EFFECT OF ETHANOLIC FRUIT EXTRACT OF *XYLOPIA AETHIOPICA* (DUNAL) A. RICH (ANNONACEAE) *AND* XYLOPIC ACID ON REPRODUCTIVE FUNCTION IN MALE RATS

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DECLARATION

The experimental work described in this thesis was carried out at the Department of Pharmacology, KNUST. This work has not been submitted for any other degree.

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HEAD OF DEPARTMENT

ABSTRACT

Xylopia aethiopica (African guinea pepper) is used mainly as a spice, postpartum tonic and to induce postpartum placental discharge by traditional birth attendants (TBA) and to enhance male fertility across the West African Sub-region especially Ghana and Nigeria. Despite of its frequent and regular use, no attempt has been made to scientifically examine the effects of the spice on reproduction as well as the toxicological profile. The present study was thus undertaken to evaluate the effect of 70% alcoholic extract of the fruits of Xylopia aethiopica and its major constituent, xylopic acid on steroid hormones, spermatogenesis and testicular histology as well as the toxicity profile in male. Extract of Xylopia aethiopica was administered (30, 100 and 300 mg/kg p.o) to different groups of male rats for sixty days. Blood samples were collected 24 hours after the last treatment by cardiac puncture for haematology parameters and for enzyme and other biochemical assays. Oral administration of the extract produced significant ($p<0.001$) increases Hb, total white blood cells and neutrophil in a dose dependent fashion. It however did not affect RBC, and HCT. The extract also caused a significant increase in serum total protein, albumin, globulin, HDL and total cholesterol levels as well as indirect and total bilirubin dose dependently while decreasing serum ALT. It did not however have a significant effect on Renal function test (urea and creatinine). The present finding indicates that Xylopia aethiopica fruit has immune boosting properties.

In order to evaluate the effect of 70% alcoholic extract of Xylopia aethiopica on reproductive function of adult male rat, ethanolic fruits extract of X. aethiopica was administered orally to groups of male Sprague Dawley rats at the doses of 30, 100 and 300 mg/kg for 60 days. The reproductive organ weights, change in animal body weight, caudal epididymal sperm count, motility and viability, histology of testes and androgenic hormonal levels were evaluated. Increase in body weight as well as weight of testis and epididymis and a significant increase in caudal sperm count was noticed. Histological sections of testis exhibited spermatogenesis. Extract treatment also induced significant increase in serum testosterone and luteinizing hormone levels. The studies clearly reveal androgenic activity of the extract and its effects on hypothalamic pituitary gonadal axis.

To evaluate the effect of xylopic acid on serum sex hormone levels and spermatogenesis in male rats, Male Sprague Dawley rats were divided into four groups of six animals each. Group I served as the control and were given distilled water (vehicle for the XA). Groups II, III and IV rats were given XA orally at the dose of 10, 30 and 100 mg kg^{-1} respectively for 28 days. Blood was collected from the saphenous veins of animals on day 7 of the treatment and on day 28 after which the rats were euthanized to remove testes and other organs for

biochemical and histological analysis. Xylopic acid did not cause any changes in body weight, but significantly decreased testicular and epididymal weight (P < 0.01). Sperm motility, viability, and epididymal sperm counts of rats treated with Xylopic acid for 28 days were significantly reduced (P < 0.01). Serum testosterone levels were significantly reduced (P < 0.01). Untreated females mated by treated males exhibited significant decrease in fertility index in dose-dependent manner. The testes of the treated rats also exhibited some degree of oxidative stress as measured by the level of MDA and glutathione peroxidase activity in the testis. There were varying degrees of damage to the seminiferous tubules. Reversal of these changes, however, occurred after two weeks of recovery. In conclusion, Xylopic acid may possess reversible antifertility activity as well as spermatotoxic properties the mechanism of which may involve direct effect on mature spermatozoa and germ cells in the testes as a result of oxidative stress causing defective sperm cells and hence reduce fertility.

To characterize potential anti-androgenic properties of xylopic acid and to elucidate the possible mechanism of the antifertility activity of xylopic acid in rats, xylopic acid was administered to orchidectomized immature rats following the Hershberger assay protocol. This evaluates the ability of a chemical to elicit biological activities consistent with androgen agonists, antagonists or 5α-reductase inhibitors. Thirty male Sprague-Dawley rats (42 days old, weighing about 60-70 g) which were either orchidectomized or sham operated. At 11 days post-castration the rats were weighed and assign to five treatment groups. The animals were treated daily for 10 days and the weight of the animals taken daily. On the day after the last treatment the rats were necropsied to isolate organs and tissues for study of androgenic or anti-androgenic effects. The endpoints evaluated were the growth/body weight, and weight of the seminal vesicles plus coagulating glands with fluid, ventral prostate, levator ani plus bulbocavernosus muscle, glans penis, Cowper's glands (bulbourethral glands), and liver all without fixation. Xylopic acid exhibited anti-androgenic activity similar to cyproterone acetate, an anti-androgenic agent by decreasing the weight of the accessory sex organ. In conclusion whereas the crude extract possesses fertility enhancing properties, the pure acid exhibits anti-androgenic properties.

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My utmost thanks go to the almighty ALLAH for his endless mercies

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Finally, my heartfelt gratitude goes to my family and all the wonderful friends who were not mentioned for want of space.

DEDICATION

This thesis is dedicated to my parents, my wife and to my children Hamdaan Wundabli and Yumzaa Nadhira.

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ABBREVIATIONS

5-HT5-hydroxytryptamine

ACH Acetylcholine

ACTH Adrenocorticotropic Hormone

ALP Alkaline Phosphatase

ALT Alanine Aminotransferase

ANOVA Analysis of Variance

AR Androgen receptor

AST Aspartate Aminotransferase

BCG Bromocresol Green

BUN Blood Urea Nitrogen

CNS Central Nervous System

D-BIL Direct Bilirubin

HDL High Density Lipoprotein

EDC Endocrine Disruption compounds

ED Erectile Dysfunction

EDTA Ethylene DiamineTetra acetic Acid

FSH Follicle-Stimulating Hormone

GABA γ-Amino butyric Acid

GGT γ-glutamyltranspeptidase

GLDH Glutamate Dehydrogenase

GnRH Gonadotropin-Releasing-Hormone

GRAN Granulocyte

hCG Human chorionic gonadotropin

HRP Horseradish Peroxidase

I-BIL Indirect Bilirubin

LDH Lactate Dehydrogenase

LDH Lactate Dehydrogenase

LDL Low Density Lipoprotein

LH Luteinizing Hormone

LYM Lymphocyte

MID Mid Cell Count

PRL Prolactin

PSS Physiological Salt Solution

RBC Red Blood Cell Count

SEM Standard Error of the Mean

SIA Sandwich Enzyme Immunoassay

TAG Triglycerides

T-BIL Total Bilirubin

T-CHOL Total Cholesterol

T-PROT Total-Protein

TST Tail Suspension test

VIP Vasoactive Intestinal Polypeptide

VLDL Very Low Density Lipoprotein

WBC White Blood Cell Count

WHO World Health Organization

X.A Xylopic Acid

X.A.E *Xylopia aethiopica* Extract

Chapter 1

INTRODUCTION

Plants and derivatives of plant play a key role in world health and have long been known to possess biological activity. According to Burns (2000), thirty percent of all modern drugs are derived from plants. Available evidence suggests that approximately 80% of Africans rely on traditional healthcare practitioners and medicinal plants for their daily healthcare needs (Johnson *et al.*, 2007; McKay *et al.*, 2007). Natural products have been an overwhelming success in our effort in fighting diseases. They have reduced pain and suffering, and revolutionized the practices of medicine. Natural products are the most important anticancer and anti-infective agents. More than 60% of approved and pre-new drug application (NDA) candidates are either natural products or related to them, not including biological such as vaccines and monoclonal antibodies(Demain, 1999). The World Health Organization (WHO) estimates that almost 75% of the world's population has therapeutic experience with herbal remedies (Liu *et al.*, 2007). This is principally because of a belief that herbal remedies may have fewer side effects and can enhance the effects of conventional agents or be an alternative treatment (Desai *et al.*, 2009).

Xylopia aethiopica is a common plant in Africa especially West and Central African with wide applications in traditional medicine in the management of several diseases. There is the need therefore for scientific research into the pharmacological

activity since its traditional usage may not necessarily carry with it any scientific evidence.

1.1 XYLOPIA AETHIOPICA

Botanical name: *Xylopia aethiopica, (Dunal) A. Rich*
Family*: Annonaceae*

1.1.1 Description

Xylopia aethiopica (Dunal) A. Rich is a slim, tall tree of about 60–70 cm in diameter that can reach up to 15– 30 m tall, with a straight stem and a slightly stripped or smooth bark growing mainly in the tropical forest of Ghana, Nigeria and Cameroon. The fruits are rather small and look like twisted bean-pods. When dry, the fruit turn dark brown, cylindrical, 2.5 to 5 cm long and 4 to 6 mm thick. The contours of the seeds are visible from outside. Each pod contains 5 to 8 kidney-shaped seeds of approximately 5 mm in length (Johnkennedy *et al.*, 2011).

1.1.2 Commercial names:

Ethiopian/Guinea pepper, Spice tree, Negro pepper

1.1.3 Common and vernacular names in Africa

Ethiopian -Konde- berbere,

Ghana – Twi; Hwenetia or Hweneteaa, Hwentia in Fante, Kimba–Hausa, So or Soo in the

Ga-Adangme, Ezinli in Nzema, Tsuo, Tso or Akatapuresas in Ewe

La Cote d'ivoire – Fonde,

Nigeria – Sesedu (Yoruba), Kimba (Hausa), uda (Igbo)

Sierra Leone – Hewe,

Togo – Akatapure,

Figure 1.1: Fruits of *Xylopia aethiopica* (Dunal) A. Rich

1.1.4 Traditional and Modern Medicinal Uses

The fruits of *Xylopia aethiopica* are most commonly used for commercial applications. Medicinally, the fruits are used as cough-remedy, calmative, purgative and repulsive to pain. The fruit is a common ingredient in most foods across West Africa (Burkill, 1985a). In Liberia, the spice is smoked and inhaled for respiratory ailments (Iwu, 1993). The dried fruits are also used in Southwest and Southeastern parts of Nigeria as spices in the preparation of two special local soups named ''obeata'' and ''isi-ewu''. In the Northern part of Ghana the fruits are used in the preparation of almost all staple foods especially in spicing Khebab and in preparing ''Hausa Koko''; a local porridge. The fruit is often incorporated in preparations for enema and external uses, calling on its repulsive properties for pains in the ribs, chest and generally for any painful area, lumbago (low back and waist pains), neuralgia (pains in the nerves) and in the treatment of boils and skin eruptions (Iwu, 1993; Sofowora, 1977). The fruit decoction is useful in the treatment of bronchitis and dysenteric conditions, and as a medicine for bulimia (eating disorder). The fruit of X. *aethiopica* are given to women after child birth as condiment in soup and tea to accelerate the production of milk (Burkill, 1985a; Iwu, 1993; Tatsadjieu *et al.*, 2003).The seeds are also mixed with other spices, rubbed on the body as cosmetic and scent, and as perfume for clothing. The crushed, powdered fruit mixed with Shea butter and coconut oil is used as creams,

cosmetic products and perfumes. The fruits mixed with its roots are used in the treatment of rheumatism (Johnkennedy *et al.*, 2011). Even the odiferous roots of the plant are employed in tinctures, administered orally to expel worms and other parasitic animals from the intestines, or in teeth rinsing and mouth wash extracts against toothaches (Ghana Herbal Pharmacopoeia, 1992). Notwithstanding all the medicinal uses, the dried fruits are use as flavourings to prepare local soups in West Africa thus the name African pepper (Iwu, 1993).

1.1.5 Geographical Distribution

Xylopia is mostly found in lowland rainforest and in moist fringe forest in the savanna zones of Africa, but largely located in West, Central and Southern Africa. These trees are widely distributed in the humid forest zones of West Africa especially along rivers in the drier area of the region (Tairu *et al.*, 1999). In tropical and highlands of Africa (from Ethiopia to Ghana), both species X. *aethiopica* and X. *striata* occur and both are used for local cooking. In South America, a third species is of interest, X. *aromatica* (burro pepper), has found similar applications among Brazilian Indios (Iwu, 1993; Tairu *et al.*, 1999). The tree prefers high rainfall areas and well-drained soils. While X. *aethiopica* thrives in the forest regions, the tree can also be found in transitional zones. Loamy and sandy loamy soils are conducive for the cultivation of the plant. The plant can successfully be intercropped with other staple food items in the first four years. In West Africa, the tree flowers twice

per year, in March to July and in October to December. After picking, the fruits are sun dried for four to seven days (Burkill, 1985a).

1.1.6 Chemical Composition

The plant contains anonaceine, alkaloid, and rutin, volatile aromatic oil and a fixed oil. The kaurene and xylopic acid has been isolated from *X. aethiopica (*Ekoag *et al., 1970)*. Other constituents isolated from the essential oil of *X. aethiopica* which are of different chemical group's are monoterpene and sesquiterpene hydrocarbons, alcohols, aldehydes and oxides. Analysis of the essential oil showed the monoterpene hydrocarbons to account for 19.5% of the oil of which beta-pinene was the most prominent (14.6%). Sesquiterpene hydrocarbons accounted for 10.6%. Alcohols represented the largest group of constituents of the oil, 41.6% and of this, 41 terpene-4-ol was found to be the major constituent (23.4%). Cuminic aldehyde, 6.5%, was identified in a moderate concentration. About 16.3% of the oxides were 1-cineole.The essential oil has been well characterized with linalool, β-trans-ocimene, α-farnesene, α-pinene, β-pinene, myrtenol, β-phellandrene, and 3-ethylphenol as the major volatile constituents (Tairu *et al.*, 1999). Studies have shown that the intense 'pepperish note' of the oil of the fruit largely comes from linalool and provides the characteristic aroma of the ground, dried, smoked fruits of *Xylopia aethiopica* (Karioti *et al.*, 2004; Tairu *et al.*, 1999) The essential oil yield varies from 2.0% to 4.5%. The essential oils of the stem bark (0.85%) and the leaves

(0.5%) of *X.aromatica* have also been investigated. The bark oil consists mainly of pinene, trans-pinocarveol, verbenone and myrtenol and differs significantly from that of the leaf oil (spathulenol, cryptone, beta-caryophyllene and limonene). Several studies have reported different chemical compositions of essential oil extracted from fruits and leaves of *X. aethiopica*. In 2003, Tatsadjieu identified β-pinene (18.3%), terpinen-4-ol (8.9%), sabinene (7.2%), α-phellandrene (7.1%) in the fruits of *X. aethiopica (*Tatsadjieu *et al., 2003).*Karioti et al (2004)also found essential oil from different organs of *X. aethiopica*; significant proportions of germacrene-D in the leaves (24.4%), fresh fruits (19.4%) and dry (25.1%) and *trans*-meta-mentha-1(7), 8-diene in the roots (30.4%) and stem bark (30.7%).The plant also contains high amounts of copper, manganese, and zinc.

1.1.7 Pharmacological activity of Xylopia aethiopica

Key constituents of the plant are diterpenic and Kaurenic compounds and these are within the fruit extracts which have shown some pharmacological activity as an antimicrobial, antifungal, anti HIV, antimalarial, Anti-proliferative agent as well as possess haematopoietic activity, immune boosting effect, androgenic and spermatogenic activity (Boakye-Yiadom *et al.*, 1977; Fleischer *et al.*, 2008; Taiwo. *et al.*, 2009; Woode *et al.*, 2011a; Woode *et al.*, 2011b). However, it has not been shown to be effective against *E. coli (Iwu, 1993; Tairu et al., 1999)*. Studies have shown that extract from the seeds of *X. aethiopica* possess great potential as hypolipidemic and antioxidative agent thus reducing the risk of atherosclerosis in research animals

(Sarah *et al.*, 2011; Woode *et al.*, 2011b). The anti-inflammatory activity of the kauranes has been shown to involve the impairment of inflammation signalling through inhibition of NF-κB activity (Castrillo *et al.*, 2001). Kaurenoic acid (e*nt*-kaur-16-en-19-oic acid), a well-known *ent*-kaurenediterpene, is reported to have different biological activities such as analgesia (Block *et al.*, 1998), diuretic, vasorelaxant, anti-inflammatory and antipyretic effects in rodents (Somova *et al.*, 2001a; Sosa-Sequera *et al.*, 2010). Xylopic acid [15β-acetoxy-(-)-kaur-16-en-19-oic acid] and its epimer, acetylgrandifloric acid [15α-acetoxy-(-)-kaur-16-en-19-oic acid), are reported to exhibit antibacterial activity (Davino *et al.*, 1989).

1.2 MALE REPRODUCTIVE SYSTEM

The male reproductive system consists of a pair of testes, epididymides and accessory sex organs which include the seminal vesicles, ductus deferens, prostate, coagulating gland, bulbourethral gland and the gland penis (figure 1.2). The testes are encapsulated ovoid organs consisting of seminiferous tubules separated by interstitial tissues containing leydig cells which are responsible for the production of testosterone. The testis has two main functions namely production of sperms and testosterone. Testosterone plays an important role in maintaining spermatogenesis, accessory sex organs and secondary sexual characters. The epididymis is a single highly convoluted duct, closely applied to the surface of the testes extending from the anterior to the posterior pole of the testis. The epididymis acts as a storage place for the spermatozoa where they become motile and acquire the capacity to fertilize. The seminal vesicles are paired, bag-shaped glands and the

internal surface consists of intricate system of folds to form irregular diverticula. The seminal vesicles secrete a viscous fluid which is expelled along with the sperms. It contains several essential nutrients required by the sperms for their development. The ventral prostate is a bilobed structure situated ventral to the urethra. It has numerous small ducts through which the secretions are discharged directly into the urethra. The secretions contain several nutrients and also serve as a lubricant for the sperms(Setchell *et al.*, 1994). Semen is thus a complex mixture of product of the testes, epididymides, ductuli deferentia and ampullae, prostate gland, vesicular glands and bulbourethral glands. Abnormal function of any of these organs can alter seminal characteristics and hence fertility (Amann, 1981).

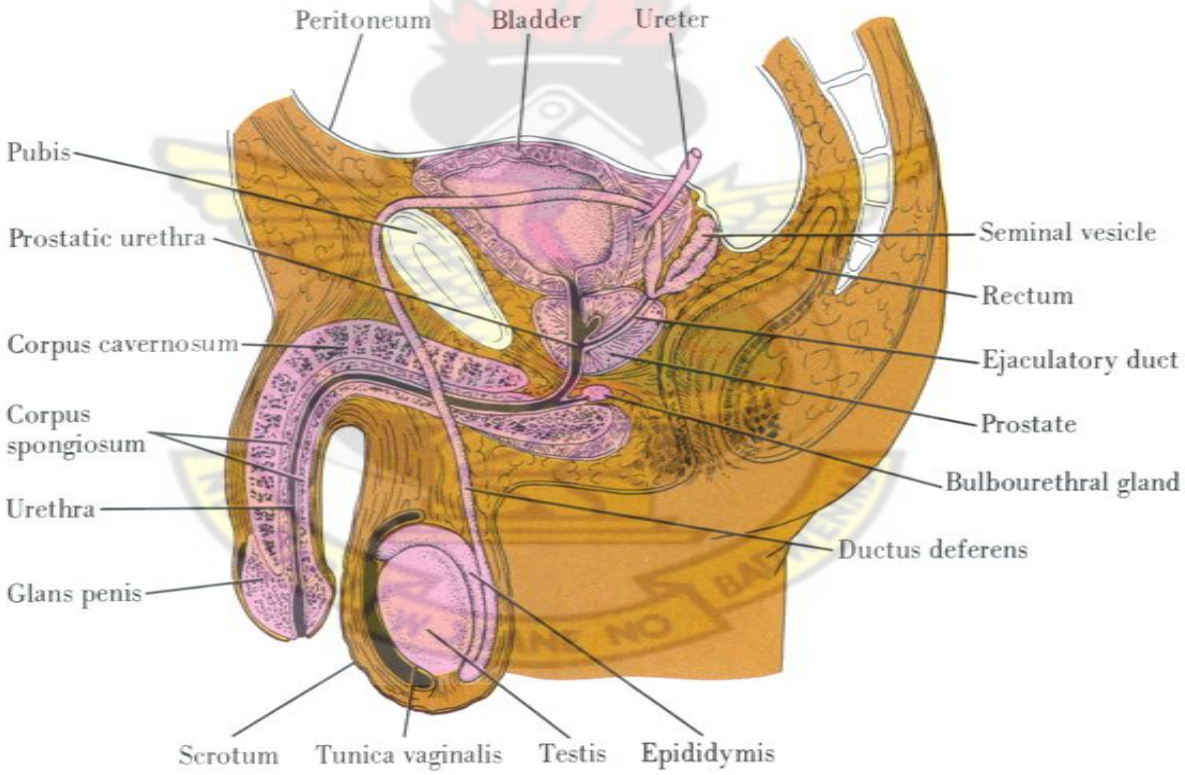


Figure 1.2: The male reproductive system

1.2.1 Regulation of the male reproductive system

Male reproduction encompasses the production of viable sperm, their delivery into the female reproductive tract, fertilization of the female oocytes, and production of

normal offspring (Lucio *et al.*, 2005; Tyl, 2001). Successful male fertility requires an adequate sperm count, adequate sperm motility, the appropriate functioning of accessory sex organs (to produce and concentrate semen and to activate and capacitate sperm), and appropriate sexual behaviour (i.e., mounting, intromission, ejaculation). The production of viable sperm (spermatogenesis) in the testes is under genetic control (a male-determining gene on the Y chromosome) and neuroendocrine regulation initiated in the brain by the hypothalamic-pituitary-gonadal (HPG) axis(Lucio *et al.*, 2005; Tyl, 2001). There is also intratesticular endocrine, autocrine, and paracrine regulation. Once the sperm are produced and delivered to the epididymis, they are stored in the cauda epididymis and moved into the vas deferens. Epididymal maturation must occur; this includes acquisition of motility and the ability to penetrate and fertilize the oocytes, and acrosomal cap activation. Semen is produced from accessory sex organs—seminal vesicles, coagulating glands, the prostate, bulbourethral (Cowper's) glands and preputial glands. The sperm in semen are moved through the blood-engorged erect penis and ejaculated into the female's vagina-cervix-uterus. Indirect influences also play a role, such as nutritional status, liver metabolism of xenobiotics and endogenous hormones, and vascularization. The testis is relatively anoxic, with low blood flow and low oxygen tension; insult can alter the permeability of the testicular vasculature and consequently affect blood flow (Miller, 1998). Regulation of the neuroendocrine system begins with specialized cells in the hypothalamus in the brain that release gonadotropin-releasing hormone (GnRH) in a pulsatile pattern. GnRH travels via the hypothalamic-pituitary portal system directly to the anterior lobe of the pituitary. In a receptor-mediated process, GnRH stimulates cells of the anterior lobe of the pituitary to secrete the gonadotropins, luteinizing hormone (LH), and follicle-stimulating hormone (FSH). LH and FSH travel via the systemic blood supply to the testes. LH binds to receptors on the interstitial cells of Leydig to stimulate Steroidogenesis (i.e., synthesis of testosterone) in these cells. FSH binds to receptors on the intratubular Sertoli cells, which act as nurse cells for the

developing germ cells and are necessary for spermatogenesis(O'Donnell *et al.*, 2001).Inhibin, produced by the Sertoli cells and testosterone (T) from the Leydig cells both exert negative feedback on the pituitary production of FSH and LH as well as the hypothalamic production of GnRH (Russell *et al.*, 1990).

Male reproductive toxicants have been classified in a number of ways, based on direct or indirect action. It has been proposed that direct-acting toxicants interact with cellular components, based on inherent chemical reactivity or via specific receptor binding, whereas indirect-acting toxicants acted via disruption of endocrine homeostasis or by metabolic activation to a direct toxicant (Tyl, 2001). Other researchers (Miller, 1998) used the direct-acting versus indirect- acting classification but proposed that a direct toxicant affects the testis without endocrine mediation, whereas an indirect toxicant acts at a non-germ-cell site (e.g., hypothalamic pituitary axis or the Leydig cell. Miller (1998) also used the direct- versus indirect-acting classification but defined direct- acting toxicants as those that produced their primary effects on testicular cells, the duct system (e.g., the epididymis), or on mature spermatozoa. This definition would include effects on non-germ-cell testicular cell types such as the Leydig or Sertoli cells. Because the testis is subject to hormonal control and regulatory feedback loops, the action of direct toxicants on testicular cell types would result in secondary disruption of endocrine homeostasis (Tyl, 2001). Indirect toxicants are defined as having a primary action on hypothalamic-pituitary neuroendocrine controls or on extra gonadal systems (Tyl, 2001). This last classification, which is based on the processes altered, does not consider whether the parent compound or a metabolite is eliciting the toxicity (Miller, 1998). Other indirect effects that could affect male reproduction include anosmia (the absence or loss of the sense of smell unable to detect pheromone signals from receptive females. They also include peripheral neuropathy, in that the male cannot mount the female due to his hind limb

weakness or achieve appropriate intromission, ejaculation, and/or stimulation of the cervix (Tyl, 2001). When aromatase enzyme, which converts T to oestrogen locally in the brain of male rats, is inhibited, it can result in altered male reproductive function. An absence or reduction in brain-localized oestrogen in the male rat will affect mating behaviour, resulting in impaired reproductive performance. Changes in neonatal thyroid hormone levels can as well result in changes in testis size and sperm production (Meistrich, 1986; Tyl, 2001).

Human epidemiological studies have suggested effects such as reduced sperm count, reduced fertility, increased incidence of testicular cancer and congenital birth defects in men, and increased incidence of breast cancer in woman (Barlow *et al.*, 1999). According to Barlow (1999), the mechanisms of action of many agents suspected of affecting reproductive function are not fully understood. A given chemical, may involve multiple sites of action and complex disturbances in the homeostatic processes. Sex hormone disrupters and their metabolites which are structurally similar to endogenous ligands, may interact directly with the physiological ligand's receptor in the cells of the gonads or accessory sex organs; which may mimic the action of the hormone, thus, resulting in receptor stimulation and resultant biological effects (agonistic) or may block or reduce the binding and biological activity of endogenous hormones (antagonistic). Alternatively it may modify post-receptor signalling pathways within cells the effects of which might also result in interference in the hypothalamus-pituitary-gonadal axis, the brain-pituitary-thyroid axis or in neurotransmitters in the central nervous system. Indirect effects could also result from the chemical inducing or inhibiting metabolic enzymes causing changes in the production or breakdown of endogenous hormones or alterations in carrier proteins in the blood (Makris *et al.*, 2010; Mitsumori *et al.*, 1994; Rao *et al.*, 1981). A chemical may be directly active or may act indirectly, requiring initial metabolic activation. Thus, disruption of endocrine secretion, binding, feedback control or target activity can be affected by action at several sites. It is not often clear when an alteration in hormone activity is

observed whether this is the result of a primary effect on hormone secretion and on subsequent receptor interaction or if the effect is a response to organ damage or some other mechanism (Barlow *et al.*, 1999; Mitsumori *et al.*, 1994; Wang *et al.*, 2010).

