ASSESSMENT OF REPRODUCTIVE FUNCTIONS IN MALE WISTAR RATS TREATED WITH *Saccharum officinarum* MOLASSES

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ABSTRACT

Refined sugar, a major product from *Saccharum officinarum*, contains mainly sucrose and possesses anti-androgenic effects. *Saccharum officinarum* Molasses (SOM), a sweet by-product obtained during sugar production, is rich in phenolic compounds, minerals and organic acids. The use of SOM as substitute sweetener is increasing because of its nutritional advantage over refined sugar. However, there is paucity of information on its effects on reproductive functions. This study was designed to investigate reproductive functions of SOM-treated male Wistar rats.

Saccharum officinarum was obtained from Karu, Nasarawa State and authenticated at the Department of Botany herbarium, University of Ibadan (UIH No.: 22613). *Saccharum officinarum* juice (SOJ) was obtained using standard procedure. The SOJ was subjected to three cycles of heating and cooling to obtain SOM. The SOM was fractionated with methanol and water and the constituents of fractions (SOMMF and SOMAqF) were identified using GC-MS. Twenty male rats (100-120 g) were grouped into four (n=5) and they received distilled water (1.0 mL/kg/day, Control), SOJ (1.0, 3.2, 10.0 mL/kg/day) orally for 8 weeks, respectively. Another thirty-five male rats (160-180 g) were grouped into seven (n=5) and they received distilled water (1.0 mL/kg/day, Control), SOMMF (1.0, 3.2, 10.0 mL/kg/day) and SOMAqF (0.6, 2.0, 6.4 g/kg/day) orally for 8 weeks, respectively. Testicular and epididymal malondialdehyde were assayed spectrophotometrically. Sperm profile, histology of testes and epididymides were assessed microscopically. Also, testicular cells from twelve rats were isolated, cultured and incubated with SOMMF, SOMAqF, components of SOMMF (Lupeol) and SOMAqF (Diethyl Phthalate). Cell livability, proliferation and testosterone in cultured cells were quantified using ELISA. Data were analyzed using ANOVA at $\alpha_{0.05}$.

The SOMMF (methanol) yielded 72.8% and SOMAqF (aqueous) 27.2%. Major constituents from SOMMF and SOMAqF were lupeol (87.2%) and diethyl phthalate (47.4%), respectively. The SOJ (10.0 mL/kg/day) significantly increased serum testosterone, sperm concentration and abnormal spermatozoa compared to control. The SOJ (10.0 mL/kg/day) significantly decreased sperm livability (38.5±4.5%) compared to

control (69.7±4.7%). The testicular malondialdehyde 2.4±0.2, 2.6±0.1 U/mg protein of SOMMF (1.0, 3.2 mL/kg/day) and 2.6±0.4 U/mg protein of SOMAqF (6.4 g/kg/day) significantly increased compared to control (1.6±0.2 U/mg protein). The SOMMF (1.0 mL/kg/day) significantly increased epididymal malondialdehyde (43.0±5.2 U/mg protein) relative to control (15.7±6.9 U/mg protein). Similarly, SOMMF (3.2, 10.0 mL/kg/day) significantly decreased sperm livability (79.2±2.4, 71.3±5.0%) compared to control (91.7±2.0%). The SOMAqF (2.0, 6.4 g/kg/day) significantly reduced sperm livability (78.8±2.1, 74.2±3.0%) compared to control (91.7±2.0%). Also, SOMAqF (2.0, 6.4 g/kg/day) significantly increased abnormal spermatozoa (13.1±1.3, 14.2±1.5%) compared to control (9.0±0.7%). Seminiferous tubules and epididymal ducts of SOMAqF-treated rats showed architectural distortion. Testicular cell proliferation significantly increased in SOMMF (2.9±0.3) compared to control (1.9±0.2). Cell livability significantly decreased in Lupeol (1.1±0.2 million/mL) and diethyl phthalate (1.1±0.1 million/mL) compared to control (1.9±0.2 million/mL). *In vitro* testosterone biosynthesis significantly reduced in diethyl phthalate (0.8±0.1 ng/mL) compared to control (1.2±0.0 ng/mL).

Saccharum officinarum molasses caused lipid peroxidation and reduced sperm quality. These actions were linked to its constituents; lupeol and diethyl phthalate. Hence, *Saccharum officinarum* molasses could adversely affect reproductive functions in male Wistar rats.

Keywords: *Saccharum officinarum* molasses, Serum testosterone level, Sperm quality, Lupeol

Word count: 496

DEDICATION

To the All-knowing and ineffable GOD my Maker and Redeemer, for His gift of life, hope of a glorious future and blessings on the way.

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To all my GOD sent friends who are part of my success story, I appreciate them all.

CERTIFICATION

I certify that this work titled: **Assessment of reproductive functions in male Wistar rats treated with *Saccharum officinarum* molasses** was carried out by Eunice Ogunwole in the Department of Physiology, College of Medicine, University of Ibadan.

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LIST OF ABBREVIATIONS

EDC	Endocrine-disrupting compounds
HFCS	High Fructose Corn Syrup
SOJ	*Saccharum officinarum* juiice
SOM	*Saccharum officinarum* molasses
DNA	Deoxyribonucleic acid
SRY	Sex-determining region Y
LH	Luteinizing hoemone
AMH	Anti-mullerian hormone
HPG	Hypothalamic-pituitary-gonadal
GnRH	Gonadotropin releasing hormone
FSH	Follicle stimulating hormone
hCG	Human chorionic gonadotropin
STDs	Sexually transmitted disesases
ED	Erectile Dysfunction
SHBG	Sex-hormone-binding-globulin
PCB	Polychlorinated biphenyl
DDT	Dichlorodiphenyltrichloroethane
Ace-K	Acesulfame potassium

UK	United Kingdom
USA	United State of America
CO_2	Carbondioxide
GRAS	Generally regarded as safe
UIH	University of Ibadan Herbarium
LLE	Liquid – liquid extraction
GC-MS	Gas chromatography - mass spectroscopy
NIST	National institute of standards and technology
ELISA	Enzyme Linked Immunosorbent Assay
H&E	Hematoxylin and Eosin
TBARS	Thiobarbituric Acid Reactive Substances
MDA	Malondialdehyde
TBA	Thiobarbituric acid
HCL	Hdrogen chloride
NaOH	Sodium hydroxide
EDTA	Ethylenediaminetetraacetic acid
SOD	Superoxide dismutase
DTNB	Dithiobis (2-nitrobenzoic acid)
tGSH	Total glutathione
GSH	Glutathione

NVRIAUCC	National Veterinary Research Institute, Animal Use and Care committee
DMSO	Dimethyl Sulfoxide
EC_{50}	Half maximal effective concentration
MF	Molasses Fraction
ROS	Reactive oxygen species
NADH	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate

CHAPTER ONE

1.0 INTRODUCTION

Infertility a disorder of reproductive system, is the inability of a couple to accomplish clinical pregnancy after having unprotected sexual intercourse regularly for a duration of twelve months or more (Zegers-Hochschild *et al*., 2009). Reports states that it affects about 8 and 12% of couples in their reproductive age across the world (Ombelet *et al.*, 2008). Problems in the male partner are the commonest single group of course (Agarwal *et al.*, 2015; Shafali and Bridget, 2016). The rise in occurrence of reproductive disorders such as infertility, subfertility, sexual dysfunction, defects in sperm characteristics and impaired growth has raised alarm to the general populace and the world of science (Sengupta, 2014). There is a lot of concern in the society on the possible harmful effects of contact with endocrine-disrupting compounds (EDCs) in our environs on animal/human health (John *et al.,* 2016; Maria *et al.,* 2017). EDCs are artificial or natural substances that reacts with hormones, found in our food sources/environs at a concentration enough to hamper the typical physiology of endocrine systems/tissues, particularly the reproductive endocrine axis (Crisp *et al.,* 1998).

Lifestyle and dietary habits are known factors that can cause antiandrogenic effects as they have been associated with the prevalence of several reproductive diseases (Strazzullo and Matarazzo, 2017). A variety of environmental contaminants that possesses antiandrogenic properties have shown some impacts on the endocrine system with consequent effect on human and animal health (Gutendorf and Westendorf, 2001; Cremonese, *et al.,* 2017). In order to maintain reproductive health, assessment of possible endocrine disrupting compounds is important, as we come in contact with these substances daily. Traditional medical practitioners recommend some plants for treatment of diseases. They also recommend other type of plants to be taken periodically for sustainance of good health in addition to provision of vital nutrients, known as functional foods/nutraceutics

(Tabassum and Hamdani, 2014). Popular examples of such are sweeteners and ingredients which do not just enhance food taste, add color or flavour to food but sometimes serve as digestive aids and prevent diseases when comsumed regularly (Anderson and Gustafson, 1988; Raben *et al.*, 2002). Sweeteners are used as sources of nutrients and epicurean facilitators for the culinary preparation of some food types (Kirtida, 2011; Lluís *et al.*, 2014). These constituents are useful for preservation, improving texture and other uses associated with enhancing food qualities (Sanjay, 2015).

Refined sugar (sucrose – the common table sugar) is a popular sweetener used widely (Phanikumar, 2011). Over the years, study showed that refined sugar is a non-lethal and is safe for use (Daniels and Roach, 1987). However, some other reports show that sugar intake may cause some adverse effects (Howard and Wylie-Rosett, 2002). Previous studies linked increased dietary intake of sugar with dental caries, problem with appendice and cancers, gall bladder, early aging, cardiovascular and autoimmune diseases and other metabolic diseases (Appleton, 1996; Howard and Wylie-Rosett, 2002).

Previous study had associated refined sugar consumption with reproductive dysfunction in males (Chiu *et al.*, 2014). Consumption of sugar sweetened snacks and drinks has been correlated with low spermatozoa concentration (Liu *et al.*, 2015). Excessive intake of high fructose corn syrup has been linked with metabolic diseases (James *et al.*, 2013). This ordeal led to the substitution of refined sugar with artificial (Non-sugar) sweeteners such as saccharin, sucralose and aspartame that replicates the effect of sugar in taste contain (Sanchari *et al.*, 2014). It presented direr effects such as cancer of the bladder, weight gain, brain tumors, among others (Tandel, 2011; Jotham, *et al.*, 2014). In order to curb these effects there is a shift to the use of natural products because of their nutritive values and believe that they have more beneficial effects on health status (Hong-fang *et al.*, 2009; Yannis *et al.*, 2015). This emphasis of the health benefits of molasses, includes its functional characteristics, nutrient and sweetening quality that support its use as a substitute for sugar (Harish *et al.*, 2009).

Saccharum officinarum (sugar cane) is cultivated world wide, it is an energy-rich food that is highly nutritious with numerous minerals, enzymes and has various medicinal and therapeutic potentials (Banerji *et al.,* 1997). Lo *et al.* (2005) stated that *Saccharum officinarum* juice has a wide biological property of elevating inherent immunity to infections. *Saccharum officinarum* molasses (SOM) is a natural sweetener obtained as a byproduct during the processing and production of refined sugar from sugarcane juice. *Saccharum officinarum* molasses is now used as an ingredient in the human diet (Joaquim *et al.,* 2011; Rahiman and Pool, 2016). Lluís *et al* (2014) reported 0.1 kg/per capita/per year as an estimated value of intake of sweeteners.

Saccharum officinarum molasses is the more concentrated byproduct, obtained from the 3rd boiling process of the sugarcane syrup, after the sucrose in the sugar is allowed to crystallize (Mackintosh, 2000). In the 20th century, molasses was imported into the United States from the Caribbean Islands. It was a popular sweetener as it was cheaper than refined sugar which was very expensive as at that time until the late 19th century (Engerman, 1983). Thailand, India, Brazil, Taiwan, the Philippines and the United States are the largest producers of molasses. Over the years this residual product has been used in feeding animals due to its ability to eradicate dust and reduce feed wastage as well as an important source of dietary energy (Blair, 2007; Heuzé, *et al.,* 2012). Molasses is also used in sweetening of hot drinks and alcoholic beverages (Rahiman and Pool, 2011). Report states that sugar consumption has an adverse effect on male reproductive physiology and SOM is been explored as a substitute natural sweetener (Rahiman and Pool 2010). Rahiman and Pool (2016) showed that *in vivo* exposure of animals to molasses produced an immunosuppressive effect. On the other hand, studies report that sugarcane molasses may be beneficial for treatment of bone defects (Martinho *et al.,* 2013), protection against deoxyribonucleic acid (DNA) oxidative damage (Guimaraes *et al.,* 2007), stimulation of testosterone and estrogen in both testicular and ovarian cell cultures (Rahiman and Pool, 2016).

Statement of problem

The prevalence of male reproductive dysfunction is on an alarming increase (Shafali and Bridget, 2016). Lifestyle and dietary habits have contributed to antiandrogenicity and hormonal imbalance in men as related to reproductive disorders. High intake of sugars has been related with occurrence of reproductive dysfunction in men (Chiu *et al.,* 2014), problems such as impotence, low sex drive and low sperm count have a direct association with hormonal imbalance (Xiaoping *et al.,* 2004).

Justification of the study

Evidence suggests that *Saccharum officinarum* and it's by products may be associated with potential endocrine disrupting effects (Pate, 1983; Cellar, 2006). *Saccharum officinarum* juice is wildly consumed and is the main source from which both refined sugar and *Saccharum officinarum* molasses are made (Banerji *et al.,* 1997), however there is a paucity of facts on its reproductive activities. *Saccharum officinarum* molasses is gaining increasing acceptability as a substitute for sugar (McDonell, 2017), but there is dearth of knowledge of its actions on male reproductive physiology. The study was therefore designed to assess the reproductive functions of male Wistar rats administered *Saccharum officinarum* molasses.

Aim

The study aimed at assessing the reproductive functions of male Wistar rats administered *Saccharum officinarum* molasses.

The objectives of the study were:

1. To evaluate the effects of *Saccharum officinarum* juice in Wistar rats on: sperm indices, serum levels of reproductive hormones and histology of male reproductive organs.
2. To determine the effects of *Saccharum officinarum* molasses in Wistar rats on: sperm indices, serum levels of reproductive hormones, oxidative status of reproductive organs and histology of male reproductive organs.
3. To investigate the possible mechanism of action of *Saccharum officinarum* molasses using testicular cell culture.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 The male reproductive system

In humans, the reproductive system in males comprise of the testes, epididymis, prostate gland, seminal vesicle and penis which play some roles in the reproductive process. Reproduction is the process whereby an organism creates its own descendants. The male reproductive system have a primary direct function which is provision of spermatozoa to fertilize the female egg (ovum). This system consists of the testicles and an array of ducts and glands. The testes produce spermatozoa and transports it via the reproductive ducts. The secretions from the glands make up the fluid (i.e semen) ejaculated from the urethra Barrett and Ganong, 2012).

2.2 Male reproductive functions

2.2.1 Spermatogenesis

Spermatogenesis is the process of division of primary germ cells of male to form spermatogonia. Each primary spermatocyte divides into 2 secondary spermatocytes and each secondary spermatocyte into 2 spermatids, which then develops into mature spermatozoa. Therefore, the primary spermatocyte gives rise to 2 cells. These 2 secondary spermatocytes sub-divides to produce 4 spermatozoa. This process oocurs in a stepwise fashion within the testes and epididymis of mammals, for humans it takes a period of about 74 days (Heller and Clermont, 1963). It is vital for sexual reproduction, begins at puberty and generally lasts until death, even though as a person grow older, there could be a slight decrease in the quantity of spermatozoa produced. Spermatogenesis is splitted into various different stages, each conforming to a specific cell type.

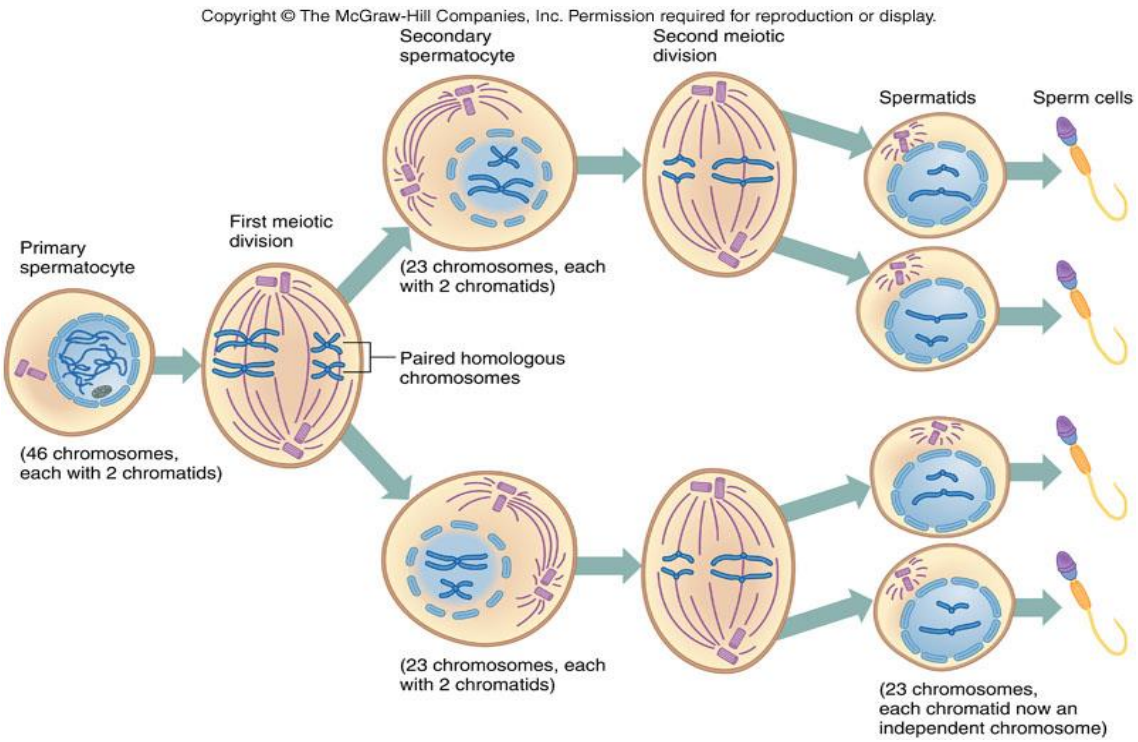

Figure. 2.1 Stages of spermatogenesis (Griswold, 2016).

2.2.2 Androgenesis

Androgens are steroid hormones primarily involved in the development of male-specific phenotype during embryogenesis, in the establishment of sexual maturation at puberty, and in the maintenance of the male reproductive function, spermatogenesis and sexual behavior during adult life (Gobinet *et al.*, 2002). Endogenous androgens, mainly testosterone and its active metabolite dihydrotestosterone (DHT), exert most of their effects by binding to the androgen receptor (AR), a ligand-activated transcription factor, which results in the control of gene transcription by the interaction of AR with coregulators and specific DNA sequences of androgen-responsive genes of the target cells (Matsumoto, *et al.*, 2008).

2.2.3 Factors influencing spermatogenesis

The process of spermatogenesis responds very quickly to any changes in the milieu, especially hormone and temperature. Testosterone is needed in great quantity to sustain the process, this is accomplished through binding of testosterone by androgen binding protein

found inside seminiferous tubules. Seminiferous epithelium is easily affected by high temperature in man and animals. Temperatures as high as normal body temperature can cause a negative effect to the Seminiferous epithelium. Thus, the testes are situated in the scrotum outside the body. The optimum temperature is kept at 2 °C – 8 °C less than body temperature. This is realized by control of flow of blood and testes decend from the effect of body heat via the dartos smooth and cremasteric muscle. Other factors that adversely affect the rate of spermatogenesis includes diets deficient in vitamins B, E and A, presence of metals, anabolic steroids, exposure to x-ray, alcohol and infectious diseases (Barrett and Ganong, 2012).

2.2.4 Sperm discharge function

The discharge of semen into the reproductive tract of the female has to do with the following steps;

2.2.5 Libido

This is known as sexual drive or the biological need for sexual activity. It is known as sex-seeking behavior. Its strength varies between individuals and within an individual over a given period. A High serum testosterone level has been linked with better sexual activity in healthy older men (Toone *et al.*, 1983).

2.2.6 Erection

This is when the penis is in a firm and enlarged state due to a multifaceted interaction of psychological, neural, vascular and endocrine factors. A penile erection ensues when the corpora cavernosa becomes filled up with venous blood usually due to parasympathetic nerve induced vasodilatation that results from any physiological stimuli. Sexual stimulation or arousal usually causes penile errection but it can also occur when the urinary bladder is full or suddenly during the course of a day or at night, usually in erotic/wet dreams. The penis swells and enlarges throughout sexual activities to take place (Fox, 2002).

2.2.7 Ejaculation

Ejaculation and erection must occur for spermatozoa to be deposited into the genital tract of the female with no technologic assistance. Ejaculation is considered as a two stage events, seminal emission - the deposition of semen into the posterior urethra and the expulsion of semen from the urethra (Silverthorn, 2007). The sympathetic nervous system controls emission and ejaculation. Emission has to do with the forceful contraction of muscles and myoid complexes adjacent the vas, ampullae of the vas, seminal vesicles and prostate. Ejaculation on the other hand, is the closing of the bladder neck to avoid backward flow of semen and contraction of the periurethral muscles, primarily the bulbocavernosus muscle (Fox, 2002).

2.2.8 Orgasm

This is a deep and generally pleasant feeling that ensues at the climax of sexual arousal followed by a drop in sexual tension. In order for an individual to have an orgasm it is dependent on different conditions, types and amounts of stimulation. Orgasm comprises of a sequence of rhythmic contractions in the genital region and pelvic organs (Barrett and Ganong, 2012).

2.3 Hypothalamic-pituitary-gonadal (HPG) axis

The hypothalamus, anterior pituitary and testes makes up the HPG axis of the male reproductive systems. It forms a finely tuned system regulated via a classic negative feedback mechanism as shown below (Vadakkadath and Atwood, 2005).

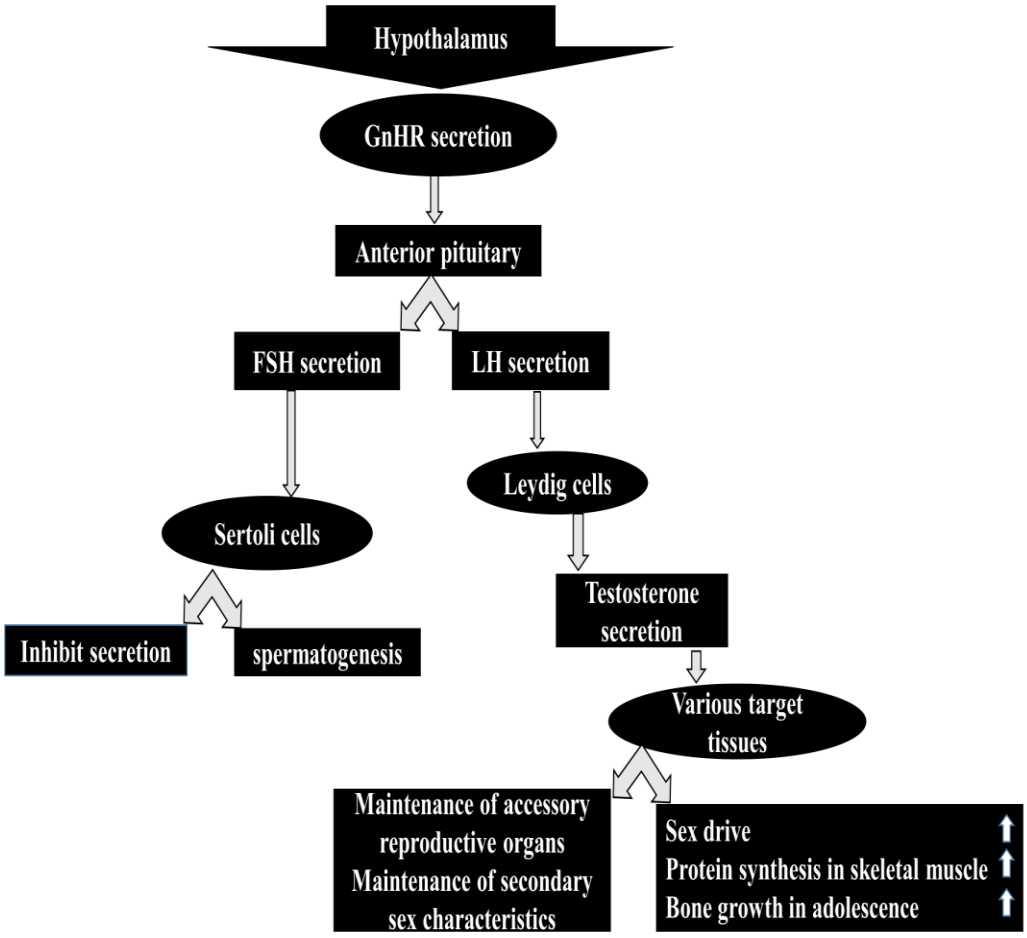

Figure. 2.2 Hypothalamic-pituitary-gonadal (HPG) axis (Vadakkadath and Atwood, 2005)

2.4 Fertility/infertility in males.

Fertility is the capacity to bring forth offspring. Human fertility depends on various factors which includes endocrinology, nutrition, sexual behavior, culture, timing, instinct, economics, emotions and way of life. The hormonal cycles in females and males is determinant of the time a female becomes pregnant and the time a man is most virile. While the male cycle is variable the female cycle is about twenty-eight days. A male is capable of producing sperm and ejaculating at any time of the month, but the sperm quality rise and fall occasionally, as corelated with their internal cycle. Clinical infertility is the inability of becoming pregnant after having sexual intercourse without protection for a period of one year. Male infertility is the incapability of a male to make a reproductive age-group female

pregnant after having regular unprotected sexual intercourse for a period of twelve months. About 40-50% of infertility are caused by male factors (Hirsh, 2003).

2.5 Causes of infertility in males

Some factors that causes male infertility includes: hormonal imbalances, psychological, physical and behavioral problems. Male infertility in most occasions is caused by testicular damage whereby the testicles are unable to produce sperm. A damage to the testicles prevents spermatogenesis from taking place, likened to menopause (nonetheless not natural like menopause) with no cure (Hirsh, 2003).

In addition to testicular impairment, low sperm production and poor sperm quality also causes infertility. Infertility in men is frequently due to deficits in the semen and the quality of the semen is used as an alternate determinant of male fecundity (Cooper *et al.,* 2010). Semen quality is a vague term that denotes one or more of several semen features assessed in a fresh ejaculate. The most vital factors usually measured in determination of the male fecundity are the total sperm count (concentration x volume millions), sperm concentration (millions/mL), motile sperm (%), sperm livability, sperm with normal morphology (%) and semen volume (mL). Furthermore, fertility is a reflection of the status of "general" health. An indidvidual living a hale and hearty life will probably produce healthy sperm and vice versa. Lifestyle choices that impact male fertility negatively includes sperm count and sperm motility are both reduced significantly in smokers, testicular shrinkage and infertility from use of anabolic steroid. high levels of adrenal steroid hormones caused by overly intense exercise, deficiency of Zinc and vitamin C in the diet, use of tightly fitting underwears increases scrotal temperature, malnourishment and anemia, infection, oxidative stress. Putting some of these behaviors into consideration and making necessary adjustment can improve a man's fertility (Seyedeh, 2015).

2.5.1 Infection as a cause of male infertility

Studies have shown that the presence of bacteria in semen may compromise sperm quality (Sanocka *et al.,* 2005). The bacteria responsible for semen contamination generally originate from the urinary tract of patients, or can be transmitted by a partner via sexual

intercourse (Benjamin *et al.*, 2014). However, it has been reported that detection of bacteria in semen does not necessarily suggest infection, as bacterial isolates ic seminal fluid may also result from sample contamination or colonization of the urethral orifice (Moretti *et al.*, 2009).

Microorganisms can affect the male reproductive function either directly, causing agglutination of motile sperm and thus reducing the ability of acrosome reactions and making alterations in cell morphology, or indirectly through production of reactive oxygen species generated by the inflammatory response to infection (Tremellen, 2008). The massive infiltration of activated leukocytes into the inflammatory site may be associated with impairment of sperm fertilizing potential due to oxidative, apoptotic, and immune processes (Fraczek and Kurpisz, 2015).

2.5.2 **Oxidative stress** and **male infertility.**

Oxidative stress describes when a system has an imbalance between oxidation and reduction reactions, leading to generation of excess oxidants or molecules that accept an electron from another reactant (Henkel, 2011). Common molecules that receive the unpaired electron are lipids in membranes and carbohydrates in nucleic acids (Tremellen, 2008). This leads to potential cellular and DNA damage when ROS are greater than the antioxidant carrying capacity. Industrial exposure is a cause of increased ROS production and sperm DNA damage. Exposure to heavy metals (e.g., cadmium, lead, iron, and copper), pesticides, phthalate, and pollution can lead to sperm damage (Tremellen, 2008). Studies have shown higher levels of ROS in patients with oligospermia (Agarwal *et al.*, 2014). High levels of ROS disrupt mitochondrial membranes, leading to activation of caspases and ultimately apoptosis (Agarwal *et al.*, 2008). To maintain physiological oxidative mechanisms whilst minimising the risk of cellular injury, a balance between ROS and antioxidants is necessary. Antioxidants are reducing agents that react with and buffer ROS and are considered a form of defence against oxidative stress (Sies, 1993). They are categorised as enzymatic or non-enzymatic agents (Sies, 1993), with non-enzymatic agents subdivided into those endogenously produced or those consumed through food or supplements. Important enzymatic antioxidants in infertility include superoxide dismutase

(SOD), glutathione peroxidase (GPX), and catalase. SOD scavenges superoxide anion and catalyses its conversion to H_2O_2 and O_2. High levels of SOD have been found in Sertoli cells, whilst germ cells have high activity levels that are maintained during spermatogenesis in developing and maturing spermatozoa (Bauché *et al.,* 1994). SOD has also been identified throughout the epididymis and in seminal plasma (Chen *et al.,* 2003). This enzyme decreases markers for oxidative stress (Kobayashi *et al.,* 1991), protects sperm against lipid peroxidation (Tavilani *et al.,* 2008) decreases DNA damage (Chen *et al.,* 2003).

2.5.3 Hormonal problems

Hormonal problem is a factor that causes a small percentage of male infertility which cannot be ignored. The hypothalamus-pituitary axis controls an array of hormonal activities that enables testes to yield and efficently transport spermatozoa. There could be a disorderliness in these activities such as, a failure or disruption in the release of gonadotrophic-releasing hormone (GnRH) by the brain can cause interruption in sperm production and testosterone synthesis, there could be a failure in the pituitary to produce enough LH and FSH, inability of the cells of Leydig to synthesise testosterone following LH stimulation, production of some substances that causes imbalance of sex-hormone in men (Bhatia *et al.,* 2014). Some hormonal disorders which can interrupt male fertility include:

Hyperprolactinemia: is a condition whereby there is a high level of prolactin observed in up to 10 – 40 % of infertile males. Higher levels of the hormone cause decreased production of spermatozoa and libido, it may lead to impotence (Fox, 2002).

Hypothyroidism: A reduction in libido, poor testicular function and semen quality can result from low thyroid hormone levels.

Congenital Adrenal Hyperplasia: A condition whereby an elevated level of adrenal androgen suppresses pituitary function. Occur in about 1% of infertile men (Bhatia *et al.,* 2014).

Hypogonadotropic Hypopituitarism: It is a situation whereby the pituitary gland secretes low levels of LH and FSH arresting sperm maturation and causing progressive germ cell depletion (Silverthorn, 2007).

Panhypopituitafism: this is a condition of complete failure of the pituitary gland wherby there is a decrease in the hormones it secretes. Using pituitary hormonal supplement may reinstate potency and the use of human chorionic gonadotropin (hCG) may stimulate testosterone and sperm production (Bhatia *et al.,* 2014).

2.6 Actions of xenoestrogens on the reproductive system

Previous studies have shown that the environment is now considered as "a virtual sea of estrogens" as it is even in drinking water (Field *et al.,* 1990: Sharpe and Skakkebaek, 1993). Most probably the estrogens were recycled from defecated man-made estrogens (birth control pills) at water board plants. They are more potent and may cause harm to the male sexual vigor as they are unable to connect with sex-hormone-binding-globulin (SHBG). Study reports increased exposure of the fetus to estrogenic substances *in utero* and during the years of reproduction as chief cause of remarkable increase in development disorders and functioning of the male reproductive system. Exposure to xenoestrogen have found a correlation between xenoestrogen exposure and low testosterone resulting in a decreased level of testosterone in men and has been related to prostate problems, impotence, low sex drive and low sperm count (Seyedeh, 2015).

Exogenous sources of estrogen which also abate male sexual vitality are food, water and air. Weakly estrogenic substances such as polychlorinated biphenyl (PCB), dioxin and dichlorodiphenyltrichloroethane (DDT) have been shown to have contaminate our environs in the past 50 years as they are resilient to biodegradation and are reprocessed in our surroundings till harbor safely in our bodies. These poisonous chemicals are known to alter spermatogenesis, and they pose adverse effects during sexual development. Experimental studies report that these estrogens impede the proliferation of Sertoli cells (Bhatia *et al.,* 2014). The quantity of produced sperm is based on number of Sertoli cell, as Sertoli cells are only able to sustain a certain amount of germ cells that will mature into

sperm. Its proliferation happens mainly during fetal life and prior to puberty. It is regulated by FSH. Reports states that earlier administration of estrogen in life inhibits FSH secretion leading to decreased quantity of Sertoli cell and low sperm count (during adulthood). The same events have been reported to occur in humans (Sharpe and Skakkebaek, 1993).

2.7 Sweeteners as sources of nourishment

Grotz and Munro (2009), reported that other than any taste sensation humans naturally attracted to sweet tastes. Newborn mammals are directed towards safety, nourishing foods and drinks as a result of their sweet taste (Sardesai and Waldshan, 1991). Hence, sweetness is an important factor in selection of beverages and food (Gifford *et al.,* 2009). Sweeteners are food condiments we use to give better taste to food substances we consume on daily bases. Natural sweeteners are obtained from natural products without using chemicals as they are produced. They are compounds with sweet taste and high nutrient value. Monosaccharides or disaccharides are the main constituent of natural sweeteners They include: honey, Maple syrup, molasses, stevia and sucrose. Artificial sweeteners on the other hand, are derivative of chemical synthesis of organic compounds that are either natural or not. They have very little or no nutrient value as they are compounds synthesized that possess high-intensities of sweetness, as a result to attain the same amount of sweetness, a little of the compound is needed. Artificial sweeteners serve in limiting caloric intake or prevention of dental cavities hence are used in food products (Walters, 2009). Some examples of artificial sweeteners include: Aspartame, Cyclamate, High fructose Corn Syrup (HFCS 55, Isoglucose), Neotame, Saccharin and Sucralose (Gifford *et al.*, 2009).

2.8 Component of Sucrose

Sucrose also known as table sugar, is a disaccharide with the molecular formula $C_{12}H_{22}O_{11}$ and a molar weight of 342.30 g/mol. Sucrose is formed from glucose and fructose connected by an α (alpha) 1 on the glucose, to a β (beta) 2 on the fructose glycosidic linkage. It has a solubility value of 1 g in 0.5 mL water and 1 g in 0.2 mL boiling water (Walters, 2009).

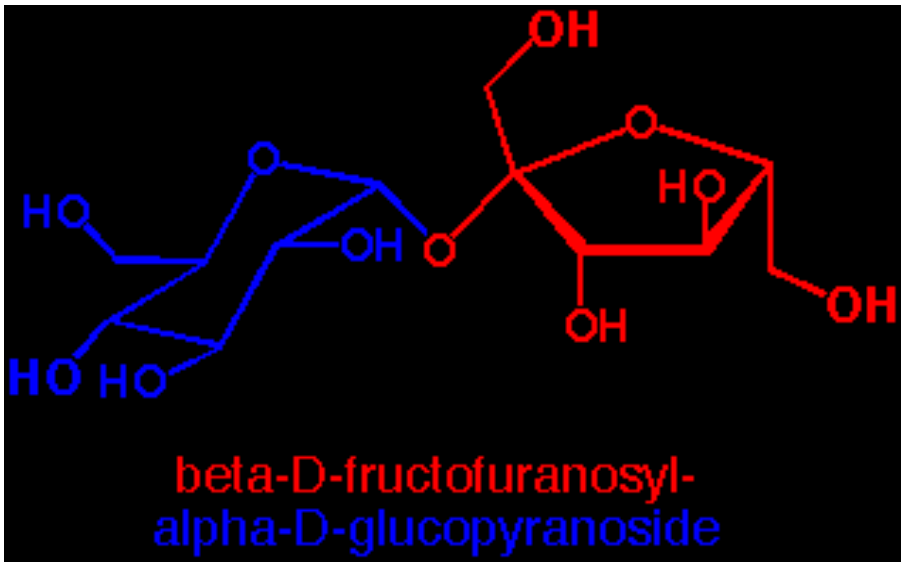

Figure. 2.3 Chemical Structure of Sucrose (Walters, 2009).

Sucrose is produced commercially from processing of sugarcane or beets. Purifying process take out the yellow-brown pigments of crude sugar to yield white crystal form (Walters, 2009).

2.9 *Saccharum officinarum* (Sugarcane) plant

Saccharum officinarum is of the genus *saccharum L* of the grass family (*poaceae*). It is a tall monocotyledonous plant that is cultivated, grows in clumps of cylindrical stalks with a diameter of 1.25 to 7.25 cm and a height of 6 to 7 m. It normally takes about 8-12 months to mature. The cane at maturity appears green, yellow, purplish or reddish and is ripe at its maximum sugary capacity (Onwueme and Sinha, 1993). It is a worldwide vital crop containing high sucrose and low fiber, useful for nutritional and economic sustenance. Sugar cane accounts for 60% of the world sugar needs while sugarbeet accounts for 40% (Onwueme and Sinha, 1999).

The most common synonyms of the sugar cane juice based on country includes; UK and USA referred to as raw sugar, brown sugar, muscovado, in Nigeria, Kenya and South Africa as jaggery, Brazil as rapdura, in Japan as Kokuto, black sugar (Kuro Sato), in India

and Pakistan as jaggery or gur. In addition to obtaining sugar, in the tropics and subtropics a lot of people delights in and consume cane juice. Indian medicine recommends eating of fresh sugar cane for sound health (Payne, 1982).

Sugarcane possess a broad variety of biological activities, its roots and stems are useful, Ayurvedic and Unani medicine for treatment of urinary tract infections or skin disorders, bronchitis, heart complications, inadequate milk production, cough, anaemia, constipation and general weakness (Elakkiya *et al.*, 2012). Extracts from sugarcane has shown immunostimulation property (El-Abasy *et al.*, 2003), anti-thrombosis property, anti-inflammatory capacity, vaccine adjuvant, regulating acetylcholine release (Barocci *et al.*, 1999) and anti-stress property. This juice also raises inborn immunity to infections (Lo *et al.*, 2005). Elakkiya *et al.*, (2012) reported that *Saccharium officinarum* peel extract, is rich in phenolic compounds, exhibit a high radical scavenging property and reducing power. Also methanol extract of *Saccharium officinarum Linn* (leave) administered on Normal cyclic female Wistar strain exerted antifertility and antiestrogenic effects in normal cyclic female rats increasing cholesterol level in the rats which were non-toxic and transient (Balamurugan *et al.*, 2009).

2.10 Sugarcane Processing

Sugarcane is produced and harvested basically for cane sugar and use as seed for subsequent plantings. Sugarcane processing has to do with the production of sucrose, other byproducts are molasses, bagasse and filtercake. Molasses is produced in two forms which are as an edible syrup and inedible for humans. Blackstrap molasses was initially used mainly as supplement for animal feed. Ethanol, compressed yeast, citric acid and rum are produced from molasses. The edible form of molasses is a blend of invert sugars, corn syrup and maple syrup.

2.10.1 Process description

This is divided into two sections, unrefined or raw sugar production stage with end product referred to as “cane sugar” and refining of sugar production stage with end product referred to as “refined sugar” (Payne 1982).

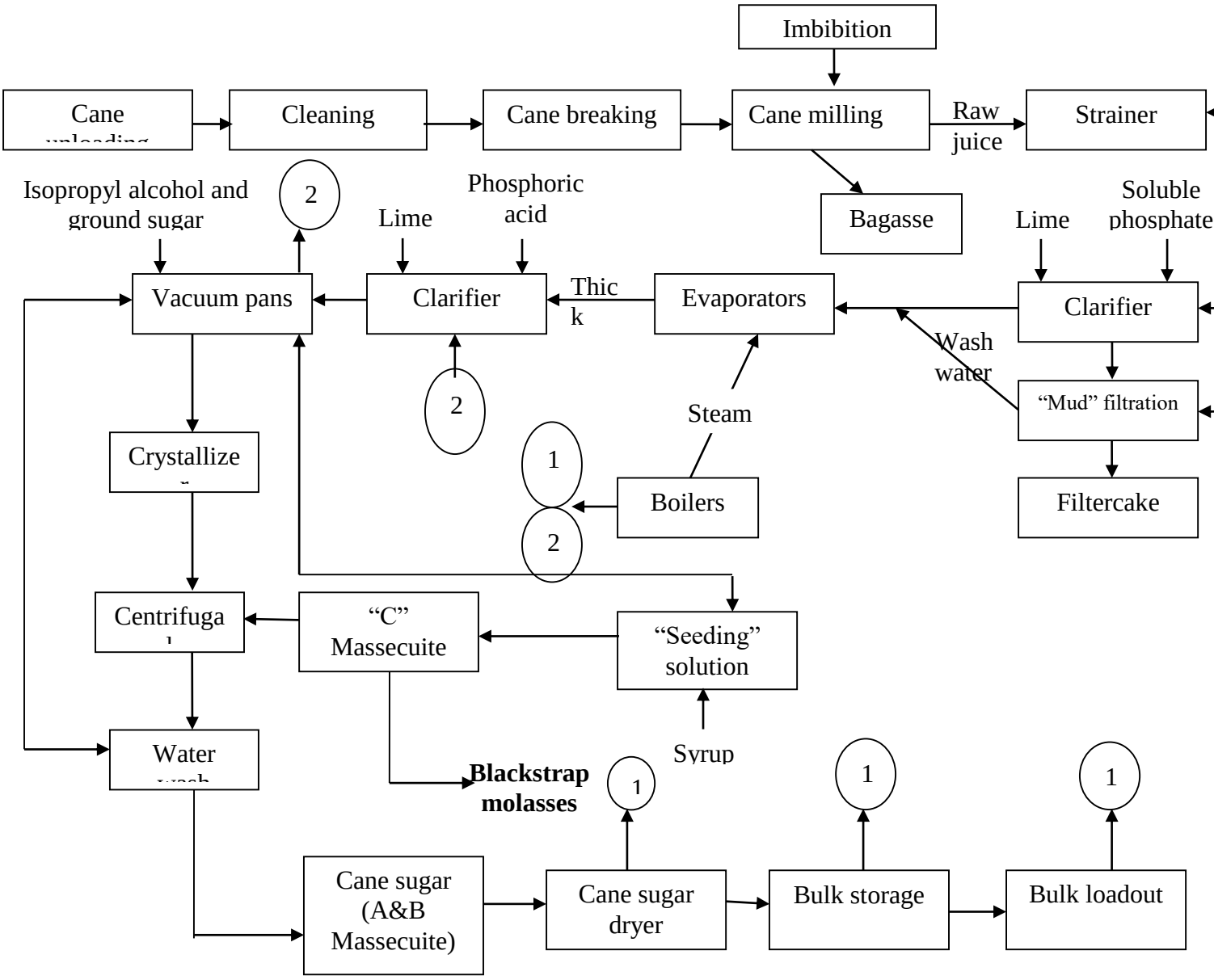

Figure 2.4 Simplified flow diagram for a typical sugar production plant (Payne, 1982)

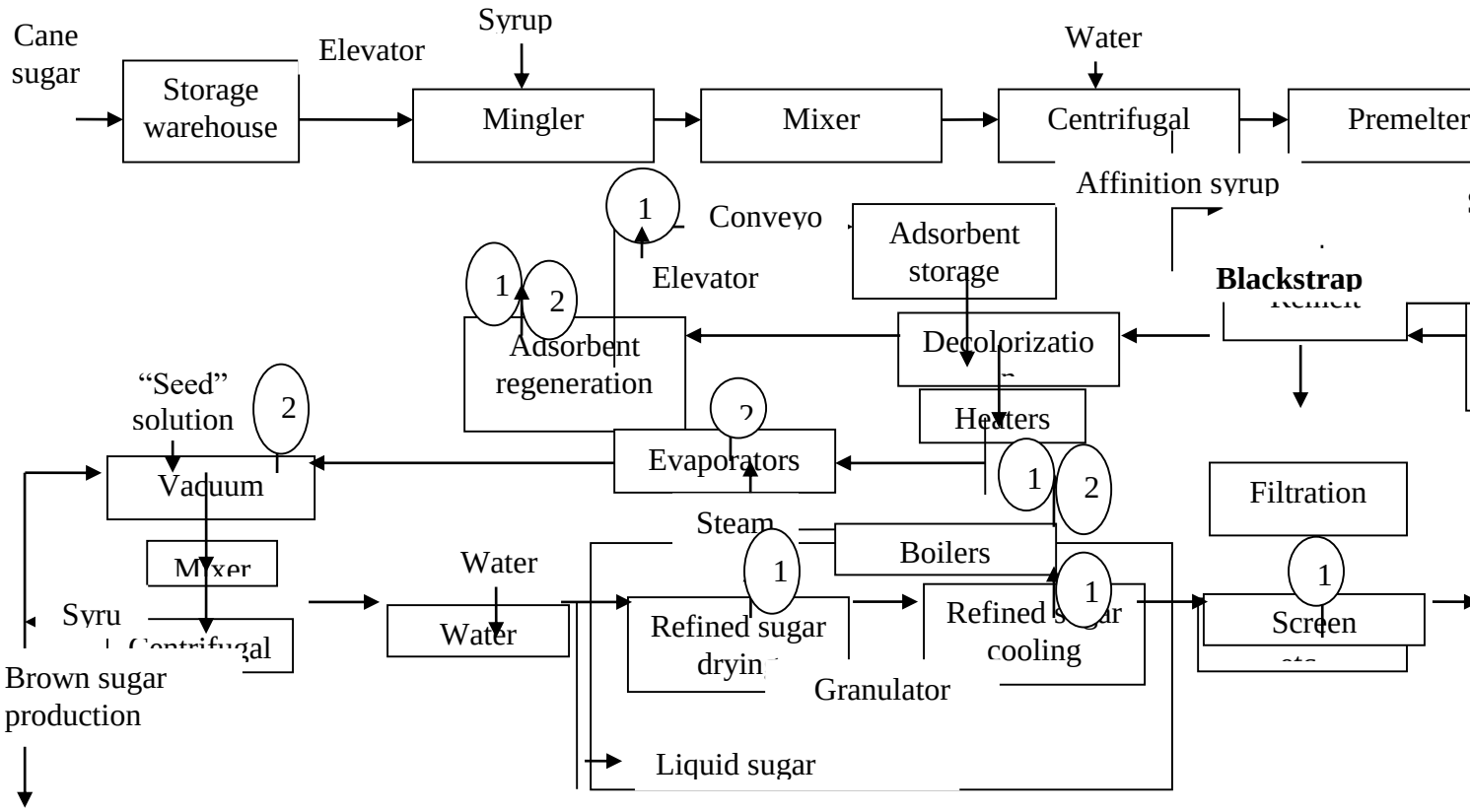

Figure 2.5 Simplified process flow diagram for refined sugar production. (Payne, 1982)

2.11 *Saccharum officinarum* (Sugarcane) molasses

Raw sugarcane juice contains 14–16 Brix obtained upon pressing of the culms. Concentrating it to 55–65 Brix yields sugarcane syrup. Additional evaporation of the juice to around 80 Brix results in molasses, a dark produce nearly as dense as bee honey (Figureueroa and Ly, 1990). Molasses is a natural and nutritious sticky byproduct as sugar is processed. Blackstrap molasses is the further concentrated byproduct, gotten from the 3rd boiling of the syrup from sugarcane, following crystallization of sucrose/sugar. The importation of molasses into the United States from the Caribbean Islands has been from the time of the early colonists. It was a well-known sweetener used until the late 19th century since it was cheaper than refined sugar that was very expensive then. Molasses is produced largely in Taiwan, Thailand, India, Brazil, the Philippines and the United States. Over the years, molasses was used in animal feeds for eradicating dust and feed wastage, now it has become a good source of dietary energy (Blair, 2007; Heuzé, *et al.*, 2012). The common names of sugarcane molasses are table or fancy molasses, refiners or blackstrap molasses, cooking molasses or syrup.

