subdivided into three crucial phases: a mitotic amplification phase, a meiotic phase, and a post-meiotic phase also known as spermiogenesis. The mitotic division produces two types of cells; Type A cells that replenish the stem cells, and Type B cells that differentiate into

spermatocytes. Two meiotic divisions from primary spermatocytes give rise to secondary spermatocytes and round spermatids, which undergo spermiogenesis (morphological differentiation) to produce mature sperm (Rathke et al, 2014, Fayomi and Orwig, 2018). The sperm migrate, by short contractions of the tubule, to the mediastinum testis and are then transported through a complex network of canals (rete testis and efferent ductules) to the epididymis for temporary storage. The sperm move through the epididymis and the spermatic duct to be stored in the seminal vesicles for eventual ejaculation with the seminal fluid (Rogers, 2011).

The Sertoli cell rests on the basement membrane of the seminiferous tubule and its cytoplasm extends to the lumen of the tubule. Sertoli cells have an extensive cytoplasm which ramifies throughout the whole germinal epithelium, enclosing all the cells of the spermatogenic series. The cytoplasmic outline of the Sertoli cell is thus highly irregular and constantly changing to permit the progressive movement of developing spermatozoa towards the luminal surface. Sertoli cells are important in the regulation of spermatogenesis and spermiogenesis. Sertoli cells form tight junctions with each other as well as with the developing germ cells. It is well established that high concentrations of androgen hormones, secreted by Leydig cells of the testicular interstitium, are essential for production and maturation of spermatogenic cells. Sertoli cells secrete an androgen-binding protein which transports testosterone and dihydrotestosterone to the lumen of the seminiferous tubule. These hormones are also necessary for function of the epithelium of the rete testis and epididymis; production of this binding protein is believed to be dependent on the pituitary gonadotrophin follicle stimulating hormone (FSH).

Leydig cells, the principal cell type found in the interstitial supporting tissue between the seminiferous tubules, synthesise and secrete the male sex hormones and other non-steroid substances. They occur singly or in clumps and are embedded in the rich plexus of blood and lymph capillaries which surrounds the seminiferous tubules. Testosterone is the main hormone secreted by Leydig cells. Testosterone is not only responsible for the development of male secondary sexual characteristics at puberty but is also essential for the continued function of the seminiferous epithelium. The secretory activity of Leydig cells is controlled by the pituitary gonadotrophic hormone luteinising hormone, sometimes called interstitial cell stimulating hormone (ICSH) in the male (Young et al, 2014).

2.2.1.1 DEVELOPMENT OF THE TESTES

The testis develops from the gonadal ridge, formed by proliferation of the coelomic epithelium and a condensation of underlying mesoderm, on the medial side of the mesonephros. Primordial germ cells from the yolk sac migrate to the gonadal ridge and become incorporated in the developing gonad. At first the testis and mesonephros are situated on the posterior abdominal wall, attached by the urogenital mesentery. As the testis enlarges its cranial end degenerates and the remaining organ lies at a more caudal location. Most of the mesonephros atrophies. Derivatives of the remaining mesonephric tubules include the vasa efferentia of the testis and the paradidymis (a small collection of tubules above the epididymis at the lower end of the spermatic cord). In the male, the mesonephric duct forms the canal of the epididymis, vas deferens, ejaculatory duct and

the appendix of the epididymis (a small appendage on the head of the epididymis) (Sinnatamby, 1999).

2.2.2 EPIDIDYMIS AND VAS DEFERENS

The epididymis is a firm structure, attached behind the testis, with the vas deferens to its medial side. It consists of a single highly coiled tube packed together by fibrous tissue. It has a large head at its upper end, connected by a body to a pointed tail at its lower end. The head is connected to the upper pole of the testis by the vasa efferentia and the tail to the lower pole by loose connective tissue. The body is partly separated from the testis by a recess which is open laterally, the sinus of the epididymis. The lateral surface of the epididymis is covered by the tunica vaginalis, which also lines the sinus. From the tail the vas deferens, a direct continuation of the canal of the epididymis, provided with a thick wall of smooth muscle, passes up medially. It enters the spermatic cord, passes through the inguinal canal, across the side wall of the pelvis just under the peritoneum, and crosses the pelvic cavity. It pierces the prostate and opens by the ejaculatory duct into the prostatic urethra. It enters the abdomen at the deep inguinal ring and passes along the side wall and floor of the pelvis to reach the back of the bladder. The epididymis is supplied by a branch of the testicular artery. Venous and lymphatic drainage are as for the testis. It is innervated by sympathetic fibres from the coeliac ganglion via the testicular artery (Sinnatamby, 1999).

2.2.2.1 DEVELOPMENT OF THE EPIDIDYMIS AND VAS DEFERENS

The whole length of the single tube constituting the epididymis and vas is a persistent and much elongated part of the mesonephric (Wolffian) duct of the embryo (Sinnatamy, 1999).

CHAPTER THREE

MATERIALS AND METHODS

3.1 COLLECTION AND IDENTIFICATION OF PLANT

The plant sample was bought from the Uselu market, Benin City fresh and it was identified by a plant taxonomist in the Plant Biology and Biotechnology department, University of Benin, Benin City, Dr. T. Akinibosun, as *Garcinia kola.*

3.2 EXTRACTION OF PLANT MATERIAL

The peel of the seed was removed and the fruit was chopped into particles then air dried at a room temperature of 30^0C for a few days then oven dried at a low temperature of 30^0C for ten minutes. It was thereafter pulverized to powder level using the British milling machine. The weight was actualized 600g and it was macerated with distilled water 900ml with constant shaking and stirring for twenty-four hours. Then filtration was

involved to separate the residue from the filtrate using filter paper, funnel and conical flask. The filtrate was thereafter concentrated in a water bath. The crude extract preserved in sample bottles in a refrigerator.

3.3 ANIMAL CARE AND MANAGEMENT

This study was carried out on twenty healthy adult male wistar rats weighing between 200g to 250g. The animals were obtained from the animal holdings of the Department of Anatomy, University of Benin, Benin City. All animals were fed with Top Feed growers mash and water ad libitum.

3.4 EXPERIMENTAL DESIGN

The animals were randomly assigned to one control group and three administration groups, each containing five rats in the following manner:

Group A was the control group.

Group B was low dose administration group (250mg/kg body weight).

Group C was intermediate dose administration group (500mg/kg body weight).

Group D was high dose administration group (1000mg/kg body weight).

The animal were acclimatized to the room condition of the animal house for a period of fourteen days then administration of the plant extract ensued for the next fifty six days. Administration of the extract was done orally using an orogastric tube once daily.

The animals were marked for ease of identification and their body weights were measured on a weekly basis using the Top loader weighing balance throughout the experimental period.

3.5 DETERMINATION OF LD_{50}

LD_{50} of *Garcinia kola* was determined from the previous work of Udenze et al (2014) to be 6741.43mg/kg.

3.6 SACRIFICE OF ANIMALS

At the end of the 56 days experimental period, each rat was anesthetized with the use of chloroform after which a transverse incision was made through the ventral wall of the abdomen of each rats. The testes and epididymis were excised and fixed in Bouin's fluid in preparation for their histological analysis. The vas deferens was immediately obtained for conduction of the sperm analysis. Blood samples were collected from the animals by cardiac puncture and placed in labelled sample bottles for the hormonal assay estimation.

3.7 SPERM ANALYSIS

Sperm cells were collected from the epididymis during the animal sacrifice by litigation of the extremities of the vas deferens to a length of about 36nm, 6ul of normal saline was then added to the ligated extremities. The ligated extremities of the vas deferens were placed on a Petri dish thereafter which the vas deferens is teased to allow the sperm cells to diffuse. The section was viewed under the microscope for sperm motility. The results were evaluated under the following variables:

(1) Progressive motility(PM)

(2) Non Progressive Motility(NPM)

(3) Percentage Immotile(IM)

Spermatozoa is picked from the Petri dish using a micro Pasteur pipette and dispenses on a clean slide. It is then covered with a cover slip and viewed under the microscope (×10 and ×40 objective lenses). The percentage motility score was evaluated for each of the samples in the groups of the experiment.

Sperm morphology was evaluated using a smear of sperm cell on a grease free slide and allowed to air dry. The sample was stained using the Bryan-Leishman technique (Besley et al, 1980) for 30 minutes. The smear was rinsed with distilled water, bloated and finally air dried. It was then viewed with oil immersion objective. The results were recorded as a percentage of normal and abnormal sperm cells. Normal spermatozoa appear with their characteristic head, normal neck and tail. The abnormal sperm cells present with bent heads, as headless, as tailless and with deformed middle piece.

The sperm count was determined by making a 1:20 dilution of the spermatozoa with 10% formal saline in test tube. The counting chamber is charged with a few drops of mixed solution into the chamber. Observation is done with the ×10 objective lens of the microscope and the sperm cells are counted and recorded in $10^6/mm^3$.

3.8 HISTOLOGICAL PROCEDURES

The testicles and epididymis were fixed in Bouin's fluid for about 24 hours and the tissues were processed by paraffin was embedment method of Drury and Wallington (1980).

The tissues were dehydrated for one hour each at room temperature by passing them through increasing concentrations of ethanol, first through 70% ethanol, 90% ethanol, absolute ethanol and then through absolute ethanol a second time.

The dehydrated tissues were cleared in two changes with xylene at room temperature for a one hour period each change.

The tissues were then infiltrated in two changes of molten paraffin wax at 60^0C for one hour each change and finally embedded in paraffin wax multi-block embedding molds.

The embedded tissues blocks were trimmed and each mounted on wooded block for sectioning on a rotatory microtome.

Sections of 5µm thickness were produced from the tissue blocks using a rotatory microtome (Bright B5143, Humtington, England). The sections were placed in a water bath at 40^0C and thereafter transferred to a slide drier to promote their adherence to the slides. Procedure for Haematoxylin and Eosin were carried out in accordance with Drury and Wellington (1980).

3.8.1 HAEMATOXYLIN AND EOSIN STAINING METHOD

The tissues were placed in xylene for about five minutes to remove excess paraffin wax from them.

The tissues were then rehydrated by passing them through descending grades of ethanol; absolute ethanol, 90% ethanol, 70% ethanol and then into water each stage about two minutes.

Staining of the tissues was done using Haematoxylin and Eosin dyes for about ten munites each followed by rinsing in water. Excess stains were removed by running tap water for about two minutes.

The tissues were differentiated in 1% acid alcohol for about one minute and washed in running tap water.

The sections were then counter-stained with 1% Eosin for about three minutes each followed by rinsing in water.

The tissues were then dehydrated by passing through increasing concentrations of ethanol; 70% ethanol, 90% ethanol and then absolute ethanol for about two minutes each.

The tissues were cleared in xylene for five minutes and finally mounted in Distrene Plasticizer and Xylene (DPX) and covered with a cover slip.

3.9 BIOCHEMICAL ASSAY

Serum Testosterone assay: Serum T was assayed from blood samples obtained from cardiac puncture. The samples were assayed in batches from a standardized curve using the enzyme-linked immunosorbent assay (ELISA) method (Teitz, 1995). The microwell kits used were from Syntro Bioresearch Inc., California USA. Using 10µl of the standard, the samples and control were dispensed into coated wells. 100µl T conjugate reagent was added followed by 50µl of anti-T reagent. The contents of the microwell were thoroughly mixed and then incubated for 90 minutes at room temperature. The mixture was washed in distilled water and further incubated for 20 minutes. The reaction was stopped with 100µ of 1M hydrochloric acid. Absorbance was measured with an automatic spectrophotometer at 450nm. A standard curve was obtained by plotting the concentration of the standard versus the absorbance and T concentration was determined from standard curve.

Luteinizing Hormone assay: The BioCheck LH ELISA is based on the principle of a solid phase enzyme-linked immunosorbent assay (Teitz, 1995). The assay system utilizes sheep polyclonal anti-LH for solid phase (microtiter wells) immobilization, and a mouse

monoclonal anti-LH in the antibody enzyme (horseradish peroxidase) conjugate solution. The test sample was allowed to react simultaneously react with the antibodies, resulting in the LH molecules being sandwiched between the solid phase and the enzyme-linked antibodies. Aftetr 45 minutes incubation at room temperature, the wells were washed to removed unbound labelled antibodies. A solution of Tetramethyl-benzidine was added and incubated for 20 minutes, resulting in the development of a blue colour. The colour development was stopped with the addition of HCl, and the resulting yellow colour measured spectrophotometrically at 450nm. The concentration of LH is directly proportional to the colour intensity of the test sample.

Follicle Stimulating Hormone assay: This assay was carried out using double antibody radio immunoassay. A rat recombinant FSH {I125} from Amersham, UK was used. The sensitivity of the assay was 0.9ng/ml (Teitz, 1995).