1.2.2 THE TESTIS

The testis is surrounded by a few connective tissues, the tunica albuginea which is a tough fibrous ring. It is composed of three layers, an outer layer of visceral peritoneum, the tunica vaginalis, the tunica albuginea proper and on the inside the tunica vasculosa (Setchell *et al.*, 1994). The testis is made up of the seminiferous tubules where the spermatozoa are formed. The tubules are two-ended, convoluted loops with both ends opening into the rete testis, through which sperms pass on their way to the excurrent duct system. It has been shown that there are 30 tubules in rats (Dym, 1976) with a diameter of between 50 and 100 μm (Setchell *et al.*, 1994; Wing *et al.*, 1982). The seminiferous epithelium is a complex stratified epithelium containing two cell populations; the spermatogenic cell (stem cell which regularly replicates and differentiates into mature sperm as they migrate toward the lumen) and the sertoli cells, non-replicating physically supporting cells (Steinberger *et al.*, 1971). The walls of the tubules are composed of four layers, an innermost layer of non cellular material, surrounded by a layer of smooth muscle-like or myoid cells which are probably responsible for the peristaltic movements of the tubules, a layer of collagen fibres and on the outside a layer of endothelial cells (Clermont, 1958). The interstitial tissue fills up the spaces between the seminiferous tubules and contains blood and lymph vessels (Clark, 1976; Fawcett, 1973). Species difference in the occurrence and significance of interstitial cell type has been reported (Fawcett *et al.*, 1973). The interstitial tissue is mainly composed of Leydig cells which are the sites of production of testicular androgens. Leydig cells contain

large amount of smooth endoplasmic reticulum, mitochondria, Golgi complex, centrioles and number of lipid droplets (Aguas, 1981). Leydig cells have been shown to be associated with blood vessels (Fawcett, 1973) or found nearer to walls of the seminiferous tubules and these cells show variation in size and structure depending on the spermatogenic stage of the adjacent tubules (Bergh, 1983). Mast cells and macrophages have also been reported in the interstitial tissue (Nistal *et al.*, 1984). Sertoli cells lie immediately inside the boundary tissue of the tubules and surround the undeveloped germinal cells before puberty.

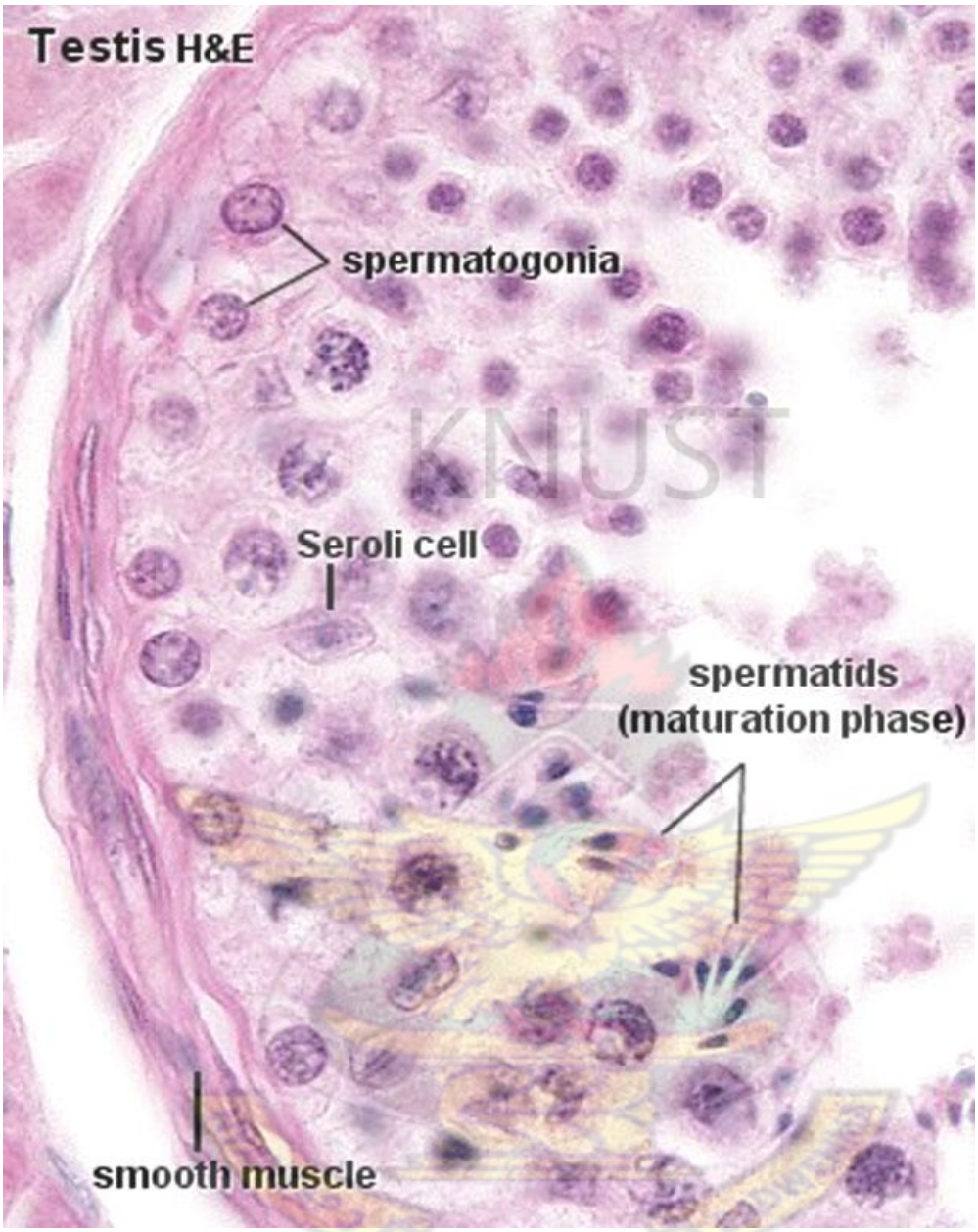


Figure 1.3. A photomicrograph of a transverse section of the testes showing the sertoli cells and spermatogonia

It has been shown that Sertoli cells do not synthesis DNA but do incorporate [^{3}H]-thymidine into DNA in rats less than 14days old (Nagy, 1972; Steinberger *et al.*, 1971). The cytoplasm of the Sertoli cells extends from the boundary tissue to the lumen of the tubule in the adult. Sertoli cells share the surface of the boundary tissue with the spermatogonia as shown in figure 1.3. Pairs of Sertoli cells and with the spermatocytes and early spermatids and they embed the late spermatids in their luminal surface (Fawcett, 1973). The intricate cytoskeleton and numerous processes of sertoli cells appear to be responsible for the positioning, movement and shaping of the spermatogenic cells. The blood – testis barrier between adjacent sertoli cells contains occluding tight junctions which prevent the free transport of molecules from the outside of the tubule into the spermatocytes and spermatids (Fawcett, 1973; Setchell, 1980). Sertoli cells have been reported to have number of specific functions such as secretion of fluid, phagocytosis, the maturation and release of spermatozoa and the synthesis of the intratubular androgen binding protein (Chemes, 1986; Clermont *et al.*, 1987).

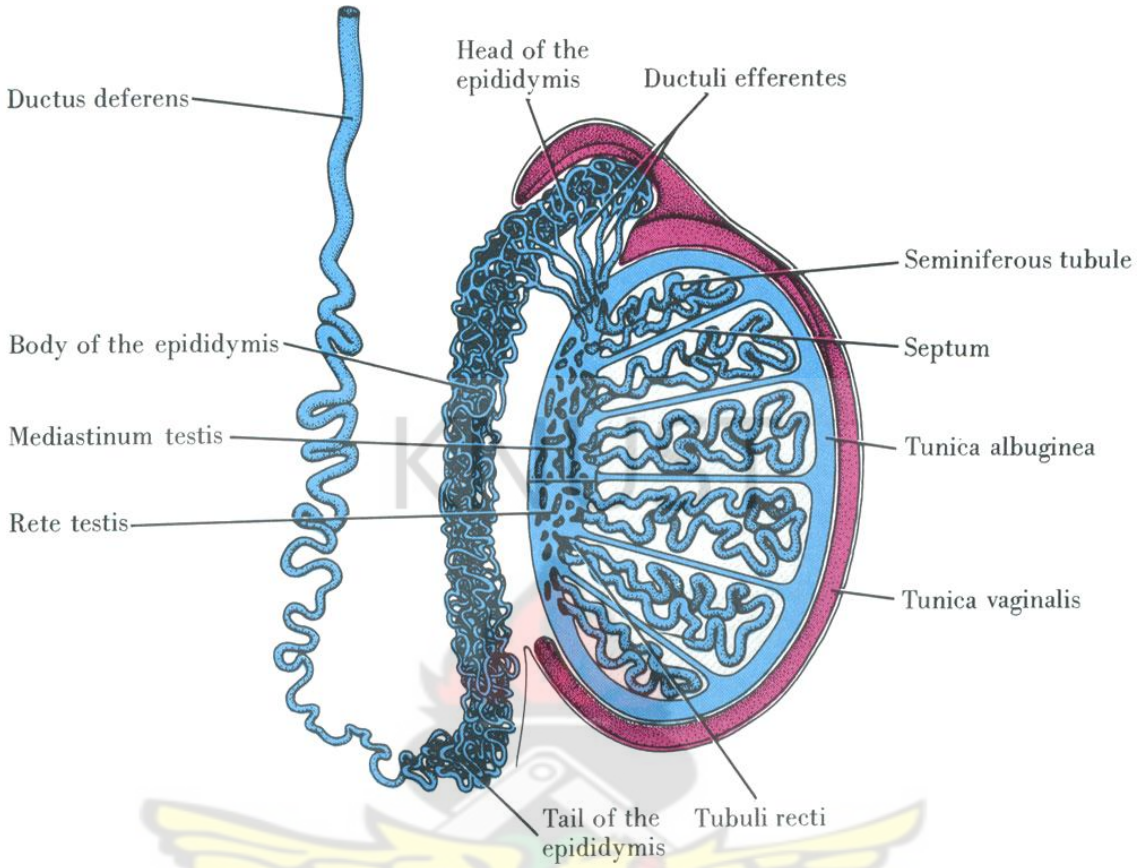


Figure 1.4: An illustration of the Testes

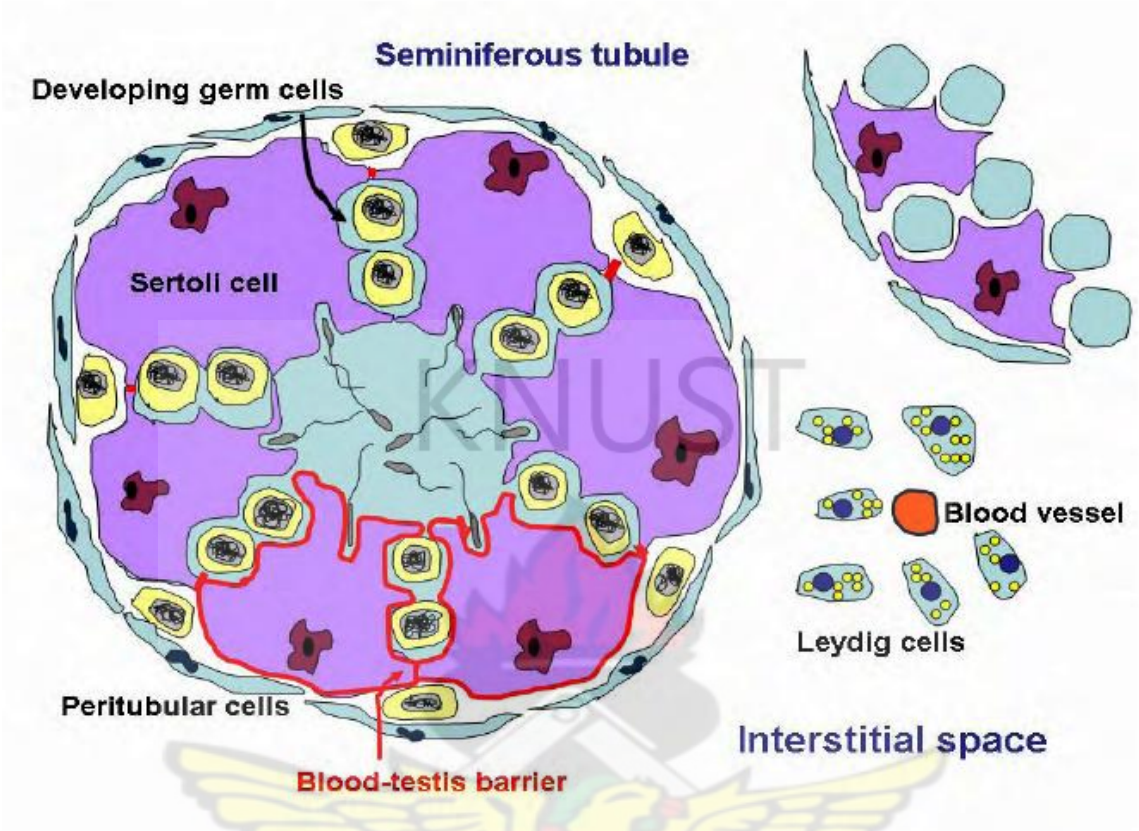


Figure 1.5: The functional organization of the testes, illustrating the seminiferous tubules and interstitial tissues as well as the basal and the ad luminal compartment

1.2.3 Spermatogenesis

Spermatogenesis is the sum of the transformations that results in formation of spermatozoa from spermatogonia while maintaining spermatogonial numbers. This process takes place within the seminiferous tubules of the testis, in close association with the somatic cells of the seminiferous epithelium; the Sertoli cells(O'Donnell *et al.*, 2001).The process involves a complex series of biochemical and morphological transformations leading to the formation of a mature spermatozoon. When germ cell development is complete, the mature spermatids are released from the Sertoli cells into the tubule lumen, and proceed through the excurrent duct system, known as the rete testis, until they enter the epididymis via the efferent ducts (Fig. 1.3). During passage through the epididymis, the spermatids undergo a series of biochemical changes to become motile spermatozoa capable of fertilization. The testicular parenchyma, consisting of seminiferous tubules and interstitial tissue (Fig.1.3), is enclosed by a capsule called the tunica (O'Donnell *et al.*, 2001). The interstitial tissue contains the blood and lymphatic vessels, which are essential for the movement of hormones and nutrients in and out of the testis. Leydig cell, the most frequently encountered cell type in the interstitium (Christensen, 1975), is primarily involved in the secretion of androgens, notably testosterone, as well as other steroids including oestrogen. Within the seminiferous tubules, the Sertoli cells reside on a basement membrane, under which are the lymphatic endothelium and the peritubular myoid cells (Dym *et al.*, 1970). The structure of the Sertoli cell is extremely complex, with numerous cup shaped processes encompassing the various germ cell types (Russell *et al.*, 1990) (Fig. 1.5). Developing germ cells form intimate associations with Sertoli cells, with multiple germ cell types in contact with one Sertoli cell. The various generations of germ cells are not randomly distributed within the seminiferous epithelium, but are arranged in strictly defined cellular associations (Leblond *et al.*, 1952b). It is the unique associations of these germ cells with Sertoli cells that constitute the cycle of the seminiferous epithelium (fig 1.7), and each particular association of germ cells is referred to as a stage (Leblond *et al.*, 1952a; O'Donnell *et al.*, 2001). The number of stages of

spermatogenesis in a particular species is thus defined by the number of morphologically recognizable germ cell associations within the testis; in the mouse there are 12 stages, in the rat there are 14, and in the human there are 6 (Clermont, 1972; Leblond *et al.*, 1952a). Germ cell development involves a complicated series of events, and the various germ cell types can be distinguished on the basis of morphology and the differential expression of proteins (O'Donnell *et al.*, 2001).

Spermatogonia are the most immature germ cells in the seminiferous tubules of the testis and are present between Sertoli cells, close to the basement membrane of the tubule and include type A spermatogonia, intermediate permatogonia (found only in rodents), and type B spermatogonia, the latter of which are considered to be committed to differentiation (Leblond *et al.*, 1952b; O'Donnell *et al.*, 2001) as shown in fig 1.6. The true stem cells of the germ cell population are considered to be a subset of the type A spermatogonial population, although their identity cannot be discerned on the basis of morphology (Russell *et al.*, 1990).

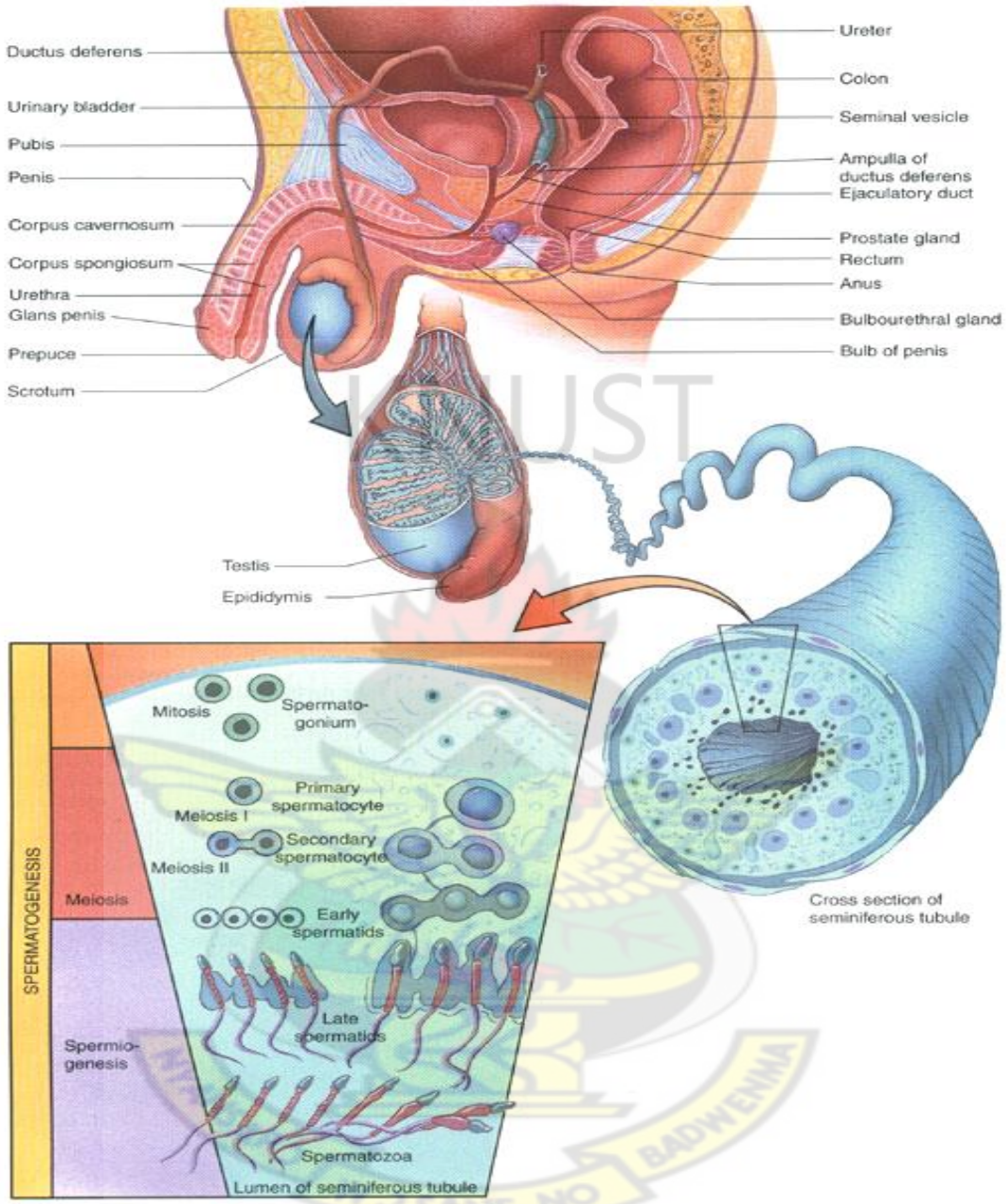


Figure 1.6: Diagrammatic representation of spermatogenesis in man

The spermatogonia undergo numerous mitotic processes to produce a large number of germ cells available for entry into meiosis. Thus, proliferation of the spermatogonial population provides the source for the millions of sperm that are produced per day (Clermont, 1972). After the last mitosis of type B spermatogonia, preleptotene primary spermatocytes are formed (Clermont, 1972). These cells replicate their DNA and hence initiate meiosis (Russell *et al.*, 1990). During the prophase of the first meiotic division, germ cells undergo morphological transitions that can be classified on the basis of nuclear size and morphology (Hess, 1990). In the zygotene phase, pairing of homologous chromosomes occurs, and cells with completely paired chromosomes are termed pachytene spermatocytes. After the pachytene phase, a brief diplotene phase follows in which the chromosome pairs partially separate and the cells then undergo the first meiotic division to yield secondary spermatocytes (Hess, 1990). These cells quickly undergo the second meiotic division to yield the haploid round spermatid. The differentiation of round spermatids into the mature elongated spermatid form takes place, with no further division, during the process known as spermiogenesis (Cheng *et al.*, 2010; O'Donnell *et al.*, 2001). Spermiogenesis briefly involves formation and development of the acrosome and flagellum, condensation of the chromatin, reshaping and elongation of the nucleus, and removal of the cytoplasm before release of the spermatid during spermiation (Leblond *et al.*, 1952b; Russell *et al.*, 1990). Acrosome formation involves the fusion of Golgi granules to form the acrosome which contains hydrolytic enzymes which enables the spermatozoa to

move through the investing layers of the oocytes. After commencement of spermatid elongation, the highly condensed spermatid nucleus becomes incapable of transcription, and immature round spermatids transcribe high levels of mRNAs that are subject to translational delay until translation is required during elongation (Braun, 1998). Spermiation is the final step of spermiogenesis and involves the release of the mature elongated spermatid from the Sertoli cell into the lumen of the seminiferous tubule (Cheng *et al.*, 2010; Russell *et al.*, 1990).

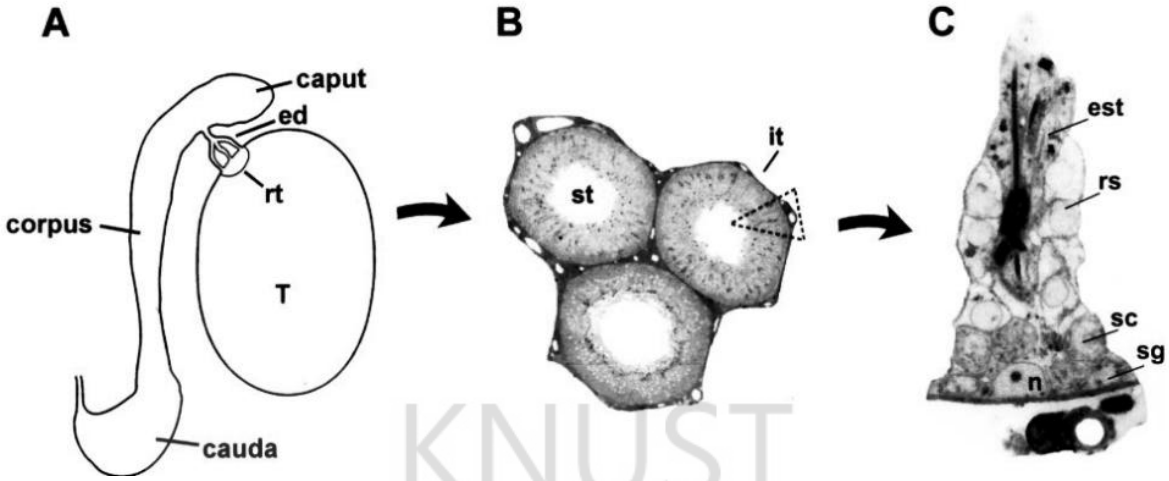


Figure 1.7. Diagram of the testis and spermatogenesis.

A, Diagram of the testis (T), rete testis (rt), efferent ducts (ed), and caput, corpus, and cauda epididymis. B, Cross-section through an adult rat testis, showing several seminiferous tubules (st) in various stages of development, and the interstitial space (it) which contains Leydig cells, blood and lymph vessels, and macrophages. C, High magnification of the seminiferous epithelium. A single Sertoli cell with a basally located nucleus (n) with a central nucleolus can be seen. The cytoplasm of the Sertoli cell surrounds germ cells at various stages of development. Spermatogonia (sg) are located closest to the base of the tubule, with spermatocytes (sc) above the spermatogonial layer. Round spermatids (rs) are visible above the spermatocyte layer and in this micrograph; elongated spermatids (est) are embedded within the Sertoli cell. The seminiferous epithelium resides on the basal lamina, which is made up of extracellular matrix and peritubular myoid cells.

The duration of spermatogenesis is the time taken from the division of type A spermatogonia to the release of spermatozoa has been reported to be approximately 50 days in rats and 64 days in men (Clermont *et al.*, 1965). It is reported that the duration of spermatogenesis requires between 4.3 and 4.7 cycles of the seminiferous epithelium which is the interval required for one complete series of cellular associations to appear at one point within the tubules (Clermont, 1972; Steinberger *et al.*, 1971). Administration of a drug or test compound as well as an unknown factors, can induce absence of all germ cells more mature than certain cell type. This condition is term as ''complete degeneration of pachytene spermatocytes'' which is usually wrongly term ''germ cell arrest''. If there were an ''arrest'' of germ cells, there would be accumulation of the most mature type (Amer *et al.*, 1997). Factors inducing complete germ cell degeneration often acts on specific type of germ cell and affected germ cells may develop for some time before degeneration (Courot, 1980). These degenerations may result in sertoli cell dysfunction.