2.12 Bioactive compounds in *Saccharum officinarum* molasses

The maturity of sugar cane, method of extraction and quantity of sugar obtained determines quality of the molasses. The molecular formula of molasses is $C_6H_{12}NNaO_3S$, molar weight 201.22 g/mol, and density 1.41 g/cm^3. Molasses comprises sucrose 35.9%, fructose 5.6%, glucose 2.6%, nitrogen 1.01%, reducing substances 11.5% and 0.78% sulfur and several non sugar organic materials (Veronica *et al.*, 2012). Some of the identified compounds in sugarcane molasses include phenolic compounds, minerals and organic acids (Wright *et al.*, 2014).

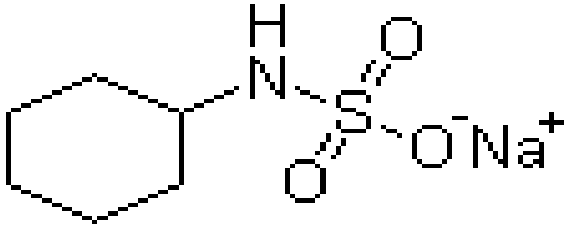

Figure 2.6 Chemical Structure of Molasses (Veronica *et al.*, 2012).

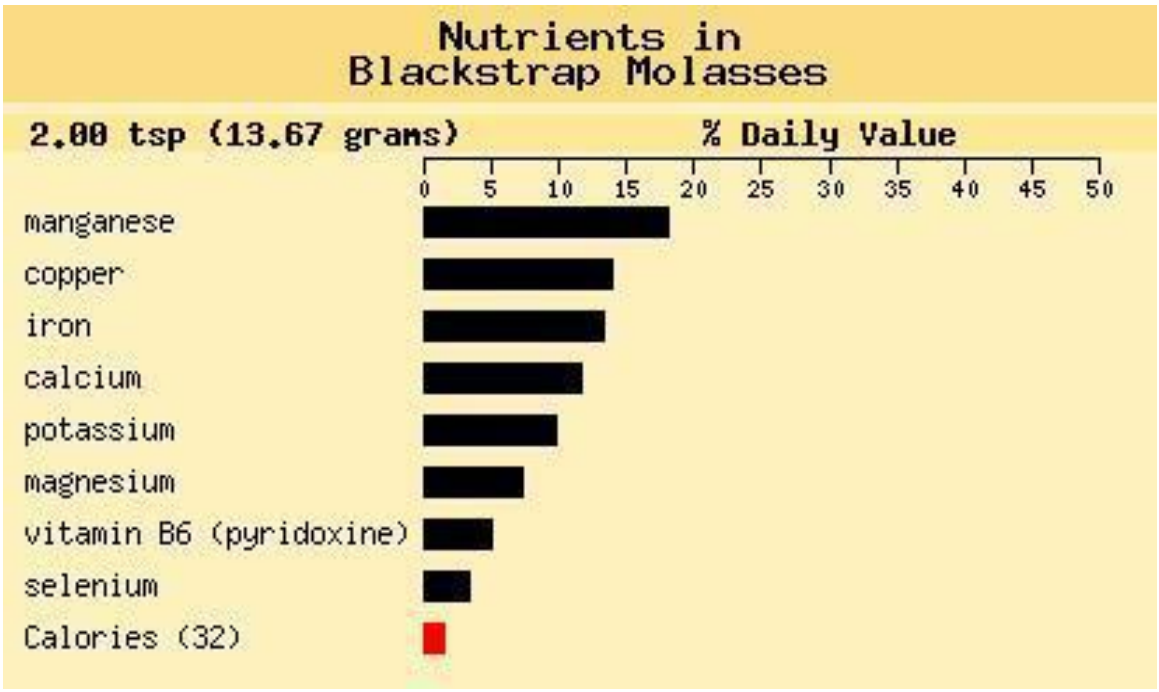

Figure 2.7 Nutrients in blackstrap Molasses (Wright *et al.*, 2014).

2.13 Processing of blackstrap molasses

Molasses are of three grades mild, dark and blackstrap. In making molasses, the sugar cane plant is harvested and its leaves are removed. A cane press is used to extract the juice which is boiled to concentration so as to promote sugar crystallization. Mild molasses containing a high sugar content is the product obtained from the first boiling and removal of the sugar crystals as only a small amount of sugar was extracted from the source. The second stage of boiling and extracting sugar results in dark molasses with a bitter tinge taste. Blackstrap molasses is a product of third boiling of the syrup. Most of the sucrose from the juice gets crystallized, but blackstrap molasses is still majorly sugar by calories. Although, it is made up of substantial amounts of minerals and vitamins, calcium, magnesium, potassium and iron, unlike refined sugar 15 mL of blackstrap molasses provides about 20% of the daily value of each of those nutrients (Veronica *et al.*, 2012).

Figure. 2.8 Blackstrap molasses

Source: <http://www.sugalik.com/molasses/composition.htmL>.

2.14 Uses of *Saccharum officinarum* molasses (blackstrap molasses).

It is noteworthy that refined sugar is a popular form of sweetener widely used, evidences of its deleterious effect on some body systems and functions results in emphasy of the possible health effects of molasses, since it has useful properties and nutritents to food, reassuring its use as an alternative for refined sugar (Harish *et al.*, 2009). Blackstrap molasses became popular in the mid-twentieth century during introduction of the health food movement. With its distinct and rich flavor, molasses is now a fundamental constituent of our diet and is presently being used as an ingredient in sweetening hot drinks and alcoholic beverages (Rahiman and Pool, 2010). Molasses is taken straight from the spoon undiluted or added to fresh lemon juice, used as sweetener to breakfast cereals, smoothies, milk, tea or coffee. It is used as a baking agent giving a distinctive flavor to bread, cookies, biscuits and cakes. Also molasses is used to baste chicken or turkey, so as to give it a rich color and taste (Reyed and El- Diwany, 2008). Molasses can also be used to preserve whole fish or meat offal without grinding (Perez, 1995) whereby, the osmotic pressure of the molasses makes the raw tissues to dry out. The fish or offal (50%) is totally covered by the molasses (50%) and kept free of air by putting a weighted grid or netting on the surface of the mixture.

2.15 Health benefits of molasses

In contrast with refined sugar and corn syrup, that has almost all its nutrients stripped off without valuable nutrients but cause health problems in some people (Howard and Wylie-Rosett, 2002; Appleton, 1996), molasses is said to be generally regarded as safe (GRAS) by U.S. Food and Drug Administration. There is a general believe that it improves health other than its special flavor and taste as it contains mineral and phenolic compounds (Guimaraes *et al.,* 2007).

Sugarcane molasses have a broad field of biological properties (Tanaki, *et al.,* 2003), especially antioxidatant (Phillips *et al.,* 2009), prophylactic and other physiological functions (Takara, *et al.,* 2002). Anecdotal reports states that molasses is beneficial for the treatment of cancers, diseases of the heart, gum and skin, strokes, arthritis, joint pains, blood pressure, diabetes, ulcers, anaemia, senility, strengthens nails, teeth, bones and hair (Weisinger *et al.,* 2011).

2.16 Adverse effects of blackstrap molasses

Molasses was reported to possess possible adverse effects on certain physiological systems. Reports claim that cattle given molasses (containing 3% urea) with small quantity of roughages had “molasses toxicity” syndrome. Study stated that molasses toxicity induces effects which includes fast breathing, excessive salivation reduced temperature of the body, lethargy in animals (Pate, 1983). Blackstrap molasses was also reported to have a link with endocrine disruption in cattles (Cellar, 2006). Furthermore, Rahiman and Pool (2016) reported an immunosuppressive effect of molasses when it was administered daily over a prolonged period.

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Plant material

Saccharum officinarum plant was obtained from Karu, Nasarawa State, Nigeria. It was authenticated at the herbarium of Botany Department, University of Ibadan and given the identification number UIH-22613. Blackstrap molasses® *Saccharum officinarum* molasses (Old English Incorporated, USA) was used for the study.

3.1.1 Extraction of *Saccharum officinarum* juice.

The cane of *Saccharum officinarum* plant was harvested and stripped off its leaves and the stalk was scrapped off its back to remove dirt. The cane was chopped into small sizes and a cane press juicer (Vevor, 110LBS/H) was used to get the juice which was filtered with muslin cloth to remove debris. The fresh liquid filtrate was administered to the animals on a daily basis using an oral canula immediately after extraction from the cane.

3.1.2 Fractionation of *Saccharum officinarum* molasses (SOM).

The principle of fractionation employed was liquid – liquid extraction (LLE). This was done on a macro-scale using a separatory funnel. The methods of Gandhi *et al.*, (2003) and Leila *et al.,* (2007) were used in extracting and fractionating SOM. Twenty grams of SOM was poured into a separatory funnel, a consecutive liquid/liquid partition with ethyl acetate, methanol and water were used for the exhaustive extraction. The ethyl acetate, methanol and residue (aqueous fraction) were concentrated to get fractions (Gandhi *et al.*, 2003). Upon solvent extraction of 20 g of sample, *Saccharum officinarum* methanol fraction (SOMMF) gave a yield of 14.56 g (72.84 %) and *Saccharum officinarum* aqueous fraction (SOMAqF) gave a yield of 5.43 g (27.14 %). This process was repeated for larger quantity of SOM to obtain the desired quantity of the fractions. The SOMMF and SOMAqF were used for the study.

3.1.3 Phytochemical screening

Phytochemical screening of *Saccharum officinarum* juice (SOJ), *Saccharum officinarum* methanol fraction (SOMMF) and *Saccharum officinarum* aqueous fraction (SOMAqF) were all performed separately using standard procedures (Odebiyi and Sofowora, 1978).

Tannins Test

Twenty mililitres (20 mL) of water was added to 0.5 mL of fraction and boiled. It was filtered and a drop of 0.1 % ferric chloride was added. Colour change was observed to either brownish green or a blue-black (Odebiyi and Sofowora, 1978).

Saponins Test

Five mililitres (5 mL) of distilled water was added to 0.5 mL of fraction. It was mixed and observed for a stable persistent froth. A drop of olive oil was added to the froth it was vortexed briskly and observed for any emulsification (Vaghasiya *et al.*, 2011).

Flavonoids Test

A drop of 1% aluminium solution was added to an aqueous filtrate of the fractions and observed for a yellow colour (Odebiyi and Sofowora, 1978).

Terpenoids Test

Two mililitres (2 mL) of fraction was added to 2 mL of chloroform, it was mixed and 3 mL of concentrated H_2S0_4 was added. It was observed for a layer with reddish brown colour at the interface (Odebiyi and Sofowora, 1978).

Alkaloids Test

The fraction (0.5 mL) was added to 10 mL of acid alcohol, boiled and filtered. Two mililitres (2 mL) of dilute ammonia was added to 5 mL of filtrate, then 5 mL of chloroform was gradually shaken to obtain the alkaloidal base. Ten (10 mL) of acetic acid was added to remove chloroform layer and divided into two. To the first portion, Mayer's reagent was added and observed for cream precipitate. To the second portion Draggendorff's reagent was added and observed for reddish brown precipitate (Senguttuvan *et al.*, 2014).

Anthraquinones Test

The fraction (0.5 mL) was boiled in sulphuric acid (10 mL), filtered and shaken with 5 mL of chloroform. The chloroform layer was aliquot into a test tube, dilute ammonia was added and observed for presence of a pink, red or violet colour in the ammonical phase implying hydroxyl anthraquinone (Vaghasiya *et al.,* 2011).

Reducing sugars Test

The fraction (0.5 mL) in 5 mL of water was added to boiling Fehling's solution (A and B) and observed for colour changes (Senguttuvan *et al.,* 2014).

Cardiac glycosides Test

Glacial acetic acid (2 mL) and a drop of ferric chloride solution were added to 5 mL of fraction; it was mixed with 1 mL of sulphuric acid. It was observed for a brown ring at interface implying a deoxysugar characteristic of cardenolides (Vaghasiya *et al.,* 2011).

3.1.4 Determination of Gas chromatography and mass spectroscopy (GCMS)

This analysis of the fractions was done with an Agilent Technologies 7890AGC interfaced to an Agilent 5973N mass selective detector. HP-5MS column with diameter of 30 x 0.25 μm x 1.0 μm was used with helium as carrier gas at a flow rate of 22 cm/sec. The temperature of gas chromatography oven was initially 50 ^{o}C held for 1 minute at 25 ^{o}C/mins, then increased to 100 ^{o}C held for 5 minutes, then to 300 ^{o}C held for 5 minutes, total run time was 48 minutes. The injector temperature was 250 ^{o}C with a split ratio of 1:30 and MS detector at 280 ^{o}C. Electronic integration was measured to get compositions with flame ionization detector at 280 ^{o}C. The peak numbers, relative abundance of constitiuents and retention time were evaluated (Chauhan *et al.,* 2014). The data was retrieved via GC-MS solution software and National institute of standards and technology (NIST) library was used to identify the corresponding peaks.

3.2 Animals

Ethical approval was obtained from the University of Ibadan Animal care and Use Research Ethics Committee (UI-ACUREC/18/0074). All procedures involving the use of animals were in accordance with the University of Ibadan Animal care and Use guideline.

Fifty-five (55) adult male (100 -180 g) Wistar rats were obtained from the Central Animal House, University of Ibadan. They were kept in well aerated clean plastic cages. Animals were fed rat chow and water *ad libitum* and acclimatized for two weeks before commencement of the study. Rats were chosen for this study because they require small space, have a short life span and are not expensive. The dosage regime used for the esperiment was in accordance with OECD guideline (2001).

3.3 Experimental design

The study was carried out in three phases:
First study protocol examined the effects of *Saccharum officinarum* juice (SOJ) on histopathology, sperm and hormonal profile of male Wistar rats.

Second study protocol examined the effects of *Saccharum officinarum* molasses (SOM) on oxidative status, histopathology, sperm and hormonal profile of male Wistar rats.

Third study protocol examined the *in vitro* effects of *Saccharum officinarum* molasses fractions and its synthetic constituents on testicular cell culture of male Wistar rats.

3.3.1 Experimental protocol for phase one

Twenty (20) adult male Wistar rats (100 - 120 g) were grouped into four (n = 5) as follows;

Group 1: Control distilled water (1 mL/kg/day)
Group 2: 1.0 mL/kg/day of SOJ
Group 3: 3.2 mL/kg/day of SOJ
Group 4: 10.0 mL/kg/day of SOJ.

Saccharum officinarum juice was given once a day for eight weeks using the oral canula. The body weights were taken once a week and on the last day of administration. Prior to sacrifice, rats were bled at the tail to determine the fasting blood glucose level.

3.3.2 Sacrifice and collection of organs

After treatment of eight weeks, rats were sacrificed under thiopental anaesthesia (40 mg/kg, i.p. Pereda *et al.,* 2006). They were cut open along the linea alba of the anterior abdominal wall to the thoracic cavity to uncover the heart and the organs. The testis, epididymis,

seminal vesicle, liver and kidney were harvested, freed from adherent tissues and immediately weighed using a digital electronic scale (model EHA501 with a capacity of 0.10-500 g). The epididymis and testes were fixed in Bouin's fluid for histological assessment.

3.4. Experimental protocol for phase two

Thirty-five (35) adult male Wistar rats (160-180 g) were grouped into seven (n=5). *Saccharum officinarum* molasses methanol fraction (SOMMF) and *Saccharum officinarum* molasses aqueous fraction (SOMAqF) were administered for a period of eight weeks using the oral canula once daily as follows;

Group 1: Control distilled water (1 mL/kg/day)

Group 2: 1.0 mL/kg/day SOMMF

Group 3: 3.2 mL/kg/day methanol fraction of SOMMF

Group 4: 10.0 mL/kg/day methanol fraction of SOMMF.

Group 5: 0.6 mg/kg SOMAqF.

Group 6: 2.0 mg/kg SOMAqF.

Group 7: 6.4 mg/kg SOMAqF.

The dosage regime was in accordance with OECD guideline (2001). The body weights were taken on a weekly basis and prior to sacrifice. Just before sacrifice, rats were bled at the tail to obtain a little portion of blood for assessment of fasting blood glucose level.

3.4.1 Sacrifice and collection of organs

Rats were sacrificed under thiopental anaesthesia (40 mg/kg, i.p. Pereda *et al.*, 2006). They were cut open along the linea alba of the anterior abdominal wall to the thoracic cavity to access the heart and the organs. The testis, epididymis, seminal vesicle, prostate, liver and kidney were harvested, freed from adherent tissues and immediately weighed with a digital electronic scale. The epididymis and testes were fixed in Bouin's fluid and the liver was fixed in 10 % formalin for histological examination.

3.5 Blood collection and serum preparation

This procedure was done for both phase one and phase two of the study. Blood was collected into plain tubes via cardiac puncture. After about 45 minutes the blood was centrifuged for 15 minutes at 3000 rpm. The serum portion was separated from the blood and stored at -20 ^{0}C for ELISA of hormones (FSH, LH and testosterone).

3.6 Determination of the fasting blood glucose level

Principle:

This test assesses the ability of a mammal to either produce or not produce enough insulin to stimulate the uptake of glucose from the blood after taking food high in glucose. This test is usually done after a duration of fasting to make sure the blood glucose level is so small so as not to cause the release of more than trivial amounts of insulin (Muktabhant *et al.*, 2012).

Materials:

Blood glucose monitor (Fine test Auto-coding ^{TM} Premium, USA), operator's manual, reagent (test) strips.

Procedure:

Blood sample was obtained from the rat tail. It was immediately allowed to drop on the Blood glucose monitor. The blood glucose level was determined and recorded using the operator's manual as a guide (Muktabhant *et al.*, 2012).

3.7 Assessment of serum hormonal level

3.7.1 Determination of testosterone

Antibody, enzyme-antigen conjugate and testosterone (native antigen) are the essential reagents needed for the enzyme immunoassay.

Procedure:

Assay was done in duplicate; two wells were prepared for each of the seven points of the calibrator curve and each sample and one for blank. The wells were mixed thoroughly and

incubation done at 25 ^{0}C for 1 hour. Thereafter content of each well was removed and washed with 350 µl of wash buffer. The washing was repeated two more times by decanting the water (Ekins, 1998).

A 100 µl of working substrate solution (mixture of substrate A and substrate B) was then added separately to the 3 wells containing calibrator, sample and blank. The wells were incubated at 28-30 ^{o}C for 15 minutes. Thereafter 50 µl of stop solution was added separately to the 3 wells containing the calibrator, sample and blank then gently mixed for 15- 20 seconds. Absorbance reading was at 450 nm against blank (Ekins, 1998).

3.7.2 Determination of Follicle Stimulating Hormone and Luteinizing Hormone

The assay (FSH or LH enzyme immunoassay) is an immunometric "sandwich" design, the important reagents are high affinity and specificity antibodies (enzyme and immobilized) with distinct epitope recognition and native antigen (serum). The FSH and LH Enzyme linked immunosorbent assay (ELISA) kits were used (Matthew *et al.,* 2000).

Procedure:

Assay was done in duplicate; two wells were prepared for each of the six points of the calibrator curve and each sample and one for blank. 0.50 mL of serum were aliquot into the assigned wells, 0.1 mL of FSH or LH – Enzyme reagent was added to all wells mixed thoroughly then incubated at 25 ^{0}C for an hour. Thereafter the content of each well was decanted and washed with 300 µl of wash buffer. Washing was repeated two more times by decanting the water (Matthew *et al.,* 2000).

A 100 µl of working substrate solution (mixture of substrate A and substrate B) was then added separately to the 3 wells containing calibrator, sample and blank. The wells were then incubated at 25-27 ^{o}C for 15 minutes. Thereafter 50 µl of stop solution was added separately to the 3 wells containing the calibrator, sample and blank and mixed gently for 15- 20 seconds. Reading of absorbance was at 450 nm (Matthew *et al.,* 2000).

3.8 Epididymal sperm profile analysis

The method of Raji *et al.,* (2000) was used to prepare the epididymal fluid for this analysis. Sperm analysis was done as described by Zemjanis (1970).

3.8.1 Epididymal progressive sperm motility

This was done instantaneously and rapidly, epididymal fluid was obtained from the opened caudal epididymis, it was placed on the slide and a drop of warm sodium citrate (2.9%) was added. A cover slip was placed on it and viewing was done with a x40 objective lens of light microscope (Olympus, Japan) to evaluate motility which was expressed in percentage.

3.8.2 Epididymal sperm viability

Eosin/nigrosin stain was used. Epididymal fluid was dropped on a slide and stained, a dense smear was made and dried. It was viewed under x40 objective lens light microscope. The sperm cells alive were not stained but dead sperm cells were stained. A minimum of 100 total of spermatozoa were counted and mean percentage of live sperm was taken.

3.8.3 Epididymal sperm morphology

This was done by using the stain of Wells and Ewa on the sperm smears on the slides which were air-dried and viewed with microscope (x100 objectives) under immersion oil. The sperm with abnormal morphologies were determined from total count of about 400 spermatozoa (Vasan, 2011).

3.8.4 Epididymal volume

This was done by dipping the epididymis in a 10 mL measuring cylinder containing 5 mL of normal saline so as to record the volume of fluid displaced.

3.8.5 Epididymal sperm concentration

The caudal epididymis was minced in 1 mL normal saline and rediluted to a total dilution factor of 200. A cover slip was fixed firmly on the Neubauer counting chamber and a drop of the homogenized epididymis was released on the counting chamber, below the cover slip. The spermatozoa were counted in five large Thoma square using a microscope (Raji

and Bolarinwa, 1997). The volume of 5 squares is 1/50 µL, the number of sperm in 1 mL was calculated and the results were presented in million/mL as shown below:

Sperm count (million/mL) = Number of cells counted x 50 x 200 (dilution factor) x 1000/mL.

3.9 Histological assessment of the organs

3.9.1 Histological assessment of epididymis and testes

The epididymis and testes were fixed in Bouin's fluid for about 20 hours, while the liver was fixed in 10 % formalin for at least 5 hours as soon as they were collected from the animals.

3.9.1.1 Dehydration

This was done by passing the tissues through graded ethanol (70%, 95%, 100%) for 2 hours each consecutively. This gradual procedure was undertaken so as to remove the inherent water content of the organ in a gradual way considering osmotic dynamics.

3.9.1.2 Clearing

The tissues were then cleared of the ethanol that the tissues had bathed in and to initiate and complete the process that would make cells transparent at microscopic level. The clearing was done with xylene for 6 hours.

3.9.1.3 Embedding

The tissues were then infiltrated by placing them in molten paraffin wax which served as support to the tissues for subsequent stage of sectioning. The tissues were infiltrated for 6 hours, thereafter the tissues were embedded; which was the positioning of the processed, infiltrated tissues in molten paraffin wax within an enclosure called mould. The embedded tissues were left until the wax solidifies. The tissues were cut into blocks and held in position by paraffin wax. The blocks were then clamped and positioned for sectioning.

3.9.1.4 Microtomy

Sectioning was done with a microtome which cut only a thin slice of the original tissues at a preset thickness of 4 µm. The satisfactory sections were picked up with microscope glass slides that had been coated on one side with glycerin egg albumin. The slides carrying the

sections were then labeled with a diamond pencil, and arranged in a slide carrier and then put in an oven to dry.

3.9.1.5 Staining

Haemotoxylin and eosin stain was placed on the slides so as to determine the over-all morphology. It was afterwards dewaxed with xylene three times for 3 minutes each and hydrated in 100% ethanol for 3 minutes. This was followed by hydration in 96% ethanol, 75% ethanol and water successively for 3 minutes each. The slides were stained in haematoxylin for 15 minutes and left-over stain was washed off. The slides were put in 1% acid for 5 seconds and washed off. The slides were thereafter counterstained in eosin for 3 secs and dehydrated in serial ethanol solutions (50 %, 70 %, 95 %, and 100 %). The stained slides were cleared in xylene before mounting on the microscope for histological assessment.

3.9.2 Histology of the Liver

The Liver of the experimental rats were fixed in 10% formalin, treated with graded alcohol and xylol, embedded in paraffin and sectioned at 4–6 m thickness. Hematoxylin and Eosin stain was used for histological study.

3.10 Biochemical Analysis

3.10.1 Assessment of tissue protein activity

Total protein level was measured with spectrophotometric assay kit (Fortress® diagnostics USA).

Reagent:
Copper II sulphate 18 mmol/l
Biuret Reagent consisting NaOH 200 mmol/l
Sodium Potassium Tartrate 32 mmol/l
Standard made up of protein solution of 60 mg/mL

Procedure:

The standard, sample and 20 µL of distilled water was added to the test tubes for, standard sample and blank respectively. A 1000 µL of Biuret reagent was added to all test tubes. Incubation was done for ten minutes at 37 ^{o}C and absorbance read at 546 nm against the blank.

Calculation:

$$\text{Total protein (mg/mL)} = \frac{\Delta \text{ Abs sample}}{\Delta \text{ Abs Strd}} \times \text{strd concentration}$$

Where

Abs = Absorbance

Abs = Absorbance

3.10.2 Evaluation of tissue lipid peroxidation.

This was done by measuring Thiobarbituric Acid Reactive Substances (TBARS/MDA) produced during lipid peroxidation as described by Buege and Aust (1978).

Procedure:

2 mL of TCA-TBA-HCL acid reagent was added to 1 mL of sample in a test tube. 3 mL of this reagent was placed in a blank test tube. The mixture was placed in boiling water bath (100^{o}C) for 15 minutes. Cooling and centrifuging was done to remove flocculent precipitate at 1000 g for 10 minutes. Absorbance of supernatant was read at 535 nm against the blank.

$$\text{TBARS activity} = \frac{\text{O.D x V x 1000}}{\text{A x v x 1 x Y}}$$

Where

O.D = absorbance of sample test at 535 nm

V = Total volume of the reaction = 3 mL

A = Molar estimation co-efficient of product = 1.56×10^{5} $m^{-1}cm^{-1}$

I = light path = 1 cm

V = volume of tissue extract used = 1 mL

Y = mg of tissue in the volume of sample used.

3.10.3 Assessment of tissue catalase activity.

This was done as described by the method of Sinha (1972).

Reagents:

5 % $K_2Cr_2O_7$ (Dichromate solution)

0.2 M H_2O_2

Stock dichromate/acetic acid solution.

Working dichromate/acetic acid solution

Phosphate buffer

Procedure:

A mixture of 0.5 mL of H_2O_2, 1.0 mL buffer and 0.4 mL water was made and 0.2 mL of enzyme was added so as to initiate reaction. Two mililitres of dichromate/acetic acid was added after 0, 30, 60 and 90 seconds of incubation. The enzyme was added to the control tube after adding the acid reagent heating was done for 10 minutes and colour formed was read at 610 nm. Catalase activity was expressed as µmole of H_2O_2 decomposed/min/mg protein.

Calculation:

$$\text{Amount of } H_2O_2 = \frac{\text{Abs test sample}}{\text{Abs of standard}} \times \text{Conc. Standard}$$

Activity = 1/time log Conc. X standard/conc. Test

Where

Abs = Absorbance

Conc. Standard = concentration

3.10.4 Assessment of tissues superoxide dismutase (SOD) activity.

This was done as described by Misra and Fridovich (1977).

Reagents:

i. 0.05 M carbonate buffer (pH 10.2).
ii. 0.03 mM Adrenaline solution (Freshly prepared).
iii. 0.005 M HCL Solution.
iv. 0.10 M HCL Solution.

Procedure:

0.2 mL distilled water was added to reference tube, while 0.2 mL of the enzyme extract was added to the sample test tubes. Carbonate buffer (2.5 mL) was added to each test tubes and equilibrated at 25 ^{o}C, 0.3 mL of 0.3 mM adrenaline solution was then added to the reference and each of the test solutions. The contents were mixed and reading was at 420 nm against a blank.

Calculation:

Δ absorbance/minutes = (A_3-A_0)

Where A_0 is absorbance after 30 seconds

A_3 is absorbance after 150 seconds

$$\% \text{ inhibition} = \frac{100 \times \Delta \text{ substrate absorbance}}{\Delta \text{ blank absorbance}} \times 100$$

However, one unit of SOD activity equals quantity of SOD needed to cause 50% inhibition of auto-oxidation of adrenaline to adrenochrome/minutes.

$$\text{SOD enzyme activity (Units /mg wet tissue)} = \frac{\% \text{ Inhibition}}{50 \text{ X } y}$$

Where y = mg of tissue in the volume of the sample used.

3.10.5 Assessment of tissue reduced glutathione concentration.

The reduced glutathione level was measured by spectrophotometric assay kit (Oxford® Biomedical Research, USA).

Reagent:

i. NADPH: Vial content was reconstituted with 500 µL Assay Buffer and added to 5.5 mL Assay vortexed and left on ice.
ii. Oxidoreductase: 30 µL of Oxidoreductase was added to 6 mL Assay Buffer and left on ice.
iii. DTNB: Vial content was reconstituted with 500 µL Assay Buffer and added to 5.5 mL Assay vortexed and left at 25^{o}C.
iv. Standard curve preparation: A GSH standard was prepared with GSH Working Stock at 0.5 to 20 µM in 5 % MPA.

Procedure:

Tissue was extracted and washed in 1x phosphate buffered saline, blotted on a filter paper and placed (20 mg to 1 g) in 10 mL of ice-cold 5 % MPA solution. The tissue was homogenized and centrifuged at 3,000 x g at 4 °C for 10 minutes. Supernatant was collected and kept at 0-4 °C and assay was performed.

A 50 µL of GSH standard, diluted or undiluted sample was added to each well followed by 50 µL each of DTNB and Oxidoreductase solutions. Incubated for 10 minutes at 25ºC and 50 µL of -NADPH solution was added to each well for reaction to begin. Plate reading was done after 1 minute with a kinetic program that monitors reaction at one minute intervals for 10 minutes. Color change was observed at 405 - 412 nm.

Calculations:

The standard curve was plotted with Mean V (the rate of color change) versus GSH concentrations. The standard curve was used to determined the total GSH concentration of samples.

Mean V = (absorbance at 10 minutes - OD at 0 minutes)/10

Mean V is proportionate to the GSH standard concentrations.

3.11 Experimental protocol for phase three

This aspect of the study was undertaken so as to get a better (mechanistic) knowledge of the toxicity posed by the sample in animals. Cell-culture system was used because it is a reliable, reproducible and relatively inexpensive experimental system used to assess chemical toxicity at the cellular level.

3.11.1 Materials

- 96 well microplate (tissue culture plate code 3861-096), flat bottom 24 well tissue culture plate.
- Micropipette (Autoclavable and UV resistant Fisher brand, Ex K08813251, Made in Japan) and multipipette (Fisher brand, 9002118).

- Microplate reader (thermo scientific (Shangai) multiskan Ex. Type: 355, Ref: 51118170, SN: 3550904177 serial RS-232C) made in china.
- Inverted microscopes - Carl Zeiss, Axiovert 40 CFL (identification number 195-040136) and Ernst Leitz GMBH Wetzlar Diavert microscope with transformer (Typ 301-211.001, Nr 16200) made in Germany. Microscope camera attached to computer monitor (Axiocam 1Cc1 (195-040316) and software name - Carl Zeiss Axiovision.
- Incubator (Schutzarts DINEN 60529-IP20 memmert GmbH-CO.kg, D-91126 Schwabach FRG) made in Germany.
- Sterilegard - Class II biological safety cabinet (model SG403 A-HE-INT, serial no-99013) the Baker Company.
- Centrifuge - Thermo fisher scientific megafuge 1 made in Germany.
- Dissecting sets, Neubauer haemacytometer, cover slips, hand gloves, cotton wool, sterile bottles (duran 100 and 500 mL), Bijou bottles, petri dish, Nose masks, Head gear, and laboratory coat.

3.11.2 Reagents

Bovine serum albumin (Sigma, USA), Glutamax, Streptomycin, Penicillin, Amphotericin B, RPMI – 1640 (Modified medium - Sigma, USA), all of which were tissue culture grade, CO_2 gas, Lupeol, Diethyl Phthalate, ethanol, Dimethyl Sulfoxide (DMSO), Testosterone (ELISA kit - Sigma, USA), Luteinizing hormone (ELISA kit - Sigma, USA), MTT Cell Proliferation Assay kit, Deionized water and 70% alcohol.

3.11.3 Procedure for testicular culture

Ethical approval was given by National Veterinary Research Institute, Animal Use and Care committee ((NVRI/EC/20/016). Another set of twelve (12) adult male Wistar rats (180-200 g) were obtained from the Small Animal House, National Veterinary Research Institute Vom. They were housed, given feed and water *ad libitum* and acclimatized for 2 weeks before commencing experiment.

The rats were sacrificed by cervical dislocation and removal of testes was done in sterilized condition (Chernecky and Saunders, 1993). The method used for the study was a modification of that adopted by Rahiman and Pool (2010). Briefly;

i. pulverized testes from each rat were placed in serum free medium (SFM) which comprised of bovine serum albumin (0.2%), glutamax (1%), 1% combination of penicillin, streptomycin and Amphotericin B for prevention of contaminants and RPMI-1640.
ii. The cells were centrifuged and supernatant containing the cells was decanted into another tube.
iii. Centrifugation was done at 1000 × g for 10 minutes, supernatant was discarded, cell pellet was suspended in SFM made up to 20 mL. Incubation was done at 37 °C and 5 % CO_2 for an hour.
iv. Cells were centrifuged for 10 minutes at 1000 x g, supernatant was discarded, cell pellet re-suspended in 20 mL SFM, incubation was done at 37 °C and 5 % CO_2 for 30 minutes.
v. The cell centrifugation was done for 10 minutes at 1000 x g, the supernatant discarded, cell pellet was re-suspended in 20 mL SFM. Incubation was done for 20 minutes at 37 °C and 5 % CO_2 to acertain baseline testosterone synthesis of cells.
vi. The cell prepared were either activated or not using luteinizing hormone enzyme conjugate at 10 mIU/mL and counted using a Neubauer hemacytometer (Melissa, 2002) for seeding of cells.
vii. Five dilution ranges (15.625 ug/mL, 31.25 ug/mL, 62.5ug/mL, 125ug/mL and 250 ug/mL) were used as follows:
    - ✓ Distilled water control at (5 µl/well).
    - ✓ *Saccharum officinarum* molasses methanol fraction in distilled water (5 µl/well).
    - ✓ *Saccharum officinarum* molasses aqueous fraction in distilled water (5 µl/well).
    - ✓ Diethyl acetate in 0.5% of DMSO in distilled water (5 µl/well).
    - ✓ Lupeol in 1% alcohol and distilled water containing 0.5% DMSO (5 µl/well).

All the above were aliquot into a 96 well culture plate, the cell suspensions were seeded (100 µl/well) (from vii i.e. the LH stimulated, and unstimulated) was aliquot into the plate and incubated for 4 hours with 5 % CO_2 at 37 °C (Mand *et al.,* 2010).

ix Fresh cell preparations were made, part of the suspension cells were stimulated with LH and the other part unstimulated. The suspension cells (100 µl/well) were pipette into culture plate and 31.25 ug/mL (EC_{50}) each of *Saccharum officinarum* molasses methanol fraction in distilled water, *Saccharum officinarum* molasses aqueous fraction in distilled water, Diethyl acetate in 0.5% of DMSO in distilled water and Lupeol in 1% alcohol and distilled water containing 0.5% DMSO were aliquot at 5 µl/well. The plates were incubated for 4 and 24 hours, after which the supernatant was apirated and assessed for testosterone with Enzyme-linked immunosorbent assay (ELISA) kits.

3.11.5 MTT Cell Proliferation Assay

In vitro tests that assess cellular toxicity are characterized as tests that measure cellular functions and tests that measure cell death. This test was done to assess the *in vitro* viability of the cells before determining the steriodogenic function of the testicular cells *in vitro* (Riss *et al*., 2013).

3.11.6 Kit Components

1. MTT Cell Proliferation Assay Reagent (One 10 mL bottle of MTT reagent).
2. Detergent Solution (One 100 mL bottle of Detergent Solution).

Procedure:

1. Cell suspension was prepared comprising 0.1-1.0 x 10^6 cells/mL in medium.
2. 100 µl of cell suspension/well was added to a 96-well cell culture plate with *Saccharum officinarum* molasses methanol fraction in distilled water, *Saccharum officinarum* molasses aqueous fraction in distilled water, Diethyl acetate in 0.5% of DMSO in distilled water and Lupeol in 1% alcohol and distilled water containing 0.5% DMSO and culture for 24 hours at 37 ^oC and 5 % CO_2 in a humidified incubator.

3. To each well, 100 µl of the CytoSelect^{TM} MTT Cell Proliferation Assay Reagent was added.
4. Incubation of plate was at 37 ^{o}C and 5 % CO_2 for 3-4 hours until purple precipitate was visible.
5. 100 µl of detergent solution was added/well.
6. Incubation was at 4 hours protected from light.
7. Cell viability was determined using spectrophotometer reading at 570 nm as the primary wavelength.

3.12 Statistical analysis

Data were presented as mean ± SEM. Mean differences were compared via one-way ANOVA with Graphpad prism 5 and statistically significant at $P \leq 0.05$.

CHAPTER FOUR

4.0 RESULTS

4.1 Phytochemical screening of *Saccharum officinarum* juice.

The result showed that the chemical constituents present in S*accharum officinarum* juice are saponins, tannins, flavonoids, terpenoid, cardiac glycosides, alkaloids and reducing sugars. Antraquinone was absent (Table 4.1).

Table 4.1 Phytochemical constituents of *Saccharum officinarum* juice.

Constituent	Observation
Saponin	++
Tannin	+
Flavonoids	+++
Terpenoid	+++
Cardiac glycosides	+
Antraquinone	-
Alkaloids	++
Reducing sugar	+++

Key: + present, - absence

\+ Low

++ Moderate

+++ High

4.2 Effect of *Saccharum officinarum* juice on fasting blood glucose level.

The fasting blood glucose level was significantly increased ($P<0.05$) in groups treated with 3.2 and 10.0 mL/kg/day juice as compared with control (Figure 4.1; Appendix 1).

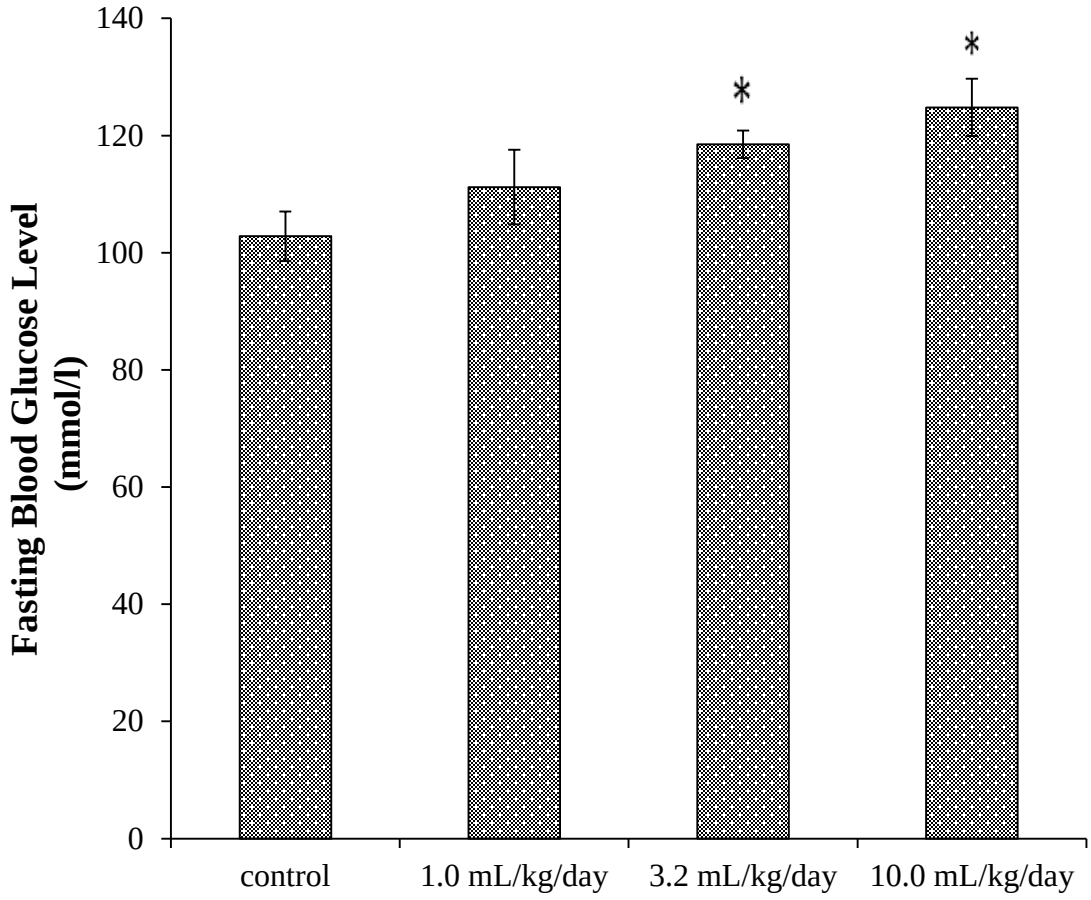

Figure. 4.1 Effect of *Saccharum officinarum* juice on fasting blood glucose level. Columns represents Mean±SEM. n=5. *P<0.05 relative to the control.

4.3 Effect of *Saccharum officinarum* juice on relative organ weights.

Saccharum officinarum juice significantly increased ($P<0.05$) in epididymal weight of rats treated with 10.0 mL/kg/day. Seminal vesicle weight was significantly increased ($P<0.05$) in groups treated with 3.2 and 10.0 mL/kg/day. There were on changes in the weights of the testes, kidney and liver (Table 4.2).

Table 4.2 Effect of *Saccharum officinarum* juice on relative organ weights (g).

Group	**Control**	**SOJ mL/kg/day**		
		1.0	**3.2**	**10.0**
Testes	0.64±0.014	0.62±0.04	0.62±0.037	0.71±0.023
Epididymis	0.08±0.006	0.08±0.005	0.09±0.01	0.13±0.02*
Seminal vesicle	0.11±0.011	0.12±0.006	0.14±0.008*	0.14±0.013*
Kidney	0.74±0.087	0.63±0.063	0.62±0.012	0.72±0.053
Liver	4.18±0.162	4.03±0.244	3.69±0.195	4.20±0.291

Data presented as Mean±SEM. n=5. *P<0.05 relative to the control.

