EVALUATIONOF THE GONADOPROTECTIVE EFFECT OF *Allanblackia floribunda*Oliver (Clusiacea) ON TESTES AND ACCESSORY ORGANS OF WISTAR RATS

OLASANTAN OLASIJI DANIEL

B.Sc. (Hons.) (Abeokuta)

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ABSTRACT

This study investigated the effect of the crude ethanolic extract and fractions of the stem bark of *Allanblackia floribunda*on the testes and accessory organs of rats. This was with a view to ascertaining the direct effects of the plant on the biochemistry and physiology of the reproductive organs of humans.

Fresh stem barks of *A. floribunda* were collected, identified, dried at room temperature for three weeks, and thenground into fine powder. Five hundred grammes (500 g) of the powdered stem bark was soaked in 2.5 l of 70% ethanol for 72 h, filtered and concentrated to dryness under reduced pressure at 40^{o}C to obtain the crude ethanolic extract.Fifty grammes (50 g) of the crude extract was later partitioned to obtain the aqueous, ethyl acetate and butanol fractions of the extract. Forty five (45)male rats of average weight between 150-200 g were distributed into nine groups of five rats per group. Rats in group 1 (control group) were administered distilled water, while groups 2 to 5 were orally administered 200 mg/kg body weight of the aqueous, ethyl acetate, butanol fractions and crude extract respectively. Also, 300 mg/kg body weight of the aqueous, ethyl acetate, butanol fractions and crude extract were administered to groups 6 to 9 respectively. On the 29th day, the animals were sacrificed; testes, epididymis, seminal vesicle and prostate glands were weighed.Catalase and glutathione peroxidaseactivities were determined in the testes. Serum acid phosphatase,alkaline phosphatase, lactate dehydrogenase and glucose-6-phosphate dehydrogenase activities were then determined using standard procedures.

The results showed that crude extract of *A. floribunda*contained alkaloids, flavonoids, saponins, tanins, triterpenes and cardiac glycosides.The median lethal dose (LD_{50}) of the crude extract was higher than 5000mg/kg body weight. All groupshad lower seminal vesicle, testicular and epididymis weights when compared with the control.Furthermore, testicular catalase (CAT)across the groups (0.28$\pm$0.05, 0.25$\pm$0.04, 0.53$\pm$0.21, 0.21$\pm$0.05, 0.29$\pm$0.06,

0.40±0.10, 0.27±0.07, 0.34±0.09) U/L and also testicular glutathione peroxidase (GPx): (0.19±0.01, 0.15±0.01, 0.18±0.01, 0.21±0.02, 0.17 ±0.01, 0.16±0.01, 0.15±0.01) U/L/mg protein were reduced when compared with the control [0.62±0.36 U/L (CAT); 0.21±0.02 U/L/mg protein (GPx)] respectively. The prostatic acid phosphatase (ACP) activities in groups 2, 3, and 9 (10.45±1.96U/L, 7.33±0.10U/L, 18.89±1.41U/L) were significantly increased when compared with the control (2.05±0.86 U/L). However, the testicular alkaline phosphatase (ALP) activities in groups 6 and 7 (230.18±18.89 U/L, 222.46±14.46 U/L); and testicular glucose-6-phosphate dehydrogenase (G6PDH) activities in groups 5, 6, 7, 8 and 9:(72.91±16.52U/L, 73.19±5.39U/L, 82.28±13.63U/L, 90.86±15.78U/L, 70.67±9.62U/L) were significantly reduced when compared with the control [362.66±21.53 U/L (ALP); 179.47±19.69U/L (G6PDH)].

The study concluded that the ethanolic extract and fractions of *A. floribunda*had deleterious effects on the testes and accessory organs of the animals, most especially at 300 mg/kg dosage of the crude ethanolic extract and therefore could not be used as an aphrodisiac.

Keywords : ethanolic extract, fractions of the stem, biochemistry and physiology.

Supervisor: Dr. O. O. Babalola

xvii, 108p

CHAPTER ONE

1.0 Introduction

1.1 Plants as Medicinal Agents in Herbal Medicine

Globally, plants are widely used for the production of medicinal agents. This usage is commonly referred to as traditional or herbal medicine and such plants used for their curative purposes are called herbs. Herbal medicines encapsulates the synthesis of therapeutic experiences of generations of practicing physicians of indigenous systems of medicine for over hundreds of years (Pal and Shukla, 2003) and involves the use of plants for the promotion of healing and maintenance of health (Hussin, 2001). It is said that the use of herbal medicines originated in Egypt back in 1550 B.C., yet many of their pharmacological effects remain poorly understood. Out of the estimated 800,000 plant species on earth (von Reis, 1977); about a quarter have been categorized and only a small fraction of these have been examined for pharmacological efficacy (Hussin, 2001).

1.2 Definition of Herbs

Herbs are categorized and defined differently by several disciplines. Culinary professionals think of herbs as vegetable products that only add flavour or aroma to food. Botanists restrict the meaning of herbs to non-woody, seed-producing plants that grow, then die, during repeating growing seasons. Medically, herbs are most accurately defined as crude drugs of vegetable origin utilized for the treatment of disease states, often of a chronic nature, or to attain or maintain a condition of improved health (Bastin, 1999). An herb, according to Hussin (2001) is defined as a plant or plant part used for its aromatic, savoury, medicinal or cosmetic properties. Generally, the whole plant or plant parts are used singly or in combination with more than one plant for the purpose of treatment.

Herbs and plants can be processed and can be taken in different ways and forms, and they include the whole herb, teas, syrup, essential oils, ointments, salves, rubs, capsules, and tablets that contain a ground or powdered form of a raw herb or its dried extract. Plants and herbs extract vary in the solvent used for the extraction, temperature, and extraction time, and

include alcoholic extracts (tinctures), vinegars (acetic acid extracts), hot water extract (tisanes), long-term boiled extract, usually roots or bark (decoctions), and cold infusion of plants (macerates) (Wachtel-Galor and Benzie, 2011).

The trend in our present day time is that many people have turned away from conventional medicines, with the belief that 'natural' substances like herbs are safer than synthetic substances. Vinod (2002) stated explicitly that the growing interest in herbs is based on the belief that the plants have a vast potential for use as a curative medicine and the widely acclaimed notion by herbal practitioners that "natural" equal 'harmless' and 'synthetic' equal 'toxic' and 'dangerous'. As such, herbal medicines are currently in demand and their popularity is increasing day by day (Verma and Singh, 2008) and there is little doubt that the use of herbal medicines is growing globally. In fact, the trend in herbal usage has been that of a progressive one in so much that it has been estimated that 80% of the world population use some form of herbal medicine (WHO, 2008). Worldwide, statistics have shown that the usage of herbal medicine increases at a rate of 10-20% annually and in Africa up to 90% and in India 70% of the population depend on traditional medicine to help meet their healthcare needs (WHO, 2008).

The most common reasons for using traditional medicine are that it is more affordable, more closely corresponds to the patient's ideology, allays concerns about the adverse effects of chemical (synthetic) medicines, satisfies a desire for more personalized healthcare, and allows greater public access to health information. The major use of herbal medicines is for health promotion and therapy for chronic, as opposed to life-threatening, conditions. However, usage of traditional remedies increases when conventional medicine is ineffective in the treatment of disease, such as in advanced cancer and in the face of new infectious diseases. (Canter and Ernst 2004; Qato *et al.* 2008; Loya *et al*, 2009; Cohen and Ernst 2010). Hence, regardless of why an individual uses herbs, it provides an important

healthcare service whether people have physical or financial access to allopathic medicine, and it is a flourishing global commercial enterprise (Engebretson 2002; Evans *et al.*, 2007).

Furthermore, herbal medicines have been shown to have good values in treating many diseases including infectious diseases, hypertension, etc. (Erah, 2002). In recent times, herbs are applied to the treatment of chronic and acute conditions and various ailments and problems such as cardiovascular disease, prostate problems, depression, inflammation, and to boost the immune system, to name but a few (Wachtel-Galor and Benzie, 2011). That they can save lives of many, particularly in the developing countries, is undisputable.

However, the major challenges of any pharmaceutical scientist are serious problems with the overall quality, safety and efficacy of herbal products. Unfortunately, most countries do not have regulatory policies that can effectively protect their citizens from the identified problems. Preservation and dosage measurement are serious problems in developing countries and that has really necessitated the continual and holistic studies being carried out by scientists in Asia (China, India) and Africa (Nigeria) especially in ascertaining the safety levels of these commonly used herbs in order to establish their potency or lethality upon consumption (Erah, 2002).

1.3 Study Plant

Allanblackia is a family of medium-sized tree species of humid forest zone of Africa producing berry-like fruits that are suspended on long pedicels, and the seeds of the genus have a very low germination success {less than 5%} (Vivien and Faure, 1996). *Allanblackia floribunda* Oliver or tallow-tree, is one out of nine identified species of the genus (Fobane *et al.*, 2014) and it is a fruit tree of Clusiaceae family or Guttiferae.

Allanblackia floribunda is found in the rain forest of South and Equatorial forest of East part of Nigeria areas, to the Central African Republic and East of the Democratic Republic of Congo, and South to Northern Angola (Orwa and Munjuga, 2007). The generic

name is after Allan Black, a 19th century Kew botanist. The specific name 'floribunda' describes the abundant flowering in this species, making showy displays (Orwa *et al.*, 2009).

1.3.1 Description

Allanblackia floribunda is an evergreen forest tree confined to tropical Africa, which grows up to 30 m tall. As shown in Figure 1.1 and 1.2, its stem bark are dark brown, patchy; slash thin, reddish at the surface, yellow beneath, exuding a sticky yellow juice. The branches are slender, drooping and often conspicuously whorled, numerous, horizontal, hollow, with longitudinal grooves, brownish black, glabrous (Fobane *et al.*, 2014).

It is evergreen, dioecious, bole fairly short, straight, cylindrical, without buttresses but sometimes basally thickened; the bark surface is reddish brown to blackish, with small irregular scales, inner bark granular, reddish or brown, exuding a little clear sap. The fruit is a large ellipsoid berry, with 5 longitudinal ridges, 40–80-seeded. The seeds are ovoid and enclosed in a pinkish aril embryo, embedded in oily endosperm. The seedlings emerge after six to eighteen months of planting with hypogeal germination (Orwa and Munjuga, 2007).

Under natural conditions, trees start flowering after about twelve years. Flowering occurs during a large part of the year, in particular from January to September. Fruits take nearly a year to mature and ripe fruits are also found during a large part of the year. The fruits are eaten by wild pigs and porcupines, which may disperse the seeds (Orwa and Munjuga, 2007).

Plate 1.1a: Fruits of ***Allanblackia floribunda***

Plate 1.1b: *Allanblackia floribunda* Stem Bark and Tree

1.3.3 Scientific Classification

Allanblackia floribunda

Scientific classification

Kingdom: Plantae

(unranked): Angiosperms

(unranked): Eudicots

(unranked): Rosids

Order: Malpighiales

Family: Clusiaceae

Genus: *Allanblackia*

Species: *floribunda*

Binomial name

Allanblackia floribunda Oliver.

1.3.4 Common Names

ENGLISH: Tallow-tree, mkanyi fat, kagne butter);

ITALIAN: Ouotera, bouandjo;

SWAHILI: Mkimbo;

CAMEROON: nsangomo (Ewondo), or matatolo (Douala).

NIGERIA: **EDO** ízéni = elephant rice (auctt.) izọkhain = grass-cutter rice

EFIK: ẹ́díáng cf. Treculia behavior, Moraceae (auctt.)

IGBO: egba, Awarra, ọcha, ala-enyī = elephant's breast

YORUBA: usonige, orogbo, egba, orógbó erin = elephant's bitter kola.

1.3.5 Ethnobotanical Uses

Allanblackia floribunda has been shown to display a wide spectrum of biological and pharmacological activities, which provide experimental support for the empirical ethno-pharmacological use of this plant in traditional medicine (Dieudonné *et al.*, 2013).

The seeds are full of enormous nutritional and therapeutic potentials: they are consumed by humans in times of starvation and by rodents (Anonyme, 2004). The fat obtained from the seed, known as 'allanblackia fat' or 'beurre de bouandjo' in Congo, is used in food preparation (Orwa and Munjuga, 2007) and also used to produce butter which contributes less cholesterol to the blood and limit the risk of cardiovascular diseases; the fatty acids inherent in their seeds are popular in cosmetics and food (Foma and Abdala, 1985; Bonanome and Grundy, 1988). The tree's fruit is edible and its seeds are a source of edible oil long used by local populations. Even, the international food industry has become interested in the fruit and its fat as a natural solid component for margarines and similar products because the fruit's slimy pulp can also be made into jams and jellies (Orwa and Munjuga, 2007).

The decoction of the leaves and fruits have also been reported for use in the treatment of malaria and toothache (Burkill, 1984; Odugbemi, 2006). The traditional uses indicate possible anti-inflammatory and antimicrobial activity (Ayoola *et al.*, 2009). In Akwa Ibom State of Nigeria, the leaves and the bark of the stem and root of the plant are used by the local communities to treat dysentery, diarrhea, skin diseases and some other microbial diseases (Ajibesin *et al.*, 2008). A decoction of the whole fruit is used in Ivory Coast to relieve scrotal elephantiasis. In Nigeria and Ghana, it is used to relieve tooth ache and rheumatism. All parts of the plant are used traditionally in the treatment of smallpox, chicken pox and measles indicating possible antiviral activity. The fatty substance of the seeds is mildly purgative (Ayoola *et al.*, 2009).

The bark is used in Cameroon for its therapeutic properties in the treatment of cough, dysentery, diarrhea and toothache (Laird, 1996) and as an aphrodisiac and pain reliever (Fobane *et al.*, 2014). In Gabon, a decoction is taken to treat dysentery and as a mouthwash for toothache (Raponda-Walter and Sillans, 1961). In Congo (Brazzaville), a decoction of the bark or the leaves is taken to treat stomach-pains, cough, asthma, toothache, bronchitis and other bronchial infections (Van der Vossen and Mkamilo, 2007; Bilanda *et al.*, 2010). The bark decoction of the stem and root is also used in Central African Republic and West Africa to treat toothache, dysentery and as an analgesic (Lewis and Elvin-Lewis, 1977). Bilanda *et al.* (2010) reported that information provided by practitioners of traditional medicine in central Cameroon also suggest that *Allanblackia floribunda* possesses useful aphrodisiac and antihypertensive properties. Consequently, it is one of the plants frequently used for the treatment of hypertension as well as sexual disorders and male fertility in acute problems (Bilanda *et al.*, 2010; Kada *et al.*, 2012).

The wood is locally used, but is of secondary importance (Orwa and Munjuga, 2007). The wood is used in Nigeria in construction of local houses i.e. hut-building and for making walls, doors and window-frames, and in Liberia for planks. In Ghana small trees are cut for poles and find use as mine pit-props and bridge-piles. The twigs are used in Ghana as candlesticks, and the smaller ones as chew-sticks and tooth-picks in Ghana and Gabon (Burkill, 1984). The inner bark contains a sticky yellow resin which has anodynal properties. In the region it is pounded and rubbed on the body to relieve painful conditions (http://plants.jstor.org/upwta/2_814). Sap squeezed from the bark is a component of a medicine used to treat urethral discharge (Orwa and Munjuga, 2007).

In a nutshell, the species is one of the most commonly used medicinal plants in Cameroon (Laird, 1996) and possibly has even greater potential. For example, Guttiferone F, an HIV-inhibitor (Fuller *et al.*, 2003) was found in the extracts of the heartwood from *A.*

floribunda (Locksley and Murray, 1971). The active compounds of its bark contain prenylated xanthone, a natural product acting against human epidermoid carcinoma of the nasopharynx cancer line (Nkengfack *et al.*, 2002).

1.4 Studies on *Allanblackia floribunda* Oliver

Bilanda *et al.* (2010) carried out a study on the antihypertensive and antioxidant effects of aqueous extract of *Allanblackia floribunda* stem bark in alcohol and sucrose-induced hypertensive rats. *Allanblackia floribunda* was found to significantly prevent increase in the mean blood pressure in both alcohol-induced and sucrose-induced hypertensive rats. Administration of the plant extract also led to the prevention of total cholesterol, HDL-cholesterol and triglycerides increase in serum lipid in alcohol-induced hypertensive rats as compared to the untreated alcohol-induced hypertensive rats. In sucrose-induced hypertensive rats, the extract significantly prevented the high concentrations of total cholesterol and triglycerides induced by sucrose treatment as compared to the untreated sucrose-induced hypertensive rats, without affecting that of HDL-cholesterol.

Allanblackia floribunda also prevented the increase in atherogenic index, bilirubin, urea, alanine transferase and aspartate transferase in alcohol-induced hypertensive rats and sucrose-induced hypertensive rats and it also significantly prevented the increase of superoxide dismutase (SOD), malondialdehyde (MDA) and catalase and the decrease of reduced glutathione (GSH) concentration in aorta, heart, kidney and liver of alcohol-induced hypertensive rats and sucrose-induced hypertensive rats (Bilanda *et al.*, 2010).

Kada *et al.* (2012) investigated the effect of aqueous extract of *Allanblackia floribunda* stem bark on sexual behavior in adult male rats. Administration of the extract resulted in the significant increase of mount frequency, intromission frequency, ejaculatory latency, penile licking frequency, computed indices of sexual behavior and wiring-touch frequency whereas the mount latency, intromission latency and post-ejaculatory interval were significantly decreased throughout the experimental period establishing the stimulating

potential of *Allanblackia floribunda* aqueous extract on sexual desire and potency of male rats (Kada *et al.*, 2012).

Dieudonné *et al.* (2013) carried out a toxicological study on aqueous extract of the stem bark of *Allanblackia floribunda* on rats where they determined the acute toxicity both orally and intraperitoneally; and sub-acute toxicity orally alone; and discovered that in acute test, the oral administration did not cause any death treatment related signs. The LD_{50} was estimated to be 125 mg kg^{-1} (intraperitoneal route). In sub-acute treatment, neither significant difference was observed on body weight, food and water consumption nor organs and haematological parameters. The biochemical analysis showed that the level of alanine transferase (ALT) dose dependently decreased at all doses in male and female rats while tissue creatinine decreased only in female rats (Dieudonné *et al.*, 2013).

Kada *et al.* (2013) further investigated the effects of *Allanblackia floribunda* aqueous and ethanol stem bark extracts and their potential mechanism on fictive ejaculation in spinal male rats. Sequential intravenous treatments of rats with extracts significantly decreased the occurrence of ejaculation induced by dopamine; the oral pre-treatment with both extracts also significantly decreased the ejaculation induced by dopamine. All had inhibitory activities on ejaculation (Kada *et al.*, 2013).

Nkengfack *et al.* (2002) discovered a novel prenylated xanthone, named allanxanthone A, which was isolated from the stem bark of *Allanblackia floribunda* in addition to known compounds, 1,5-dihydoxyxanthone, 1,5,6-trihydroxy-3,7-dimethoxyxanthone, stigmasterol and stigmasteryl-3-O-b-d-glucopyranoside. The structure of the new compound was assigned as 1, 3, 5-trihydroxy-2-(3-methylbut-2-enyl)-4-(1, 1-dimethylprop-2-enyl) xanthone, by means of spectroscopic analysis. The NMR spectral data of 1, 5-dihydroxyxanthone was also reported as well as the *in vitro* cytotoxic activity of xanthone metabolites against the KB cell line (Nkengfack *et al.*, 2002).