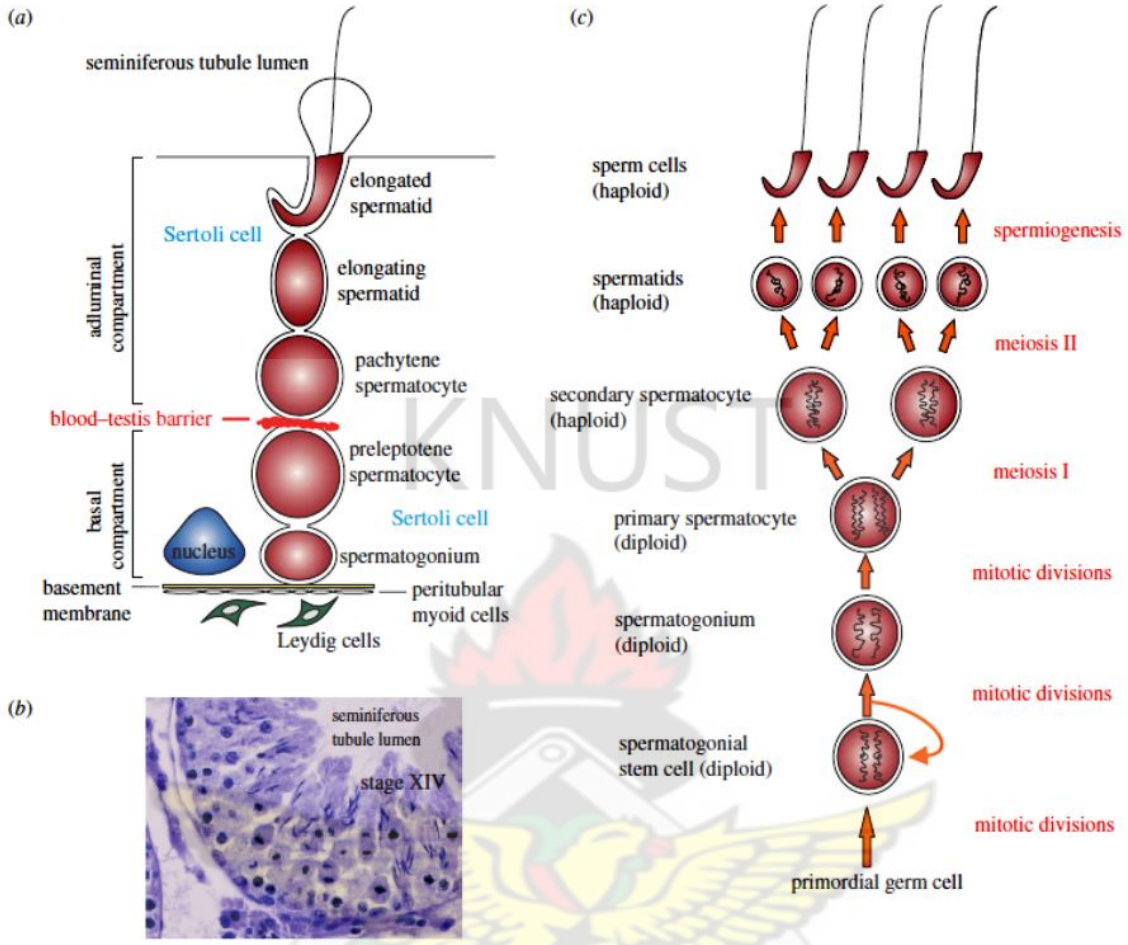


Figure 1.8: The biology of spermatogenesis in the rat

(a) Schematic drawing of the seminiferous epithelium from a seminiferous tubule in the adult rat testis, illustrating the morphological features of different germ cells during development and their intimate relationship with the Sertoli cell. Leydig cells that produce testosterone and oestradiol-17b via steroidogenesis are restricted to the interstitium. The blood–testis barrier (BTB) comprising tight junctions, basal ectoplasmic specializations and desmosome-gap junctions physically divides the seminiferous epithelium into a basal and an apical (ad luminal) compartment. (b) Cross-section of a stage XIV tubule from an adult rat testis during which time meiosis occurs. Several newly formed spermatids from secondary spermatocytes at anaphase are clearly visible in this stage. (c) Drawing depicting the process that occurs in male germ cells during spermatogenesis.

1.2.4 Hormonal Regulation of Spermatogenesis

Germ cell development depends on a highly coordinated interaction with the Sertoli cell. Germ cells and Sertoli cells can communicate directly via ligand/receptor-mediated interactions or paracrine factors. The production and secretion of many Sertoli cell proteins involved in germ cell development occur in a stage-dependent manner (Parvinen, 1982), reflecting the ability of the Sertoli cell to adapt to the changing needs of the germ cell. For many years it was presumed that Sertoli cells were the major controlling factor in the timing of germ cell development; however, studies investigating rat-to-mouse spermatogonial transplantation clearly demonstrated that rat germ cells in contact with mouse sertoli cells develop according to the kinetics of rat spermatogenesis, thus highlighting the role of germ cells in controlling their own fate (Franca *et al.*, 1998). As well as the production of spermatozoa, the testis is involved in the production of hormones that are required for various functions in the body, including maintenance of secondary sexual functions, and feedback on the hypothalamus and the pituitary to control the secretion of the gonadotropins LH and FSH. It is well known that the gonadotropins are the major endocrine regulators of spermatogenesis (McLachlan *et al.*, 2002b; Sharpe, 1994; Weinbauer *et al.*, 1993b). LH targets the Leydig cell to stimulate the secretion of androgens, namely testosterone, which in turn acts on androgen receptors in the seminiferous epithelium to control spermatogenesis. FSH targets receptors within the Sertoli cell to regulate spermatogenesis by stimulating the production of numerous Sertoli cell factors. The roles of testosterone and FSH in the testis have been studied extensively, yet relatively little is known about how these hormones act within the Sertoli cell to stimulate and maintain spermatogenesis (McLachlan *et al.*, 1996; Sharpe, 1994; Weinbauer *et al.*, 1993b). Androgens alone have been shown to stimulate all phases of germ cell development in the hypogonadal (hpg) mouse, which is congenitally deficient in GnRH and therefore LH and FSH (Singh *et al.*, 1995), highlighting the requirement of spermatogenesis for androgen. The question

of whether FSH was essential for spermatogenesis in mice was answered by the generation of transgenic mice possessing targeted disruptions of the FSH receptor gene (Dierich *et al.*, 1998) or the FSH b-subunit gene (Kumar *et al.*, 1997). Males of both transgenic models are fertile and display all stages of germ cell development, as are the androgen-treated hpg mice (Singh *et al.*, 1995), suggesting that FSH is not an absolute requirement for fertility. However, in both cases the testes are smaller, and less sperm are produced (Dierich *et al.*, 1998; Kumar *et al.*, 1997; Singh *et al.*, 1995), due to the requirement for FSH during the neonatal period of Sertoli cell division (Krishnamurthy *et al.*, 2000; Singh *et al.*, 1996). More recent quantitative studies on FSH receptor knock-out mice also demonstrated defects in sperm development, leading to the production of poor quality sperm (Krishnamurthy *et al.*, 2000). Thus while FSH is not essential for spermatogenesis, it is clearly essential for quantitatively normal spermatogenesis and fertility. In terms of the endocrine regulation of spermatogenesis by FSH, LH, and androgens, it is clear that the initiation and maintenance of quantitatively normal spermatogenesis and thus full fertility rely on the delicate balance of the hypothalamo-pituitary-testis axis. Inhibin, produced by the Sertoli cells and testosterone (T) from the Leydig cells both exert negative feedback on the pituitary production of FSH and LH. T also exerts negative feedback on the hypothalamic production of GnRH (Russell *et al.*, 1990). Sertoli cells have receptors for both FSH and T for additional coordination between the Sertoli and Leydig cell populations (Russell *et al.*, 1990).

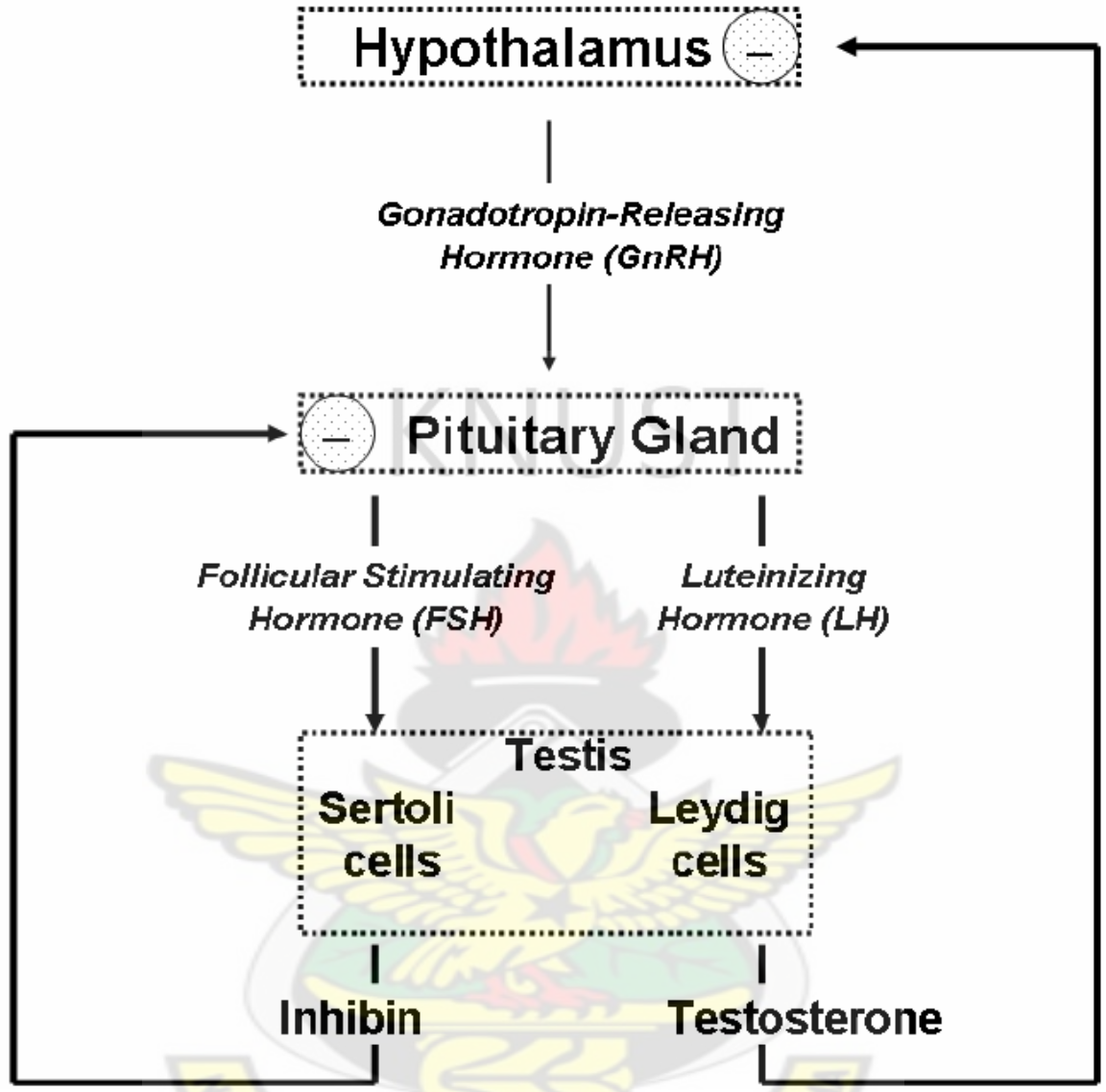


Figure 1.9: Feedback regulation of testosterone production by the Leydig cells via the hypothalamic-pituitary-gonadal axis

The pituitary hormones; follicle stimulating hormone (FSH) and luteinizing hormone (LH) have been shown to stimulate the seminiferous tubules and restore spermatogenesis in hypophysectomised rats (Greep *et al.*, 1937). A reduction in the number of B spermatogonia and primary spermatocytes has been reported in hypophysectomised adult rats (Clermont *et al.*, 1967). The preleptotene andpachytene spermatocytes have been reported to degenerate in hypophysectomised rats (Russell *et al.*, 1977) and upon testosterone or dehydrotestosterone administration spermatogenesis was restored (Mainwaring, 1977). FSH was reported to be necessary for the restoration of spermatogenesis in testis following hypophysectomy (Steinberger *et al.*, 1971). The effects of FSH on Sertoli cells in culture have been reported (Fritz *et al.*, 1975). Synergistic action of FSH and testosterone for the maintenance of spermatogenesis has also been reported(Steinberger *et al.*, 1971). A high concentration of testosterone within the seminiferous tubules by stimulation of LH has been shown to play a role in the inhibition and maintenance of sperm production (Fritz *et al.*, 1975). Initiation of spermatogenesis in monkeys by testosterone alone has been reported which was associated with a 2-4 fold elevation of intratesticular androgen concentration (Marshall *et al.*, 1984). Testosterone alone has been shown to maintain the spermatogenesis qualitatively in a dose dependent fashion in monkeys (Weinbauer *et al.*, 1993a).

1.2.5 Steroidogenesis

Steroidogenesis - the production of Testosterone (T) and dihydrotestosterone (DHT) from cholesterol by a series of P450 enzymes in the Leydig cells of the testis—is necessary for both spermatogenesis and for development of secondary sex characteristics. In utero, T (produced locally by the interstitial cells of Leydig, which are regulated by LH) is responsible for the differentiation of the Wolffian ducts into the epididymides, vasa deferentia, seminal vesicles, and the growth of

the levator ani-muscle and bulbocavernosus gland (the LABC complex).DHT (produced locally in the testis by the conversion of T using the enzyme 5-α-reductase) is responsible for the differentiation of the genital tubercle into the external genitalia and the urogenital sinus into the prostate and Cowper's glands, and for the regression of nipple anlagen in male foetuses. The Sertoli cells are also necessary for spermatogenesis as it produces inhibin (also called the Müllerian inhibiting substance) which is regulated by FSH. It causes the Müllerian ducts (which are initially present in both sexes in utero and which form oviducts and the uterus in female foetuses) to regress, thereby suppressing female reproductive development. At puberty, there is another surge of FSH and LH and, therefore, T, which initiates preputial separation (PPS) in the rodent, development of tertiary sex characteristics, and spermatogene (Miller, 1998).

The role of the testis as has been explained is to produce fertile sperm for procreation and steroid hormones for sexual and reproductive function. Follicle-stimulating hormone (FSH) stimulates male germ cells (spermatogonial cells) to develop into mature sperm by spermatogenesis. These stem cells are continuously renewed by mitosis for most of the lifetime of human males. FSH binds to FSH receptors on Sertoli cells and stimulates the cAMP-mediated second messenger pathway resulting in the activation of various factors required for successful spermatid production. Luteinizing hormone (LH) binds to its receptor on the Leydig cell membrane, which is also coupled to the cAMP signalling pathway, to stimulate the production of testosterone de novo from cholesterol. In concert with the actions of FSH, this testosterone is required for optimal sperm production, as well as for sexual function. LH induces the various cytochrome P450 enzymes and dehydrogenases involved in testosterone synthesis in Leydig cells, including CYP17 17, 20-lyase, the key activity directing the biosynthesis of steroids toward the sex hormones (Dharia *et al.*, 2004; Sasano *et al.*, 1989). The weak androgen androstenedione is converted to testosterone by 17b-hydroxysteroid dehydrogenase (17b-HSD), and the balance between these androgens depends on

the activity and type of 17b-HSD present. 17b-HSD types 3 and 5 catalyse the conversion of androstenedione to testosterone and are expressed in the testis Leydig cells (Mindnich *et al.*, 2004), whereas type 2 (found in prostate and placenta among others) performs the opposite reaction (Luu-The, 2001). Leydig, Sertoli, and germ cells further express low levels of aromatase, which converts testosterone originating from the Leydig cells into oestradiol, a step that appears to be necessary for the successful initiation of spermatogenesis and mitosis of spermatogonia (Carreau *et al.*, 2003). It was found that treatment of bonnet monkeys (Macacaradiata) with a triazole-containing aromatase inhibitor related to letrozole blocked spermatid development (Shetty *et al.*, 1997). The most potent endogenous androgen dihydrotestosterone (DHT) is formed from testosterone by steroid 5a-reductase. This reaction is weak in the adult testis but is predominant in the epididymis and prostate where DHT has important physiological roles in maintaining sexual function. It is also expressed in other peripheral tissues such as skin and liver. Increased expression of steroid 5a-reductase has been associated with benign prostate hyperplasia and prostate cancer.

Some studies have suggested that incubation of testicular slices with [^{14}C] acetate produced labelled testosterone (Brady *et al.*, 1951; Hall *et al.*, 1962). Conversion of [^{3}H] cholesterol into testosterone by testicular slices has also been shown (Hall, 1966). It has been reported that acetate is first converted to cholesterol and the cholesterol then converted to pregnenolone by successive oxidations under the influence of C_{20}-C_{22}desmolase complex (cytochrome P450) in mitochondria in the presence of NADPH and oxygen (Burstein *et al.*, 1971). Testicular homogenates of rat convened pregnenolone more to progesterone than that of 17-hydroxypregnenolone. Progesterone was shown to be converted to 17-hydroxyprogesterone, proandrostenedione and testosterone in the incubation of slices or homogenates of testis of hypophysectomised or immature rats treated with human chorionic gonadotropin (hCG) to increase the volume of the interstitial cells (Slaunwhite *et al.*, 1956).

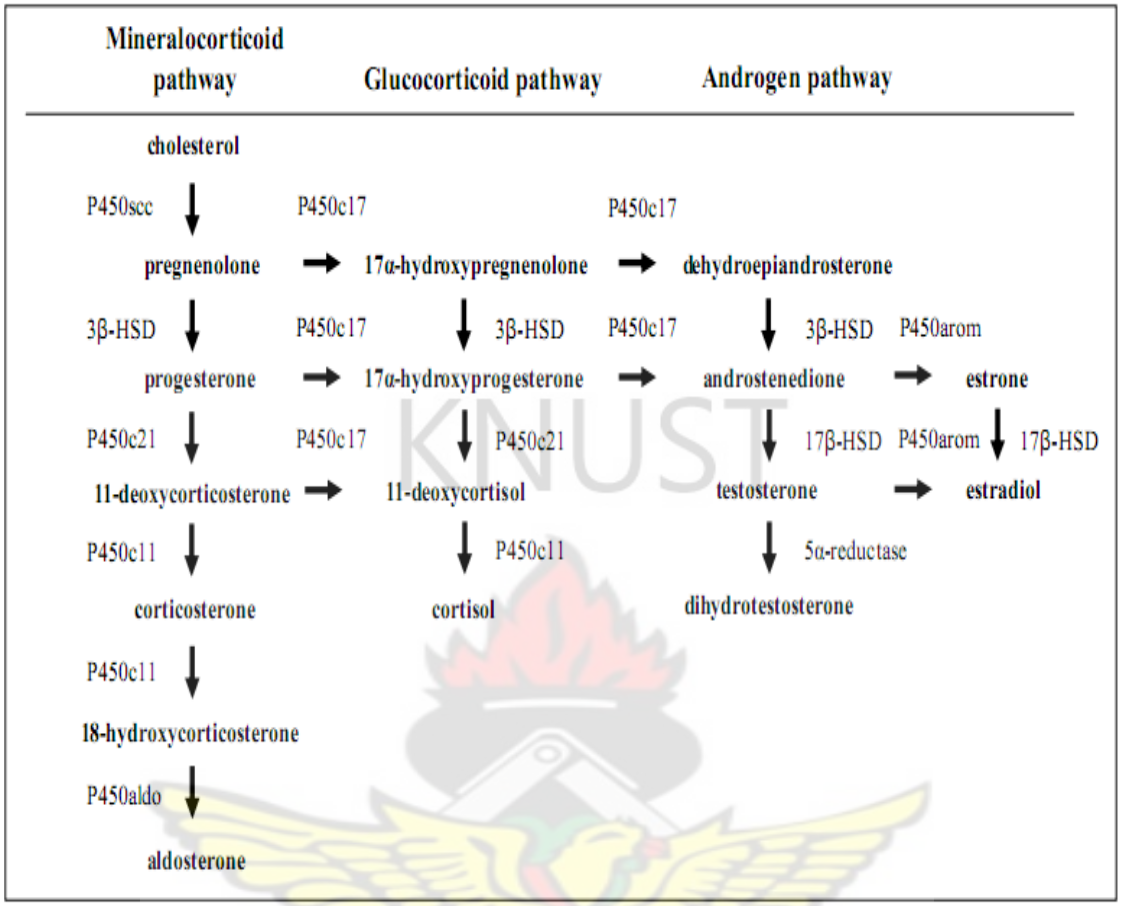


Figure 1.10 Major pathways in steroid biosynthesis.

P450scc, cytochrome P450 cholesterol side chain cleavage; 3β-HSD, 3β-hydroxysteroid dehydrogenase; P450c17, cytochrome P45017α-hydroxylase /17,20-lyase; 17β-HSD, 17β-hydroxysteroid dehydrogenase; P450c21cytochrome P450 21-hydroxylase; P450c11, cytochrome P450 11β-hydroxylase; P450 arom, cytochrome P450 aromatase; P450aldo, aldosterone synthase cytochrome P450.

Steroid hormones can be categorized into five different groups according to the receptors to which they bind: mineralocorticoids, glucocorticoids, androgens, oestrogens and progestagens. The biosynthesis of steroid hormones begins with the cleavage of the side chain of cholesterol to form pregnenolone (Figure 1.10). This reaction takes place in the matrix side of the inner mitochondrial membrane by the enzyme the P450scc (Simpson *et al.*, 1966). To keep up steroidogenesis, the cell must provide a supply of substrate cholesterol to P450scc enzyme within the mitochondria for subsequent production of steroid hormones. The delivery of cholesterol to the inner mitochondrial membrane by Steroidogenic Acute Regulatory protein (StAR) is the rate-limiting step in the steroidogenic pathway (Stocco, 2000). StAR protein is acutely regulated and the expression of protein is critically dependent on trophic hormone stimulation (e.g. LH and ACTH). This makes it susceptible to environmental toxicants: several xenobiotic [e.g. 4-tert-octylphenyl and pesticides Lindane (1,2,3,4,5,6-hexachlorocyclohexane) and glyphosate Roundup (2-(phosphonomethylamino) acetic acid)] have been reported to disrupt Steroidogenesis by inhibiting the expression of the StAR protein (Walsh *et al.*, 2000a; Walsh *et al.*, 2000b). Mutations in the StAR gene are known to cause the disease lipoid congenital adrenal hyperplasia (lipoid CAH) (Bose *et al.*, 1996; Lin *et al.*, 1995). Lipoid CAH is an autosomal recessive lethal condition in which cholesterol and cholesterol esters accumulate and the new born child is unable to synthesize a sufficient amount of steroids. StAR knockout mice display a phenotype that is very similar to lipoid CAH in humans providing a good model in which to study the mechanism of StAR protein's essential contribution to Steroidogenesis and endocrine development (Caron *et al.*, 1997). Pregnenolone, the precursor of all steroid hormones, diffuses across the mitochondrial membranes to the cytoplasm in which it is further metabolized by the enzymes associated with the smooth endoplasmic reticulum. 3β-hydroxysteroid dehydrogenase (3β-HSD), one of the few non-CYP450 enzymes involved in Steroidogenesis, converts Δ5-3β-hydroxysteroid to Δ4-3-ketosteroids. The enzyme 3β-HSD is widely expressed in

steroidogenic and non-steroidogenic tissues such as the tests, prostate, skin and brain (Simard *et al.*, 2005). In the rat, four isozymes of 3β-HSD have been characterized showing differential and tissue-specific expression (Simard *et al.*, 1993).The cytochrome P450 17α-hydroxylase/17-20lyase (P450c17) has two distinct catalytic activities. It mediates 17α-hydroxylation of pregnenolone or progesterone (P4) resulting in the production of hydroxypregnenolone or hydroxyprogesterone. II). The enzyme also mediates the cleavage of the C17, 20 bond of these compounds forming a weak androgen, dehydroepiandrosterone (DHEA) or androstenedione. The C17, 20-lyase is highly expressed in the gonads and it is essential for directing the biosynthesis of steroids towards the sex hormones (Miller *et al.*, 1997). Androstenedione is converted to testosterone by the enzyme 17β-hydroxysteroid dehydrogenase (17β-HSD). Several types of 17β-HSD have been characterized. 17β-HSD type 3 is primarily expressed in the tests (Andersson, 1995). Androgens (androstenedione and T) can be irreversibly transformed into oestrogens by cytochrome P450 aromatase (P450arom). In the mammalian tests, low levels of P450arom are expressed not only in Leydig cells, but also in Sertoli cells and germ cells (Carreau *et al.*, 2003). Dihydrotestosterone (DHT), the most potent endogenous androgen, is formed from testosterone by 5α-reductase. There are two isozymes of 5α-reductase that have differential biochemical and molecular features (Imperato-McGinley *et al.*, 2002): type 1 has a neutral to basic pH optimum and it is mainly expressed in the peripheral tissues (e.g. skin, kidneys, intestine and liver) and type 2 that has an acidic pH optimum and is predominantly expressed in the prostate, epididymis and seminal vesicles (Imperato-McGinley *et al.*, 2002; Jenkins *et al.*, 1992; Normington *et al.*, 1992). DHT induces the differentiation of male accessory sex organs (Wilson *et al.*, 2004). Thus, 5α-reductase is a critical determinant of androgen activity in these tissues. Rats and mice do not express P450c17 in the adrenal cortex as mentioned earlier (Le Goascogne *et al.*, 1991; Perkins *et al.*, 1988). They use corticosterone as a glucocorticoid instead of cortisol. In the adrenal cortex, the enzymes P450c21 catalyse the 21-hydroxylaton of both

glucocorticoids and mineralocorticoids. In the zona fasciculata and zona reticularis, mitochondrial cytochrome P450c11 mediates the conversion of 11-deoxycortcosterone to corticosterone (Ho *et al.*, 1993; Ogishima *et al.*, 1992). The synthesis of cortisol, the main glucocorticoid produced in humans, also involves the catalysing action of P450c11. Corticosterone can be further metabolized to aldosterone which is the most potent steroid regulating electrolyte balance. This reaction is catalysed by mitochondrial P450aldo in the zona glomerulosa (Ogishima *et al.*, 1992).

1.2.6 Effect of Drugs on Male Reproductive System

A large number of compounds have been shown to induce anti-spermatogenic effects in several mammalian species (Fody *et al.*, 1985; Neumann *et al.*, 1970). The inhibitory effects of low and moderate doses of androgen on testis have been demonstrated (Yasuda *et al.*, 1965). Oligospermia has been reported in men receiving large doses of testosterone (Bardin and Paulsen, 1981). Oestrogens have been shown to inhibit testicular functions to a much higher degree than androgens due to the reported inhibition of gonadotropin release (Boas *et al.*, 1950). Azoospermia, gynecomastia and loss of libido have also been reported following oestrogen treatment (Ewing *et al.*, 1978). It has been shown that loss of libido and fertility and inhibition of spermatogenesis following high doses of oestrogen could be maintained by intratesticular implantation of testosterone in rats (Hohlweg *et al.*, 1961). The 19-nortestosterone derivatives namely norethindrone, norethynodrel, norgestrel have been shown to inhibit spermatogenesis (Neumann *et al.*, 1970). Hyperprolactinemia has been reported to cause impotence in men (Andersson *et al.*, 1981; Barkley, 1979). Antiandrogens possessing antigonadotrophic activities such as Cyproterone acetate have been shown to inhibit spermatogenesis in animals and men within 3 to 4 weeks in a dose dependent manner (Neumann *et al.*, 1970). Pure antiandrogens, with no progestational or antigonadotrophic properties, flutamide for instance, exerted

stimulatory influence on testicular functions (Mathur *et al.*, 1982). Gossypol, a phenolic compound has been shown to suppress Leydig cell testosterone synthesis and sperm counts in men (Qian *et al.*, 1984). Cimetidine, a histamine H2 receptor antagonist, has been shown to cause hypospermia and decreased testosterone levels in men and decreased weights of prostate and seminal vesicles in rats (Strauss, 1982; Winters *et al.*, 1979). Dibromodichloropropane (DBCP), a chlorinated hydrocarbon has been shown to cause azoospermia with loss of germ cells in men exposed for more than 10 years. Men with 2 to 10 years exposure to DBCP showed oligospermia and hypospermatogenesis (Biava *et al.*, 1978; Strauss, 1982). Colchicine and its derivatives have been shown to arrest mitosis in the metaphase and thereby decreasing sperm counts (Merlin, 1972). Cyclosporine A, an immunosuppressive agent has been shown to impair spermatogenesis, testicular Steroidogenesis, epididymal sperm maturation and fertility in adult and pre-pubertal rats (Seethalakshmi *et al.*, 1987). Drugs used in chemotherapy have been reported to have adverse effects on reproductive potential. The different classes of anticancer drugs have been shown to affect testis in different ways (Chapman, 1983).