4.4 Effect of *Saccharum officinarum* juice on percentage change in body weight.

At the end of treatment period (eight weeks), the percentage change in body weight of groups treated with 1.0 and 3.2 mL/kg/day *Saccharum officinarum* juice (SOJ) were significantly decreased compared with the percentage change in body weight observed in the control. There was significant decrease ($P<0.05$) in percentage change in body weight of groups treated with 10.0 mL/kg/day SOJ compared with the percentage change in body weight of the control. Also, the percentage change in body weight of group treated with 3.2 mL/kg/day SOJ was significantly decreased compared with the percentage change in body weight of groups treated with 1.0 and 10.0 mL/kg/day SOJ (Figure 4.2; Appendix 2).

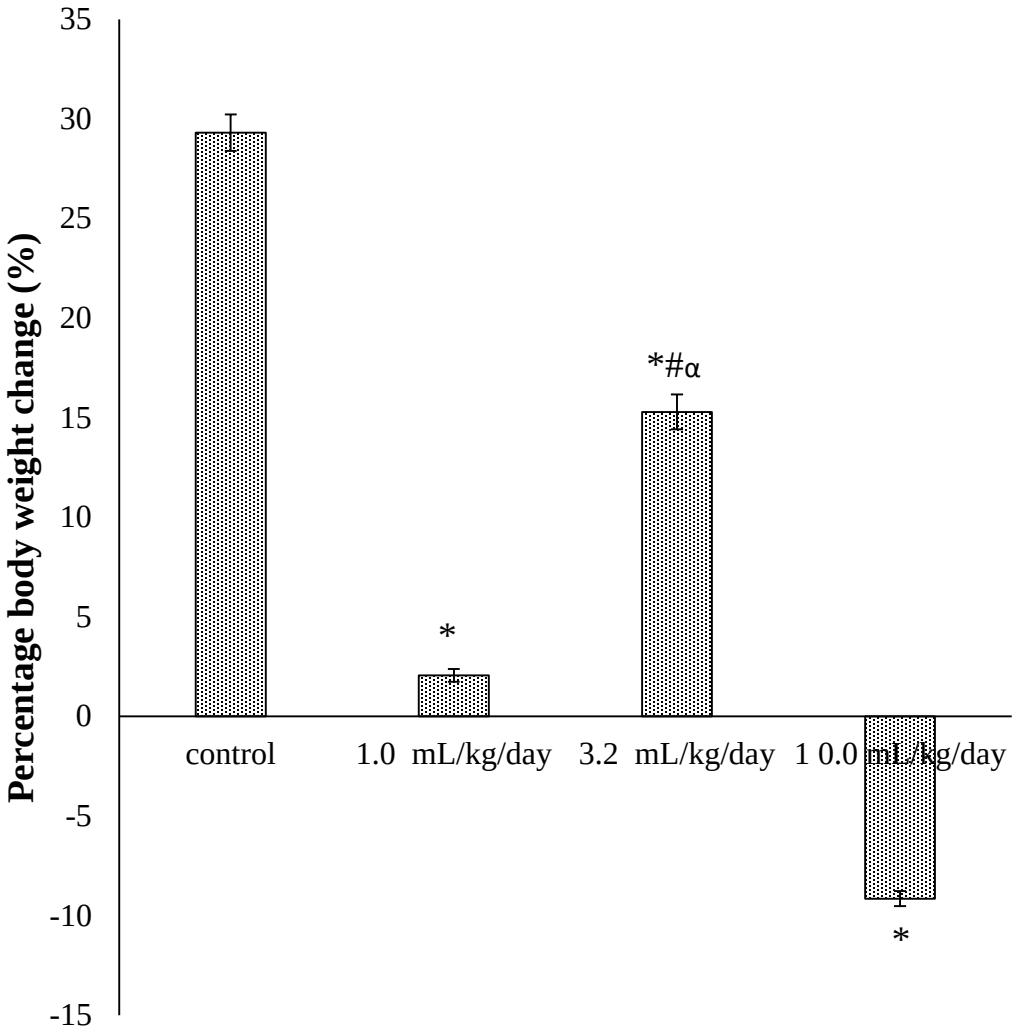

Figure 4.2 Effect of *Saccharum officinarum* juice on percentage change in body weight. Data presented as Mean±SEM. n=5. *P<0.05 compared with control. $^{\#}$P<0.05 compared with 1.0 mL/kg/day. $^{\alpha}$P<0.05 compared with 10.0 mL/kg/day.

4.5 Effect of *Saccharum officinarum* juice on serum hormonal profile.

There were no significant changes in the serum levels of FSH and LH of *Saccharum officinarum* juice (SOJ) treated groups compared with the control (Figure 4.3; Appedix 3 and 4). Serum testosterone level significantly increased in 10.0 mL/kg/day SOJ relative to the control, 1.0 and 3.2 mL/kg/day SOJ (Figure 4.4; Appendix 5).

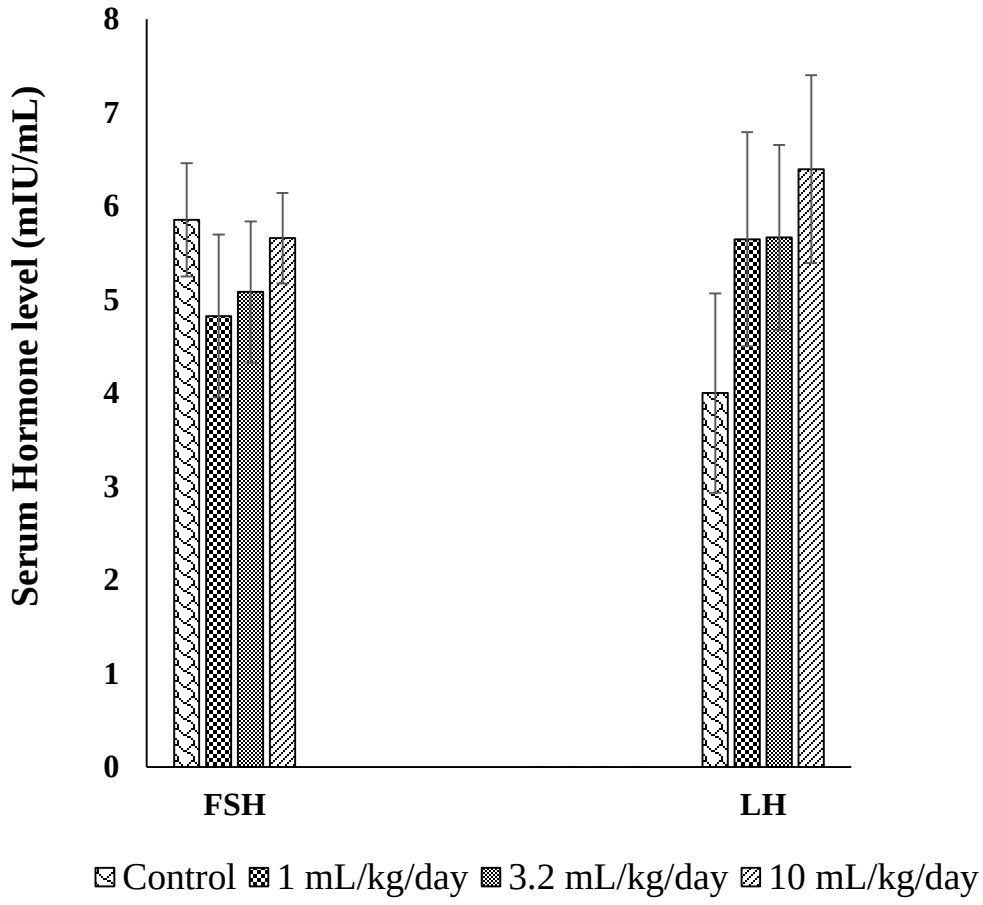

Figure. 4.3 Effect of *Saccharum officinarum* juice on serum Follicle stimulating hormone and Luteinizing hormone level. Columns represents Mean±SEM. n=5.

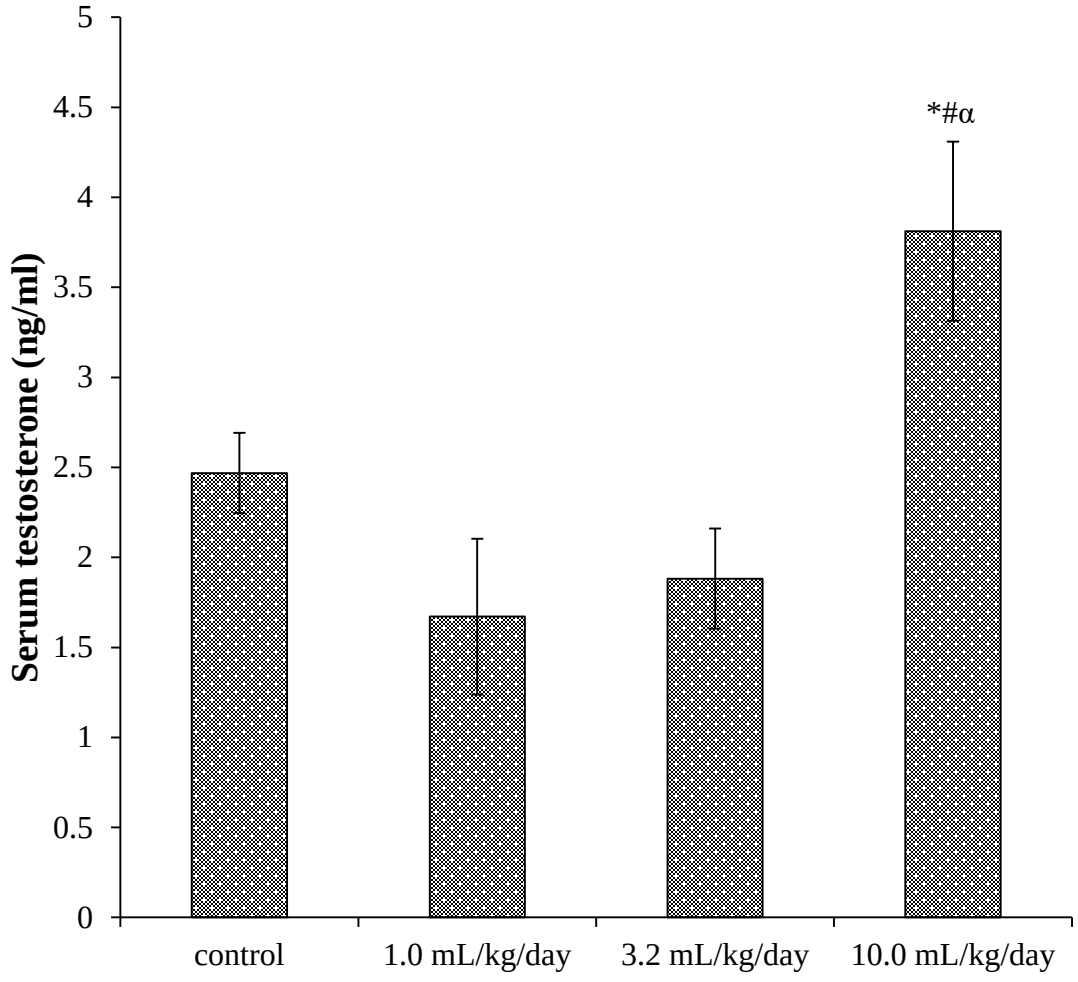

Figure. 4.4 Effect of *Saccharum officinarum* juice on serum testosterone level. Columns represents Mean±SEM. n=5. *P<0.05 compared with control. $^{\#}$P<0.05 compared with 1.0 mL/kg/day. $^{\alpha}$P<0.05 compared with 3.2 mL/kg/day.

4.6 Effect of *Saccharum officinarum* juice on epididymal sperm characteristics.

The sperm concentration and percentage abnormal sperms were significantly increased ($P<0.05$) in the group treated with 10.0 mL/kg/day *Saccharum officinarum* juice (SOJ) (Figures 4.5 and 4.6; Appendix 6 and 8; Plate 1). Whereas figure 4.7 (Appendix 8) show that 10.0 mL/kg/day SOJ significantly decreased ($P<0.05$) sperm viability relative to control and 1.0 and 3.2 mL/kg/day SOJ. The sperm motility of groups treated with 1.0 and 3.2 mL/kg/day were significantly decreased ($P<0.05$) compared with control and 10.0 mL/kg/day SOJ (Figure. 4.8; Appendix 9).

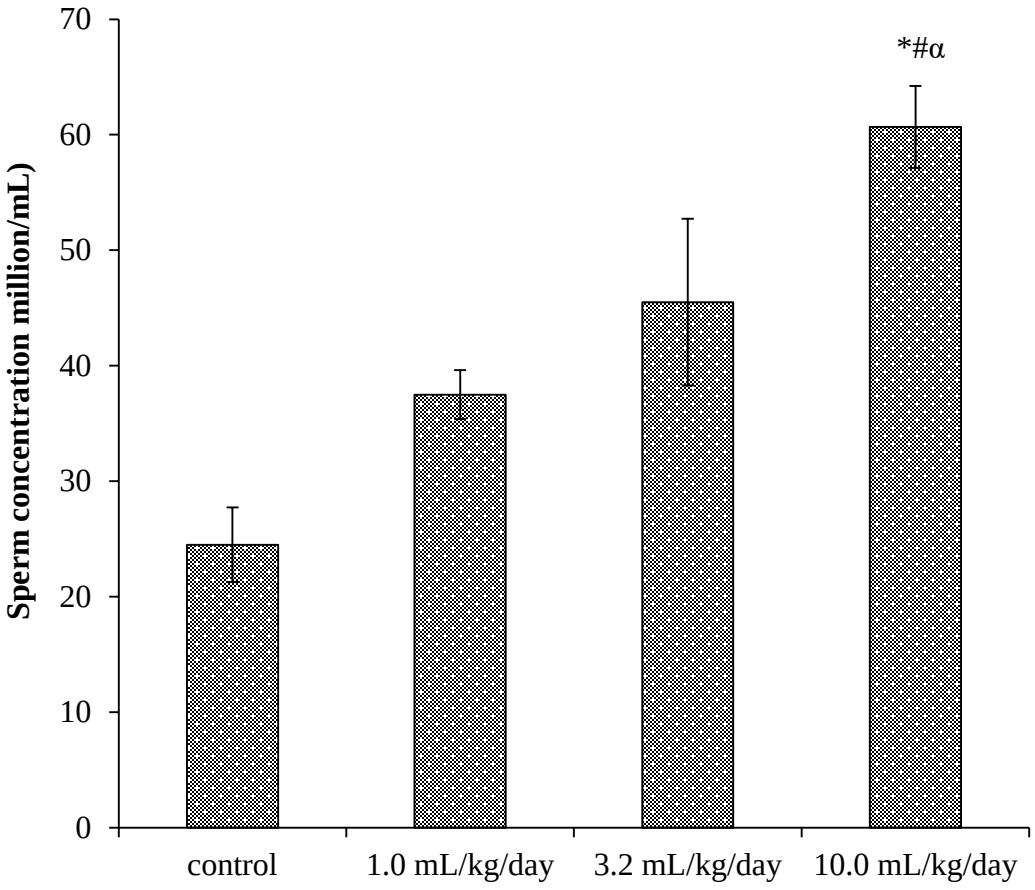

Figure. 4.5 Effect of *Saccharum officinarum* juice on sperm concentration.
Columns represents Mean±SEM. n=5. *P<0.05 as related to control. $^{\#}$P<0.05 compared with 1.0 mL/kg/day. $^{\alpha}$P<0.05 compared with 3.2 mL/kg/day.

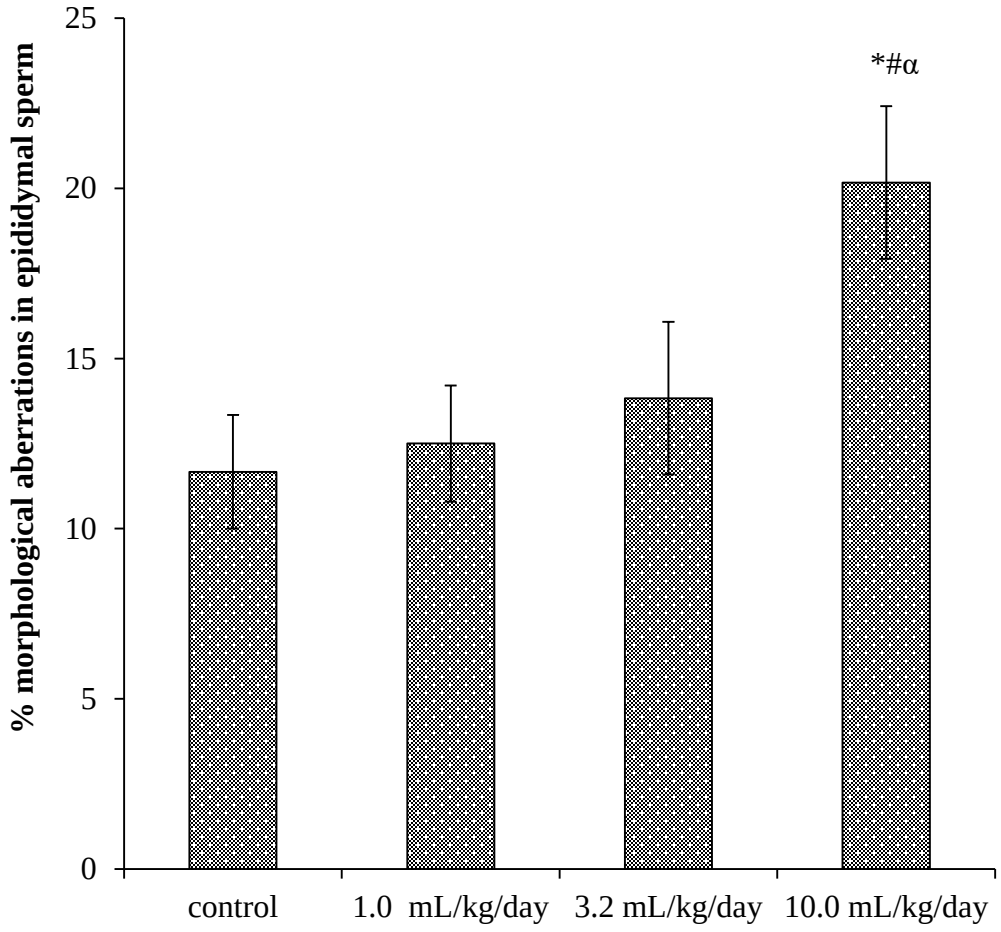

Figure. 4.6 Effect of *Saccharum officinarum* juice on percentage morphological aberrations in epididymal sperm. Columns represents Mean±SEM. n=5. *P<0.05 as compared to control. #P<0.05 compared to 1.0 mL/kg/day. αP<0.05 compared to 3.2 mL/kg/day.

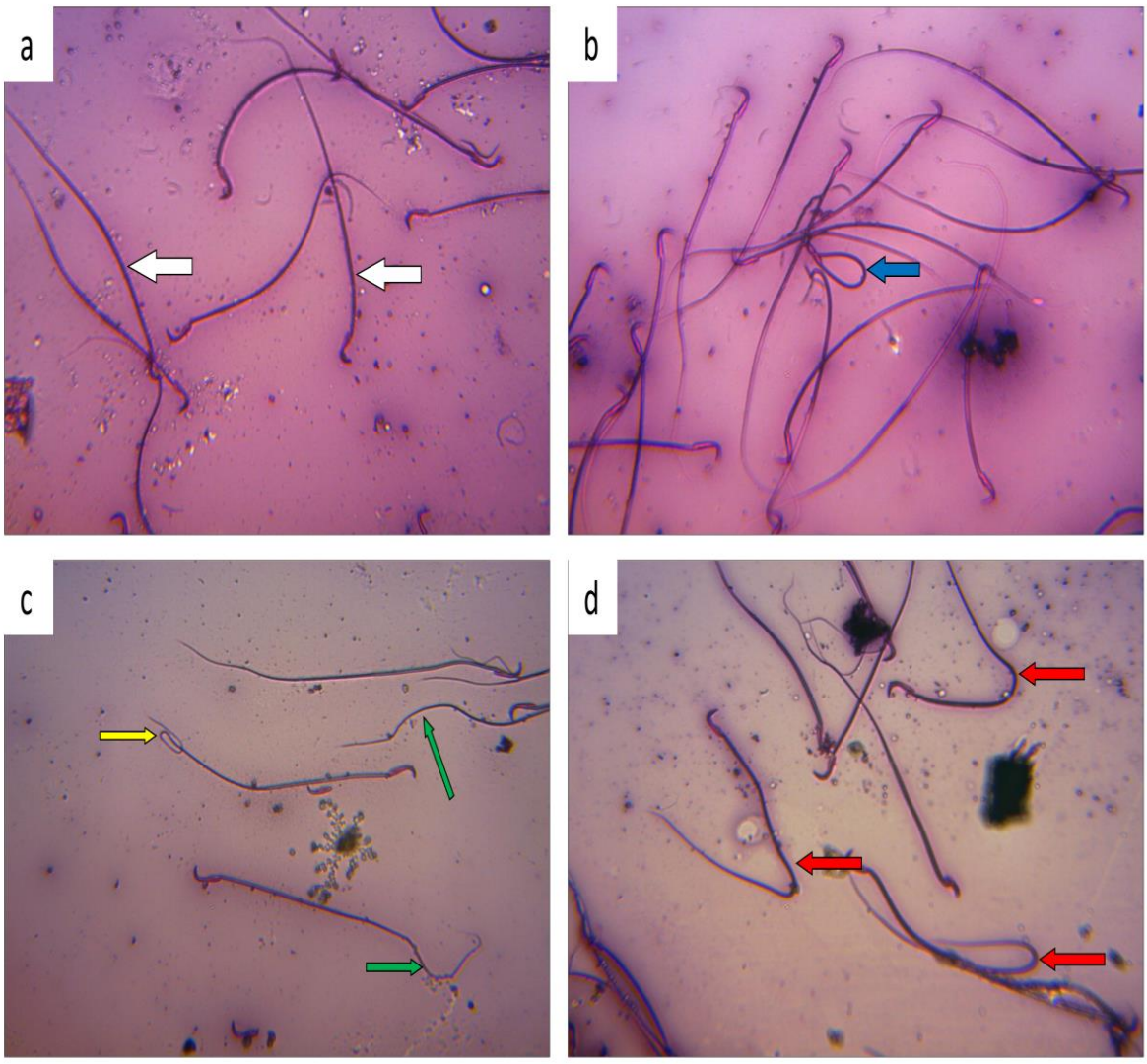

Plate 1. Morphology of spermatozoa of rat treated with *Saccharum officinarum* juice (SOJ)

Stained by eosin nigrosin, presented at x400 magnification.

a) Morphology of spermatozoa of control rat, show normal head midpiece and tail (white arrows).

b) Morphology of spermatozoa of rat treated with 1.0 mL/kg/day SOJ, show coiled midpiece (blue arrow)

c) Morphology of spermatozoa of rat treated with 3.2 mL/kg/day SOJ, show coiled tail (yellow arrow)

d) Morphology of spermatozoa of rat treated with 10 mL/kg/day SOJ, show Bent midpiece (red arrows).

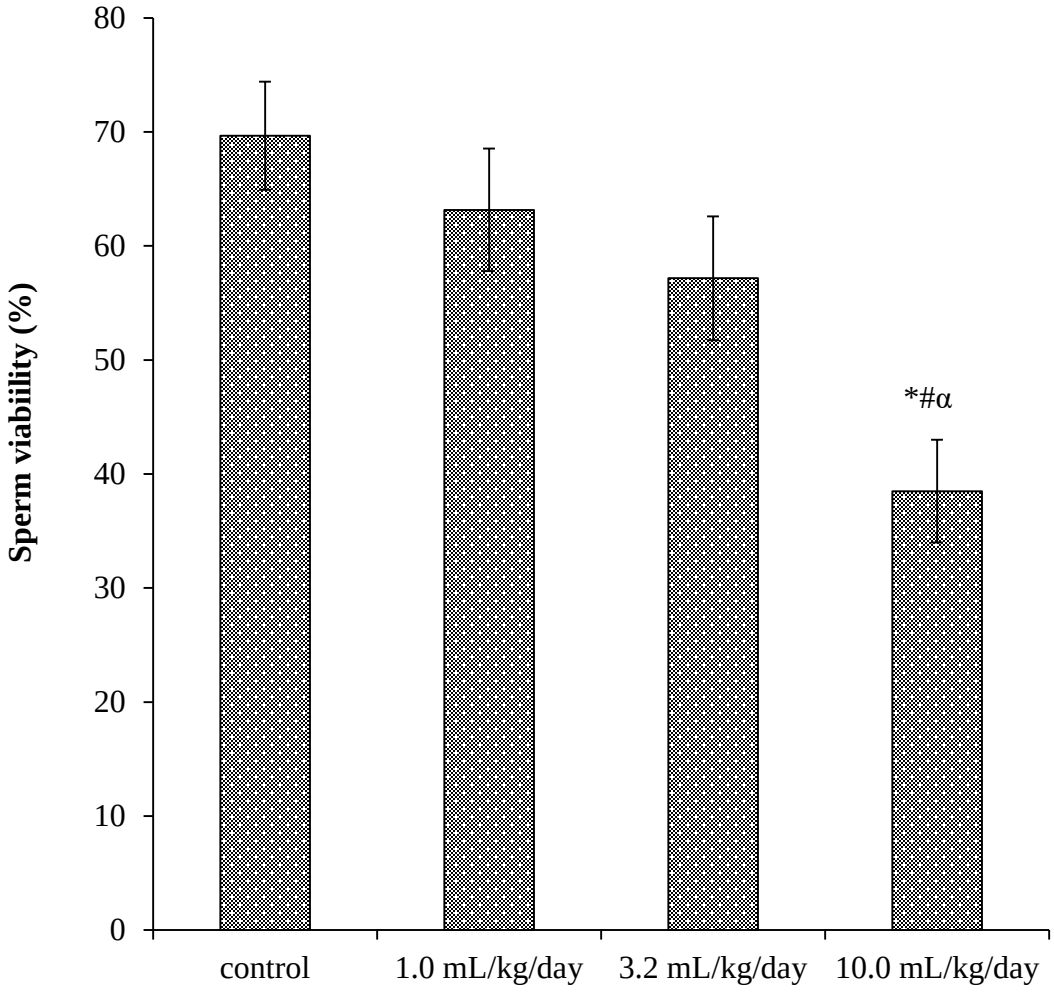

Figure. 4.7 Effect of *Saccharum officinarum* juice on sperm viability.
Columns represents Mean±SEM. n=5. *P<0.05 compared with the control, #P<0.05 compared with 1 mL/kg/day, αP<0.05 compared with 3.2 mL/kg/day.

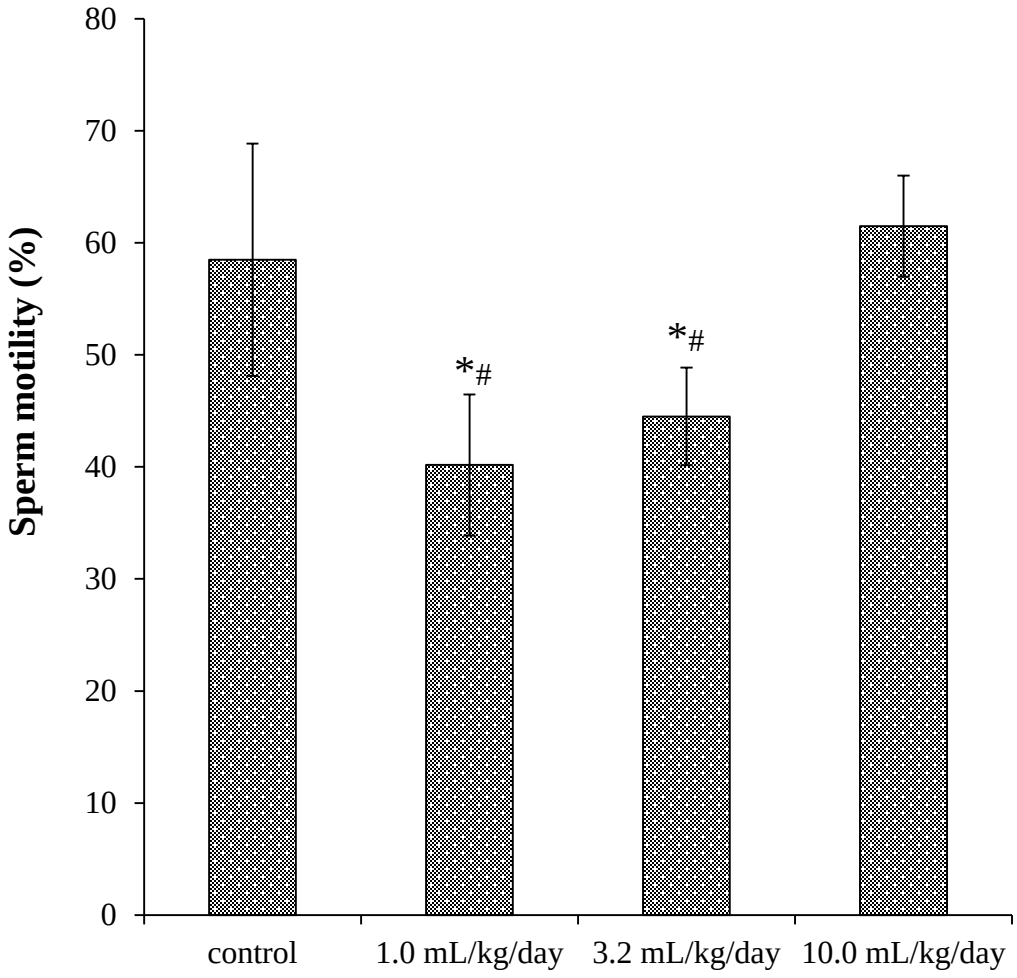

Figure. 4.8 Effect of *Saccharum officinarum* juice on sperm motility. Columns represents Mean±SEM. n=5. $^{*}P<0.05$ compared with control. $^{\#}P<0.05$ compared with 1.0 mL/kg/day.

4.7 Effect of *Saccharum officinarum* juice on histology of testes.

Testicular sections from group treated with 1 mL/kg/day *Saccharum officinarum* juice, showed few Leydig cells with wide and empty interstitial spaces. The groups administered 3.2 and 10 mL/kg/day *Saccharum officinarum* juice showed testicular sections with mild interstitial vascular congestion (Plate 1).

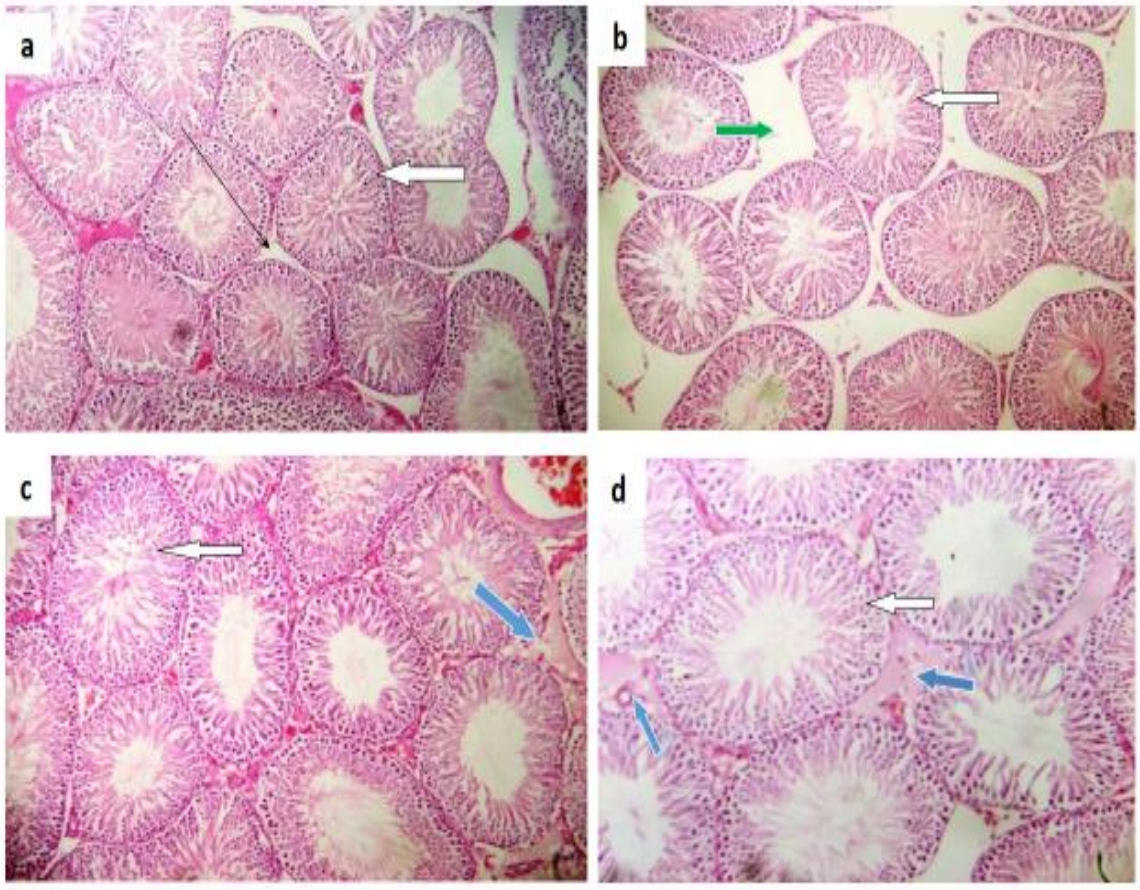

Plate 2. Photomicrograph of testicular section of rat treated with *Saccharum officinarum* juice (SOJ).

Tissues stained by H&E, presented at x100 magnification.

a) Testicular section of control rat, shows normal seminiferous tubules. Spermatogonia, Sertoli cells and lumen (white arrow) appear normal. The Leydig cells and interstitium appear normal (black arrow).

b) Testicular section of rat treated with 1 mL/kg/day SOJ show normal seminiferous tubules. Spermatogonia, Sertoli cells and lumen (white arrow) appear normal. The interstitium appear wide and empty with few Leydig cells (green arrow).

c) Testicular section of rat treated with 3.2 mL/kg/day SOJ show normal seminiferous tubules the spermatogonia, Sertoli and germ cell layer appear with normal maturation stages (white arrow). There is mild vascular congestion in the interstitium (blue arrow).

d) Testicular section of rat treated with 10 mL/kg/day SOJ show normal seminiferous tubules the spermatogonia Sertoli cells appear normal and germ cell layer show normal maturation stages (white arrow) and interstitium show mild vascular congestion (blue arrows)

4.8 Effect of *Saccharum officinarum* juice on histology of epididymis.

Epididymal section from 10 mL/kg/day *Saccharum officinarum* juice (SOJ) show wide interstitium with mild infiltration of inflammatory cells; all other treated groups show normal architecture (Plate 2).

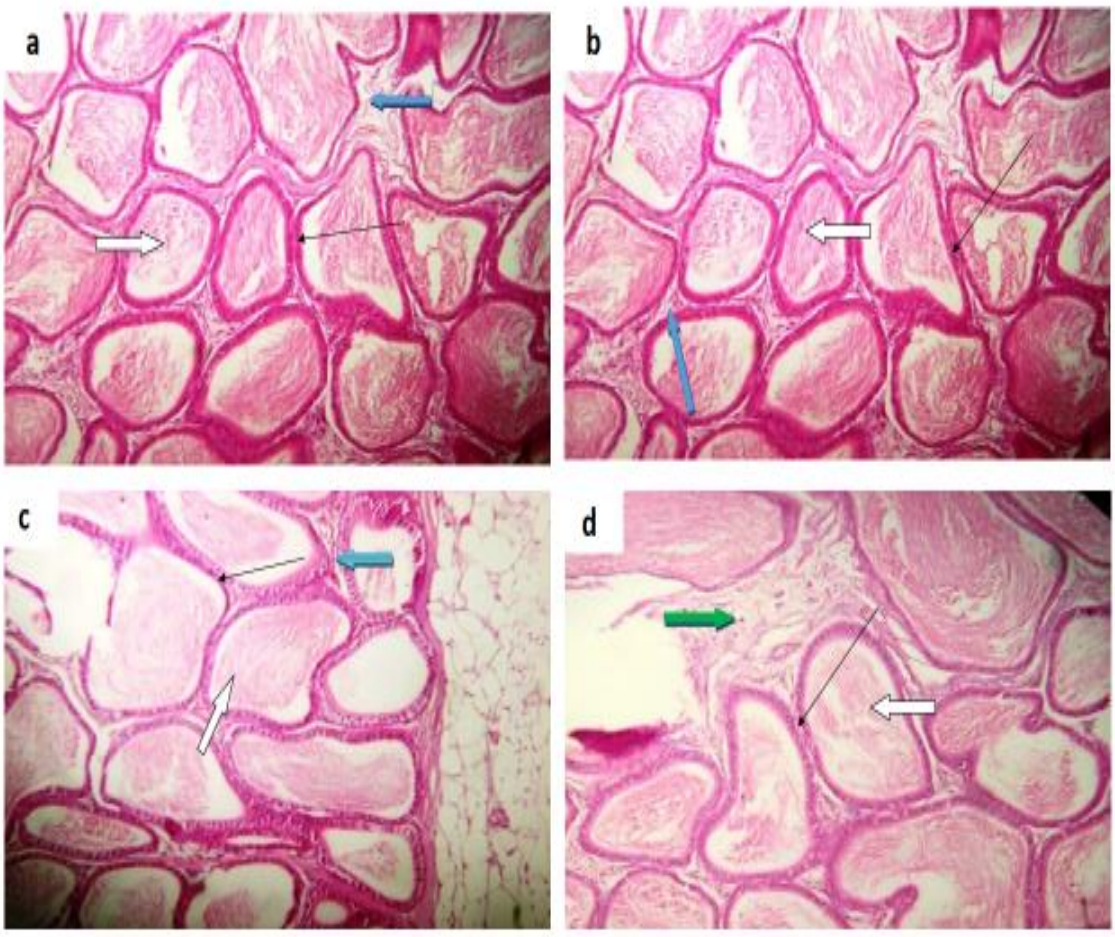

Plate 3. Photomicrograph of epididymal section of rat treated with *Saccharum officinarum* juice (SOJ).

Tissues stained by H&E, presented at x100 magnification.

a) Epididymal section of control rat shows normal epididymal ducts, epithelial layers and normal smooth muscle (black arrow), lumen contains spermatozoa (white arrow). The interstitial cells and spaces appear normal (blue arrow).

b) Epididymal section of rat treated with 1 mL/kg/day SOJ show normal epididymal ducts, smooth muscle and epithelial layer (black arrow), the lumen contains spermatozoa (white arrow). The interstitium appear normal (blue arrow).

c) Epididymal section of rat treated with 3.2 mL/100/day SOJ show normal smooth muscle, epithelial layers and epididymal ducts, (black arrow), lumen contains spermatozoa (white arrow). The interstitium appear normal (blue arrow).

d) Epididymal section of rat treated with 10 mL/100g/day SOJ show normal epithelial layers, epididymal ducts and smooth muscle (black arrow), lumen contains spermatozoa (white arrow). Widen interstitium with mild infiltration of inflammatory cells (green arrow).

4.9 Phytochemical Screening of fractions from *Saccharum officinarum* molasses.

The crude *Saccharum officinarum* molasses (SOM) consist of saponins, flavonoids alkaloids and reducing sugars (Table 4.3). *Saccharum officinarum* molasses methanol fraction (SOMMF) consist majorly of saponins, flavonoids, terpenoid, cardiac glycosides and reducing sugars (Table 4.3). Tannins, antraquinone and alkaloids were absent. The *Saccharum officinarum* molasses aqueous fraction (SOMAqF) consist of saponins and flavonoids (Table 4.3).

Table 4.3 Phytochemical constituents of fractions from *Saccharum officinarum* molasses.

Phytochemical	**Crude *Saccharum officinarum* molasses (SOM)**	***Saccharum officinarum* molasses methanol fraction (SOMMF)**	***Saccharum officinarum* molasses aqueous fraction (SOMAqF)**
Saponin	+	++++	++
Tannin	-	-	-
Flavonoids	+	++	++
Terpenoid	-	++	-
Cardiac glycosides	-	++++	-
Antraquinone	-	-	-
Alkaloids	+	+	-
Reducing sugar	+	++++	-

Key: + present, - absence

\+ Low

++ Moderate

+++ High

4.10 Gas chromatography and mass spectroscopy (GCMS) of *Saccharum officinarum* molasses methanol and aqueous fractions.

Figures 4.9 and 4.11 show GCMS of fractions from *Saccharum officinarum* molasses showing relative abundance of chemical constituents with retention time. The predominant constituents identified in *Saccharum officinarum* molasses methanol fraction (SOMMF) was Lup-20(29)-en-3-ol, acetate, (3. beta) at retention time 44.62s (Table 4.5). Nine constituents were identified in *Saccharum officinarum* molasses aqueous fraction (SOMAqF), the most abundant constituent identified was Diethyl Phthalate at retention time 13.44s at retention time 43.55s and least abundant constituent was Octasiloxane, 1,1,3,3,5,5,7,7,9,9, 11,11,13,13,15,15-hexadecamethyl- (Table 4.7). Figures 4.10 and 4.12 show the fragmentation patterns and structures of Lup-20(29)-en-3-ol, acetate, (3. beta.) and Diethyl Phthalate respectively.

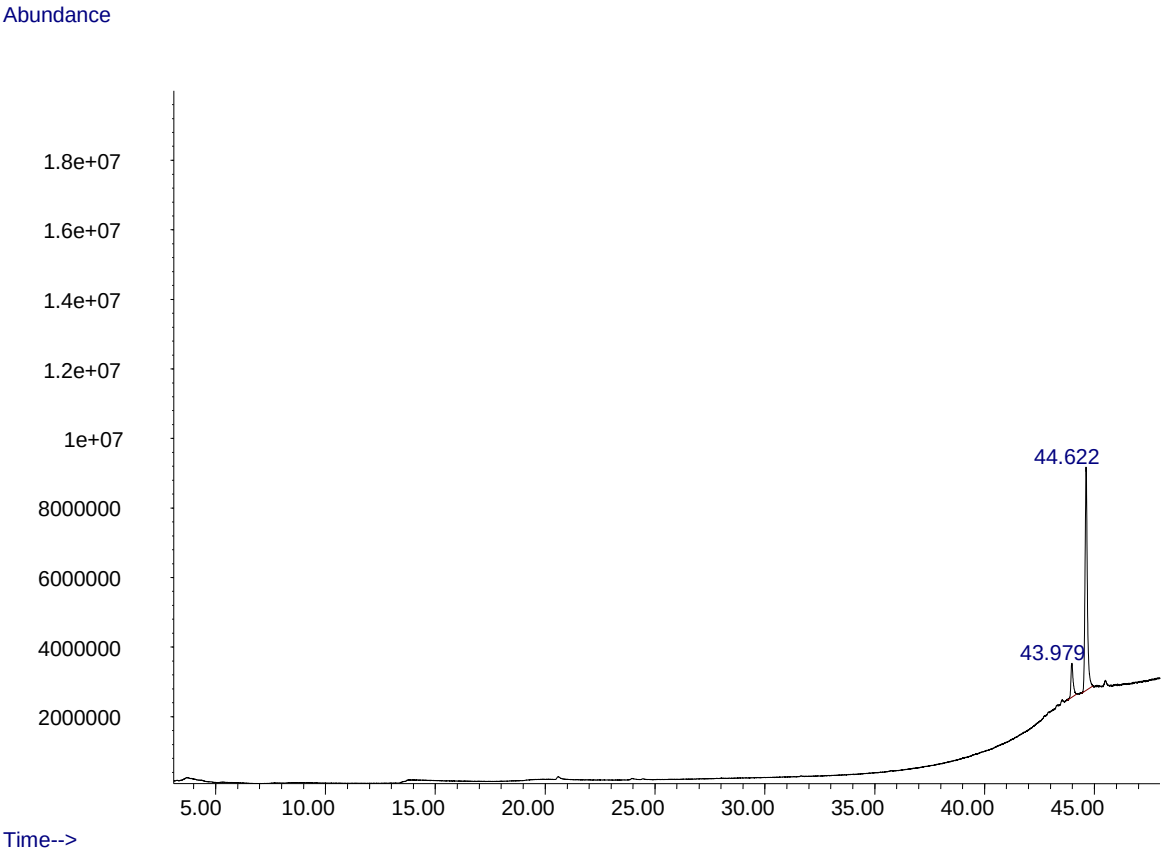

Figure. 4.9 Gas chromatogram of the chemical constituents in *Saccharum officinarum* molasses methanol fraction.

Table 4.4 Retention time and percentage of total constituents of *Saccharum officinarum* molasses methanol fraction.

Peak #	R.T. min	first scan	max scan	last scan	PK TY	Peak height	corr. area	corr. % max	% of total
1	43.979	7107	7147	7184	VV 2	974168	68661877	14.69%	12.810%
2	44.622	7222	7259	7312	PV 8	6426753	467334402	100.00%	87.190%

Sum of corrected areas: 535996279

Table 4.5 Nomenclatures of individual constituents in *Saccharum officinarum* molasses methanol fraction.