1. 5 Statement of Research Problems

Infertility issues affect approximately 15% of all couples globally, and male factor is the sole or contributing factor in roughly half of all these cases. However, there has been an increased usage of natural herbs in the treatment of male sexual dysfunctions. *Allanblackia floribunda* is widely used in West and Central Africa to treat myriads of ailments and it is one of the plants that has been reportedly used by some locals in boosting sexual performance (aphrodisiac).

Despite the acclaimed sexual enhancing potentials of the stem and root decoction of this plant, there is dearth of information on the effects of this plant on the testicular function parameters as related to sexual enhancement. This study is thus set to investigate the effects of ethanolic extract of the stem bark of *Allanblackia floribunda* on the testicular function of male rats at similar doses used previously for the demonstration of its aphrodisiac potentials by previous authors. Several authors (Gonzales *et al.*, 2001; Manna *et al.*, 2004; Sharma *et al.*, 2001; Gupta and Kumar, 2004) have used parameters like percentage testes–body weight ratio, activities of alkaline phosphatase, acid phosphatase, of the testes to assess the testicular function in male rats, hence, their use in this study.

1.6 Specific Objectives of Research

The specific objectives of the research are to

(a) obtain an ethanolic extract of the plant;

(b) carry out phytochemical screening on the ethanolic extract;

(c) partition the extract into different fractions of ethyl acetate, aqueous, and n-butanol;

(d) investigate the acute toxicity of the crude extract and establish the median lethal dose (LD_{50}); and

(e) investigate the possible effects of the extracts on the gonads and accessory organs of the rats.

1.7 Expected Contribution to Knowledge

This study will provide information on the possible deleterious or therapeutic effects of *Allanblackia floribunda* on the male reproductive organs.

CHAPTER TWO

2.0 Literature Review

2.1 Male Sexual Function

Sexual health is a state of physical, emotional, mental and social well-being in relation to sexuality; it is not merely the absence of disease, dysfunction or infirmity (Yakubu *et al.*, 2007). Sexuality on the other hand is a complex, multi-dimensional phenomenon that incorporates biological, psychological, interpersonal and behavioural dimensions and sexual stimulation of the human male results in a series of psychological, neuronal, vascular, and local genital changes. For there to be a normal sexual intercourse and sexual fulfilment in males, the male sexual organs (the copulatory organ, the penis) and factors relating to erection must function normally (Yakubu *et al.*, 2007).

Males reach peak sexual capacity in the late teens. With advancement of age, a gradual decrease in sexual responsiveness then occurs (Schiavi and Rehman, 1995), characterized by a prolongation of the time required to achieve full erection and decrease in the effectiveness of psychic and tactile stimuli. The maintenance of erection requires continuing direct genital stimulation. Orgasm and the feeling of ejaculatory inevitability frequently become less intense. Penile detumescence occurs more rapidly and the refractory period is more prolonged. Also, the ejaculatory volume decreases with age (Kandeel *et al.*, 2001).

The effects of age on male reproductive physiology as reviewed by Kandeel *et al.* (2001) found that aging is associated with decreased total serum and bioavailable testosterone concentrations, decreased testosterone to estradiol ratio, increased sex hormone-binding-globulin (SHBG) leading to increased plasma protein binding of circulating testosterone and decreased testosterone clearance, decreased Luteinizing Hormone pulse frequency, and diminished accumulation of 5 α-reduced steroids in reproductive tissues.

Furthermore, disorders of sexual function have been reported to be common among men irrespective of their ages, ethnicities, and cultural affiliations. It has even been estimated that more than 152 million men worldwide experienced erectile dysfunction in 1995, and that this number will rise by 170 million, to approximately 322 million by the year 2025 (Kandeel *et al*., 2001). This sounds a little bit disturbing for males in particular, for the male gender places more emphasis on performance as a proof of masculinity or virility and this has been a common denominator in that men look for any available means to improve their performance during sexual intercourse.

At least three different classifications for the changes that occur in the human male during sexual intercourse have been described. Kolodny *et al.* (1979) described a psychosexual response cycle that consists of four phases: excitement, plateau, orgasm, and resolution. Another classification by Govier *et al.* (1995) and Lue and Tanagho (1988) based on penodynamic changes during the sexual cycle divides each of the psychosexual phases into two interrelated events as excitement into latency and tumescence; plateau into erection and rigidity; orgasm into emission and ejaculation; and resolution into detumescence and refractoriness. The third classification by Walsh and Wilson (1987) focuses on the functional activities during the sexual cycle by adding an initial phase of desire or libido. Thus, the normal male sexual response cycle can be functionally divided into five interrelated events that occur in a defined sequence: libido, erection, ejaculation, orgasm, and detumescence.

2.1 Libido or Sexual Desire

Libido is defined as the biological need for sexual activity (the sex drive) and frequently is expressed as sex-seeking behaviour. Its intensity is variable between individuals as well as within an individual over a given time. Higher serum testosterone appears to be associated with greater sexual activity in healthy older but not younger men (Toone *et al.*, 1983).

2.2 Erection

Erection is the enlarged and rigid state of the sexually aroused penis sufficient enough for vaginal penetration. It results from multiple psychogenic and sensory stimuli arising from imaginative, visual, auditory, olfactory, gustatory, tactile, and genital reflexogenic sources (Yakubu *et al.*, 2007).

Further, erection is associated with significant psychological and physical changes, including heightened sexual arousal, full testicular assent and swelling, dilatation of the urethral bulb, an increase in glans and coronal size, cutaneous flush over the epigastrium, chest, and buttocks, nipple erection, tachycardia and elevation in blood pressure, hyperventilation, and generalized myotonia (Kolodny *et al.*, 1979; Skakkebaek *et al.*, 1981).

2.3 Ejaculation

Ejaculation is the act of ejecting semen. It is a reflex action that occurs as a result of sexual stimulation. It is made up of two sequential processes. The first process called emission is associated with deposition of seminal fluid into the posterior urethra (Yakubu *et al.*, 2007). Simultaneous contractions of the ampulla of the vas deferens, the seminal vesicles, and the smooth muscles of the prostate (Walsh and Wilson, 1987; Wagner, 1981) mediate emission, while the second process is the true ejaculation, which is the expulsion of the seminal fluid from the posterior urethra through the penile meatus.

2. 4 Orgasm

This is the climax of sexual excitement. The entire period of emission and ejaculation is known as the male orgasm (Guyton and Hall, 2000). Male orgasm is marked by the following physiological events: smooth muscle contraction of the accessory sex organs; buildup and release of pressure in the posterior urethra; sensation of the ejaculatory inevitability; contraction of the urethral bulb and perineum; rhythmic contractions of the pelvic floor muscles; semen emission and ejaculation; and finally, the reversal of the generalized physiological changes and sexual tension. Sensory cortical neurons perceive

these events as pleasurable. Factors that influence the subjective sensation of orgasmic pleasure include the degree of sexual excitement, recency of sexual activity, and the psychosexual makeup of the individual (Kandeel *et al.*, 2001).

2. 5 Detumescence

This is the subsidence of an erect penis after ejaculation (Wagner, 1981). It is the point where the penis returns to the flaccid state. Vasoconstriction of the arterioles and reversal of arousal events divert the blood away from the penile sinuses and allow an increase in the venous drainage of their contents. Initially, the rate of blood outflow increases by about 10-fold, then it is followed by a progressively decreasing rate until it reaches the pretumescence level (Wagner**,** 1981) and a period of inhibition to resumption of erectile and ejaculatory functions (Kandeel *et al.*, 2001).

2.6 Male Sexual Dysfunction (MSD)

According to Yakubu *et al.* (2007), sex disorders of the male are basically classified into disorders of sexual function, sexual orientation, and sexual behaviour. In general, several factors must work in harmony to maintain normal sexual function. Such factors include neural activity, vascular events, intracavernosal nitric oxide system and androgens (Guay *et al.*, 2003). Thus, malfunctioning of at least one of these could lead to sexual dysfunction of any kind. Sexual dysfunction in men refers to repeated inability to achieve normal sexual intercourse. It can also be viewed as disorders that interfere with a full sexual response cycle.

These disorders make it difficult for a person to enjoy or to have sexual intercourse. While sexual dysfunction rarely threatens physical health, it can take a heavy psychological toll, bringing on depression, anxiety, and debilitating feelings of inadequacy. Unfortunately, it is a problem often neglected by the healthcare team who strive more with the technical and more medically manageable aspects of the patient's illness (Salmon, 1983).

In its peculiarity, sexual dysfunction is more prevalent in males than in females and thus, it is conventional to focus more on male sexual difficulties (Guay *et al.*, 2003). It has

been discovered that men between 17 and 96 years of age could suffer sexual dysfunction as a result of psychological or physical health problems (Joe, 1990). Generally, a prevalence of about 10% occurs across all ages.

Due to the fact that sexual dysfunction is an inevitable process of aging, the prevalence is over 50% in men between 50 and 70 years of age (Rendell *et al.*, 1999). As men age, the absolute number of Leydig cells decreases by about 40%, and the vigour of pulsatile luteinizing hormone release is dampened. In association with these events, free testosterone level also declines by approximately 1.2% per year. These have contributed in no small measure to prevalence of sexual dysfunction in the aged (Guay *et al.*, 2003).

Besides the age factor, Male Sexual Dysfunction (MSD) could be caused by various factors. These include: psychological disorders (performance anxiety, strained relationship, depression, stress, guilt and fear of sexual failure), androgen deficiencies (testosterone deficiency, hyperprolactinemia), chronic medical conditions (diabetes, hypertension, vascular insufficiency (atherosclerosis, venous leakage), penile disease (Peyronie's, priapism, phinosis, smooth muscle dysfunction), pelvic surgery (to correct arterial or inflow disorder), neurological disorders (Parkinson's disease, stroke, cerebral trauma, Alzheimer's spinal cord or nerve injury), drugs (side effects) (anti-hypertensives, central agents, psychiatric medications, antiulcer, antidepressants, and anti-androgens), life style (chronic alcohol abuse, cigarette smoking), aging (decrease in hormonal level with age) and systemic diseases (cardiac, hepatic, renal pulmonary, cancer, metabolic, post-organ transplant) (Guay *et al.*, 2003; Feldman *et al.*, 1994; Kandeel *et al.*, 2001).

Also, sexual dysfunction takes different forms in men. A dysfunction can be life-long and always present, acquired, situational, or generalized, occurring despite the situation. A man may have a sexual problem if he ejaculates before he or his partner desires, does not ejaculate, or experiences delayed ejaculation, is unable to have an erection sufficient for

pleasurable intercourse, feels pains during intercourse, lacks or loses sexual desire. Male sexual dysfunction can be categorized as disorders of desire, disorders of orgasm, erectile dysfunction, disorders of ejaculation and failure of detumescence (Yakubu *et al.*, 2007).

2.6.1 Disorders of Desire

Disorders of desire can involve either a deficient or compulsive desire for sexual activity. Dysfunctions that can occur during the desire phase include:

(i) Hypoactive sexual desire (HSD), which according to American Psychiatric Association (1994) is defined as persistently or recurrently deficient (or absent) sexual fantasy and desire for sexual activity leading to marked distress or interpersonal difficulty. It results in a complete or almost complete lack of desire to have any type of sexual relation. A critical level of blood androgens is required for the maintenance of normal sexual desire, and non-erotic penile erections in most men (Kandeel *et al.*, 2001). In actual fact, a certain concentration of androgens is required for initiation and maintenance of spermatogenesis and for maximum stimulation of growth and function of the prostate and seminal vesicles (Walsh and Wilson, 1987; Myers, 1985). However, the amount of androgens required for these latter effects is greater than that needed for maintenance of libido. Therefore, total or free testosterone levels may not be an adequate measure of sexual drive, at least in some populations (Kandeel *et al.*, 2001).

(ii) Compulsive sexual behaviours (CSBs) constitute a wide range of complex sexual behaviours that have strikingly repetitive, compelling, or driven qualities. They usually manifest as obsessive-compulsive sexuality (*e.g.* excessive masturbation and promiscuity), excessive sex-seeking in association with affective disorders (*e.g.* major depression or mood disorders), addictive sexuality (*e.g.* attachment to another person, object, or sensation for sexual gratification to the exclusion of everything else), and sexual impulsivity (failure to resist an impulse or temptation for sexual behaviour that is harmful to self or others such as exhibitionism, rape, or child molestation) (Kaplan, 1996).

2.6.2 Erectile Dysfunction (ED)

This is best defined as persistent failure to generate sufficient penile body pressure to achieve vaginal penetration and/or the inability to maintain this degree of penile rigidity until ejaculation (Wagner, 1981). Laumann *et al.* (1999) defined erectile dysfunction as the difficulty in achieving or maintaining an erection sufficient for sexual activity or penetration, at least 50% of the time, for a period of six months. It results in significant psychological, social and physical morbidity (Monga, 1999), and annihilates his essence of masculinity (Bosch *et al.*, 1991).

Causes of erectile dysfunction can be physiological, drug related, individual habit and lifestyle e.g. smoking, and organic (Kandeel *et al.*, 2001). The organic causes of erectile dysfunction can be grouped into systemic diseases and endocrine, neurological, vascular, or local penile disorders (Walsh and Wilson, 1987) and of all the groups, vascular insufficiency is probably the most common cause of organic male sexual dysfunction (Myers, 1985; Meuleman and Diemont, 1995).

2.6.3 Disorders of Ejaculation

There exists a spectrum of disorders of ejaculation ranging from mild premature to severely retarded or absent ejaculation. These include:

(i) Premature ejaculation which is the most common male sexual dysfunction (Aizenberg *et al.*, 1997) and can be any of the following: persistent or recurrent ejaculation with minimum sexual stimulation that occurs before, upon, or shortly after penetration and before the person wishes it; marked distress or interpersonal difficulty; and the condition does not arise as a direct effect of substance abuse. Premature ejaculation and sexual desire disorders were the frequent reported problems in young adult males with adverse familial relationship (Kinzl *et al.*, 1996).

(ii) Painful ejaculation which results from side effect of tricyclic antidepressants (Metz *et al.*, 1991) is a persistent and recurrent pain in the genital organs during ejaculation or immediately afterwards.

(iii) Inhibited or retarded ejaculation: This is when ejaculation does not occur at all.

(iv) Retrograde ejaculation: This is when ejaculation is forced back into the bladder rather than through the urethra and out of the end of the penis at orgasm.

2.6.4 Disorders of Orgasm

Male orgasmic disorder is defined as a persistent or recurrent delay in, or absence of orgasm after a normal sexual excitement phase during sexual activity (American Psychiatric Association, 1994).

2.6.5 Failure of Detumescence

This is a prolonged erection usually lasting for between 4 hours or greater. It is painful and always unaccompanied by sexual desire despite the fact that it is often preceded by usual sexual stimuli. Diagnostic options for male sexual dysfunction include: patient's history which embodies medical history (evaluating historical events like chronic disease, pharmacological agents, endocrine disorders, surgeries and trauma), psychological history (assessing individual's upbringing relationships, early sexual experiences, inadequate sexual information and general psychological health), sexual history (to ascertain the time and manner of onset, its course, current status, and associated medical or psychological problems), physical examination (entails general and systemic evaluation, assessment of gonadal function, vascular competence, neurological integrity, and genital organ normalcy), diagnosis testing (include blood tests, vascular assessment, sensory testing and nocturnal penile tumescence and rigidity testing) (Kandeel *et al.*, 2001; Guay *et al.*, 2003).

2.7 Management Options of MSD

Management options of MSD include:

Psychological/behavioural (therapy with a trained counsellor aimed at helping people to address feelings of anxiety, fear and guilt that may have an impact on sexual function); drug therapy (use of testosterone replacement therapy for cases of androgen insufficiency and other pharmacological agents);

Non-surgical devices which include vacuum pump (expands the penis and reduces pressure within the cavernous sinusoidal space) and constrictive rings (external device used for managing erectile dysfunction in patients with mild to moderate venous leakage);

Surgical treatment which include venous ligation (used to correct leakage of blood from the veins); penile prosthesis (creates adequate space within the tissue of each cavernosal body);

Penile implants (involves inserting a malleable or rigid substance into the penis to effect a semi-rigid state) and phytotherapy (involves the use of herbs (medicinal plants)) (Grenier and Byers, 1995; Michael and Owen, 1997; Ogah, 1999).

2.8 Aphrodisiac

Aphrodisiac was named after *Aphrodite*, the Greek goddess of sexual love, beauty and fruitfulness identified in Roman Mythology with the goddess Venus (Yakubu *et al*., 2007).

An aphrodisiac can therefore be described as any substance that enhances sex drive and/ or sexual pleasure. Aphrodisiac can also be viewed as any food, drug, scent or device that can arouse or increase sexual drive or libido (Rosen and Ashton, 1993). Most aphrodisiacs also heighten other aspects of sensory experience such as light, touch, smell, taste and hearing; and this enhanced sensory awareness contributes to sexual arousal and pleasure (Taberner, 1985).

Through history, a wide range of characteristics has qualified different substances as aphrodisiac (Davis, 1985). However, two possible approaches include the cultural and scientific.

Several primary non-scientific themes have arisen that have echoed through multiple cultures and times. First, the genitals have often been deemed to be aphrodisiacs. Another popular belief by hunters of those eras was consumption of specific parts of their prey in order to gain characteristics of those organs (Rosen and Ashton, 1993).

In England, it was believed that plants with any phallic-like features such as asparagus, parsnips and carrots were likely to be aphrodisiac in their effect (Basry, 1989). Ukrainians celebrate carrots and celery as folk aphrodisiac. In Chinese culture, much of the aphrodisiac power of ginseng and rhinoceros horn comes from their appearance rather than their chemical composition (Basry, 1989).

Based on their mechanism of actions, aphrodisiacs can be divided into three categories which include:

(a) Aphrodisiacs that simply provide a burst of nutritional value, thereby improving the immediate health or well-being of the consumer and consequently improving sexual performance and libido. This simple improvement in general health can lead to a burst of energy and translate into an increased sexual appetite (Barry *et al.*, 1996). For example, in Chinese tradition, the use of rhinoceros horn as an aphrodisiac may lie in the fact that rhino horn consists of fibrous tissue with large proportions of elements like calcium and phosphorus; beyond the fact that rhino horn resembles an erect penis. Deficiency in these elements could lead to muscle weakness and general fatigue while large doses of these elements could lead to general increased vigour and stamina (Davis, 1985).

(b) The second group are those with specific physiological effect. They may affect blood flow; mimic the burning of fire of sex and intercourse and increase the duration of sexual activity. An example is Spanish fly made of dried and crushed beetles of the *cantharis* and *mylabris* genus. The active ingredient in Spanish fly, a crystallized lactone, cantharidin (Taberner, 1985), when applied topically, causes burning sensation at the point of blistering

(Yakubu *et al.*, 2007). Its consumption has also been reported to cause increased blood flow in the body (Yakubu *et al.*, 2007). Other physiologically active drugs used to sustain erection, help to limit the influence of sympathetic nervous system e.g. Sildenafil citrate (viagra) and yohimbine from *Pausinystalia yohimbe* (Taberner, 1985).