1.3 MEDICINAL PLANTS

The influences of culture are present in different areas of human health, as is the case with reproductive behaviours and fertility. It is an ancient believe that the fertility of the soil all species is determined by divine will. This believes is very strong among people living in communitarian societies and strong religious communities as is the case in Africa. Parenthood is a major transition in the lives of young couples in these societies and failure to conceive a child is a serious personal challenge (Sundby, 1997). Many of these couple therefore do not admit failure and seek medical help and prefer to suffer silently due to the social stigma attach to infertility (Ikechebelu *et al.*, 2003). A great part of the stigma associated with

infertility depends on cultural aspects. Social norms play an important role in determining behaviour and have special relevance to issues of parenthood, fertility and sexuality. The fragile balance between what the individual needs and what society finds to be normal can have a profound impact on personality, coping, wellbeing and sexual behaviour. Furthermore, the cultural differences can impact the sex drive in an infertility context (Papreen *et al.*, 2000). While many couples presenting for infertility treatment experience high levels of psychological distress associated with the diagnosis of infertility, the process of assisted reproduction itself is also associated with increased levels of anxiety, depression and stress. The origin of infertility may reside in the female or the male of both. But in Africa and in Ghana in particular, a visit to fertility centre would reveal that it is mainly the women who go to medical help. Most men shy away from seeking conventional medical treatment for their infertility status even though a random sample of 2179 case in district hospital in Ghana reveal a prevalence of infertility as 11.8% among women and 15.8% among men (Geelhoed *et al.*, 2002).

Infertility in Ghana and other sub-Sahara countries deserves more recognitions as a public health problem. In urban and more advance societies there are assisted medical technologies to help infertile couples to have children. A paradox about these medical treatments is that they often only serve to reinforce existing socio-cultural practices, norms and values. In the rural areas people are more dependent on the natural environment than in the urban areas. The environment is used to fulfil their basic needs by agriculture, hunting, fishing and to collect medicinal plants for health problems. People in the rural area live closer to nature, and they, therefore, have a greater knowledge of the flora and fauna surrounding them (Runganga *et al.*, 1992).

In Ghana, the traditional healers collects plants for general diseases and infertility management base on the perceive cause of the problem. Male fertility is addressed with numerous plants used as aphrodisiacs and to enhance sperm production. Plants that have been used for the management of male infertility includes, *Strophanthus hispidus, Alchornea cordifolia, Terminalia ivorensis, sphenocentrum jollyanum Pierre, and Xylopia aethiopica* (Burkill, 1985b; Burns, 2000). The parts of these plants that are used include the roots, bark and fruits which are either boiled or taken as drinks or chew in the raw state. There has been significant scientific evaluation of the efficacy, potency and safety of some of these plants on male reproductive function. According to Owiredu et al., (Owiredu *et al.*, 2007), root extract of *sphenocentrum jollyanum Pierre* exhibited fertility enhancing activity by increasing sexual behaviour such as mounting frequency, intromission frequency and prolonged ejaculation latency. In the same study the extract also showed central stimulatory sexual effect by increasing the level of serum testosterone in treated animals (Owiredu *et al.*, 2007). The findings of Owiredu et al., (2007) and other similar studies have shown that they may be scientific evidence supporting the traditional usage of some of these plants in the management of male infertility in Ghana and there is therefore the need to scientifically validate some of these plants and their extract for possible usage in the management of male fertility.

1.4 INFERTILITY

Parenthood is undeniably one of the most universally desired goals in adulthood, and most people have life plans that include children. However, not all such couples who desire a child will achieve one spontaneously and a proportion of couples will need medical help to resolve underlying fertility problems (Gerais *et al.*, 1992; Gerrits, 1997). The reproductive processes in humans require both excellent integrity of and excellent interactions between the female and male reproductive systems. Thus the origin of infertility is equally due to inefficient male or female factors. In approximately 40% of infertility cases, where the aetiology has been determined, they are due to female factors, another 40% to male factors, and the remaining 20% are due to both male and female factors (Hatasaka *et al.*, 1997). Infertility is defined as the inability of a non-contraceptive user but sexually active woman to have a live birth after 12 months of unprotected and regularly and properly timed intercourse at least three times a week (Pressat *et al.*, 1985). This childlessness can be caused by primary infertility in which case the couple has never had a live birth or secondary infertility where the couple has had at least one live birth. Globally, WHO (1997) estimates that sixty to eighty million couples experience unwanted infertility. It has been estimated that about 20-30% of marriages in Sub Saharan Africa and 10-15% in the Western world are childless (Cates *et al.*, 1985; Okonofua, 1999). The average infertility in Africa is 10.1% of couples, with a high of 32% in some countries such as Cameroon, Central African Republic and Gabon (Gerais *et al.*, 1992). Infertility is an important problem in African Society, and indeed worldwide. Reproductive failure therefore has very far-reaching social and economic implications in Ghana, where the main reason for marriage is child-bearing. Children are seen as a means of perpetuating the family name and also as a form of insurance cover for the parents against old age and

disability, in the absence of any national social security (Sundby, 1997; Sungree, 1987). A childless marriage, therefore, has lost all these perceived benefits. Infertility is a life crisis with invisible losses, and its consequences are manifold. Childless couples experience social stigma and isolation. Infertility can threaten a person's identity, status and economic security and consequently, be a major source of anxiety leading to lowered self-esteem and a sense of powerlessness (Okonofua, 1997). Studies have shown that in resource-poor countries, where children are highly valued for cultural and economic reasons, childlessness often creates enormous problems for couples; especially for the women who are generally blamed for the infertility (Okonofua, 1997).

Because of the serious social and economic impact of childlessness on couples, a variety of treatments are sought though a pattern of treatment-seeking has not clearly emerged. Couples may delay seeking medical advice because of the fear of a final definite diagnosis, emotional stress, the physical discomfort of the tests they would have to undergo and admitting failure in their efforts to conceive (Bunting *et al.*, 2007). Couples therefore seek varied traditional methods and religious practices, including visits to temples and prayer camps (Gerais *et al.*, 1992; Gerrits, 1997). In Africa and most of the developing world including Ghana, Patterns of treatment-seeking depend on the couple's socio-economic status, decision-making within the family, the level of information and accessibility of treatment (Bunting *et al.*, 2007; Lampic *et al.*, 2006). High costs sometimes results in discontinuation of treatment or resort to unqualified practitioners (Mehta *et al.*, 2008). Treatment for male infertility usually depends on the cause of the infertility. Fertility in men requires normal functioning of the hypothalamus and pituitary gland and the testes. A variety of conditions can therefore lead to infertility in men. In about 30 to 40 percent of causes are due to problems in the testes, 10 to 20 percent are due to a

blockage in the pathway that sperm use to exit the testes during ejaculation, 1 - 2 % of causes are due to pituitary/hypothalamic problems and up to 50 % of male infertility the cases have no identifiable cause(Ikechebelu *et al.*, 2003). In Africa however, the major cause of infertility in men are azoospermia (no spermatoazoa), oligospermia (low sperm count) and asthenozoospermia (abnormal sperm morphology) (Ikechebelu *et al.*, 2003; Imade *et al.*, 1993).

Currently, the therapy for male factor infertility in the developed countries focuses on microsurgery to correct varicoceles or obstruction of the male reproductive ductal system. Additionally, there has been a strong shift away from evaluating and treating the man and proceeding directly to expensive artificial reproductive technologies(Crimmel *et al.*, 2001). Prior attempts at medical therapy for male factor infertility have included hormonal therapy (GnRH agonists and antagonists, gonadotropins, anti-estrogens, testosterone, and aromatase inhibitors), antioxidant therapy, antibiotics, corticosteroids, methyl xanthene's, vitamins, minerals and amino acids (zinc and arginine), and angiotensin-converting enzyme inhibitors (Crimmel *et al.*, 2001). The observation that no medication is approved by the United States Food and Drug Administration for treatment of male infertility confirms the conclusion that adequate controlled studies of potential therapeutic agents are either lacking or failed to elicit a significant improvement in fertility (Burns, 2000). Many men shy away from conventional therapies because of concerns about side effects and lack of efficacy. Long-term satisfaction with current therapies ranges from 40% to 70% (Jarow *et al.*, 1996).Alternative or complementary medicine has become an increasingly popular option for many patients (Crimmel *et al.*, 2001). This trend is reflected in the 47.3% increase in total visits to alternative medicine practitioners, from 427 million in 1990 to 629 million in 1997 (Eisenberg *et al.*, 1998). During this same period of time, the estimated

expenditures for alternative medical professional services increased 45.2%, with an estimated $21.2 billion spent, including at least $12.2 billion paid out-of pocket (Crimmel *et al.*, 2001). In Africa however most people rely on plants and their extracts for the management of diseases including fertility (Gerais *et al.*, 1992; Gerrits, 1997).

1.5 JUSTIFICATION

Plants and their components have played a remarkable role in health care since antique. Plant-based traditional medicines are still of great importance to people living in developing countries and also serve as source to discovery of new drug for a variety of diseases affecting man(Dattner, 2003). In developed countries herbal medicine are extensively used because they are considered natural and relatively safe (Gurib-Fakim, 2006). These medicines contain plant materials as their pharmacologically active components(Pribitkin, 2005). Plants and derivatives of plant played a key role in world health and have long been known to possess biological activity. According to Burns (2000), thirty percent of all modern drugs are derived from plants. Available evidence suggests that approximately 80% of Africans rely on traditional healthcare practitioners and medicinal plants for their daily healthcare needs (Johnson *et al.*, 2007; McKay *et al.*, 2007). Natural products have been an overwhelming success in our effort in fighting diseases. They have reduced pain and suffering, and revolutionized the practices of medicine. Natural products are the most important anticancer and anti-infective agents. More than

60% of approved and pre-new drug application (NDA) candidates are either natural products or related to them, not including biological such as vaccines and monoclonal antibodies(Demain, 1999).Indigenous African herbal medicines are widely used despite an apparent lack of scientific evidence for their quality, safety and efficacy (Johnson *et al.*, 2007). Pharmacological exploration of some medicinal plants of African and Ghanaian origin has shown that many of these medicinal plants possess therapeutic attributes which are safer than the current conventional medicines used for the treatment of erectile dysfunction and infertility most of which are associated with serious side effects including headache, facial flushing, Hypotension, myalgia and dyspepsia(Supuran *et al.*, 2006). One plant that has shown beneficial therapeutic effect in the treatment and management of several diseases in Ghana and Africa is *Xylopia aethiopica* [Dun.] A. Rich, family Annonaceae

The fruits of Xylopia *aethiopica* and other parts of the plant as has been described are used as spices in several African societies. It is also a common medicinal plant used in the treatment of several diseases including fertility enhancing in both male and females. Although antimicrobial, cardiovascular, diuretic, cytotoxic, anti-inflammatory, anti-proliferative, antimalarial and antioxidant activities have been reported for various extracts of this plant and its components (Adaramoye *et al.*, 2011a; Boakye-Yiadom *et al.*, 1977; Somova *et al.*, 2001b), no study has examined the effects of alcoholic extract of Xylopia *aethiopica* and its major component; xylopic acid on sex hormones, spermatogenesis and reproductive function in rats. The aim of the present study was therefore to scientifically evaluate the effect of ethanolic fruit extract of Xylopia *aethiopica* and it major constituent; xylopic acid on reproductive function in male rats.

1.5.1 OBJECTIVES

The objective of the present study is to carry out pharmacological evaluation of the ethanolic fruit extract of *Xylopia aethiopica* and its major constituent Xylopic acid on male reproductive function using animal models

Specific objectives included evaluating the extract and xylopic acid for:

- spermatogenic activity
- Steroidogenesis
- fertility and antifertility activity
- androgenic and anti-androgenic activity
- Testicular effect of the extract and the acid
- Blood biochemical and haematological profile

Chapter 2 PLANT COLLECTION, EXTRACTION AND PHYTOCHEMICAL ANALYSIS

2.1 PLANT COLLECTION AND EXTRACTION

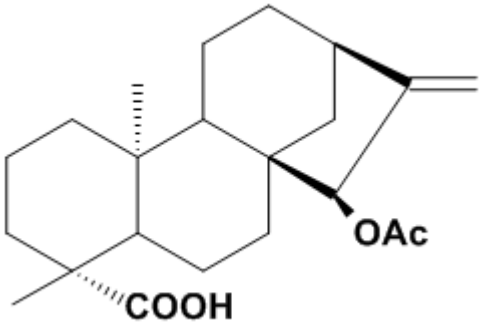
The fruits of the *Xylopia aethiopica* [Dun.] A. Rich, were collected from the KNUST Botanical garden in February 2008, and authenticated by Dr. Abraham Mensah of the Department of Pharmacognosy, KNUST, Kumasi, Ghana. A voucher specimen (FP/08/76) has been deposited in the herbarium of the faculty.

2.1.1 Preparation of extract

Fruits were sun dried and pulverized in a hammer mill. The pulverized fruits (3.5 kg) were placed in cylindrical jars and macerated with 70% v/v ethanol for 72 h. Using a vacuum rotary evaporator, the ethanol filtrate was concentrated at a low temperature, under reduced pressure. This resulted in a greenish solid mass of an ethanolic extract of *Xylopia aethiopica* with a percentage yield of 34.8 % (w/w). The extract was stored in a refrigerator from which fresh solution were prepared using distilled water when required.

2.1.2 Isolation of Xylopic Acid (15β-Acetoxy-(-) - kaur-16-en-19-oic Acid)

Xylopic acid was isolated using the method described by Ekon and Ogan (1968). The dried fruits (0.36 kg) were pulverized and soaked in petroleum ether (40-60 ° C) for three days. The petroleum ether extract was drained and concentrated using rotary evaporator at a temperature of 50 °C. Ethyl acetate (5.0 ml) was added to the concentrate to facilitate crystallization of xylopic acid. Crystals which formed after three days were washed with petroleum ether 40 - 60 °C repeatedly. Xylopic acid was purified using recrystallization by dissolving it in ethanol (BDH Chemicals, Pooled, England). The resulting solution was filtered and left to stand for three days to recrystallized, yielding 1.41% (5.1 g) of xylopic acid with 95% purity.

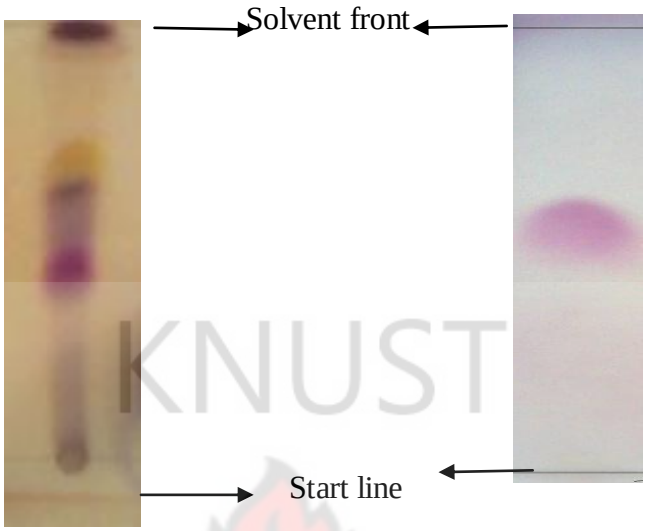


Molecular weight:360.487 g/mol
Molecular formula:$C_{22}H_{32}O_4$

Figure 2.1Structure of Xylopic Acid

2.1.3 Thin Layer Chromatography of xylopic acid (X.A) and extract (X.A.E)

A solvent system of ethyl acetate: petroleum ether (2:18) was prepared and allowed to stand for 30 minutes. After dissolving XA in chloroform it was spotted on a thin plate and allowed to stand in the Chroma tank until 10 cm mark was attained by the solvent front. The plate was then sprayed with anisaldehyde. The extract revealed several spots which indicate the presence of several compounds (Plate 2.1a). On the contrary, xylopic acid revealed a single spot indicating the presence of a single compound (Plate2.1b). The spot for xylopic acid occurred at 5.3 ± 0.01 for both the isolated xylopic acid and the xylopic acid present in the extract after the solvent front had been allowed to travel a total distance of 10 cm.



(a) (b)

Plate 2-1: TLC results of (a) extract revealing several spots indicating the presence of several compounds and (b) xylopic acid showing a single spot indicating the presence of a single compound.

2.1.4 Determination of purity using High Performance Liquid Chromatography (HPLC)

The chromatograph consisted of LC-10AT Shimadzu pump with programmable absorbance detector (783A Applied Bio systems) and Shimadzu CR501 chromatopac. Phenomenexhypersil 20 micron C18 200 × 3.20 mm column was used. The mobile phase consisted of methanol and water (9:1) eluted isocratically at 0.5 ml min^{-1}. Portions of 20 µl of a suitable concentration of pure XA were loaded and injected unto the column after dissolving in the mobile phase at 60 ^{o} C. The eluent was monitored at 206 nm. Portions of the XAE and XA were loaded and injected. The peak(s) were noted as component(s) of the XAE and XA.

2.1.5 Determination of some properties of xylopic acid

Melting point determination was carried out on the crystals as well as the solubility of crystals in petroleum ether, ethanol, methanol, ethyl acetate and chloroform

2.2 RESULTS

High performance liquid chromatography analysis of XAE revealed several peaks (Fig 2.2)corresponding to the various compounds such as essential oil, volatile oil, resin, arocene, a rutheroside fat, bitter principles, alkaloids, glycosides, carbohydrate, protein free fatty acid, mucilages, kaurenoic and xylopic acid (Burkill, 1985b; Igwe *et al.*, 2003; Somova *et al.*, 2001a) which have been reported to be present in the fruits. Xylopic acid, however, showed a single peak (Fig 2.3) indicating the purity of the isolated xylopic acid.

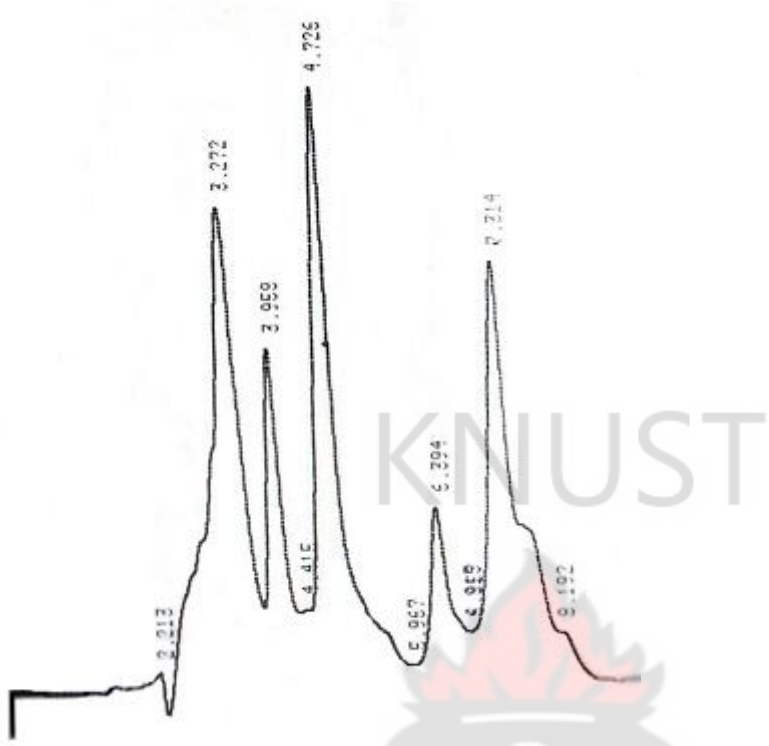


Figure 2.2: HPLC finger print of the extract showing peaks of the various compounds in the extract

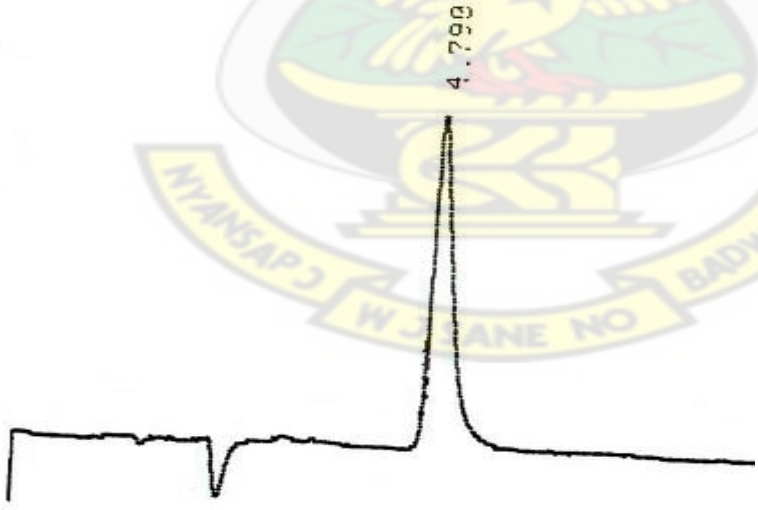


Figure 2.3: Chromatogram of XA showing a single peak corresponding to the isolated XA.

2.2.1 Determination of some properties of xylopic acid

Melting point of the crystals (XA) was determined to be 260-261°C. The crystals were also sparingly soluble in petroleum ether, ethanol, methanol, ethyl acetate and soluble in chloroform.

2.3 DISCUSSION

The presence of several peaks from the HPLC analysis of the fruit extract *of X. aethiopica* as shown in Fig 2.1 indicates that the extract contains several constituents. Studies have shown that as many as forty one (41) different compounds have been identified from *X. aethiopica*, with xylopic acid being the major component. other constituents that have been identified from oil obtained from *X. aethiopica* aresabinene (36%); 1,8-cineole or eucalyptol (12.7%) and terpinen-4-ol (6.98%) (Chalchat *et al.*, 1995). According to Chalchat et al., (1995) other constituents from different chemical groups such as monoterpene and sesquiterpene hydrocarbons, alcohols, aldehydes and oxides have been isolated from the essential oil of *X. aethiopica*. Several of these components within the fruit extracts have been shown to have some pharmacological activity as an antimicrobial, antifungal, anti HIV, antimalarial, Anti-proliferative agent as well as possess haematopoietic activity, immune boosting effect, androgenic and spermatogenic activity (Boakye-Yiadom *et al.*, 1977; Fleischer *et al.*, 2008; Taiwo. *et al.*, 2009; Woode *et al.*, 2011a; Woode *et al.*, 2011b).

2.4 CONCLUSION

In conclusion, the study have shown by way of HPLC analysis that ethanolic fruit extract from X. *aethiopica* contains several compound with Xylopic acid been the major constituent.

Chapter 3

EFFECT OF ETHANOLIC FRUIT EXTRACT OF XYLOPIA AETHIOPICA ON REPRODUCTIVE FUNCTION OF MALE RATS

3.1 INTRODUCTION

In Africa especially south of the Sahara, the use of plants and its extract for the treatment and management of diseases has been in existence since ancient times. Factors such as poverty and illiteracy still militate against availability and accessibility of conventional medical services. A larger number of these tropical plants and their extract have shown beneficial therapeutic effects including fertility-enhancing and contraceptive compounds, anti-oxidant, anti-inflammatory, anti-cancer, anti-microbial and aphrodisiac (Raji *et al.*, 2006)). Among the promising medicinal plants, *X. aethiopica*, an angiosperm of the Annonaceae family is a tropical evergreen tree growing up to 20 metres bearing aromatic seeds. Dry fruits of X. *aethiopica* are black berry containing 4-9 peppery seeds both which are used as spices in the preparation of special dishes in most West African Countries especially in Ghana, Nigeria, Liberia, Benin and Cameroon thus the name African pepper (Adaramoye *et al.*, 2011b; Barminas *et al.*, 1999; Tairu *et al.*, 1999; Taiwo. *et al.*, 2009). The fruit of *X. aethiopica* are given to women after child birth as condiment in soup and tea to accelerate the production of the milk (Burkill, 1985a; Iwu, 1993; Tatsadjieu *et al.*, 2003). It is also used to treat bronchitis, asthma, and in the treatment of male infertility and rheumatism (Burkill, 1985a). Traditional medicine practitioners and traditional birth attendants (TBA) also uses decoction of the seeds to induce placental discharge postpartum and according to Burkhil (1985a) because of its (*X.aethiopica*) traditional usefulness after child birth, it was employed in government hospitals in Ghana and was deemed to have abortificient properties. Despite frequent and regular use, there is no scientific work done to examine its effect on male fertility and spermatogenesis. The present study was

intended to bridge the gap in our continued efforts to establish the effects of local food spices or medications on male reproductive function.

3.2 MATERIALS AND METHODS

3.2.1 Animals

All experiments were performed with male Sprague-Dawley rats weighing 150-200 *g* bought from Noguchi Memorial Institute for Medical Research, University of Ghana, Accra and kept at the Animal House Facility of the Department of Pharmacology, KNUST. The animals were allowed to acclimatize to the laboratory condition (temperature 24-27 °C and 12 hour light-dark cycle) for two week before commencement of the experiment. The rats were allowed free access to solid pellet diet and water *ad libitum* throughout the study. Prior permission was obtained from the ethical committee of the Pharmacology Department, KNUST. All the animals were treated according to the National Institute of Health Guidelines for the care and use of laboratory animals (NIH, Department of Health and Human Services Publication no. 85-23, revised 1985).

3.2.2 Experimental design

Twenty four male rats were divided into four groups of six animals each. Group I served as the control and were given distilled water (vehicle for the extract). Groups II, III and IV rats were given 30, 100 and 300 mg kg^{-1} respectively, fruit extract of *X. aethiopica*. The vehicle and extract were given orally for 60 days. Blood samples were collected from each rat under anaesthesia by cardiac puncture into plain bottles for hormone, enzyme and other biochemical assays after which the rats were euthanized to removed testes and other organs.

3.2.3 Fertility test

From day 53 to 60 of treatment, the rats were transferred to individual cages and mated with untreated and fertile females in natural oestrus (sexually receptive). The presence of sperm on the vaginal lavage fluid or copulatory plug was

designated as the first day of pregnancy (1 DP). Pregnant rats were allowed to complete their pregnancy to term. The day of parturition was designated as day 1 of postnatal life. After birth the following reproductive parameters were analysed: offspring/dam ratio, fertility index (number of pregnant rats/number of mated females x100), viability index (number of live pups on day 4 of postnatal life/number of live offspring born x100), lactation index (number of live pups on day 21 of postnatal life/number of live pups on day 4 of postnatal life x100).