3.10 STATISTICAL ANALYSIS

The data obtained from the experiment were analyzed using descriptive and inferential statistics; all values were presented as Mean ± Standard Error of Means (S.E.M). The significance of the difference in the mean of all parameters was determined using one-way analysis of variance (ANOVA; 95% confidence interval). Least Square Differences (LSD), post-hoc tests were carried out for all groups. All statistical analysis was carried out using Statistical Package for Social Sciences version 22 developed by International Business Machine Corporation (IBM) in Armonk, New York.

3.11 PHOTOMICROGRAPHY

The sections examination was carried out using a Leica DM750 research microscope with a digital camera Leica ICC50 attached. Photomicrographs of the tissues were obtained at various magnifications.

CHAPTER FOUR

RESULTS

4.1 EFFECT ON BODY WEIGHT

At the end of the 8th week (day 56) of the experiment, animals in the four groups exhibited both decrease and increase in weight at various point along the course of the experiment. These changes in weight showed no significant difference after analysis (P>0.05) between the groups administered to when compared with the control animals.

4.2 EFFECT ON ORGAN WEIGHT

There was no statistically significant changes in the relative organ weight (testes and epididymis) of the groups administered *Garcinia kola* to when compared to the control group at the end of the 8th week (day 56).

4.3 EFFECT ON TOTAL SPERM COUNT

At the end of the 8th week (day 56), the total sperm count of the control group A was 397.50 ± 4.78 ×10^{6} cells/mm^{3}. There is no significant difference (P>0.05) in the total sperm counts of rats in low dose group B (380.00 ± 4.08 ×10^{6} cells/mm^{3}), in the intermediate dose group C (375.00 ± 2.88 ×10^{6} cells/mm^{3}) and in the high dose group D (367.00 ± 4.78 ×10^{6} cells/mm^{3}) when compared to control

4.4 EFFECT ON SPERM MOTILITY

At the end of the 8th week (day 56), there is no statistical significance ($P>0.05$) in the number of progressively motile sperm cells of animals in low dose administration group B (80.00 ± 0.00%), group C administered intermediate dose (70.00 ± 0.00%) and group D administered high dose (70.00 ± 0.00%) when compared with control (80.00 ± 0.00%). There was no statistical significance ($P>0.05$) in the percentage of non-progressively motile sperm cells in low dose group B (10.00 ± 0.00%), intermediate dose group C (10.00 ± 0.00%) and high dose group D when compared with control group A (10.00 ± 0.00%). There was no statistical significance ($P>0.05$) in the percentage of immotile sperm cells in low dose group B (10.00 ± 0.00%), intermediate dose group C (20 ± 0.00%) and high dose group D (20 ± 0.00%) when compared to control (10.00 ± 0.00%).

4.5 EFFECT ON SPERM MORPHOLOGY

At the end of the 8th week (day 56), the percentage of sperm cells with normal morphology in control group A was 92.50 ± 1.44%. There was significant decrease ($P<0.05$) in the percentage of sperm cells with normal morphology in animals in low dose group B (75.00 ± 2.88%), intermediate dose group C (76.25 ± 2.40%) and high dose group D (80.00 ± 0.00%). There was significant increase ($P<0.05$) in the percentage of abnormal sperm cells in animals in low dose group B (25.00 ± 2.88%), intermediate dose group C (23.75 ± 2.39%) and high dose group D (20.00 ± 0.00%) when compared to control group A of (7.5 ± 1.44%).

4.6 EFFECT ON TESTICULAR HISTOLOGY

At the end of the 8th week (day 56), there was no significant difference in the histological structure of the testes of the animals in the experiment. However, the testicular tissues of the animals in low dose group B, intermediate dose group C and high dose group D showed varying degree of vascular congestion, spermatozoa maturation arrest and Leydig cell hyperplasia.

4.7 EFFECT ON HISTOLOGY OF EPIDIDYMIS

At the end of the 8th week (day 56), there was no significant difference in the histological structure of the epididymis of the animals. However, the epididymal lumen of the animals in low dose group B, intermediate dose group C and high dose group D showed varying degree of mild depletion in spermatozoa population.

4.8 EFFECT ON SERUM CONCENTRATION OF FOLLICLE STIMULATING HORMONE (FSH)

At the end the 8th week, the serum concentration of follicle stimulating hormone (FSH) of the control group was 0.6450 ± 0.1250 mu/ml. there was no significant difference ($P>0.05$) in serum concentration of FSH in the animals of low dose group B (1.0350 ± 0.0150 mu/ml) when compared to control, but there was significant increase ($P<0.05$) in intermediate dose group C (1.2550 ± 0.0255 mu/ml) and high dose D (1.5200 ± 0.0220 mu/ml) when compared to control.

4.9 EFFECT ON SERUM CONCENTRATION OF LUTEINISING HORMONE

At the end of the 8th week, the serum concentration of luteinizing hormone of the control group was 0.5100 ± 0.0424 mu/ml. There was no significant difference ($P>0.05$) in the serum concentration of luteinizing hormone of animals in low dose group B (0.735 ± 0.0212 mu/ml). However, there was significant increase ($P<0.05$) in animals of intermediate group C (0.9600 ± 0.0282 mu/ml) and high dose group D (1.2600 ± 0.0565 mu/ml) when compared to control.

4.10 EFFECT ON SERUM CONCENTRATION OF TESTOSTERONE

At the end of the 8th week, the serum concentration of testosterone of the control group was 0.6900 ± 0.0500 nmol/L. There was no significant difference ($P>0.05$) in the serum concentration of testosterone of animals in low dose group B (1.0150 ± 0.0150 nmol/L) when compared to control. However, there was significant increase ($P<0.05$) in animals of intermediate group C (1.8250 ± 0.0250 nmol/L) and high dose group D (2.2200 ± 0.0200 nmol/L) when compared to control.

ORGAN WEIGHTS

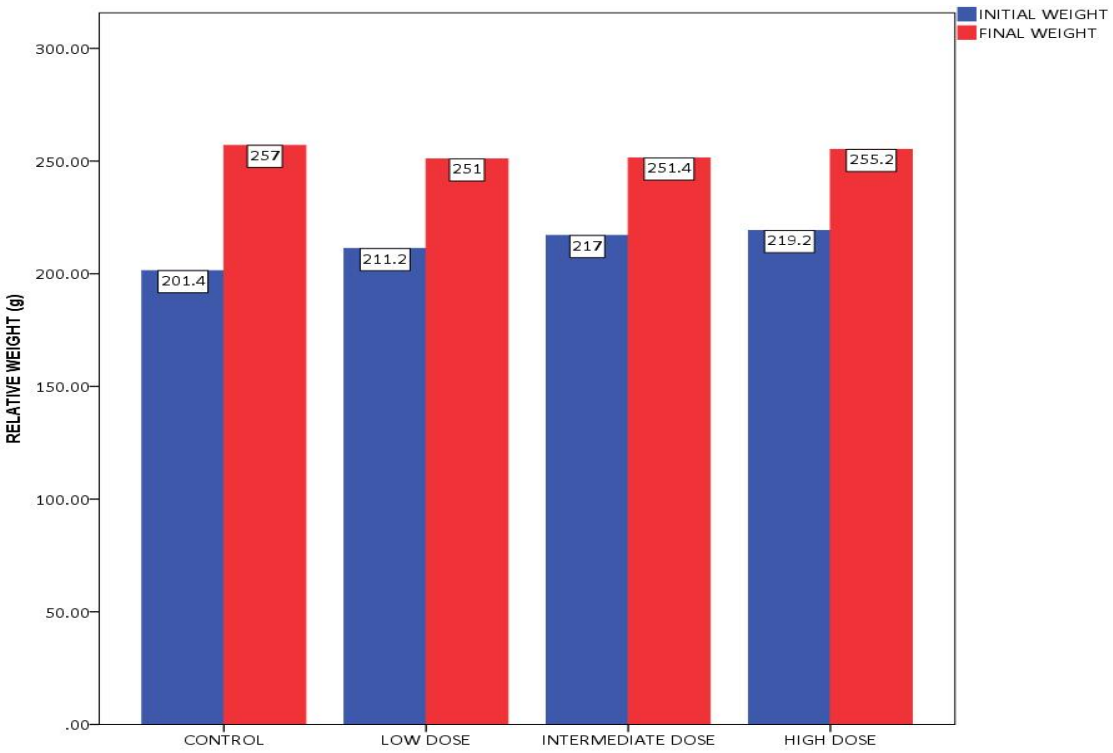

FIGURE 4.1: Effect of *Garcinia kola* On the Initial and Final Body Weight of Adult Male Wistar Rats for 8 Weeks.

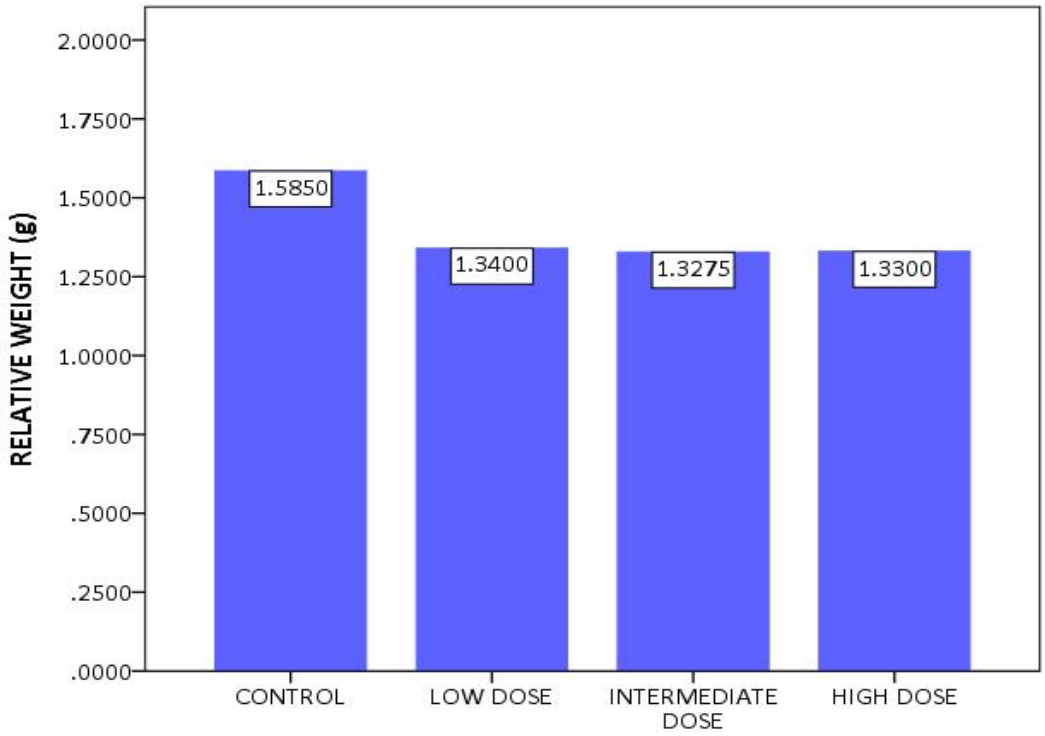

FIGURE 4.2: Effect of *Garcinia kola* On the Relative Organ Weight of the Testes of Adult Male Wistar Rats for 8 weeks.