(c) The third group of biologically active aphrodisiacs are those that are psychologically active in nature. They actually cross the blood brain barrier and mimic or stimulate some areas of sexual arousal. Examples include hormones, pheromones and a wide variety of neurotransmitters (Yakubu *et al.*, 2007).

2.9 Some Local Plants Used as Aphrodisiacs

It is a common knowledge that the use of herbs is very common in developing countries, particularly in rural settings. However, an increase in the use of plants has been observed in metropolitan areas of developed countries (Harnack *et al.*, 2001). Plants are extensively used to relieve sexual dysfunction. Ginseng, for example, is an essential constituent in traditional Chinese medicine (Kim *et al.*, 1976) and at least 6 million Americans use the root of this slow-growing perennial plant (Nocerino *et al.*, 2000). Another root, known as Maca (*Lepidium meyenii*), has traditionally been used by Peruvian inhabitants living at high altitudes as a nutrient, an energizer, as aphrodisiac and/or fertility-enhancing agent. It has been proved to be effective in improving sexual desire in men (Yakubu *et al.*, 2011), and sexual behaviour in male rats and mice (Zheng *et al.*, 2000; Cicero *et al.*, 2001; Cicero *et al.*, 2002).

Similarly, other authors have lent scientific credence to the use of *Fadogia agrestis* (English: black aphrodisiac, Hausa: Baakin gagai) stem as an aphrodisiac by increasing the concentration of serum testosterone made possible by its saponin content (Yakubu *et al.*, 2011). Traditional herbs have also been a revolutionary breakthrough in the management of sexual inadequacies (sexual dysfunction) and have become widely acclaimed as an “instant” treatment (Adimoelja, 2000). Some of these herbs include *Terminalia cattapa* seeds (Almond

fruit), root of *Garcina kola* Heckel (Yoruba: orogbo), stem bark and twig of *Carpolobia albe* (Yoruba: osunsun, osun), whole plant of *Euphorbia hirta* L (Yoruba: egele) and leaves, roots and fruits of *Musa parasidiaca* L (plantain) (Gill, 1992; Ratnasooriya and Dharmasiri, 2000). Other indigenous medicinal plants, have been claimed to improve potency.

Allanblackia floribunda could be considered as a substantial part into the categorization of aphrodisiac substances that increase sexual activity after delaying ejaculation (Sandroni, 2001). According to the traditional claims, the stem bark of *A. floribunda* is orally consumed by men seeking an increase of their sexual activities (Laird, 1996; Noumi, 1998). In line with this belief, Kada *et al.* (2013) showed that it increased the ejaculatory latency and inhibited provoked ejaculation. This suggests that the plant contains bioactive agents which may exert an aphrodisiac effect on several different levels (central nervous system, testosterone production, blood vessel relaxation) (Kada *et al.*, 2012).

2.10 Reactive Oxygen Species and Sperm Quality

Spermatogenesis is fundamental to male fertility and involves a high intricate process of cellular proliferation, differentiation and apoptosis. It takes place in the seminiferous tubules of testis where spermatogonial stem cells (germ cells) give rise to mitotically active spermatogonia that eventually become spermatocytes. Spermatocytes undergo meiosis and a series of differentiation steps to produce the haploid spermatids with the support and close association of somatic sertoli cell population. It is however interesting to note that in many testicular insults, it is the germ cell population that is most sensitive and most often respond by undergoing apoptosis while other cell populations appear relatively unchanged. (Lysiak and Turner, 2007)

The generation of reactive oxygen species (ROS) in the male reproductive tract has become a real concern because of their potential toxic effects at high levels on sperm quality and function (Saleh and Agarwal, 2002). ROS are highly reactive oxidizing agents belonging to the class of free radicals (Aitken and Fisher, 1994) and a free radical is defined as ‘‘any

atom or molecule that possesses one or more unpaired electrons'' (Warren *et al.*, 1987). Free radicals are highly reactive chemical molecules and they oxidatively modify biomolecules they encounter (Agarwal and Sekhon, 2010). Reacting almost immediately with any substance in their surrounding area, they begin a chain reaction leading to cellular damage (Warren *et al.*, 1987). Superoxide anion, hydroxyl radical and hydrogen peroxide are major reactive oxygen species (ROS) present in seminal plasma.

Reports have indicated that high levels of ROS are detected in semen samples of 25% to 40% of infertile men (de Lamirande *et al.*, 1995; Padron *et al.*, 1997). 'Male factor' infertility is seen as an alteration in sperm concentration and/or motility and/or morphology in at least one sample of two sperm analyses (Agarwal and Sekhon, 2010). Spermatozoa, like all cells living in aerobic conditions, constantly face the oxygen (O_2) paradox: O_2 is required to support life, but its metabolites such as ROS can modify cell functions, endanger cell survival, or both (de Lamirande and Gagnon, 1995), hence, ROS must be continuously inactivated to keep only a small amount necessary to maintain normal cell function. It is not surprising that a battery of different antioxidants is available to protect spermatozoa against oxidants (Sies, 1993).

2.11 Oxidative Stress and Male Infertility

Male germ cells at various stages of differentiation have the potential to generate ROS and low physiologic levels are needed to regulate sperm capacitation, acrosome reaction and sperm–oocyte fusion (Agarwal and Saleh, 2002; Agarwal *et al.*, 2004). To maintain normal cell function, excess ROS must be continuously inactivated by seminal plasma antioxidants. These block the formation of new ROS or act as scavengers and remove ROS already generated. Natural antioxidant enzyme systems include catalase, glutathione peroxidase and superoxide dismutase (Baker *et al.*, 1996).

As shown in Figure 2.1, a delicate balance exists between physiological ROS and antioxidants in the male reproductive tract in healthy men (Sikka, 2001). Oxidative stress

(OS) arises when excess free radicals overwhelm the antioxidant defense of the male reproductive tract (Sharma and Agarwal, 1996; Kemal *et al.*, 2000).

2.12 Defence Mechanisms of the Male Reproductive System

Seminal plasma and sperm themselves are well endowed with an array of protective antioxidants (Fujii *et al.*, 2003; Garrido *et al.*, 2004). These antioxidants are either enzymatic or non- enzymatic antioxidants.

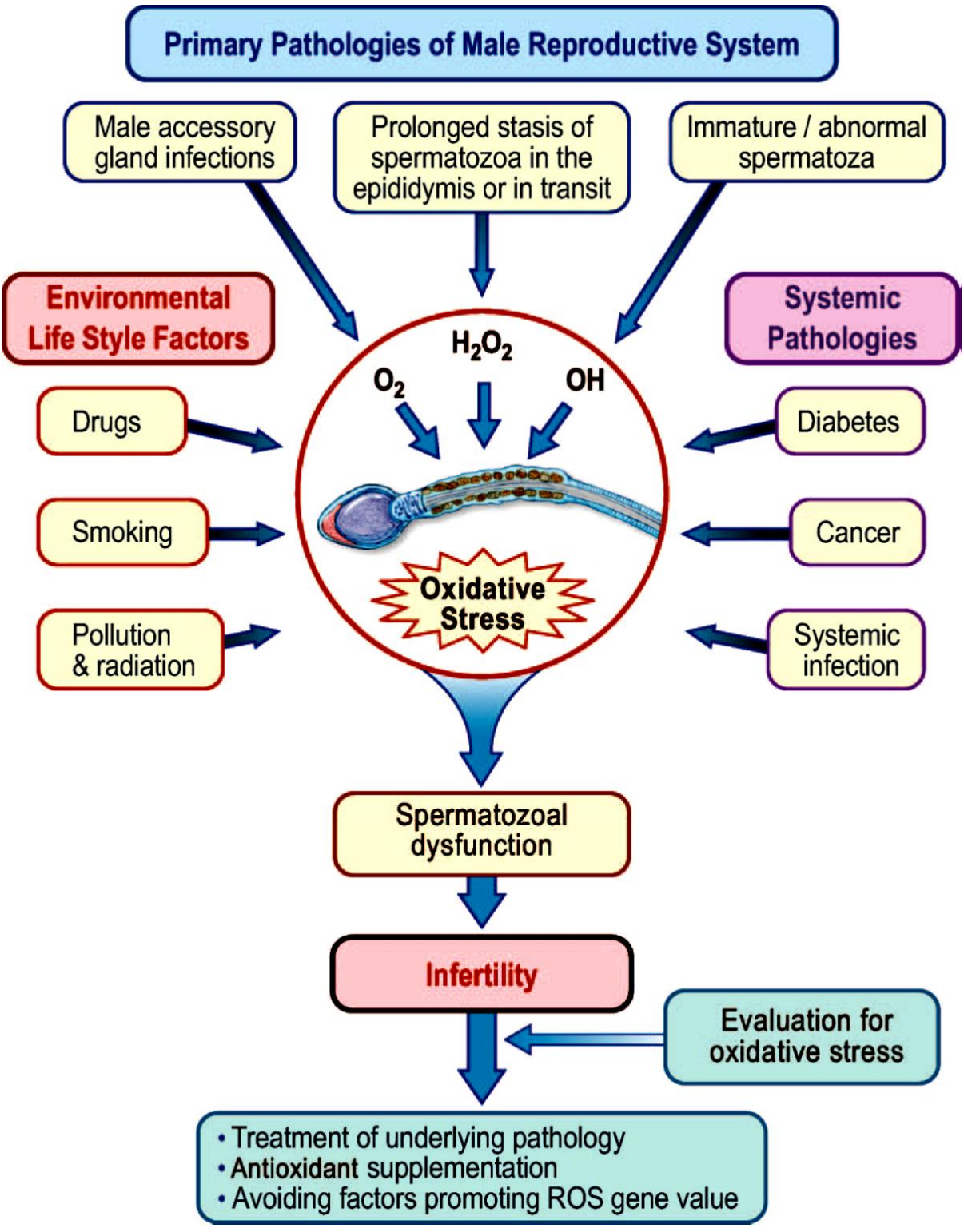


Figure 2.1 Factors Contributing to Oxidative Stress-Induced Male Infertility (Agarwal and Sekhon, 2010). OH^- - hydroxyl radical, O_2 - oxygen, H_2O_2 – hydrogen peroxide

2.12.1 Enzymatic Antioxidants

Three groups of enzymes play significant roles as oxidant scavengers. Superoxide dismutase (SOD), catalase, and glutathione peroxidase (GPx) (Mahanta *et al.*, 2012). Superoxide dismutases (SOD) are metal-containing enzymes that catalyze the conversion of two superoxides into oxygen and hydrogen peroxide, which is less toxic than superoxide. Catalase, an enzyme found in peroxisomes, degrades hydrogen peroxide to water and oxygen, thereby completing the reaction started by SOD (Kefer *et al.*, 2009). Figure 2.2 shows how these antioxidant enzymes interplay in systemic protection.

Glutathione peroxidase (GPx) is the final member of the seminal enzymatic antioxidant triad. GPx consists of a family of antioxidants (GPx1-5) that are involved in the reduction of hydroperoxides using glutathione as an electron donor. The GPxs are located within the testis, prostate, seminal vesicles, vas deferens, epididymis, seminal plasma and spermatozoa themselves (Vernet *et al.*, 2004). GPx must really be playing an important protective role against oxidative attack since it was found out that its specific inhibition *in vitro* leads to a large increase in sperm lipid peroxidation (Twigg *et al.*, 1998). Male factor infertility has been linked with a reduction in seminal plasma (Giannattasio *et al.*, 2002) and spermatozoa (Garrido *et al.*, 2004) GPx activity, further supporting an important role for this enzyme in male fertility. In addition, men exhibiting leukospermia-associated oxidative stress have been reported to have significantly reduced GPx activity within their spermatozoa (Therond *et al.*, 1996).

Finally, the continued activity of GPx depends on the regeneration of reduced glutathione (GSH) by glutathione reductase (GTR). Selective inhibition of GTR reduces the availability of reduced glutathione for maintaining GPx activity, thereby exposing sperm to oxidative stress. Hence, the coordinated activity of GPx, GTR and glutathione clearly play a pivotal role in protecting sperm from oxidative attack (Williams and Ford, 2004).

Other enzymes, such as glutathione transferase, ceruloplasmin or hemoxygenase may also participate in enzymatic control of oxygen radicals and their products (Tremellen, 2008).

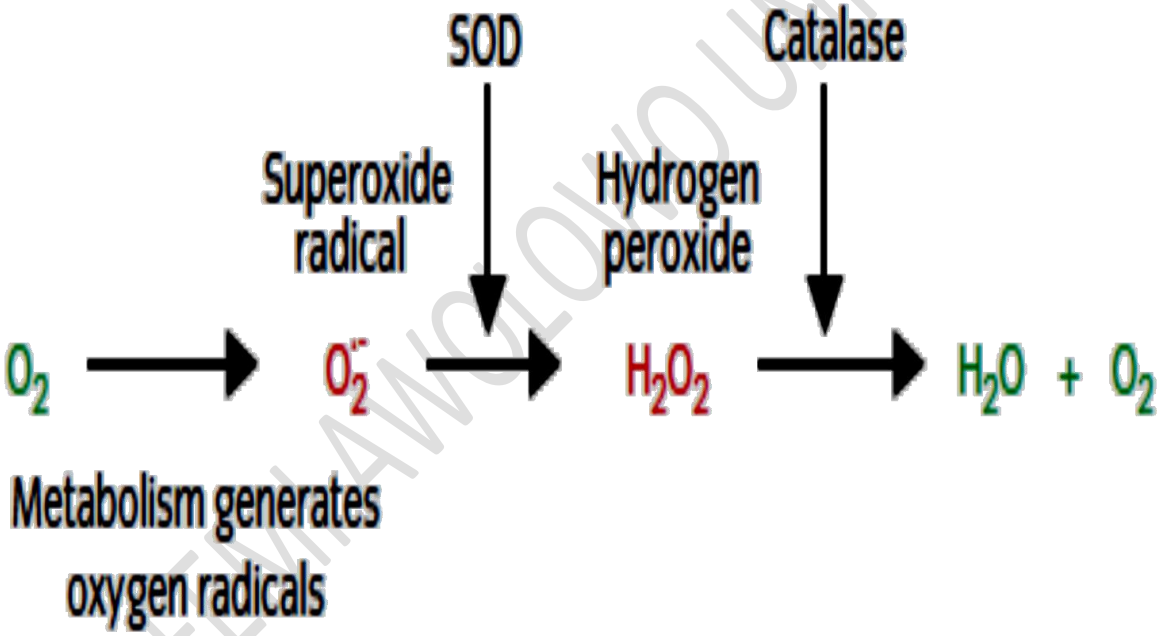


Figure 2.2 Antioxidant Scavenging Pathways of Free Radicals by Superoxide Dismutase and Catalase (Kefer *et al.*, 2009). SOD – superoxide dismutase, $O_2^{\cdot-}$ - superoxide radical, O_2 - oxygen, H_2O - water, H_2O_2 –hydrogen peroxide,.

As shown in Figure 2.3, Glutathione peroxidase (GPx) also scavenges hydrogen peroxide (H_2O_2), along with glutathione (GSH), which becomes oxidized and is reduced/regenerated by glutathione reductase (GR) and nicotinamide adenine dinucleotide phosphate (NADPH) to complete the cycle and allow further antioxidant function.

2.12.2 Non-Enzymatic Antioxidants

The non-enzymatic antioxidant glutathione may well be the most important intracellular defense against ROS. Glutathione is a tripeptide, composed of glutamate, cysteine, and glycine. The cysteine subunit provides an exposed free sulfhydryl group (SH) that directly scavenges free radicals. Once oxidized, glutathione is then regenerated/reduced by glutathione reductase and nicotinamide adenine dinucleotide phosphate (NADPH) to complete the cycle (Agarwal *et al.*, 2007). Vitamins E and C also play critical roles as non-enzymatic antioxidants. Vitamin E plays a vital role in protecting cell membranes from oxidative damage trapping and scavenging free radicals within cellular membranes.

Vitamin C is a water-soluble antioxidant that reduces radicals from a variety of sources, and also serves to recycle oxidized vitamin E (Kefer *et al.*, 2009). Under normal conditions, these enzymatic and non-enzymatic antioxidants act to maintain an overall low level of oxidative stress in the semen, allowing for normal oxidant-dependent cell signaling processes and normal spermatic function, while concomitantly avoiding oxidant-induced cell damage. In contrast, the pathological effects of oxidative stress arise under conditions where levels of unscavenged ROS increase, or the antioxidant buffering capacity of the system decreases, thus perturbing the delicate oxidant/antioxidant balance. These free radicals induce sperm cell injury through several pathways, and can significantly impact both sperm quality and function. (Warren *et al.*, 1987; Agarwal *et al.*, 2007; Tremellen, 2008). This oxidative stress-induced sperm damage has been suggested to be a significant contributing factor in 30–80% of all cases of male infertility. (Warren *et al.*, 1987; Tremellen, 2008).

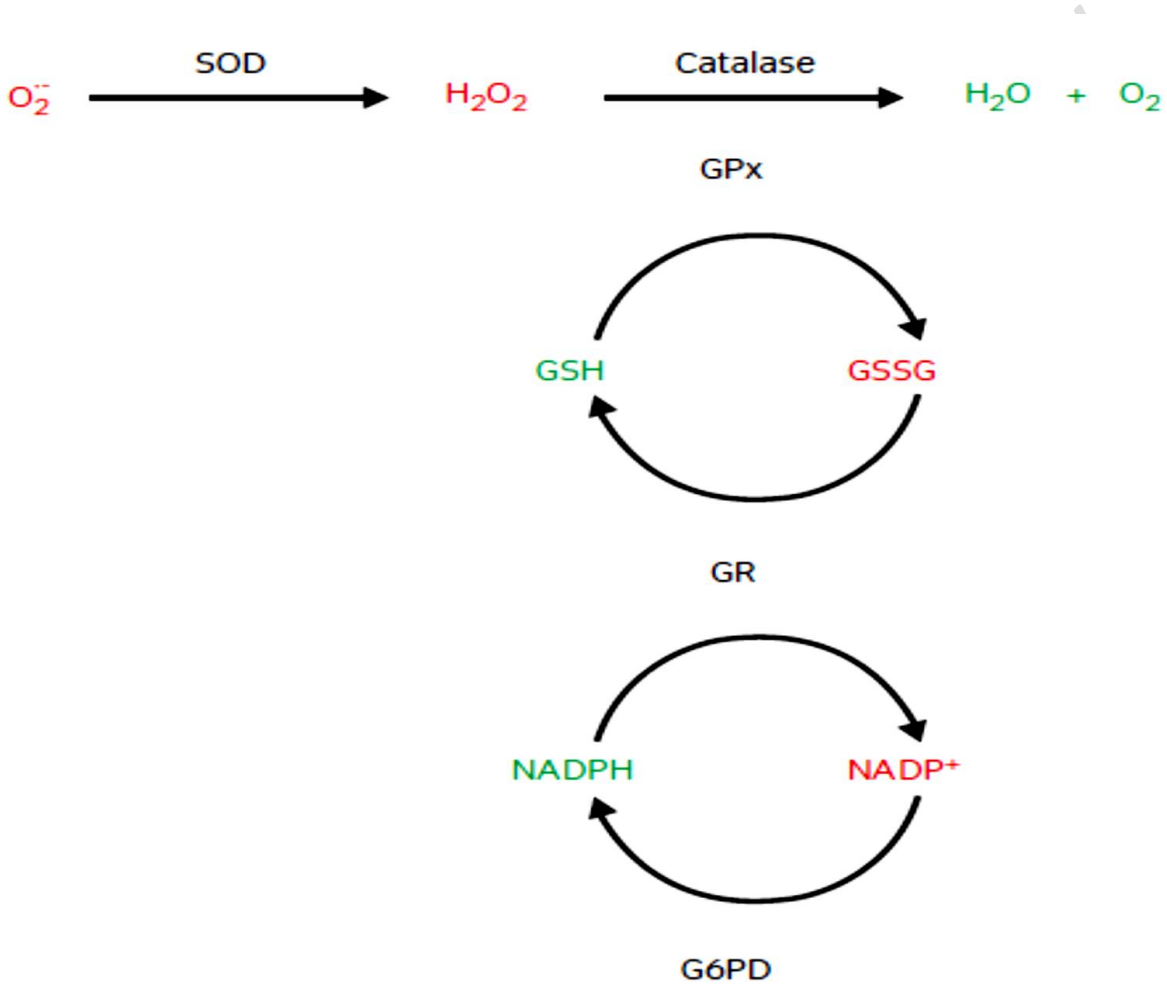


Figure 2.3 Antioxidant Scavenging Activity of Glutathione Peroxidase (GPx) (Kefer *et al.*, 2009). O_2^- - superoxide radical, SOD – superoxide dismutase, H_2O_2 – hydrogen peroxide, GPx – glutathione peroxidase, GSH – reduced glutathione, GSSG – glutathione disulfide, GR – glutathione reductase, NADPH - nicotinamide adenine dinucleotide phosphate, $NADP^+$ - nicotinamide adenine dinucleotide phosphate, G6PD – glucose-6-phosphate dehydrogenase

2.13 Mechanism of Antioxidant Protection in Semen

The endowment of seminal plasma with a battery of antioxidant mechanisms compensate for the deficiency in cytoplasmic enzymes in sperm (Donnelly *et al.*, 1999). Antioxidant defense mechanisms include three levels of protection:

(1) prevention,

(2) interception, and

(3) repair (Sies, 1993).