Pk #	R.T. min	Area %	Library/ID	Ref#	CAS#	Qual
1	43.982	12.81	Olean-12-en-3-ol, acetate, (3.beta)	261153	001616-93-9	90
			4,4,6a,6b,8a,11,11,14b Octamethyl-1,4,4a,5,6, 6a,6b,7,8,8a,9,10,11,12,12a,14,14a,14b -octadecahydro-2H-picen-3-one	248690	1000194-62-4	90
			12-Oleanen-3-yl acetate, (3.alpha)	261151	033055-28-6	90
2	44.623	87.19	Lup-20(29)-en-3-ol, acetate, (3.beta.)	261156	001617-68-1	99
			A'-Neogammacer-22(29)-en-3-ol, acetate, (3.beta, 21.beta.)	261167	002085-25-8	64
			Urs-12-en-24-oic acid, 3-oxo-, methyl ester, (+)-	261139	020475-86-9	64

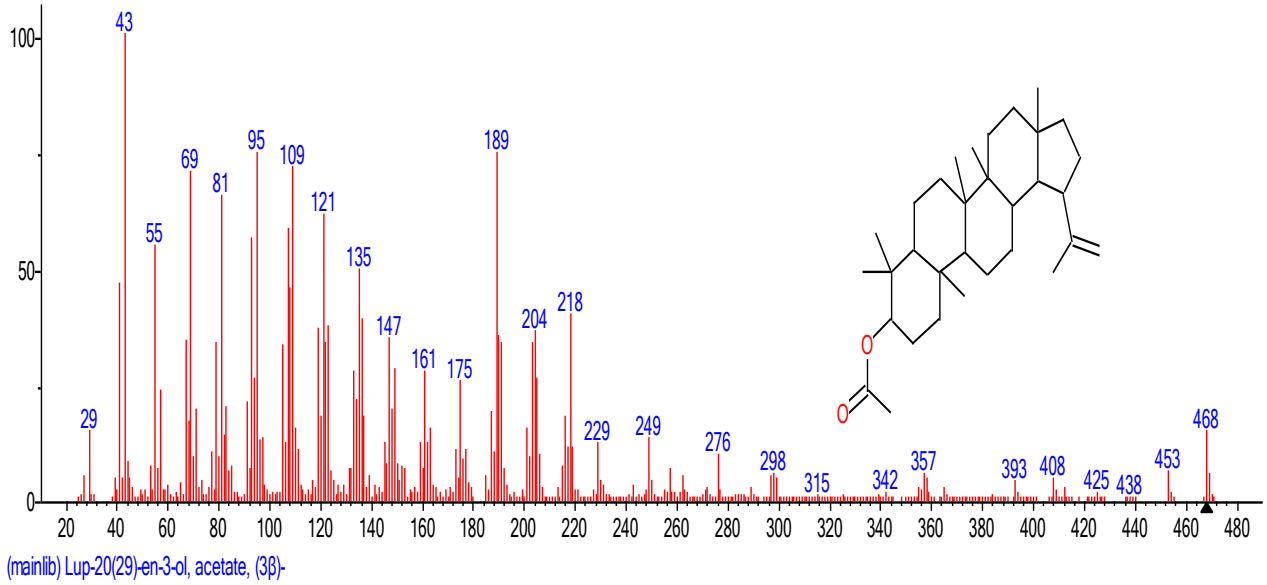

Figure. 4.10 Fragmentation pattern and structure of Lup-20(29)-en-3-ol, acetate, (3.beta).

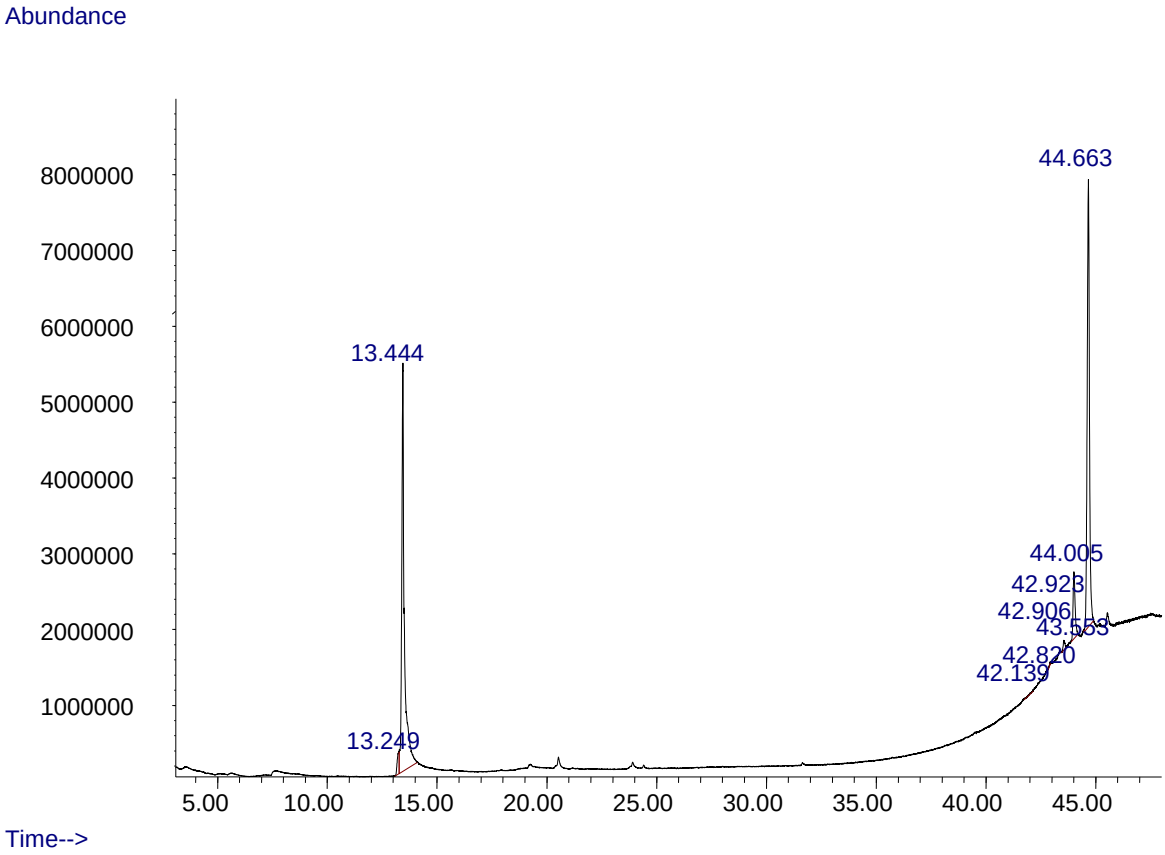

Figure. 4.11 Gas chromatogram of the chemical constituents in *Saccharum officinarum* molasses aqueous fraction.

Table 4.6 Nomenclature of individual constituents in *Saccharum officinarum* molasses aqueous fraction.

Pk #	R.T. min	Area %	Library/ID	Ref#	CAS#	Qual
1	13.249	1.67	Diethyl Phthalate	85001	000084-66-2	98
2	13.444	47.43	Diethyl Phthalate	84999	000084-66-2	98
3	42.139	0.15	2-Ethylacridine	71643	055751-83-2	55
4	42.820	0.10	(Z)-Decyl icos-9-enoate	256742	1000414-43-4	58
5	42.906	0.07	Octasiloxane,1,1,3,3,5,5,7,7,9,9,11,11,13,13,1515-hexadecamethyl-	272253	019095-24-0	74
			4-Dehydroxy-N-(4,5-methylenedioxy-2-nitro benzylidene) tyramine	157264	1000111-66-9	74
6	42.923	0.03	Octasiloxane, 1,1,3,3,5,5,7,7,9,9, 11,11,13,13,15,15-hexadecamethyl-	272253	019095-24-0	53
7	43.553	0.74	Octasiloxane, 1,1,3,3,5,5,7,7,9,9, 11,11,13,13,15,15-hexadecamethyl-2 -	272253	019095-24-0	58
8	44.005	5.97	Olean-12-en-3-ol, acetate, (3.beta)	261153	001616-93-9	93
9	44.663	43.85	Lup-20(29)-en-3-ol, acetate, (3.beta.)	261156	001617-68-1	99

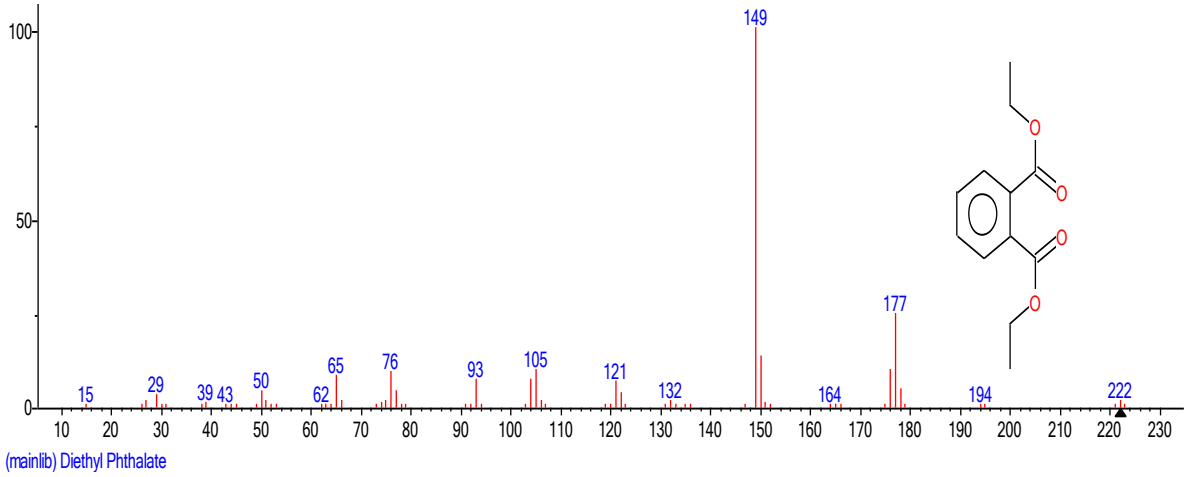

Figure. 4.12 Fragmentation pattern and structure of Diethyl phthalate.

4.11 Effect of *Saccharum officinarum* molasses methanol fraction on fasting blood glucose level.

Saccharum officinarum molasses treated rats did not show significant changes in fasting blood glucose levels when compared with the control and between the treated groups (Figure. 4.13; Appendix 11).

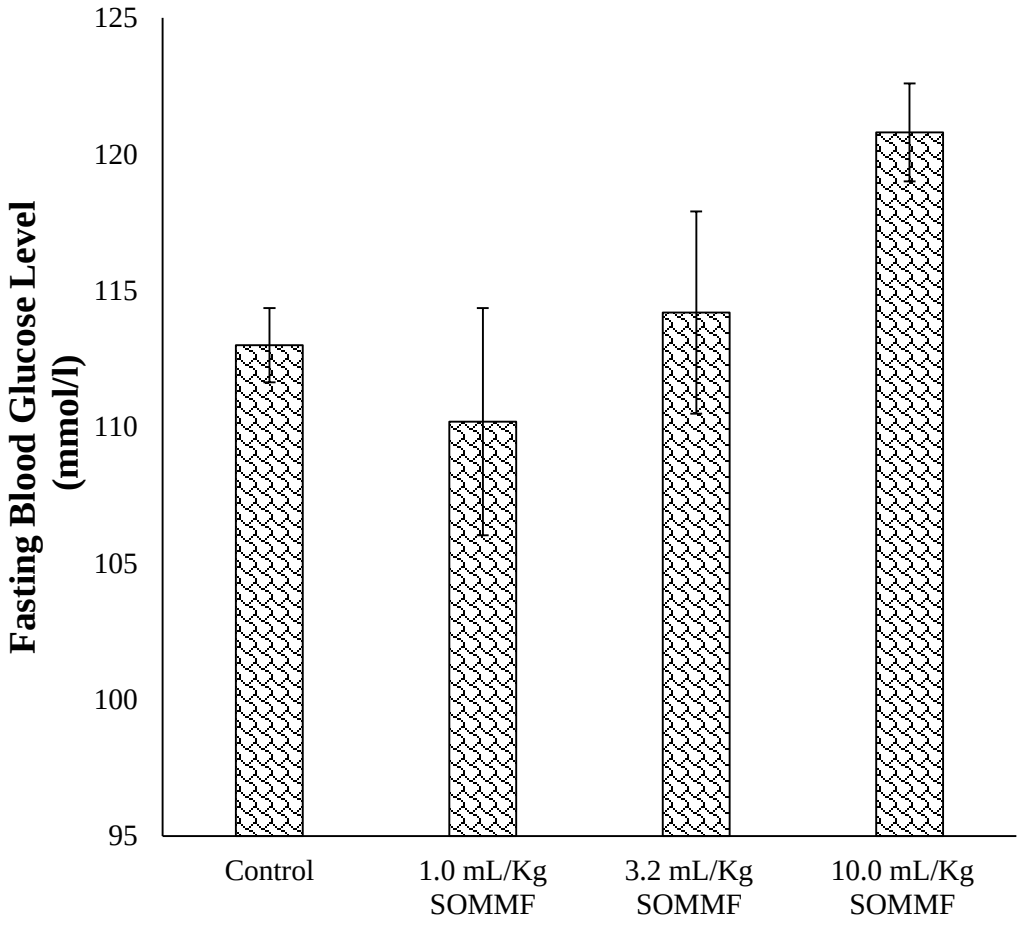

Figure. 4.13 Effect of *Saccharum officinarum* molasses methanol fraction on fasting blood glucose level. Columns represents Mean±SEM. n=5.
Saccharum officinarum molasses methanol fraction (SOMMF)

4.12 Effect of *Saccharum officinarum* molasses methanol fraction on percentage body weight change.

There were no significant differences in the percentage body weight change of *Saccharum officinarum* molasses treated rats when compared with the control and between other treated groups (Figure 4.1; Appendix 12).

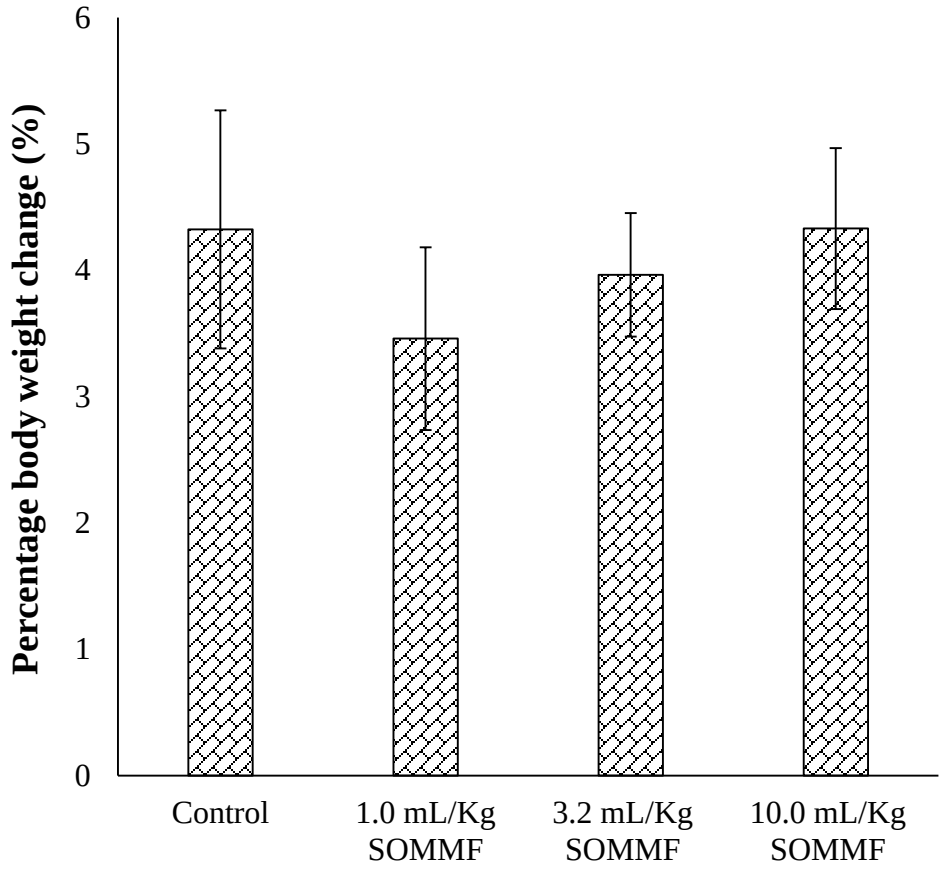

Figure. 4.14 Effect of *Saccharum officinarum* molasses methanol fraction on percentage body weight change. Columns represents Mean±SEM. n=5.
Saccharum officinarum molasses methanol fraction (SOMMF)

4.13. Effect of *Saccharum officinarum* molasses methanol fraction percentage body weight change.

The weights of the organs were not significantly altered relative to the control and between other treated groups (Table 4.7).

Table 4.7 Effect of *Saccharum officinarum* molasses methanol fraction on relative organ weights

		mL/kg/day SOMMF		
Group	**Control**	**1.0**	**3.2**	**10.0**
Testes	0.509±0.02	0.521±0.03	0.475±0.02	0.5±0.04
Epidydimis	0.241.0.01	0.222±0.02	0.0216±0.01	0.232±0.02
Seminal vesicle	0.413±0.02	0.391±0.08	0.353±0.051	0.453±0.03
Prostate gland	0.153±0.01	0.129±0.01	0.106±0.01	0.138±0.01
Kidney	0.545±0.01	0.541±0.01	0.488±0.02	0.522±0.02
Liver	2.851±0.08	3.011±0.11	2.626±0.11	2.81±0.189

Data presented as Mean±SEM. n=5.

Saccharum officinarum molasses methanol fraction (SOMMF).

4.14 Effect of *Saccharum officinarum* molasses methanol fraction (SOMMF) on lipid peroxidation and antioxidant enzymes.

In the liver, alterations in levels of malondialdehyde (MDA) and glutathione were not significant, but SOD and catalase activities were significantly decreased ($P<0.5$) in the group treated with 1.0 mL/kg/day SOMMF as compared with control, 3.2 and 10 mL/kg/day SOMMF (Table 4.8).

Testicular MDA level increased significantly ($P<0.5$) in 3.2 and 10 mL/kg/day SOMMF treated rats as compared with control and 1.0 mL/kg/day SOMMF. The activity of testicular superoxide dismutase (SOD) also increased significantly ($P<0.5$) in 3.2 and 10 mL/kg/day SOMMF compared with control (Table 4.9).

Epidydimal MDA level and SOD activity significantly increased ($P<0.5$) in group treated with 10 mL/kg/day SOMMF as compared to control, 3.2 and 10 mL/kg/day SOMMF. There were no significant changes in catalase activity and tissue glutathione level in relation to control and between other treated groups (Table 4.10).

Table 4.8 Effect of *Saccharum officinarum* molasses methanol fraction on lipid peroxidation and antioxidant enzymes of the liver.

		mL/kg/day SOMMF		
Group	**Control**	**1.0**	**3.2**	**10.0**
MDA (U/mg)	4.572±0.52	3.131±0.28	3.878±0.79	4.92±0.87
SOD (U/mg)	560.7±147.8	220.7±53.22$^{*\#\alpha}$	430±103.2	345.9±148.4
CATALASE (IU/L)	1455±162.3	1666±354	1847±263.5	1375±117.7
GSH (uM/mg)	4.715±0.60	4.352±0.28	4.783±0.20	4.197±0.33

Data presented as Mean±SEM. n=5. *P<0.05 relative to the control. $^{\#}$P<0.05 relative to 3.2 mL/kg/day SOMMF and $^{\alpha}$P<0.05 relative to 10 mL/kg/day SOMMF.

Saccharum officinarum molasses methanol fraction (SOMMF).

Table 4.9 Effect of *Saccharum officinarum* molasses methanol fraction on lipid peroxidation and antioxidant enzymes of the testes.

		mL/kg/day SOMMF		
Group	**Control**	**1.0**	**3.2**	**10.0**
MDA (U/mg)	1.647±0.24	1.38±0.011	2.369±0.18*#	2.56±0.13*#
SOD (U/mg)	174.8±23.72	214.7±49.21	329.4±54.64*	290.2±34.93*
CATALASE (IU/L)	698.2±62.94	539.3±44.62	676±65.25	771.5±158.6
GSH (uM/mg)	2.876±0.31	2.928±0.27	3.182±0.43	2.708±0.29

Data presented as Mean±SEM. n=5. *P<0.05 relative to the control, #P<0.05 compared with 1.0 mL/kg/day SOMMF.

Saccharum officinarum molasses methanol fraction (SOMMF)

Table 4.10 Effect of *Saccharum officinarum* molasses methanol fraction on lipid peroxidation and antioxidant enzymes of the Epididymis.

		mL/kg/day SOMMF		
Group	**Control**	**1.0**	**3.2**	**10.0**
MDA (U/mg)	15.69±6.89	13.26±3.05	19.94±8.16	43.01±5.20*$^{\#\alpha}$
SOD (U/mg)	1620±379.2	2350±550.5	3513±1089	5383±1479*$^{\#\alpha}$
CATALASE (IU/L)	3669±898.3	3338±771,8	6558±1716	6446±1707
GSH (uM/mg)	9.08±1.97	7.13±1.99	13.38±6.31	15.76±4.17

Data presented as Mean±SEM. n=5. *P<0.05 relative to the control. $^{\#}$P<0.05 compared with 1.0 mL/kg/day SOMMF. $^{\alpha}$P<0.05 compared with 3.2 mL/kg/day SOMMF. *Saccharum officinarum* molasses methanol fraction (SOMMF)

4.15 Effect of *Saccharum officinarum* molasses methanol fraction on epididymal sperm characteristics.

There were significant decreases ($P<0.05$) in sperm concentration and viability of groups treated with 3.2 and 10 mL/kg/day SOMMF in relation to control (Figure 4.15 and 4.16; Appendix 13 and 14).

Saccharum officinarum molasses methanol fraction (SOMMF) of 10.0 mL/kg/day caused significantly decrease ($P<0.05$) in sperm motility relative to control and 10.0 mL/kg/day SOMMF (Figure 4.17; Appendix 15).

The epididymal volume increased significantly ($P<0.05$) in 10 mL/kg/day SOMMF treated group as compared with control, 1.0 and 3.2 mL/kg/day SOMMF (Figure 4.18; Appendix 13).

The percentage abnormal sperm morphology was not significantly altered relative to the control and other treated groups (Figure 4.19; Appendix 17).

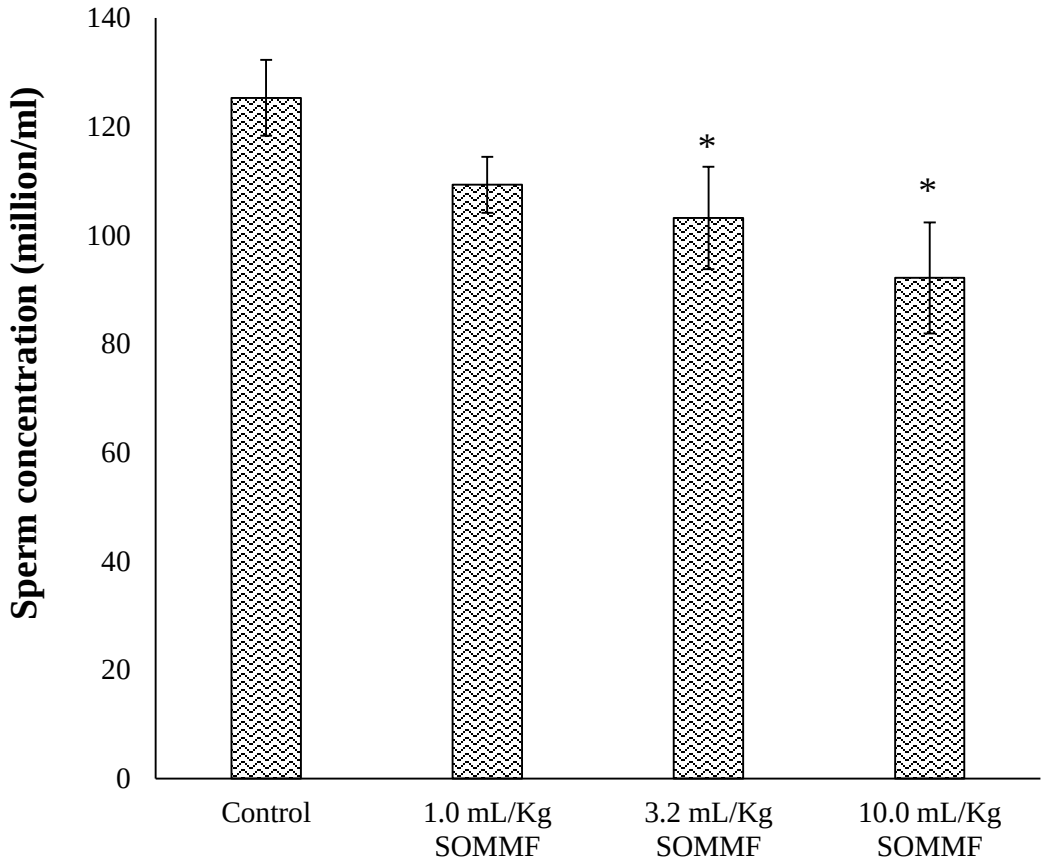

Figure. 4.15 Effect of *Saccharum officinarum* molasses methanol fraction on sperm concentration.

Columns represents Mean±SEM. n=5. *P<0.05 relative to control.

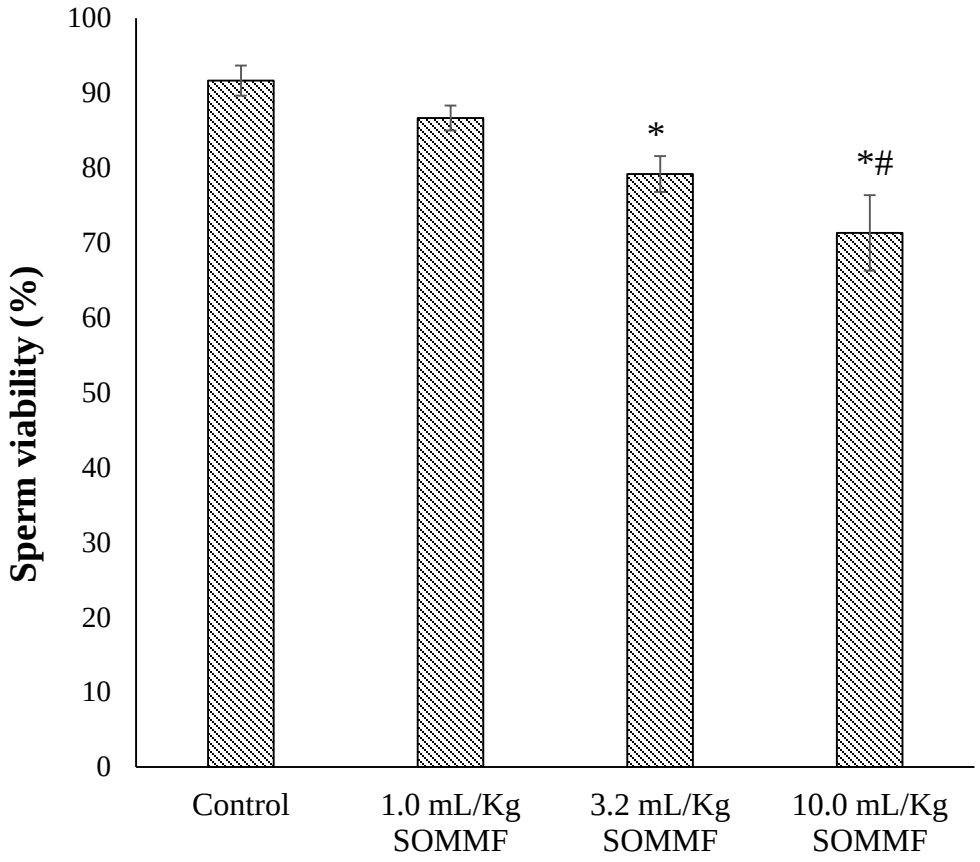

Figure. 4.16 Effect of *Saccharum officinarum* molasses methanol fraction (SOMMF) on sperm viability.

Columns represents Mean±SEM. n=5. *P<0.05 as related to the control, $^{\#}$P<0.05 relative to 1.0 mL/kg/day SOMMF.

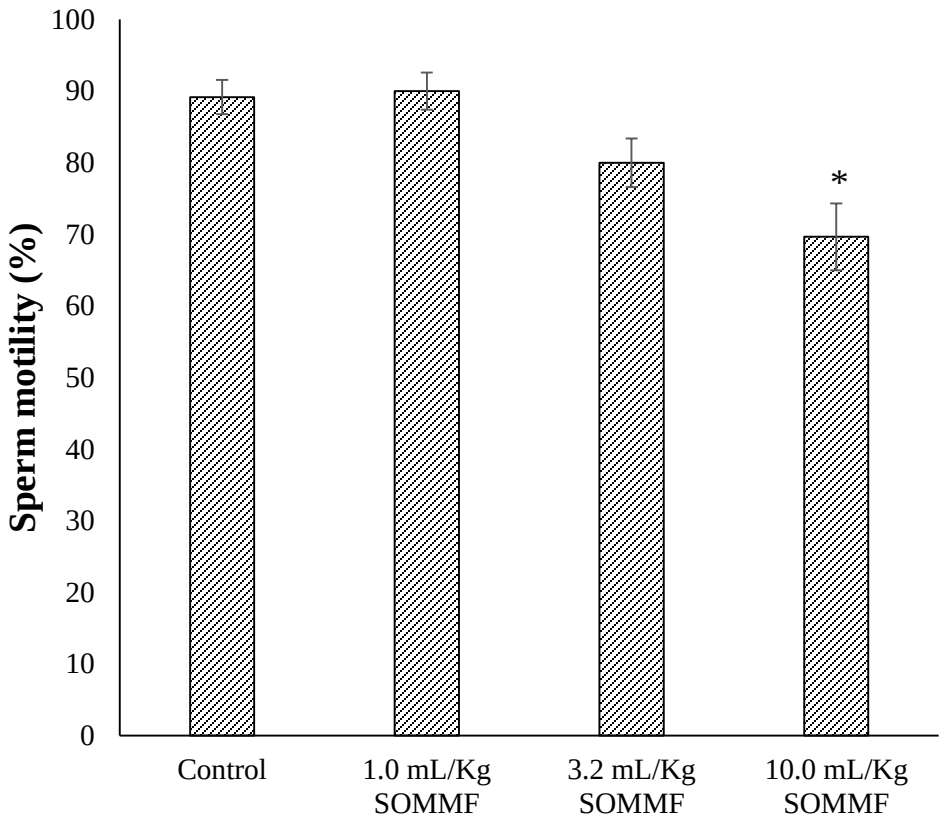

Figure. 4.17 Effect of *Saccharum officinarum* molasses methanol fraction (SOMMF) on sperm motility.

Columns represents Mean±SEM. n=5. *P<0.05 as related to control.

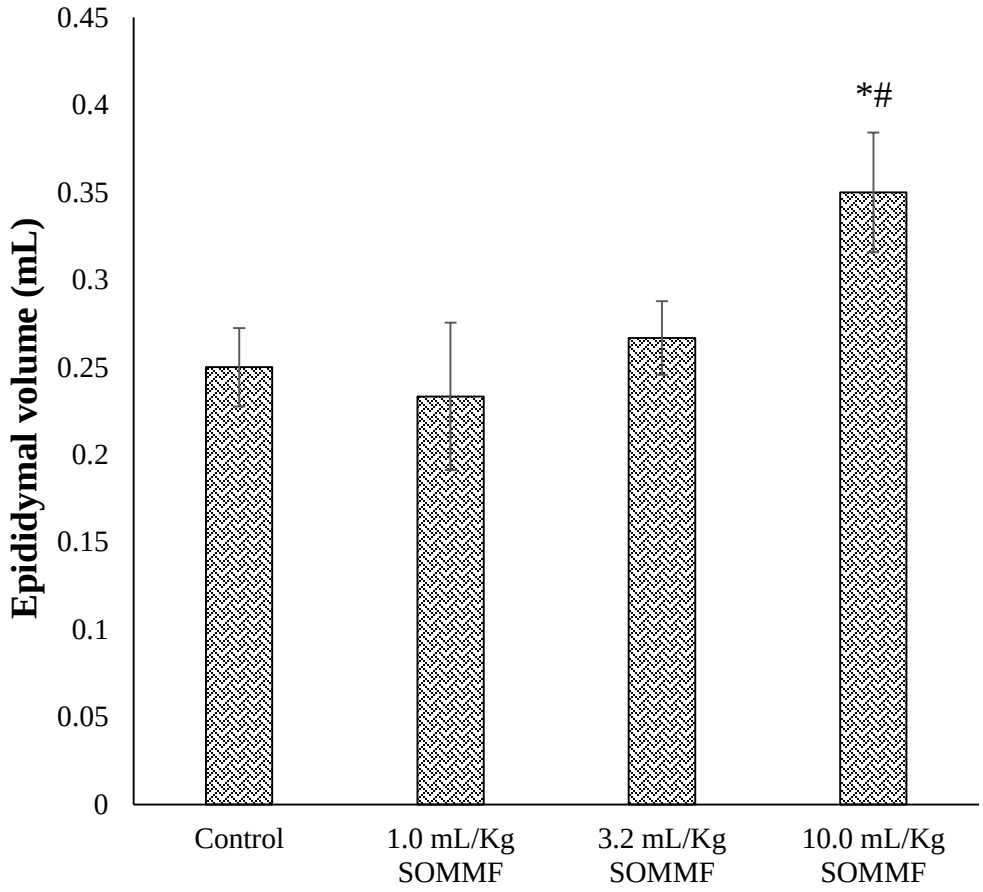

Figure. 4.18 Effect of *Saccharum officinarum* molasses methanol fraction (SOMMF) on epididymal volume.

Columns represents Mean±SEM. n=5. *P<0.05 relative to the control. #P<0.05 relative to 1.0 mL/kg/day SOMMF.

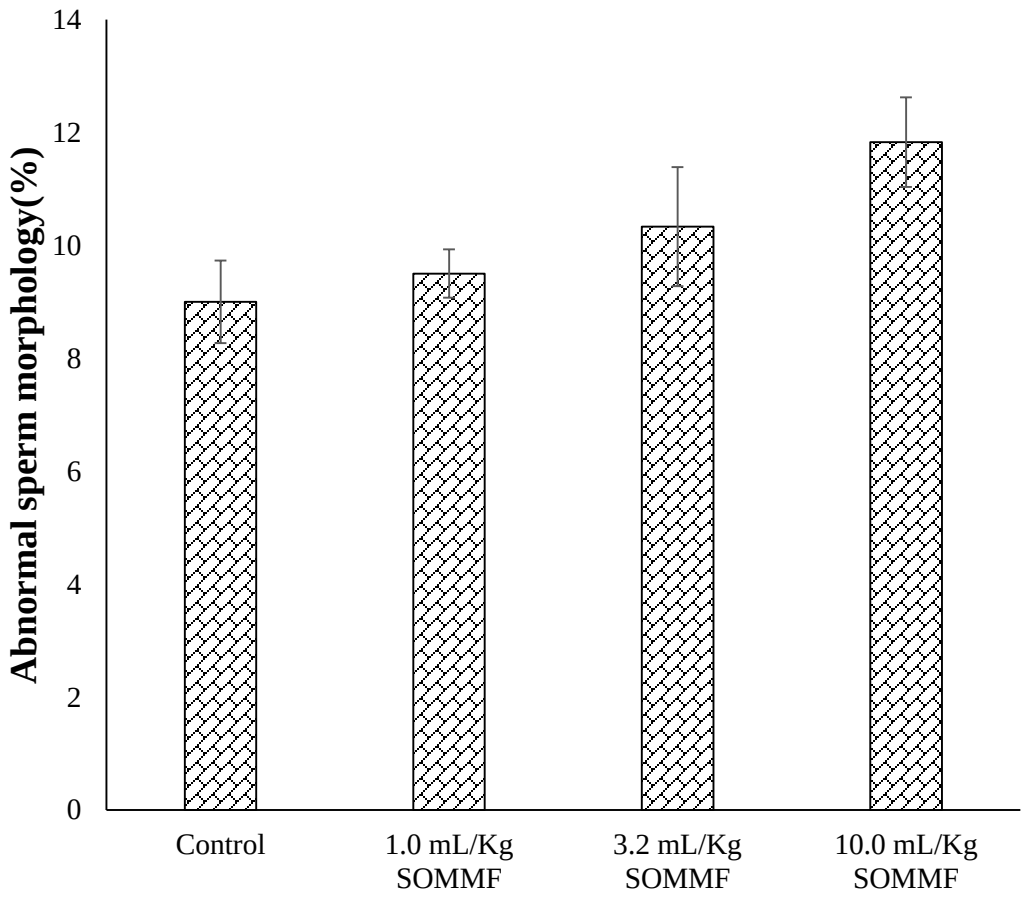

Figure. 4.19 Effect of *Saccharum officinarum* molasses methanol fraction (SOMMF) on abnormal sperm morphology. Columns represents Mean±SEM. n=5.

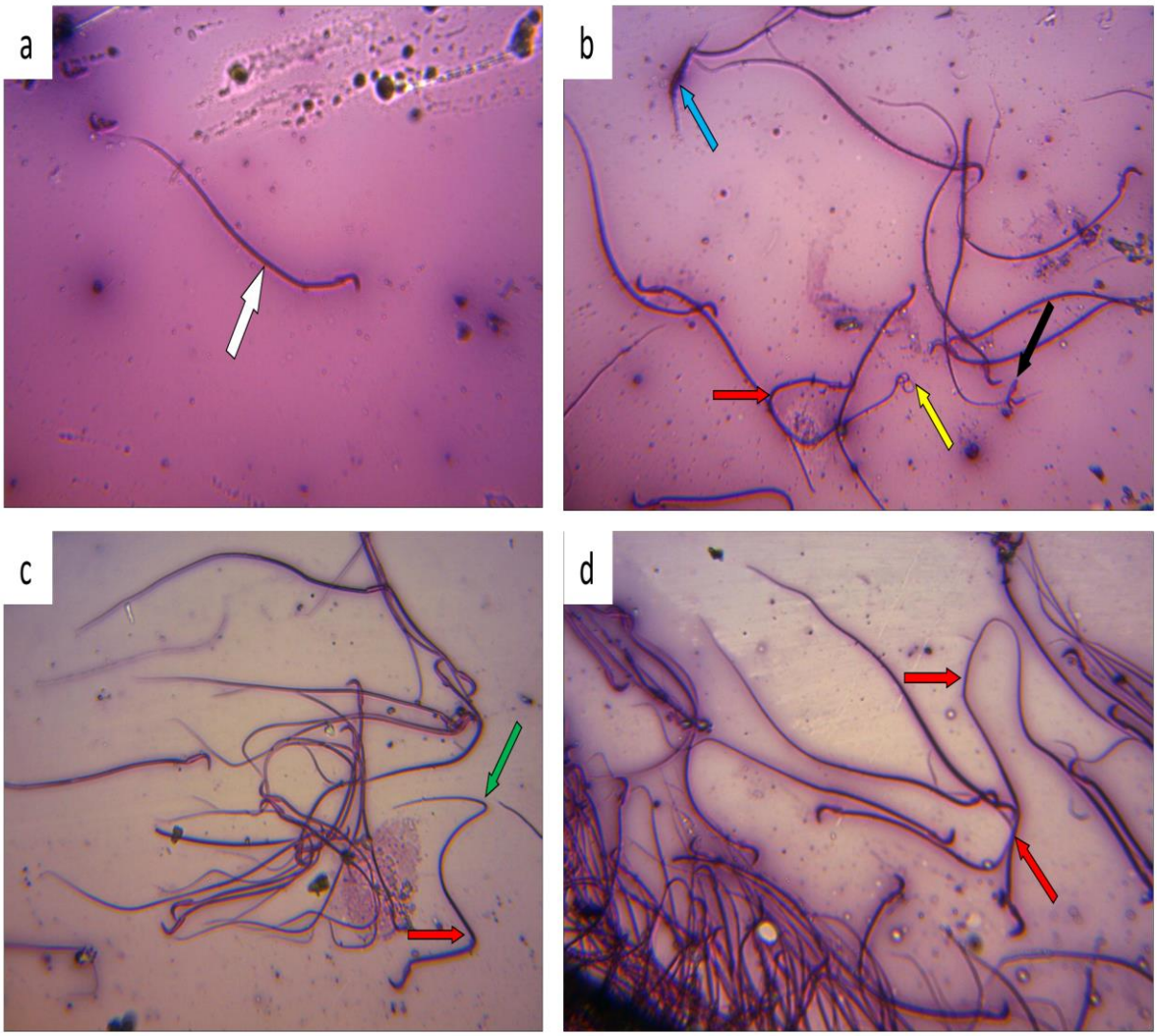

Plate 4. Morphology of spermatozoa of rat treated with *Saccharum officinarum* molasses methanol fraction (SOMMF).

Stained by eosin nigrosin, presented at x400 magnification.

a) Morphology of spermatozoa of control rat, show normal head midpiece and tail (white arrows).

b) Morphology of spermatozoa of rat treated with 1.0 mL/kg/day SOMMF, show Coiled tail (yellow arrow), bent midpiece (red arrow) triple headed (black arrow) and swollen head (blue arrow)

c) Morphology of spermatozoa of rat treated with 3.2 mL/kg/day SOMMF, show bent midpiece (red arrow) and curved tail (green arrow).

d) Morphology of spermatozoa of rat treated with 10 mL/kg/day SOMMF, show bent midpiece (red arrow).

4.16 Effect of *Saccharum officinarum* molasses methanol fraction on serum hormonal profile.

The levels of serum FSH and LH were unaltered significantly when compared with control and between other treated groups (Figure. 4.20; Appendix 18). Also, there was no significant change in serum testosterone level as compared with control and between other treated groups (Figure. 4.21; Appendix 19).

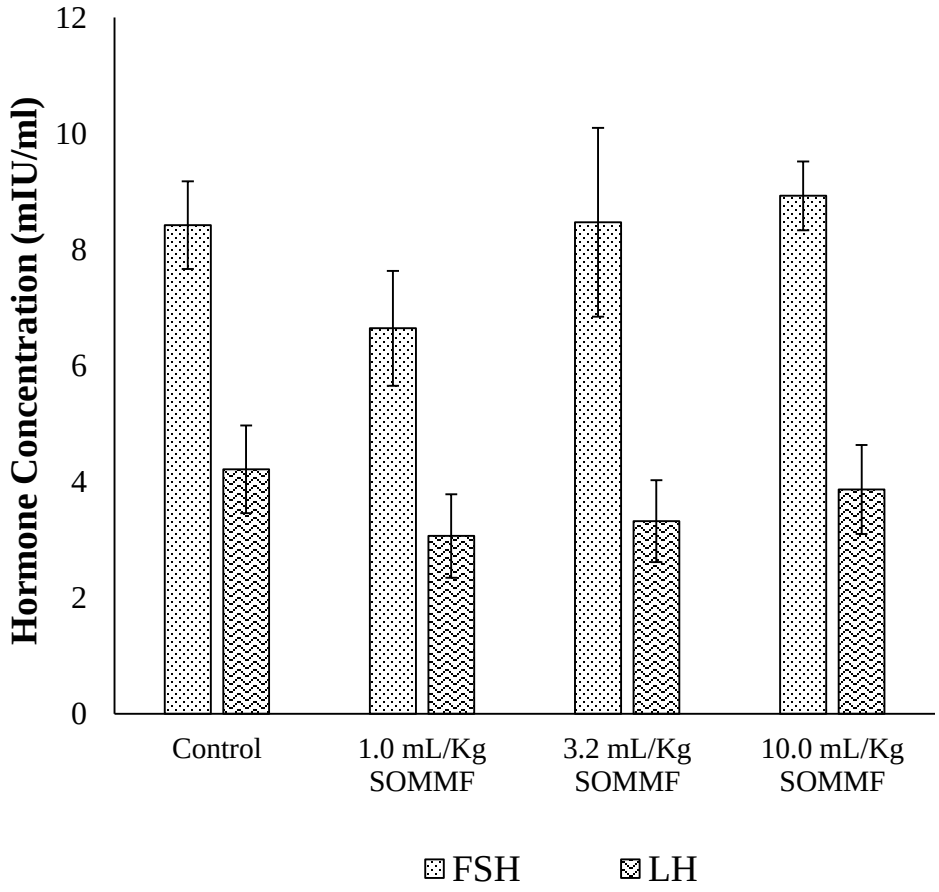

Figure. 4.20 Effect of *Saccharum officinarum* molasses methanol fraction (SOMMF) on serum follicle stimulating hormone and luteinizing level. Columns represents Mean±SEM. n=5.