3.2.4 Semen collection and analysis

The left testis was removed along with its epididymis and fatty tissues trimmed off. The caudal epididymis was then separated from the testis and lacerated to collect semen onto a microscope slide for sperm characteristic evaluation according to methods describe by Tyl., (2001) and WHO, (2010). Briefly, two drops of warm 2.9% sodium citrate was added to the semen on the microscope slide and examined under the microscope at ×100 magnification for progressive sperm motility. Sperm viability was evaluated with the eosin–nigrosin stain technique. Semen was mixed with two drops of the stain. A thick smear was prepared and air-dried. The stained slides were examined under the microscope at ×100 magnification. Viable (live) sperm cells appeared unstained while the non-viable (dead) sperm absorbed the stain. The viable and non-viable sperm were counted and the percentage of each calculated. Cauda epididymis from the right side was placed in a petri dish containing 10 ml of phosphate buffered saline (PBS) pre-warmed to 35-37 °C and split with surgical blade to open the epididymal duct to release its contents. The petri dish was then swirled to achieve a uniform sperm suspension from which a sperm count was carried out in the improved Neubauer haemocytometer as shown in Fig 3.1

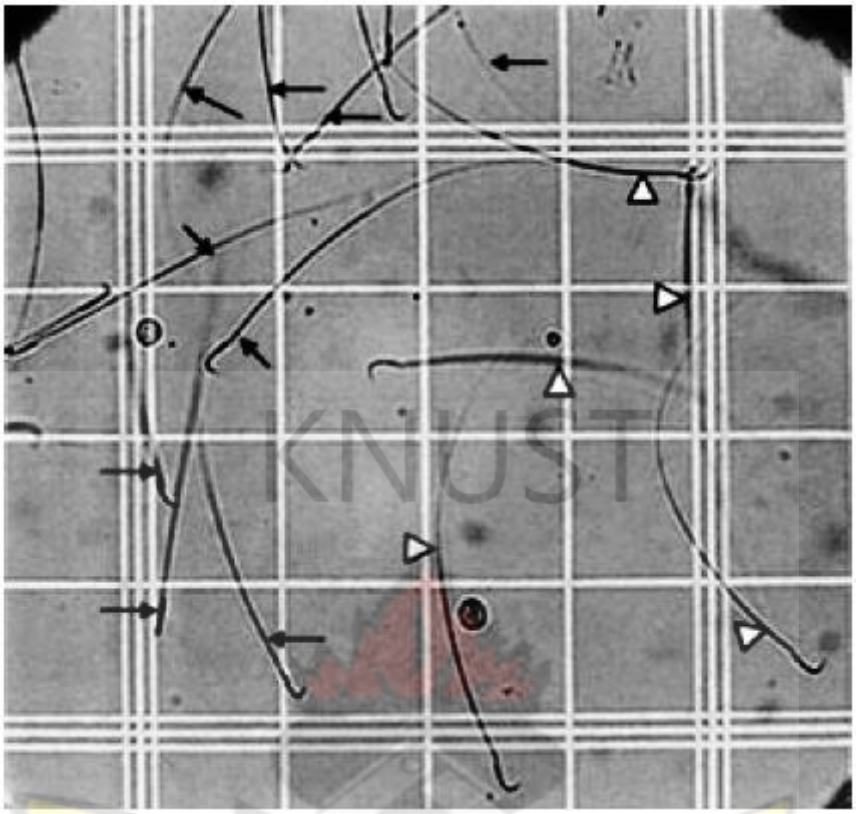


Figure 3.1: Microscopic photograph of the secondary square (area within the triple line border) of a haemocytometer.

The black arrows indicate the nine sperm that will be counted; five white arrows identify the sperm that will not be counted.

Total sperm concentration was calculated using methods described by Tyl, (2001) as follows;

To calculate the total number of sperm per cauda epididymis based on secondary count:

Total sperm = mean count x dilution factor

Where:

Mean count = (count 1 + count 2)/2

And

$$\text{Dilution factor} = \frac{\text{total vol.PBS in dish}}{\text{transferred vol}} \times \frac{\text{total vol.test tube}}{\text{vol.secondary square} \times \text{no.squares}}$$

For this protocol, transferred volume is 0.5 ml and the volume of a secondary square is $4x10^{-16}$ ml

To calculate the epididymal sperm count per milligram cauda (sperm density):

Sperm density = (mean count x dilution factor)/cauda weight (mg) or

Sperm density = total number of sperm per cauda/cauda weight (mg)

3.2.5 Estimation of serum FSH, LH and Testosterone

Serum testosterone, follicle-stimulating hormone (FSH), and luteinizing hormone (LH) were determined by sandwich enzyme immunoassay (SIA) using NoviWell™ assay kits (HySkill Diagnostics, Bahlingen, Germany). Assays were carried out as described by the manufacturer. The assay is based on simultaneous binding of hormone to two monoclonal antibodies; one is immobilized on the

microplate, the other is soluble and conjugated with horseradish peroxidase (HRP). Briefly, 2 µl aliquots of standards and samples were dispensed into their respective wells in ready-to-use micro titre plates precoated with anti-hormone IgG antibodies. After the addition of 100 µl anti-hormone HRP conjugate (1:100 dilutions) to each well, the plates were incubated for 30 min at room temperature. The contents of the well were then aspirated and the wells washed three times with 200 µl of wash solution. The enzyme reaction was started by addition of the chromogen (tetramethylbenzidine/hydrogen peroxide system) into each well. Plates were then incubated for 10 min. The reaction was stopped by addition of 100 µl of 0.15 M H_2SO_4. Absorbance was measured at 450 nm in an ELx800™ Microplate Reader (Bio-Tek Instruments, Winooski, VT, USA). Within-assay coefficient of variation was 6.1% for FSH, 5.4% for LH, and 6.2% for testosterone. The analytic sensitivities of the assays were 1.0 mIU/ml for FSH and LH and 0.1 ng/ml for testosterone as provided by the manufacturer.

3.2.6 Histology

Testes and epididymides of control and extract-treated rats were removed and weighed and fixed in Bouin's fluid for 6 h before they were transferred into 10% formalin for histological processing. The sections were processed for light microscopic study using the wax-embedding method. Briefly, the tissues were washed in phosphate buffer and dehydrated through a graded series (70%, 80%, 90% and 95%) of ethanol (BDH Chemical Limited, Poole, England) for one and half hours each. It was then passed through two changes of absolute ethanol (99.7%) for an hour each. The tissues were cleared in xylene (BDH Chemical Limited, Poole, England) and impregnated with molten wax overnight. Molten blocks were

sectioned using the Rotary type microtome (Bright Instrument Company limited, Huntingdon, England) at approximately 5µm thick. Sections were thereafter stained with haematoxylin and eosin and examined under the microscope for histological evaluation. Photomicrographs of the slide preparation were taken.

3.2.7 Statistical analysis

Results are expressed as mean ± SEM. The difference between the means were determined by one-way analysis of variance (ANOVA) followed by Newman-Keuls's post-hoc test. In all statistical tests, a value of $P<0.05$ was considered significant. All analysis was performed using SigmaPlot for Windows, Version 11.0, (Systat Software, Erkrath, Germany; www.systat.com).

3.3 RESULTS

3.3.1 Effects of X.A extract on body and reproductive organ weights in male rats

As shown in Table 3.1., the extract significantly increased the animal body weight [$F_{3, 12} = 6.496$; $p = 0.0074$], testicular weight [$F_{3, 13} = 8.133$; $p = 0.0026$] as well as epididymal weight [$F_{3, 16} = 66.46$; $p < 0.0001$] in a dose-dependent manner compared to the respective controls.

Table 3.1: Effects *of X. aethiopica* extract on Body and reproductive organ weights in male rats

PARAMETER	CONTROL	30 mg/kg	100 mg/kg	300 mg/kg
Bdywt (g)	161 ± 5.10	162.5 ± 5.20	173.3 ± 1.67	185 ± 2.89*
Testis. Wt (mg)	3449 ± 94.78	3794 ± 109.10	4188 ± 121.60*	4398 ± 252.20**
Epidid.Wt (mg)	135.6 ± 10.74	322.8 ± 15.54***	400.8 ± 28.29***	512 ± 18.83***

Results are express as mean ± SEM, *P ≤ 0.05, ** P ≤ 0.01, ***P ≤ 0.001 compared to respective controls (one-way ANOVA followed by Newman-Keuls post hoc).

3.3.2 Effect on sperm count, sperm motility and viability

Administration of extract of *X. aethiopica* for 60 days significantly increased the epididymal sperm count ($F_{3,16}$ = 69.16, P < 0.001) dose-dependently compared to the control group. The extract-treated animals however did not exhibit any significant change in sperm motility, viability and morphology compared to the control group (Table3.2).

Table 3.2: Effect of X. aethiopica extract on sperm characteristics

PARAMETER	CONTROL	30 mg/kg	100 mg/kg	300 mg/kg
Sperm Count (million/mL)	67.70 ± 4.01	90.5 ± 6.54	153.40 ± 20.41**	325.60 ± 17.64 ***
Sperm Motility (%)	83.20 ± 2.35	83.00 ± 2.30	84.00 ± 3.11	77.60 ± 2.46
Viability (%)	66.40 ± 1.86	61.80 ± 2.75	62.20 ± 0.86	62.80 ± 1.72

Results are express as mean ± SEM, *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001 compared to respective controls (one-way ANOVA followed by Newman-Keuls post hoc).

3.3.3 Effect of extract on male Fertility

The number of rats having successful mating was unaffected in all groups giving a mating index of 100%. However, the fertility of these rats was significantly influenced by the extract treatments in terms of fertility index, offspring/dam ratio, and the number of live foetuses by the cohabited female rats. The percentage of pregnant females was 60%, 80%, 80% and 75% for control, 30 mg/kg, 100 mg/kg and 300 mg/kg groups respectively. Female rats cohabited with treated male rats bore significantly higher ($P < 0.05$) number of litters as shown in Table 3.3. Viability index and lactation index as well as offspring weight was unaffected by extract treatment as shown in Table 3.3

Table 3.3: Effect of X*ylopia aethiopica* fruit extract on male fertility in rats

parameter	Control	30 mg/kg	100 mg/kg	300 mg/kg
Treated males	6.0	6.0	6.0	6.0
Mated females	5.0	5.0	5.0	4.0
Pregnant females	3.0	4.0	4.0	3.0
Offspring/Dam ratio	4.0	7.0	7.5	6.0
Number of live foetuses	12.0	28.0	30.0	18.0
Number of dead foetuses	0.0	0.0	0.0	0.0
Mating index (%)	83.3	83.3	83.3	66.7
Fertility index (%)	60.0	80.0	80.0	75.0
Viability index (%)	100.0	100.0	100.0	100.0
Lactation index (%)	100.0	100.0	100.0	100.0
offspring weight (g)	1.7 ± 0.01	1.7 ± 0.03	1.7 ± 0.02	1.5 ± 0.01

Fertility index = number of male impregnating a female rats/number of males cohabitated' ×100.
Viability index = number of live pups on day 4 of postnatal life/number of live offspring born ×100
Lactation index = number of live pups on day 21 of postnatal life/number of live pups on day 4 of postnatal life ×100
Mating index = Number of male with confirmed mating/number cohabitated x 100

3.3.4 Effect on serum FSH, LH and testosterone

Elevated testosterone and LH levels were observed in rats treated with ethanolic fruit extract of *X.aethiopica*. Serum FSH level increased significantly after treatment with 30 mg of the extract. Follicular stimulating hormone level however decreased significantly in animals treated with 100 mg/kg and 300 mg/kg of the extract ($F_{3, 16} = 59.081$; $p<0.001$) as shown in figure 3.2. The serum testosterone and luteinizing hormone were significantly increased in all the treated groups compare with the controls ($F_{3, 16} = 34.921$; $p<0.001$) and ($F_{3, 16} = 31.788$; $p<0.001$), respectively. It was noted that the change in hormone level was dose dependent (Fig 3.2).

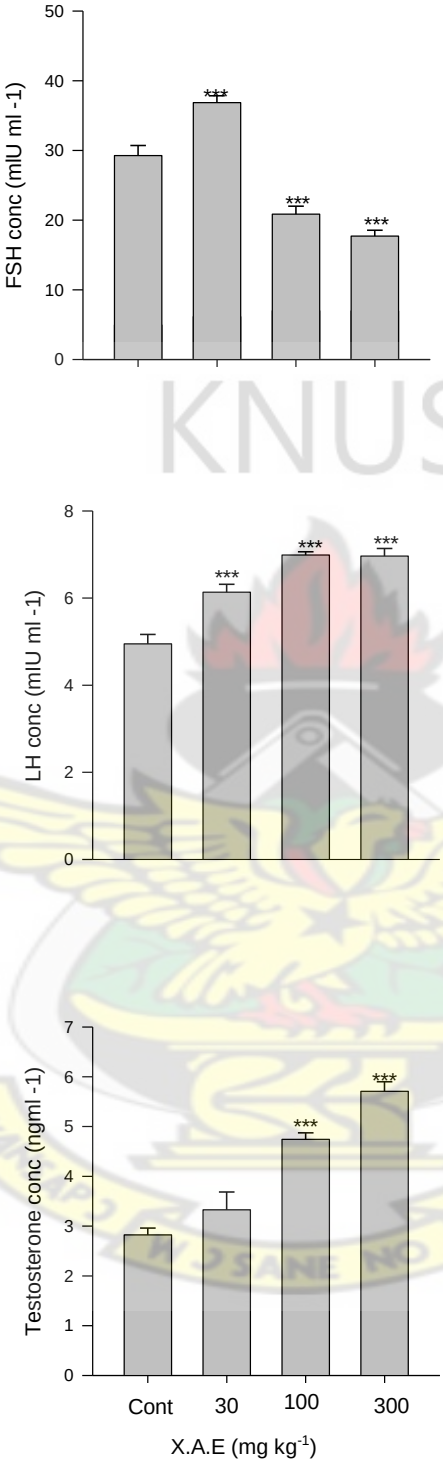


Figure 3.2: Effect of *X.aethiopica* fruit extract on reproductive hormones in male rats.

Results are presented as means ± SEM. *$P \leq 0.05$, ** $P \leq 0.01$, ***$P \leq 0.001$ compared to respective controls (one-way ANOVA followed by Newman-Keuls*post hoc*).

3.3.5 Testicular Histology

Transverse sections of testes of control group animals showed normal histoarchitecture. Sertoli and Leydig cells of normal size were present. The number of seminiferous tubules was normal with bundles of normal spermatozoa. In the transverse sections of extract-treated group animals, increase in diameter of seminiferous tubules was observed. Extract treatment also improved spermatogenesis in all groups as compared with control group as shown in the figure below.

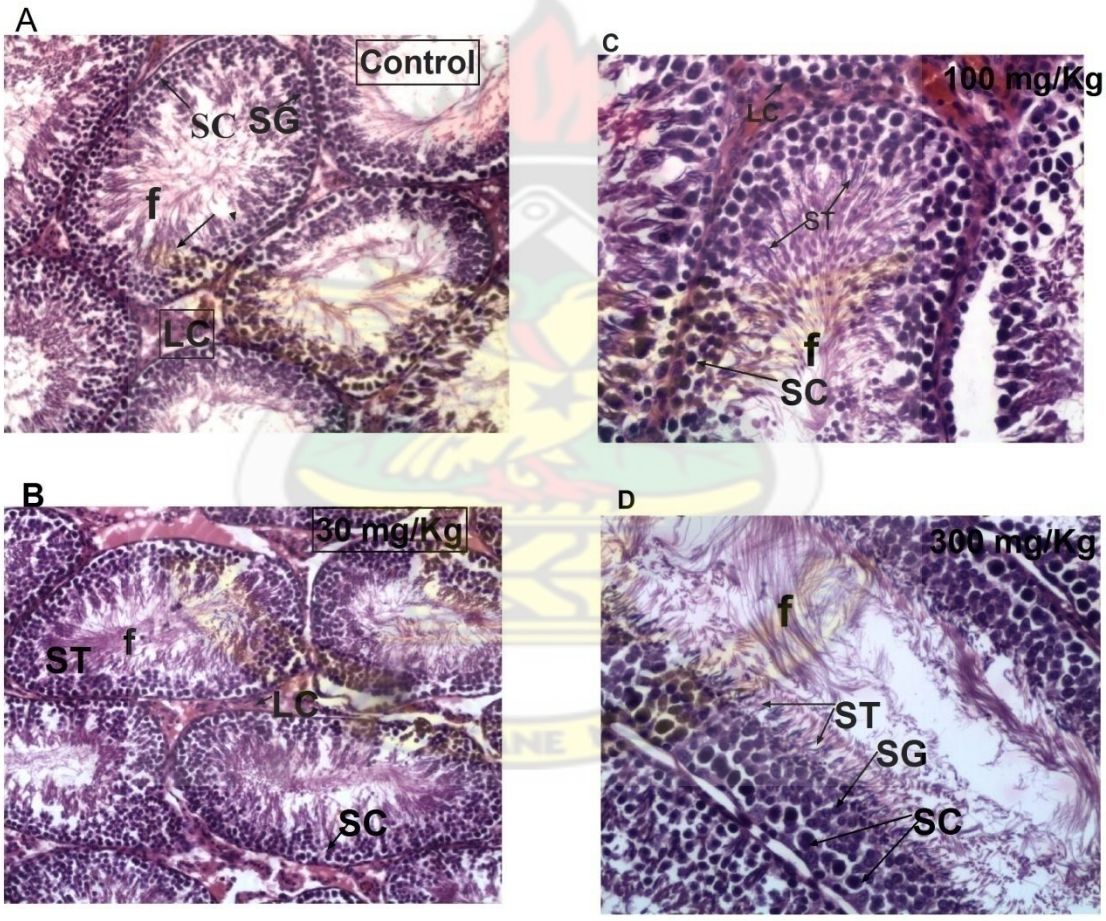


Figure 3.3: Histology of testis

(a) control group where (b) ethanolic extract 30 mg/kg, (c) ethanolic extract 100 mg/kg, and (d) ethanolic extract 300 mg/kg treated groups: increase in number of spermatids cells, spermatogonia with increase in diameter of seminiferous tubule (F, flagellae; LC, Leydigs cell; SG, spermatogonia; GC, Germinal cell ST, spermatid, Sc, Sertoli cell.). H&E stain. X400

3.4 DISCUSSION

The finding of the study indicates that ethanolic extract of *X. aethiopica* causes increase in animal body weight as well as weights of sexual organs such as testis and epididymis dose-dependently. The weight, size and secretory function of testes, epididymides and seminal vesicle are closely regulated by androgens (Agrawal *et al.*, 1986). The number of sperms in epididymis was also markedly increased. The increased sperm counts as well as increased weight of sexual organs are indicative of improved fertility because of extract treatment. In order to understand these observations measurements of testosterone, FSH and LH levels after treatment was undertaken. Elevated levels of testosterone (T) were noticed in treated animals. Testosterone is the main male gonadal hormone produced by the interstitial cells of the Leydig in the testis. It is also the major index of androgenecity. A certain concentration of androgens is also required for the initiation and maintenance of spermatogenesis and for the stimulation of growth and function of the glands penis (GP), coagulating gland (CG), prostate, levator ani bulbocavanous muscle, and seminal vesicles. Testosterone also helps in maintaining body shape, and increasing muscle mass and strength (Chauhan *et al.*, 2010). The levels of FSH and LH were also increased after treatment with the extract. Follicle stimulating hormone level however decreased with increasing dose of the extract possibly due to negative feedback by the increase in LH and testosterone. Testosterone is produced by Leydig cells of the testes in response to LH, under the control of the hypothalamic–pituitary–testis axis (Chauhan *et al.*, 2007). It can be said that the elevation in serum steroid hormone levels by administration of the extract may be accountable for the observed effect on spermatogenesis. The finding of the present study is in conformity with the work of Oguike and Archbong (2011) who observed an increase in testicular and epididymal weight, improved sperm count, motility and morphology in rabbit

bucks treated with *X. aethiopica* fruit extract. Both testosterone and FSH have an important function in spermatogenesis (Chauhan *et al.*, 2010). The result of the male fertility also confirms the fertility enhancing potential of fruit extract of *X. aethiopica* in male rats. The evaluation of fertility and sexual performance provides important data about the effect of chemicals on the reproductive system. These tests evaluate parameters such as fertility index, offspring/dam ratio and offspring viability. Alterations in such parameters may indicate impairment of reproductive function. The results demonstrated that treatment with fruit extract of *X. aethiopica* may increase fertility. The finding of the present study thus validates the use of this plant to enhance fertility in men in traditional medicine.

Interestingly, it was observed that sperm morphology, motility and viability remained unaltered in control- as well as extract treated animals. Follicle stimulating hormone has key roles in the development of a normal complement of functional Sertoli cells (and thus adult spermatogenic potential), in the maturation of Sertoli cells at puberty and the maintenance of their cytoskeleton and cell junctions, and in the maintenance of spermatogonial development. Together, FSH and T support meiosis, exhibit an anti-apoptotic action on spermatocytes and round spermatids, and act co-operatively to promote spermatid maturation and sperm release (Chauhan *et al.*, 2010). The androgenic and spermatogenic effect of the fruit extract of *X. aethiopica* is also evident in the histological sections which show an increase in germ cells and mature spermatozoa in the seminiferous tubules. Reduced numbers of spermatozoa, malformed spermatozoa or their reduced or insufficient motility are the leading causes of disturbed fertility or infertility in patient. The extract may thus provide an alternative for management of infertility due to reduced

spermatogenesis. Further studies are necessary to elucidate the compounds of the ethanolic extract responsible for enhance spermatogenesis in rats.

3.5 CONCLUSIONS

In conclusion, ethanolic extract of *X. aethiopica* increased LH and Testosterone levels as well as sperm count, the mechanism of which appears to be through stimulation of gonadotropin probably by hypothalamus activation. The extract also did not have any toxic effect on spermatozoa and germs cell as well as sperm characteristic such as morphology, viability and motility and may be potentially useful in the management of fertility problem in men especially those with low sperm count, a validation of its traditional use as a fertility improving agent in Ghana

Chapter 4 EFFECT OF XYLOPIC ACID ON REPRODUCTIVE FUNCTION AND SPERMATOGENESIS

4.1 INTRODUCTION

Plants and plant products have been used the world over for treatment of various human ailments since ancient times. The use of medicinal plants and its extract in traditional medicine is still prevalent in developing countries attributable in part to poverty and illiteracy which militate against availability and accessibility to conventional medical service (Raji *et al.*, 2006). Many plant species from tropical forests have been identified as containing fertility enhancing and contraceptive compounds. About370 of the plants have shown to offer promise of safer and more effective male and female contraceptive agents (Maurya *et al.*, 2004). In addition, more than 600 plant species appear to offer potential as abortificients (World Health Organisation, 1981).The fruit extract of *X. aethiopica* has been shown to have antimicrobial, antifungal activity as well as possess haematopoietic activity, immune boosting effect, and increases LH and testosterone levels as well as fertility in rats (Boakye-Yiadom *et al.*, 1977; Taiwo. *et al.*, 2009; Woode *et al.*, 2011a; Woode *et al.*, 2011b). Although antimicrobial, cardiovascular, diuretic, cytotoxic, androgenic and spermatogenic activities have been reported for various extracts of this plant (Adaramoye *et al.*, 2011a; Boakye-Yiadom *et al.*, 1977; Somova *et al.*, 2001b), no study has examined the effects of the major constituent; xylopic acid, on spermatogenesis and reproductive function. The present study was thus intended to evaluate the effect of xylopic acid, a major constituent of the fruits of *X. aethiopica* on serum sex hormone levels, spermatogenesis and testicular histology in male rats.

4.2 MATERIALS AND METHODS

4.2.1 Drugs and chemicals

Testosterone, follicle-stimulating hormone (FSH), prolactin (PRL) and luteinizing hormone (LH) assay kits were obtained from HySkill Diagnostics (Bahlingen, Germany).

4.2.2 Experimental design

Male Sprague Dawley rats were divided into four groups of six animals each. Group I served as the control and were given distilled water (vehicle for the XA). Groups II, III and IV rats were given XA orally at the dose of 10, 30 and 100 mg kg^{-1} respectively for 28 days. Blood was collected from the saphenous veins of animals on day 7 of the treatment and on day 28 after which the rats were euthanized to remove testes and other organs according to approved protocols as described by Tyl (2001).

4.2.3 Semen collection and analysis

The left testis was removed along with its epididymis and fatty tissues trimmed off. The caudal epididymis was then separated from the testis and lacerated to collect semen onto a microscope slide for sperm characteristic evaluation according to methods described by Tyl., (2001) and WHO, (2010) as stated in the previous chapter.

4.2.4 Estimation of serum Prolactin, FSH, LH and Testosterone

Serum testosterone, follicle-stimulating hormone (FSH), and luteinizing hormone (LH) were determined by sandwich enzyme immunoassay (SIA) using NoviWell™ assay kits (HySkill Diagnostics, Bahlingen, Germany). Assays were carried out as described by the manufacturer. The assay is based on simultaneous binding of hormone to two monoclonal antibodies; one is immobilized on the

microplate, the other is soluble and conjugated with horseradish peroxidase (HRP). Briefly, 2 µl aliquots of standards and samples were dispensed into their respective wells in ready-to-use micro titre plates precoated with anti-hormone IgG antibodies. After the addition of 100 µl anti-hormone HRP conjugate (1:100 dilutions) to each well, the plates were incubated for 30 min at room temperature. The contents of the well were then aspirated and the wells washed three times with 200 µl of wash solution. The enzyme reaction was started by addition of the chromogen (tetramethylbenzidine/hydrogen peroxide system) into each well. Plates were then incubated for 10 min. The reaction was stopped by addition of 100 µl of 0.15 M H_2SO_4. Absorbance was measured at 450 nm in an ELx800™ Microplate Reader (Bio-Tek Instruments, Winooski, VT, USA). Within-assay coefficient of variation was 6.1% for FSH, 5.4% for LH, and 6.2% for testosterone. The analytic sensitivities of the assays were 1.0 mIU/ml for FSH and LH and 0.1 ng/ml for testosterone as provided by the manufacturer.

4.2.5 Histology

Testes and epididymides of control and extract-treated rats were removed and weighed and fixed in Bouin's fluid for 6 h before they were transferred into 10% formalin for histological processing. The sections were processed for light microscopic study using the wax-embedding method. Briefly, the tissues were washed in phosphate buffer and dehydrated through a graded series (70%, 80%, 90% and 95%) of ethanol (BDH Chemical Limited, Poole, England) for one and half hours each. It was then passed through two changes of absolute ethanol (99.7%) for an hour each. The tissues were cleared in xylene (BDH Chemical Limited, Poole, England) and impregnated with molten wax overnight. Molten blocks were sectioned using the Rotary type microtome (Bright Instrument Company limited, Huntingdon, England) at approximately 5µm thick. Sections were thereafter stained with Haematoxylin and Eosin and examined under the microscope for histological evaluation. Photomicrographs of the slide preparation were taken.

4.2.6 Estimation of lipid peroxidation as measured by malondialdehyde (MDA) concentration

Testes from each rat was homogenized separately at a tissue concentration of 50 mg/ml in 0.1M of ice-cold phosphate buffer (pH 7.4) and centrifuged at 1000 g at 4^{o}c for 5 min. The homogenate (0.5 ml) was mixed with 0.5 ml of normal saline (0.9% NaCl) and 2 ml of TBA-TCA mixture (0.32 g TBA in 75 ml of 0.25N HCl with 15 g TCA). The mixture of the volume was made up to 100 ml by 95% ethanol) and boiled at 100 ^{o}c for 15 min. The mixture was then cooled at room temperature and centrifuge at 4000 *g* for 15 min. Absorbance of the supernatant was measured using Bio-Teck spectrophotometer (Bio-teck instrument, Winooski, VT, USA) at 535 nm. The MDA concentration was calculated using extinction coefficient of 1.34x10 ^{5}mol/cm^{3}.