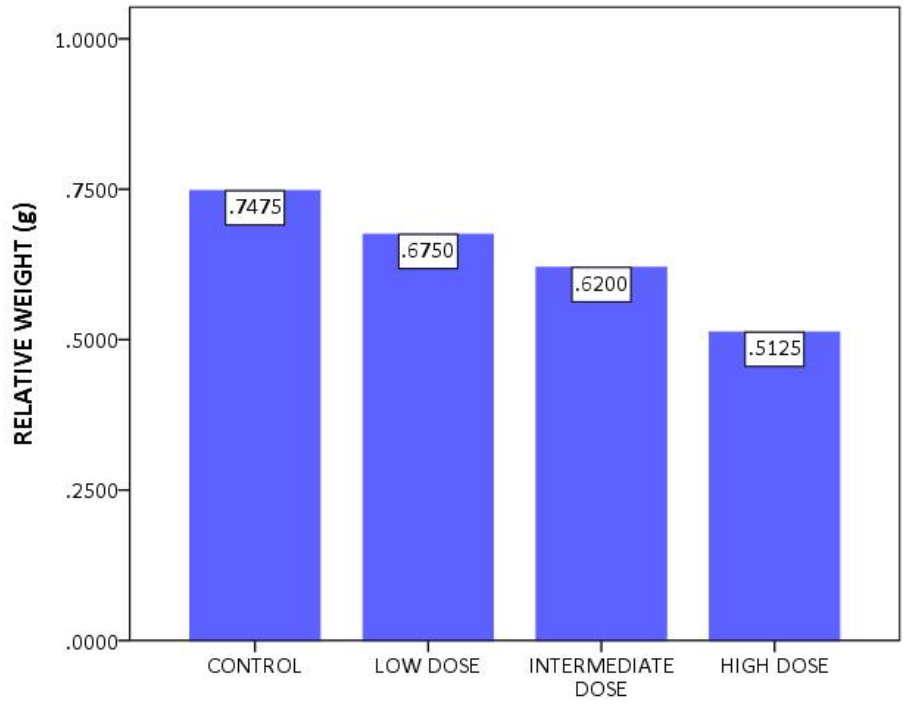

FIGURE 4.3: Effect of *Garcinia kola* On the Relative Organ Weight of the Epididymis of Adult Male Wistar Rats for 8 weeks

TOTAL SPERM COUNT

($\times 10^6$ cells/mm^3)

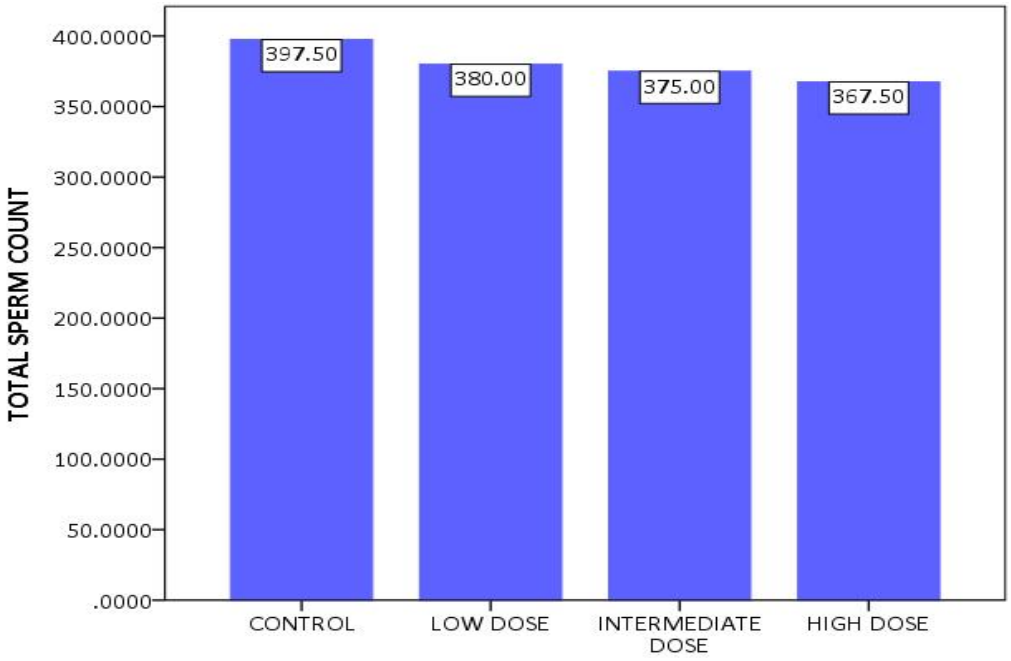

FIGURE 4.4: Effect of *Garcinia kola* On the Total Sperm Count of Adult Male Wistar Rats for 8 weeks

SPERM MOTILITY

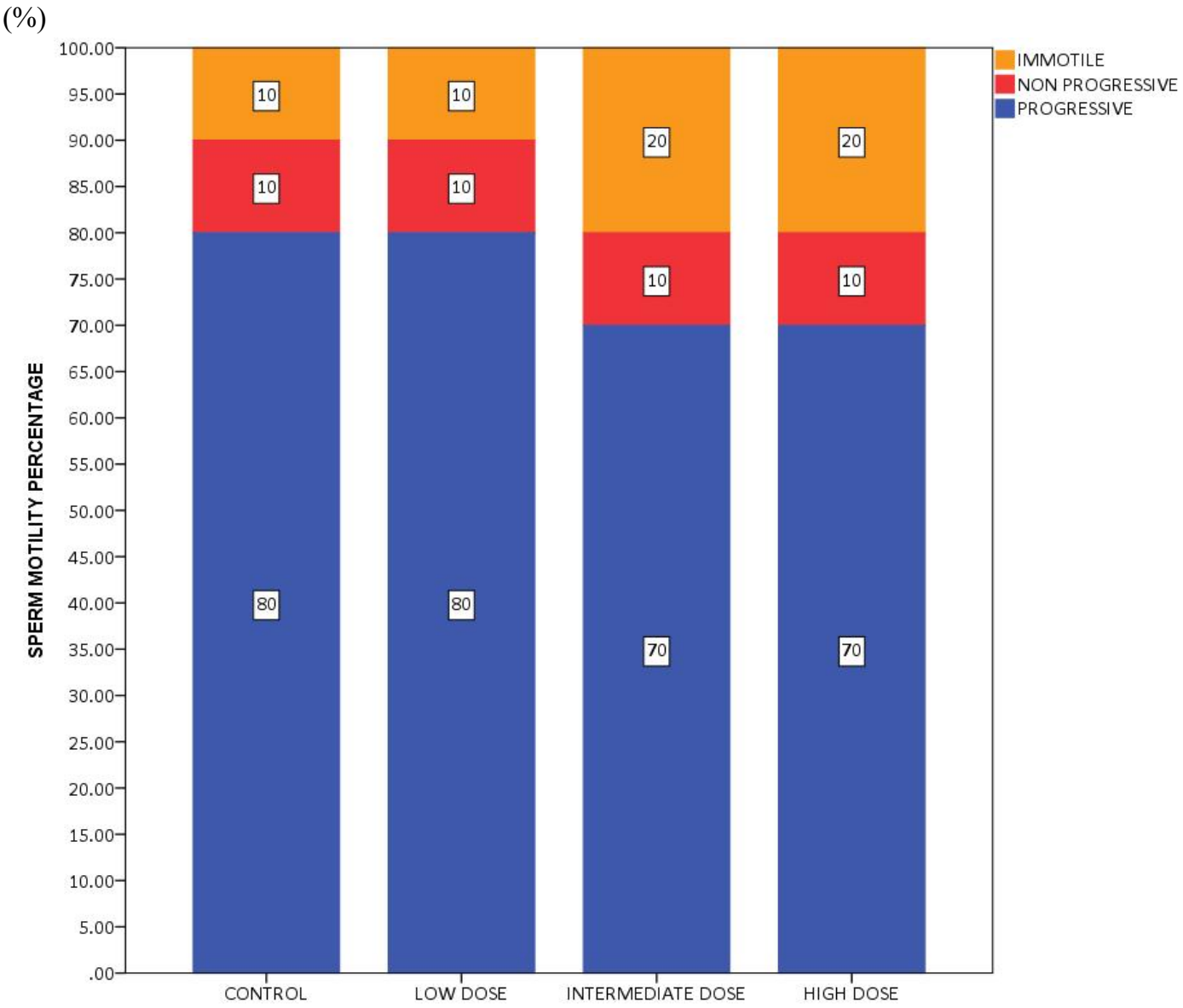

FIGURE 4.5: Effect of *Garcinia kola* On the Sperm Motility (Progressive, Non-Progressive and Immotile) of Adult Male Wistar Rats for 8 weeks

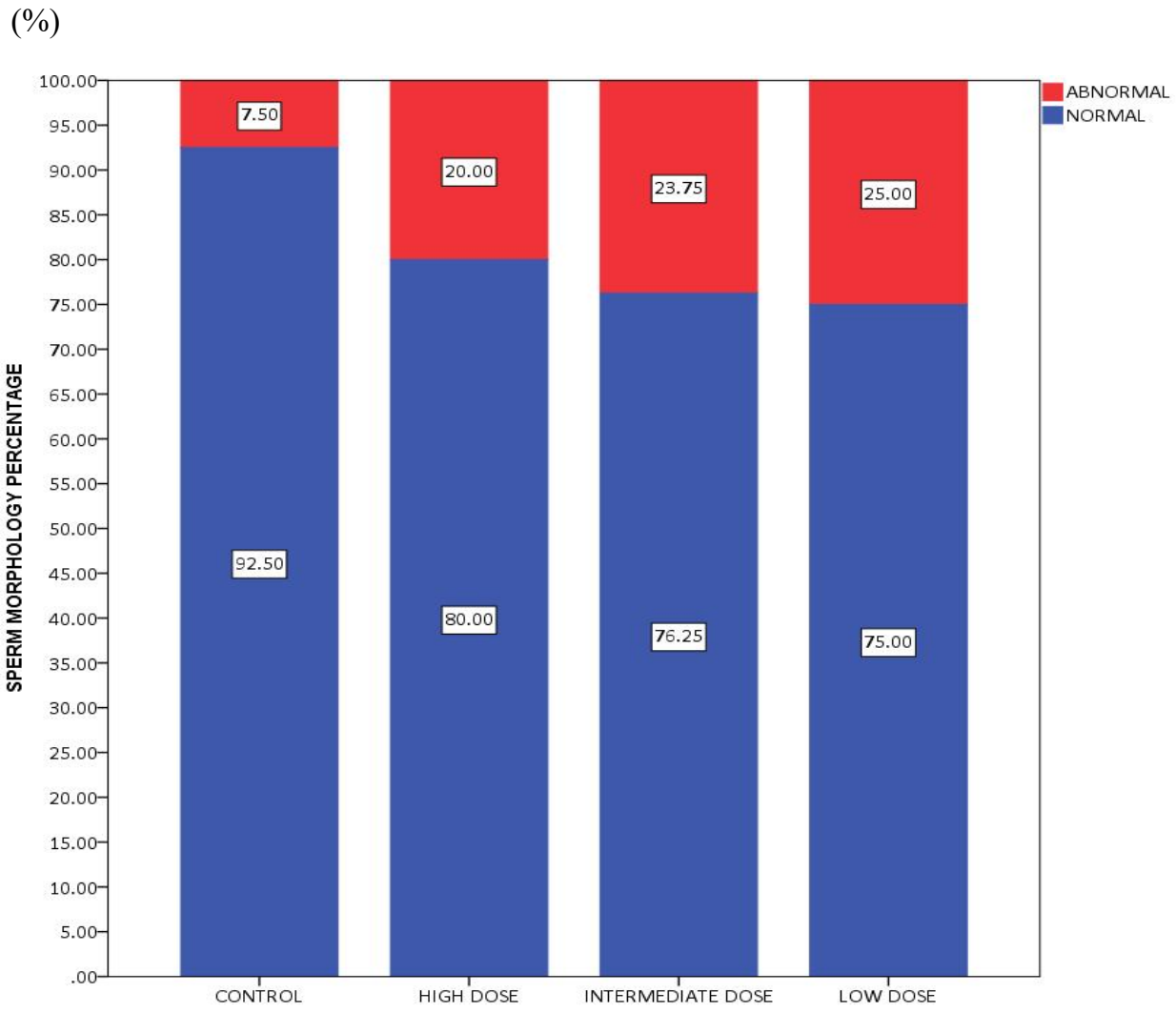

FIGURE 4.6: Effect of *Garcinia kola* On the Sperm Morphology of Adult Male Wistar Rats for 8 weeks

HORMONAL PROFILE

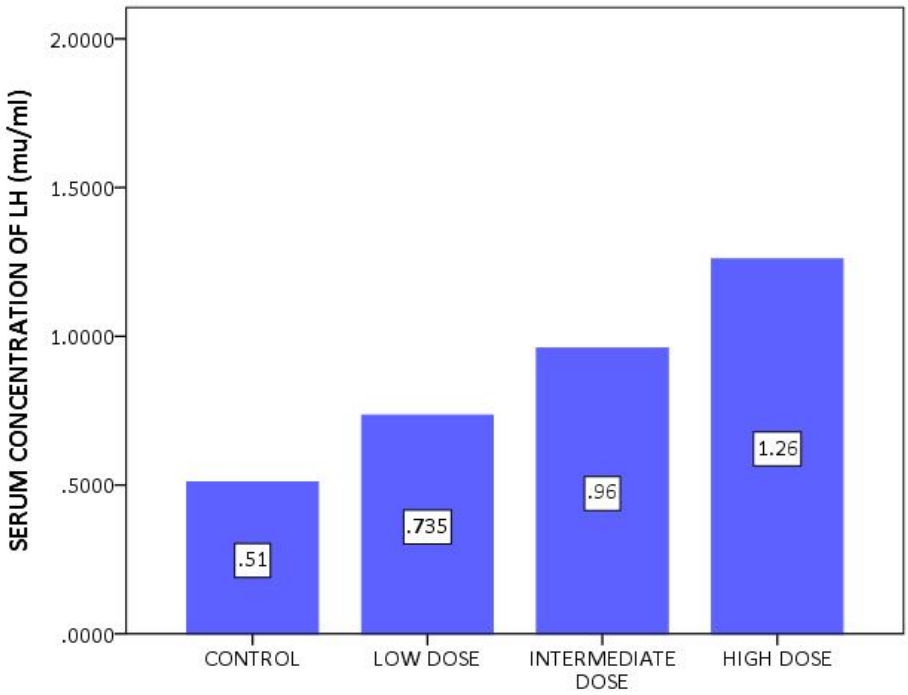

FIGURE 4.7: Effect of *Garcinia kola* On the Serum Concentration of Luteinizing Hormone of Adult Male Wistar Rats for 8 weeks