2.13.1 Prevention

Prevention of ROS formation is the first line of defense against oxidative insult. An example is the binding of metal ions, iron, and copper ions in particular, which prevents them from initiating a chain reaction (Sies, 1993). Chelation of transition metals is a major means of controlling sperm lipid peroxidation (LPO) and DNA damage. When transition metals become loosely bound to oxygen reduction products, they can produce secondary and more reactive oxidants, particularly the hydroxyl radical ($OH^{-}x$) (Halliwell, 1990).

2.13.2 Interception

Free radicals have a tendency toward chain reaction (i.e. a compound carrying an unpaired electron will react with another compound to generate an unpaired electron, ‘‘radical begets radical’’). Hence, the basic problem is to break this chain reaction by the formation of non-radical end products (Sies, 1993). The peroxyl radicals are major reaction partners because their half-life extends into the range of seconds (7 seconds). In contrast, the hydroxyl radical, with its high reactivity and extremely short half-life (1029 seconds), cannot be intercepted with reasonable efficiency (Sies *et al.*, 1992).

2.13.3 Repair

In some situations, the damage caused by oxidants may be repaired. Unfortunately, spermatozoa are unable to repair the damage induced by oxidative stress (OS) because they

lack the cytoplasmic enzyme systems that are required to accomplish this repair. This is one of the features that make spermatozoa unique in their susceptibility to oxidative insult (Alvarez *et al.*, 1987; Aitken *et al.*, 1989).

2.14 Internal Sources of Excessive Reactive Oxygen Species (ROS) Production in Semen

Within semen there are two principal sources of production of free radicals; leukocytes and sperm (Tremellen, 2008). Morphologically abnormal spermatozoa and seminal leukocytes have been implicated as the main sources of high ROS production in human ejaculates (Aitken and West, 1990; Kessopoulou *et al.*, 1992). Virtually every human ejaculate is contaminated with potential sources of ROS (Aitken and Fisher, 1994). It follows that some sperm cells will incur oxidative damage and a concomitant loss of function in every ejaculate. Thus, the impact of oxidative stress (OS) on male fertility is a question of degree rather than the presence or absence of pathology.

The ability of sperm to produce ROS inversely correlates with their maturational state. During spermatogenesis there is a loss of cytoplasm to allow the sperm to form its condensed, elongated form. Immature teratozoospermic sperm are often characterized by the presence of excess cytoplasmic residues in the mid-piece. These residues are rich in the enzyme glucose-6-phosphate dehydrogenase, an enzyme which controls the rate of glucose flux and intracellular production of β-nicotinamide adenine dinucleotide phosphate (NADPH) through the hexose monophosphate shunt. This NADPH is used to fuel the generation of ROS via NADPH oxidase located within the sperm membrane (Gomez *et al.*1996; Fisher and Aitken, 1997; Said *et al.*, 2005). As a result, teratozoospermic sperm produce increased amounts of ROS compared with morphologically normal sperm.

Spermatozoa may generate ROS in two ways:

(a) The NADPH oxidase system at the level of the sperm plasma membrane (Aitken *et al.*, 1992), and

(b) The NADH-dependent oxidoreductase (diphorase) at the level of mitochondria (Gavella and Lipovac, 1992).

The mitochondrial system is the major source of ROS in spermatozoa in infertile men (Plante *et al.*, 1994). When spermatogenesis is impaired, the cytoplasmic extrusion mechanisms are defective, and spermatozoa are released from the germinal epithelium carrying surplus residual cytoplasm. Under these circumstances, the spermatozoa that are released during spermiation are believed to be immature and functionally defective (Hauser *et al.*, 2007). As shown in Figure 2.4, retention of residual cytoplasm by spermatozoa is positively correlated with ROS generation via mechanisms that may be mediated by the cytosolic enzyme Glucose-6-phosphate dehydrogenase (Aitken *et al.*, 1989).

The underlying pathology behind free radicals ability to reduce sperm motility was first reported by Jones *et al.* (1979). They reported that ROS-induced peroxidation of the sperm membrane decreasing its flexibility and therefore tail motion. Sperm membranes are vulnerable to this type of damage as they contain large amounts of unsaturated fatty acids. Direct ROS damage to mitochondria, decreasing energy availability, may also impede sperm motility (de Lamirande and Gagnon, 1992; de Lamirande *et al.*, 1997; 1998). By either mechanism, oxidative stress impairs sperm motility and will result in less sperm reaching the oocyte for fertilization (Whittington *et al.*, 1999; Kao *et al.*, 2007).

2.15 External Origins of Oxidative Stress

The origins of sperm oxidative stress are summarized in Figure 2.5. While pathologies such as genitourinary tract infection and varicocele are well established causes of oxidative stress, others such as hyper-homocysteinaemia and diabetes are only now just becoming recognized as possible causes (Tremellen, 2008). Also, lesser well established potential causes of male oxidative infertility are discussed below.

2.15.1 Idiopathic

Idiopathic male factor infertility has been linked with oxidative stress by several research groups. One of the principal causes of this association is the observation that morphologically abnormal sperm have an increased capacity to generate ROS, but also a reduced antioxidant capacity (Gomez *et al.*, 1996; Garrido *et al.*, 2004; Said *et al.*, 2004; 2005). As approximately one-third of infertile men exhibit teratozoospermia (Thonneau *et al.*, 1991), it is not surprising that sperm oxidative stress is commonly identified in the idiopathic infertile male population. Even men with normozoospermic idiopathic infertility exhibit significantly higher seminal ROS production and lower antioxidant capacity than fertile men (Pasqualotto *et al.*, 2001; Agarwal *et al.*, 2006), for as yet unknown reasons.

2.15.2 Iatrogenic

The use of assisted reproductive technologies (ART) has the potential to exacerbate sperm oxidative stress. During IVF and IUI treatment semen is centrifuged to separate sperm from seminal plasma. This exacerbates oxidative stress as centrifugation increases sperm ROS production many fold, while removing sperm from protective antioxidants within seminal plasma (Potts *et al.*, 2000). In addition cryopreservation of sperm, another commonly used technique in ART, is associated with an increase in sperm oxidative stress (Watson, 2000).

Drugs such as the chemotherapy agent cyclophosphamide have been linked with sperm oxidative stress. Administration of cyclophosphamide to animals is reported to increase testicular malondialdehyde (MDA) levels and produce a fall in testicular catalase, implying the presence of oxidative stress (Das *et al.*, 2002; Ghosh *et al.*, 2002). Drugs such as aspirin and paracetamol (acetaminophen) can also produce oxidative stress by increasing cytochrome P_{450} activity, thereby boosting ROS generation (Agarwal and Said, 2005).

2.15.3 Lifestyle

Smoking results in a 48% increase in seminal leukocyte concentrations and a 107% increase in seminal ROS levels (Saleh *et al.*, 2002). Smokers have decreased levels of seminal plasma antioxidants (Mostafa *et al.*, 2006), placing their sperm at additional risk of oxidative damage. Also, dietary deficiencies have been linked with sperm oxidative damage by several research groups. A study carried out to examine the self-reported dietary intake of various antioxidants and nutrients (vitamins C and E, β-carotene, folate and zinc) in a group of 97

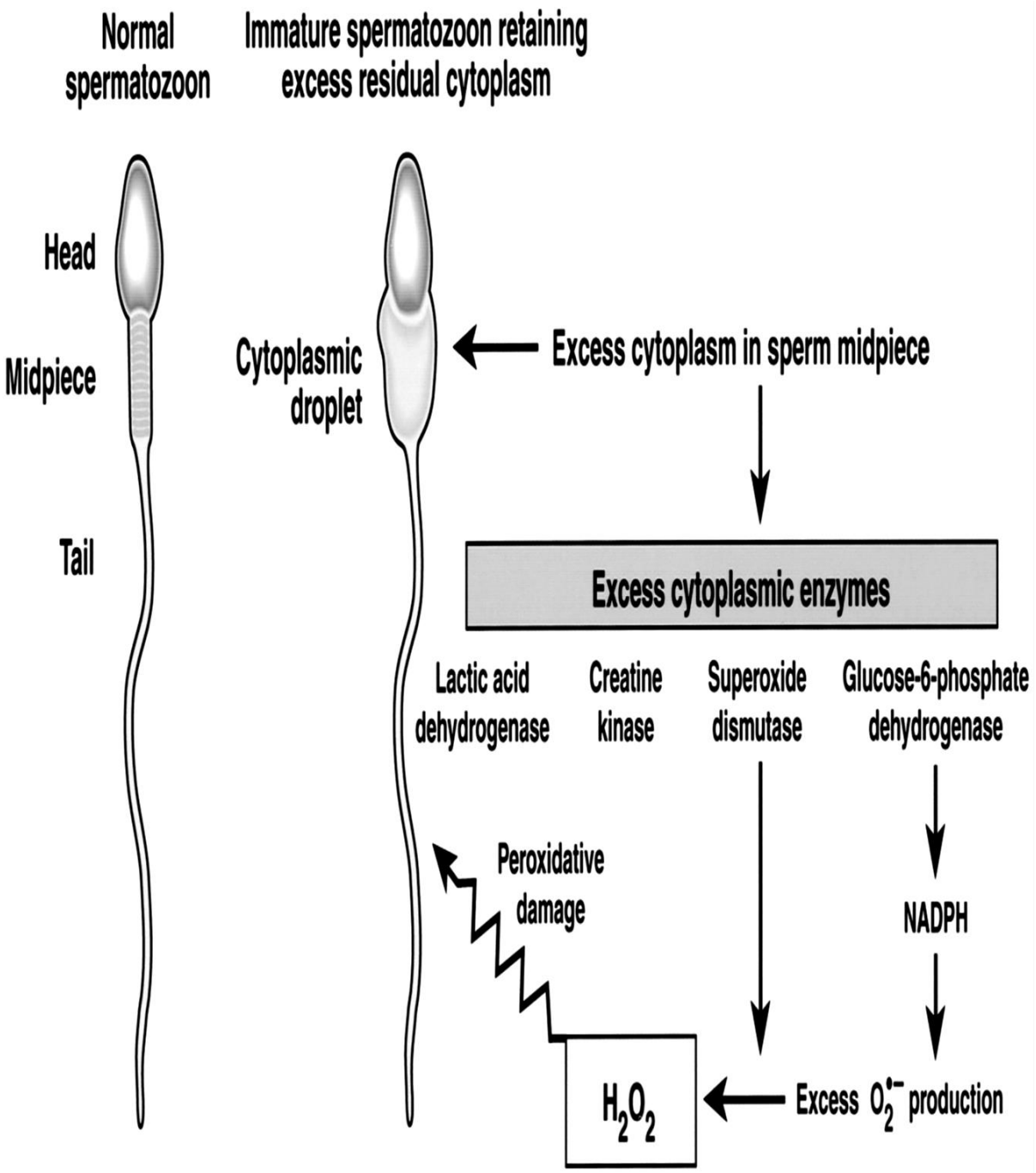


Figure 2.4 Mechanism of Increased Production of Reactive Oxygen Species (ROS) by Abnormal Spermatozoa (Spermatozoa with Cytoplasmic Retention) (Saleh and Agarwal, 2002). H_2O_2 – Hydrogen peroxide, $O_2^{\cdot-}$ - Superoxide radical

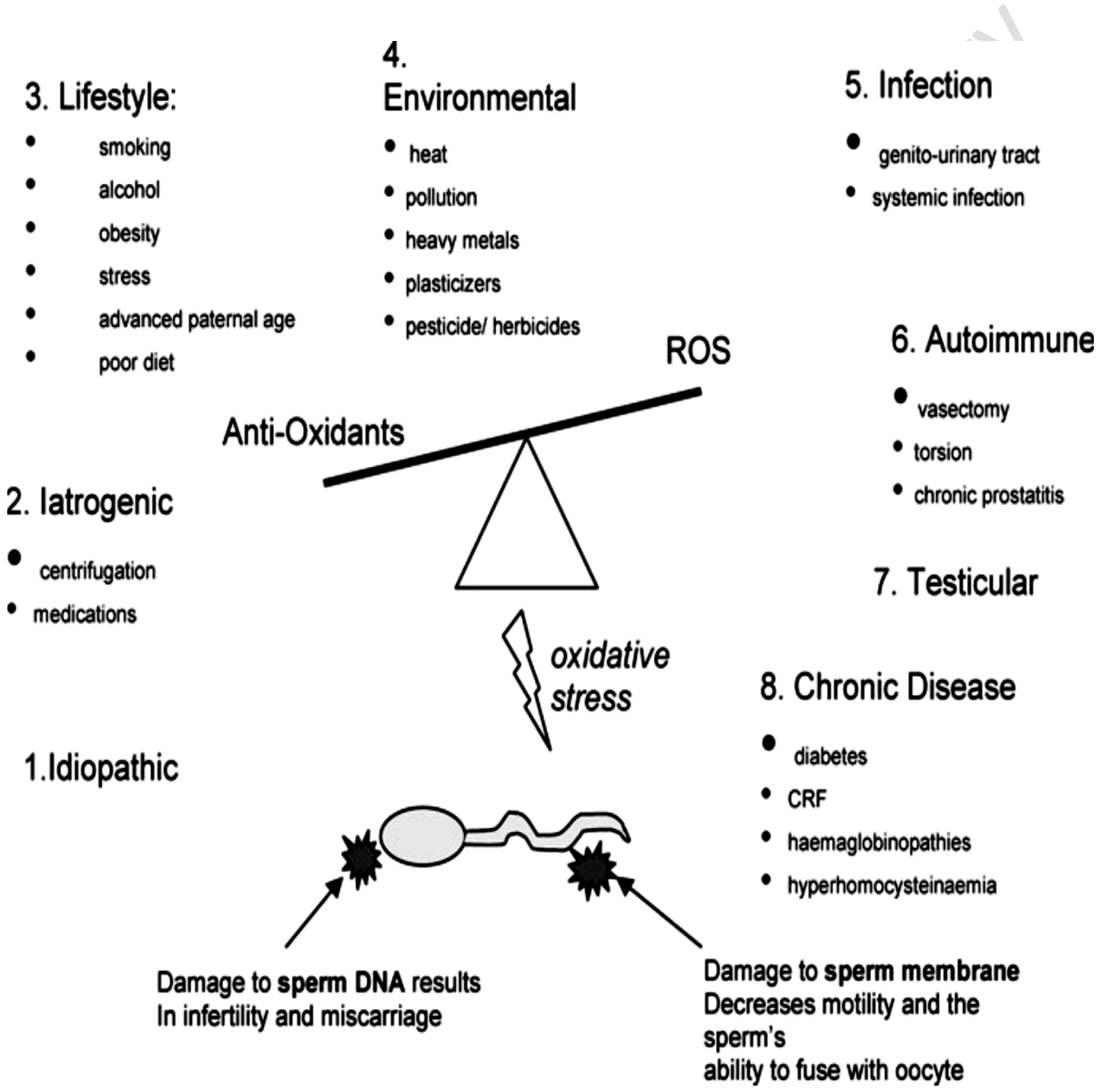


Figure 2.5. The Oxidative Stress Balance (Tremellen, 2008). ROS – Reactive oxygen species; CRF – Chronic Renal Failure

healthy non-smokers and correlated this with sperm quality (Eskenazi *et al.*, 2005) did observe a significant correlation between vitamin C intake and sperm concentration and between vitamin E intake and total progressively motile sperm. This is also consistent with earlier reports of a significant link between seminal plasma vitamin E levels and an increase in percentage of motile sperm (Therond *et al.*, 1996). Other researchers had linked low seminal plasma vitamin C levels with increased sperm DNA damage (Fraga *et al.*, 1991; Song *et al.*, 2006). Song *et al.* (2006) correlated sperm DNA damage with dietary antioxidant intake in infertile men.

Not surprisingly, excessive alcohol consumption causes an increase in systemic oxidative stress as ethanol stimulates the production of ROS, while many alcohol abusers have diets deficient in protective antioxidants (Wu and Cederbaum, 2003; Koch *et al.*, 2004). A study on a number of alcoholic men of reproductive age has suggested the presence of oxidative stress within the testicle by reporting a significant reduction in plasma testosterone, increase in serum lipid peroxidation byproducts and a drop in antioxidants (Maneesh *et al.*, 2006)..

Extremes of exercise activity, at both ends of the spectrum, have as well been linked with oxidative stress. It is not surprising that high impact exercise is linked with oxidative stress since muscle aerobic metabolism creates a large amount of ROS (Peake *et al.*, 2007). In a rodent model, increasing levels of exercise are linked with a reduction in sperm count and motility and a corresponding increase in biochemical signs of testicular oxidative stress (Manna *et al.*, 2004). Conversely, obesity produces oxidative stress as adipose tissue releases pro-inflammatory cytokines that increase leukocyte production of ROS (Singer and Granger, 2007). Furthermore, accumulation of adipose tissue within the groin region results in heating

of the testicle which has been linked with oxidative stress and reduced sperm quality (Banks *et al.*, 2005; Ishii *et al.*, 2005; Perez-Crespo *et al.*, 2007).