4.2.7 Biochemical assay of Glutathione peroxidase

The Glutathione peroxidase activity was measured biochemically in the testicular homogenate. The testicular tissue was homogenized in phosphate buffered saline at a concentration of 50 mg/ml which was then centrifuge at 1000 g for 10 min. the reaction mixture was made up of 2.3 ml of 0.1 M Tris-Hcl buffer (pH 7.4), 75 µl of 0.04 M of reduced glutathione (GSH), 100 µl of 0.1 M nicotinamide adenine dinucleotide phosphate (NADPH) and 100 µl of glutathione reductase. The homogenate (100 µl) was added to 2.8 ml of reaction mixture and incubated for 5 min at 25 ^{o}c. the reaction was initiated by the addition of 100 µl of 0.75 mM/l H_2O_2, and the absorbance was measured at 340 nm for 5 min. The activity of glutathione peroxidase was expressed as nM NADPH oxidized/mg tissue/min using a molar extinction coefficient of 6.22x10^{3} (mM/l)/cm.

4.2.8 Statistical analysis

Results were expressed as mean ± SD. The significance of difference between the means was determined by one-way analysis of variance (ANOVA) with Newman-Keuls's as *post-hoc* test. Difference between subject factors (time and dose treatment) was analysed using two-way analysis of variance as a within-subject factor followed by Bonferroni's as *post hoc* test. The relationship between semen characteristic, MDA and glutathione peroxidase activity was performed using linear regression. In all statistical tests, a value of $P<0.05$ was considered significant. All analysis was performed using Sigma Plot for Windows, Version 11.0, (Systat Software, Erkrath, Germany; www.systat.com).

4.3 RESULTS

4.3.1 Effect of XA on body and organ weight of male rats

Xylopic acid treatment caused no significant change in animal body weight compared with the control. However, the weight of the testis and epididymis both showed a dose dependent reduction in the treated animals ($p < 0.001$) compared with control animals. The seminiferous tubular diameter also caused a significant ($P < 0.001$) reduction in all treated rats compared to control rats as shown in table 4.1.

Table 4.1: Effect of XA on animal body weight, and reproductive organ weight

Treatment Group	change in body wt (g)	testicular wt (g)	wt of epididymis (g)	Seminiferous tubular D (µm)
Control	70.70 ± 17.7	0.96 ± 0.2	0.20 ± 0.0	135.20 ± 18.9
10 mg kg^{-1}	69.40 ± 9.2	0.825 ± 0.2	0.19 ± 0.0	115.50 ± 11.8*
30 mg kg^{-1}	76.90 ± 4.9	0.60 ± 0.1**	0.14 ±0.0***	106.60 ± 6.6***
100 mg kg^{-1}	68.90 ± 11.0	0.60 ± 0.1***	0.12 ± 0.0***	83.28 ± 11.5***

Data are express as mean ± SD, *$P \leq 0.05$, ** $P \leq 0.01$, ***$P \leq 0.001$ compared to respective controls (one-way ANOVA followed by Newman-Keuls*post hoc*), wt = weight and D = diameter.

4.3.2 Effect on Prolactin, FSH, LH and Testosterone

Serum prolactin concentration was significantly reduced ($P<0.05$) when rats were treated with XA (10, 30 and 100 mg kg^{-1}) as compared to the controls group for both 7 days and 28 days. Treatment for 7 days showed no significant change in the serum FSH level compared with the control group. However, a significant increase was noted in FSH when treatment was continued for 28 days ($P<0.05$). Duration of treatment had significant [$F_{3, 16;}$ = 22.78; P = 0.002] incremental effect on serum FSH at 100 mg kg^{-1} dose level. Luteinizing hormone levels showed a significant dose dependent increase after 7 days of treatment ($P<0.05$) as well as 28 days (P = 0.001). Two-way analysis of variance showed that duration had significant effect [$F_{3, 16}$ = 19.62; P = 0.0004] as well as concentration [$F_{3, 16}$ = 15.96; P < 0.0001] on LH levels. Generally, there was a significant ($P < 0.05$) increase in serum testosterone levels in

rats treated with XA (10 and 30 mg kg^{-1}) for 7 days (Fig 4.1). However, continuous treatment for 28 days significantly (P = 0.05) decreased serum testosterone levels at all dose levels compared with the control groups. Concentration and duration of dosing both significantly decrease serum testosterone level in rats [$F_{3, 12}$; P = 0.0004] as shown in Fig 4.1.

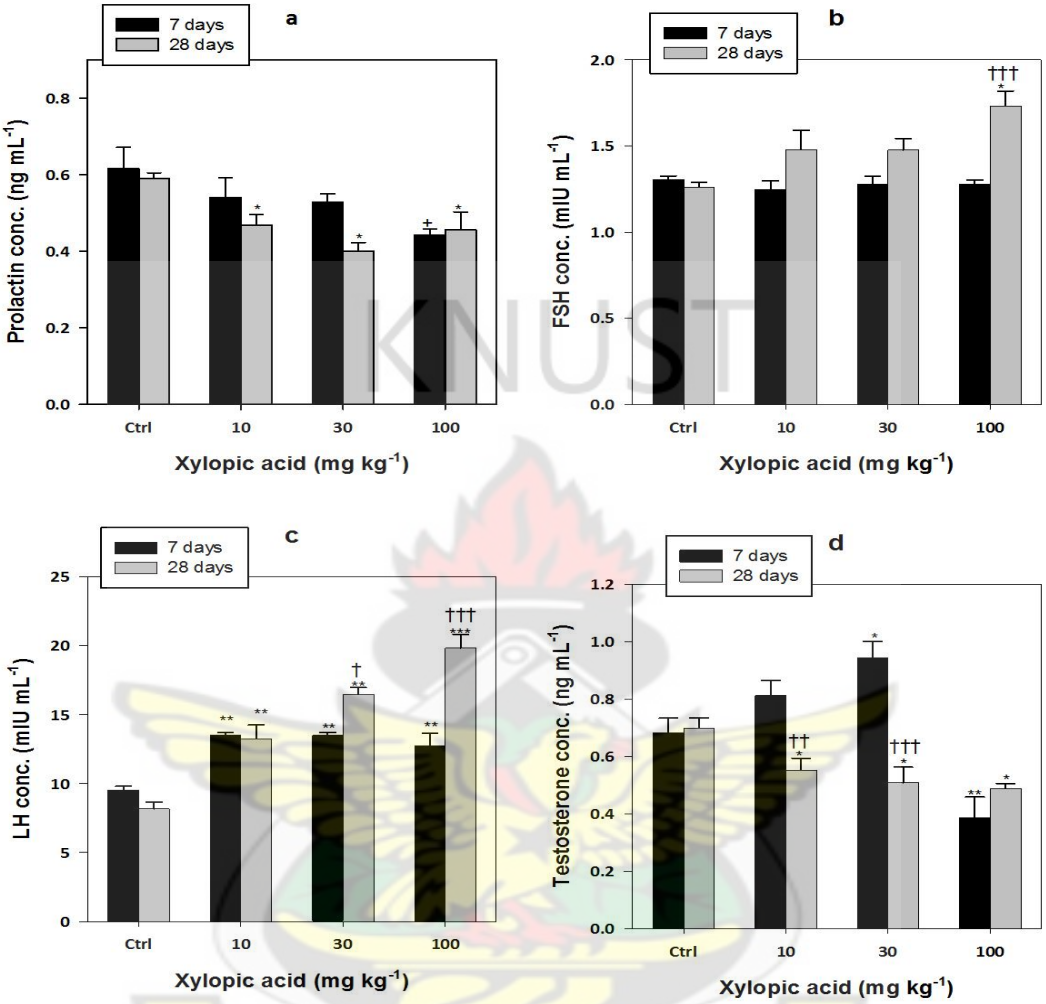


Figure 4.1: Effect of xylopic acid on reproductive hormones in male rats.

Results are presented as means ± SD. *$P \leq 0.05$, ** $P \leq 0.01$, ***$P \leq 0.001$ compared to respective controls (one-way ANOVA followed by Newman-Keuls*post hoc*); †$P \leq 0.05$, ††$P \leq 0.01$, †††$P \leq 0.001$ (two-way repeated measures ANOVA followed by Bonferroni's post *hoc*).

4.3.3 Effect of xylopic acid on sperm count, sperm motility and viability

Administration of XA significantly decreased epididymal sperm count ($F_{3, 16}$ = 69.16, p < 0.001) dose-dependently in treated rats compared with control group. The treated animals also exhibited a significant decrease in sperm motility ($F_{3, 16}$ = 43.95, P < 0.001) and sperm viability ($F_{3.16}$ = 15.68, P < 0.001) compared with the control group (Table 4.2).

Table 4.2: Effect of Xylopic acid on sperm characteristic

Parameter	Control	10 mg kg^{-1}	30 mg kg^{-1}	100 mg kg^{-1}
Sperm count ($X10^6$/ml)	129.8 ± 13.1	93.0 ± 6.1***	64.3 ±1.7***	55.8 ± 1.8***
Sperm motility (%)	75.2 ± 3.6	50.6 ± 8.3***	13.2 ± 4.3***	0.0 ± 0.0***
Viability (%)	69.0 ± 2.6	47.6 ± 5.7***	15.6 ± 2.9***	0.0 ± 0.0***
GPX activity	189.1 ± 9.1	236.7 ± 12.2	313.1 ± 50.9	636.1 ± 65.0**

Data are express as mean ± SD, *$P \leq 0.05$, ** $P \leq 0.01$, ***$P \leq 0.001$ compared to respective controls (one-way ANOVA followed by Newman-Keuls*post hoc*)

4.3.4 Effect of Xylopic acid on lipid peroxidation

The level of lipid peroxidation as measured by malondialdehyde (MDA) concentration showed a significant increase in the treated groups (F3.12 = 7.494, P = 0.0044) compared to MDA levels in the control group. The antioxidant enzyme; glutathione peroxidase activity also showed a significant increase in the treated group (F3, 8 = 23.05, P= 0.0003) compared to the control as shown in table 4.2. The relationship of lipid peroxidation in the testes as measured by MDA levels with sperm motility in rats treated with extract of XA is shown in figure 4.2. There was a significant but negative correlation between MDA concentration in the testes and sperm motility (r = -0.7720, p = 0.0005) as well as sperm viability (r = -0.5617, p =

0.052) in the treated animals. For every increase in MDA concentration there was 0.04 ± 0.01 decrease in motility and 0.03 ± 0.02 decrease in viability.

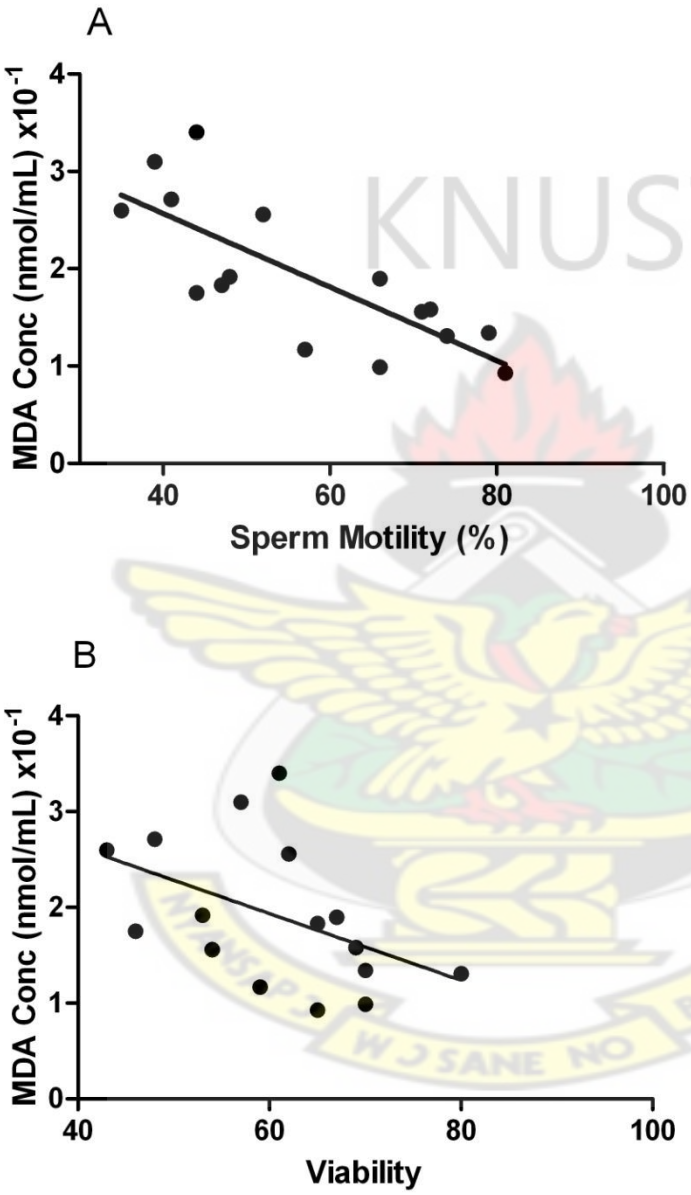


Figure 4.2: Regression analysis between MDA concentration and sperm motility as well as sperm viability in rat treated with XA.

4.3.5 Histology of the testis

Histological analysis of the testes of rats in the control showed normal morphological appearance with seminiferous tubules showing all the various stages of spermatogenesis (Fig 4.3A). Administration of XA (10, 30 and 100 mg kg^{-1}) caused visible cytotoxic activity by clearing all matured spermatozoa, germ cells and other cell in the seminiferous tubules compared with the control rats (Fig 4.3). The treated group showed depleted germ cell layer, disorganization of the normal regular layering of the various stages of spermatogonia and spermatocytes as shown in fig 4.3B and C. Regeneration of germinal epithelium and restructuring of the germinal interstitium occurred in the recovery rats as shown in fig 4.3D.

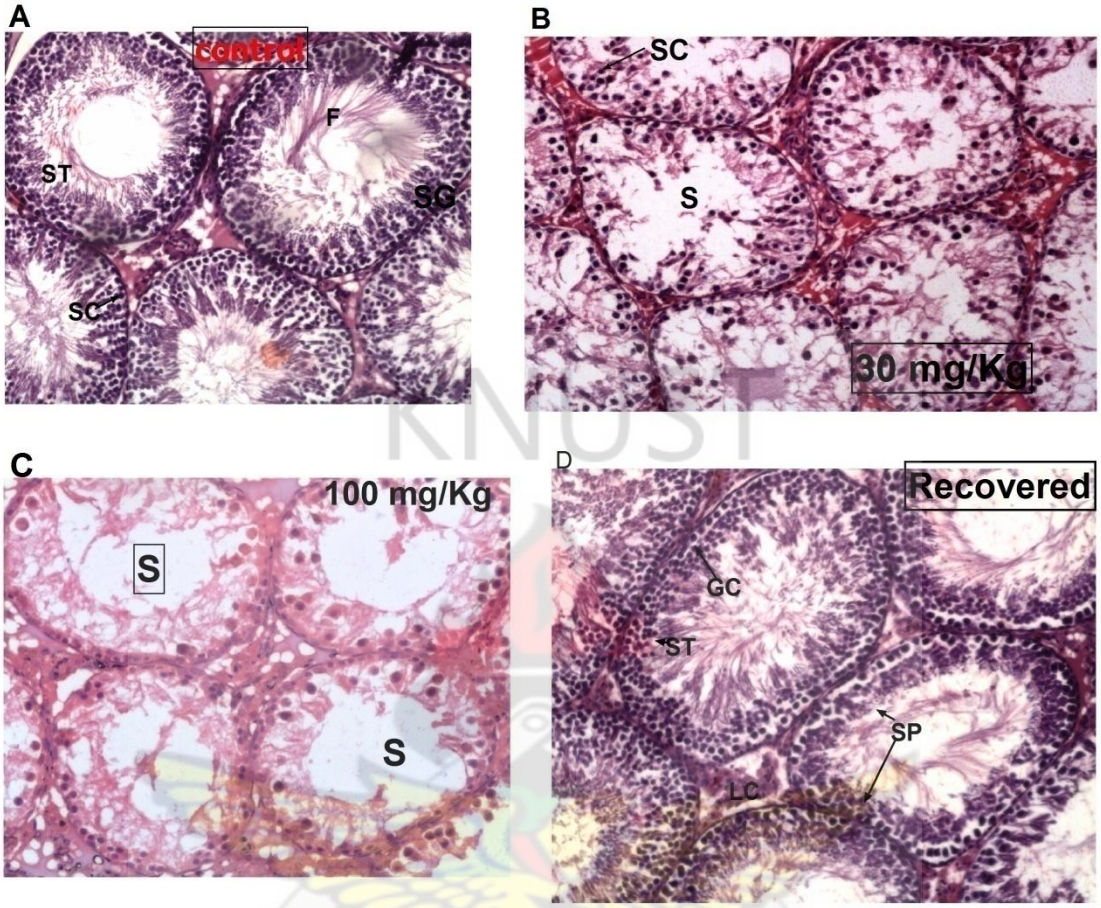


Figure 4.3: Light photomicrograph of testes;

Showing testes of A) control rat seminiferous tubules showing various stages of spermatogenesis; B) testes of rat treated with XA (30 mg kg^{-1}) showing irregular shape seminiferous tubules; C) testes of rats treated with XA 100 mg kg^{-1}showing seminiferous tubules with various degrees of degeneration. D) Testis of rats from the recovery group showing various stages of spermatogenesis. H&E stain, x 250. LC, Leydig cell; GC, Germinal cell; ST, Spermatids; SP, Spermatozoa; SG, spermatogonia; F, Flagellae; S, Space; Sc, Sertoli cell.

4.4 DISCUSSION

In testis, a significant proportion of the weight is associated with spermatogenic function(McLachlan *et al.*, 2002a); hence the suppression of testicular weight observed in this study following XA treatment might result from the significant decrease in the sperm count and content of spermatids and spermatozoa seen in the present study as evident in the results of the histology which showed a marked reduction of these cells in the seminiferous tubules. The significant reduction in the reproductive organ weights of the rats in this study may be attributed to the decrease in testosterone levels. It has been shown that in rats Leydig cell number per testis increases in parallel with testicular weight following birth, accompanied by increases in testosterone level (Meistrich *et al.*, 1999).

Gonadotropins and testosterone are the prime regulators of germ cell development. The successful and complete male germ cell development is dependent on the balanced endocrine interplay of hypothalamus, pituitary and the testis (de Krester, 1974). FSH binds with receptors in the sertoli cells and stimulates spermatogenesis. LH stimulates the production of testosterone in Leydig cells, which in turn may act on the Sertoli and peritubular cells of the seminiferous tubules and stimulates spermatogenesis (O'Donnell *et al.*, 1999). It has been hypothesized that an elevation of intratesticular testosterone contributes to the maintenance of testicular atrophy and that agents reducing the intratesticular testosterone levels stimulate the recovery of spermatogenesis (Meistrich, 1999). This hypothesis could be related to the result obtained in the present study where there was an increase in the serum testosterone level after administration of 30 mg kg^{-1} body weight of XA for 7 days. This level however, reduced with the administration of higher doses of the XA (100 mg kg^{-1}). The dose dependent reduction in serum testosterone level when duration of treatment was extended from 7 days to 28 days has further shown that XA could be acting as testosterone antagonist/anti-androgen at higher dose. The increased serum LH and FSH levels

observed may probably be due to suppression of negative feed-back inhibition of anterior pituitary which may secondarily increase the level of these hormones as seen in the 21 day treated rats (Fujisawa *et al.*, 1994).

The result of the present study also demonstrated that the number of Leydig cells decreased in the rats treated with XA. This could be the cause of testosterone shortage, which is needed along with the Sertoli cells to carry out spermatogenesis (Kanai *et al.*, 1986). There are a number of probable mechanisms for the anti-gonadal/anti-androgenic activities of XA; it may exert a direct inhibitory action on the testis; or it may affect the pituitary, causing changes in gonadotropins concentrations and thus subsequent spermatogenic impairment. Studies have shown that XA exhibits cytotoxicity and antiproliferative activity in human cervical cancer cells (Adaramoye *et al.*, 2011b). The spermatotoxicity and the dose dependent degeneration of the seminiferous tubules as well as reduction in tubular diameter in the testis of rats treated with XA in the present study is thus in conformity with the earlier studies indicating the cytotoxicity of XA (Adaramoye *et al.*, 2011b; Asekun *et al.*, 2004)The decrease in epididymal sperm count and high number of morphologically abnormal sperm indicates interference of XA with testicular spermatogenesis as observed in the histological section of the testis. Decrease in sperm motility and viability suggests alteration of sperm maturation in the epididymides (Sathiyraj *et al.*, 2010). Reduced number of spermatozoa, malformed spermatozoa or their reduced motility and viability are the leading causes of disturbed fertility or infertility in patients (Chauhan *et al.*, 2008). Among plant based contraceptives, inhibition of male fertility after administration of natural substances has been related to decreased spermatozoa density (Sharma *et al.*, 2001). Seminiferous tubular cytoarchitecture were, however, restored to normal in the recovery groups.

Reduced sperm motility, viability and malformation have been shown to correlate with the state of oxidative stress in the testis. The result presented in this study

indicates that lipid peroxidation correlates inversely with percentage motile sperm. Motility of spermatozoa depends largely on the integrity of the mitochondrial sheath of the spermatozoa, of which polyunsaturated fatty acids (PUFA) are a major component (Aitken *et al.*, 1995; Aitken *et al.*, 1989). An alteration in the lipid membrane structure has been stated as the cause of sperm pathology in many andrological diseases (e.g. variocoele, germ-free genital tract inflammation). Polyunsaturated fatty acids and cholesterol are the main targets for free radical damage, and an inverse relationship between lipid peroxidation and sperm motility has been demonstrated (Aitken *et al.*, 1989; Suleiman *et al.*, 1996). Oxidative damage is common for spermatozoa during epididymal maturation and storage. Human and rodents spermatozoa are highly susceptible to oxidative injury but are naturally protected from such injury by the antioxidant properties of seminal plasma (Iwasaki *et al.*, 1992; Zalata *et al.*, 1995). Reactive oxygen species (ROS) generated by spermatozoa play an important role in normal physiologic processes, such as sperm capacitation, acrosome reaction, oocyte fusion and stabilization of the mitochondrial capsule in the midpiece (Agarwal *et al.*, 2003; Desai *et al.*, 2009).

The results of the present study is similar to previous studies that show increasing levels of lipid peroxidation as measured by MDA concentration in sperms and seminal plasma in males with asthenozoospermia, asthenoteratozoospermia and oligoasthenoteratozoospermia(Fraczek *et al.*, 2005; Nakamura *et al.*, 2002). It is also in conformity with the findings that excessive ROS production causes oxidative stress, resulting in decrease sperm motility, viability and increase midpiece sperm defects that impair sperm function (Agarwal *et al.*, 2003; Gagnon *et al.*, 1999). Agarwal *et al.*, (2003) also reported that increased ROS formation is correlated with a reduction of sperm motility. The increase in glutathione peroxidase activity in the treated animals in this study goes to confirm the onset of oxidative stress in testes

of these animals. During the early onset of oxidative stress there is an initial rise in the activity of antioxidant enzymes like glutathione peroxidase to combat the increasing reactive oxygen species until saturation when it begins to decline.

4.5 CONCLUSION

In conclusion, the results of the present study suggest that XA possesses reversible antifertility, spermatotoxic and anti-androgenic properties the mechanism of which may involve direct effect on germ cells and other cells in the testes causing oxidative stress and a possible hypothalamic-pituitary-gonadal axis involvement.

Chapter 5 EVALUATION OF ANTI-ANDROGENIC ACTIVITY OF XYLOPIC ACID

5.1 INTRODUCTION

Xylopic acid [15-β-acetoxy-(-)-kaur-16-en-19-oic acid], a diterpene kaurane derivative obtained upon extraction of the fruits of *Xylopia aethiopica* with petroleum ether caused a reduction in serum testosterone and LH levels as well as a significant reduction in testicular weight, epididymal sperm count, motility and viability in rats as stated earlier in this study. Reduction in sperm count and sperm quality as well as reproductive organ weight reduction are mostly associated with androgen antagonist or anti-androgens (Kelce *et al.*, 1995; Thompson *et al.*, 2003). Generally, accessory sex glands and tissues are dependent upon androgen stimulation to gain and maintain weight during or after puberty. If endogenous testicular source of androgens are removed, exogenous source of androgens are needed to increased or maintain the weight of these tissues (Ashby *et al.*, 2000; Yamada *et al.*, 2000). There is increasing anxiety that reproductive development and function in human and other species may be affected by chemicals that behave like steroid hormones which are linked to the increasing incidence of reproductive cancers and a worldwide decline of semen quality (Toppari, 1996).

Evaluation of the androgen and antiandrogen activity of xylopic acid is thus invaluable in our quest to establish its effect and mechanism of action on the

male reproductive system. The present study was carried out to characterize potential anti-androgenic properties of xylopic acid and to elucidate the possible mechanism of the antifertility activity of xylopic acid exhibited in rats as earlier reported.

5.2 MATERIALS AND METHODS

5.2.1 Drugs and chemicals

Pentobarbitone was obtained from Sigma-Aldrich Inc., St. Louis, MO, USA. Testosterone propionate was a gift from Abeth Consult Limited (Kumasi, Ghana); Cyproterone acetate was obtained from Bayer Australia Ltd (Melbourne, Australia).

5.2.2 Hershberger assay

5.2.2.1Animals

All experiments were performed with immature male Sprague-Dawley rats weighing 60-70 g bought from Noguchi Memorial Institute for Medical Research, University of Ghana, Accra and kept at the Animal House Facility of the Department of Pharmacology, KNUST, Kumasi. The animals were allowed to acclimatize to laboratory condition (Temperature 24-26°C and 12 hour light-dark cycle) for two weeks before commencement of the experiment with free access to solid pellet diet (GAFCO Trading company, Tema) and water ad libitum throughout the study. Prior permission was obtained from the ethical committee of the Pharmacology Department, KNUST. All the animals were

treated according to the National Institute of Health Guidelines for the care and use of laboratory animals (NIH, Department of Health and Human Services Publication no. 85-23, revised 1985).

5.2.3 Experimental Procedure

The experiment was carried out by Hershberger assay (1953) as modified by Dorfman (1962). Thirty male Sprague-Dawley rats (42 days old, weighing about 60-70 g) which were either orchidectomized or sham operated. The rats were anaesthetized with pentobarbitone and the testes were exteriorized via a midline incision. The testicular blood vessels were clamped and ligated and each testis was removed. The midline musculature was sutured and the skin was auto clipped. The condition of the animals was checked on a daily basis and the clips were removed from the healed wound 7 days after the operation. At 11 days post-castration the rats were weighed and assigned to five treatment groups as follows; Group 1 received 0.4mg/kg/day of testosterone propionate (s.c.), group 2 received 0.4mg/kg/day of Testosterone propionate (s.c.) plus 10 mg/kg of XA orally, group 3 received 0.4mg/kg/day of testosterone propionate (s.c.) plus 30 mg/kg of XA orally, group 4 received 0.4mg/kg/day of testosterone propionate (s.c.) plus 100 mg/kg of XA orally, group 5 (sham operated) received only distilled water. The oral administration of XA was done 30 minutes after the subcutaneous injection of testosterone propionate. The animals were treated daily for 10 days and the weight of the animals taken daily. On the day after the last treatment, blood samples were taken by cardiac puncture and the rats were

then necropsied to isolate organs and tissues for study of androgenic or anti-androgenic effects. The endpoints evaluated were the growth/body weight, and weight of the seminal vesicles plus coagulating glands with fluid, ventral prostate, levator ani plus bulbocavernosus muscle, glans penis, Cowper's glands (bulbourethral glands), and liver all without fixation.