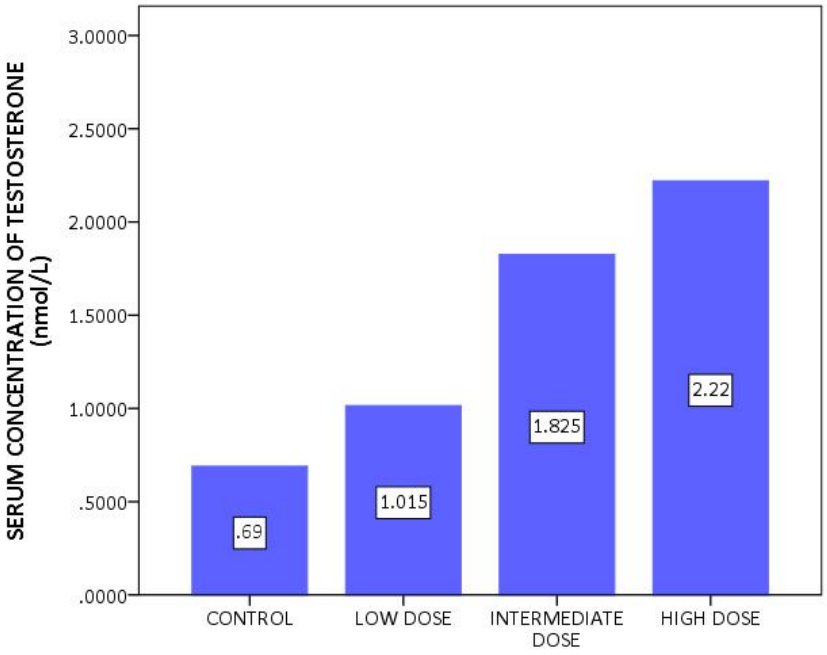

FIGURE 4.8: Effect of *Garcinia kola* On the Serum Concentration of Testosterone of Adult Male Wistar Rats for 8 weeks

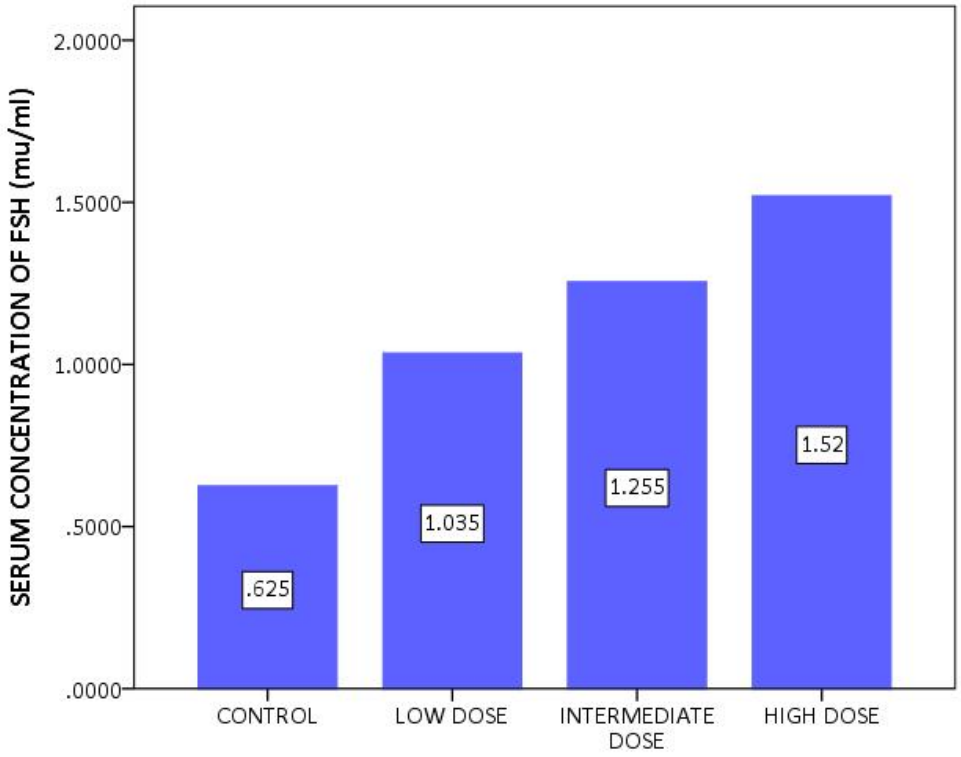

FIGURE 4.9: Effect of *Garcinia kola* On the Serum Concentration of Follicle Stimulating Hormone of Adult Male Wistar Rats for 8 weeks

TESTICULAR HISTOLOGY

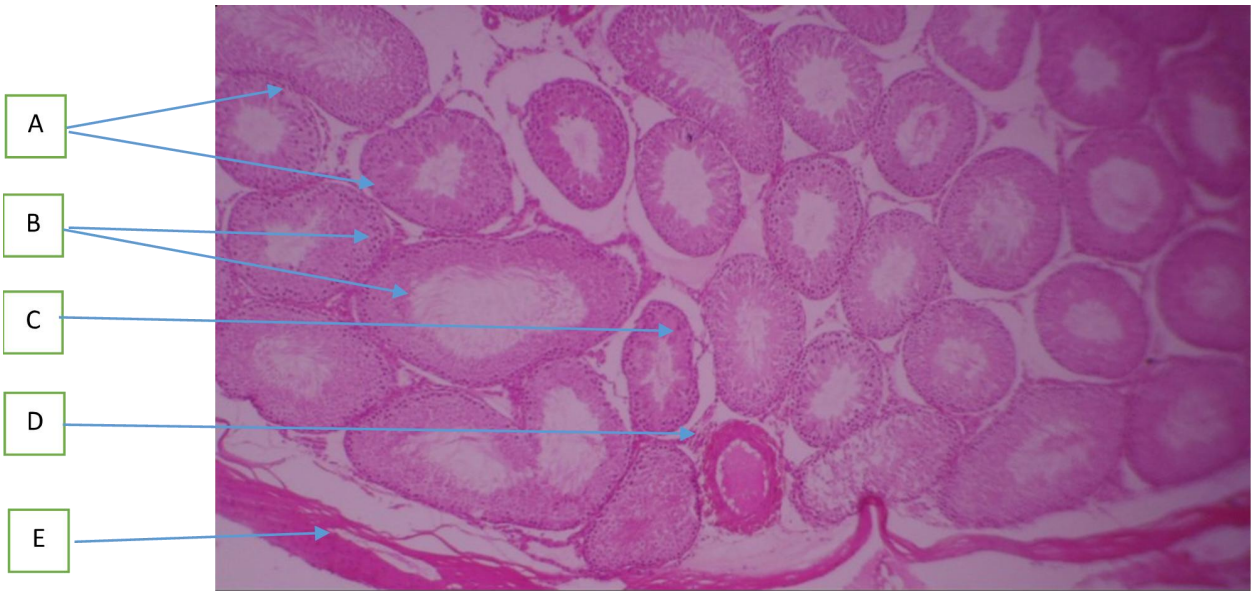

FIGURE 4.10: Testes of control A = seminiferous tubules lined by spermatocytes = B, C = sertoli cells. D = interstitial space and E = tunica albuginea (H&E X40)

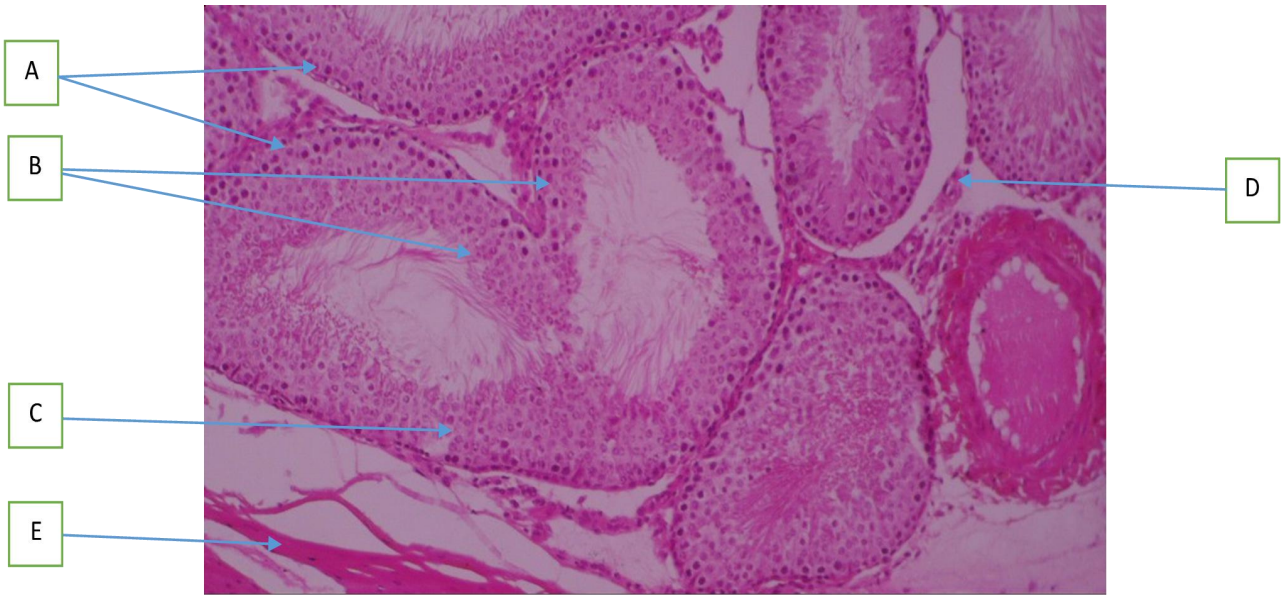

FIGURE 4.11: Testes of control higher magnification A, B, C, D, E (H&E X100)

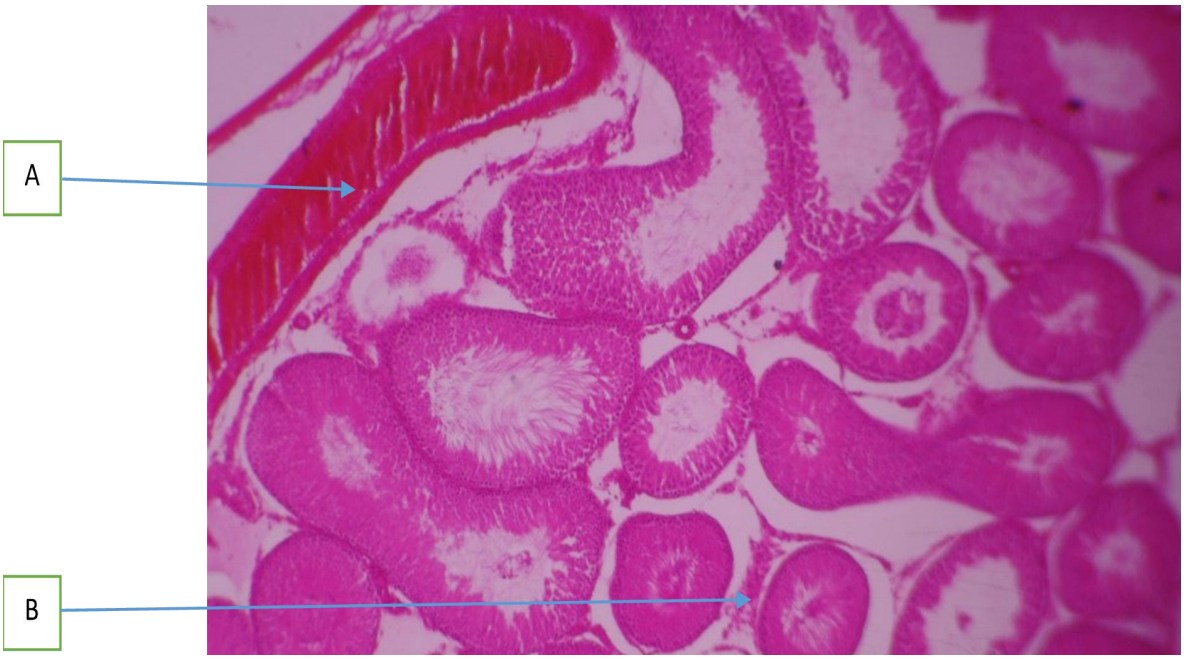

FIGURE 4.12: Testes of rat given low dose of *Garcinia kola* extract
A = vascular congestion B = Leydig cell hyperplasia (H&E X40)