Psychological stress is another factor that produces a reduction in semen quality; with the underlying mechanism previously felt to be related to a central impairment of gonadotropin drive (Fenster *et al.*, 1997). However, studies have linked a period of psychological stress with a reduction in sperm quality mediated by an increase in seminal plasma ROS generation and a reduction in antioxidant protection (Eskiocak *et al.*, 2005; 2006).

Several studies have reported that sperm DNA damage increases with advancing age in both fertile and infertile men (Singh *et al.*, 2003; Moskovtsev *et al.*, 2006). It is possible that an increase in oxidative sperm DNA damage is the underlying pathology. A large observational study has confirmed that systemic oxidative stress increases with age (Junqueira *et al.*, 2004). Animal studies using the Brown Norway rat, an established model of male reproductive aging, confirm that sperm from older animals produce more free radicals than from young animals and have a reduced enzymatic antioxidant activity, resulting in an increase in ROS-mediated sperm DNA damage (Zubkova *et al.*, 2005; Weir and Robaire, 2007).

2.15.4 Environmental

Industrial waste products have a wide impact on human and environmental health, and the impact of these compounds on fertility has been investigated by several groups; an example of which are phthalates. Phthalates are chemicals used as a plastic softener and are contained in a wide range of food packaging and personal care products. Exposure to phthalates can occur via dietary consumption, dermal absorption or inhalation and has been linked with impaired spermatogenesis and increased sperm DNA damage (Kasahara *et al.*, 2002; Hauser *et al.*, 2007). Oral administration of phthalate esters to rats is reported to

increase the generation of ROS within the testis and a concomitant decrease in antioxidant levels, culminating in impaired spermatogenesis (Lee *et al.*, 2007).

Several other environmental pollutants have also been linked with testicular oxidative stress. Pesticides such as lindane, methoxychlor and the herbicide dioxin-TCDD have all been linked with testicular oxidative stress in rodent models (Tremellen, 2008). The commonly used preservative sulfur dioxide has also been shown to produce testicular oxidative stress in laboratory animals (Meng and Bai, 2004) and air pollutants such as diesel particulate matter act as potent stimuli for leukocyte ROS generation (Gonzalez-Flecha, 2004; Alaghmand and Blough, 2007). The relationship between airborne pollutants and testicular oxidative stress can be explained in the light that the oxidative insult caused by the pollutants is responsible for the increase in sperm DNA damage recorded following periods of airborne pollution (Tremellen, 2008).

Also, heavy metal exposure has been conclusively linked with sperm oxidative damage. Both cadmium and lead are linked with an increase in testicular oxidative stress and a resultant increase in sperm DNA oxidation (Xu *et al.*, 2003; Naha and Chowdhury, 2006). The increase in infertility and miscarriage observed in the partners of welders and battery/paint factory workers, recorded by Gennart *et al.* (1992) and Bonde (1993) may be due to oxidative damage to sperm DNA initiated by the inhalation of metal fumes.

2.15.5 Infection

2.15.5.1 Genitourinary Tract Infection

Men prone to recurrent genitourinary tract infections, such as paraplegics, have been confirmed to have high degrees of sperm oxidative pathology (Padron *et al.*, 1997; Brackett *et al.*, 2008). Bacteria responsible for genitourinary tract infection may originate from the urinary tract or can be sexually transmitted (Fraczek and Kurpisz, 2007; Fraczek *et al.*, 2008). Typical non-sexually transmitted pathogens include Streptococci (*S. viridans* and *S. pyogens*), coagulase-negative Staphylococci (*S. epidermidis, S. haemolyticus*), gram-negative bacteria

(*E. coli*, *Proteus mirabilis*) and atypical mycoplasma strains (*Ureaplasma urealyticum*, *Mycoplasma hominis*). All of these pathogens will create an acute inflammatory response with an influx of leukocytes into the genital tract and a resulting increase in ROS production (Ochsendorf, 1999; Potts *et al.*, 2000). Segnini *et al.* (2003) has also established the linkage between current or past Chlamydia infection and an increase in oxidative damage to sperm.

2.15.5.2 Systemic Infection

Several chronic systemic infections have been linked with increased oxidative stress throughout the body. Human immunodeficiency virus (HIV) infection is associated with an increase in leukocyte number and activation within semen (Umapathy *et al.*, 2001). Hepatitis B and C infection has also been correlated with significant hepatic oxidative stress (Chen and Siddiqui, 2007; Seronello *et al.*, 2007); and impaired sperm motility has been seen in hepatitis B and C patients (Durazzo *et al.*, 2006; Vicari *et al.*, 2006). Finally, chronic infections such as tuberculosis, leprosy, malaria and Chagas disease have all been linked with elevated degrees of systemic oxidative stress; all having both direct and indirect effects on the semen quality (Tremellen, 2008).

Viral infections may also initiate oxidative damage to sperm. Herpes simplex virus (HSV) has been found to have a possible role in the initiation of oxidative damage to sperm. Herpes simplex DNA is found in 4–50% of infertile men's semen, with IgM antibodies towards HSV being associated with a 10-fold increase in the rate of leukospermia (Bezold *et al.*, 2007; Krause *et al.*, 2002; 2003). Given the well-recognized link between leukospermia and seminal ROS levels, together with the observation of a reduction in sperm motility in men positive for seminal HSV DNA (Kapranos *et al.*, 2003), it is likely that HSV is a viral pathogen involved in oxidative stress unlikely that the male reproductive tract would be spared from this systemic oxidative insult.

2.15.6 Autoimmune

Chronic non-bacterial prostatitis (NIH Category III) is a chronic inflammation of the prostate in the absence of infection and has been reported by several groups to be associated with considerably elevated oxidative stress within semen (Pasqualotto *et al.*, 2000; Shahed and Shoskes, 2000; Potts and Pasqualotto, 2003). In the majority of cases of chronic non-bacterial prostatitis it is reported that an adverse autoimmune response to seminal or prostate antigens is responsible for the pathology, leading to an increase in pro-inflammatory cytokines and activated ROS producing leukocytes within the semen (Batstone *et al.*, 2002; Motrich *et al.*, 2005; 2007). It is therefore not surprising to see the majority of studies linking chronic nonbacterial prostatitis with a significant reduction in sperm density, motility, morphology and membrane integrity (Engeler *et al.*, 2003; Motrich *et al.*, 2005; Henkel *et al.*, 2006); although this is refuted by some groups (Pasqualotto *et al.*, 2000; Ludwig *et al.*, 2003). Oxidative stress has been proposed as a significant cause for infertility after vasectomy reversal. It is believed that vasectomy disrupts the normal blood-testis barrier, leading to a loss of immune privilege and activation of immune responses against sperm (Filippini *et al.*, 2001). Several studies have documented an increase in seminal leukocytes, pro-inflammatory cytokines and free radical production within semen following vasectomy reversal (Kolettis *et al.*, 1999; Sharma *et al.*, 1999; Nandipati *et al.*, 2005).

2.15.7 Testicular

Oxidative stress is now widely believed to be the principal underlying pathology linking varicocele with male infertility (Saleh *et al.*, 2003; Agarwal *et al.*, 2006). The increase in varicocele-related ROS production is strongly correlated with a reduction in sperm DNA integrity when assessed by either TUNEL (Smith *et al.*, 2006) or 8-hydroxy-20-deoxyguanosine DNA oxidative metabolite levels (Chen *et al.*, 2004).

Cryptorchidism is a common cause for male factor infertility in which the primary pathology is hypo-spermatogenesis due to deficient maturation of gonocytes to type-A

spermatogonia (Huff *et al.*, 1991). However, it has been reported that men with cryptorchidism surgically treated with orchidoplexy early in life still have markedly elevated sperm ROS production and DNA fragmentation compared with fertile controls (Smith *et al.*, 2007). Torsion of the spermatic cord has long been recognized as a cause of male infertility, even when this torsion is unilateral. It is now generally accepted that oxidative stress related to ischemia reperfusion injury is the underlying cause of damage to both the torted and contra-lateral testis. A prolonged period of ischemia followed by surgical or spontaneous restoration of blood flow leads to an influx of activated leukocytes into both testis (Turner *et al.*, 2004) and a consequent increase in generation of free radicals (Filho *et al.*, 2004). Oxidative stress then leads to necrosis of the germinal cells with resulting subfertility or infertility.

2.15.8 Chronic Disease

Diabetes has long been recognized to impair male fertility by interfering with both spermatogenesis and erectile function. Recently it has been reported that diabetic men have significantly higher levels of sperm DNA fragmentation than normal controls (Agbaje *et al.*, 2007). While this study did not directly measure oxidative stress, the authors proposed that to be the most likely mechanism. The toxic accumulation of homocysteine may cause reproductive dysfunction and oxidative stress within the testis (Forges *et al.*, 2007; Sonmez *et al.*, 2007). For the observed increase in sperm DNA damage was an increase in oxidative stress as this is now recognized as a key pathology underlying many chronic complications of diabetes. In support, studies using the Streptozotocin-induced diabetic rat model have found a significant increase in testicular oxidative stress within 6 weeks of initiation of the diabetic state (Shrilatha and Muralidhara, 2007).

Chronic inflammation and oxidative stress are highly prevalent in patients with chronic kidney disease and end-stage renal disease (Oberg *et al.*, 2004). Surprisingly, even when uraemia is reversed by haemodialysis, a persisting state of chronic inflammation and

oxidative stress persists (Danielski *et al.*, 2003; Pupim *et al.*, 2004). Furthermore, renal transplant patients with stable renal function and no obvious signs of immune rejection of their graft also have elevated levels of oxidative stress (Moreno *et al.*, 2005). Patients with haemaglobinopathies such as beta-thalassemia major have high degrees of systemic oxidative stress (Livrea *et al.*, 1996), with this oxidative damage confirmed to involve sperm (Carpino *et al.*, 2004). The likely cause of oxidative stress is iron overload from multiple blood transfusions. Iron is a potent pro-oxidant capable of redox cycling when not safely bound to transferrin in the blood or stored as ferritin in tissue.

CHAPTER THREE

3.0: Materials and Methods

3.1: Materials

3.1.1: Collection and Identification of Plant Material

Fresh stem barks of *Allanblackia floribunda* were collected from J4 forest reserve in Ogun State, Nigeria. The plant was identified and authenticated by Mr. G. A. Ibhanesebor at Ife Herbarium, Department of Botany, Obafemi Awolowo University, Ile-Ife, Nigeria. The voucher specimen was deposited in the Herbarium of the same department and the specimen identification number is IFE 7379.

3.1.2: Reagents and Chemicals

All the reagents used in the study were of analytical grade. The reagents/chemicals were obtained from reputable sources such as British Drug House (BDH) Chemicals Limited, London, Sigma Fine Chemicals Limited, Upsalla, Sweden. Diagnostic kits from Randox Laboratories Ltd, United Kingdom. All solutions, buffers and reagents were prepared with distilled water and stored in the refrigerator at 4^0C.

3.1.3: Equipment

The equipment used were Vis-Spectrophotometer (Model S23A, Gulfex Medical and Scientific, England), Bench centrifuge (Model 90-2, Microfield instrument, Essex England), Edward High Vacuum Pump (Model ED-100; Edward Vacuum Components, Crawley, England), Neubauer's counting chamber and pH meter (Techmel and Techmel, U.S.A).

3.1.4: Experimental Animals

The male Wistar rats used in this study were obtained from the animal house, Faculty of Pharmacy, Obafemi Awolowo University, Ile-Ife, Nigeria. The animals were acclimatized for two weeks, fed with standard commercial pellet diet (Ladokun Feeds, Ibadan) and given access to water *ad libitum*. The rats were housed under standard conditions, at natural light and dark cycle.

3.2: Methods

3.2.1: Preparation of Ethanolic Extract of *A. floribunda*

The dried barks of the *Allanblackia floribunda* (1.5 kg) was ground into fine smooth powder using impact mill. The powdered stem bark (500 g) was soaked in 70% ethanol (2.5 l) for 72 h, and filtered afterwards with a double layered cheese cloth and concentrated to dryness using a rotary evaporator Model ED-100.

3.2.2 Partitioning into Fractions

The ethanolic extract (50 g) was taken up in hot distilled water (200 ml). The filtrate was partitioned sequentially with hexane, ethyl acetate and butanol in capped separating funnel. The content was vigorously shaken, allowed to settle down and carefully separated. The process was repeated several times until the colour of the solvent remained unchanged. The same fractions were combined and concentrated in rotary evaporator separately. The fractions and aqueous residue were dried in a desiccator, weighed, labeled and kept in the deep freezer at -4^{O} C until needed for further analyses.

3.2.3: Phytochemical Screening of the Aqueous Extract

The phytochemical screening of the extract was carried out by a procedure that was based on the earlier reports of Trease and Evans, (1978) and Sofowora (2006).

3.2.3.1: Test for Flavonoids

The extract of *A. floribunda* (5.0 mg) was dissolved separately in 5.0 ml of distilled water, followed by filtration. To 1.0 ml of the filtrate, few drops of ethanolic potassium hydroxide solution were added. The resulting solution was examined for formation of suspension, cloudiness or precipitation.

3.2.3.2: Test for Tannins

The extract (0.5 g) was dissolved in 20 ml of distilled water in a test tube and then filtered. Few drops (2-3) of 0.1% (w/v) ferric chloride in glacial acetic acid solution was added to the filtrate. The mixture was observed for a brownish green or blue-black precipitate for the confirmation of the presence of tannins.

3.2.3.3: Test for Alkaloids

Acidic solution of the extract was prepared by accurately weighing 50 mg into three separate test tubes and 10 ml of 10% (v/v) HCl was added. The test tubes were heated and filtered. Then, to each of the filtrates, 1.0 ml of Mayer's reagent, Wagner's reagent, and Drangendorff reagent were added respectively. The mixtures were examined for colour change, turbidity or formation of precipitate. Equal volume of 10% (v/v) HCl was used as parallel control.

3.2.3.4: Test for Saponins (Frothing Test)

The extract (0.05 g) suspended in 2.0 ml distilled water in a test tube was vigorously shaken and noted for froth. Then, the tube was warmed gently at about 70^{o}C and shook vigorously again. The appearance and persistence of frothing before and after warming was noted.

3.2.3.5: Test for Anthraquinone

The extract (0.5 g) was boiled in 2.0 ml of dilute sulphuric acid and filtered while it is still hot. To the filtrate, 2.5 ml of benzene was added and the mixture was vigorously shaken. The benzene layer was separated and to half its volume, 1ml of 10% (v/v) ammonia solution was added. The ammonia layer was observed for either a pink, red or violet colouration.

3.2.3.6: Test for Steroids

Acetic anhydride (2 ml) and 2 ml of conc. H_2SO_4 were added to 0.5 g of the extract and shaken. A colour change from violet or light brown to deep viscous brown indicates the presence of steroids.

3.2.3.7: Test for Cardiac Glycosides

The extract (0.5 g) was extracted with 2 ml chloroform and filtered into a clean test tube. Concentrated sulphuric acid was carefully added to form a lower layer. A reddish brown colour ring at the chloroform/sulphuric acid interphase indicates the presence of a steroidal ring or glycine of the cardiac glycosides.

3.2.3.8: Test for Xanthoproteins

The extract (0.1 g) was dissolved in 5 ml distilled water and filtered. The filtrate was shaken with drops of dilute nitric acid and ammonia solution. Formation of coloured precipitate indicates the presence of xanthoproteins.

3.2.3.9: Test for Phlobatannins

The aqueous extract (0.5 g) was dissolved in 10 ml of water in a test tube and then filtered. The filtrate (1 ml) was shaken with 10% HCl and then observed for the deposition of red precipitate which indicates of the presence of phlobatannins.

3.2.3.10: Test for Triterpenes

The extract (0.5 g) was gently warmed with 5 ml of chloroform and filtered. Concentrated H_2SO_4 (2 ml) was carefully added to the filtrate and a reddish brown colouration formed at the interface indicated the presence of triterpenes.

3.3: Acute Toxicity Study (LD_{50} Determination)

Acute toxicity study was carried out according to the method of Lorke (1983) in two phases. Albino mice (18) weighing between 18-35 g were distributed into three groups of three mice per group. In phase one, nine (9) mice randomly divided into three groups were administered single dose of 10, 100 and 1000 mg/kg body weight of the aqueous extract respectively. The general behavior of the mice was observed continuously for one hour (hr) after the treatment and then intermittently for 4 hours over a period of 24 hours for any signs of toxicity, deaths and the latency of death. Based on the results obtained from phase one study, the procedure was repeated (phase II) using another set of nine (9) mice randomly divided into three groups and were orally administered 1600, 2900 and 5000 mg/kg body weight, respectively. The LD_{50} was estimated from the plot of percentage mortality versus logarithm of concentrations.

3.4: Sub-Chronic Toxicity Study

The study was carried out as described by Biswas *et al.* (2010). A total of forty five albino rats of average weight between 150-250 g were randomly distributed into nine groups

of five rats per group. Group 1 served as control and were administered normal saline, while rats in groups 2 to 9 were orally administered 200 and 300 mg/kg body weight of the aqueous, ethyl acetate, butanol and crude extract respectively, once daily for 28 days. The rats were weighed before the commencement of treatment and thereafter weighed weekly throughout the duration (28 days) of the study. On the 29th day, after administration of the extract, the animals were sacrificed. The blood samples were collected by ocular puncture into plain bottles for estimation of biochemical parameters and the organs such as the testes, epididymis, liver, brain and kidney were excised, weighed, rinsed with normal saline and stored for further biochemical and histological studies.

3.4.1:Preparation of Blood Serum

The blood collected in plain bottle was allowed to clot for 30 minutes and later centrifuged at 3000 rpm for 10 min. The supernatant (serum) was stored in sterile vial and kept in freezer for biochemical analyses.

3.4.2 Preparation of Tissue Homogenate

Testes were surgically removed and a 10% (w/v) tissue homogenate was prepared by homogenizing the testes in phosphate buffer solution, pH 7.4 using mortar and pestle over ice. The homogenates were centrifuged at 10,000xg using a cold centrifuge and the supernatant was collected as a source for the determination of marker enzyme activity, and anti-oxidants.

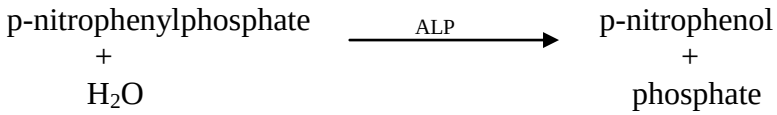
3.5: Biochemical Assays:

3.5.1:Estimation of Alkaline Phosphatase (ALP) Activity

The activity of ALP in serum was assayed according to the recommendations of the Deutsche Gesellschaft fur Klinische Chemie (Rec. GSCC DGKC) (1972), using Randox Diagnostic Kit. The kit contained a Substrate vial (p-nitrophenylphosphate) and buffer system consisted of 100 mol/L Diethanolamine buffer, pH 9.8 and 0.5 mmol/L magnesium chloride.

Principle:

Alkaline Phosphatase catalyses the hydrolysis of p-nitrophenylphosphate to form p-nitrophenol. The rate of formation of p-nitrophenol was monitored and measured spectrophotometrically at 405 nm which is proportional to ALP activity.