5.3 CHICKEN COMB BIOASSAY

5.3.1 Animals

The Cockerels (Single White Leghorn) were obtained 1-day post-hatch from Asamoah and Yamoah farms (Kumasi, Ghana) and were housed in stainless steel cages (34×57×18 cm3) at a population density of 8 chicks per cage. Food (Chick Mash, GAFCO, Tema, Ghana) and water were readily available.

5.3.2 Experimental design

The chicken comb method as described by Dorfmann (1962) is based on the response of the comb of chicken to androgens and antiandrogens. Single comb White Leghorn chicks (7 to 10 day old) were used for the experiment. At the beginning of the assay, sum of the length and height of the comb were calculated. Chickens were distributed into ten different groups of 8 animals each. Group 1 received only distilled water and served as the untreated control. Group 2, 3 and 4 were injected intramuscularly with 0.02, 0.1 and 1.0 mg/kg of testosterone propionate in olive oil as a standard androgen. Group 5, 6 and 7

were given 3, 10 and 30 mg/kg of cyproterone acetate orally to serve as a standard anti-androgen. Group 8, 9 and 10 were given xylopic acid at the doses of 10, 30 and 100 mg/kg orally. All animals were treated daily for 7 consecutive days. Twenty four hours after the last treatment, the chicken combs were measured again and the growth of the comb expressed as the sum of the height and length in millimetres.

5.4 STATISTICAL ANALYSIS

Results are expressed as mean ± SD. The significant of difference between the means was determined by one-way analysis of variance (ANOVA) with Newman-Keuls's as post-hoc test. In all statistical tests, a value of $P<0.05$ was considered significant. All analysis was performed using Sigma plot for Windows, Version 11.0, (Systat Software, Erkrath, Germany; www.systat.com).

5.5 RESULTS

5.5.1 Hershberger assay

The body weight of animals that received xylopic acid did not differ significantly from vehicle control group and the reference control that received only TP as shown in figure 5.1. Xylopic acid treatment to castrated testosterone-treated male rats reduced significantly both absolute and relative weight of the following tissues, seminal vesicle (14.4%), prostate (26.6%), Glands penis (22.3%), LABC (7.7%), Cowper's gland (14.6%). The number in parentheses refers to reduction of absolute weight caused by 10 mg/kg of XA. The weight reduction were even more pronounced at the middle dose of 30 mg/kg and at the highest dose of 100 mg/kg of XA compared to the control and the testosterone propionate treated group as shown in figure 5.2. In contrast, testosterone administration to castrated animals increase significantly both absolute and relative weight of the androgen dependent tissues as follows seminal vesicle (7.8%), prostate (4.6%), Glands penis (9.7%), LABC (1.4%), Cowper's gland (6.6%) compared to the vehicle treated animals as shown in Figure 5.2. Subcutaneous administration of TP had the expected stimulatory effect on the androgen dependent tissues as stated above. Co-administration of xylopic acid with TP, as observed essentially abolished the stimulatory effects of the standard androgen on the tissues. The weight of the liver in rats receiving TP plus various doses of xylopic acid was significantly increased in a dose dependent manner compared to control and TP administered rats as shown in Figure 5.2.

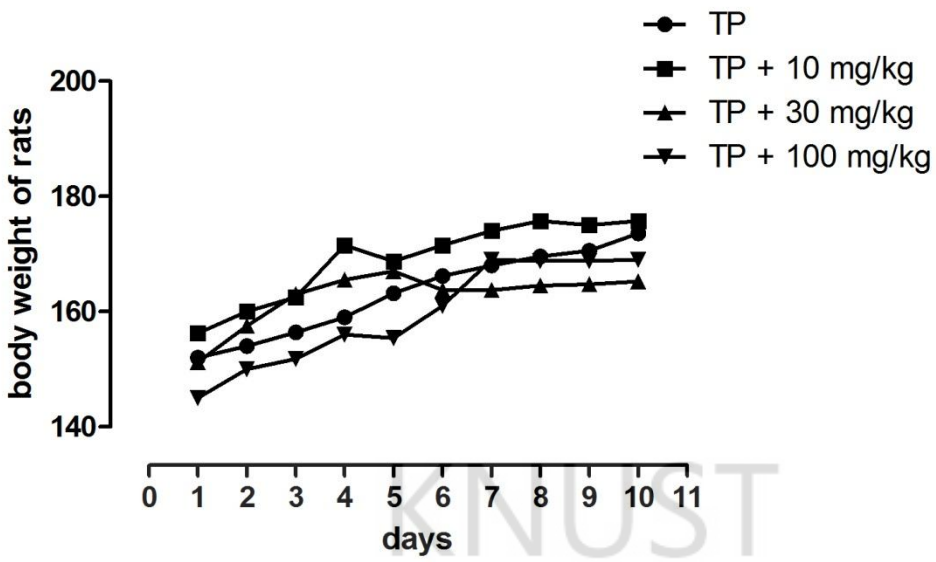


Figure 5.1: Effect of X.A on animal body weight

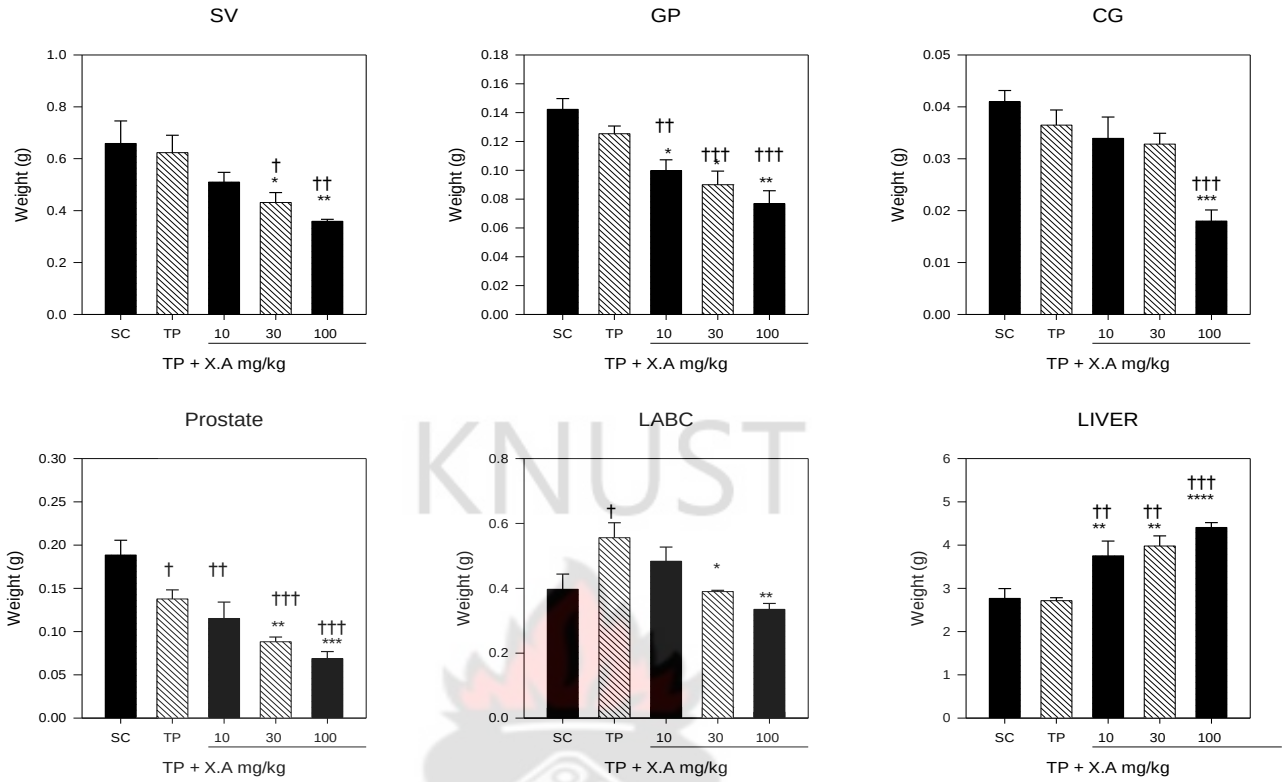


Figure 5.2: Relative weight of androgen dependent organs.

Seminal Vesicles (SV), Gland Penis (GP), Coagulating Gland (CG), Prostate, Levator ani Bulbocavanous Muscle (LABC), Liver from sham castrated and castrated rats treated with Testosterone Propionate (0.4 mg/kg sc) with or without Xylopic Acid (X.A) at doses of 10, 30 and 100 mg/kg given orally. Results are presented as means ± SEM. *P ≤ 0.05, ** P ≤ 0.01, ***P ≤ 0.001 compared to TP treated. (One-way ANOVA followed by Newman-Keuls post hoc); †P ≤ 0.05, ††P ≤ 0.01, †††P ≤ 0.001 compared to sham castrated control rats (one-way ANOVA followed by Newman-Keuls post hoc).

5.5.2 Chicken comb bioassay

From the results presented while testosterone propionate (0.02, 0.1 and 1.0 mg/kg) significantly increased the growth of the chicken comb dose dependently, cyproterone as expected (3, 10, and 30 mg/kg) dose dependently inhibited the growth of the chicken comb ($p < 0.001$) compared to the control and the standard androgen (TP) treated groups (Fig 5.3). The growth of the comb in chicken receiving various dose of xylopic acid (10, 30 and 100 mg/kg) was inhibited significantly ($p < 0.01$) compared to the TP treatment group similarly to standard anti-androgen, cyproterone which was used as shown in figure 5.3. Administration of XA to TP treated chicken inhibited the growth of the chicken comb. The growth inhibition exhibited by XA on TP treated chicken was qualitatively comparable, although weaker to the action of cyproterone on TP treated chicken (Fig 5.3). Plate 5-1 shows the growth of the comb of chicks treated with various doses of Testosterone propionate and Cyproterone while plate 5-2 shows the growth of the comb of chicks treated with various doses Testosterone propionate and XA.

Plate 5-1: Photographs of White leghorn chicks treated with various doses Testosterone propionate and Cyproterone showing the growth of the comb (arrows)

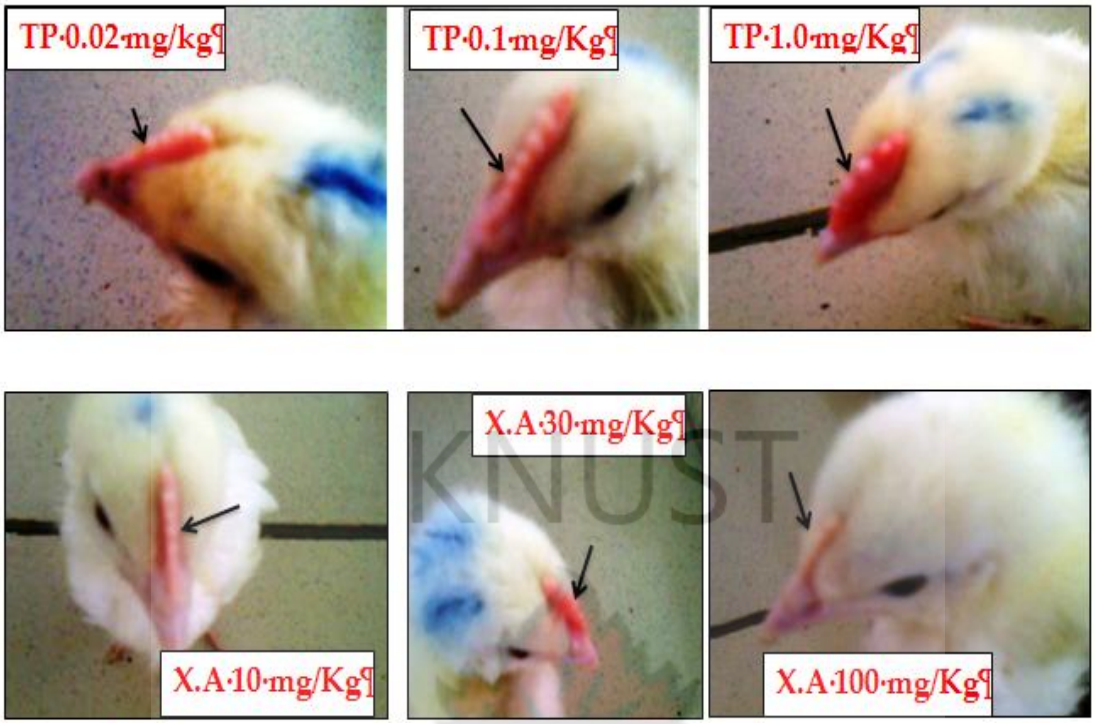


Plate 5-2: Photographs of White leghorn chicks treated with various doses Testosterone propionate and X.A. showing the growth of the comb (arrows)

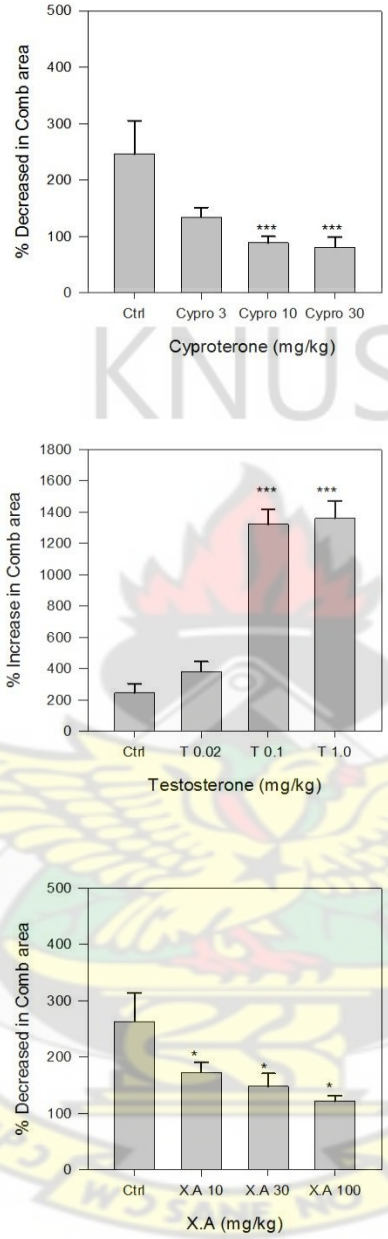


Figure 5.3: The percentage change in the comb area of chicken treated with Testosterone Propionate, Cyproterone, and various doses of XA.

5.6 DISCUSSION

Various xenobiotics and naturally occurring compounds have been found to disrupt the endocrine system of animals (Toppari *et al.*, 1996). Reduction in androgen-dominance to oestrogens and interference with androgen action are apparent mechanisms causing demasculinization and fertility decline in males (McKinnell *et al.*, 2001; Rivas *et al.*, 2002; Williams *et al.*, 2001).The United States Food Quality Protection and Safe Drinking Water Acts of 1996 led to the US Environmental Protection Agency issuing the EDSTAC report in 1998. Among the several requirements of that report was the need for rodent assays for the detection of anti-androgens/androgens and agents affecting Steroidogenesis. In the present study, when XA was administered to immature male rats orally, it exhibited antiandrogenic activity as seen by the significant decrease in the weight of the seminal vesicles, ventral prostate, LABC, gland penis, and the Cowper's gland, as these organs are dependent on androgens. This antiandrogenic action further support the earlier reports presented in this thesis that administration of XA to adult male rats resulted in a significant reduction in sperm count, motility, viability and significantly increase abnormal sperm morphology as well as decrease the number of Leydig cells and the seminiferous tubular diameter. To further confirm the antiandrogenic action of XA, various doses (10, 30 and 100 mg/kg) were administered to day- old chicks and the effect of XA on the chicken comb growth were observed and compared to standard androgen (testosterone propionate) as well as standard antiandrogen (cyproterone acetate). Xylopic acid as expected exhibited antiandrogenic effect on the chicken comb similar to cyproterone acetate by suppressing the growth of the comb whose growth is under the influence of androgens. The stimulatory effect of the testosterone propionate was also suppressed when it was administered together with XA.

Anti-androgens may exhibit their activity both peripherally on androgen-dependent tissues and by feedback action at a central site (Mainwaring, 1977; Moguilewsky *et al.*, 1979; Neumann, 1985; Neumann *et al.*, 1977; Raynaud *et al.*,

1979). They may compete with the peripheral androgen receptors and thus inhibit the effect of endogenous or exogenous androgens. Centrally, they inhibit gonadotropin secretion and thereby diminish testosterone production by the gonads. In addition to their effects on reproduction and accessory sexual organs, anti-androgens inhibit sebum production (anti-acne drugs) and delay androgen-dependent hair loss (Hans, 2007; Neri, 1977; Neumann *et al.*, 1970).

Inhibition of 5α-reductase, an enzyme located in tissues such as the prostate, is one pharmacological approach to inhibit benign prostate hyperplasia in men. Such inhibitors reduced the conversion from testosterone to 5α-dihydrotestosterone (DHT).Testosterone is converted to 5α-dihydrotestosterone (DHT) by the enzyme 5α-reductase which is specifically localized in some androgen-responsive target tissues(e. g., prostate, seminal vesicle, epididymis, skin and sebaceous glands), whereas in other androgen sensitive tissues, such as the skeletal muscles and the central structures, the androgenic hormone is testosterone. Inhibition of 5α-reductase provides a selective approach to androgen deprivation in DHT-target tissues, such as the prostate. The 5α-reductase inhibitors are applied in the therapy of benign prostate hyperplasia(Hans, 2007).

The results of the present study therefore suggest that X.A has anti-androgenic properties which can interfere with androgen signalling by two mechanisms. Firstly the action of the substance can be the inhibition of androgen binding to androgen receptor and secondly the inhibition of enzymes involved in the production of sex hormones such as 5α-reductase and aromatase.

5.7 CONCLUSION

In conclusion, Xylopic acid exhibited anti-androgenic activity similar to Cyproterone acetate, a known anti-androgenic agent which acts by blocking androgen receptors which prevents androgens from binding to them and

suppresses luteinizing hormone which in turn reduces testosterone levels thus suppressing the actions of testosterone and its metabolite dihydrotestosterone on tissues. The results thus confirm the earlier report of antifertility activity of Xylopic acid in male rats.

Chapter 6 EFFECT OF ETHANOLIC FRUIT EXTRACT OF XYLOPIA AETHIOPICA ON HAEMATOLOGICAL NAD BIOCHEMICAL PARAMETERS IN RATS

6.1 INTRODUCTION

The use of *X. aethiopica* as a herb is very common in Ghana, especially in the rural areas where access to conventional medicine is still very low due to non-availability and/or affordability. The fruit is often incorporated in preparations for enema and external uses, calling on its repulsive properties for pains in the ribs, chest and generally for any painful area, lumbago (low back and waist pains), neuralgia (pains in the nerves) and in the treatment of boils and skin eruptions (Iwu, 1993; Sofowora, 1977). The fruit decoction is useful in the treatment of bronchitis and dysenteric conditions, and as a medicine for bulimia (eating disorder) and to enhance fertility in both males and females. The fruit of *X. aethiopica* are given to women after child birth as condiment in soup and tea to accelerate the production of milk (Burkill, 1985a; Iwu, 1993; Tatsadjieu *et al.*, 2003). The seeds are also mixed with other spices, rubbed on the body as cosmetic and as perfume for clothing. The crushed, powdered fruit mixed with Shea butter and coconut oil is used as creams, cosmetic products and perfumes. The fruits mixed with its roots are used in the treatment of rheumatism (Johnkennedy *et al.*, 2011). Even the odorous roots of the plant are employed in tinctures, administered orally to expel worms and other parasites from the intestines, or in teeth rinsing and mouth wash extracts against toothaches (Ghana Herbal Pharmacopoeia, 1992). Notwithstanding all the medicinal uses, the dried fruits are used as flavourings to prepare local soups in West Africa thus the name African pepper (Iwu, 1993). Despites its uses there has been no comprehensive study on the effect of Xylopia *aethiopica* on haematological and biochemical parameters. It is therefore of interest to examine whether Xylopia *aethiopica* influences haematological and biochemical indices. The present study was thus aimed at finding the effect of the

biochemical and haematological profiles of rat treated with ethanolic fruit extract of ***Xylopia*** *aethiopica* and it major constituent Xylopic acid.

6.2 MATERIALS AND METHODS

6.2.1 Animals

All experiments were performed with male Sprague Dawley rats weighing 150-200 g bought from Noguchi Memorial Institute for Medical Research, University of Ghana, Accra and kept at the Animal House Facility of the Department of Pharmacology, KNUST. The animals were allowed to acclimatize to the laboratory condition (temperature 24-27^{o}C and 12 hour light-dark cycle) for two weeks before commencement of the experiments. The rats were allowed free access to solid pellet diet (GAFCO, Tema) and water ad libitum throughout the study. All the animals were treated according to the National Institute of health Guidelines for the care and use of laboratory animals (NIH, department of Health and Human services publication no. 85-23, revised 1985).

6.2.2 Experimental design

In all forty eight rats were used for this study. Twenty four of the rats were divided into four equal groups of six rats each. Group I served as the control and the rats were given distilled water (vehicle for the extract). Groups II, III and IV rats were given 30, 100 and 300 mg kg−1 respectively, of *Xylopia aethiopica* fruit extract. In another experiment twenty four were also divided into four equal groups of six rats. Group 1 served as control and were given distilled water; groups 2, 3 and 4 were given 10, 30 and 100 mg kg−1 respectively of xylopic acid. The vehicle, extract and the xylopic acid were given daily (po) for 60 days. Twenty four hours after the last treatment the rats were put under light ether anaesthesia and blood samples taken by cardiac puncture into EDTA tubes for haematology parameters and plain tubes for the biochemical assays.

6.2.3 Analysis of Haematological variables

Various haematological parameters including white blood cell count (WBC), lymphocyte count (LYM), mid cell count (MID), granulocyte count (GRAN), red blood cell count (RBC), haemoglobin concentration (HGB), packed cell volume (PCV), mean cell volume (MCV), mean cell haemoglobin (MCH), mean cell haemoglobin concentration (MCHC), red cell distribution width (RDW), and platelet concentration (PLT) were determined in an automated blood analyser CELLDYN 1700®, version 1.08, (Abbott Diagnostics, Abbott Park, Illinois, USA). CELL-DYN haematology auto analyser relies primarily on flow cytometry to determine the WBC count and five-part differential count.

6.2.4 Biochemical assays

Serum biochemistry was performed on the ATAC 8000 Random Access Chemistry System (Elan Diagnostics, Smithfield, RI, USA). Parameters that were determined included: liver function tests - aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), glutamyltranspeptidase (GGT), lactate dehydrogenase (LDH), total bilirubin (T-BIL), direct bilirubin (DBIL), indirect bilirubin (I-BIL), total-protein (T-PROT), albumin globulin,; renal function tests -serum sodium (Na+), serum potassium (K+), blood urea nitrogen (BUN), serum creatinine, serum uric acid, a Anion gap. Also lipid profile which include total cholesterol (T-CHO), triglycerides (TAG), high density lipoprotein (HDL), very low density lipoprotein (VLDL), low density lipoprotein (LDL) and coronary risk were determined. All reagents were from JAS™ Diagnostics, Inc; Miami Florida, USA).

6.2.4.1Albumin (BCG)

At a controlled pH, bromocresol green (BCG) forms a coloured complex with albumin. The intensity of colour at 630 nm is directly proportional to albumin content. The instantaneous initial absorbance is obtained as suggested by Webster, (1977). The method used by the JAS™ albumin reagent is based on that of Doumas et al., (1971).

$$BCG + Albumin \rightarrow Green\ BCG/Albumin\ complex$$

6.2.4.2Bilirubin

Most methods currently used for assaying bilirubin are based on the reaction between bilirubin and diazotized sulphanilic acid solutions. In aqueous solution only the direct (conjugated) bilirubin will react in this manner. The JAS™ direct bilirubin reagent uses an acid diazo method. Conjugated bilirubin reacts with diazotized sulphanilic acid to produce an acid azobilirubin, the absorbance of which is proportional to the concentration of direct bilirubin in the sample and can be measured at 550 nm. The JAS™ total bilirubin method is based on a modification of the Pearlman and Lee, (1974) method in which a surfactant is used as a solubilizer. Sodium nitrite is added to sulphanilic acid to form diazotized sulphanilic acid. Bilirubin in the sample reacts with the diazotized sulphanilic acid to produce azobilirubin which absorbs strongly at 550 nm. The absorbance measured at 550 nm is directly proportional to the total Bilirubin concentration in the sample.

6.2.4.3Total Protein (Biuret)

The present method is based on the modification of Gornall et al., (1949). Protein in serum forms a blue coloured complex when reacted with cupric ions in an alkaline solution. The intensity of the violet colour is proportional to the amount of protein present when compared to a solution with known protein concentration.

$$Protein + Cu^{2+} \xrightarrow{Alkaline} Coloured\ Complex$$

6.2.4.4Gamma glutamyltransferase (GGT)

The JAS™ method is based on the kinetic photometric test, according to the International Federation of Clinical Chemistry and Laboratory Medicine (IFCC) (Shaw *et al.*, 1983). GT in the sample catalyses the transfer of the glutamyl group from L-Gamma-glutamyl-3-carboxyl-4-nitroanalide to glycylglycine according to the reaction below. The amount of 5-amino-2-nitrobenzoate

formed is proportional to GT activity and may be measured kinetically at 405 nm by the increasing intensity of the yellow colour formed.

$$L-\gamma-glutamyl-3-carboxyl-4-nitroanalide+glycylglycine \xrightarrow{Gamma\ -GT} l-y-glutamyl-glycylglycine+5-amino-2-nitrobenzoate$$

6.2.4.5 Aspartate aminotransferase (AST)

The present method is based on IFCC recommendations. AST catalyses the transfer of the amino group from L-aspartate to 2-oxoglutarate to yield oxaloacetate and L-glutamate. The oxaloacetate undergoes reduction with simultaneous oxidation of NADH to NAD+ in the malate dehydrogenase (MDH) catalyses reaction. The resulting rate of decrease in absorbance at 340 nm is directly proportional to the AST activity. Lactate dehydrogenase (LDH) is added to prevent interference from endogenous pyruvate which is normally present in serum.

$$L-aspatate+2-Oxoglutarate \xrightarrow{AST} Oxaloacetate+L-glutamate$$

$$Oxaloacetate+NADH \xrightarrow{MAH} l-Malate+NAD^{+}$$

6.2.4.6Alanine aminotransferase (ALT)

The procedure described herein is based on the method of Bergmeyer and Hørder,(1980). Alanine aminotransferase (ALT) catalyses the transfer of the amino group from L-alanine to 2-oxoglutarate to yield pyruvate and L-glutamate. Lactate dehydrogenase (LDH) catalyses the reduction of pyruvate and simultaneous oxidation of NADH to NAD+. The resulting rate of decrease in absorbance at 340 nm is directly proportional to the ALT activity. Endogenous sample pyruvate is rapidly and completely reduced by LDH during the initial incubation period so that it does not interfere with the assay.

$$L - Alanine + 2 - oxoglutarate \xrightarrow{ALT} Pyruvate + L - glutamate$$

$$Pyruvate + NADH \xrightarrow{LDH} L - Lactate + NAD^{+}$$

6.2.4.7Alkaline phosphatase (Alk.Phos.)