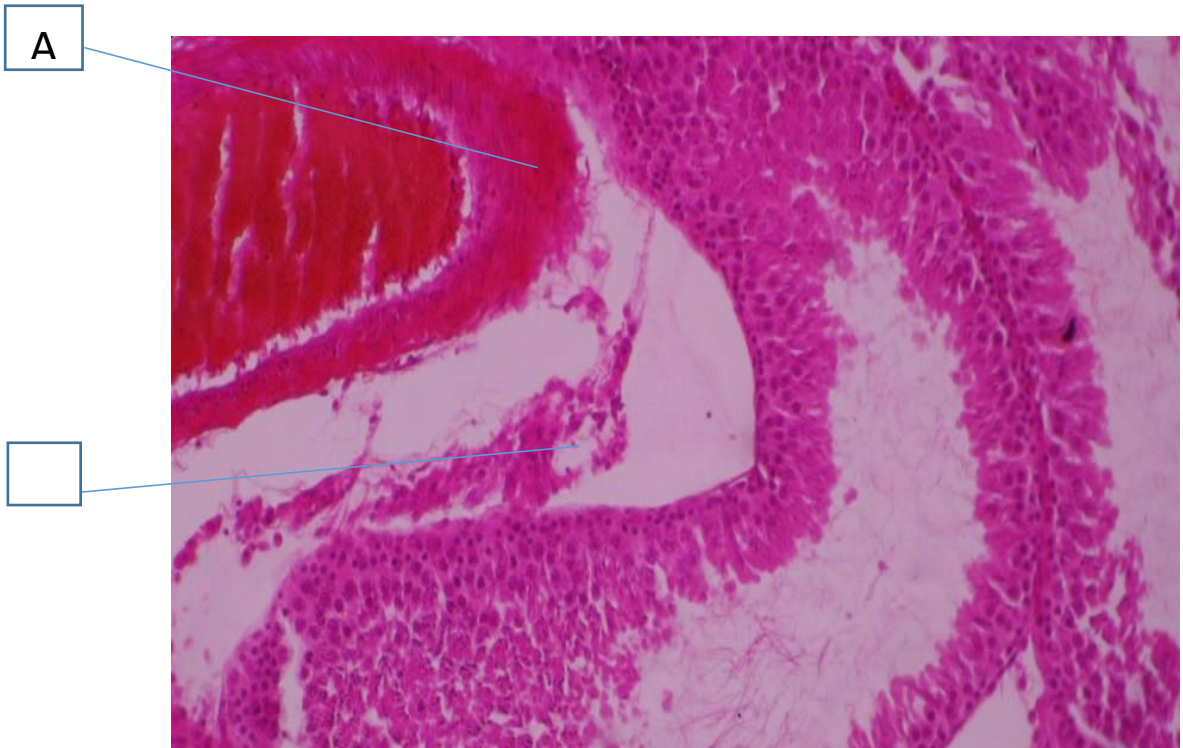

FIGURE 4.13: Low dose higher magnification A, B (H &E ×100)

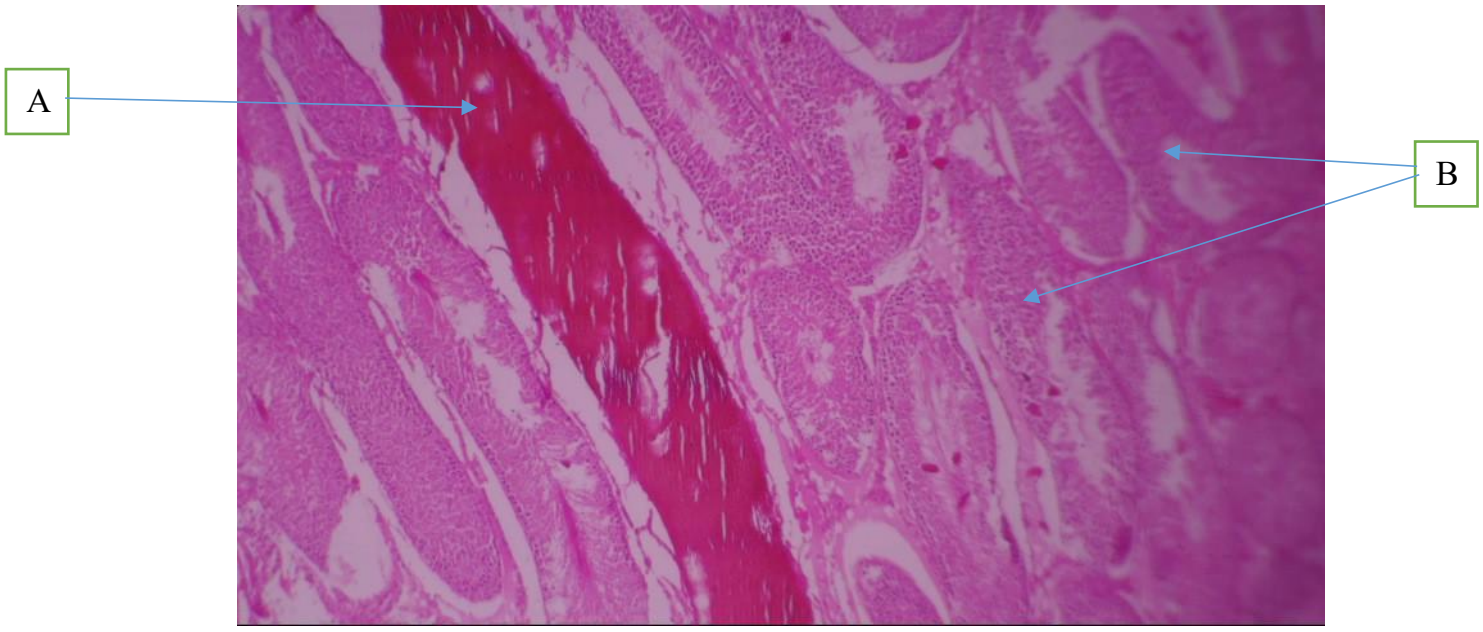

FIGURE 4.12: Testes of rat given intermediate dose extract: A = vascular congestion and dilatation, B = necrosis (H&E x 40)

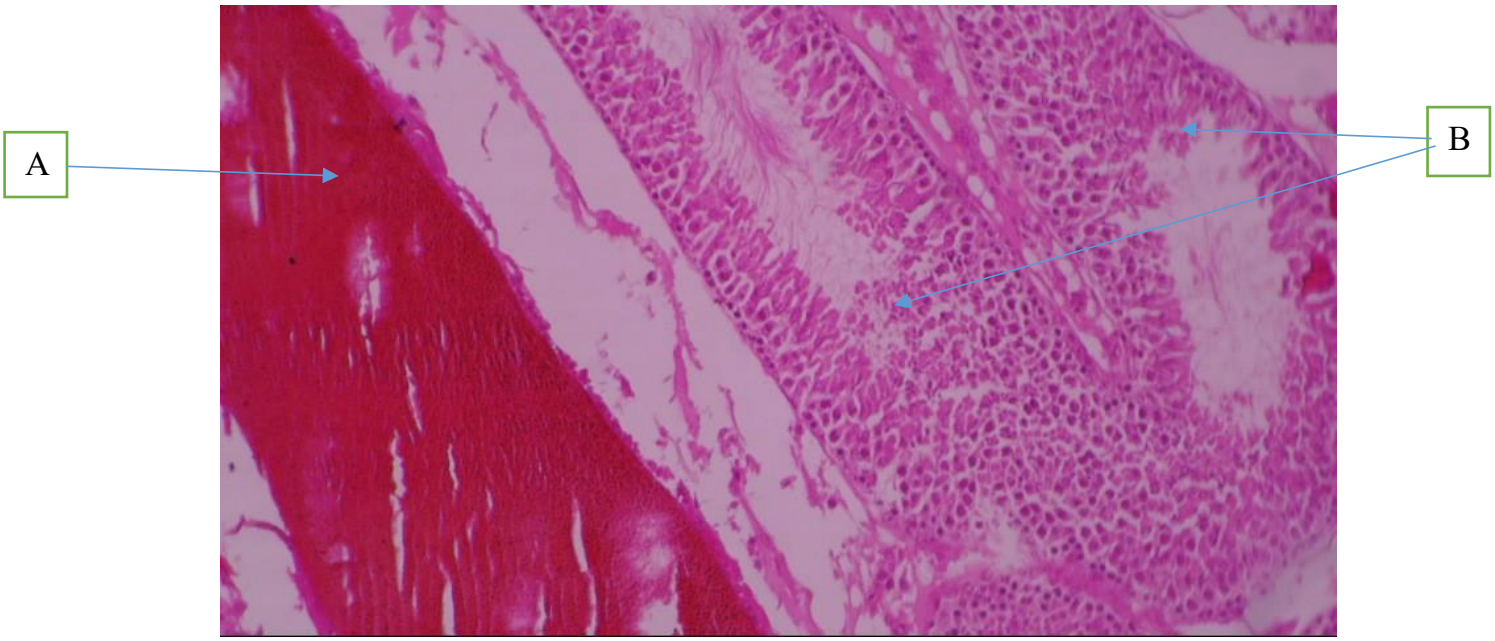

FIGURE 4.15: Intermediate dose higher magnification A, B (H&E x 100)

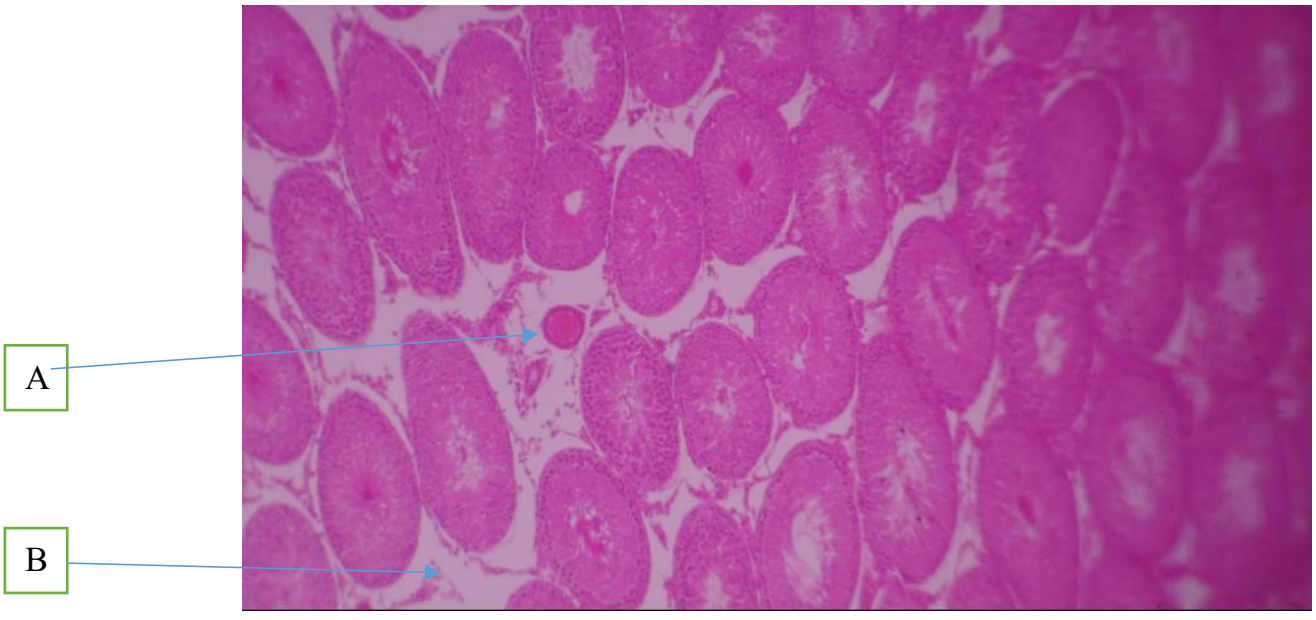

FIGURE 4.16: Testes of rat given high dose extract: A = vascular congestion and B = interstitial oedema (H&E x 40)