Procedure:

Serum (0.02 ml) was pipetted into 1 cm cuvette at room temperature ($25^{O}C$) and the reaction was initiated by addition of 1.0 ml substrate. The initial absorbance was taken immediately at 405 nm and repeated for the first, second and third minutes consecutively so as to obtain the change in absorbance/min. ALP activity was calculated by the expression;

$$\text{ALP activity (U/L)} = 2760 \times \Delta\text{A } 405 \text{ nm/min}$$

One unit of alkaline phosphatase will catalyze the hydrolysis of 1.0 micromole of p-nitrophenylphosphate to form p-nitrophenol and phosphate per minute at pH 7.0, at $25^{O}C$.

3.5.2 Assay for Prostatic Acid Phosphatase (PACP) Activity

The activity of PACP in plasma was assayed according to the method of Seiler, (1983), using Randox Diagnostic Kit. The kit contained two substrate vials, one to assay for total acid phosphatase (1-naphthylphosphate and 4-chloro-2-methylhenyldiazonium salt) and the other to assay for non-prostatic acid phosphatase (Sodium Tartrate); and a buffer system of Citrate buffer 75 mmol/L, pH 5.2.

Principle:

Acid Phosphatase catalyzes the hydrolysis of 1-naphthylphosphate to form 1-naphthol and inorganic phosphate. The 1-naphthol further reacts with 4-chloro-2-methylhenyldiazonium salt to form an azo dye. The rate of formation of the azo dye was monitored and measured spectrophotometrically at 405 nm which is proportional to ACP activity.

1-naphthyl phosphate + H_2O $\xrightarrow{ACP}$ 1-naphthol + phosphate

1-naphthol + 4-chloro-2-methylhenyldiazonium salt $\longrightarrow$ azo dye

Procedure:

Serum (0.025 ml) was pipetted into 1 cm cuvette at room temperature ($25^{O}C$) and the reaction was initiated by the addition of 0.5 ml substrate. The initial absorbance was taken immediately at 405 nm and repeated for the first, second and third minutes consecutively so as to obtain the change in absorbance/min. PACP activity was calculated by the expression;

$$\text{PACP activity (U/L)} = 743 \times (\Delta\text{A TACP 405 nm/min} - \Delta\text{A NPAP 405 nm/min})$$

One unit of acid phosphatase will catalyze the hydrolysis of 1.0 micromole of 1-naphthylphosphate to form 1-naphthol and inorganic phosphate per minute at pH 7.0, at $25^{O}C$.

3.5.3 Assay for Glucose – 6 - Phosphate Dehydrogenase Activity

The activity of G-6-PDH in testes was assayed according to the method of Seiler, (1983), using Randox Diagnostic Kit. The kit contained two substrate vials, R2 ($NADP^+$) and R3 (Glucose-6-Phosphate); and a buffer system of EDTA, 3.2 mmol/L and Triethanolamine buffer 31.7 mmol/L, pH 7.6.

Principle:

Glucose-6-phosphate dehydrogenase catalyzes the oxidation of Glucose-6-phosphate in the presence of $NADP^+$ to give Gluconate-6-phosphate. The rate of absorbance change due to the reduction of $NADP^+$ is then measured at 340nm.

Glucose-6-Phosphate + $NADP^+$ $\xrightarrow{G\text{-}6\text{-}PDH}$ Gluconate-6-Phosphate + NADPH + H^+

Procedure

Buffer (1.00 ml) was pipetted into 1 cm cuvette at room temperature ($37^{O}C$) followed by the addition of $NADP^{+}$. The reaction was initiated by the addition of 0.015 ml substrate (testes tissue homogenate). The solution was mixed and left to incubate for 5 minutes at room temperature, then 0.015ml of substrate (Glucose-6-Phosphate), was added. Initial absorbance was taken immediately at 340 nm and repeated for the first, second and third minutes consecutively so as to obtain the change in absorbance/min. G-6-PDH activity was calculated by the expression;

$$\text{G-6-PDH activity } (mU/g/dL) = 33650 \times \Delta A\ 340\ nm/min$$

One unit of Glucose-6-phosphate dehydrogenase catalyzes the oxidation of 1.0 micromole of Glucose-6-phosphate in the presence of $NADP^{+}$ to give Gluconate-6-phosphate per minute at pH 7.0, at $25^{O}C$.

3.5.4 Assay for Lactate Dehydrogenase Activity

The activity of LDH in serum was assayed according to the recommendations of Deutsche Gesellschaft fur Klinische. Chemie (Rec. GSCC DGKC, 1970), using Randox Diagnostic Kit. The kit contained a Substrate vial (NADH) and buffer system consisted of 50 mmol/L phosphate buffer, pH 7.5 and 0.6 mmol/L pyruvate.

Principle:

LDH catalyses the conversion of pyruvate to L-lactate. The rate of formation of L-lactate was monitored and measured spectrophotometrically at 340 nm which is proportional to LDH activity.

$$\text{Pyruvate} + \text{NADH} + H^{+} \xrightarrow{\text{LDH}} \text{L-lactate} + NAD^{+}$$

Procedure:

Serum (0.02 ml) was pipetted into 1 cm cuvette at room temperature ($25^{O}C$) and the reaction was initiated by addition 1.0 ml substrate. The initial absorbance was taken after

12 seconds at 340 nm and repeated for the first, second and third minutes consecutively so as to obtain the change in absorbance/min. LDH activity was calculated by the expression;

$$\text{LDH activity (U/L)} = 4127 \times \Delta A\ 340\ \text{nm/min}$$

One unit of Lactate dehydrogenase will catalyze the reduction of 1.0 micromole of pyruvate to L-lactate per minute at pH 7.0, at 25^{O}C.

3.5.5 Assay for Catalase Activity

The catalase activity was determined according to the direct UV assay method of Aebi (1973).

Principle

The principle of the assay was based on the disappearance of H_2O_2 in the presence of the catalase.

Procedure:

The reaction mixture consisted of 50 µl of the test sample, 950 µl of (50 mM phosphate buffer, pH 7.0). The reaction was initiated by the addition of 500 µl of freshly prepared 30% (v/v) hydrogen peroxide (H_2O_2). The rate of decomposition of the peroxide was measured by reading the absorbance at 240nm. The absorbance was read at 5 seconds interval for 30 seconds. The activity of the enzyme was calculated from the following expression and expressed as units/min/mg protein:

$$\text{Catalase activity (units/min)} = \frac{[\Delta A/\text{min (blank)} - \Delta A/\text{min (sample)}] \times d \times k}{V \times 0.0436}$$

d = dilution of original sample for catalase reaction

v = sample volume in catalase reaction in ml

0.0436 = extinction coefficient for catalase

K = total reaction volume in ml

One unit of catalase will decompose 1.0 micromole of hydrogen peroxide to oxygen and water per minute at pH 7.0, at 25^{O}C.

3.5.6 Estimation of Total Protein Concentration.

Total protein test kit (Randox Diagnostic Laboratories, Ltd) was used for the estimation of the plasma total protein according to the biuret method, as reported by Tietz (1995). The kit contained Biuret reagent (consisted of 100 mM/L NaOH, 16 mM/L Na-K-tartarate, 15 mM/L Potassium iodide and 6 mMol/L Cupric sulphate) and a blank reagent (consisted of 100 mM/L NaOH and 16 mM/L Na-K-tartarat).

Principle:

Proteins, in alkaline medium, bind with the cupric ions present in the Biuret reagent to form a blue-violet coloured complex. The intensity of the colour formed is directly proportional to the amount of proteins present in the sample.

$$\text{protein} + Cu^{++} \rightarrow \text{Blue Violet Coloured Complex}$$

Procedure

The plasma (0.01 ml) was separately pipetted into clean test tubes in triplicates and 0.5 ml Biuret reagent was added and mixed thoroughly. The standard contained 0.01 ml Biuret reagent and 0.5 ml standard solution. Each of the mixtures was incubated for 30 min. at $25^{O}C$ and the absorbance of the sample and standard were measured against the sample at 546 nm against a sample blank prepared with distilled water (0.01 ml) in place of sample. The total protein concentration was estimated from the expression below;

$$\text{Total protein concentration (g/dL)} = \frac{\text{Abs}_{\text{sample}}}{\text{Abs}_{\text{standard}}} \text{ X Standard concentration (g/dL)}$$

3.5.7 Estimation of Glutathione Peroxidase Activity

The activity of glutathione peroxidase (GPx) was determined by the method of Rotruck *et al.*, (1973).

Principle:

Glutathione peroxidase converts reduced glutathione (GSH) into oxidized form using hydrogen peroxide during its reaction. The amount of GSH utilized is estimated by measuring

it in the assay mixture before and after the enzyme activity. GSH reacts with DTNB to give yellow colour, which was then measured at 412nm.

Procedure:

Buffer (0.5 ml), 0.1 ml sodium azide, 0.2 ml reduced glutathione, 0.1 ml H_2O_2, and 0.5 ml 1:10 diluted aliquot of the tissue homogenate were taken and the total volume was made up to 2.0 ml with distilled water. The tubes were incubated at 37^OC for 3 minutes and the reaction was terminated by the addition of 0.5 ml 10% (w/v) TCA. To determine the residual glutathione content, the supernatant was removed after being centrifuged at 1500 rpm for 8 minutes, and to this 4.0 ml of disodium hydrogen phosphate (0.3 M) solution and 1 ml of the DTNB reagent were added. The colour developed was read at 412 nm against a reagent blank containing only phosphate solution and DTNB reagent. Suitable aliquots of the standard were also treated similarly. One unit of glutathione peroxidase will catalyze the conversion of 1.0 micromole of reduced glutathione into oxidized form per minute at pH 7.0, at 37^OC.

3.6: Histopathological Analysis

Portions of the tissue from testes were used for histopathological examination.

Tissue Processing

The tissues were fixed in 10% formo-saline to prevent putrefaction and autolysis and they were processed as follows:

Dehydration: After fixation, the tissues were transferred into increasing concentrations of alcohol for dehydration at room temperature as follows: 70% alcohol, 80% alcohol, 90% alcohol, Absolute alcohol I, Absolute alcohol II.

Clearing: Dehydrated tissues were cleared at room temperature in two changes of xylene to replace the alcohol.

Infiltration: The tissues were then infiltrated in two changes of molten paraffin wax.

Embedding: Infiltrated tissues were embedded in paraffin wax to provide rigid support for microtomy. Sections of 5 μm thickness were produced from the tissues using a LEICA rotary microtome (Bright B5143 Huntington, England). These sections were floated in a water bath (45^{O}C) to allow spreading of the folded sections. These sections were mounted on new clean glass slides. The sections were then dried at 40^{O}C on a slide drier to enhance adherence of the sections to the slide.

Staining Method

Sections on the slide were de-waxed in xylene, treated with descending grades of alcohol as follows: Absolute alcohol, 90% alcohol, 80 % alcohol, 70 % alcohol, 50 % alcohol. Sections were stained with alum haematoxylin (Harris's). Excess stains were removed by rinsing in water. Sections were differentiated in a mixture of (1% hydrochloric acid in 70 % alcohol) to remove excess dye from the tissues. Sections were washed until nuclei were blue.

Stained with eosin and rinsed in water. Following a brief wash in water, sections were dehydrated rapidly in ascending grades of alcohol as follows: 50 % alcohol, 70 % alcohol, 80 % alcohol, 90 % alcohol, Absolute alcohol. Cleared in xylene and mounted in DPX (Distrene Plasticizer and Xylene) with a coverslip. The stained tissues were observed under the microscope (LEICA DM750) interfaced with a LEICA (ICC_{50}) camera.

3.7 Sperm Motility

The motility of sperm was evaluated directly using the Neubauer's counting chamber, after mincing in drop of sperm suspension, microscopically. Non-motile sperm numbers were first determined, followed by counting of total sperm. Sperm motility was expressed as percent of motile sperm of the total sperm counted.

3.8 Sperm Viability by Eosin Stain

This technique was used to differentiate between live and dead sperms. A drop of the Eosin stain added into sperm suspension on the slide and allowed to stand for 5 min. at 37°C,

then examined under microscope. The head of dead spermatozoa were stained red, while, the live spermatozoa remained unstained with Eosin. Sperm viability was expressed as percentage of live sperm of the total sperm counted.

3.9 Sperm Morphology

A drop of Eosin stain was added to the sperm suspension and kept for 5 min. at 37°C. Following this, a drop of sperm suspension was placed on a clean slide and spread gently to make a thin film. The film was air dried and then observed under a microscope for changes in sperm morphology. The criteria chosen for head abnormality were; no hook, excessive hook, amorphous, pin and short head. For tail, the abnormalities recorded were; coiled flagellum, bent flagellum, bent flagellum tip. The result are the percentage overall abnormal form.

3.10: Statistical Analysis

Data were expressed as Mean ± SEM, $n = 5$. The presence of significant differences among means of groups was determined by One-way Analysis of Variance (ANOVA), using GraphPad Prism (GraphPad Software, San Diego, CA, USA). Differences were considered to be significant if $p < 0.05$.

CHAPTER FOUR

4.0: Results

4.1: Yield of Ethanol Crude Extract of *A. floribunda*

The total yield of the extract weighed 277.03 g which represents 18.5 % of the starting material.

4.2: Phytochemical Constituents of Ethanol Extract and Other Fractions

Table 4.1 shows the summary of the phytoconstituents of the ethanolic extract, ethyl acetate, butanol and aqueous fractions of *A. floribunda*. The ethanolic extract test positive for the presence of flavonoids, tannins, alkaloids, saponins, cardiac glycosides and triterpenes while phlobatannins and anthraquinones were absent.

4.3: Acute Toxicity and Behavioral Effect of Ethanol Extract

Table 4.2 showed the acute toxicity effect of ethanolic *A. floribunda* in mice. There was no mortality recorded in animals treated with a single dose of 10 - 5000 mg/kg body weight. The median lethal dose (LD_{50}) of aqueous extract in the experimental mice was estimated to be greater than 5000 mg/kg body weight.

4.4: Subacute Toxicity Studies

4.4.1: Effect of Ethanolic Extract of *A. floribunda* and other Fractions on the Body

Weight of Experimental Rats

The body weight of each group was recorded before the initial administration of the different extracts and at a weekly interval as shown in (Table 4.3). The body weights of the rats at extract dose of 200 mg/kg body weight were observed to increase progressively throughout the weeks of the study with the exception of rats administered the ethyl acetate fraction which showed percentage change in the mean body weight of -1.93 %. However, at 300 mg/kg body weight, the weights of the rats increased but not as profound as those of the lower dosages with ethyl acetate and crude fractions recording a percentage body weight change of -1.65 and -3.55 % respectively.

Table 4.1: Phytochemical Constituents of the Ethanolic Extract and Fractions of *A. floribunda*

Phytochemical constituents	Ethanolic Extract	Aqueous fraction	Ethyl acetate fraction	Butanol fraction
Alkaloid	++	++	+	+
Cardiac glycoside	+++	+	+	+++
Flavonoids	++	++	++	+
Saponins	+++	+++	-	++
Tannins	+++	+	-	+++
Triterpenes	+++	+	+	+++
Phlobatanins	-	-	-	-

(+) represent positive result and (-) represent negative result

Table 4.2: Acute Toxicological Effect of Ethanolic Extract of *A. floribunda* in Mice

Dose (mg/kg)	Mortality
First Phase	
10	0/3
100	0/3
1000	0/3
Second Phase	
1600	0/3
2600	0/3
5000	0/3

Table 4.3: Effect of Ethanolic Extract of *A. floribunda* and other fractions on the Body Weight of Experimental Rats

	Body Weight Change of Rats (g)		
GROUPS	**INITIAL**	**FINAL**	**%ΔBWt.**
Control	158.46±5.13	211.04±8.52	33.54
200 mg/kg			
Aqueous	157.48±8.30	199.08±7.67	26.75
Ethyl Acetate	207.08±10.22	203.64±11.77	-1.93
Butanol	150.62±10.44	200.12±9.36	33.33
Crude	153.04±8.30	208.16±8.38	35.95
300 mg/kg			
Aqueous	164.32±19.19	170.36±14.44	3.66
Ethyl Acetate	182.46±13.90	179.98±15.07	-1.65
Butanol	159.65±12.73	182.50±14.95	14.47
Crude	169.36±28.40	163.76±24.23	-3.55

Values are presented as mean ± SEM of five (5) replicates. %ΔBWt. – Percentage Body Weight

4.4.2: Effects of Ethanolic Extract and other Fractions of *A. floribunda* on Relative Weights of Testes and Accessory Organs in Male Wistar Rats.

Table 4.4 shows the summary of the effects of the crude ethanolic extract, aqueous, butanol and ethyl acetate fractions of *A. floribunda* on relative weight of the testes, prostate, seminal vesicle and epididymis. With the exception of Group 7 (300 mg/kg Ethyl Acetate), other groups have slightly lower testicular weights when compared with the Control. Relative weights of prostate of all the other groups were slightly higher than that of the Control but not significantly.

Also, relative weights of seminal vesicle of the groups were lower than that of the Control but not significantly. The same trend was observed with the relative weights of the epididymis as well; all the groups with the exception of Group 7 had lower relative epididymis weight when compared with the control.

In general, Group 9 (300 mg/kg Crude) had the highest relative prostate weight and lowest relative testes, seminal vesicle and epididymis weights.

4.4.3:Effect of Ethanolic Extract and other Fractions of *A. floribunda* on Testicular Marker Enzymes of Male Wistar Rats

The summary of the effects of the crude ethanolic extract, aqueous, butanol and ethyl acetate fractions of *A. floribunda* on testicular marker enzymes are shown on Table 4.5. In general, there was increase in Acid Phosphatase (ACP) activity across all other groups when compared with control; with Group 2 (200 mg/kg Aqueous Fraction), Group 3 (200 mg/kg Ethyl Acetate Fraction) and Group 9 (300 mg/kg Crude extract) having a significant increase of 80.38%, 72.03% and 89.15% respectively.

However, there was a general decrease in Alkaline Phosphatase (ALP) activity across the groups when compared with the control group. The decrease observed was dose

dependent with Groups 6, 7, 8 and 9 having a lower activity when compared with Group 2, 3, 4 and 5.

Table 4.4: Relative Organ:Body Weight Ratio (%)

Organ / Group	Testes	Prostate	Seminal Vesicle	Epididymis
Group 1 (Control)	0.16±0.02	0.14±0.01	0.59±0.09	0.16±0.02
Group 2 (AQF 200 mg/kg bwt)	0.14±0.12	0.18±0.01	0.53±0.08	0.14±0.01
Group 3 (EAF 200 mg/kg bwt)	0.15±0.01	0.17±0.01	0.53±0.04	0.15±0.01
Group 4 (BUT 200 mg/kg bwt)	0.13±0.01	0.17±0.01	0.46±0.01	0.13±0.01
Group 5 (CRU 200 mg/kg bwt)	0.13±0.01	0.16±0.01	0.56±0.06	0.13±0.01
Group 6 (AQF 300 mg/kg bwt)	0.15±0.01	0.18±0.02	0.45±0.07	0.15±0.01
Group 7 (EAF 300 mg/kg bwt)	0.16±0.01	0.18±0.01	0.39±0.04	0.16±0.01
Group 8 (BUT 300 mg/kg bwt)	0.14±0.01	0.16±0.02	0.39±0.04	0.14±0.01
Group 9 (CRU 300 mg/kg bwt)	0.12±0.01	0.19±0.02	0.34±0.07	0.11±0.01

Values are expressed as mean ± SEM, n = 5 replicates. Values with (*) are statistically significant at $p < 0.05$ when compared to the control group. AQF represents (Aqueous Fraction), EAF (Ethyl Acetate Fraction), BUT (Butanol Fraction), CRU (Crude Extract).