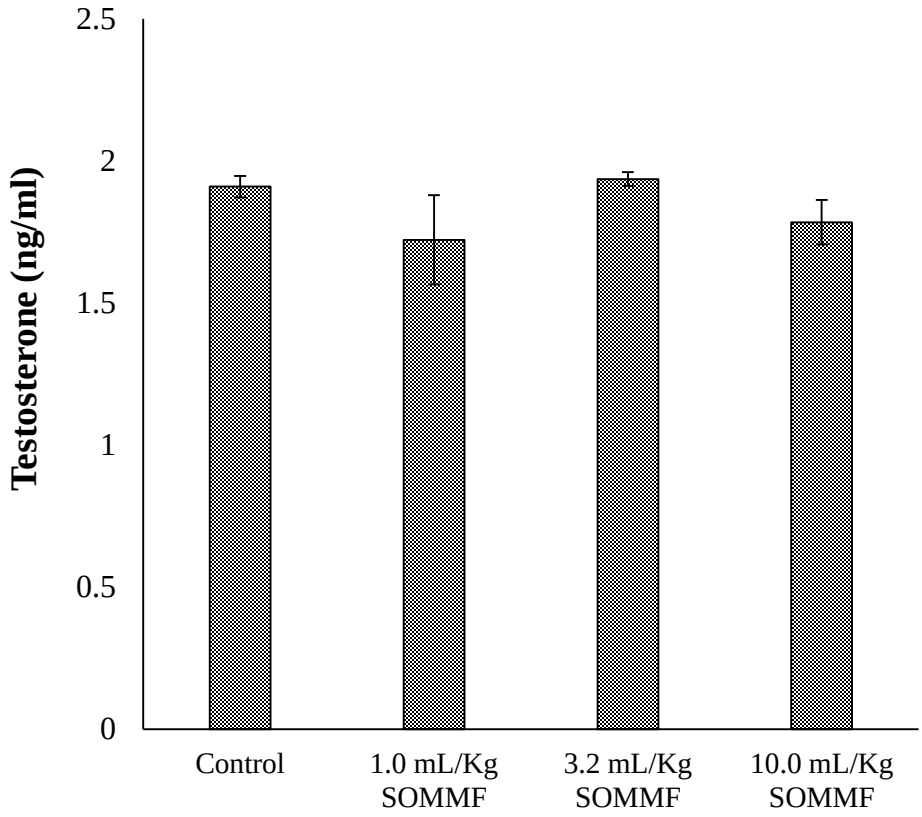

Figure 4.21 Effect of *Saccharum officinarum* molasses methanol fraction (SOMMF) on serum testosterone level. Columns represents Mean±SEM. n=5.

4.17 Effect of *Saccharum officinarum* molasses methanol fraction on histology of liver.

The liver sections of 1.0 mL/kg/day *Saccharum officinarum* molasses methanol fraction (SOMMF) treated group show mildly dilated sinusoids and infiltration of inflammatory cells (Plate 3). The liver sections of 3.2 and 10.0 mL/kg/day SOMMF treated group show normal architecture (Plates 4 and 5).

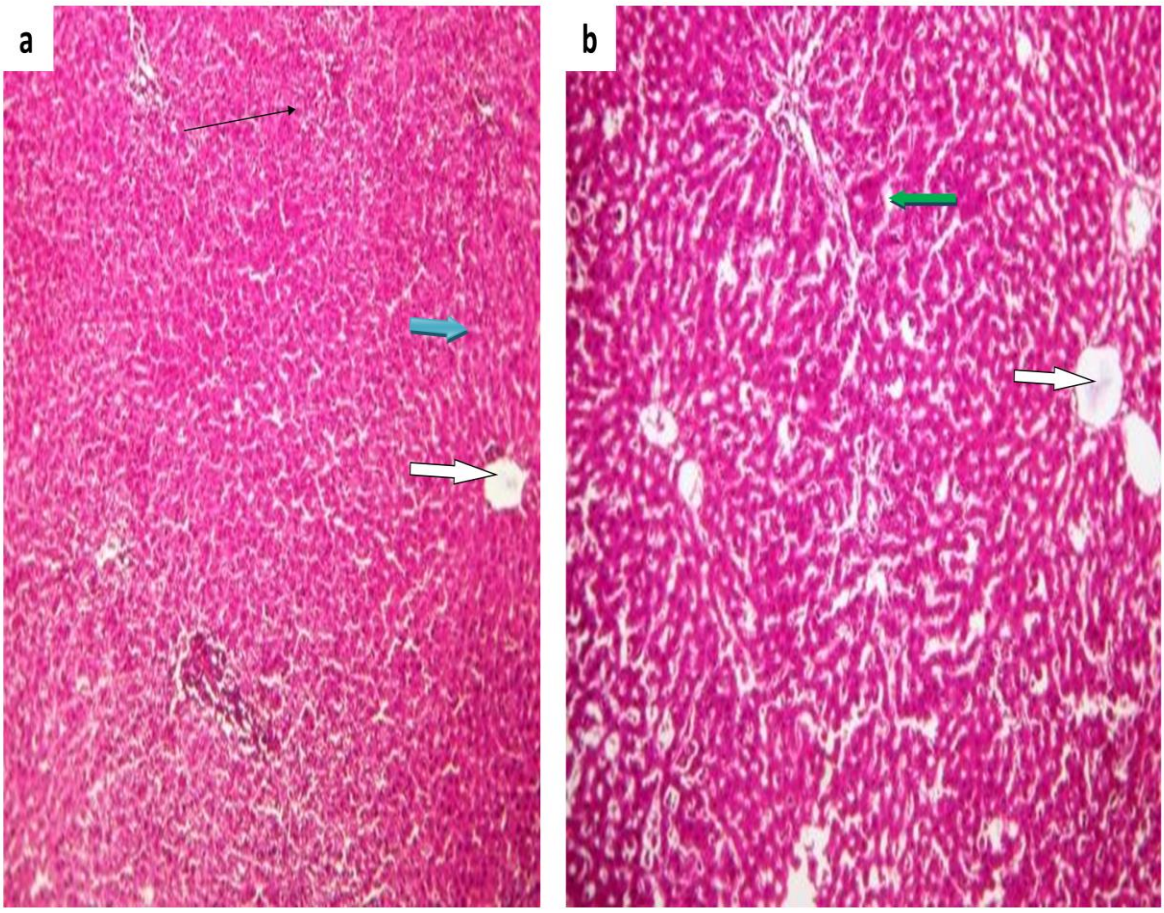

Plate 5. Photomicrograph of liver section of rat treated with *Saccharum officinarum* molasses methanol fraction (SOMMF) 1.0 mL/kg/day.

Tissues stained by H&E presented at x100 magnification

a) Liver section of control rat showing normal architecture, central venules and portal tract (white arrow), sinusoids (blue arrow) and hepatocytes (slender arrow).

b) Liver section of rat treated with SOMMF of 1.0 mL/kg/day showing normal central venules and portal tracts (white arrow), mildly dilated sinusoids with infiltration of inflammatory cells (blue arrow). The hepatocytes are normal (slender arrow).

(SOMMF - *Saccharum officinarum* molasses methanol fraction).

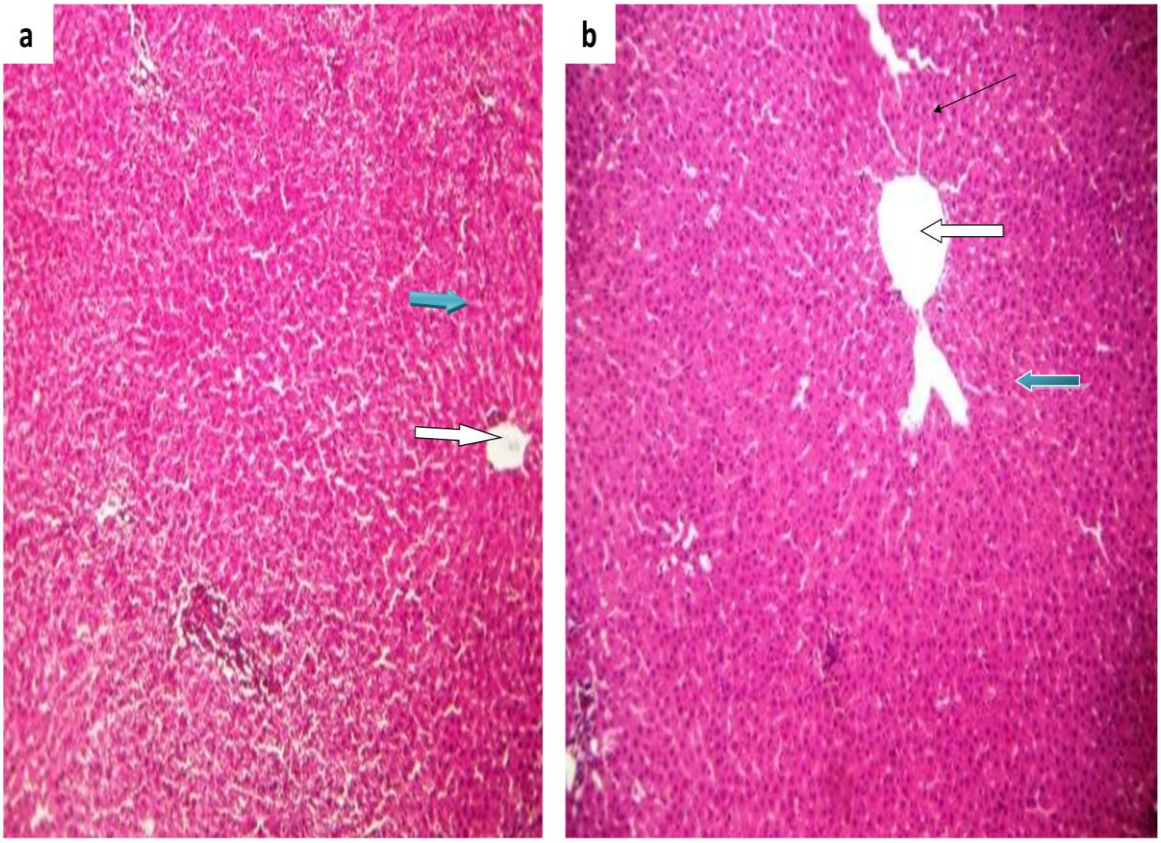

Plate 6. Photomicrograph of liver section of rat treated with *Saccharum officinarum* molasses methanol fraction (SOMMF) 3.2 mL/kg/day.

Tissues stained by H&E presented at x100 magnification.

a) Liver section of control rat showing normal architecture, central venules and portal tract (white arrow), sinusoids (blue arrow) and hepatocytes (slender arrow).

b) Liver section of rat treated with SOMMF of 3.2 mL/kg/day showing normal architecture, the central venules and portal tracts (white arrow), sinusoids (blue arrow), hepatocytes (slender arrow).

(SOMMF - *Saccharum officinarum* molasses methanol fraction).

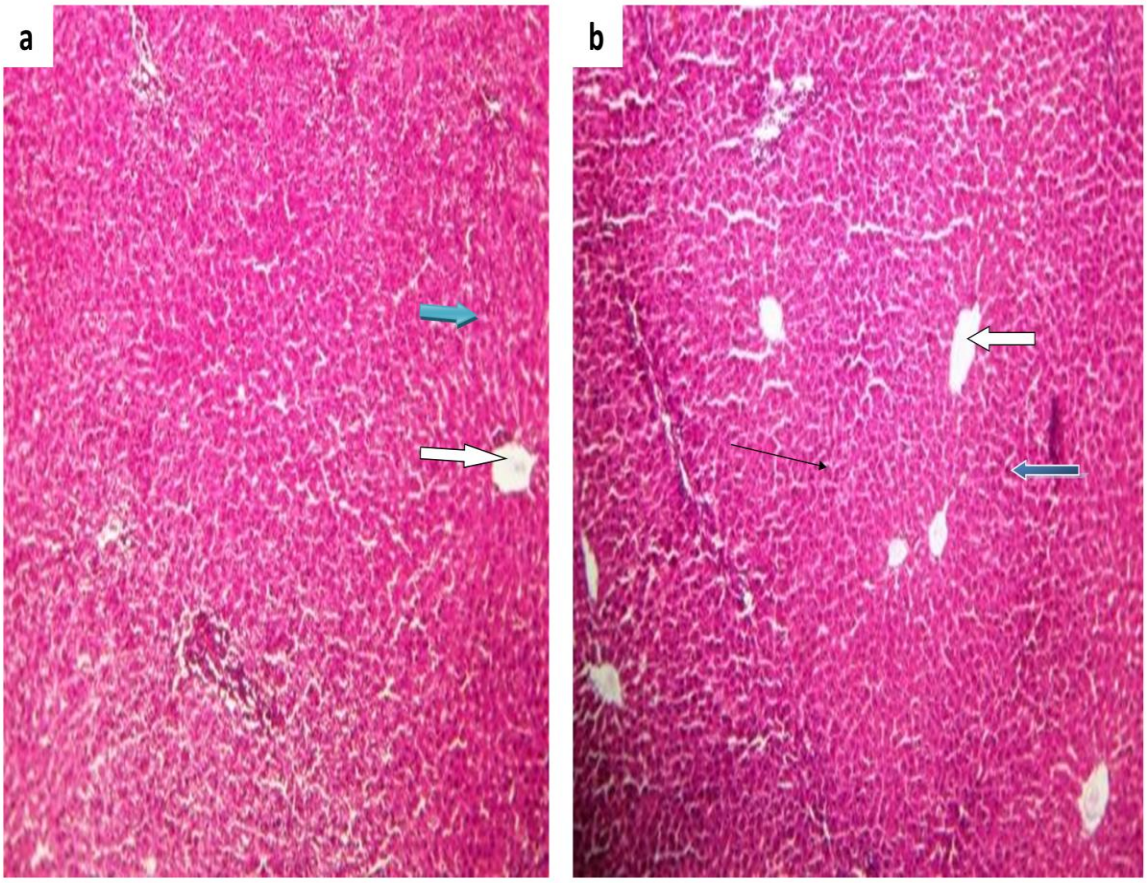

Plate 7. Photomicrograph of liver section of rat treated with *Saccharum officinarum* molasses methanol fraction (SOMMF) 10.0 mL/kg/day.

Tissues stained by H&E presented at x100 magnification.

a) Liver section of control rat showing normal architecture, central venules and portal tract (white arrow), sinusoids (blue arrow) and hepatocytes (slender arrow).

b) Liver section of rat treated with SOMMF of 10.0 mL/kg/day showing normal architecture, central venules and portal tracts (white arrow) sinusoids (blue arrow) and hepatocytes (slender arrow).

(SOMMF - *Saccharum officinarum* molasses methanol fraction).

4.18 Effect of *Saccharum officinarum* molasses methanol fraction on histology of testes.

Testicular sections of 1.0 and 3.2 mL/kg/day SOMMF treated groups appear normal compared with the control (Plate 6 and 7). Testicular sections of 10.0 mL/kg/day *Saccharum officinarum* molasses methanol fraction (SOMMF) treated group show maturation arrest in some seminiferous tubules with distorted and degenerated germinal cells, congested interstitial spaces and Leydig cells (Plates 8).

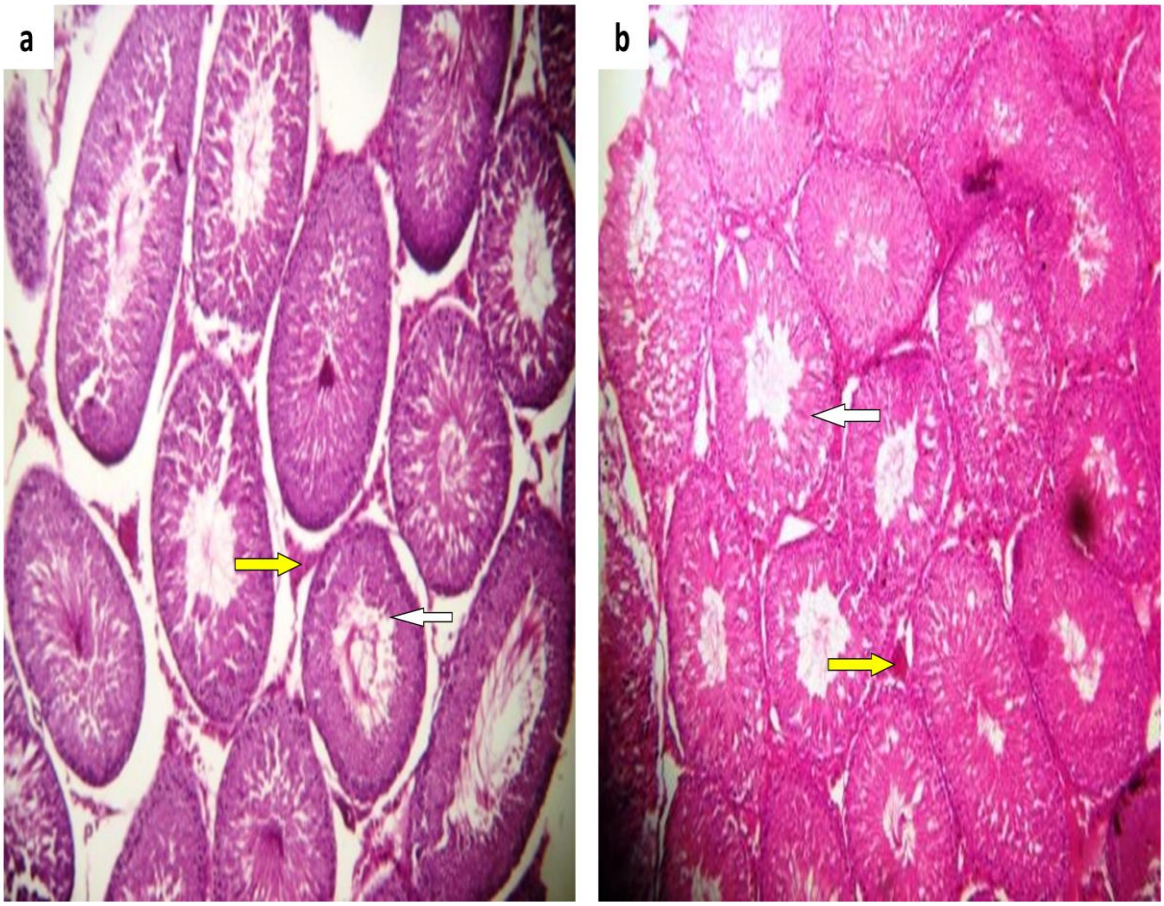

Plate 8. Photomicrograph of testicular section of rat treated with *Saccharum officinarum* molasses methanol fraction (SOMMF) 1.0 mL/kg/day.

Tissues stained by H&E presented at x100 magnification.

a) Testicular section of control rat, seminiferous tubules appear normal with maturation stages of germ cell layer (black arrow), spermatogonia, Sertoli cells and lumen appear normal (white arrow). Interstitium and Leydig cells appear normal (yellow arrow).

b) Testicular section of rat treated with SOMMF of 1.0 mL/kg/day shows normal seminiferous tubules (white arrow), germ cell layers appear with normal maturation stages (black arrow). Interstitium and Leydig cells appear normal (blue arrow).

(SOMMF - *Saccharum officinarum* molasses methanol fraction).

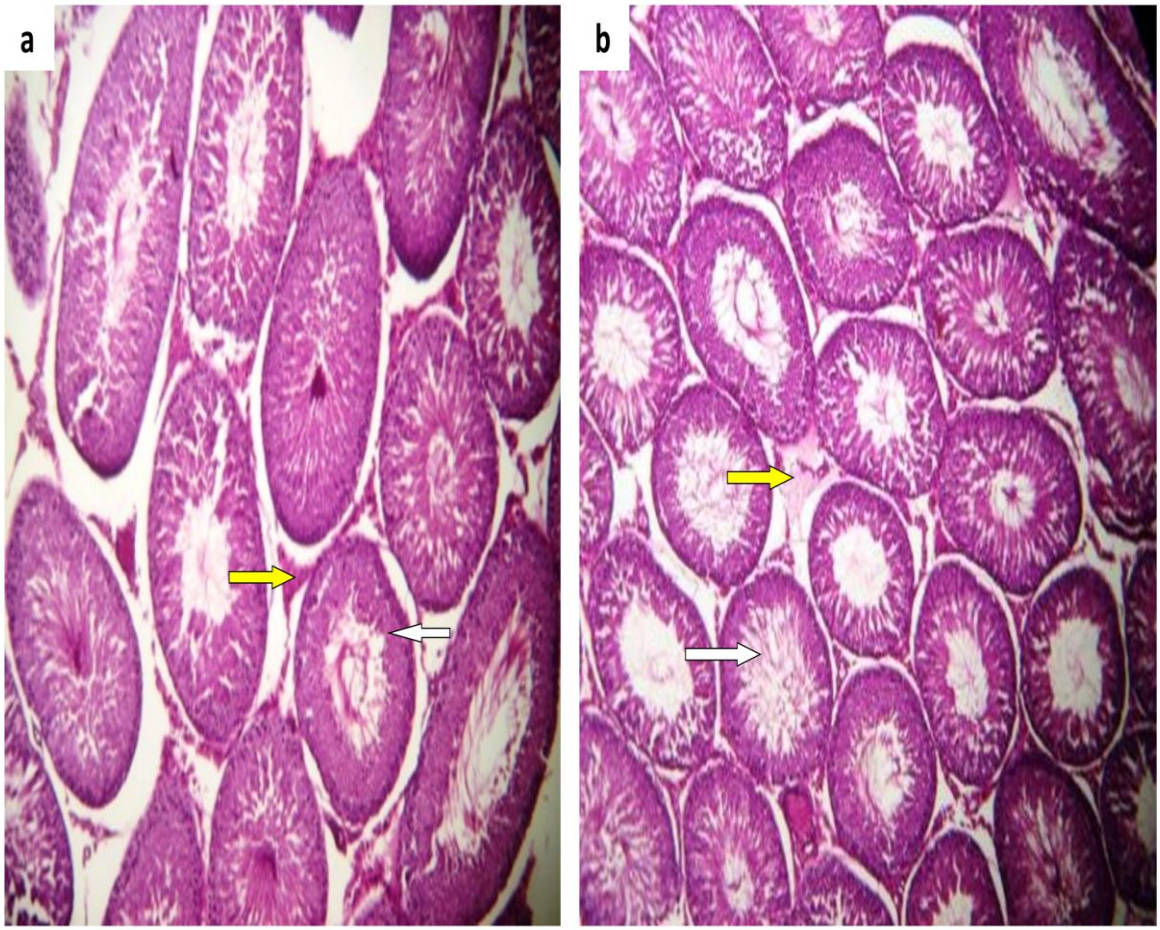

Plate 9. Photomicrograph of testicular section of rat treated with *Saccharum officinarum* molasses methanol fraction (SOMMF) 3.2 mL/kg/day.

Tissues stained by H&E presented at x100 magnification.

a) Testicular section of control rat, seminiferous tubules appear normal with maturation stages of germ cell layer (black arrow), spermatogonia, Sertoli cells and lumen appear normal (white arrow). Interstitium and Leydig cells appear normal (yellow arrow).

b) Testicular section of rat treated with SOMMF of 3.2 mL/kg/day shows normal seminiferous tubules (white arrow). The Leydig cells and interstitium appear normal (red arrow).

(SOMMF - *Saccharum officinarum* molasses methanol fraction).

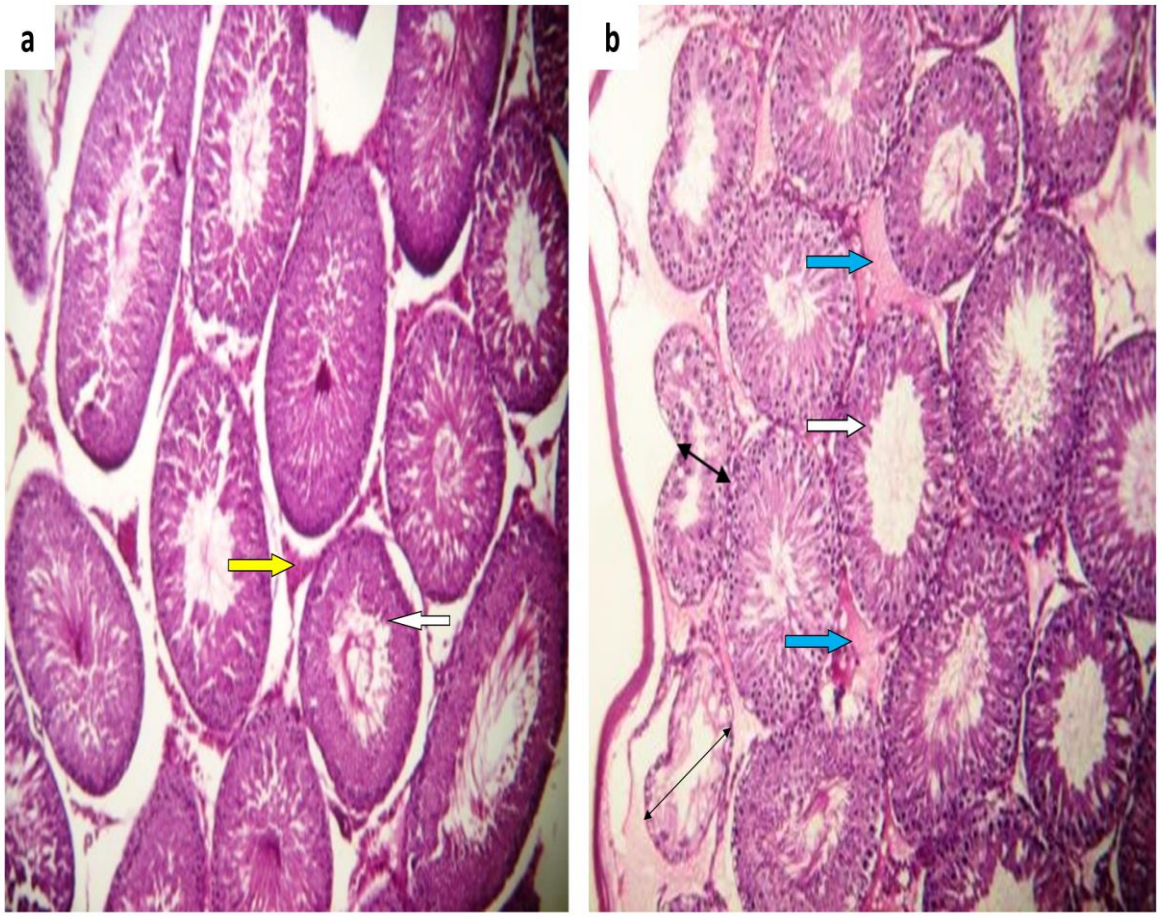

Plate 10. Photomicrograph of testicular section of rat treated with *Saccharum officinarum* molasses methanol fraction (SOMMF) 10.0 mL/kg/day.

Tissues stained by H&E presented at x100 magnification.

a) Testicular section of control rat, seminiferous tubules appear normal with maturation stages of germ cell layer (black arrow), spermatogonia, Sertoli cells and lumen appear normal (white arrow). Interstitium and Leydig cells appear normal (yellow arrow).

b) Testicular section of rat treated wit SOMMF of 10.0 mL/kg/day shows some seminiferous tubules with distorted, degenerated germinal cells and maturation arrest (spanning arrows). Interstitial spaces and Leydig cells appear congested (blue arrows).

(SOMMF - *Saccharum officinarum* molasses methanol fraction).

4.19 Effect of *Saccharum officinarum* molasses methanol fraction on histology of epididymis.

The epididymal sections of *Saccharum officinarum* molasses methanol fraction (SOMMF) of 1.0 and 3.2 mL/kg/day treated rats showed normal epididymal ducts, epithelial layers and smooth muscle, very few ducts with lack of spermatozoa storage within the lumen (Plate 9 and 10). The group treated with 10.0 mL/kg/day SOMMF show interstitium with aggregates of inflammatory cell (Plate 11).

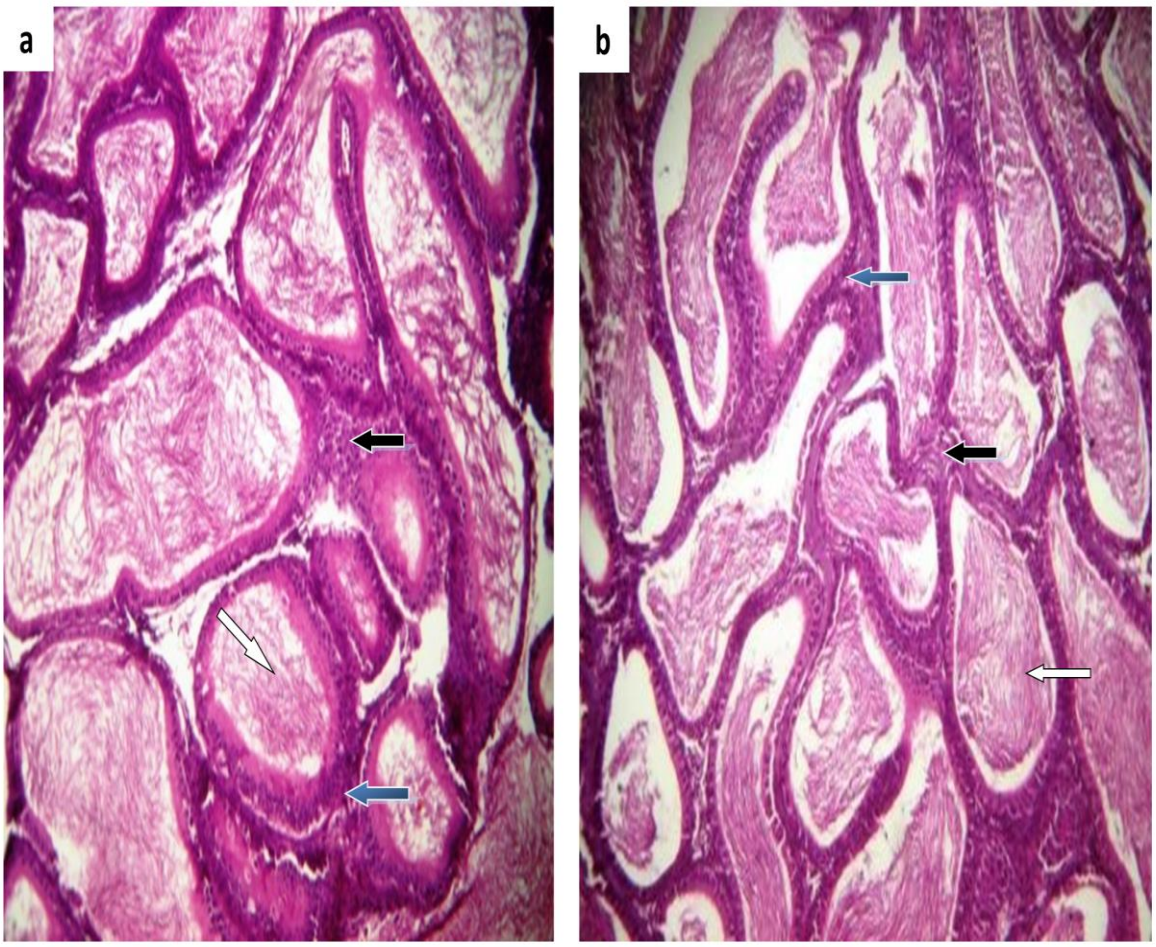

Plate 11. Photomicrograph of epididymal section of rat treated with *Saccharum officinarum* molasses methanol fraction (SOMMF) 1.0 mL/kg/day.

Tissues stained by H&E presented at x100 magnification.

a) Epididymal section of control rat shows normal epididymal ducts and contents (blue arrows), lumen contains spermatozoa (white arrow) and normal interstitium (black arrow).

b) Epididymal section of rat treated with SOM of 1.0 mL/kg/day, shows epididymal ducts appear normal (blue arrow), ducts contain spermatozoa (white arrow). Interstitium appear normal (black arrows).

(SOMMF - *Saccharum officinarum* molasses methanol fraction).

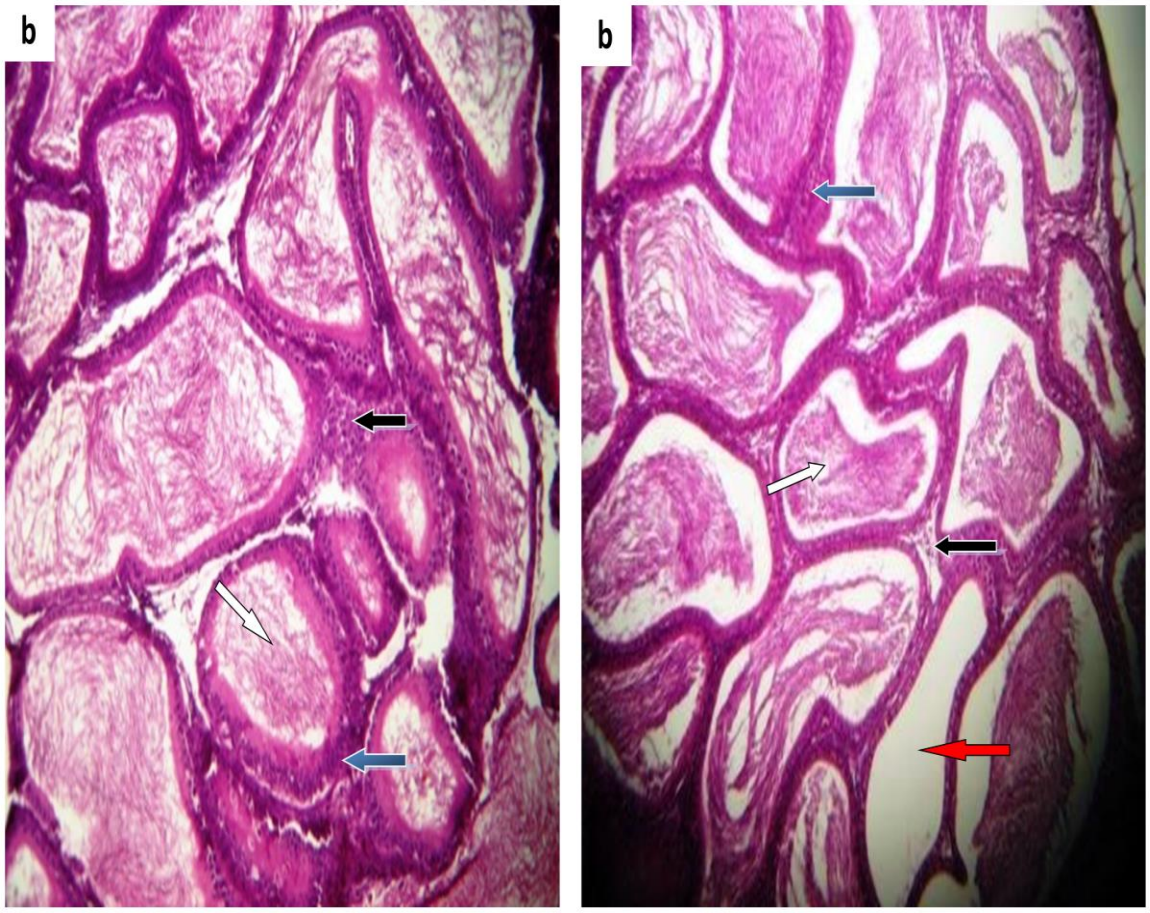

Plate 12. Photomicrograph of epididymal section of rat treated with *Saccharum officinarum* molasses methanol fraction (SOMMF) 3.2 mL/kg/day.

Tissues stained by H&E presented at x100 magnification.

a) Epididymal section of control rat shows normal epididymal ducts and contents (blue arrows), lumen contains spermatozoa (white arrow) and normal interstitium (black arrow).

b) Epididymal section of rat treated with SOM of 3.2 mL/kg/day, showing normal epididymal ducts with contents (blue arrow), few ducts are empty (white arrow). Interstitium appear normal (black arrow).

(SOMMF - *Saccharum officinarum* molasses methanol fraction).

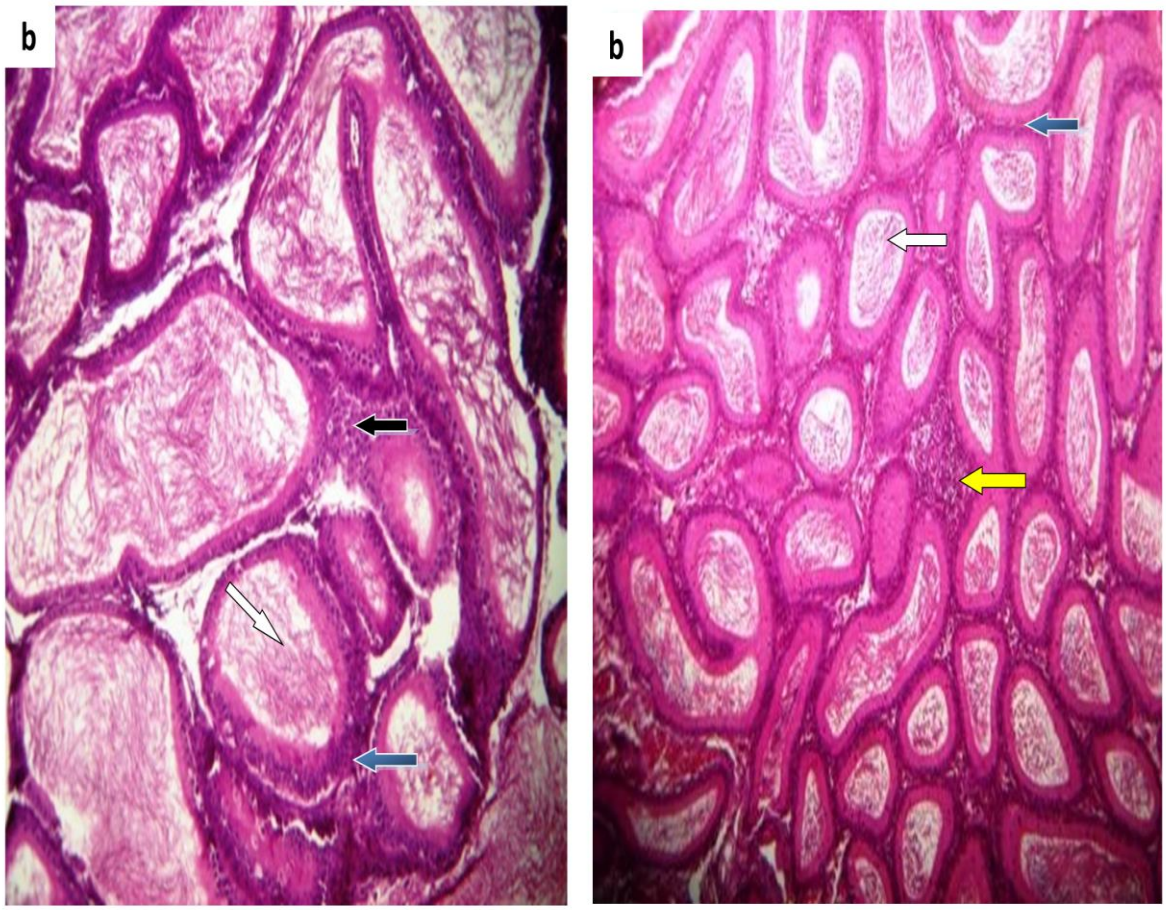

Plate 13. Photomicrograph of epididymal section of rat treated with *Saccharum officinarum* molasses methanol fraction (SOMMF) 10.0 mL/kg/day.

Tissues stained by H&E presented at x100 magnification.

a) Epididymal section of control rat shows normal epididymal ducts and contents (blue arrows), lumen contains spermatozoa (white arrow) and normal interstitium (black arrow).

b) Epididymal section of rat treated with SOM of 10.0 mL/kg/day, showing epididymal ducts with normal epithelial layers and smooth muscle (blue arrow), ducts contain spermatozoa (white arrow). Interstitium show aggregate of inflammatory cells (yellow arrow).

(SOMMF - *Saccharum officinarum* molasses methanol fraction).

4.20 Effect of *Saccharum officinarum* molasses aqueous fraction on fasting blood glucose level.

There were no significant differences in fasting blood glucose level of the *Saccharum officinarum* molasses aqueous fraction treated groups when compared with the control (Figure. 4.22; Appendix 20).

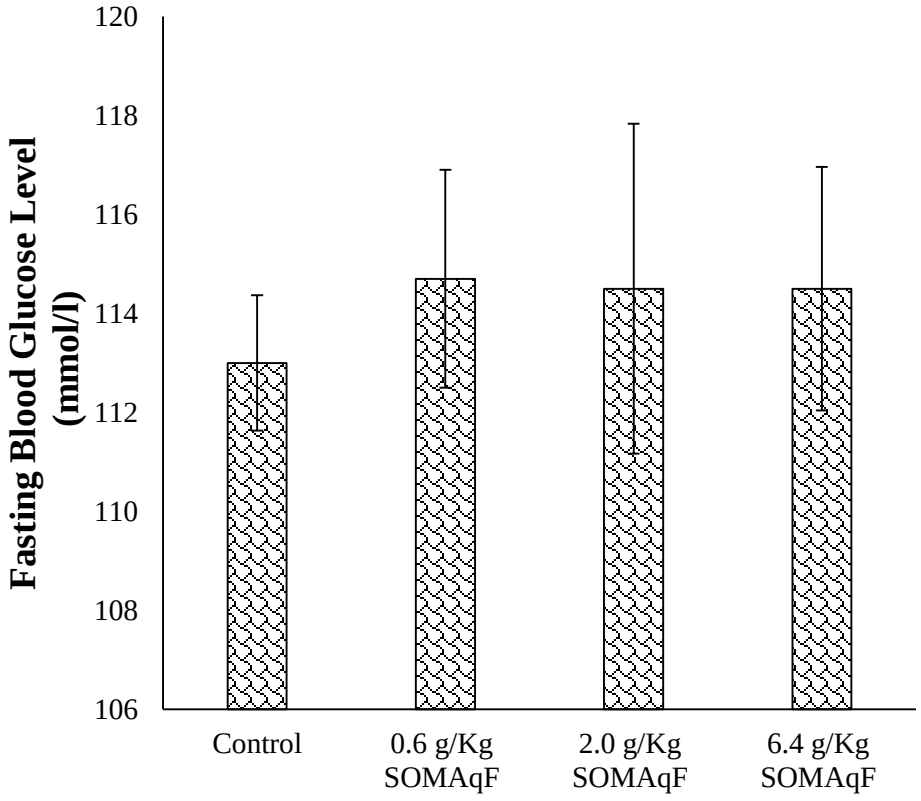

Figure. 4.22 Effect of *Saccharum officinarum* molasses aqueous fraction (SOMAqF) on fasting blood glucose level. Columns represents Mean±SEM. n=5.

4.21 Effect of *Saccharum officinarum* molasses aqueous fraction on percentage body weight change.

There was a significant decrease in the percentage body weight change of group treated with 2.0 g/kg/day *Saccharum officinarum* molasses aqueous fraction (SOMAqF) when compared with the control, 0.6 g/kg/day and 6.4 g/kg/day (SOMAqF) (Figure. 4.23; Appendix 21).

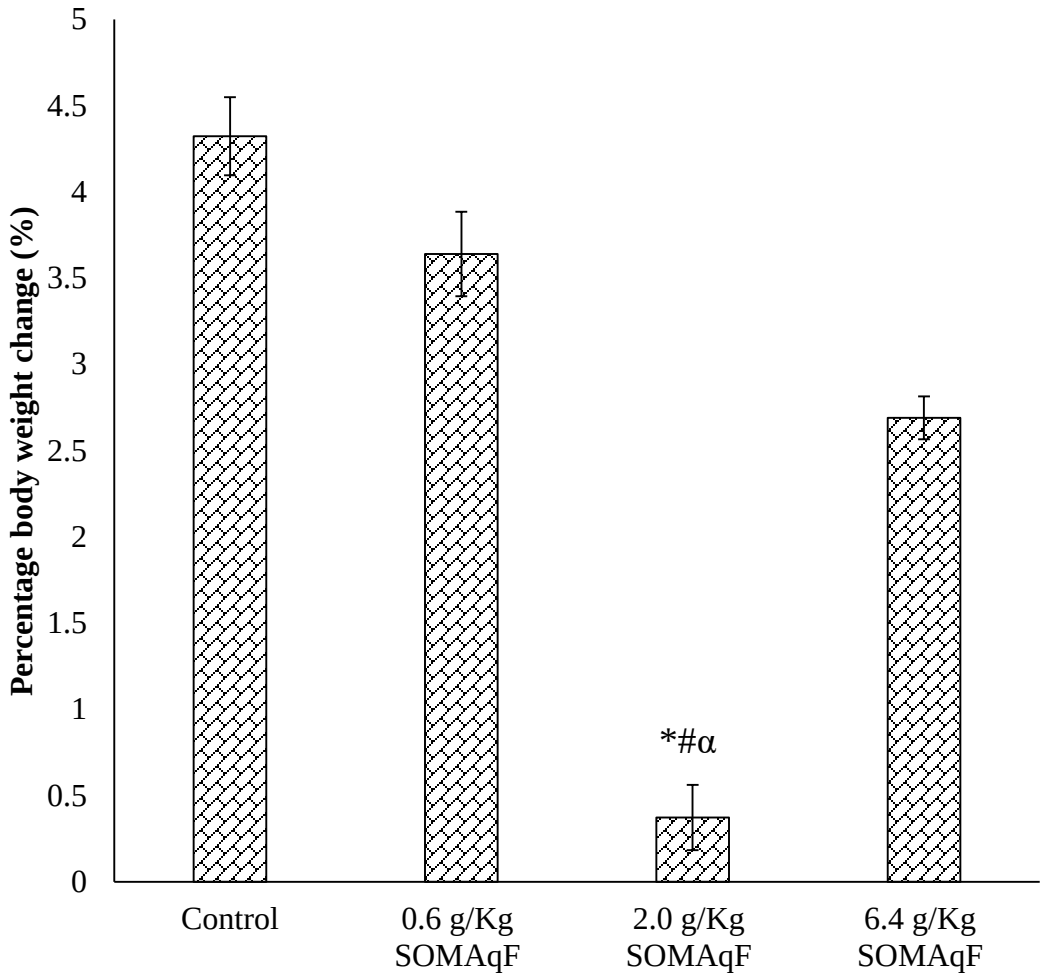

Figure. 4.23 Effect of *Saccharum officinarum* molasses aqueous fraction on percentage body weight change. Columns represent Mean±SEM. n=5. *P<0.05 compared with control. $^{\#}$P<0.05 compared with 1.0 mL/kg/day SOMAqF. $^{\alpha}$P<0.05 compared 10.0 mL/kg/day SOMAqF. *Saccharum officinarum* molasses aqueous fraction (SOMAqF).