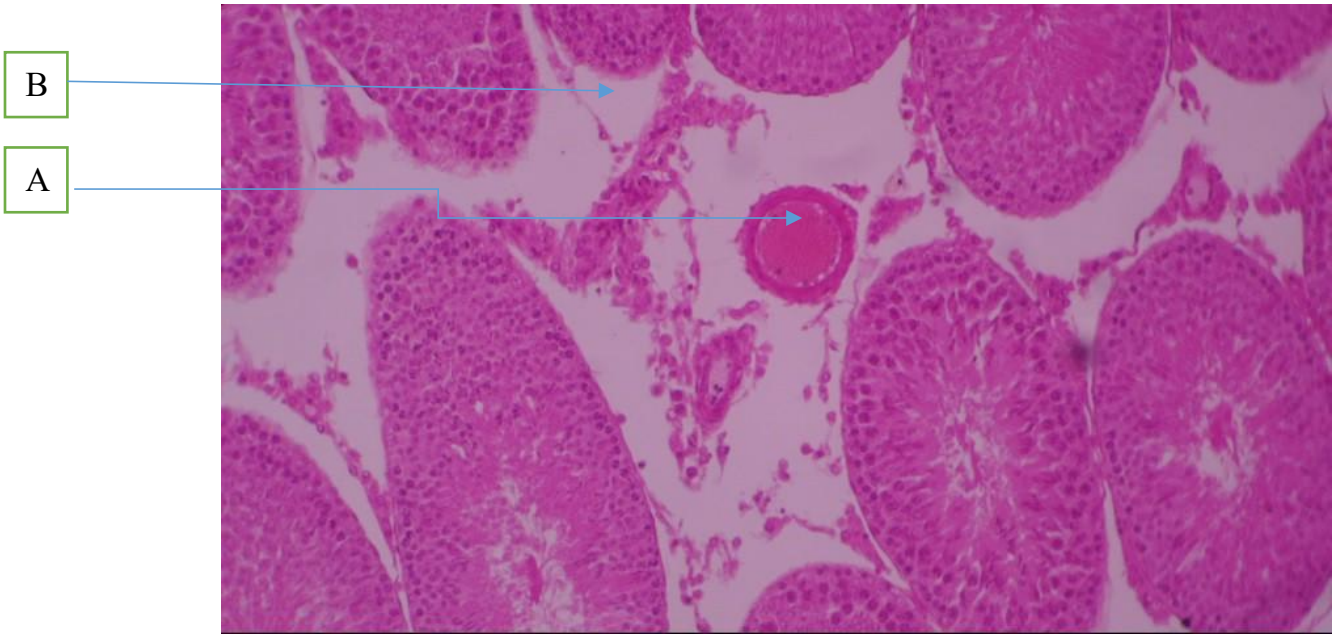

FIGURE 4.17: High dose higher magnification A, B (H&E x 100)

EPIDIDYMAL HISTOLOGY

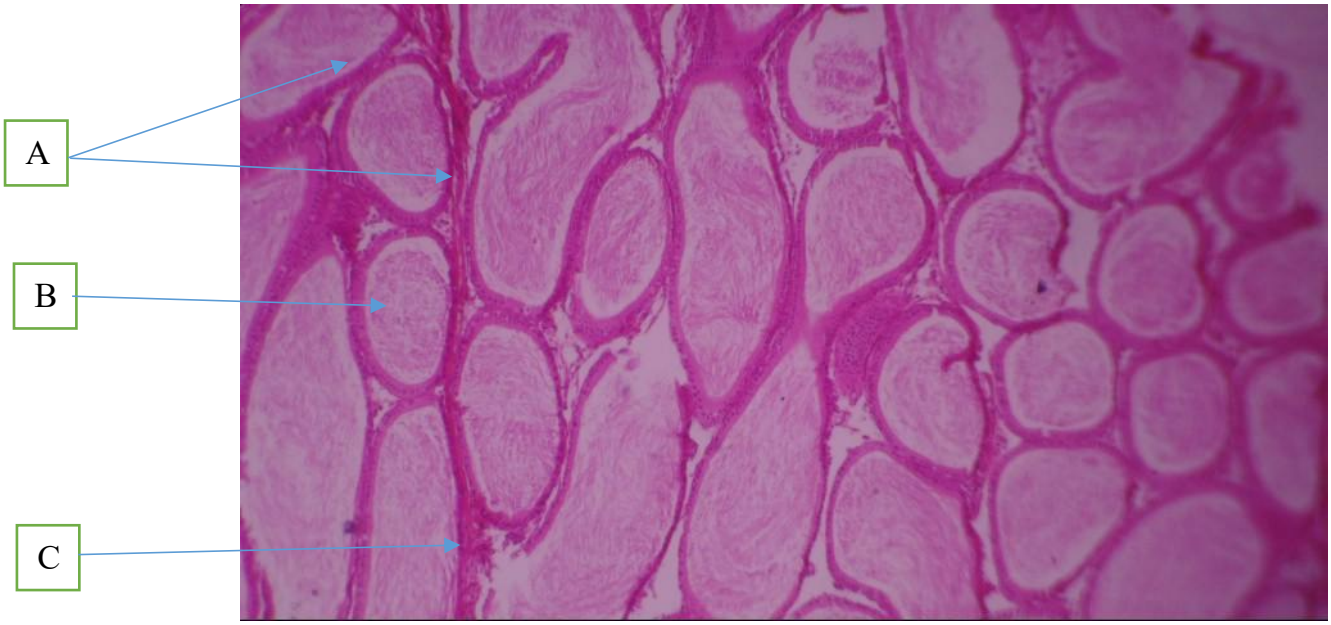

FIGURE 4.18: Epididymis of Control: A = ducts, B = lumen packed with spermatozoa and C = interstitial space (H&E x 40)

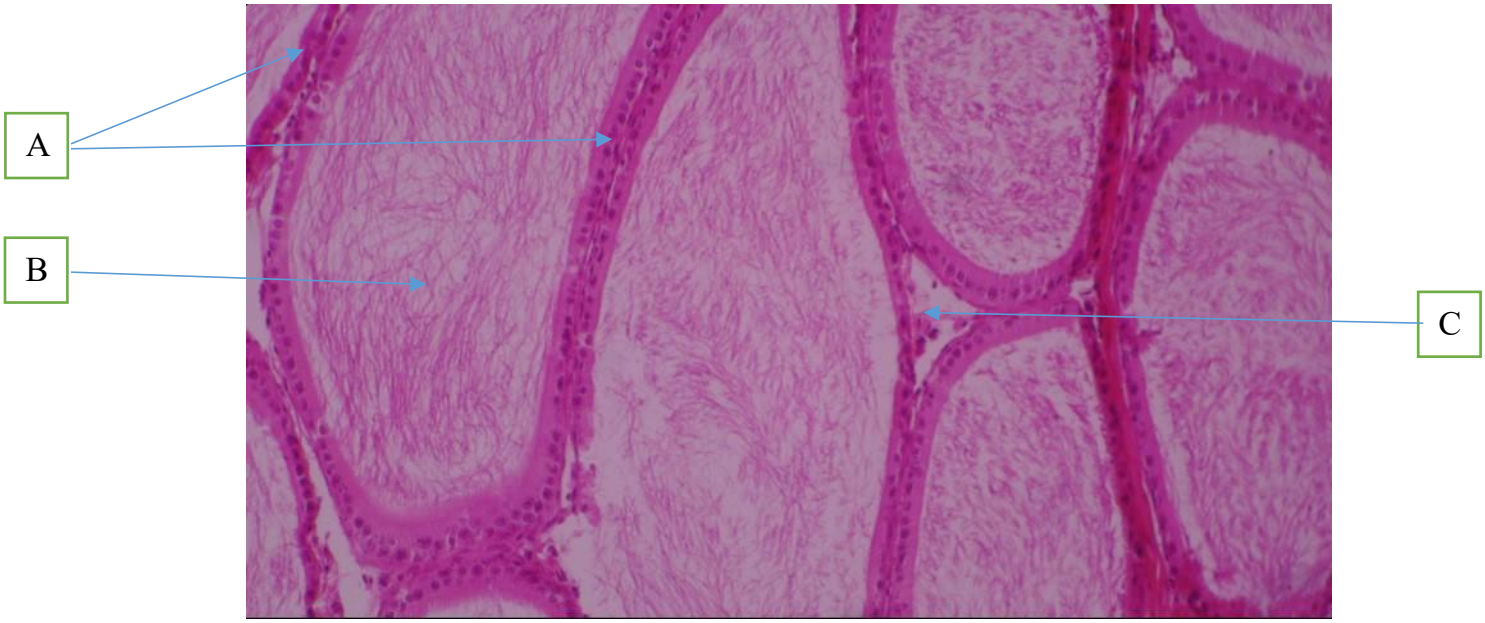

FIGURE 4.19: Epididymis of control higher magnification A, B, C (H&E x 100)

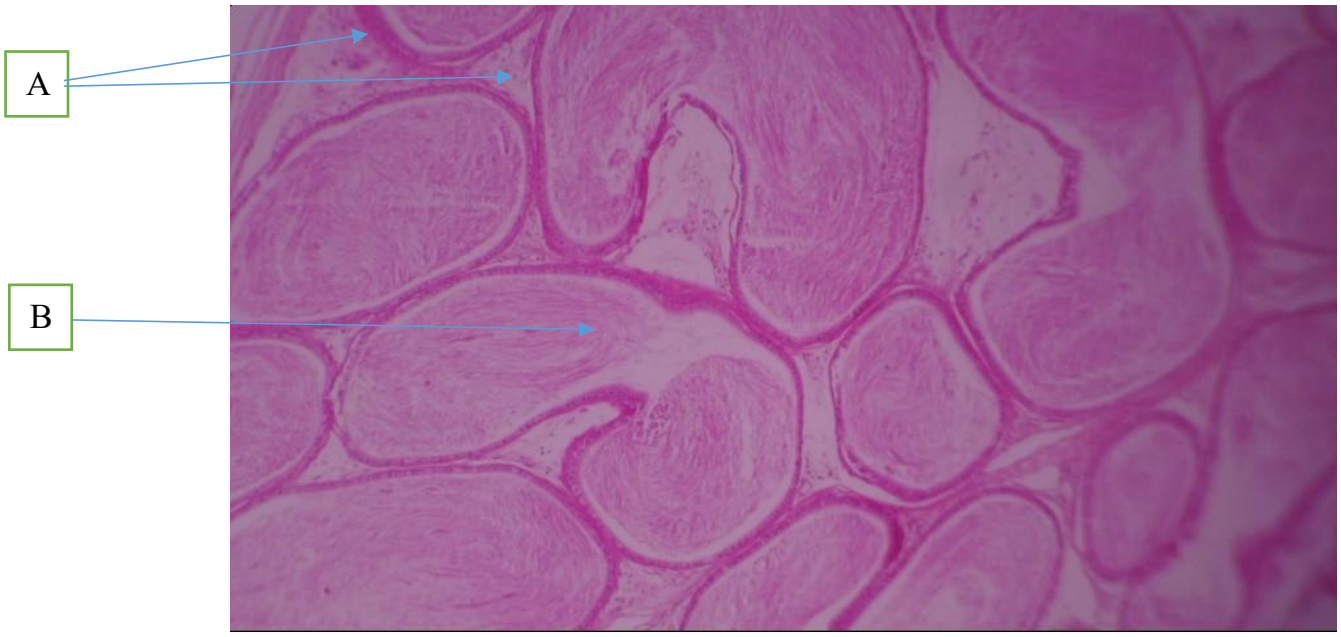

FIGURE 4.20: Epididymis of rat given low dose extract: A = ducts with B = lumen fairly filled with spermatozoa, (H&E x 40)

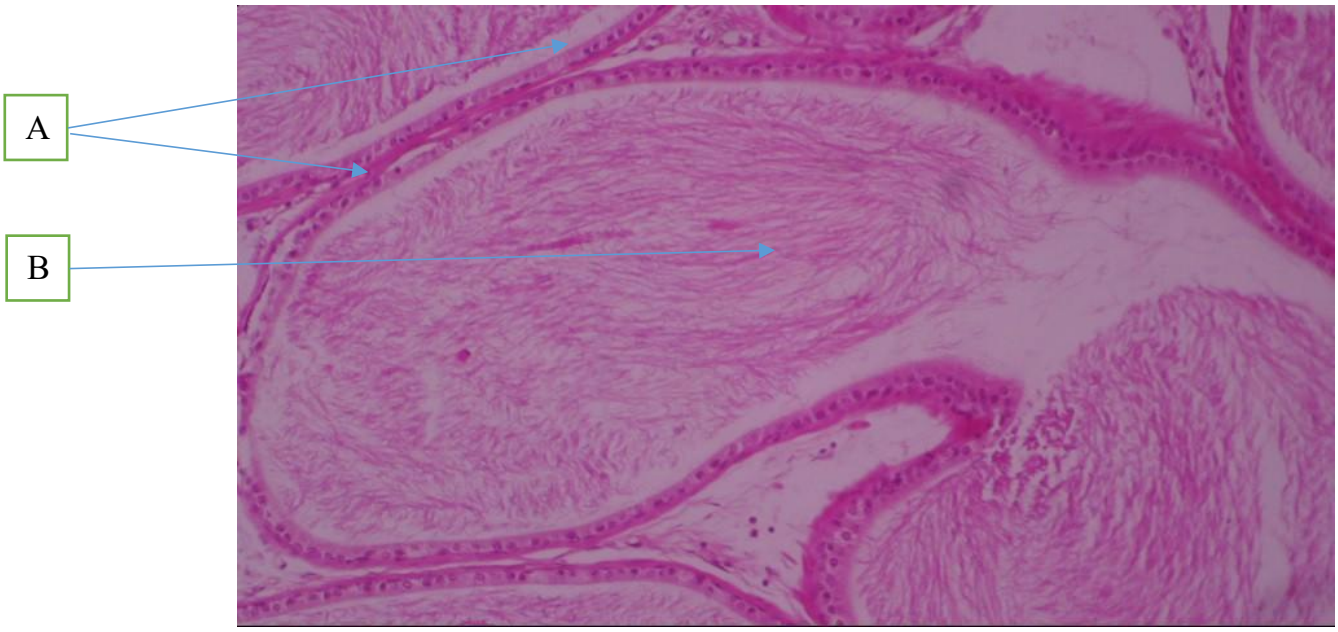

FIGURE 4.21: Epididymis of low dose higher magnification A, B, (H&E x 100)