Alkaline phosphatase in serum is determined by measuring the rate of hydrolysis of various phosphate esters under specified conditions. p-Nitrophenyl Phosphate is one such phosphate ester and was introduced as a substrate by Fujita, (1969). Bessey, Lon and Brock published an endpoint procedure (Bessey *et al.*, 1946) while Bowers and McComb, (1966) reported a kinetic procedure. The JAS™ method is based on the kinetic photometric test, according to the IFCC. Absorbance is read at 405 nm.

$$p - nitrohenylphosphate + H_2O \xrightarrow{Alk.phos} Phosphate + p - nitrophenol$$

6.2.4.8Cholesterol

The present method utilizes a phenol substitute (4-aminoantipyrine (4-AAP)) that performs like phenol but without being corrosive. The intensity of the red colour produced is directly proportional to the total cholesterol in the sample when read at 500 nm.

$$\boldsymbol{Cholesterol\ Ester + Cholesterol\ esterase \rightarrow Cholesterol + Fatty\ Acid}$$

$$\boldsymbol{Cholesterol + O_2 \xrightarrow{Cholesterol\ oxidase} Cholest - 4 - en - one + H_2O}$$

6.2.4.9Triglycerides

The present method uses a modified Trinder (Trinder, 1969) colour reaction to produce a fast, linear, endpoint reaction (Fossati *et al.*, 1982). Triglycerides in the sample are hydrolyzed by lipase to glycerol and fatty acids. The glycerol is

then phosphorylated by ATP to glycerol-3-phosphate (G3P) and ADP in a reaction catalysed by glycerol kinase.G3P is then converted to dihydroxyacetone phosphate (DAP) and hydrogen peroxide by glycerophosphate oxidase (GPO). The hydrogen peroxide then reacts with 4-aminoantipyrine (4-AAP) and 3, 5-dichloro-2-hydroxybenzen (3,5-DHBS) in a reaction catalysed by peroxidase to yield a red coloured quinoneimine dye. The intensity of the colour produced is directly proportional to the concentration of triglycerides in the sample.

$$Triglyceride + H_2O \xrightarrow{Lipase} Glycerol + fatty\ aicd$$

$$Glycerol + ATP \xrightarrow{glycerol\ \ kinase} G3P + ADP$$

$$G3P + O_2 \xrightarrow{glycerop\ hosphate\ oxidase} DAP + H_2O_2$$

$$H_2O_2 + 4 - AAP + 3{,}5 - DHBS \xrightarrow{peroxidase} Quinoneimine\ dye\ (red\ dye) + 2H_2O$$

6.2.4.10 HDL-Cholesterol

The method employed herein is a two reagent format. The first reagent contains anti human B-lipoprotein antibody which bind to lipoproteins (LDL, VLDL and chylomicrons) other than HDL. The second reagent contains enzymes which then selectively react with the cholesterol present in the HDL particles. Consequently only HDL cholesterol is subject to cholesterol measurement. The primary reading was done at 600 nm and secondary at 700 nm.

6.2.4.11 Urea Nitrogen (BUN)

The present procedure is based on a modification of the method of Talke and Schubert, (1965). Urea is hydrolyzed in the presence of water and urease to produce ammonia and carbon dioxide. The liberated ammonia reacts with A-

ketoglutarate in the presence of NADH to yield glutamate. An equimolar quantity of NADH undergoes oxidation during the reaction catalysed by Glutamate dehydrogenase (GLDH) resulting in a decrease in absorbance (340 nm) that is directly proportional to the urea nitrogen concentration in the sample.

$$Urea + H_2O \xrightarrow[Urease]{} 2NH_3 + CO_2$$

$$NH_3 + \alpha - ketoglutarate + NADH \xrightarrow[GLDH]{} L - glutamate + NAD^+$$

6.2.4.12 Creatinine

This method is based on a modification of the kinetic procedure which is fast, simple and avoids interferences (Fabiny *et al.*, 1971), incorporating a surfactant and other ingredients to minimize protein and carbohydrate interferences. Creatinine reacts with picric acid in alkaline conditions to form a colour complex (yellow-orange) which absorbs at 510 nm. The rate of formation of colour is proportional to the creatinine in the sample.

$$Creatinine + Sodium\ picrate \xrightarrow[Alkali]{} Creatinine - picrate\ complex$$

6.2.4.13 Uric Acid

The JAS™ procedure uses uricase, peroxidase and the chromogen TBHB to yield a colorimetric end product. Uric acid is oxidized by Uricase to allantoin and hydrogen peroxide. TBHB + 4-aminoantipyrine + hydrogen peroxide, in the presence of peroxidase, produce a quinoneimine dye that is measured at 520 nm. The colour intensity at 520 nm is proportional to the concentration of Uric Acid in the sample.

6.3 RESULTS

6.3.1 Haematological parameters in the extract treated rats

From the results in table 6.1, there was a significant increased in total white blood cell count ($F_{3, 12} = 4.755$, P = 0.0208) and differential neutrophil count (F 3,11,= 6.880, P = 0.0071) in the extract treated group compared to the control group using one way ANOVA. There was also a significant increase in the haemoglobin concentration in the treated rats compared to the controls (F3, 13 = 4.559, P = 0.0216). There was however no significant change in red blood cell count, mean cell volume and haematocrit between the treated animals and the control. There was no significant change in red cell distribution width, MCH and MCHC when the extract treated rats were compared to the respective controls (Table 6.1).

6.3.2 Serum biochemical indices in the extract treated group

The mean serum total protein ($F_{3, 16} = 9.800$, $P = 0.0007$), albumin ($F_{3, 16} = 7.060$, $P = 0.0031$) and globulin ($F_{3, 16} = 10.87$, $P = 0.0004$) were significantly increased in the *Xylopia aethiopica* extract treated rats compared with the control group (Table 6.2). There was also a dose-dependent significant increase in the mean concentration of indirect bilirubin ($F_{3, 15} = 13.03$, $P = 0.0002$) and total bilirubin ($F_{3, 15} = 11.76$, $P = 0.0003$) in the treatment groups compared to the control (Table 6.2). However, there was no significant change in the mean concentrations of serum direct bilirubin, creatinine, urea and uric acid in rats treated with X.A. extract and the control group (Table 6.2).

Table 6.1: Haematological parameters of animals treated for 60 days with X.A extract

PARAMETER	CONTROL	30 mg	100 mg	300 mg
WBC ($x10^9$L)	4.45 ± 0.54	5.35 ± 0.44	7 ± 0.46*	6.34 ± 0.63
RBC ($x10^{12}$/L)	7.30 ± 0.211	7.57 ± 0.523	7.41 ± 0.18	7.79 ± 0.11
Hb (g/dL)	13.20± 0.39	13.07± 0.65	14.40± 0.27	14.68 ± 0.31*
HCT (%)	45.54 ± 1.45	46.7 ± 3.47	45.04 ± 1.56	47.96 ± 0.98
MCV (fl)	62.4 ± 0.65	61.62 ± 0.50	60.72 ± 0.85	61.58 ± 0.59
MCH (pg)	18.78 ± 0.50	19.2 ± 0.16	18.9 ± 0.23	18.86 ± 0.38
MCHC (g/dL)	30.08 ± 0.91	31.22 ± 0.39	31.1 ± 0.30	30.6 ± 0.48
PLT ($x10^3$/µL)	985.3 ± 108	666.3 ± 80.99	664.7 ± 131.4	605 ± 63.04
LYM (%)	80.62 ± 3.13	80.8 ± 5.68	75.98 ± 2.48	78.48 ±2.84
NEUT (%)	14.77 ± 1.59	13.53 ± 0.39	18.68 ± 2.6	24 ± 1.80**
RDW-CV (%)	12.44 ±0.31	12.4 ± 0.15	12.48 ± 0.33	11.92 ± 0.16

Data are express as mean ± SEM, ***P* ≤ 0.05, ****P* ≤ 0.001 compared to respective controls (one-way ANOVA followed by Newman-Keuls*post hoc*)

6.3.3 Serum lipid profile

Rats treated with X.A extract showed a significant increase ($F_{3, 14}$ = 7.063, p = 0.0040) in their serum total cholesterol level compared to the control group. There was also a dose dependent and significant increase ($F_{3, 16}$ = 8.422, P = 0.0014) in serum HDL levels in the treated group compared to the control group. However there was no significant difference in the mean concentrations of serum LDL, triglycerides and the coronary risk ratio between the treated group and the controls (Table 6.3)

6.3.4 Serum enzymes

From the one-way analysis of variance; treatment of rats with the extract of Xylopia *aethiopica* induce a significant decrease ($F_{3, 13}$ = 4.617, P = 0.0207) in the activity of serum ALT compared to the control group (Table 2). However, the extract did not cause any significant changes in levels of serum AST, alkaline phosphatase (ALK. Phos) and **γ** glutamyltranferase (Table 6.2).

Table 6.2: Serum liver and renal function test following 60 days of exposure to XA.extract

PARAMETER	CONTROL	30 mg	100 mg	300 mg
Albumin (g/L)	38.54 ± 0.96	39.24 ± 1.24	39.98 ± 0.83	44.94 ± 1.27**
Globulin (g/L)	30.02 ± 1.04	34.04 ± 1.28**	26.84 ± 0.99	33.94 ± 0.84
T. Protein (g/L)	68.54 ± 1.84	73.26 ± 1.27	66.84 ± 1.73	78.88 ± 1.98**
ALK. Phos (U/L)	700.4 ± 52.77	964.8 ± 206.1	562.4 ± 33.87	709.2 ± 78.88
ALT (U/L)	148.3 ± 8.83	128 ± 7.11	118.4 ± 5.63	114.9 ± 4.11*
AST (U/L)	3.45 ± 0.85	3.2 ± 2.0	3.3 ± 1.40	0.95 ± 0.56
GGT (U/L)	0.02 ± 0.02	0.44 ± 0.25	0.74 ± 0.45	11.68 ± 7.45
DBIL (µmol/L)	1.08 ± 0.04	1.04 ± 0.05	0.98 ± 0.06	1.05 ± 0.05
INDBIL (µmol/L)	0.344 ± 0.10	0.58 ± 0.04	0.91 ± 0.08*	1.27 ± 0.20**
TBIL (µmol/L)	1.44 ± 0.09	1.66 ± 0.07	1.88 ± 0.10	2.33 ± 0.17***
Creatinine (µmol/L)	53.54 ± 4.20	56.6 ± 2.99	55.96 ± 1.50	62.76 ± 4.38
UREA (mmol/L)	7.48 ± 0.36	6.58 ± 0.90	6.65 ± 0.61	7.46 ± 0.65
URIC acid (mmol/L)	64.98 ± 3.53	58.8 ± 9.51	51.5 ± 4.66	52.7 ± 5.36

Results are expressed as mean ± SEM, ***P* ≤ 0.05, ** *P*≤ 0.01, ****P* ≤ 0.001 compared to respective controls (one-way ANOVA followed by Newman-Keuls*post hoc*)

Table 6.3: Lipid profile (mean ± SEM) following 60 days of exposure to X.A. extract

Parameter	Control	30 mg	100 mg	300 mg
Cholesterol(mmol/L)	2.06 ± 0.14	2.47 ± 0.05*	2.1 ± 0.08	2.39 ± 0.01
Triglyceride(mmol/L)	0.95 ± 0.09	0.77 ± 0.09	0.83 ± 0.03	0.86 ± 0.11
HDL (mmol/L)	2.59 ± 0.13	3.11 ± 0.04**	3.14 ± 0.10**	3.05 ± 0.06*
LDL (mmol/L)	0.84 ± 0.07	0.99 ± 0.05	0.90 ± 0.04	0.99 ± 0.06
Coronary risk (ratio)	1.16 ± 0.02	1.10 ± 0.02	1.12 ± 0.01	1.08 ± 0.03

Results are expressed as mean ± SEM, ***P* ≤ 0.05, ** *P*≤ 0.01, ****P* ≤ 0.001 compared to respective controls (one-way ANOVA followed by Newman-Keuls*post hoc*)

6.4 EFFECT OF XYLOPIC ACID ON BLOOD AND BIOCHEMICAL PARAMETERS

As shown in Table 6.4 administration of X.A to the rats for 60 days did not have any significant effect on haematological parameters except the total white blood cell count and haemoglobin levels which significantly increased in the 30 and 100 mg/kg group ($P < 0.05$) compared to the control group. There was an increase in blood platelet level dose-dependently although not statistically significant. There was however no significant change in red blood cell count, mean cell volume and haematocrit between the treated animals and the control. There were also no significant change were seen in red cell distribution width, MCH and MCHC when the extract treated rats were compared to the respective controls.

Table 6.4 Haematological parameters of animals treated for 60 days with *Xylopic Acid*

Parameter	Control	10 mg/kg	30 mg/kg	100 mg/kg
WBC ($x10^9$/L)	5.08 ± 2.40	6.20 ± 1.93	8.90 ± 0.84*	9.35 ± 0.52*
RBC ($x10^{12}$/L)	7.88 ± 0.74	7.72 ± 0.30	7.86 ± 0.58	7.893 ± 0.25
Hb g/dL	13.43 ± 0.93	14.43 ± 0.57	15.10 ± 0.61*	15.40 ± 0.29**
HCT (%)	50.90 ± 3.55	50.65 ± 1.27	50.48 ± 2.40	52.68 ± 0.74
MCV (fl)	63.48 ± 1.70	65.60 ± 1.29	64.33 ± 2.24	66.78 ± 1.14
MCH (pg)	17.95 ± 1.05	18.68 ± 0.26	19.28 ± 0.87	19.50 ± 0.24
MCHC (g/dL)	28.30 ± 1.82	28.48 ± 0.74	29.93 ± 0.34	29.23 ± 0.21
PLT (($x10^3$/µL)	582.50 ± 54.27	585 ± 72.97	655.8 ± 103.8	709.80 ± 54.19
LYM (%)	71.13 ± 8.55	77.53 ± 3.04	72.85 ± 14.84	71.95 ± 5.89
NEUT (%)	24.78 ± 3.79	24.38 ± 1.23	27.35 ± 2.11	30.13 ± 3.88

Data are express as mean ± SD, ***P* ≤ 0.05, ****P* ≤ 0.01 compared to respective controls (one-way ANOVA followed by Newman-Keuls*post hoc*)**

Serum total protein and globulin as shown in Table 6.5 significantly decrease in rats treated with X.A only at the highest dose (100 mg/kg) levels (P< 0.05) compared to the control group which received only distilled water. Serum AST were significantly high in rats that received X.A at doses of 30 mg/kg and 100 mg/kg (P<0.001) compare to the AST level in the control rats. Serum urea level in the treated group was also significantly decreased at the 30 and 100 mg/kg dose levels. Serum albumin, ALT, ALkPhos, total bilirubin, direct bilirubin and indirect bilirubin as well as creatinine and uric acid were affected by the xylopic acid administration as shown in Table 6.5.

Table 6.5: Serum liver and renal function test following 60 days of exposure to *Xylopic Acid*

PARAMETER	CONTROL	30 mg	100 mg	300 mg
Albumin (g/L)	17.77 ± 5.06	15.68 ± 3.13	16.45 ± 2.62	20.50 ± 4.79
Globulin (g/L)	7.74 ± 1.77	8.82 ± 2.98	8.118 ± 2.83	3.17± 1.75*
T. Protein (g/L)	25.61 ± 9.81	22.87 ± 6.72	18.22 ± 6.38	11.73 ± 2.96*
ALK. Phos (U/L)	198.50± 80.25	228.4± 38.57	169.10 ± 156.1	149.70 ± 99.19
ALT (U/L)	80.74± 16.22	75.80 ± 24.97	48.09 ± 27.85	57.44 ± 29.54
AST (U/L)	85.21 ± 27.12	61.36 ± 31.38	29.03 ± 5.23***	26.55 ± 3.2***
DBIL (µmol/L)	0.85 ± 0.24	0.92 ± 0.39	0.60 ± 0.32	0.49 ± 0.49
INDBIL (µmol/L)	0.60 ± 0.12	0.55 ± 0.19	0.52 ± 0.13	0.48 ± 0.25
TBIL (µmol/L)	1.29 ± 0.36	1.40 ± 0.64	1.17 ± 0.80	0.93 ± 0.74
Creatinine (µmol/L)	37.23 ± 9.33	32.07 ± 11.19	28.83 ± 14.50	27.47 ± 14.21
UREA (mmol/L)	7.91 ± 0.98	6.88 ± 0.73	5.77 ± 1.75*	4.91 ± 1.60*
URIC acid (mmol/L)	41.50 ± 11.76	34.00 ± 4.06	32.40 ± 3.58	27.40 ± 3.58

Data are express as mean ± SD, ***P≤ , 0.05, ****P* ≤ 0.01, , *****P* ≤ 0.001 compared to respective controls (one-way ANOVA followed by Newman-Keuls*post hoc*)**

The results as shown in Table 6.6 indicates that xylopic acid administration caused a significant reduction in total cholesterol levels in rats in the 30 and 100 mg/kg dose (P<0.01 and P<0.001) levels respectively while significantly increasing HDL cholesterol level in this same group of rats compare to the respective controls. The serum triglyceride and LDL cholesterol levels did not change significantly compare to the control animals.

Table 6.6: Lipid profile (mean ± SD) following 60 days of exposure to *Xylopic Acid*

Parameter	Control	30 mg	100 mg	300 mg
Cholesterol (mmol/L)	0.55 ± 0.2	0.58 ± 0.05	0.27 ± 0.13**	0.19 ± 0.07***
Triglyceride (mmol/L)	0.27 ± 0.07	0.24 ± 0.05	0.26 ± 0.10	0.24 ± 0.11
HDL (mmol/L)	0.38 ± 0.11	0.39 ± 0.11	0.54 ± 0.12*	0.56 ± 0.12*
LDL (mmol/L)	0.07 ± 0.07	0.07 ± 0.03	0.06 ± 0.04	0.04 ± 0.02
Coronary risk (ratio)	2.23 ± 0.46	2.26 ± 0.28	2.36 ± 0.39	2.12 ± 0.38

Results are expressed as mean ± SD, ***P* ≤ 0.05, ** *P* ≤ 0.01, *****P* ≤ 0.001 compared to respective controls (one-way ANOVA followed by Newman-Keuls*post hoc*)**

6.5 DISCUSSION

The treatment of rats with ethanolic fruit extract of *X. aethiopica* for 60 days significantly increased the body weight of the animals in a dose-dependent manner. This increased in body weight is an indication that the extract did not interfere with growth but rather promoted growth possibly by increasing the synthesis of proteins as shown in the increased in the serum protein level. Extract of *X. aethiopica* has shown to exhibit androgenic properties (Woode *et al.*, 2011a) and androgens are known to possess anabolic properties (Choudhary *et al.*, 1975) and this could therefore be responsible for the significant increase in body weight of the animals in the present study.

The extract also caused a significant increased in the level of Hb, WBC and neutrophil count in the treated animals. The increase in these parameters could be as a result of a direct effect of the extract on haemopoiesis in these animals. According to Eteng *et al*., (1999), alkaloids, which is one of the phytochemical components of X. *aethiopica*, is known to cause similar effect by inhibiting phosphodiesterase leading to the accumulation of cAMP which in turn stimulates protein synthesis. Locally in Ghana, decoction of dry fruits of *X. aethiopica* is taken as a postpartum tonic as well as an immune booster in individuals with low immunity (Burkill, 1985a). Neutrophils are the main type of White blood cells that is responsible for fighting infectious agents by phagocytosis. The findings of the present study is in conformity with the work of Taiwo et al., (2009) , who also found that aqueous extract of *Xylopia aethiopica* was able to significantly increase the levels of Hb, PCV,WBC and neutrophil in rats. These findings thus validate the traditional use of the extract as a tonic and immune booster.

The extract had also induced a significant increased in total protein, albumin and globulin in the treated animals compared to the control group. The significant increase in the level of these parameters is an indication that the extract stimulated their synthesis in the liver. Serum protein levels are regulated via synthesis in the liver and its levels thus reflect the synthetic

ability of the liver (Okokon *et al.*, 2010). The extract also causes a significant reduction in the level of serum ALT but did not affect the levels of AST, GGT and alkaline phosphatase. An increase in the enzymatic activity of ALT, AST and ALP in the serum may reflects hepatocellular damage (Benjamin *et al.*, 1978). The results of the enzyme analysis therefore suggest that extract of *X. aethiopica* has no hepatotoxicity at the doses used in this study. This finding is similar to the result of Taiwo et al., (2009). There were no significant changes in the serum total and direct bilirubin levels. However there was a significant increase in the serum levels of indirect bilirubin in the treated rats compared to the control animals. An increase in indirect serum bilirubin (unconjugated bilirubin) could be due to increased intravascular breakdown of red cell or decreased uptake by hepatocytes. Increase in the serum unconjugated bilirubin in this study may arise from intravascular haemolysis rather than liver injuries since the liver enzymes, total protein, albumin and total cholesterol levels were not affected. Serum urea and creatinine levels are an indication of kidney function both in man and in rodents (Jesse, 1982). The levels of urea and creatinine in the treated rats did not show any significant difference with respect to the control values, an indication that fruit extract of *X. aethiopica* is not nephrotoxic at the dose levels used in this study.

Xylopic acid administration caused a similar effect on total white blood cells and haemoglobin levels in the treated rats as the ethanolic extract by increasing their levels significantly, although the effect was more pronounced in the extract treated group. The acid unlike the extract did not affect the differential white count. With respect to serum biochemical parameters like total protein, albumin and globulin, whereas the extract significantly increased these parameters, the acid significantly caused a reduction in total protein and globulin in the treated rats. Xylopic acid also caused an increased in serum AST level which may be a marker of liver damage. According to other researchers, xylopic acid exhibits cytotoxic activity both in animal models and *in vitro* cell lines. These cytotoxic activities of the acid could be responsible for the results

obtained in the present study. Consistent with this finding, an increased level of p21 and p53 gene transcripts was also observed in XA treated cells by Adaramoye et al., (2011b). p21 is known to play a critical role in cell cycle progression (Harper *et al.*, 1993). It has been reported that cell growth arrest at G1-, G2-or S- phase is associated with an increased level of p21 expression (Radhakrishnan *et al.*, 2004). It is known that p21 may arrest cell division by interfering with cyclin/cdk2 activity (Harper *et al.*, 1993). In addition, p21 can block DNA synthesis by binding to proliferating nuclear antigen (Waga *et al.*, 1994). On the other hand, p53 is the principal tumor suppressor protein which is elevated in cells in response to stress signals.Volatile oil from the fruits of X. aethiopica has also been found to be cytotoxic to HepG-2 carcinoma cells (Asekun *et al.*, 2004).

Xylopic acid treatment decreases serum total cholesterol while increasing HDL cholesterol. This significant finding is similar to the activity of the extract which also decreased both total cholesterol and LDL cholesterol as reported by Woode et al., 2011. LDL molecules are the major transporters of cholesterol in the bloodstream and are considered ''bad cholesterol'' because they carry fats out of the liver to the blood vessels and seem to encourage the deposition of cholesterol in the arteries. The significant decreased in LDL-C, total cholesterol, and triacylglyceride, which in essence increases HDL-C levels, points to this plant as a potential hypolipidemic agent.

Treatment of rats with extract of *X. aethiopica* in the present study resulted in a significant increased in total cholesterol and HDL-cholesterol with a concomitant decreased in coronary risk ratio. Serum lipid levels have been shown to be a strong risk factor for coronary heart diseases (Edem, 2002). Other workers have also shown strong correlations between increased plasma total cholesterol, low density lipoprotein cholesterol and increased incidence of coronary heart disease (Edionwe *et al.*, 2001; Kamisako *et al.*, 2005). The

significant increased in total cholesterol observed in this study is probably due to the significant increased in high density lipoprotein (HDL) since there was no corresponding significant increased in the low density lipoproteins (LDL). According to Eder and Gidez (1982), an increased in HDL can solely be responsible for the observed increased in total cholesterol since the extract might be affecting the metabolism of HDL in the liver. This is an indication that the extract does not pose any cardiovascular risk since it only increased HDL (good cholesterol) without affecting the "bad cholesterol" (LDL), but rather can be exploited in the management of certain diseased conditions. This study is in conformity with the work of Sarah *et al.,* (2011) who also found that extract of *X. aethiopica* possess therapeutic potential in preventing the development of atherosclerosis and cardiovascular disorders by exhibiting hypocholesteroleamic effect in experimental animals.

6.6 CONCLUSION

In conclusion, the crude extract of *Xylopia aethiopica* showed haematopoietic activity by increasing formed element in blood (WBC, Hb and RBCs) in rats which could be as a result of the high iron content in the extract. It also has the ability to boost immunity and growth by increasing the plasma protein and albumin levels while possessing the antiatherogenic activity by increasing HDL (good cholesterol) and decreasing bad cholesterol (LDL), thus reducing the risk of coronary artery diseases. However, xylopic acid whiles increasing haemoglobin level and HDL-cholesterol in the treated rats, may be toxic to the liver since treatment had increased the cytosolic liver enzymes (AST). This study therefore pharmacologically authenticates the used of the crude extract in treating diseases in Ghana. The study also underscores the point that when extracts are taken in the pure form as in the case of xylopic acid it may exhibits toxicity.

Chapter 7

CONCLUSION AND RECOMMENDATION

7.1 CONCLUSIONS

The results of the present study have provided overwhelming evidence to conclude that extract of *Xylopia aethiopica* possesses immune boosting properties and that the effect could be due in part to increase haemopoietic activity and increase protein as well as immunoglobulin synthesis.

The study also provided evidence to show that the extract possesses spermatogenic activity which is mediated through stimulation of gonadotropin probably by hypothalamus activation thus increasing fertility in the treated animals. Testicular histology as well as sperm characteristics was not affected by the extract treatment.

The major component of the fruit of *Xylopia aethiopica,* Xylopic acid unlike the extract however exhibited antifertility activity possibly due to the direct effect of the acid on the spermatozoa causing oxidative stress and thus effective sperm motility and viability and hence the fertility of the animals. The antifertility of xylopic acid could also be due to the acid behaving as an anti-androgen there by blocking the effect of endogenous androgens on androgen dependent accessory sex organ as evident from the result of the present study.It is thus clear from this study that the use of this plant in the crude form may be more beneficial and safer than the purified compounds from fruits of *Xylopia aethiopica* with respect to the reproductive system of animals and possibly man.

7.2 RECOMMENDATIONS

- Effect of the extract on bone marrow and erythropoietin should be investigated

- The effect of the extract and its major constituent on female fertility should be investigated
- Effects of the minor constituents of the fruit extract on male fertility needs to be carried out.

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