4.22 Effect of *Saccharum officinarum* molasses aqueous fraction on relative organ weight.

There were significant increases ($P<0.5$) in relative weight of kidney of rats treated with 0.6 g/kg/day *Saccharum officinarum* molasses aqueous fraction (SOMAqF) as compared with control and 6.4 g/kg/day SOMAqF. The liver weights were significantly increased ($P<0.5$) in rats treated with 0.6 g/kg/day SOMAqF as compared with control and 6.4 g/kg/day SOMAqF. The weights of the reproductive organs were not significantly altered relative to the control and between other treated groups (Table 4.11).

Table 4.11 Effect of *Saccharum officinarum* molasses aqueous fraction on relative organ weight

		g/kg/day SOMAqF		
		0.6	**2.0**	**6.4**
Group	**Control**			
Testes	0.509±0.02	0.473±0.02	0.602±0.05	0.52±0.05
Epidydimis	0.241.0.01	0.249±0.01	0.304±0.03	0.22±0.022
Seminal vesicle	0.413±0.02	0.306±0.03	0.396±0.043	0.474±0.04
Prostate gland	0.153±0.01	0.22±0.052	0.174±0.02	0.146±0.02
Kidney	0.545±0.01	0.615±0.02*$^{\#}$	0.592±0.01	0.542±0.02
Liver	2.851±0.08	3.627±0.17*$^{\#}$	3.231±0.272	2.701±0.11

Data presented as Mean±SEM. n=5. *P<0.05 relative to the control. $^{\#}$P<0.05 compared with 6.4 g/kg/day SOMAqF.

Saccharum officinarum molasses aqueous fraction (SOMAqF)

4.23 Effect of *Saccharum officinarum* molasses aqueous fraction on lipid peroxidation and antioxidant enzymes.

In the liver, alterations in levels of malondialdehyde (MDA) and glutathione were not significant, but SOD and catalase activities were significantly decreased (P<0.5) in *Saccharum officinarum* molasses aqueous fraction (SOMAqF) of 0.6 mL/kg/day (Table 4.12).

Testicular MDA level increased significantly (P<0.5) in 6.4 g/kg/day *Saccharum officinarum* molasses aqueous fraction (SOMAqF) treated rats as compared with control. The SOD activity of rats treated with 0.6 g/kg/day SOMAqF increased significantly (P<0.5) as compared with control, 2.0 g/kg/day and 6.4 g/kg/day SOMAqF treated groups. Activity of catalase significantly increased (P<0.5) in 0.6 g/kg/day SOMAqF compared to the control, 2.0 g/kg/day and 6.4 g/kg/day SOMAqF. Testicular glutathione concentration was unaltered significantly relative to control and between other treated groups (Table 4.13).

There was no significant change in catalase activity and glutathione in relation to control and between other treated groups (Table 4.13).

Table 4.12 Effect of *Saccharum officinarum* molasses aqueous fraction (SOMAqF) on lipid peroxidation and antioxidant enzymes of the liver.

		g/kg/day SOMAqF		
Group	**Control**	**0.6**	**2.0**	**6.4**
MDA (U/mg)	4.572±0.52	2.752±0.52	2.288±0.41	3.75±0.88
SOD (U/mg)	560.7±147.8	265.6±60.35*	288.7±64.81	609.8±82.1
CATALASE (IU/L)	1455±162.3	879.5±192.1*	1292±261	1474±83.87
GSH (uM/mg)	4.715±0.60	3.607±0.94	3.589±0.4	5.452±0.41

Data presented as Mean±SEM. n=5. *P<0.05 relative to the control.
Saccharum officinarum molasses aqueous fraction (SOMAqF)

Table 4.13 Effect of *Saccharum officinarum* molasses aqueous fraction on lipid peroxidation and antioxidant enzymes of the testes.

Group	Control	g/kg/day SOMAqF		
		0.6	**2.0**	**6.4**
MDA (U/mg)	1.647±0.24	2.156±0.20	2.145±0.26	2.619±0.44*$^{\#}$
SOD (U/mg)	174.8±23.72	3299±2.84*$^{\#\alpha}$	270.6±34.36	260.5±34.02
CATALASE (IU/L)	698.2±62.94	1101±87.77*$^{\#\alpha}$	792.7±63.14	833.9±111
GSH (uM/mg)	2.876±0.31	4.43±0.95	3.28±0.86	3.940±0.42

Data presented as Mean±SEM. n=5. *P<0.05 relative to the control. $^{\#}$P<0.05 compared with 2.0 g/kg/day SOMAqF. $^{\alpha}$P<0.05 compared with 6.4 g/kg/day SOMAqF

Saccharum officinarum molasses aqueous fraction (SOMAqF)

Table 4.14 Effect of *Saccharum officinarum* molasses aqueous fraction on lipid peroxidation and antioxidant enzymes of the epididymis.

Group	Control	g/kg/day SOMAqF		
		0.6	2.0	6.4
MDA (U/mg)	15.69±6.89	13.17±2.36	23.89±3.94	22.89±2.0
SOD (U/mg)	1620±379.2	1729±361.8	1989±594.1	2317±912.1
CATALASE (IU/L)	3669±898.3	2144±467.6	3970±1721	3553±1070
GSH (uM/mg)	9.080±1.97	5.559±2.12	9.175±1.49	7.744±1.97

Data presented as Mean±SEM. n=5.

Saccharum officinarum molasses aqueous fraction (SOMAqF)

4.24 Effect of *Saccharum officinarum* molasses aqueous fraction on epididymal sperm characteristics.

There were significant decreases (P<0.5) in sperm concentration and viability of groups treated with 2.0 and 6.4 g/kg/day SOMAqF in relation to control (Figure 4.24 and 4.24; Appendix 22 and 23).

The *Saccharum officinarum* molasses aqueous fraction (SOMAqF) did not show significant change in sperm motility of all treated groups (Figure 4.26; Appendix 24).

The epididymal volume increased significantly (P<0.5) in 6.4 g/kg/day SOMAqF as compared with control. (Figure 4.27; Appendix 25).

The percentage abnormal sperm morphology was significantly increased (P<0.5) in 2.0 and 6.4 g/kg/day SOMAqF as compared to the control. Also, the percentage abnormal sperm morphology of 6.4 g/kg/day SOMAqF treated rats was significantly increased (P<0.5) compared to the percentage abnormal sperm morphology of 0.6 g/kg/day SOMAqF (Figure 4.28; Appendix 26).

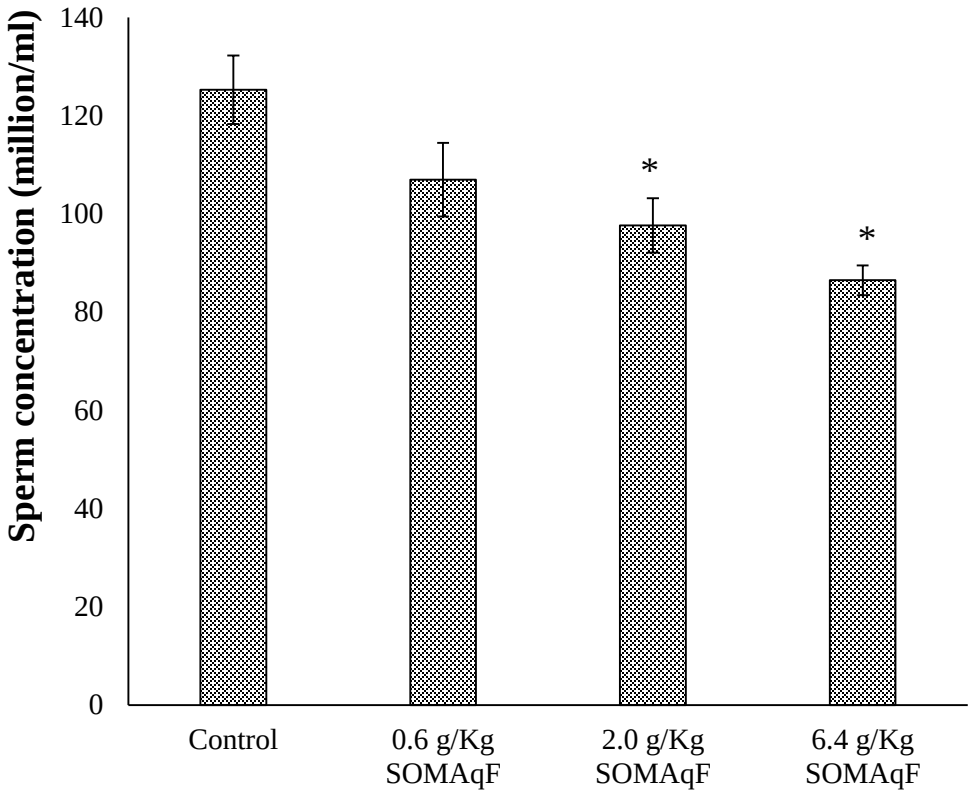

Figure. 4.24 Effect of *Saccharum officinarum* molasses aqeuous fraction on sperm concentration.

Columns represents Mean±SEM. n=5. *P<0.05 relative to control.

Saccharum officinarum molasses aqueous fraction (SOMAqF)

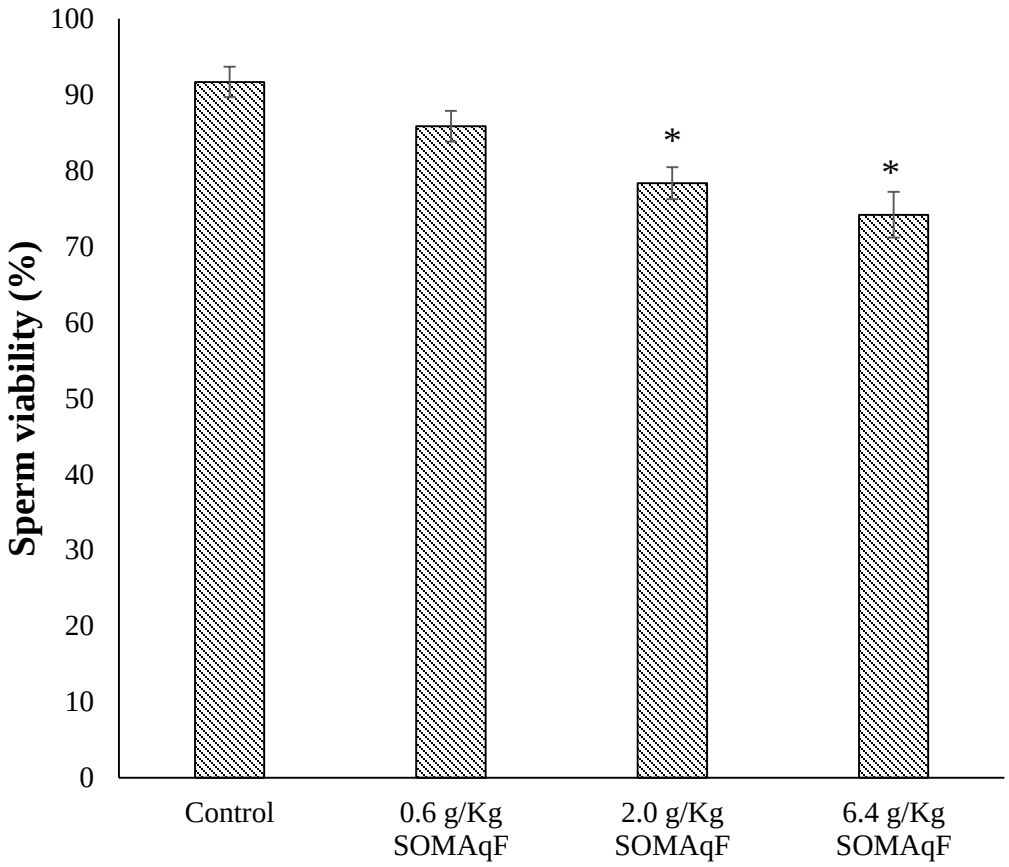

Figure. 4.25 Effect of *Saccharum officinarum* molasses aqueous fraction on sperm viability.

Columns represents Mean±SEM. n=5. *P<0.05 as related to the control.

Saccharum officinarum molasses fraction (SOMAqF)

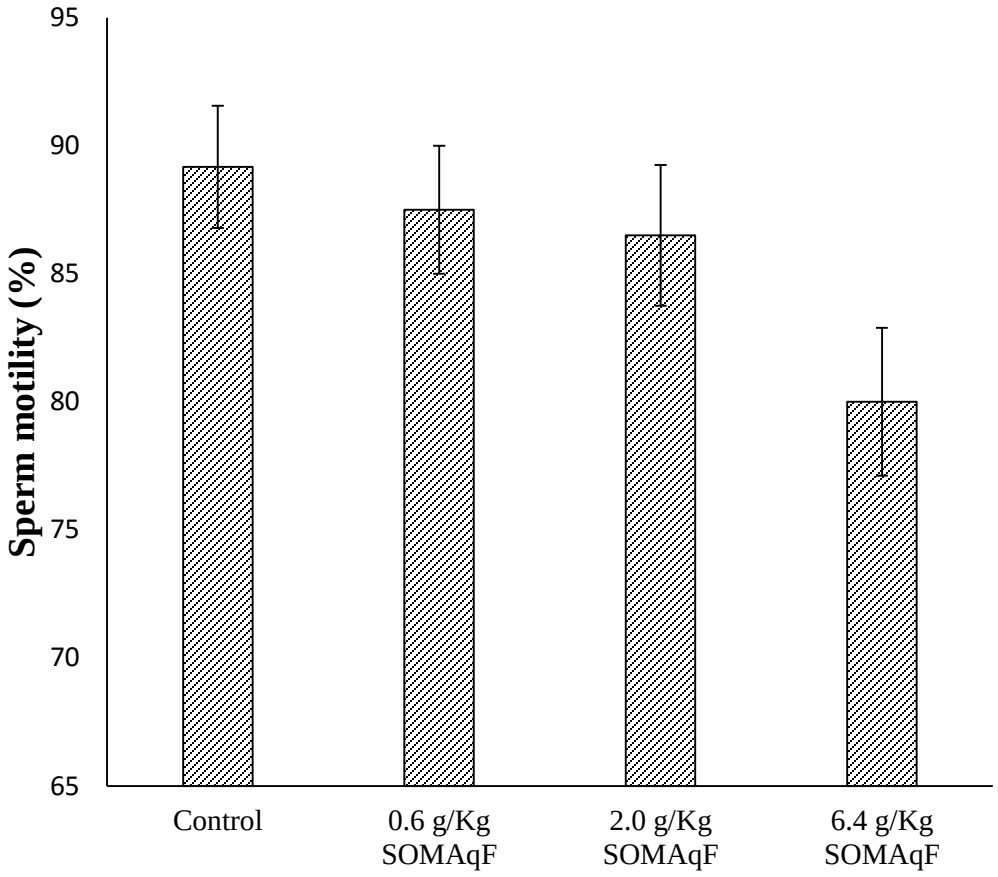

Figure. 4.26 Effect of *Saccharum officinarum* molasses aqueous fraction on sperm motility. Columns represents Mean±SEM. n=5.
Saccharum officinarum molasses aqueous fraction (SOMAqF)

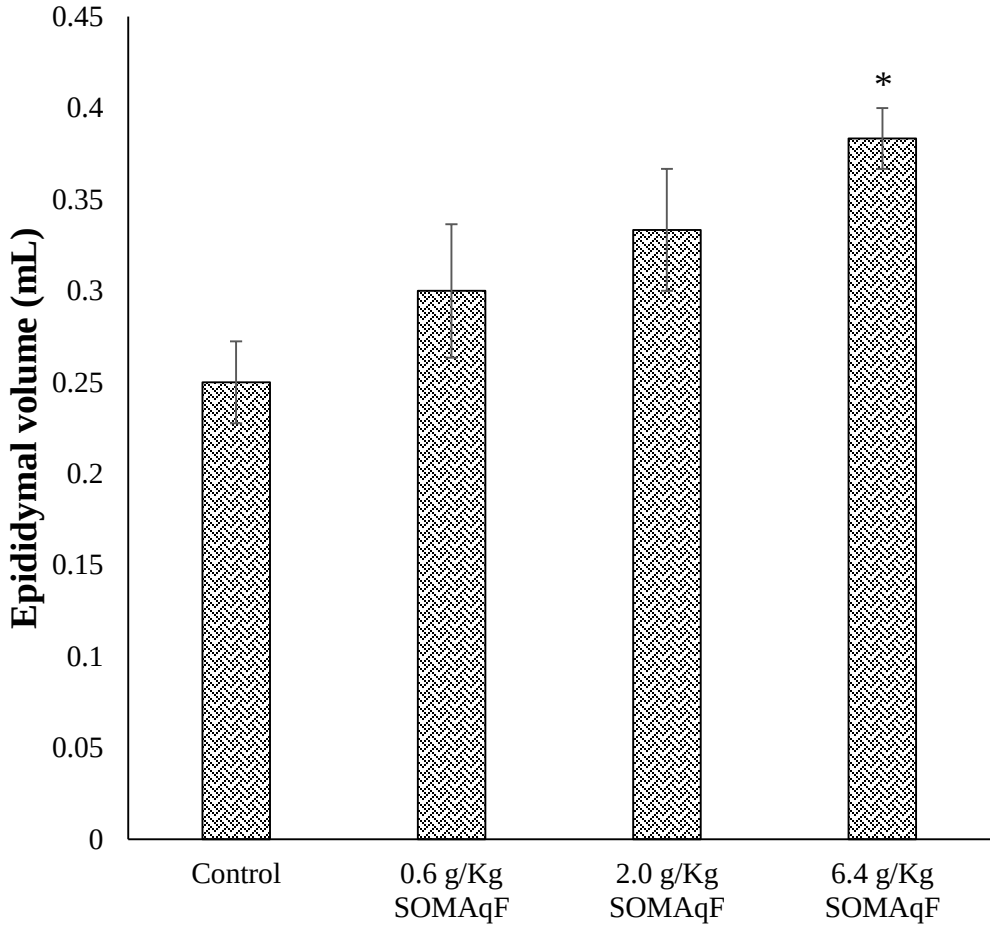

Figure. 4.27 Effect of *Saccharum officinarum* molasses aqueous fraction on epididymal volume. Columns represents Mean±SEM. n=5. *P<0.05 relative to the control. *Saccharum officinarum* molasses aqueous fraction (SOMAqF)

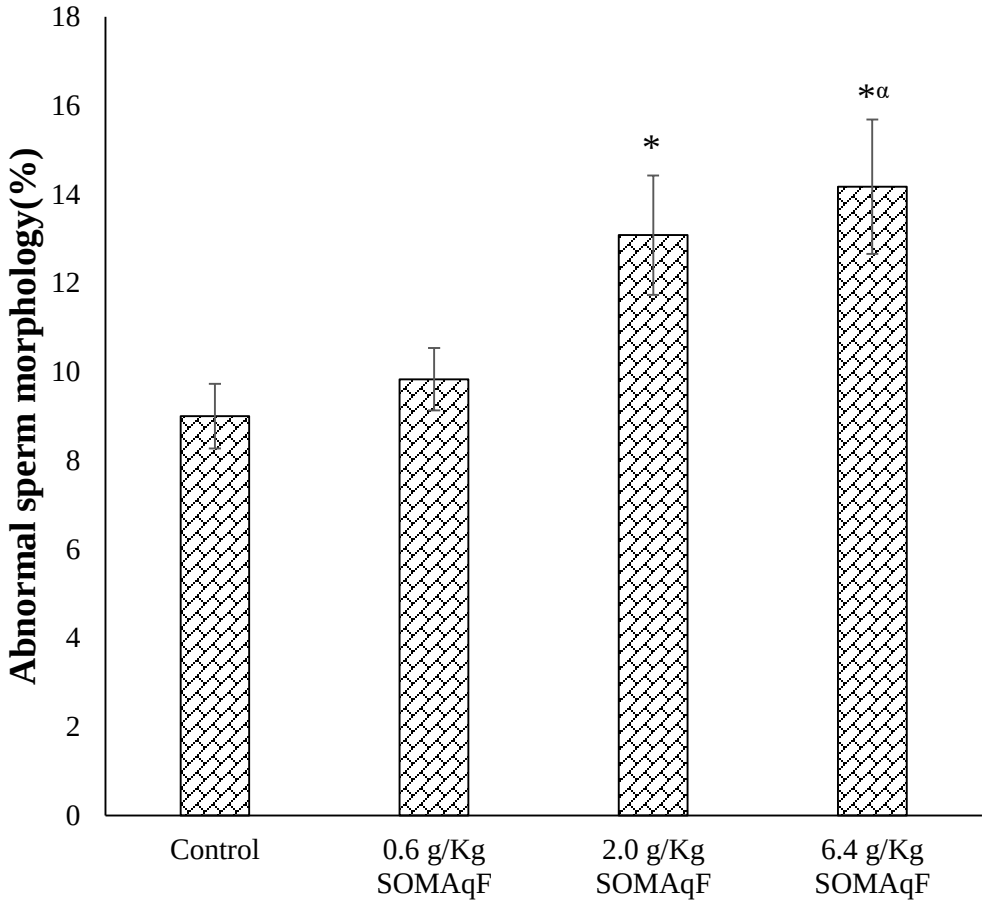

Figure. 4.28 Effect of *Saccharum officinarum* molasses aqueous fraction on abnormal sperm morphology. Columns represents Mean±SEM. n=5. *P<0.05 relative to the control, ᵅP<0.05 relative to 0.6 g/kg/day SOMAqF.

Saccharum officinarum molasses aqueous fraction (SOMAqF)

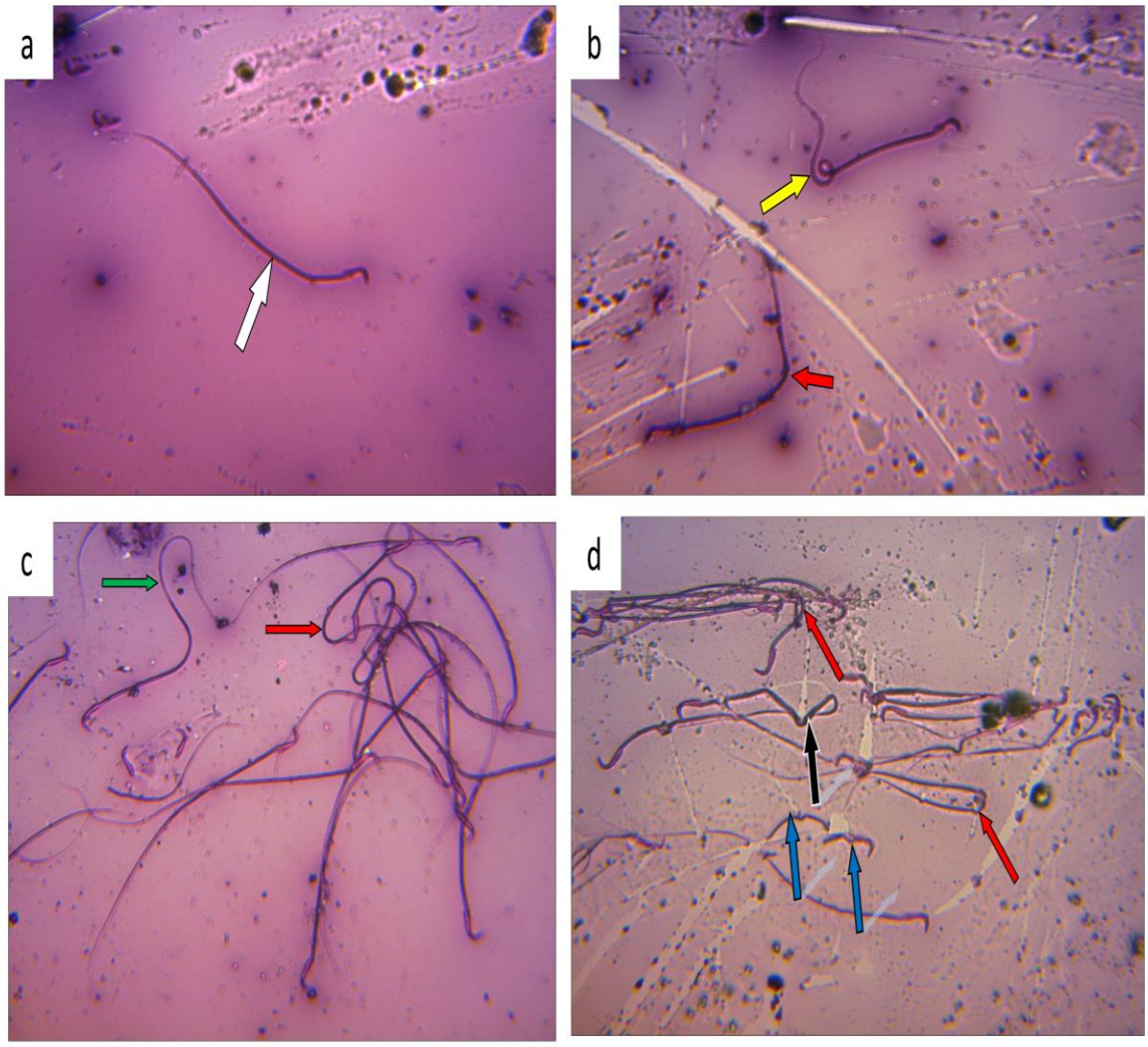

Plate 14. Morphology of spermatozoa of rat treated with *Saccharum officinarum* molasses aqueous fraction (SOMAqF).

Stained by eosin nigrosin, presented at x400 magnification.

a) Morphology of spermatozoa of control rat, show normal head midpiece and tail (white arrows).

b) Morphology of spermatozoa of rat treated with 1.0 mL/kg/day SOMAqF show coiled midpiece (yellow arrow) and bent midpiece (red arrow.

c) Morphology of spermatozoa of rat treated with 3.2 mL/kg/day SOMAqF, show bent midpiece (red arrow) and curved tail (green arrow).

d) Morphology of spermatozoa of rat treated with 10 mL/kg/day SOMAqF, show Bent midpiece (red arrow), bent head (black arrow) and detached head (blue arrow).

4.25 Effect of *Saccharum officinarum* molasses aqueous fraction on serum hormonal profile.

The levels of serum follicle stimulating hormone (FSH) and luteinizing hormone (LH) were unaltered significantly when compared with control and treated groups (Figure. 4.29; Appendix 27). Serum testosterone level significantly reduced ($P<0.5$) in 6.4 g/kg/day *Saccharum officinarum* molasses aqueous fraction (SOMAqF) treated group when compared with control and 0.6 g/kg/day SOMAqF treated group. (Figure 4.30; Appendix 28).

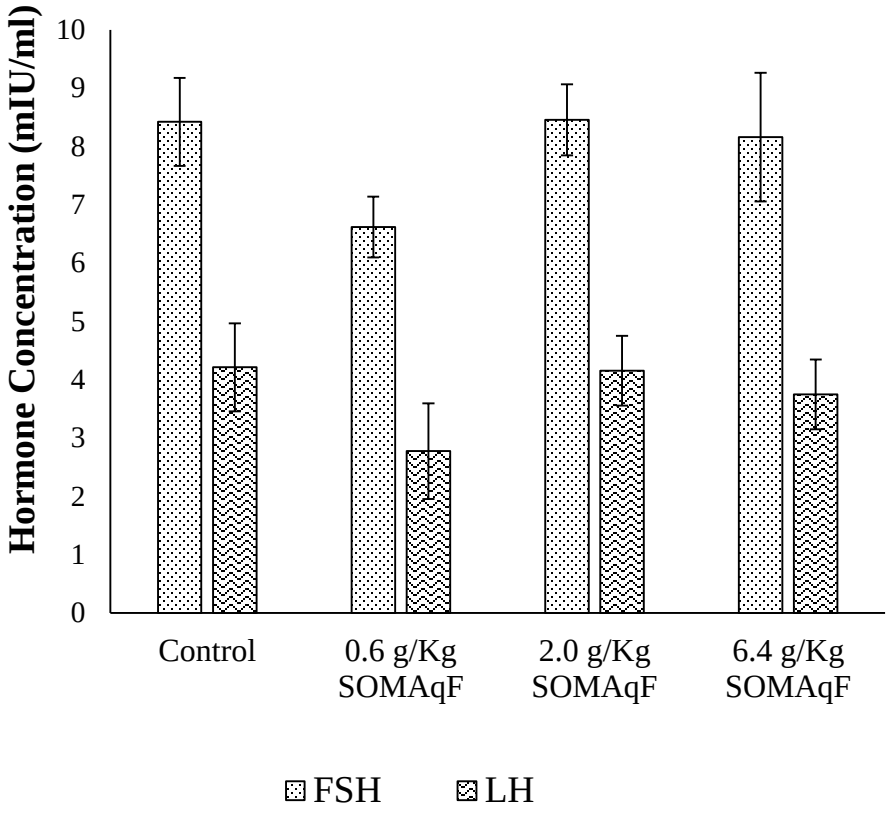

Figure. 4.29 Effect of *Saccharum officinarum* molasses aqueous fraction on serum follicle stimulating hormone and Luteinizing level. Columns represents Mean±SEM. n=5. *Saccharum officinarum* molasses aqueous fraction (SOMAqF)

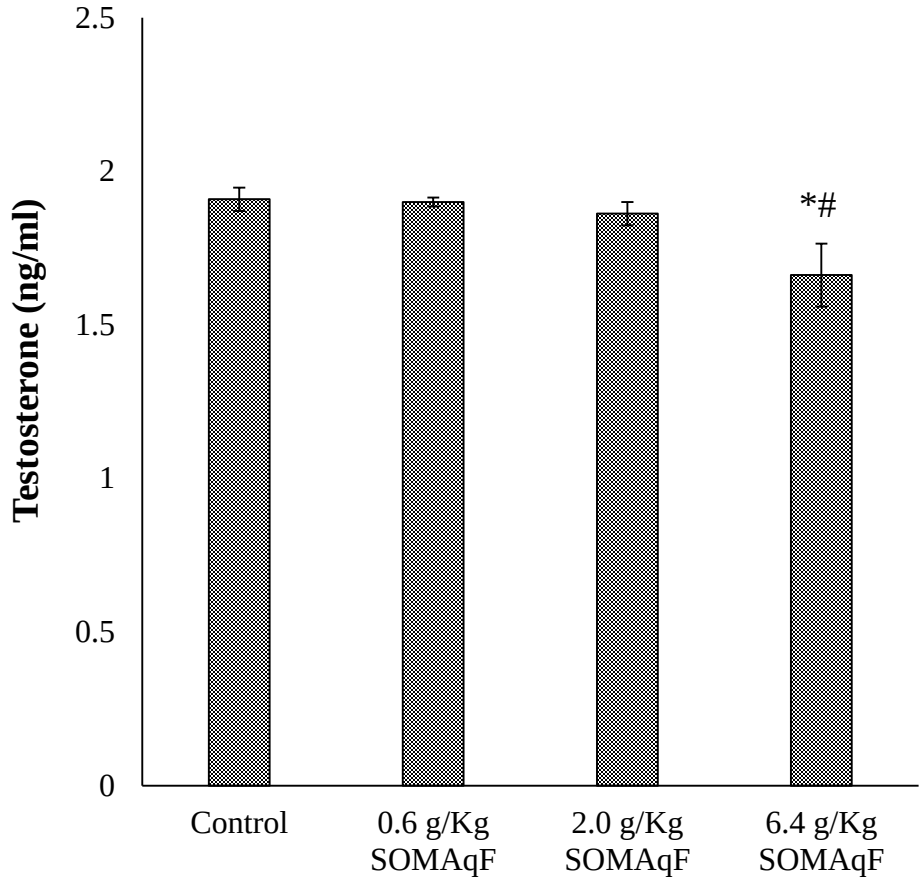

Figure. 4.30 Effect of *Saccharum officinarum* molasses aqueous fraction on serum Testosterone level. Columns represents Mean±SEM. n=5. *P<0.05 relative to the control, #P<0.05 relative to 0.6 g/kg/day SOMAqF.

Saccharum officinarum molasses aqueous fraction (SOMAqF)

4.26 Effect of *Saccharum officinarum* molasses aqueous fraction on histology of liver.

Liver section of group treated with 0.6 g/kg/day SOMAqF, show portal tracts mildly infiltrated - mild triaditis and mildly dilatated sinusoids infiltrated by inflammatory cells (Plate 12).

Liver section of 2.0 g/kg/day *Saccharum officinarum* molasses aqueous fraction **(**SOMAqF) treated group show mild peri vascular and periportal infiltration of inflammatory cells (Plate 13).

Liver section of 6.4 g/kg/day SOMAqF treated group show the liver parenchyma having a focal area of lymphocytes aggregate and sinusoids with scanty inflammatory cells (Plate 14).

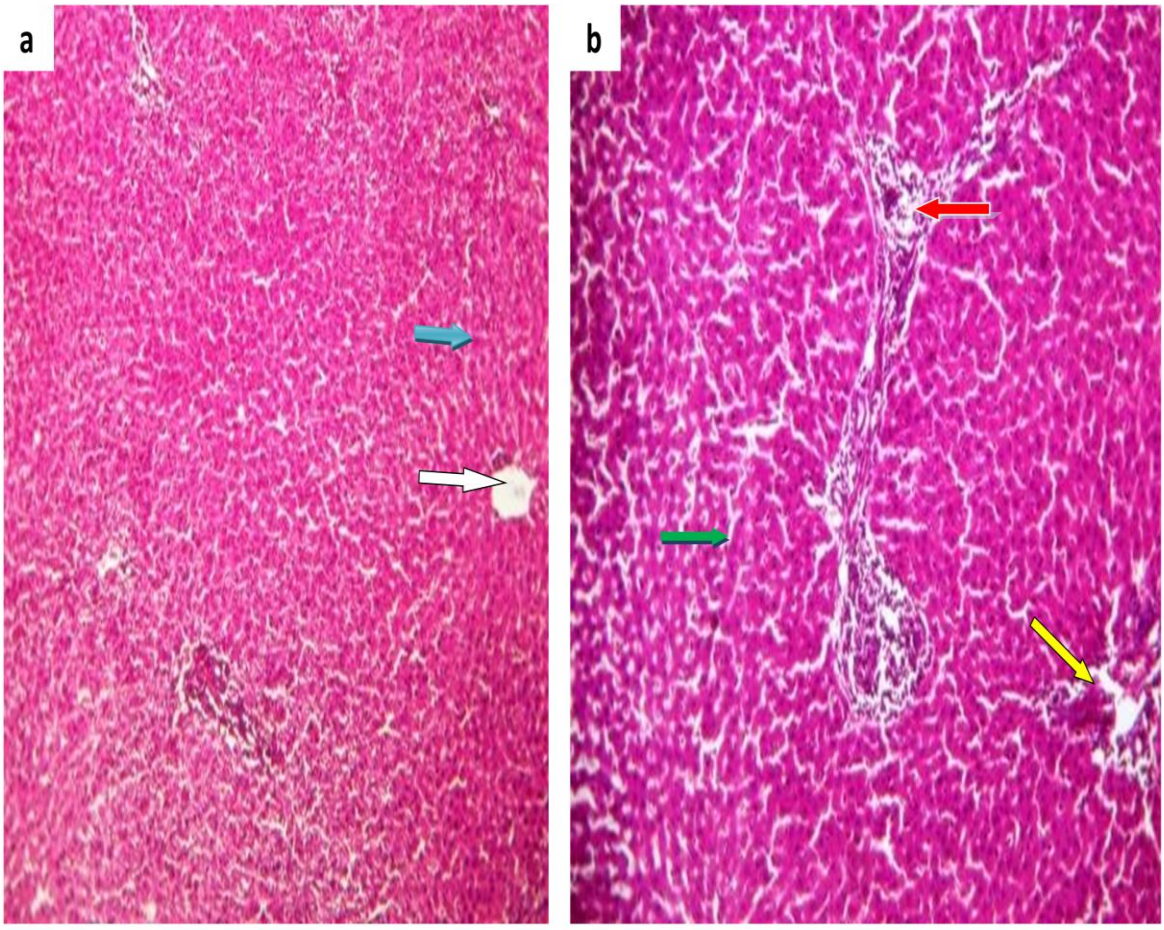

Plate 15. Photomicrograph of liver section of rat treated with *Saccharum officinarum* molasses aqueous fraction **(**SOMAqF) 0.6 g/kg/day SOMAqF.

Tissues stained by H&E presented at x100 magnification.

a) Liver section of control rat showing normal architecture, central venules and portal tract (white arrow), sinusoids (blue arrow) and hepatocytes (slender arrow).

b) Liver section of rat given 0.6 g/kg/day SOMAqF, shows portal tracts mildly infiltrated - mild triaditis (white arrow), the sinusoids mildly dilatated and infiltrated by inflammatory cells (blue arrow). Normal hepatocytes (slender arrow).

(SOMAqF - *Saccharum officinarum* molasses aqueous fraction)

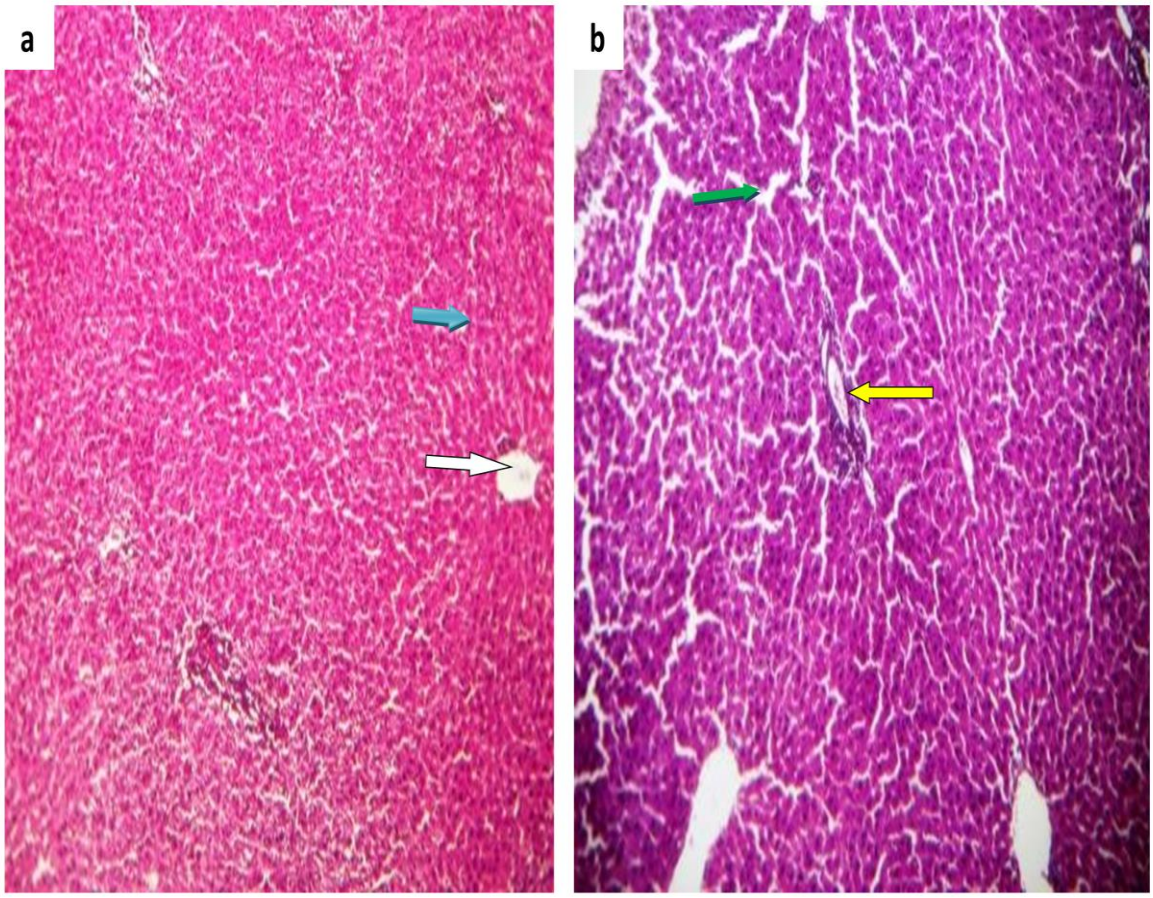

Plate 16. Photomicrograph of liver section of rat treated with *Saccharum officinarum* molasses aqueous fraction **(**SOMAqF) 2. g/kg/day SOMAqF.

Tissues stained by H&E presented at x100 magnification.

a) Liver section of control rat showing normal architecture, central venules and portal tract (white arrow), sinusoids (blue arrow) and hepatocytes (slender arrow).

b) Liver section of rat given 2.0 g/kg/day SOMAqF showing the central venules show mild peri vascular infiltration of inflammatory cells and periportal infiltration of inflammatory cells (white arrow). Normal sinusoids (blue arrow), hepatocytes appear normal (slender arrow).

(SOMAqF - *Saccharum officinarum* molasses aqueous fraction).

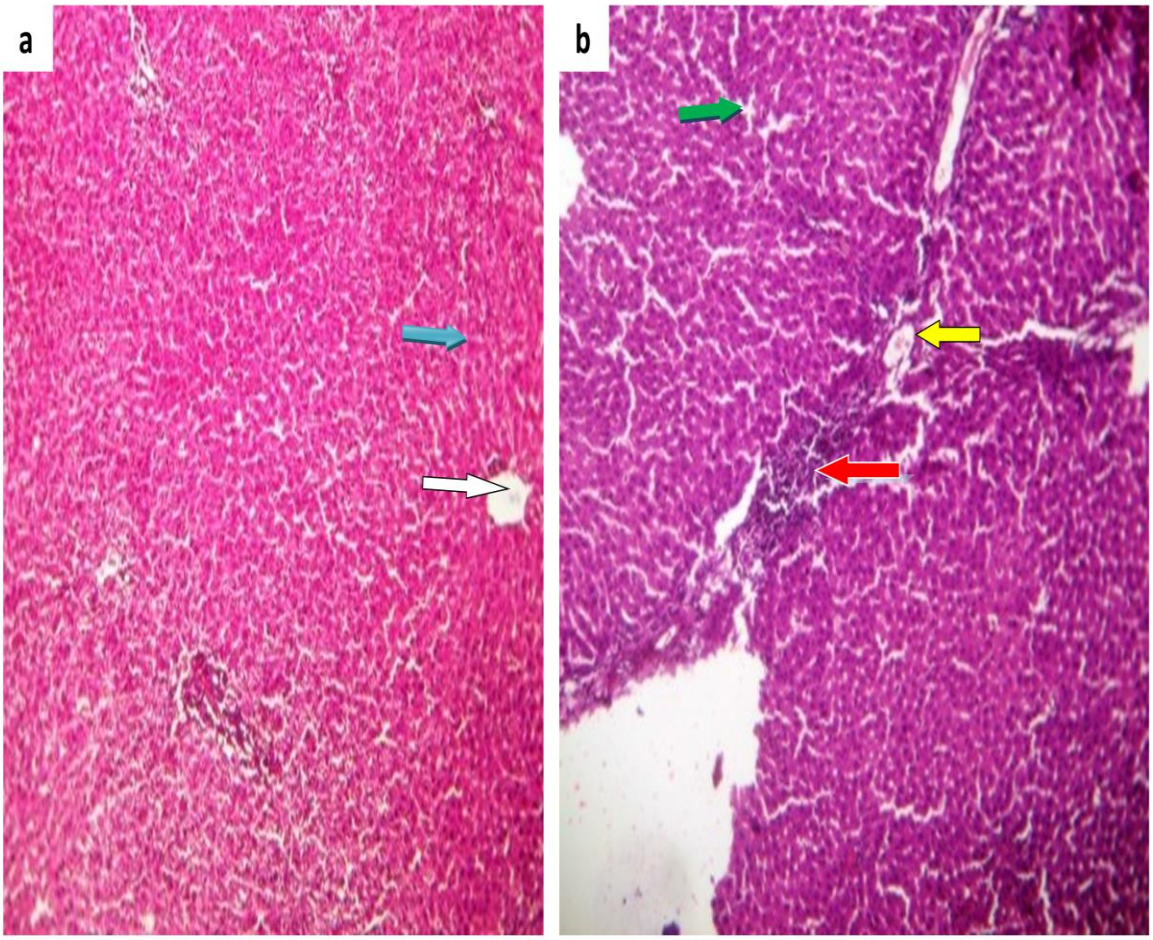

Plate 17. Photomicrograph of liver section of rat treated with *Saccharum officinarum* molasses aqueous fraction **(**SOMAqF) 6.4 g/kg/day SOMAqF.

Tissues stained by H&E presented at x100 magnification.

a) Liver section of control rat showing normal architecture, central venules and portal tract (white arrow), sinusoids (blue arrow) and hepatocytes (slender arrow).

b) Liver section of rat given 6.4 g/kg/day SOMAqF, the liver parenchyma show a focal area of lymphocytes aggregate (red arrow), sinusoids show scanty inflammatory cells (blue arrow). The hepatocytes are normal (slender arrow).

(SOMAqF - *Saccharum officinarum* molasses aqueous fraction).