Table 4.5: Effects of Aqueous, Butanol, Ethanolic and Ethyl Acetate Extract of *A. floribunda* on Testicular marker enzymes in Rats

Parameter / Group	ACP (U/L)	ALP (U/L)	LDH (U/L)	G6PDH (U/L)	TOTAL PROTEIN (g/dl)
Group 1 (Control)	2.05±0.86	362.66±21.53	103.62±14.21	179.47±19.69	5.46±0.29
Group 2 (AQF 200 mg/kg bwt)	10.45±1.96*	327.89±13.95	170.64±27.10*	98.71±5.94	6.02±0.24
Group 3 (EAF 200 mg/kg bwt)	7.33±0.10*	272.14±29.70	153.00±16.62	103.47±9.94	5.76±0.20
Group 4 (BUT 200 mg/kg bwt)	2.75±1.32	300.29±21.37	143.77±13.96	123.38±12.94	6.14±0.30
Group 5 (CRU 200 mg/kg bwt)	3.11±1.10	332.30±41.88	154.78±21.30	72.91±16.52*	4.60±0.16
Group 6 (AQF 300 mg/kg bwt)	4.77±0.37	230.18±18.89*	96.17±15.48	73.19±5.39*	5.05±0.74
Group 7 (EAF 300 mg/kg bwt)	7.13±0.70	222.46±14.46*	106.37±7.61	82.28±13.63*	5.47± 0.23
Group 8 (BUT 300 mg/kg bwt)	8.30±1.70	249.23±14.24	96.94±11.81	90.86±15.78*	5.31± 0.36
Group 9 (CRU 300 mg/kg bwt)	18.89±1.41*	265.51±47.22	130.17±18.23	70.67±9.62*	5.86±0.24

Values are expressed as mean ± SEM, n = 5 replicates. Values with (*) are statistically significant at $p < 0.05$ when compared to the control group. ACP represents (Acid Phosphatase), ALP (Alkaline Phosphatase), LDH (Lactate Dehydrogenase), G6PDH (Glucose-6-Phosphate Dehydrogenase). AQF (Aqueous Fraction), EAF (Ethyl Acetate Fraction), BUT (Butanol Fraction), CRU (Crude Extract).

Group 6 and 7 has a significant decrease of 57.56% and 63.02% respectively, compared with the control.

The activity of Lactate Dehydrogenase (LDH) showed some groups having lower values when compared with the Control Group, while other group had higher values.

Specifically, Group 6 and 8 had a non-significant lower activity while Groups 3, 4, 5, 7 and 9 had increased activity that was not significant. Group 2 however had a significant increase of 39.28% in LDH activity.

The Glucose-6-Phosphate Dehydrogenase activity (G6PDH) in all the groups were lower when compared with the Control Group with Groups 5, 6, 7, 8 and 9 having a significant decrease of 146.15%, 118.12%, 97.52% and 153.96% respectively.

Finally, there was no significant increase in Total Protein concentration (TP) in Groups 2, 3, 4, 7 and 9 while Groups 5, 6 and 8 had a non-significant decrease in Total Protein concentration when compared with the Control Group.

4.4.4: Effect of Ethanolic Extract and other Fractions of *A. floribunda* on Testicular Antioxidant Enzymes

The summary of the effects of the crude ethanolic extract, aqueous, butanol and ethyl acetate fractions of *A. floribunda* on testicular antioxidant enzymes are shown on Table 4.6. There was no significant decrease in Catalase activity (CAT) in all the groups when compared with the Control which had an activity of 0.62±0.36 U/L. Amongst the other Groups, Group 4 (200 mg/kg Butanol Fraction) had the highest Catalase Activity of 0.53±0.21 U/L and Group 5 (200 mg/kg Crude extract) had the lowest value of 0.21±0.05 U/L. Also, Glutathione Peroxidase activity (GPx) varied across all groups with Groups 2, 3, 4, 7, 8, 9 having slightly lower activity values that are not significant when compared with the Control Group.

e Table 4.6: Effect of Ethanolic Extract and other Fractions of *A. floribunda* on

Groups	Catalase (U/L)	Glutathione peroxidase (U/L/mg protein)
Group 1 (Control)	0.62±0.36	0.21±0.02
Group 2 (AQF 200 mg/kg bwt)	0.28±0.05	0.19±0.01
Group 3 (EAF 200 mg/kg bwt)	0.25±0.04	0.15±0.01
Group 4 (BUT 200 mg/kg bwt)	0.53±0.21	0.18±0.01
Group 5 (CRU 200 mg/kg bwt)	0.21±0.05	0.21±0.02
Group 6 (AQF 300 mg/kg bwt)	0.29±0.06	0.22±0.06
Group 7 (EAF 300 mg/kg bwt)	0.40±0.10	0.17±0.01
Group 8 (BUT 300 mg/kg bwt)	0.27±0.07	0.16±0.01
Group 9 (CRU 300 mg/kg bwt)	0.34±0.09	0.15±0.01

Values are expressed as mean ± SEM, n = 5 replicates. Values with (*) are statistically significant at $p < 0.05$ when compared to the control group.

AQF (Aqueous Fraction), EAF (Ethyl Acetate Fraction), BUT (Butanol Fraction), CRU (Crude Extract).

Group 6 had a slightly increased enzyme activity compared with the Control Group and Group 5 had same values of enzyme activity with the Control Group. All the values are not significantly different when compared with the Control group.

4.4.5: Effects of Aqueous, Butanol, Ethanolic and Ethyl Acetate Extract of *A. floribunda*

on Sperm Count, Sperm Motility and Live/Dead ratio in Wistar Rats.

Table 4.7 shows the summary of the effects of the crude ethanolic extract, aqueous, butanol and ethyl acetate fractions of *A. floribunda* on Sperm Count, Sperm Motility and (virility) Live/Dead ratio in the experimental Animals. All groups had lower sperm counts, motility and virility compared to the Control Group. However, Group 9 (300 mg/kg Crude) had the lowest values for the parameters measured as most of the sperm cells were observed to be dead (Plate 4.1).

4.4.6: Effects of Effect of Ethanolic Extract and other Fractions of *A. floribunda* on

Histopathology of the Testicular tissue.

Plate 4.2 shows the effects of Aqueous, Butanol, Ethanolic and Ethyl Acetate Extract of *A. floribunda* on histopathology of the testicular tissue. Normal seminiferous were noted in Groups 1, 6 and 7 with full complement of spermatogenic (Spermatogonia; Spermatocyte; Spermatid) and Leydig cells. Interstitial spaces (IS) were obscured by whitish deposit in Groups 2, 3, 4, 5, 8 and 9. Extension of whitish deposits was noted in the Seminiferous tubule of Group 5 obscuring the epithelium and cellular components of the Seminiferous Tubule. Distortion and degeneration of Seminiferous Tubule and Interstitial spaces was noted in Group. 9.

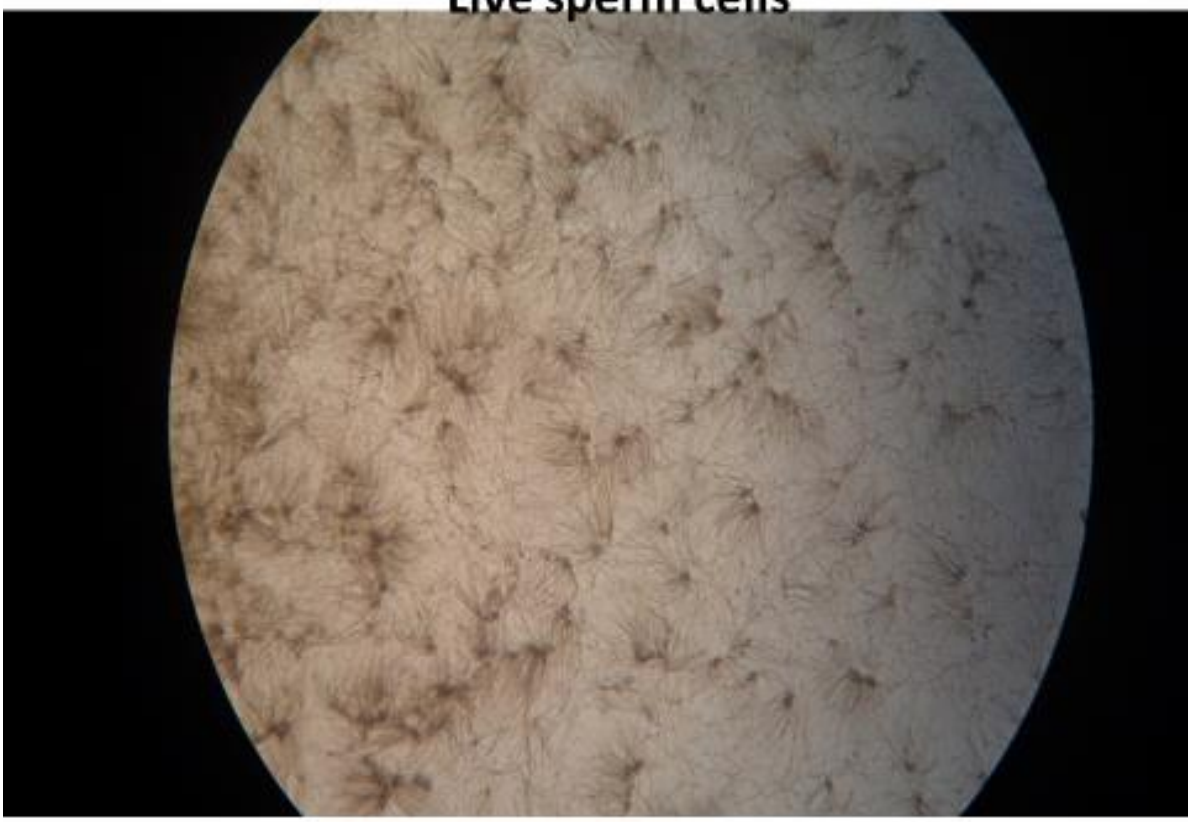
Table 4.7: Effects of Ethanolic Extract and other Fractions of *A. floribunda* on Sperm Count, Motility and Virility

Groups	Sperm Count (millions)	Sperm Motility (%)	Live:Dead ratio
Group 1 (Control)	121.80±5.37	91.00±1.00	96.80±0.73
Group 2 (AQF 200 mg/kg bwt)	97.60±7.70	74.00±2.45	96.20±0.73
Group 3 (EAF 200 mg/kg bwt)	93.80±2.96	76.00±2.45	96.80±0.73
Group 4 (BUT 200 mg/kg bwt)	82.60±3.23	68.00±2.00	93.60±2.23
Group 5 (CRU 200 mg/kg bwt)	75.40±3.28	64.00±2.45	94.20±2.40
Group 6 (AQF 300 mg/kg bwt)	97.60±5.15	83.00±4.36	96.80±0.73
Group 7 (EAF 300 mg/kg bwt)	84.20±3.98	72.00±2.00	94.20±2.40
Group 8 (BUT 300 mg/kg bwt)	95.25±4.92	77.50±2.50	94.00±3.08
Group 9 (CRU 300 mg/kg bwt)	53.00±7.20	30.00±15.28	51.67±26.19

Values are expressed as mean ± SEM, n = 5 replicates. Values with (*) are statistically significant at $p < 0.05$ when compared to the control group.

AQF (Aqueous Fraction), EAF (Ethyl Acetate Fraction), BUT (Butanol Fraction), CRU (Crude Extract).

Live sperm cells



Dead sperm cells

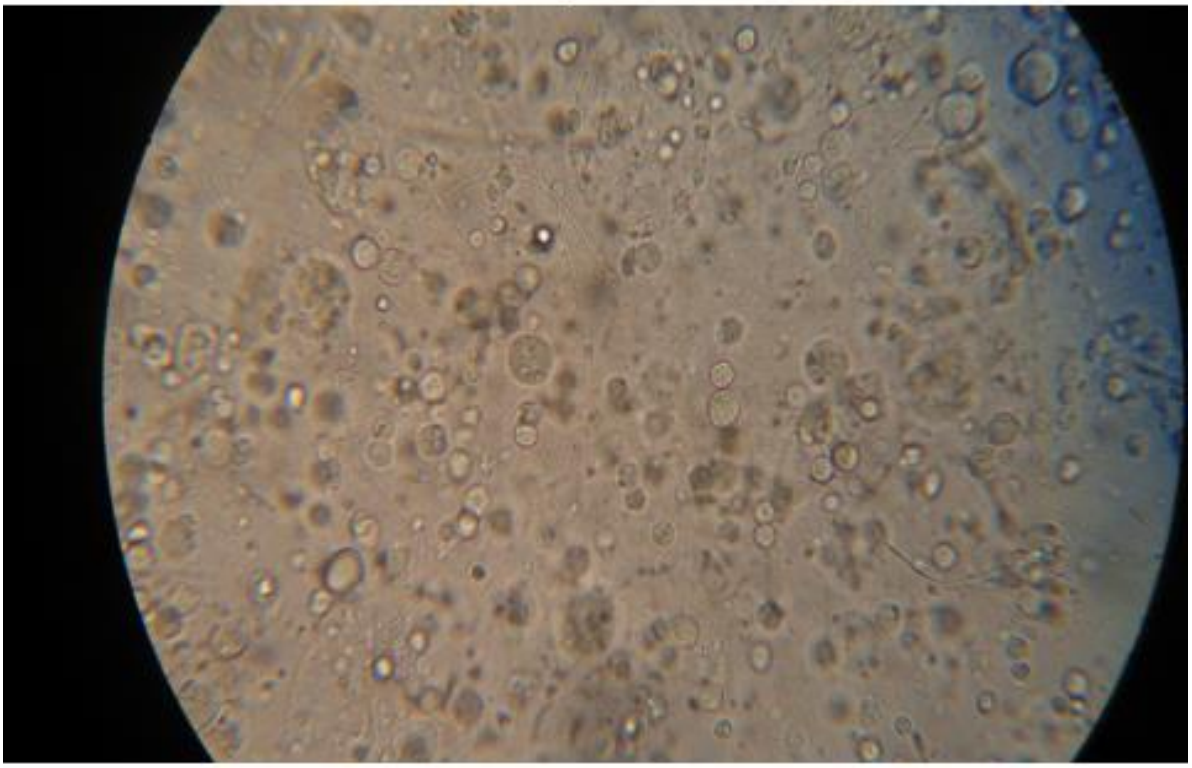


Plate 4.1. Live and Dead Sperm Cells viewed under microscope

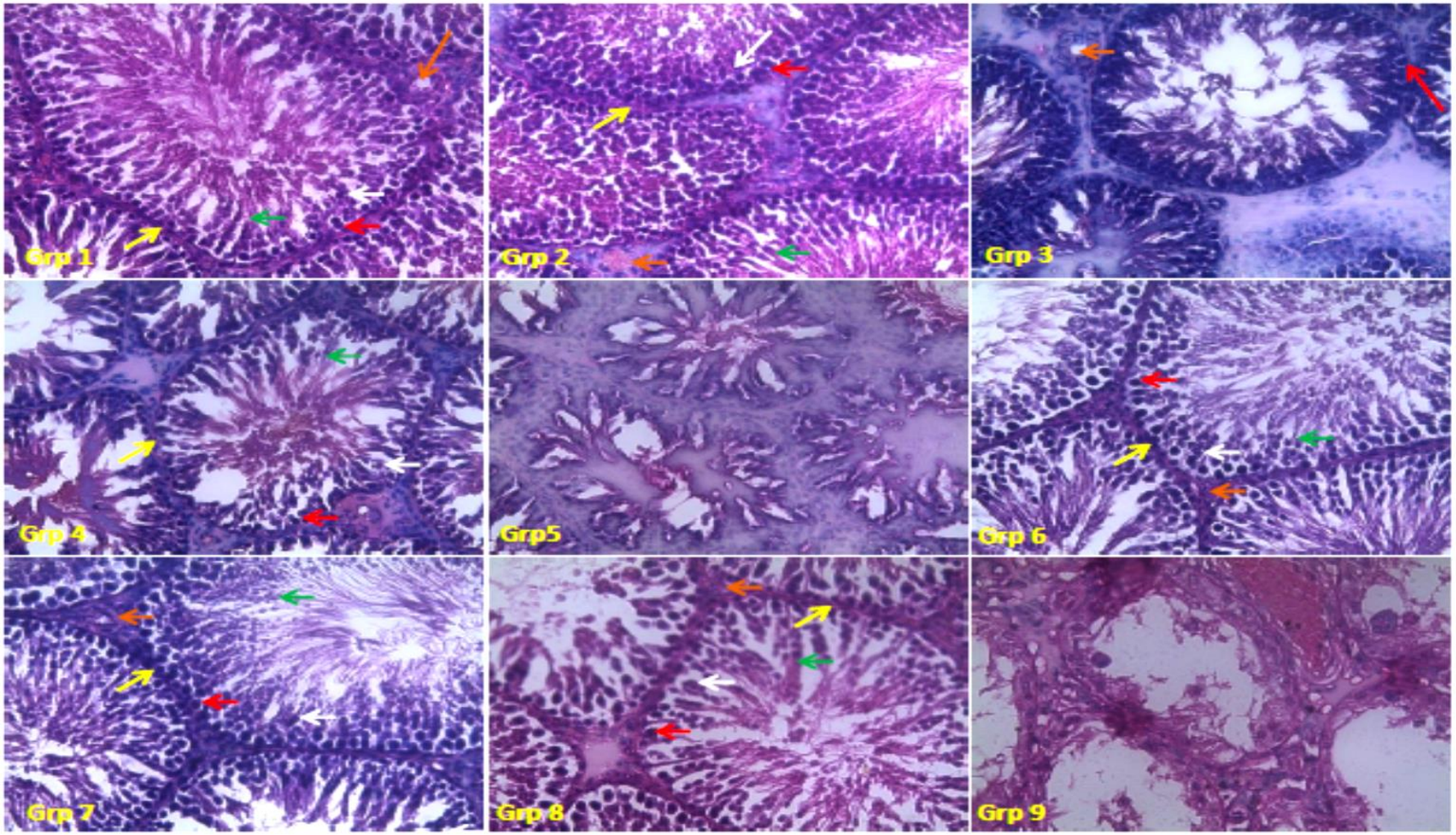


Plate 4.1: Photomicrographs of the testes showing the seminiferous tubules (ST) of the different groups. Stain H&E. Mag. X400. Normal seminiferous were noted in Groups 1, 6 and 7 with full complement of spermatogenic (Spermatogonia-Red arrow; Spermatocyte-White arrow; Spermatid-Green arrow) and Leydig cells

(Brown arrow). Interstitial spaces (IS) were obscured by whitish deposit in Groups 2, 3, 4, 5, 8 and 9. Extension of whitish deposits was noted in the Seminiferous tubule of Group 5 obscuring the epithelium (represented with Yellow arrow)

CHAPTER FIVE

5.0 Discussion and Conclusion

5.1 Discussion

It is a widely accepted fact that consumption of plant as foods or their use in herbs in adequate amounts is associated with numerous health benefits rooted in their various physiological effects as a result of their phytochemical and nutritional constituents (Saalu *et al.*, 2010). In fact the increasing use of medicinal plants and their components in traditional medicine has led to the development of new pharmaceutical drugs and it has been reported that approximately 25% of the active substances prescribed in the United States come from plant materials - representing nearly 20,000 species from several families (Céspedes *et al.*, 2006).