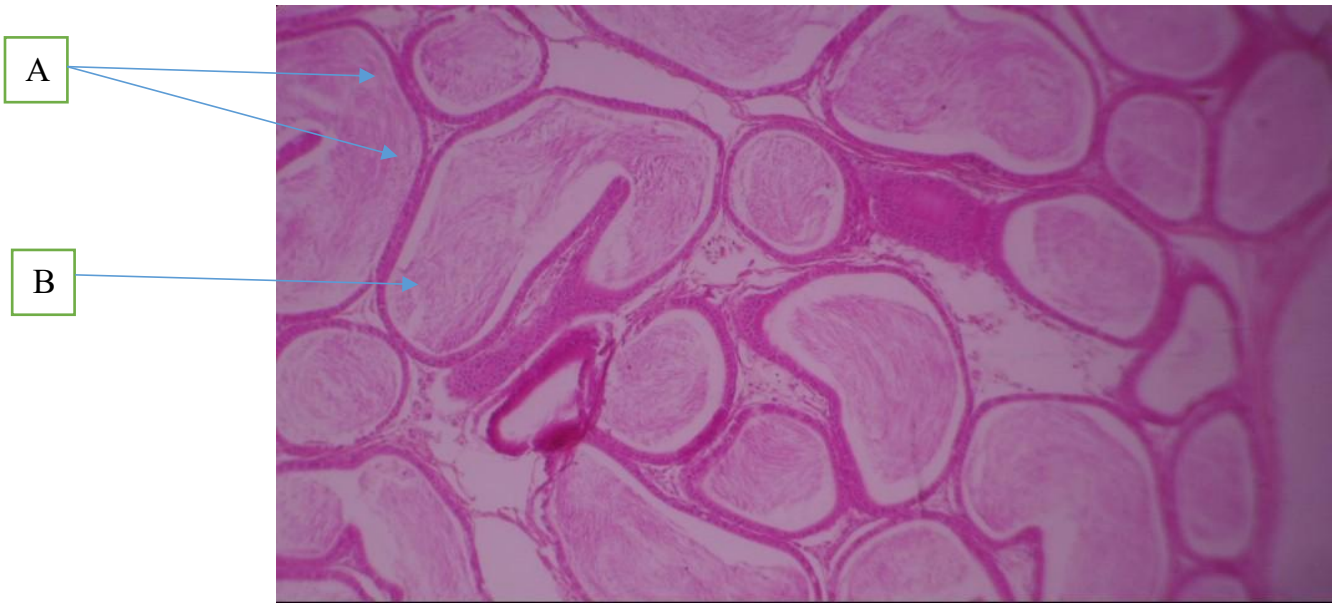

FIGURE 4.22: Epididymis of rat given intermediate dose extract: A = ducts and B = lumen mildly filled with spermatozoa and (H&E x 40)

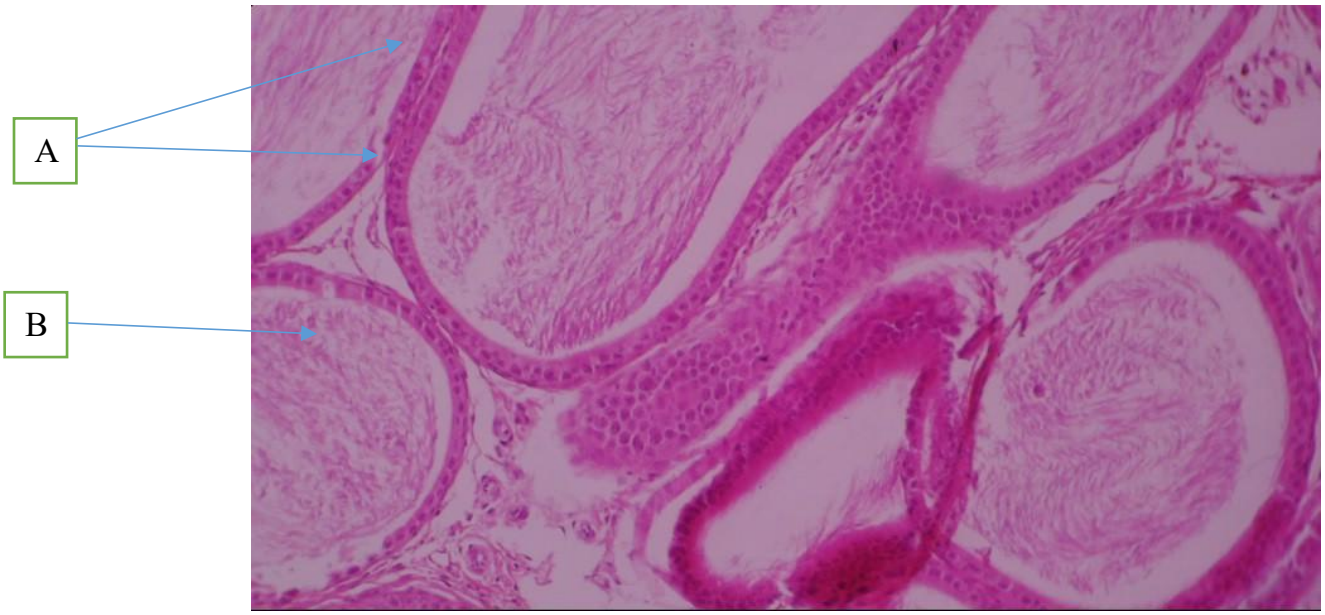

FIGURE 4.23: Epididymis of intermediate dose higher magnification A, B (H&E x 100)

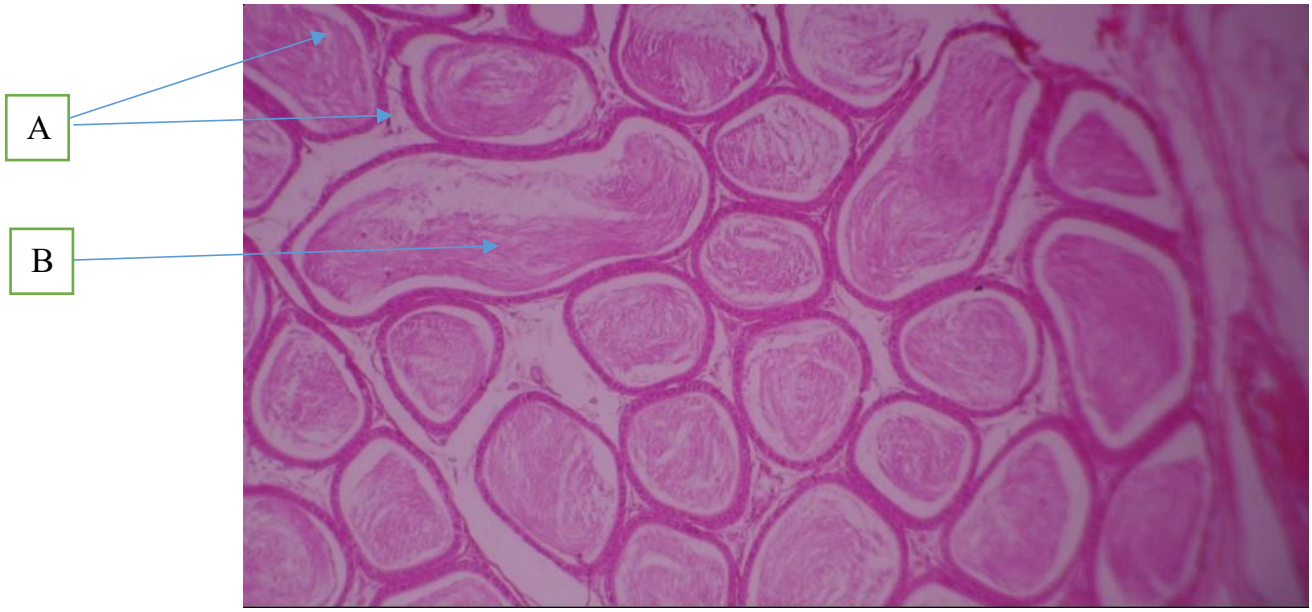

FIGURE 4.24: Epididymis of rat given high dose extract: A = ducts and B = lumen mildly filled of spermatozoa (H&E x 40)

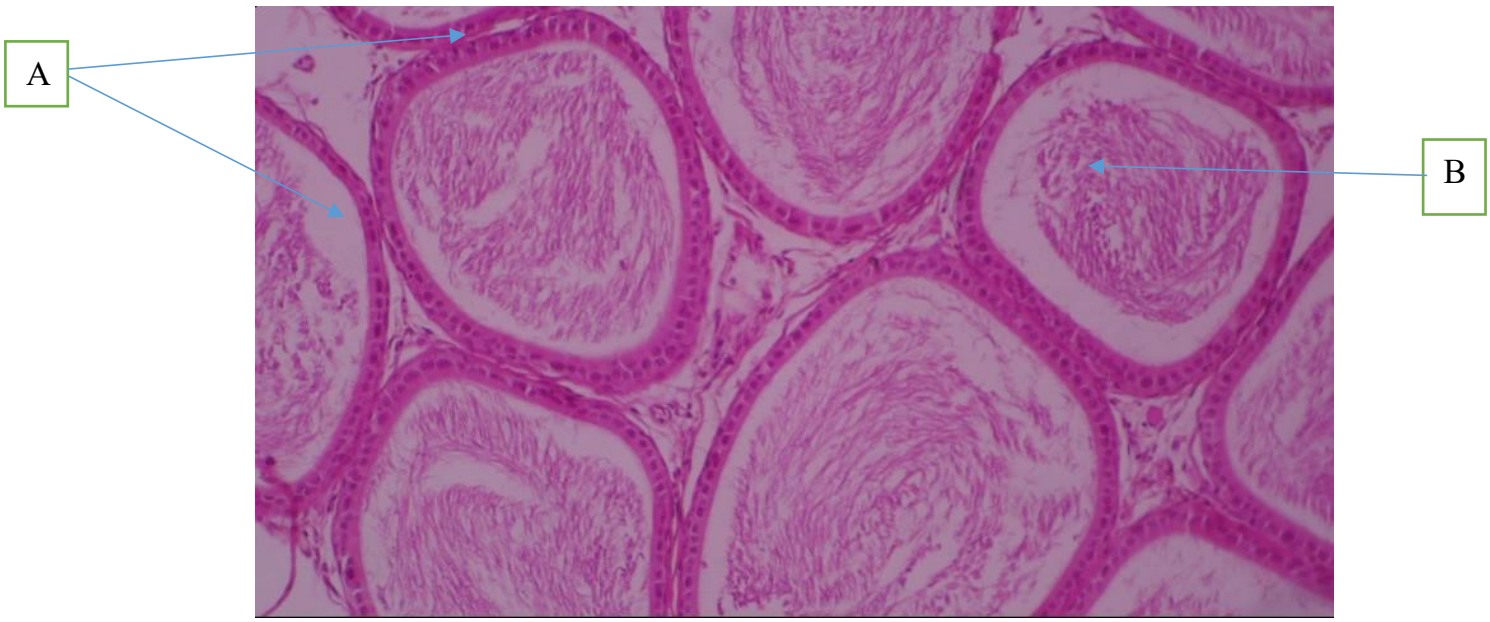

FIGURE 4.25: Epididymis of high dose higher magnification A, B (H&E x 100)

SPERM MORPHOLOGY

GROUP A (CONTROL)