4.27 Effect of *Saccharum officinarum* molasses aqueous fraction on histology of testes.

The testicular section of 0.6 g/kg/day *Saccharum officinarum* molasses aqueous fraction (SOMAqF) treated group show maturation arrest and asece of defined germ cell levels and lumen in some seminiferous tubules (Plate 15).

The testicular section of 2.0 g/kg/day SOMAqF treated group show wide seminiferous tubules with distorted and degenerated germ cells. The Leydig cells show hyperplasia (Plate 16).

The testicular section of 6.4 g/kg/day SOMAqF treated group show several seminiferous tubules with degenerated germ cells and maturation arrest at the primary level (Plate 17).

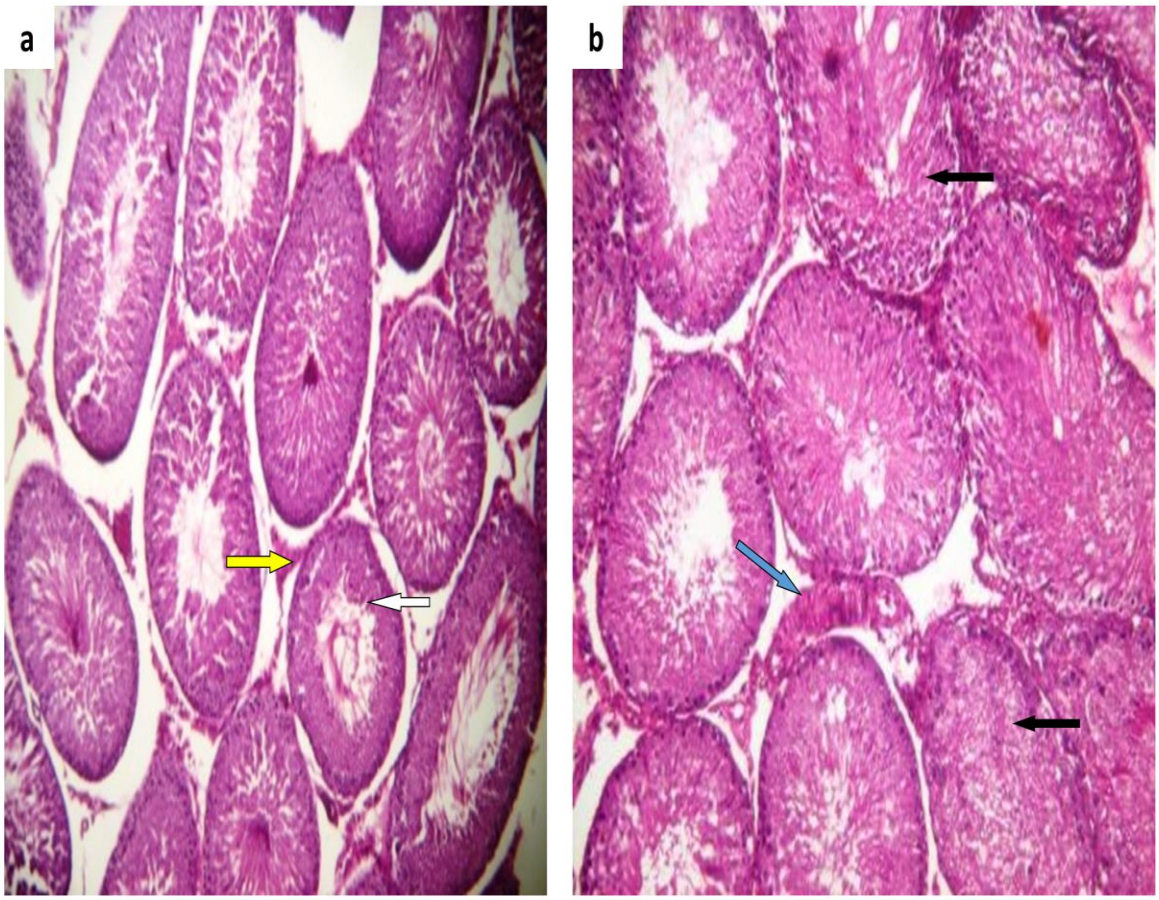

Plate 18. Photomicrograph of testicular section of rat treated with *Saccharum officinarum* molasses aqueous fraction **(**SOMAqF) 0.6 g/kg/day SOMAqF.

Tissues stained by H&E presented at x100 magnification.

a) Testicular section of control rat, seminiferous tubules appear normal with maturation stages of germ cell layer (black arrow), spermatogonia, Sertoli cells and lumen appear normal (white arrow). Interstitium and Leydig cells appear normal (yellow arrow).

b) Testicular section of rat given 0.6 g/kg/day SOMAqF shows some abnormal seminiferous tubules appearing with maturation arrest, no defined germ cell levels and lumen (black arrows). The interstitial spaces appear congested (red arrow).

(SOMAqF - *Saccharum officinarum* molasses aqueous fraction).

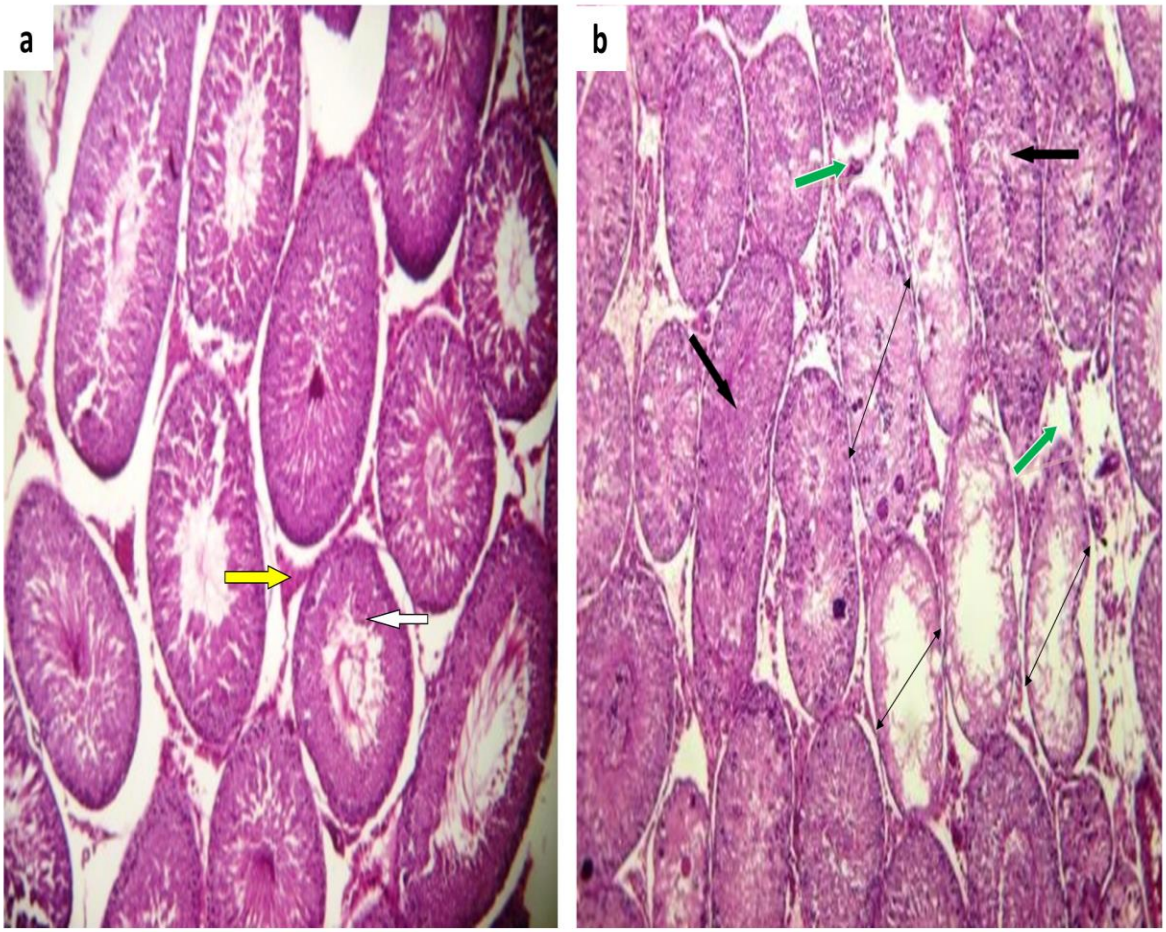

Plate 19. Photomicrograph of testicular section of rat treated with *Saccharum officinarum* molasses aqueous fraction **(**SOMAqF) 2.0 g/kg/day SOMAqF.

Tissues stained by H&E presented at x100 magnification.

a) Testicular section of control rat, seminiferous tubules appear normal with maturation stages of germ cell layer (black arrow), spermatogonia, Sertoli cells and lumen appear normal (white arrow). Interstitium and Leydig cells appear normal (yellow arrow).

b) Testicular section of rat given 2.0 g/kg/day SOMAqF shows some seminiferous tubules with degenerated germ cells, improper germ cell layer and maturation arrest at the primary level (black arrows), wide and empty lumen (white arrows). The Leydig cells show hyperplasia (green arrows).

(SOMAqF - *Saccharum officinarum* molasses aqueous fraction).

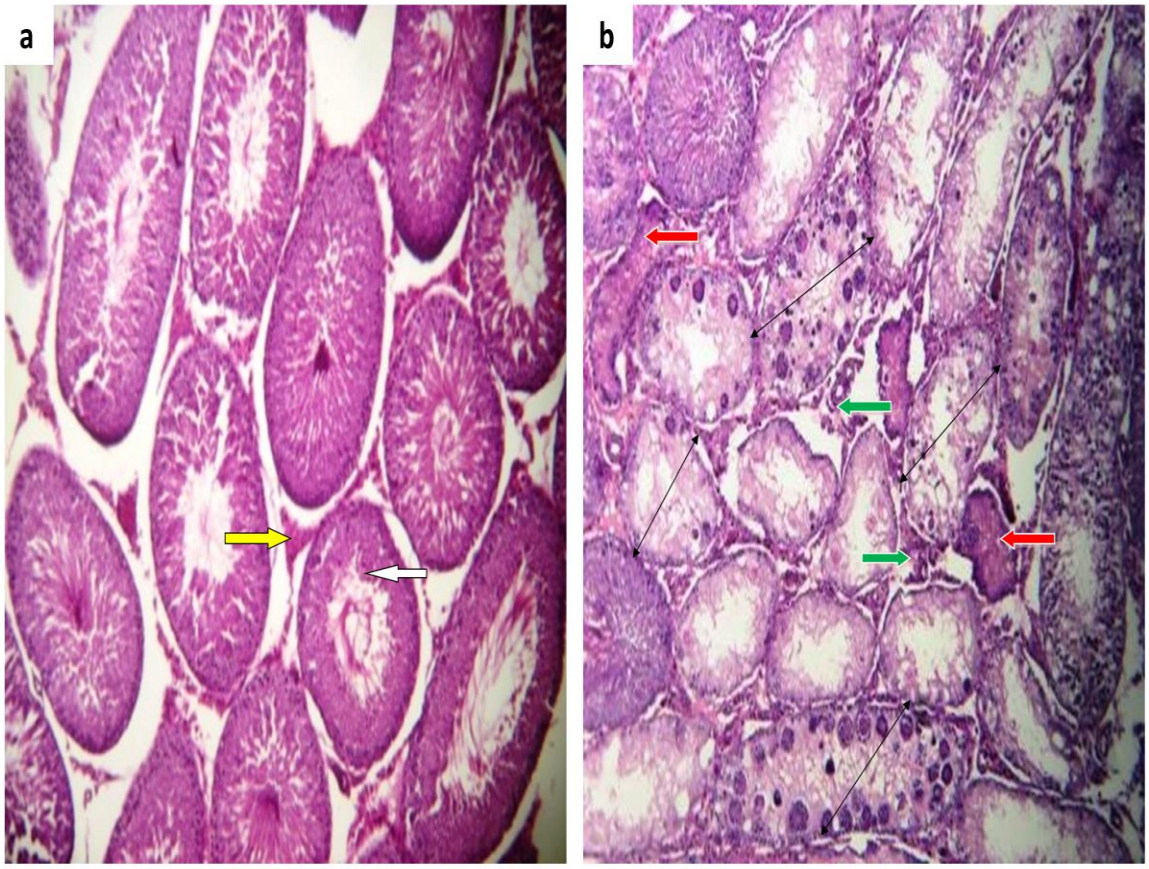

Plate 20. Photomicrograph of testicular section of rat treated with *Saccharum officinarum* molasses aqueous fraction **(**SOMAqF) 6.4 g/kg/day SOMAqF.

Tissues stained by H&E presented at x100 magnification.

a) Testicular section of control rat, seminiferous tubules appear normal with maturation stages of germ cell layer (black arrow), spermatogonia, Sertoli cells and lumen appear normal (white arrow). Interstitium and Leydig cells appear normal (yellow arrow).

b) Testicular section of 6.4 g/kg/day SOMAqF treated group show several seminiferous tubules with degenerated germ cells with lack of germ cell layer and maturation arrest at the primary level (spanning arrows). The connective tissues enveloping the tubules are thickened (red arrow). The Leydig cells show hyperplasia (green arrows).

(SOMAqF - *Saccharum officinarum* molasses aqueous fraction).

4.28 Effect of *Saccharum officinarum* molasses aqueous fraction on histology of epididymis.

Epididymal section of 0.6 g/kg/day *Saccharum officinarum* molasses aqueous fraction (SOMAqF) treated group appear normal (Plate 18). Epididymal section of 2.0 g/kg/day SOMAqF show epididymal ducts with thickened smooth muscle and epithelial layers (Plate 19). Epididymal section of 6.4 g/kg/day SOMAqF show few ducts that are empty with fibrotic interstitial tissues (Plate 20).

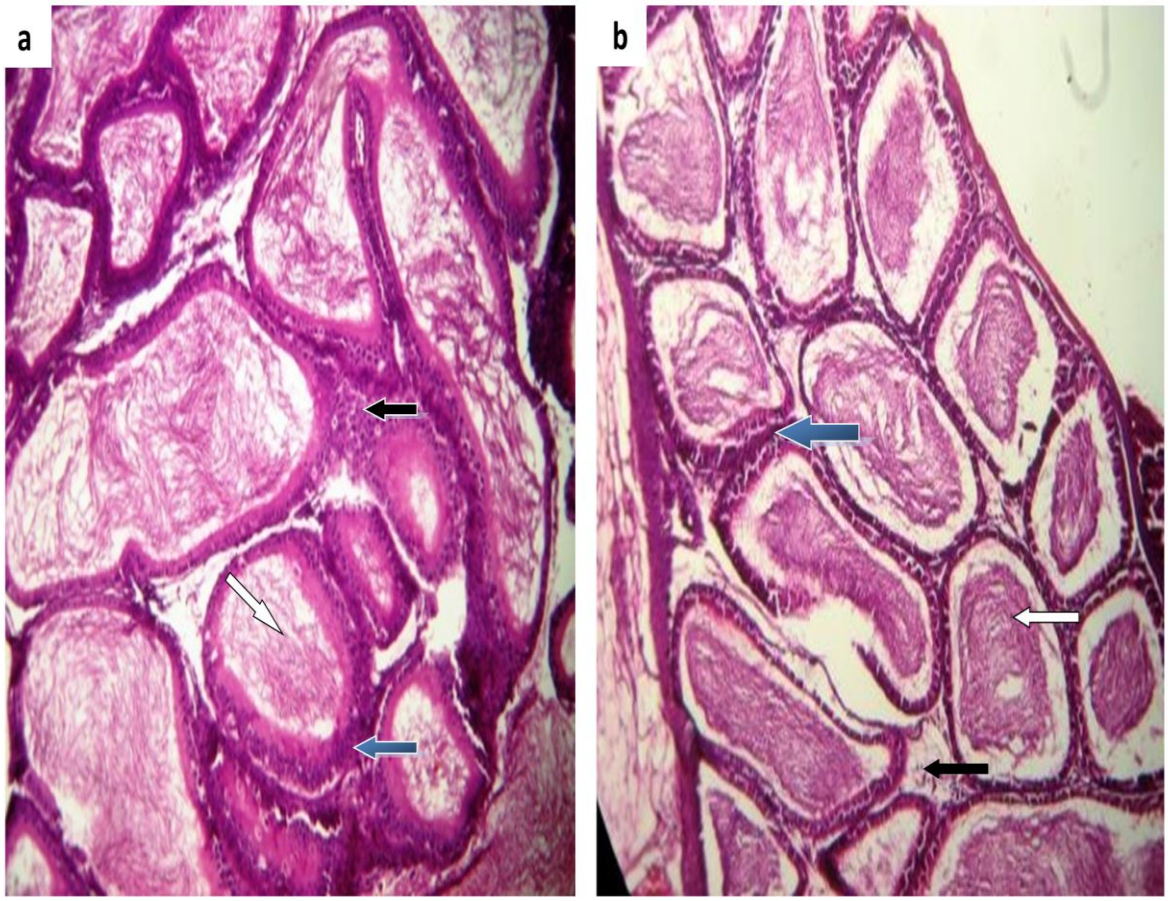

Plate 21. Photomicrograph of epididymal section of rat treated with *Saccharum officinarum* molasses aqueous fraction **(**SOMAqF) 0.6 g/kg/day SOMAqF.

Tissues stained by H&E presented at x100 magnification.

a) Epididymal section of control rat shows normal epididymal ducts and contents (blue arrows), lumen contains spermatozoa (white arrow) and normal interstitium (black arrow).

b) Epididymal section of rat given 0.6 g/kg/day SOMAqF showing normal smooth muscle epithelial layers and epididymal ducts (blue arrow), duct contains spermatozoa (white arrow). Interstitium appear normal (black arrow).

(SOMAqF - *Saccharum officinarum* molasses aqueous fraction).

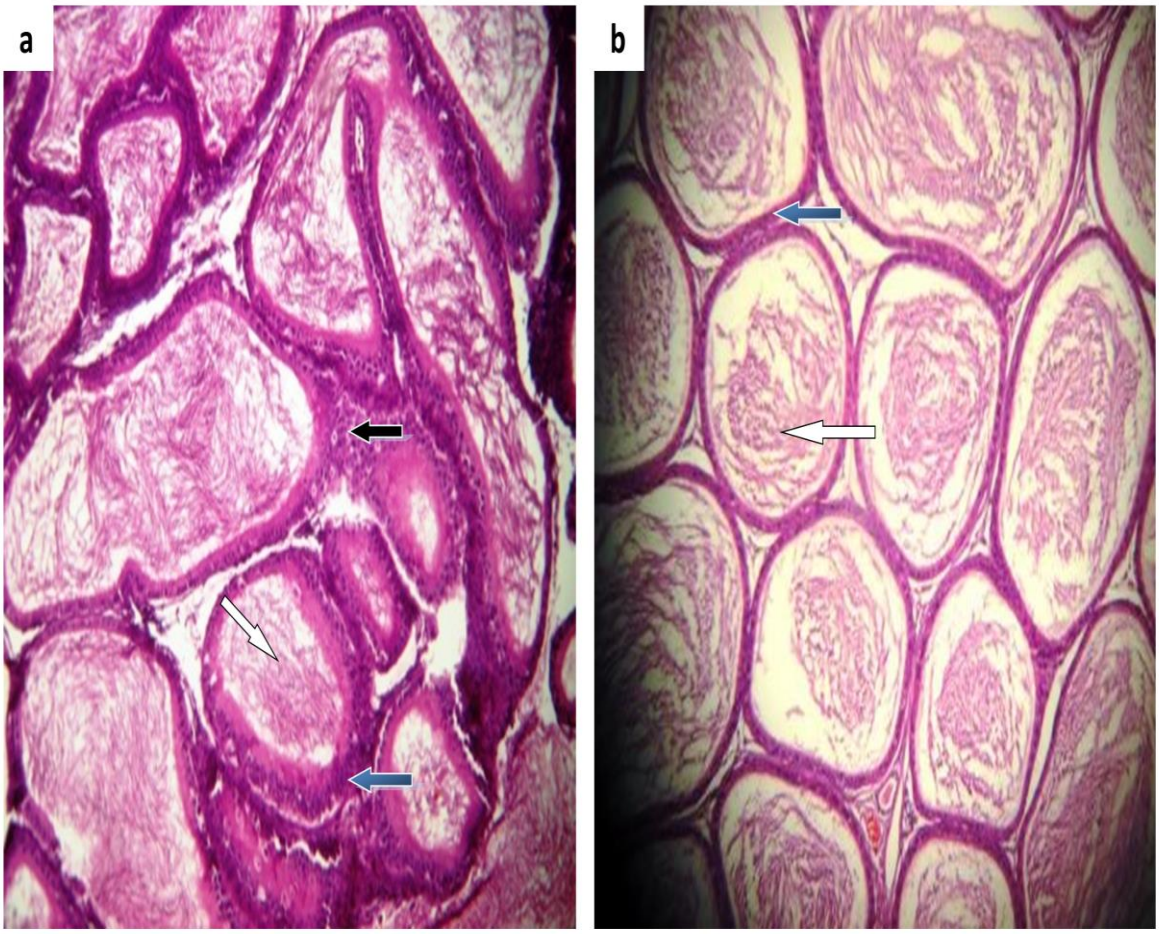

Plate 22. Photomicrograph of epididymal section of rat treated with *Saccharum officinarum* molasses aqueous fraction **(**SOMAqF) 2.0 g/kg/day SOMAqF.

Tissues stained by H&E magnification x100

a) Epididymal section of control rat shows normal epididymal ducts and contents (blue arrows), lumen contains spermatozoa (white arrow) and normal interstitium (black arrow).

b) Epididymal section of rat given 2.0 g/kg/day SOMAqF showing epididymal ducts with thickened smooth muscle and epithelial layers (blue arrow), duct contains spermatozoa (white arrow).

(SOMAqF - *Saccharum officinarum* molasses aqueous fraction).

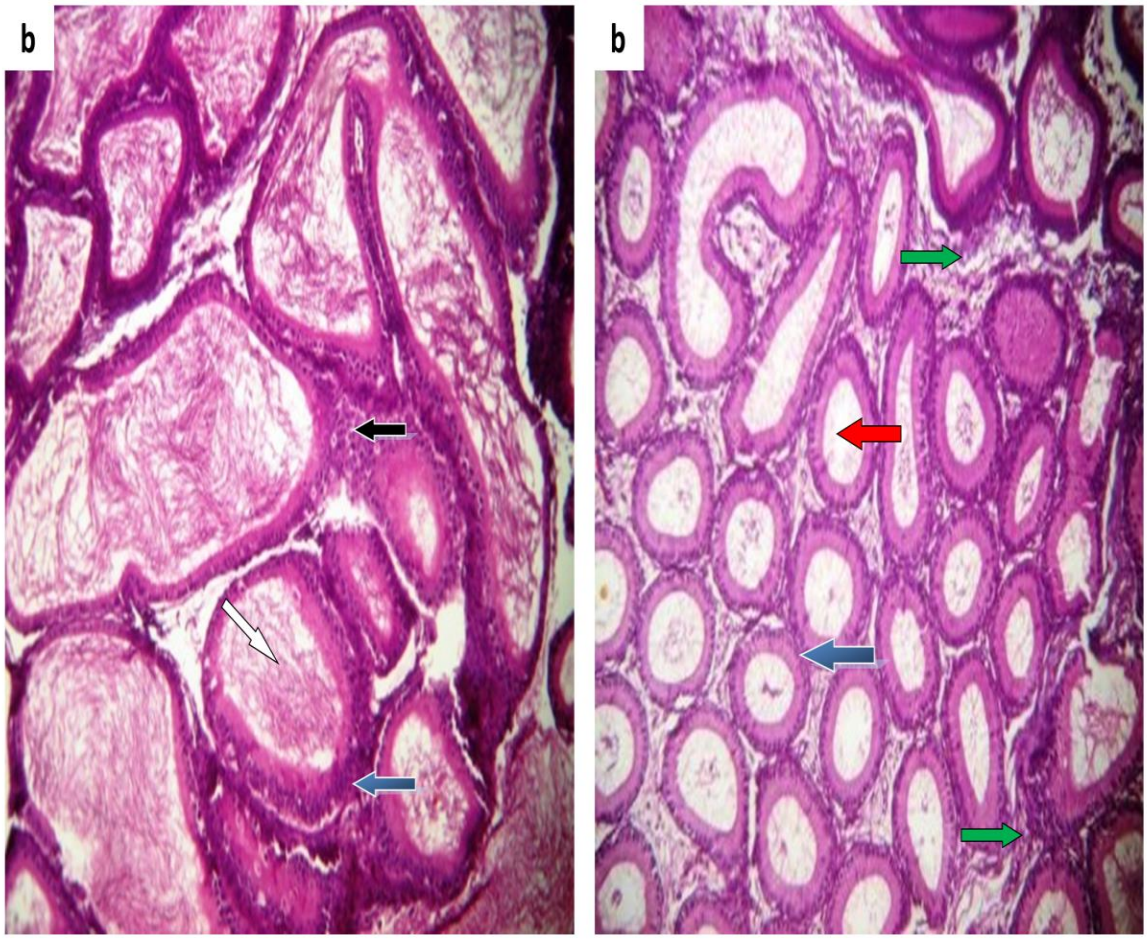

Plate 23. Photomicrograph of epididymal section of rat treated with *Saccharum officinarum* molasses aqueous fraction **(**SOMAqF) 6.4 g/kg/day SOMAqF.

Tissues stained by H&E magnification x100

a) Epididymal section of control rat shows normal epididymal ducts and contents (blue arrows), lumen contains spermatozoa (white arrow) and normal interstitium (black arrow).

b) Epididymal section of rat given 6.4 g/kg/day SOMAqF showing normal epididymal ducts with smooth muscle and epithelial layers (blue arrow), few ducts are empty (white arrow). The interstitial tissues appear fibrotic (green arrows).

(SOMAqF - *Saccharum officinarum* molasses aqueous fraction).

4.29 Effects of *Saccharum officinarum* molasses methanol and aqueous fractions, diethyl phthalate and lupeol administration on cell proliferation and viability.

Cell proliferation shows a directly proportionate significant increase ($P<0.05$) to the increase in number of cells per well (Figure 4.31; Appendix 29). At 250 ug/mL administration, *Saccharum officinarum* molasses methanol fraction (SOMMF) significantly increased ($P<0.05$) cell proliferation and viability. Diethyl phthalate and lupeol significantly reduced ($P<0.05$) the number of viable cells but no alteration observed with *Saccharum officinarum* molasses aqueous fraction (SOMAqF) as related to control i.e. cells alone (Figure 4.32; Appendix 30). At 62.5 ug/mL administration SOMMF showed a significant increase ($P<0.05$) in cell proliferation, other samples did not show significant difference (Figure 4.33; Appendix 31).

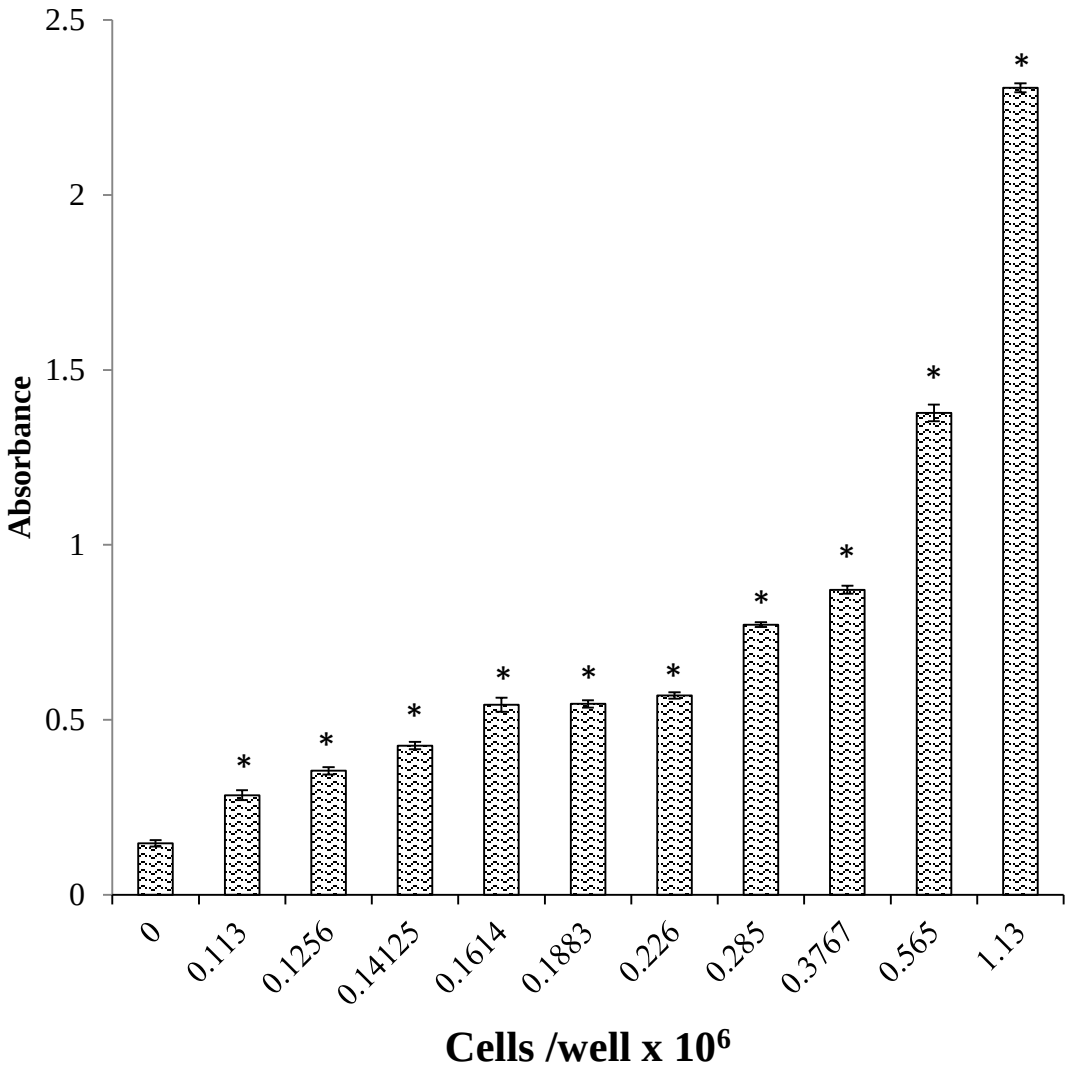

Figure. 4.31 Effects of MTT (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) on cell proliferation by varying number of cells seeded per well after 24 hours of incubation. Columns represents Mean±SEM. Number of replicates (N) =16, *P<0.05 compared with the medium (0).

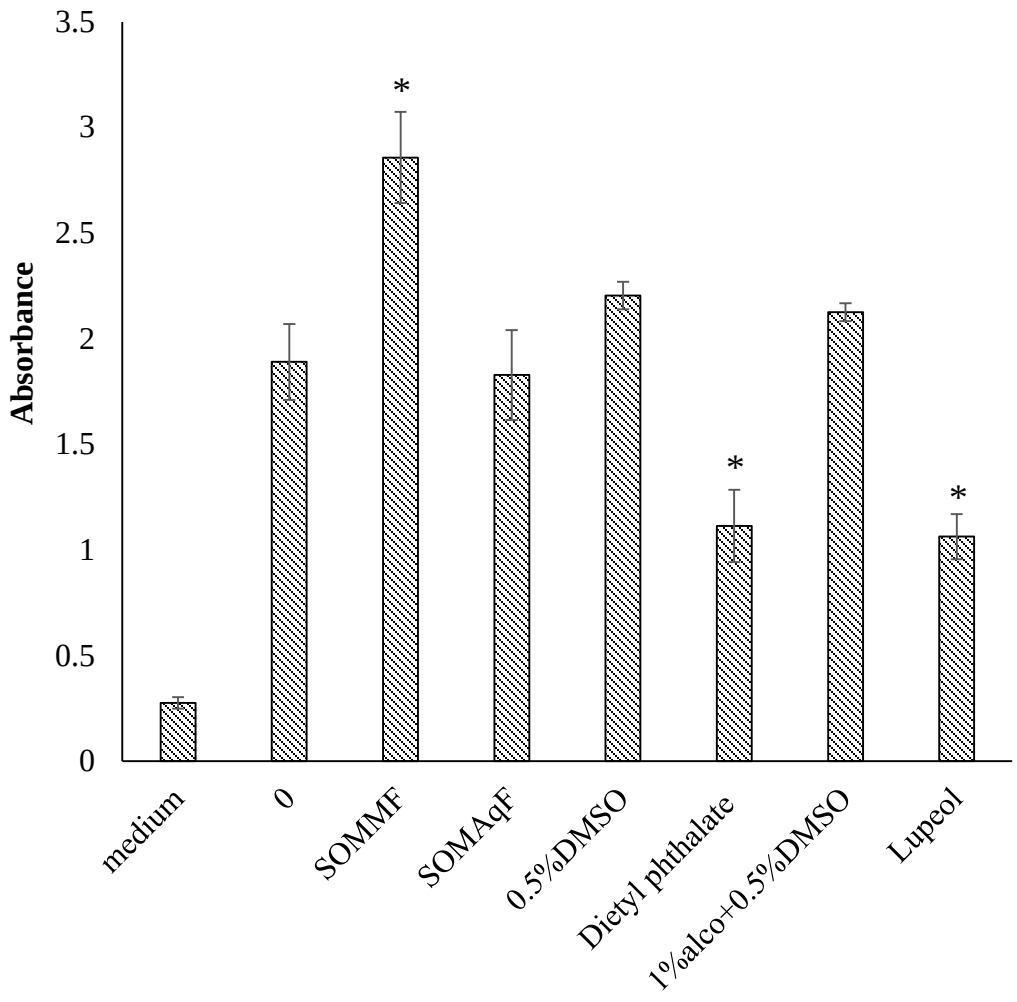

Figure. 4.32 Effects of *Saccharum officinarum* molasses methanol and aqueous fractions, diethyl phthalate and lupeol administration at 250 ug/mL on cell proliferation seeded at 1.26×10^6 cells/mL after 24 hours of incubation. Columns represents Mean±SEM. Number of replicates (N) =8, *P<0.05 compared with the cells alone (control=0).

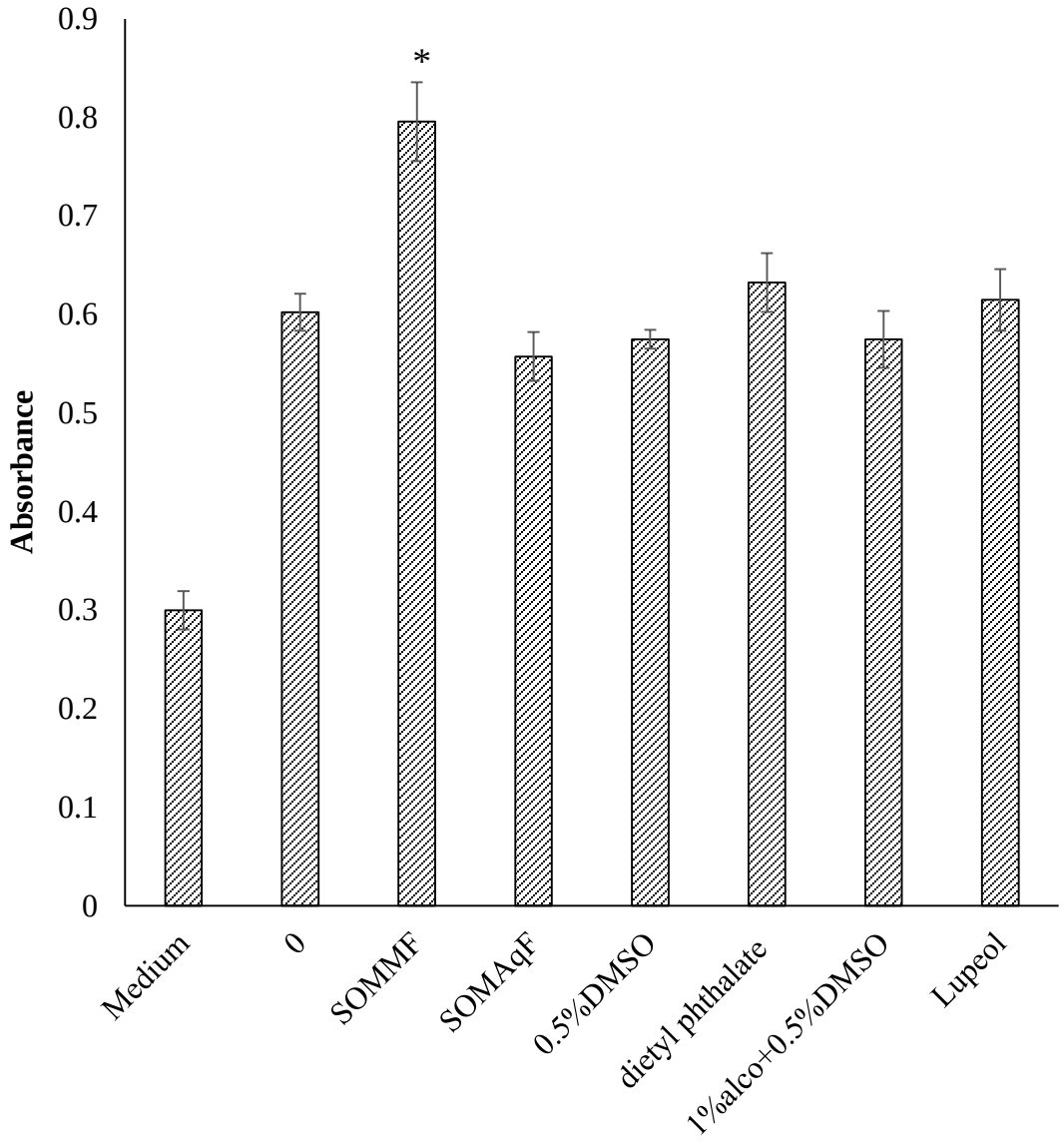

Figure. 4.33 Effect of *Saccharum officinarum* molasses methanol and aqueous fractions, diethyl phthalate and lupeol administration at 62.5 ug/mL on cell proliferation seeded at 315 x 10^3 cells/mL after 24 hours of incubation. Columns represents Mean±SEM Number of replicates N=8, *P<0.05 compared with the cells alone (control =0).

4.30 Effects of varying dilutions of *Saccharum officinarum* molasses methanol (SOMMF) and aqueous fractions (SOMAqF), diethyl phthalate and lupeol on *in vitro* testosterone biosynthesis.

Significant decreases ($P<0.05$) were noted in both LH-stimulated and unstimulated cells that treated with SOMMF dilutions ranging from 62.5-250 ug/mL compared to the control group (Figure 4.34; Appendix 32). Lupeol significantly decreased ($P<0.05$) testosterone biosynthesis of the LH-stimulated cells at dilution ranges of 62.5 and 125 ug/mL dilutions (Figure 4.35; Appendix 33). The SOMAqF and diethyl phthalate both significantly decreased ($P<0.05$) in testosterone biosynthesis of the LH-stimulated cells administered at dilution ranges of 62.5 – 250 ug/mL (Figure 4.36). Diethyl phthalate significantly reduced ($P<0.05$) testosterone biosynthesis of unstimulated cells that treated with 125 ug/mL dilution relative to control (Figure 4.37; Appendix 34 and 35).

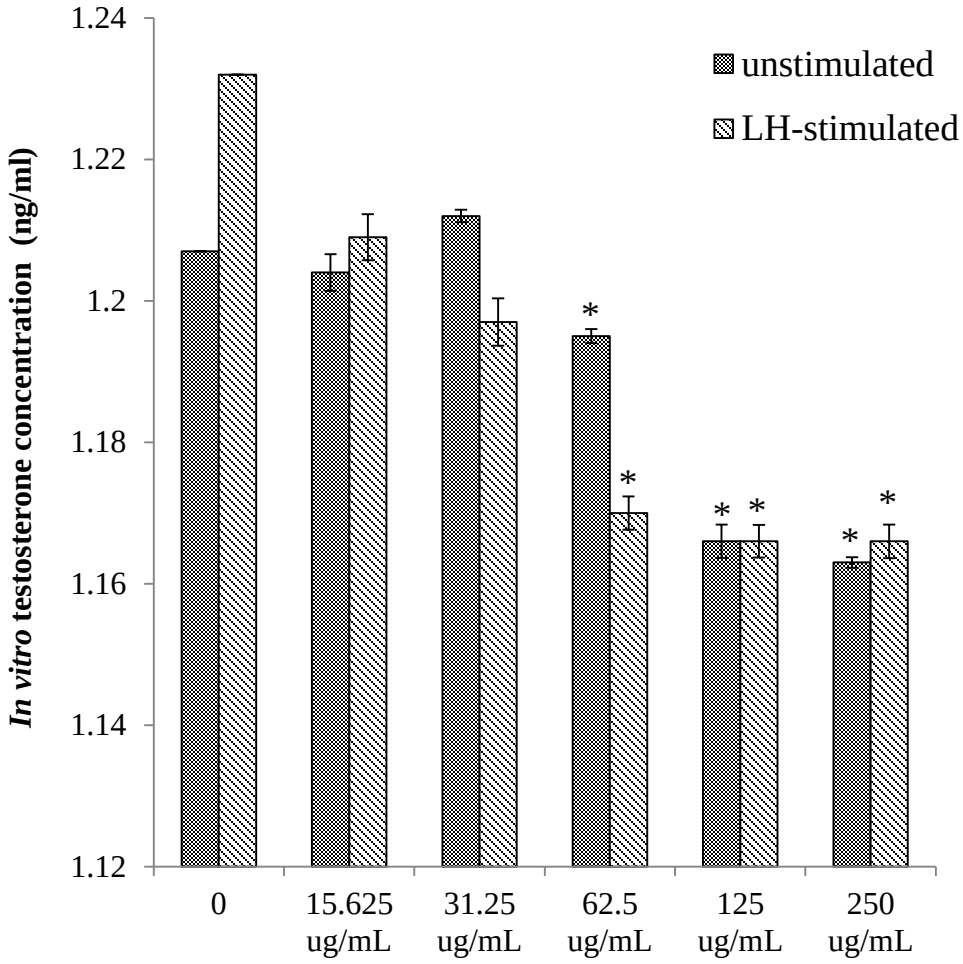

Figure. 4.34 Effects of varying dilutions of *Saccharum officinarum* molasses methanol fraction on *in vitro* testosterone biosynthesis.

Columns represents Mean±SEM. Number of replicates (N) =4, *P<0.05 compared with the medium (0). Unstimulated= Cells not activated with hormone before incubation.

LH-Stimulated= Cells activated with luteintizing hormone enzyme conjugate before incubation.

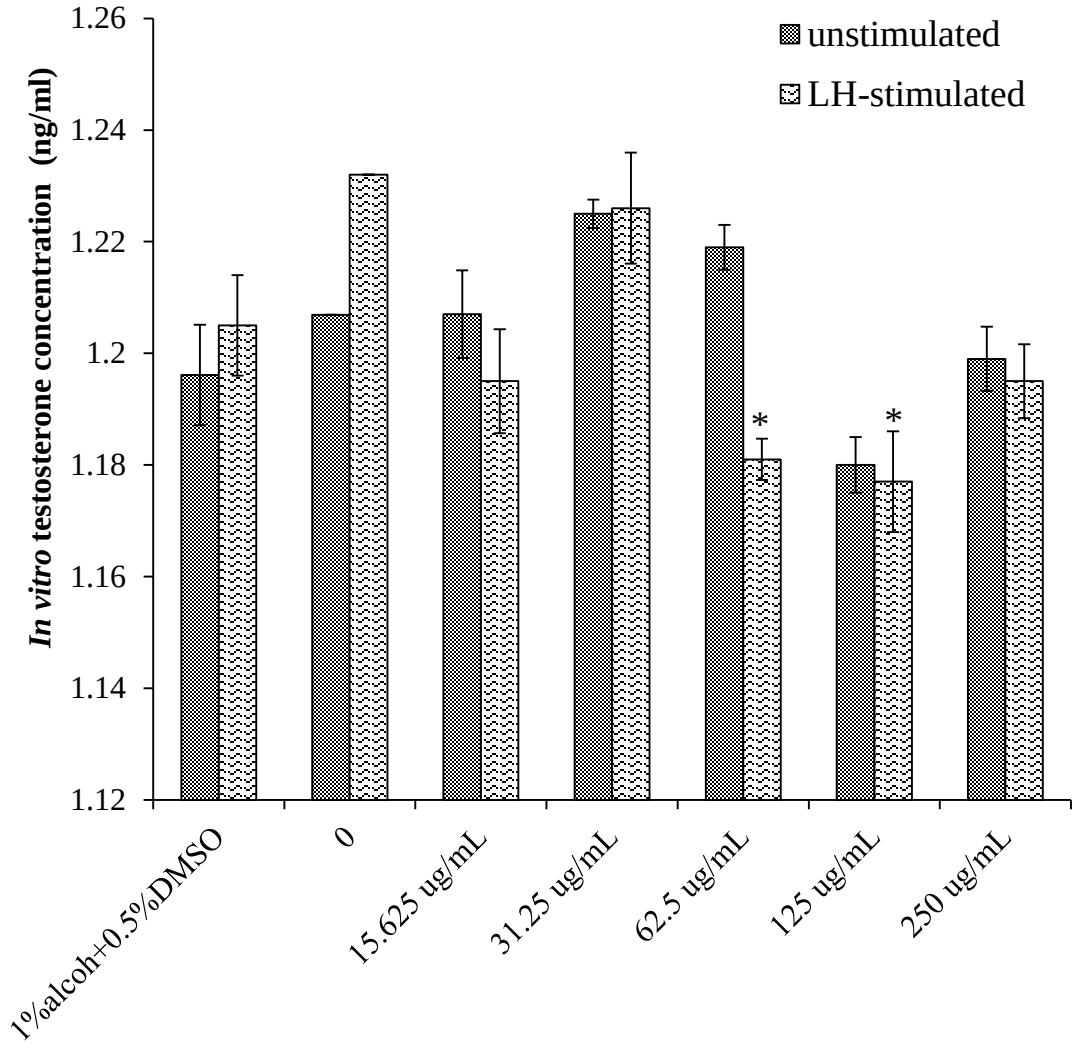

Figure. 4.35 Effects of varying dilutions of Lupeol on *in vitro* testosterone biosynthesis. Columns represents Mean±SEM. Number of replicates (N) =4, *P<0.05 compared with the control (cells alone). 1% alcoh+0.5% DMSO = Vehicle for lupeol.