Allanblackia floribunda has been shown to display a wide spectrum of biological and pharmacological activities, which provide experimental support for the empiric ethno-pharmacological use of this plant in traditional medicine. Its stem bark has also been established to contain myriads of phytochemicals and metabolites such as benzophenones, xanthones, biflavonoids etc. (Dieudonné *et al.*, 2013).

In this study, the result of the phytochemical screening of the ethanolic extract of *A. floribunda* stem bark showed the presence of alkaloids, flavonoid, cardiac glycosides, tannins, saponin, terpenes, and steroids. The results obtained here are very similar to those earlier reported by Kada *et al.*, (2013) where the ethanol extract of *A. floribunda* was shown to contain phenolics, lipids, flavonoids, glucosides, cardiac glucosides, tannins, alkaloids, lipids and anthraquinones. Further High-Performance Liquid Chromatography-Mass Spectrometry (HPLC-MS) analysis of *A. floribunda* indicated a rich content of phenolics such as rutoside in substantial amount and gentisic acid phenolics (p-coumaric acid and ferulic acid) and flavonoids (quercitrin and kaempherol) were present in trace amounts. (Kada *et al.*, 2013). The presence of these

phytochemicals in the ethanolic extract of *A. floribunda* further supports its use and pharmacological significance in the treatment of ailments.

Flavonoids, for example have been shown to have antibacterial, anti-inflamatory, antiallergic, antineoplastic, antiviral, anti-thrombotic and vasodilatory activities and its intake has been associated with a reduced risk of several chronic diseases (Miller, 1996). The presence of flavonoids in extract of bark of *Allanblackia floribunda* has been said to be responsible for its antioxidant properties and protective role on organs (Dieudonné *et al.*, 2013). Tannins also are astringent polyphenols that bind or precipitate proteins non-specifically either by hydrogen or covalent bonding to amino ($-NH_2$) group of proteins. They have traditionally been considered anti-nutritional (may decrease the digestibility of proteins) but it may be employed medicinally in antidiarrheal, hemostatic, and anti-hemorrhoidal compounds (Lamy *et al*., 2010). Saponins have various biological activities such as the expectorant, the diuretic and adaptogenic activities associated with them. They are also responsible for the characteristic bitter taste of most plants (Barr *et al*., 1998). Cardiac glycosides are cardio-active compounds belonging to triterpenoids class of compounds their biological importance is primarily in the treatment of cardiac failure. They result in an increase in cardiac output by increasing the force of contraction as a result of their ability to increase intracellular calcium concentrations (Xie and Askari, 2002).

The effect of ethanolic extract of *A. floribunda* when administered to mice showed that no animal died within 48-hour after treatment with extract. There were no signs of weakness and/or loss of appetite even up to doses of 5000 mg/kg body weight. Thus, the lethal dose (LD_{50}) was estimated to be higher than 5000 mg/kg body weight. The LD_{50} being greater than 5000 mg/kg body weight is thought to be safe as suggested by Lorke (1983). Therefore, the ethanolic extract of *A. floribunda* is practically non-acutely toxic. This correlates with results of earlier works carried out on the plant.

The present investigation on the sub-chronic administration of the ethanolic extract and partitioned fractions of *A. floribunda* has provided information on the influence of the extract on testicular functions of the test animals. This is because alterations in some testicle enzyme activities have been widely used as biomarkers to evaluate the function of organs due to their important role in energy production and biotransformation (Zhang and Lin, 2009).

The acid phosphatases (ACP) are a group of enzymes capable of hydrolyzing esters of orthophosphoric acid in an acid medium (El-Kashoury *et al.,* 2009) Acid phosphatase activity is widely distributed in human tissues and acid phosphatases represent a heterogeneous group of enzymes containing many isoenzymes, each specific for one type of tissue. The human prostate is particularly rich in this enzyme and serum enzyme levels have been used as one of the markers of prostatic cancer (Uboh *et al.*, 2010). Acid phosphatase activity/concentration has been reported to be elevated in the sera of males with metastatic prostatic cancer (Saito *et al.*, 2006; Fang *et al.*, 2008). The results of this investigation showed significant increase in ACP activity across groups Group 2 (200 mg/kg Aqueous Fraction), Group 3 (200 mg/kg Ethyl Acetate Fraction) and Group 9 (300 mg/kg Crude extract), when compared with control; with 2, 3 and 9 having a significant increase of 80.38%, 72.03% and 89.15% respectively. However, in contrast to Group 9, Group 4 (200 mg/kg Butanol Fraction) had comparatively similar activity with the control. The increase in acid phosphatase activity is an indication of lytic activity in the prostate, causing prostate toxicity and predisposition to prostate cancer as similar results were reported by Zhang and Lin (2009) where administration of 3,4-dichloroaniline caused a significant increase in the acid phosphatase activity of rats. Furthermore, the increases in activity of acid phosphatases and prostatic phosphatase represent specific toxicity in the prostate gland and a possible threat to the well-being of the testes and physiology of the sperm as this may result in autolysis and consequently cell death (Yakubu *et al.*, 2008). Obianime and Roberts

(2009) showed that there exists a correlation between the increases in prostatic acid phosphatases and histopathological damage, showing an indication of reproductive dysfunction, cell death and apoptosis leading to atrophy in testes and accessory sex organ tissues such as the prostate.

Alkaline Phosphatase is a 'marker' enzyme for plasma membrane and endoplasmic reticulum and is frequently used to assess the integrity of the plasma membrane; such that any alteration in the activity of the enzyme in the tissue and serum would indicate likely damages to the external boundary of the cell (plasma membrane) (Ananthan and Kumaran, 2013). Testicular alkaline phosphatase is involved in the intra and intercellular transport, which is needed for the metabolic reactions to channelize the necessary inputs for steroidogenesis (Latchoumycandane *et al.*, 1997). Specifically, it is involved in mobilizing carbohydrates and lipid metabolites to be utilized either within the cells of the accessory sex structure or by the spermatozoa in the seminal fluid (Ramalingam and Vimaladevi, 2002); showing its importance in steroidogenesis (Latchoumycandane *et al.*, 1997). In this investigation, there was a general decrease in alkaline phosphatase (ALP) activity across the groups when compared with the control group, which had an activity of 362.66 $\pm$ 21.53 U/L. The decrease observed was dose dependent with Groups 6, 7, 8 and 9 having a lower activity when compared with Group 2, 3, 4 and 5. Group 6 and 7 has a significant decrease of 57.56% and 63.02% respectively, compared with the control.

The reduction in the testicular ALP activity observed in this investigation could be as a result of the disruption of the ordered lipid bilayer of the membrane, inhibition of the enzyme activity (Ananthan and Kumaran, 2013) or it may also be due to a reduction in concentration or total absence of specific phospholipids required by this membrane-bound enzyme to express its full activity (Yakubu *et al.*, 2002). This could hamper the normal transportation of required ions or molecules across the membrane. It may also affect other metabolic processes where the

enzyme is involved; such as synthesis of nuclear proteins, nucleic acids and phospholipids as well as in the cleavage of phosphate esters to release metabolites for use by accessory sex structure (Yakubu *et al.*, 2008). Moreover, this might affect the testicular mobilization of carbohydrates and lipids which are to be utilized either within the cells of the accessory sex structure or by spermatozoa in the seminal fluid (Ramalingam and Vimaladevi, 2002). Similar depletion in alkaline phosphatase was also observed in the testis of rats as reported by Chitra *et al.* (1999), Joshi *et al.* (2007) and El-Kashoury *et al.* (2010) who reported that the reduction in the activity of this enzyme when treated with chlorpyrifos, mancozeb and endosulphan is due to the decreased metabolic activities. El-Kashoury *et al.* (2010) further linked the reduction to decrease in steroidogenesis and reduction in intercellular transport.

The normal adult testis of man, rat, rabbit, mouse, dog, guinea-pig, bull, and pigeon, contain unusual isoenzymes of lactate dehydrogenase, designated LDH-X and distinct from other LDH isoenzymes. The testis-specific isoenzymes of LDH are a convenient metabolic marker for the spermatogenic activity of the testis, as they are associated with the spermatogenic cycle from the stage of the pachytene primary spermatocytes. Hence, Lactate dehydrogenase has been used as a marker for active spermatogenesis (Elkington *et al.*, 1972). Lactate rather than glucose is the preferred substrate for glycolysis in primary spermatocyte and lactate is generated from glucose in the Sertoli cells under the influence of Follicle Stimulating Hormone (Jutte *et al.*, 1983). The activity of LDH in the testes reflects the production and/or utilization of the substrate – lactate. Thus, the changes in testicular lactate dehydrogenase could give an indication of testicular energy status, spermatogenesis and the rate of transformation of spermatocyte to spermatozoa (Nurudeen and Ajiboye, 2012). Except groups 6 and 8, all the groups had higher LDH values with group 2 having a significant increase of 39.28% in LDH activity. The elevated LDH activity in this study may reflect testicular degeneration and/or damage to seminiferous

epithelium, since the enzyme is closely associated with spermatogenesis and testicular development. This is in agreement with the observation of Olayinka and Ore (2014) who reported that increase in the activity of LDH in the chlorambucil-treated rats resulted from adaptation to improve spermatogenesis and testicular development after oxidative damage.

Glucose-6-Phosphate Dehydrogenase (G6PDH) is an X chromosome-linked enzyme which catalyzes the first step in the pentose phosphate pathway in which D-glucose 6-phosphate is converted to 6-phosphogluconolactone (Salati and Amir-Ahmady, 2001). This oxidation/reduction reaction transfers hydride from glucose 6-phosphate to $NADP^+$ in order to reduce the coenzyme $NADP^+$. As a result, pentose phosphate pathway will generate NADPH, a coenzyme that has an important role as an electron donor which restores reduced glutathione (GSH). NADPH and GSH will help in the maintenance of tissue integrity and protect body cells (such as testicular cells) from oxidative stress by converting harmful hydrogen peroxide to water with the help of glutathione peroxidase. In the male, Leydig cells which are present in the interstitium of the testis express all of the enzymes essential for the conversion of cholesterol to androgens and estrogens and Glucose-6-phosphate dehydrogenase, present in leydig, sertoli, and spermatogenic cells, is more active in leydig cells. Actually, the activity of G6PDH is associated with the function of leydig cells in that it provides required reducing power (NADPH) necessary for steroidogenesis and biosynthesis of testosterone – the male reproductive hormone.

The decrease observed in the Glucose-6-phosphate dehydrogenase activity (G6PDH) could be as a result of injury to the function of leydig cells and free radical attack on the sulfhydryl groups of the enzyme as reported by Farombi *et al.* (2008); who reported that the diminishing enzymatic activity of G6PDH upon treatment with tetracycline may decrease the generation of NADPH to a level that is below the demand of the GSH cycle. This injury inflicted on the leydig cells would translate to an alteration or reduction in the biosynthesis of testosterone

resulting in infertility in men. The male generative glands also contain Sertoli cells which require testosterone for spermatogenesis. (Yakubu *et al*., 2008). Hence a disruption in the biosynthesis of testosterone affects the production of sperm calls in the Sertoli cells.

The testis is sensitive to a variety of stressors and exposure to agents, hence this organ has fairly high concentrations of antioxidant (Maneesh *et al.*, 2005). These facts indicate that the defense against oxidative stress plays critical roles in the maintenance of spermatogenesis and prevention of testicular atrophy. Reactive Oxygen Species (ROS), are regularly formed during the process of normal respiration. However, the production is kept at physiologically low levels by intracellular free radical scavengers. It had demonstrated that the major sources of ROS in semen were derived from the spermatozoa and infiltrating leukocytes (Sharma *et al*, 2001). Spermatozoa and seminal plasma have their own anti-oxidative mechanisms to protect ROS-induced cellular damage. Catalase (CAT) is a heme protein which catalyses the reduction of hydrogen peroxides and protects the tissues from hydroxyl radicals. Glutathione peroxidase (GPx) also, plays a significant role in the peroxyl scavenging mechanism and in maintaining functional integration of the cell membranes, spermatogenesis, and sperm morphology and motility. It is suggested that the metabolic pathway of testosterone biosynthesis requires protection against peroxidation and will be affected by a decrease in the activity of this enzyme (Maneesh *et al*, 2005). Therefore reduction in the activity of these enzymes (GPx, CAT) may result in a number of deleterious effects due to the accumulation of superoxide anion and hydrogen peroxide (Sajeeth *et al.*, 2011).

Testicular proteins are required for spermatogenesis and sperm maturation (Nurudeen and Ajiboye, 2012). The testicular fluid normally contains stimulatory and inhibitory factors that selectively alters the protein secretions. Thus, changes in protein suggests a reduction in the synthetic activity in testes (El-Kashoury *et al*., 2009). The increase in Total Protein

concentration (TP) in Groups 2, 3, 4, 7 and 9 is as a result of accumulation of protein in testes due to androgen deprivation to target organs (El-Kashoury *et al*., 2009). This observation synced with that recorded by El-Kashoury *et al.* (2010), where elevation in testicular protein was found to cause a reduction in testicular and cauda epididymis sperm population, loss of motility and increase in the number of abnormal spermatozoa, thereby manifesting 100 % failure in treated animals. However, decrease in Total Protein concentration observed in Groups 5, 6 and 8 might be due to decrease in synthesis of the protein as a consequence of the constituents of the extract (Yakubu *et al.*, 2008).

The weight of testes and reproductive accessory organs is largely dependent on the mass of differentiated spermatogenic cells and the reduction in the weight of testes may be due to reduced tubule size, decreased number of germ cells and elongated spermatids (Sanhez *et al.*, 2004). In this study, a general reduction in the testicular and seminal vesicle weight was observed in the groups. The reduction in the testicular weight observed suggests the degenerating capacity of the extracts. Reduction in the weight of the testis could be due to inhibition of seminiferous tubule fluid formation and loss of germ cell by direct inhibition on spermatogenesis (Ananthan and Kumaran, 2013) and is in agreement with earlier studies on rats which have well established that the structural and functional integrity of the male accessory sex glands are androgen dependent (Ananthan and Kumaran, 2013). In the present study, the decreased weight of male accessory sex organs might be due to decreased bioavailability androgens and estrogenic and antiandrogenic activities of *A. floribunda* extract. The same reduction in weight was observed in the relative weights of the epididymis as well; all groups with the exception of Group 7 had lower relative epididymis weight when compared with the control. The decrease in testicular, epididymis and seminal vesicle weights could be due to reduced tubular size which resulted from degeneration and atrophy of seminiferous tubules (Olorunshola *et al.*, 2011).

The varying degrees of distortion in the testicular seminiferous tubules following the administration of the plant extract at the doses when compared with the control further point to the toxicity risk of the plant extract as revealed by the biochemical parameters investigated in this study. However, the severe tubular distortion and degeneration of the testes corroborated by microscopic pictures of the sperm cells (Plate 4.1) observed in group 9 strongly suggest a deleterious influence of the crude ethanolic extract at 300mg/kg body weight on the male fertility. Gametogenesis occurs in the seminiferous tubules while the interstitial cells secrete the testicular hormone, mainly testosterone. Therefore, any alteration in the seminiferous tubules as observed in the histopathological studies will have its consequential effect on gametogenesis (Yakubu *et al*., 2008).

5.2 Conclusion

From the results of this study, it is concluded that ethanolic extract and fractions of *A. floribunda* are not safe at the doses investigated, most especially at the higher dose of the crude ethanolic extract. This could be the explanation for the reduction in libido observed by Kada *et al.* (2012) when the rats were given 300 mg/kg doses of the extract.

Due to earlier works done by Kada *et al.* (2012) and (2013) *A. floribunda* was confirmed to be a potent aphrodisiac. However, sexual performance is not synonymous with potency. *A. floribunda* possibly exerts its aphrodisiac functions through either of two ways:

(a) Specific physiological effect. It may affect blood flow where it could mimic the burning of fire of sex and intercourse and increase the duration of sexual activity. An example of an aphrodisiac that acts in this manner is Spanish fly made of dried and crushed beetles of the *cantharis* and *mylabris* genus. The active ingredient in Spanish fly, a crystallized lactone, cantharidin (Taberner, 1985), when applied topically, causes burning sensation at the point of blistering and its consumption has also been reported to cause increased blood flow in the body

(Yakubu *et al.*, 2007). Other physiologically active drugs used to sustain erection include Sildenafil citrate (Viagra) and yohimbine from *Pausinystalia yohimbe* (Taberner, 1985).

(b) The phytochemicals may actually cross the blood brain barrier and mimic or stimulate some areas of sexual arousal just like hormones, pheromones and a wide variety of neurotransmitters (Yakubu *et al.*, 2007).

Therefore, high doses should be avoided and users should not rule out completely the possibility of chronic toxicity to the reproductive organs developing with continual usage of the plant.

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APPENDIX

1. Preparation of 2 Litres of 70% Ethanol

1400 ml of absolute Ethanol was made up to 2000ml with 600ml of distilled water.

2. Preparation of 500 ml of 0.05 M Potassium Phosphate Buffer, pH 7.4

2.177 g of sodium di-hydrogen phosphate and 30.86 g of di-sodium hydrogen phosphate was carefully weighed, dissolved with 250 ml water in a 500 ml beaker. The mixture was then transferred into a 500 ml measuring cylinder and then made up to volume with distilled water. The pH was also balanced using a pH meter.

3. Preparation of 0.2 M Sodium Phosphate Buffer, pH 8.0

61.63 g of Na_2HPO_4 and 4.35 g NaH_2PO_4 dissolved in 990 ml of distilled water. The pH adjusted with 1 M NaOH and 1 M HCl to pH 7.0 and distilled water added to make up to 1litre.

4. Preparation of 500 ml of 0.1 M Sodium Phosphate Buffer, pH 7.4

5.616 g of sodium di-hydrogen phosphate and 1.988 g of di-sodium hydrogen phosphate was carefully weighed, dissolved with 250 ml water in a 500 ml beaker. The mixture was balanced with a pH meter. It was then transferred into a 500 ml measuring cylinder and then made up to volume with distilled water.

5. Preparation of 100 ml of 10 mM NaN_3

0.065 g of NaN_3 was dissolved in 80 ml of distilled water and made up to 100ml.

6. Preparation of 100ml of 2.5 mM H_20_2

28µl of 30% H_20_2 was dissolved in 100ml of distilled water. Freshly prepare the solution always when needed for every assay.

7. Preparation of 50 ml of 4 mM Reduced Glutathione

0.0615 g of Reduced Glutathione (GSH) was dissolved in 30 ml of phosphate buffer and then made up to 50 ml with same phosphate buffer.