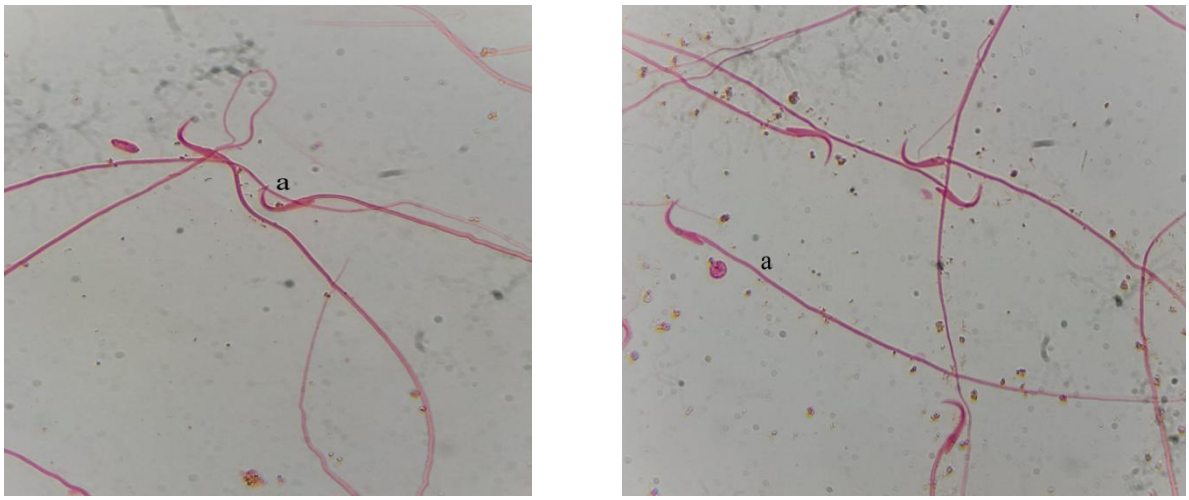

FIGURE 4.26: Sperm morphology of control group (Bryan-Leishman x40)

(1) Normal morphology, normal shape size and density **(a)**
(2) Normal sperm cell, normal shape, size and density, normal count

GROUP B (LOW DOSE)

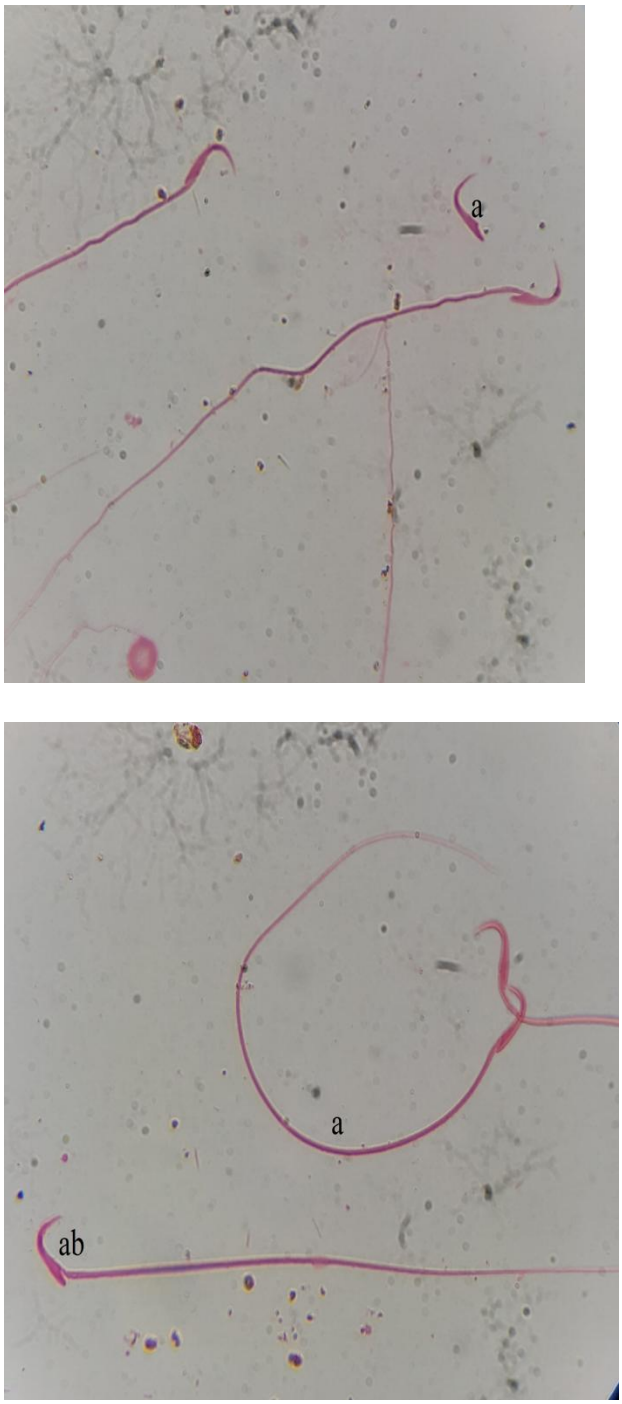

FIGURE 4.27: Sperm morphology of low dose group (Bryan-Leishman x40)

(1) Abnormal sperm cell, tailless sperm cells **(a)**
(2) Bent headed sperm cells **(ab)**

GROUP C (INTERMEDIATE DOSE)

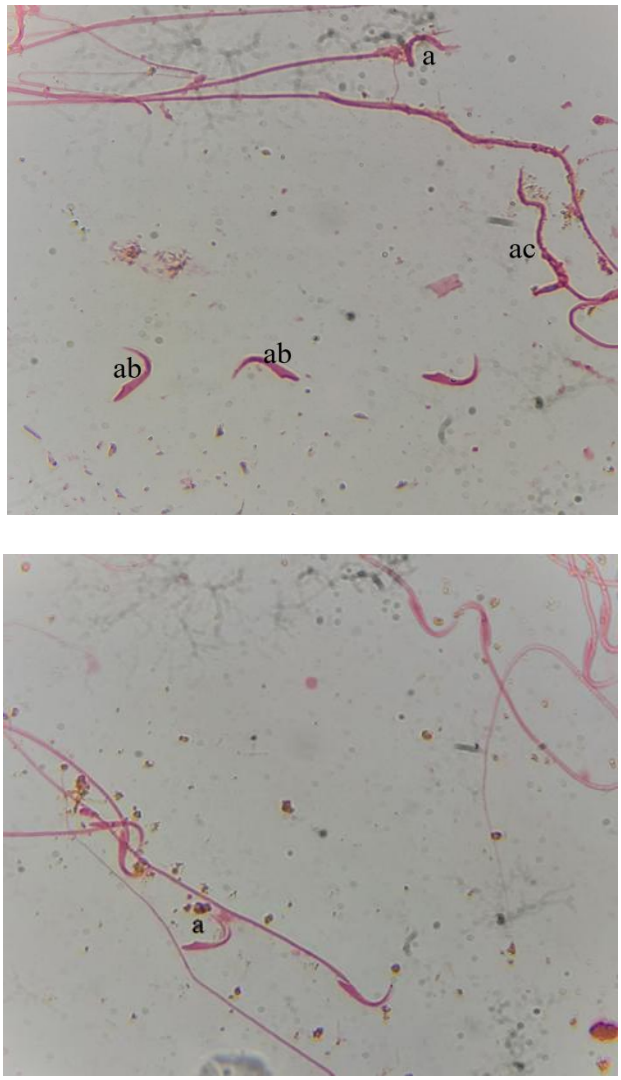

FIGURE 4.28: Sperm morphology of intermediate dose group (Bryan-Leishman x40)

(1) Deformed headed sperm cells **(a),** tailless sperm cells **(ab),** bent headed sperm cell **(ac)**
(2) Poorly formed headed sperm cells

GROUP D (HIGH DOSE)

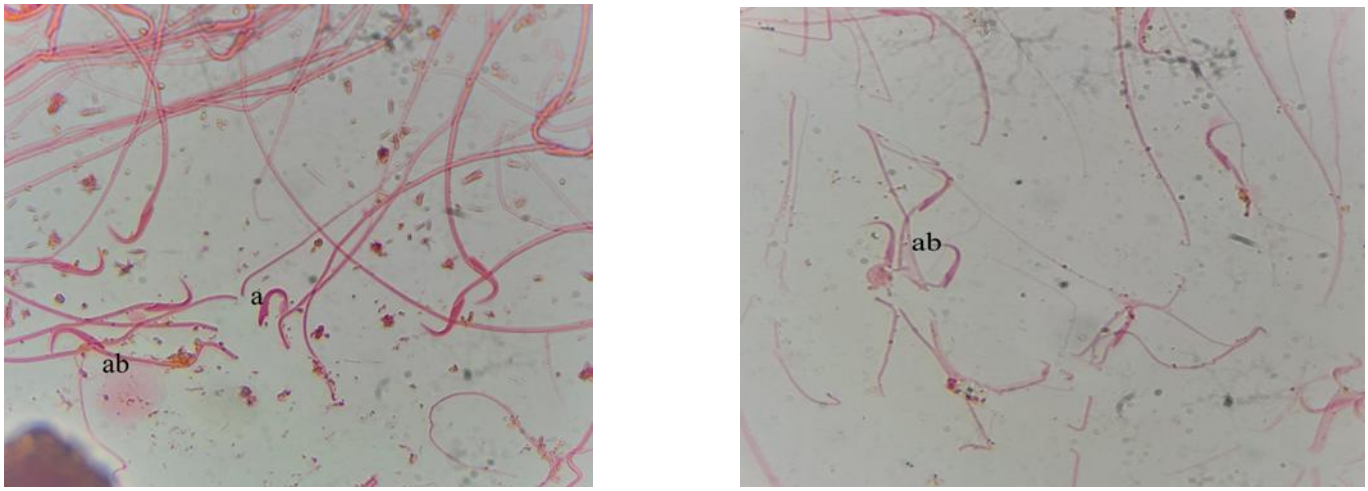

FIGURE 4.29: Sperm morphology of high dose group (Bryan-Leishman x40)

(1) Tailless sperm cells **(a),** Short tailed sperm cell **(ab)**
(2) Tailless sperm cells, **(ab)**

CHAPTER FIVE

DISCUSSION AND CONCLUSION

5.1 DISCUSSION

Sperm analysis has an important role in the routine evaluation of idiopathic male infertility, usually manifested as low sperm counts, impaired sperm motility, or absence of sperm, and remains the most common single diagnostic tool (Omu, 2013). A previous work done on *Garcinia kola* showed that it had antifertility effect however in the study *Garcinia kola* was administered for 30 days (Abu et al, 2013) which is in contrast with understanding that the spermatogenesis process in rats require about 52 days (Clouthier et al, 1996). Another work showed that extract of *Garcinia kola* seed possesses potent

aphrodisiac activity in male albino rats with resultant increase in sperm count and testosterone levels (Sewani-Rusike et al, 2016).

In this study, it was observed that *Garcinia kola* did not cause any significant changes in body weight and in the organ weight of the testes and epididymis in all administration groups as compared to the control at the end of the experiment. This is in contrast to previous study where it was shown to cause decrease in relative organ weight (Abu et al, 2013) and shown to cause increase in testicular weight (Sewani-Rusike et al, 2016).

The findings in this study also indicates that *Garcinia kola* did not cause significant changes in total sperm count when compared to control at the end of the experiment. This is in contrast to previous study where it was shown to cause decreased total sperm count (Abu et al, 2013) and shown to cause increased total sperm count (Sewani-Rusike et al, 2016).

The findings in this study also indicates that *Garcinia kola* caused significant decreases in the sperm motility and normal morphology percentages in a dose-dependent manner when compared to the control. This is in agreement with previous study (Abu et al, 2013) and in contrast with the previous study which showed no significant change in motility and normal morphology (Sewani-Rusike et al, 2016).

The findings in this study also indicates that *Garcinia kola* caused significant increase in serum concentration of testosterone, FSH and LH in a dose-dependent manner when compared to control at the end of the experiment. This is in contrast with previous work which showed decrease in the concentration of hormones (Abu et al, 2013) and in

agreement with previous that showed increase in concentration of hormones (Sewani-Rusike et al, 2016).

Histological findings in the testes and epididymis did not reveal significant differences in overall histology which is in agreement with previous study that showed no differences in organ histology (Sewani-Rusike et al, 2016). However histological findings in this showed vascular congestion and increase in Leydig cell population.

Vascular congestion has been shown to be an indication of testicular injury (Huang et al, 1990). Elevated serum FSH is a reliable indicator of germinal epithelial damage (Martin-du Pan, 1993). Increased levels of LH further promotes development of more Leydig cells (Young et al, 2014)

5.2 CONCLUSION

From the study carried out, *Garcinia kola* showed antifertility characteristics in a dose dependent manner after 56 days. Although this results might not adversely affect chances of fertilization, it is recommended that further study be carried out to investigate the mechanism of action causing these damages and further evaluate its effects in longer term.

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