Unstimulated= Cells not activated with hormone before incubation.

LH-Stimulated=Cells activated with luteintizing hormone enzyme conjugate before incubation.

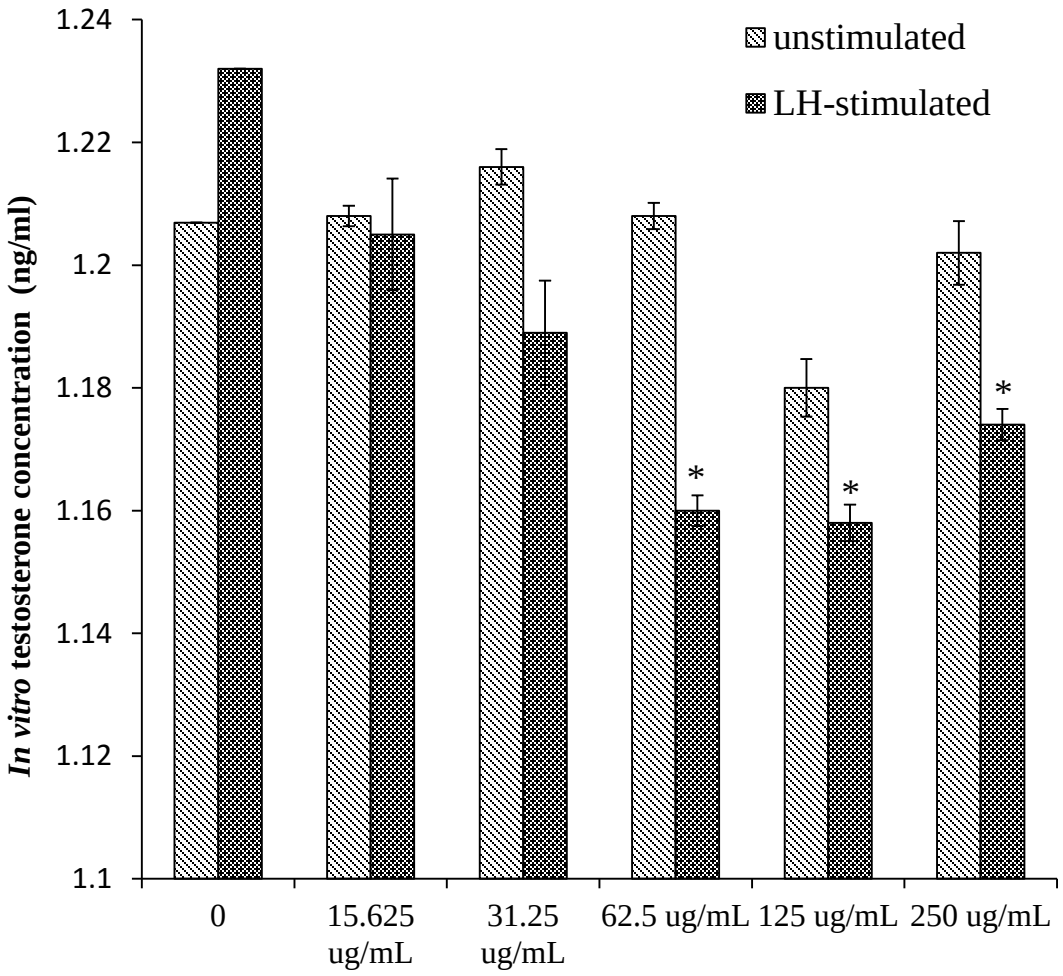

Figure. 4.36 Effects of varying dilutions of SOMAqF on *in vitro* testosterone biosynthesis. Columns represents Mean±SEM. Number of replicates (N) =4, *P<0.05 in comparism with the cells alone (0). Unstimulated= Cells not activated with hormone before incubation. LH-Stimulated=Cells activated with luteinizing hormone enzyme conjugate before incubation.

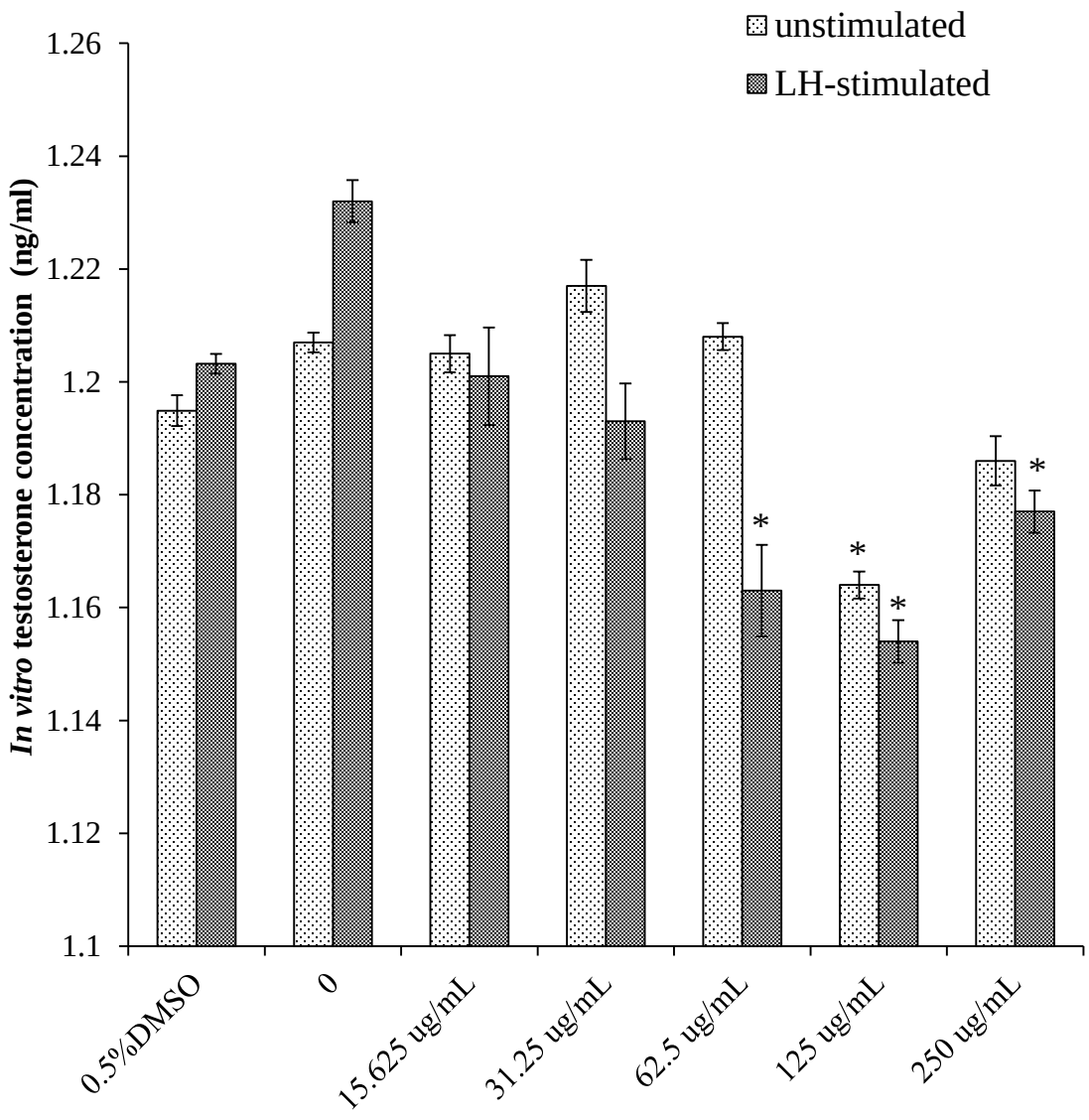

Figure. 4.37 Effects of varying dilutions of Diethyl phthalate on *in vitro* testosterone biosynthesis.

Columns represents Mean±SEM. Number of replicates (N) =4*P<0.05 as related to the cells alone (0), Unstimulated= Cells not activated with hormone before incubation. LH-Stimulated= Cells activated with luteintizing hormone enzyme conjugate before incubation.

4.31 Effects of *Saccharum officinarum* molasses methanol and aqueous fractions, diethyl phthalate and lupeol administration (31.25 ug/ml) on *in vitro* testosterone biosynthesis after 4 hours and 24 hours of incubation.

There were significant decreases ($P<0.05$) in the testosterone biosynthesis of unstimulated cells that treated with the *Saccharum officinarum* molasses aqueous fraction (SOMAqF) and lupeol after 4 hours of incubation, this was insignificant after 24 hours of incubation in all treated groups relative to control (Figure 4.38; Appendix 36). Testosterone biosynthesis of LH-stimulated cells in all treated groups at 4 hours of incubation was insignificant, but after 24 hours a significant reduction ($P<0.05$) was seen in group given Lupeol in relation to control (Figure 4.39; Appendix 37).

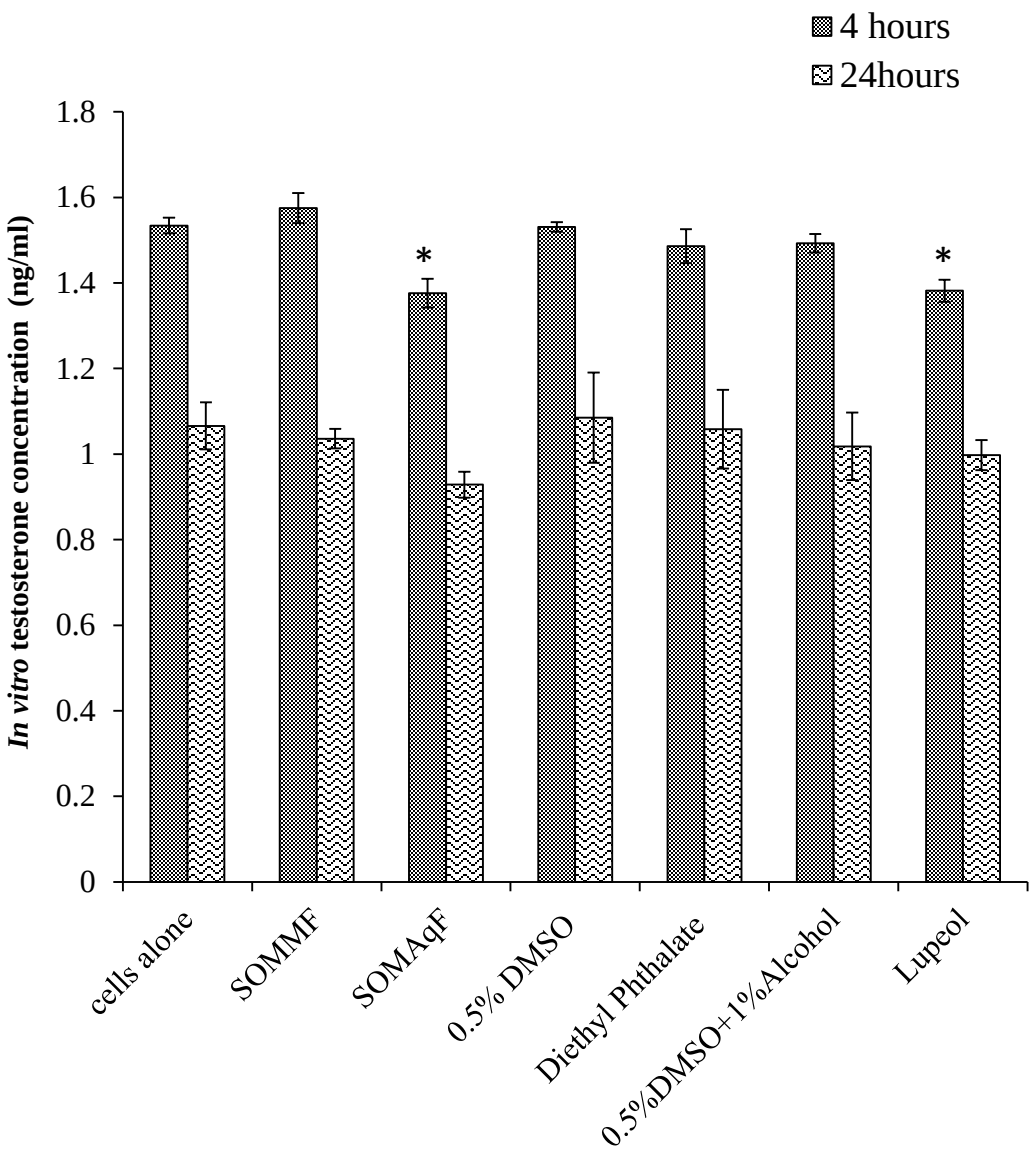

Figure. 4.38 Effect of *Saccharum officinarum* molasses methanol and aqueous fractions, diethyl phthalate and lupeol administration (31.25 ug/ml) on *in vitro* testosterone biosynthesis of unstimulated cells after 4 and 24 hours of incubation. Columns represents Mean±SEM of mean, Number of replicates (N) =4, *P<0.05 compared with cells alone.

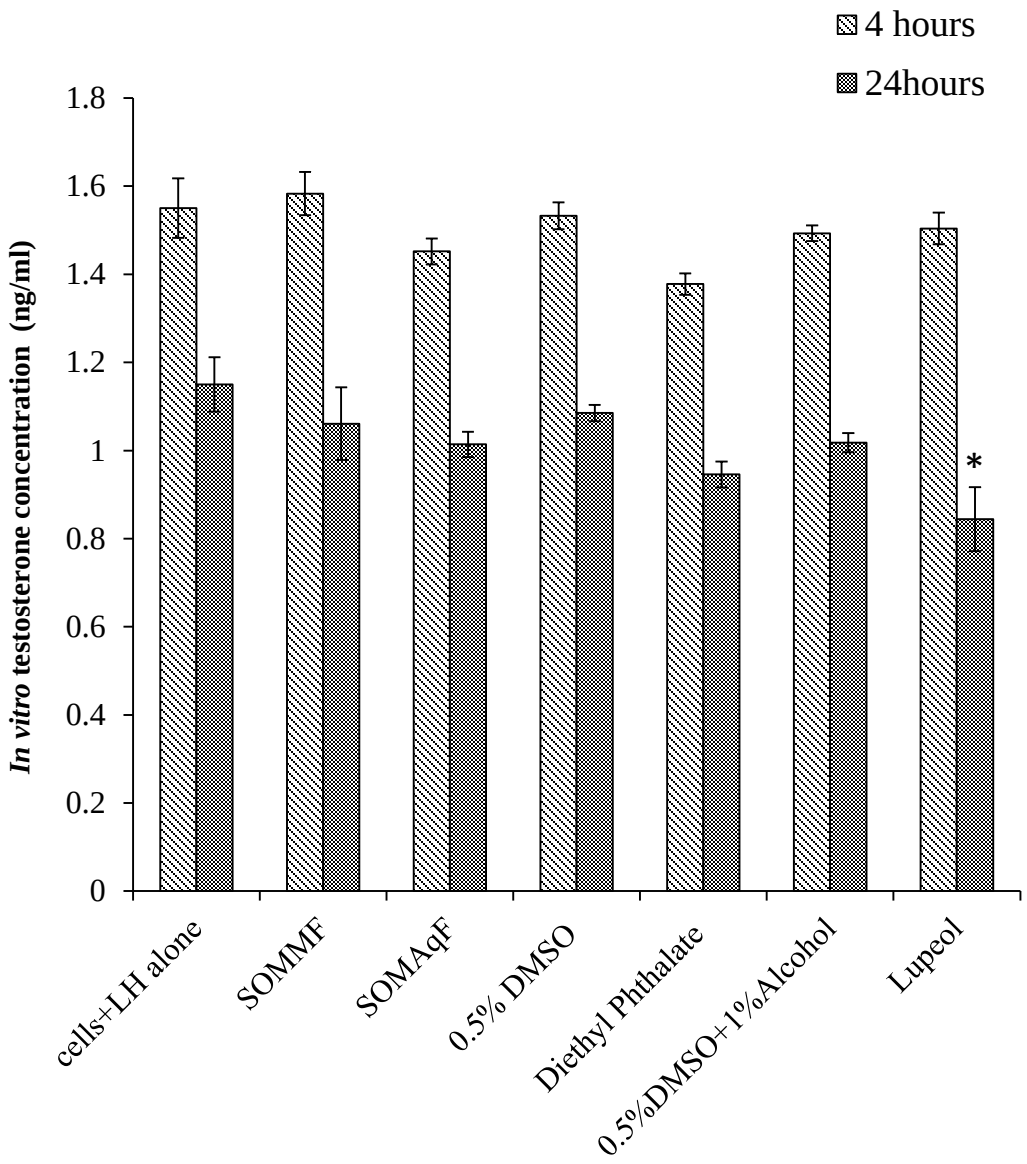

Figure. 4.39 Effect of *Saccharum officinarum* molasses methanol and aqueous fractions, diethyl phthalate and lupeol administration (31.25 ug/ml) on *in vitro* testosterone biosynthesis of LH- stimulated cells after 4 and 24 hours of incubation. Columns represents Mean±SEM of mean, Number of replicates (N)=4, *P<0.05 in comparism with the cells activated with LH enzyme conjugate.

CHAPTER FIVE

5.0 DISCUSSION

5.1. Phytochemical study

The results on chemical constituents presented corroborates the findings of Maurício *et al.*, (2006) and Ashade *et al.* (2014) that *Saccharum officinarum* is a rich source of antioxidants. The polyphenols in *Saccharum officinarum* juice together with vitamins, minerals and other cofactors all functions to decelerate the absorption of its sugars into the bloodstream, giving rise to a low glycemic index (Duke and Wain, 1981). Studies have also shown that the phenolic compounds have a potential analgesic, antimicrobial and anti-inflammatory activities (Shumaik *et al.,* 1998).

The phytochemicals present in the *Saccharum officinarum* molasses methanol fraction (SOMMF) includes flavonoid, saponins, terpenoid, cardiac glycosides and reducing sugars. The *Saccharum officinarum* molasses methanol fraction (SOMAqF) showed presence of saponins and flavonoids. Smith and Paton (1985) reported that flavonoids are the major source of colorants that are basically considered impurities during processing sugar from sugarcane. Flavonoids are known antioxidants and together with Polyphenolic compounds are suggested to have therapeutic capacities for the treatment of a lot of pathologies (Graf *et al.,* 2005) with beneficial effect on male reproductive functions (Ciftci, *et al.,* 2012; Ige and Akhigbe, 2012). Saponins have been suggested to have hypolipidemic effect and it is beneficial for treatment of cardiovascular diseases (Rodrigues *et al.,* 2005), it also has anti-inflammatory and anti-pyretic activity (Adiukwu *et al.,* 2013). Cardiac glycosides and terpenoids are reported to be useful in treating several diseases, including cancer (Evan and Vousden, 2001). Studies show they have anti-microbial, anti-fungal, anti-parasitic, anti-viral, antiallergenic, anti-spasmodic, anti-hyperglycemic, anti-inflammatory and immunomodulatory properties (Wagner and Elmadfa 2003; Rabi and Bishayee, 2009). This study confirms the fact that *saccharum officinarum* can be use in treatment of some

diseases as it contains potent phytochemicals. Reducing sugars contains sucrose, glucose and fructose (Stephen *et al.,* 2012). Previous studies have linked sugars to increased risk of male reproductive functions and cardiovascular mortality (Chang *et al.,* 2001; Chiu *et al.,* 2014).

Gas Chromatography and Mass Spectrometry of SOMMF show presence of two compounds, Lup-20(29)-en-3-ol, acetate, (3. beta) was the abundant constituent and the SOMAqF show presence of nine compounds with Diethyl phthalate being more abundant than other constituents. The report in this study was the first on the GC-MS of this brand of molasses used as a sweetener in human diet. Lup-20(29)-en-3-ol, acetate (Lupeol acetate) is a non-polar compound and a triterpene with antiprotozoal, anti-inflammatory, antitumor and anti-prostate cancer activities among others (Magareth *et al.,* 2009). Gupta *et al.,* (2005) reported that it has potential anti-fertility effects. Diethyl phthalate has being shown to have anti-microbial activity (Premjanu and Jaynthy, 2014). Phthalates have been reported to have carcinogenic, teratogenic, hepatotoxic and endocrine effects (Ahmed, 2013), study reports its negative effects on germ cell development (Heger *et al*., 2012; Johnson *et al.,* 2012).

5.2. Glycemic index, body and organ weight

This study revealed the presence of reducing sugars which are a major source of energy. A precise energy balance is necessary to sustain a steady body weight. *Saccharum officinarum* juice caused significant increases in fasting blood glucose level, organ weight as well as varying increases in the progressive body weight that demonstrates a dependence on the dose level and duration of administration. *Saccharum officinarum* juice (SOJ) is the source of refined sugar which has already been linked with high risk of cardiovascular mortality, insulin resistance and alteration in body weight (Raben *et al.,* 2002). Sweetening improves the taste of food and may result in overconsumption (Drewnowski *et al.,* 1992). This study predicts that overconsumption of SOJ for a prolong period may cause both increases in blood glucose levels and bodyweights that may pose a negative effect on the health status in the long run. On the other hand, the fasting blood glucose level and

reproductive organ weights of all the *Saccharum officinarum* molasses treated rats were insignificantly altered, corroborating Rahiman and Pool (2011) and our previous study on the comparism of the effects of crude *Saccharum officinarum* molasses and Dangote sugar on male Wistar rat reproductive functions with reports that *Saccharum officinarum* molasses may be useful in maintaining body weight as it did not show negative effects on the body. It was further shown that SOM may keep the blood glucose level normal as against Dangote sugar.

5.3. Oxidative status

Increased amount of unsaturated fatty acids and the generation of free radicals as potential reactive oxygen species (ROS) generating systems within the testes endangers it to oxidative stress (Aitken and Roman 2008; Kheradmand *et al.,* 2009). Although these ROS possess harmful effects, a regulated low ROS level is vital in normal testicular functions, which are basically spermatogenesis and steroidogenesis (Mathur and D'Cruz, 2011). *Saccharum officinarum* molasses methanol fraction and aqueous fraction caused significant increases in lipid peroxidation within the testes, this may be due to increased ROS generation. Aitken and Baker, (2006) reported that increase in generation of ROS results in lipid peroxidation and production of cyto-toxic substance such as MDA. It triggers harmful events that can disrupt sperm chromatin integrity and increase the frequency of DNA strand breaks (Aitken and Krausz, 2001).

In order to overcome oxidative stress, the testes consist of an intricate collection of antioxidant enzymes and free radical scavengers which ensures that spermatogenic and steroidogenic functions are unaltered by oxidative stress. These antioxidant defense systems are relevant as peroxidative damage is presently seen as an important cause of impaired testicular function, in support of occurrence of a broad range of diseases such as testicular torsion, diabetes, inflammation, toxicity and endocrine disruption by chemicals in the environs (Aitken and Roman 2008; Kalaiselvi *et al.,* 2014). Although, SOMMF significantly increased the activity of testicular superoxide dismutase (SOD), it did not cause significant differences in activity of catalase and concentration of glutathione (GSH) within the testes. Meanwhile SOMAqF significantly increased SOD and catalase activities

but did not alter glutathione concentration within the testes. Saradha and Mathur, (2006) reported that overexposure to environmental toxicants can impair testicular pro-oxidant/antioxidant balance which will inturn alter function of the testes. The result revealed that there might have been a disparity between pro-oxidant and antioxidant system which induced testicular oxidative stress despite the increases in activities of testicular antioxidants. It is possible that SOM may have caused a disturbance in breakdown of ROS by increasing its generation within the testes, in support of the findings of Desai *et al.* (2009) who stated that whenever ROS production exceeds/overwhelms the scavenging ability of antioxidants, oxidative stress results. Also, the treatment was given for a period of eight weeks, perhaps this may have been a long enough time to cause overexposure of the animals to both SOMMF and SOMAqF which probably caused the impairment of the pro-oxidant/antioxidant balance in the testes.

Within the epididymis, while SOMMF caused significant increases in both MDA and SOD of the same group but showed no significant changes in catalase activity and glutathione concentration, the SOMAqF did not alter MDA and antioxidant activities. The presence of MDA, a stable by-product of lipid peroxidation has been used to quantify lipid peroxidation and it is a popular mechanism of cellular injury in animals (Agarwal and Prabakaran, 2005). The increase observed in this study is suggestive of oxidative stress in relation to the abnormal semen parameters that was observed in accordance with Khosrowbeygi and Zarghami (2007). They stated that increase MDA causes an increase in abnormal semen parameters in humans. Superoxide dismutase, glutathione and catalase are the main oxidant scavenging enzymes in seminal plasma (Tremellen, 2008). Superoxide dismutase acts by catalysing change of superoxide to oxygen and hydrogen peroxide, while catalase breaks down hydrogen peroxide to oxygen and water (Tremellen, 2008). Glutathione peroxidase mops up hydrogen peroxide together with glutathione oxidizing/reducing it (Kefer *et al.,* 2009). From the study, although SOD activity was increased, it is plausible that the quantity and the rate of MDA liberated from lipid peroxidation may have overwhelm the function of SOD, at the same time submerging or masking the activity of catalase and glutathione such that they could not have any significant impact.

Malondialdehyde level in the liver were unaltered significantly relative to control. This infers the possibility that the amount of ROS were low enough and could not cause oxidative stress. The SOMMF and SOMAqF caused significant decreases in SOD activity, glutathione concentration was unaltered but SOMAqF increased catalase activity implying that the antioxidant effect of SOM was observed in the liver.

5.4. Sperm characteristics and Hormonal Profile.

The hypothalamus, anterior pituitary, and testes forms the hypothalamic-pituitary-gonadal (HPG) axis, a finely tuned system that is regulated by a typical negative feedback mechanism. As testosterone level in the blood rises, the anterior pituitary respond less to stimulation by gonadotropin releasing hormone (GnRH), causing reduced secretion of luteinizing hormone (LH) and follicle stimulating hormone (FSH). The prime regulators of germ cell development and sperm production are FSH, LH and testosterone (Bhale and Mahat, 2013). The FSH initiates spermatogenesis by acting directly on the seminiferous tubules via stimulation of mitosis and meiotic DNA synthesis in spermatogonia, whereas LH functions by stimulating spermatogenesis indirectly via testosterone (Anderson *et al.*, 1997).

Saccharum officinarum juice and SOMMF did not change serum levels of FSH and LH in all groups. *Saccharum officinarum* juice significantly increased serum testosterone level but the SOMAqF caused an opposite effect (i.e. a significantly reduced serum testosterone level) in group that received the highest dose. It is possible that the actions of SOJ and SOM may not be through the gonadotropins, but may be directly on the testes. Testosterone is the primary androgen important in development, growth and maintenance of sexual characteristics in males (Tristan and William, 2011). It is produced by interstitial cells, and is needed in large local quantity for maintaining spermatogenesis (O'shauhgnessy *et al.,* 2012). *Saccharum officinarum* juice caused an increased serum testosterone. It is plausible that the actions of SOJ was not through the gonadotropins, but may have been directly on the testes or epididymides. *Saccharum officinarum* molasses on the other hand decreased serum testosterone level, an indication that it may adversely alter the

reproductive functions. *Saccharum officinarum* juice may have the potential to increase libido in older men, since testosterone is a key factor in libido and probably serve as a potential aphrodisiac in the future.

Usually FSH acts on the Sertoli cells to facilitate the last stage of spermatid maturation, after the formation of mature spermatozoa, they are detached from the shielding Sertoli cells of the seminiferous tubule by the process spermiation into the epididymis. At this stage they are non-motile and are expected to gain motility for fertilization to be possible. However, the result of this study showed that SOM significantly increased both the sperm concetration and percentage of abnormal sperms which may have caused the decreased sperm viability that was observe. The SOMMF significantly reduced the concentration, viability and motility of the epididymal spermatozoa on increasing the dose. Concurrently, the SOMAqF significantly reduced the sperm concetration and viability with an increase in percentage of abnormal sperm as the dose was increased. This reduced sperm concentration suggests that there may have been alteration in the process of spermiation from the testes to the epididymis (Esteves, 2015). It is also possible that the insignificant change in FSH may be the reason for the sperm morphology abnormalities noted (detached head, bent/coiled midpiece, curved/coiled tail) and viability, as it revealed that there were alterations in the process of maturation within the epididymis. The non-motile sperm transported to the epididymis should gain motility and become capable of fertilization, but this was not so with SOMMF rather it caused a decrease in motility, whereas SOJ and SOMAqF did not alter sperm motility in all the treated groups. All of these sperm characteristics observed corroborates the findings from our previous study on effect of crude SOM on male reproductive functions of Wistar rats. It is implicit that SOJ and SOM may be a potential causal factor of infertility in males in the long run.

5.5. Histology of Liver, Testes and Epididymis

The SOMMF showed normal liver histology, the central venules and portal tracts appear normal with no congestion or infiltration by inflammatory cells, the sinusoids and hepatocytes show normal morphology, except in the lowest dose of extract that the

sinusoids appear mildly dilated. The SOMAqF caused mild dilation of the sinusoids, perivascular infiltration and infiltrations of the portal tracts and it further showed a focal area of lymphocytes aggregate in the group that received the highest dose. While the perivascular infiltration and lymphocytes aggregate may be due to inflammation of cells such as neutrophils, eosinophils, lymphocytes, plasmacytes, macrophages and mast cells which infiltrates around the blood vessels, the infiltration of inflammatory cells of the portal tracts may be caused by deposits of blood clots or bleeding in the blood vessels (Patrick-Olivier *et al.,* 2011). The presence of inflammatory cells in the hepatic tissue implies that SOM probably contain constituents that could interact with proteins and enzymes of the hepatic interstitial tissue that can interfere with the antioxidant defense mechanism causing the production of ROS that may in turn mimic an inflammatory reaction (Johar *et al.,* 2004). The mild dilation of the sinusiod reported may be caused by perisinusoidal fibrosis nodular regenerative hyperplasia or hepatoportal sclerosis which has been related to xenobiotics (Hillaire *et al.,* 2002).

Saccharum officinarum juice showed intact semineferous tubule in all treated groups but caused a mild vascular congestion in the interstitial spaces of the testes of groups given higher doses of the juice. This congestion may be as a result of intratesticular hemorrhage, which probably occured spontaneously within the interstitium (Evans *et al.,* 1985) contrasting Ashade *et al.,* (2014) that showed a relatively intact histological integrity of the testes on administration of extract of *saccharum officinarum* peel. Sertoli cells are component of the seminiferous epithelium with a pivotal role in testicular homeostasis. They help to make up the blood testis barrier, support and synchronize germ cells as they differentiate and mature (Clermont *et al.,* 1993). The most resistant cells of the testes are Sertoli cells (Stallard and Griswold, 1990), but from this study SOMMF and SOMAqF caused presence of abnormal cells and lack of Sertoli cells in some seminiferous tubules. Richburg and Boekelheide (1996) stated that alterations in Sertoli cells causes severe damage to spermatogenesis. These abnormal sperm cells may be the toxicant effect posed by the administration of SOM in support of Clermont *et al.* (1993), who noted that when Sertoli cells are subjected to toxicant, they manifest a variety of changes in their morphology which may cause a massive death of germ cells that they were meant to sustain.

Previous report has shown that germ cells as they are being nursed by the Sertoli cells concurrently play a feedback role of regulating the actions of the Sertoli cells (O'shauhgnessy *et al.,* 2008). The SOMAqF caused degeneration of germ cells and their layers, this may be the reason for the abnormal Sertoli cells that were formed, in line with Griswold (1995) that death of germ cells can lead to functional and morphological changes of Sertoli cells. It is plausible that the changes noted in the Sertoli cells due to the SOM may be the secondary effects of the germ cell (Stallard and Griswold, 1990). There were lack of spermatogonia, maturation arrest at the primary level with wide and empty lumen in some of the tubules. Saunders *et al.,* (2003) stated that arrest of spermiogenesis may be the reason for the wide and empty lumen shown as matured elongating spermatids were not released into the tubular lumen (Clermont *et al.,* 1993; Saunders *et al.,* 2003). The thickened connective tissues enveloping the tubules and the hyperplasia of the interstitial Leydig cells shown may be a compensatory response to the decreased intratesticular testosterone levels (Ettlin *et al.,* 1992). All these histological observations corroborates report from our previous study in which crude SOM was administered and it revealed that SOM acts directly in the testes and not through the gonadotropins. Thus SOM may be considered as a potential Sertoli cell toxicant.

The epididymal histology showed that both SOJ and SOMMF caused infiltration and aggregation of inflammatory cells in the interstitial spaces. The SOMMF and SOMAqF caused empty epididymal ducts and SOMAqF further caused fibrosis of the interstitial tissues. Infiltration of inflammatory cells are usually seen in interstitial spaces of tissues. Cell junctions between epididymal epithelial cells maintains sequestration of foreign antigens present on spermatozoa from the immune system. Any disruption of the barrier function, frequently due to ischemia of the epididymis, causes inflammation (Creasy, 2001). It is also possible that the inflammatory changes noted in this study may be due to difference in the dynamic equilibrium amid immune tolerance and toxicant facilitated activation of inflammation in the epididymis that was posed by the biological effect of the juice and SOM administered.

Testosterone deprivation causes a reduction in the quantity of normal sperm entering the epididymis. Androgens function in maintaining epididymal outflow of mature spermatids and supports its maturation process (Fan and Robaire, 1998; Foley, 2001). Several activities take place within specific parts of the epididymis and this includes protein synthesis, resorption of fluid, secretion and hormonal modulation (Orgebin-Crist, 1967; O'shauhgnessy *et al.,* 2012). The disruption of these processes or decreased secretion of androgen can pose a negative impact on fertility. The inflammatory cells seen in the interstitial tissues may be from breach of the epididymal tubule caused by increased intraluminal pressure and outburst of sperm into the interstitial spaces, then a responsive inflammation forming a node that encapsulates the sperm (Creasy *et al.,* 2012). The fibrosis observed may be from degeneration caused by ischemia after a rupture of the blood vessels within the interstitial spaces (Honore, 1978).

5.6. Cell proliferation and viability

Assessing cell proliferation and viability is a basis for several *in vitro* assays of a cell population's reaction to external stimuli (Verena *et al.,* 2010). Cellular metabolic activity and cell membrane integrity are characteristics of cell proliferation. One method reliable in assessing cell proliferation or measuring of metabolic activity is incubation of the cells with a tetrazolium salt. This yellow tetrazolium MTT (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) is reduced by metabolically active cells in active mitochondria, partly by actions of dehydrogenase enzymes, to produce reducing equivalents such as Nicotinamide adenine dinucleotide (NADH) and Nicotinamide adenine dinucleotide phosphate (NADPH) (Edmondson *et al.,* 1988). The resulting intracellular purple formazan is solubilized and measured using a spectrophotometer. This Assay measures the cell proliferation rate, on the other hand, it measures the reduction in cell viability, whenever metabolic events lead to apoptosis/necrosis.

In this study, cell proliferation increased in response to increase in number of cells seeded per well after 24 hours of culture, implying that the more the number of cells seeded per well, the higher its proliferation rate per well as it was observed 24 hours after culture in the absence of any external factors. Diethyl phthalate and lupeol are synthetic compounds

that were administered individually in order to compare their effects *in vitro* with that of the two samples (SOMMF and SOMAqF) that were used in the *in vivo* study. Previous result from this study on phytochemicals in SOMMF showed that it contains flavonoids and terpenoids. These constituents are beneficial to the male reproductive functions (Ciftci, *et al.,* 2012; Ige and Akhigbe, 2012). The GC-MS analysis of SOMMF showed that Lup-20(29)-en-3-ol, acetate, (3. beta) was predominant. It is a triterpene and is present in various plants (Connolly and Hill, 2008). The GC-MS of SOMAqF showed diethyl phthalate as its predominant constituent. While the effect exerted on the cells by the SOMMF was compared to that of lupeol, the effect exerted on the cells by the SOMAqF was compared to that of diethyl phthalate. At dilutions of 62.5 ug/mL and 250 ug/mL, SOMMF caused a significant increase in cell proliferation but lupeol caused significant decrease in cell proliferation only at 250 ug/mL. It infers that SOMMF may have increased the amount of metabolically active cells which reduced the yellow tetrazolium to purple formazan leading to a high absorbance.

Studies have shown that the pharmacological activities of lupeol includes antiprotozoal (Srinivasan *et al*., 2002), antimicrobial (Freire *et al.,* 2002) and anti-inflammatory (Bani *et al.,* 2006) among others. The increased proliferation caused by SOMMF reveals that it probably stimulates the activities of all types of cells in the testes as they were cultured together (i.e co-culture). However, this study had earlier shown that this same SOMMF caused an opposite effect *in vivo* which was a depletion of some of these testicular cells and disruption of the process of spermatogenesis as well as steriodogenesis. Hence, SOMMF exerts an adverse effect on testicular cells *in vivo* and an opposite effect *in vitro* which may be beneficial, since the testicular cells in culture still retained their viability as they proliferate.

At 250 ug/mL administration, the SOMAqF did not alter cell proliferation but diethyl phthalate caused a significant decrease in proliferation. The adverse effects posed by these synthetic compounds (diethyl phthalate and lupeol) may be due to the higher doses that were used, which probably led to decreased growth rate resulting in apoptosis or necrosis, whereby a lesser amount of the yellow tetrazolium were converted to formazan resulting

in low amount of metabolically active cells and absorbance value interpreted as decreased cell viability. Previous study has shown that phthalates alter germ cell development (Heger *et al*., 2012: Johnson *et al.,* 2012).

5.6.1. *In vitro* testosterone biosynthesis

Gunnarsson (2008) stated that for the male reproductive tract to grow and properly develop, it requires the presence of testosterone. Biosynthetic pathway is moderated by gonadotropins, steroidal hormones and important enzymes which become the target of various endocrine disruptors (Whitehead and Rice, 2006). Parameters which modifies these regulatory mechanisms/enzymes, can as well alter the synthesis of hormones causing a negative effect on male reproductive function. SOM caused a general decrease in testosterone biosynthesis which was significant at higher concentrations (i.e. 62.5–250 ug/mL) in both the LH-stimulated and unstimulated cells after 4 hours of incubation. It is probable that the action of SOMMF is determined by its duration of incubation with cells as well as dose administered, since higher doses showed significant response within a shorter period of incubation. Also, it decreased the biosynthesis of testosterone in both LH-stimulated and unstimulated cells in culture. This suggests that its actions were both dependent on gonadotropin as well as directly on the testicular cells, since the same effects were observed in the presence or absence of Luteinizing hormone in contrast with Rahiman and Pool (2010) who showed that molasses increased production of testosterone by LH activated cells from testes alone.

At 4 hours of incubation, lupeol caused a significant decrease in testosterone biosynthesis of unstimulated cells. It caused significant decreases at both 4 and 24 hours of incubation in the LH-stimulated cells. This ability of lupeol to alter testosterone level *in vitro* is implicit that its actions are either indirectly (through gonadotropin) or directly on the testicular cells and may not depend on period of incubation with cells but may be dose dependent, since the response observed was significant at higher concentration irrespective of time. This study agrees with Gupta *et al.,* (2005) who showed that lupeol acetate as an active constituent has an antifertility effect in male Wistar rats.

The SOMAqF and diethyl phthalate at higher concentrations caused significant decrease in testosterone biosynthesis in both LH-stimulated and unstimulated cells after 4 hours of incubation, but at 24 hours of incubation no significant difference was observed. They probably exerted time dependent actions through the gonadotropin or directly on the testicular cells (Ahmed, 2013), since there were no significant alterations at a longer period of incubation of the samples with cells. It is also plausible that they both have a short half life, since the GC-MS result show that diethyl phthalate is the abundant constituent present in the SOMAqF and may possess similar chemical characteristics.

5.7. Conclusion

The *in vivo* study revealed that both *Saccharum officinarum* juice and molasses negatively altered the reproductive functions of male Wistar rats. These alterations were implicated more in the testes and epididymis, implying that they both acts directly on the testes and epididymis and not necessarily through the gonadotropins. The *in vitro* study, showed that the actions of *Saccharum officinarum* molasses may likely be through the gonadotropins as well as directly on the testes. This research show that *Saccharum officinarum* molasses possess endocrine disrupting properties with evidence that it has an anti-gonadotropic as well as anti-gonadal effects that altered reproductive functions in males.

5.8 Contribution to Knowledge

To the best of my knowledge, this is the first study to report effects of fresh *Saccharum officinarum* juice on male reproductive functions in experimental rats.

Saccharum officinarum juice caused increase in the fasting blood glucose level, serum testosterone level, organ and body weights, but altered the cytoarchitecture of the testes and epididymis.

Saccharum officinarum molasses exhibited endocrine disrupting activity by reducing the serum testosterone level, altering the epididymal sperm characteristics and impairing the cytoarchitecture of the testes, epididymis and liver. *Saccharum officinarum* molasses further caused lipid peroxidation with imbalance in the proxidant/antioxidant system of testes and epididymis.

Saccharum officinarum molasses showed normal morphology of testicular cells in culture with increase in proliferation, but synthetic lupeol and diethyl phthalate reduced cell proliferation, viability and caused cell necrosis. *Saccharum officinarum* molasses methanol fraction, aqueous fraction, lupeol and diethyl phthalate all caused reduction in *in vitro* testosterone biosynthesis.

This study has shown that *Saccharum officinarum* molasses may have anti-gonadal as well as anti-gonadotropic effects. It is advised that caution should be applied while using it as a substitute sweetener for refined sugar.

5.9 Further studies

To isolate the individual active principles of *Saccharum officinarum* Molasses and investigate its possible mechanism of actions on the functions of male Wistar Rats both *in vitro* and *in vivo*.

To isolate the Sertoli and Leydig cells and investigate the possible mechanism of actions of the individual active principles of *Saccharum officinarum* molasses on their specific functions *in vitro.*

To investigate the effects of *Saccharum officinarum* Molasses and its possible mechanism of actions on reproductive functions in females.

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APPENDICES

1. Effect of *Saccharum officinarum* juice on fasting blood glucose level.

Group	Fasting blood glucose (mmol/l)
Control	102.8±4.214
1 mL/kg/day	111.2±6.358
3.2 mL/kg/day	118.5±2.349*
10 mL/kg/day	124.8±4.895*

Data presented as mean ± SEM. n=5. *P<0.05 compared with control

2. Effect of *Saccharum officinarum* juice on percentage change in body weight.

Group	Percentage body weight change (%)
Control	29.31±0.91
1 mL/kg/day	2.06±0.33*
3.2 mL/kg/day	15.29±0.87*
1 0.0 mL/kg/day	-9.15±0.38*

Data presented as mean ± SEM. n=5. *P<0.05 *P<0.05 compared with control

3. Effect of *Saccharum officinarum* juice on serum Follicle stimulating hormone level.

Group	**Follicle stimulating hormone (mIU/mL)**
Control	5.854±0.6074
1 mL/kg/day	4.824±0.8707
3.2 mL/kg/day	5.082±0.7540
10 mL/kg/day	5.657±0.4847

Data presented as mean ± SEM. n=5.

4. Effect of *Saccharum officinarum* juice on serum Luteinizing hormone level.

Group	**Serum luteinizing hormone (mIU/mL)**
Control	4.0±1.068
1 mL/kg/day	5.646±1.146
3.2 mL/kg/day	5.667±0.9894
10 mL/kg/day	6.396±1.005

Data presented as mean ± SEM. n=5

5. Effect of *Saccharum officinarum* juice on serum testosterone level.

Group	Serum testosterone (ng/mL)
Control	2.468±0.2232
1 mL/kg/day	1.671±0.4331
3.2 mL/kg/day	1.882±0.2788
1mL mL/100 g	3.811±0.4977*#α

Data presented as mean ± SEM. n=5. *P<0.05. $^{\#}$P<0.05 compared with 1.0 mL/kg/day. $^{\alpha}$P<0.05 compared with 3.2 mL/kg/day.

6. Effect of *Saccharum officinarum* juice on sperm concentration.

Group	**Sperm concentration (million/mL)**
Control	24.5±3.233
1 mL/kg/day	37.5±2.110
3.2 mL/kg/day	45.5±7.219
10 mL/kg/day	60.67±3.547*$^{\#\alpha}$

Data presented as mean ± SEM. n=5. *P<0.05. $^{\#}$P<0.05 compared with 1.0 mL/kg/day. $^{\alpha}$P<0.05 compared with 3.2 mL/kg/day.

7. Effect of *Saccharum officinarum* juice on percentage morphological aberrations in epididymal sperm.

Group	Percentage morphological aberrations in epididymal sperm (%)
Control	11.67±1.667
1 mL/kg/day	12.5±1.708
3.2 mL/kg/day	13.83±2.242
10 mL/kg/day	20.17±2.242*$^{\#\alpha}$

Data presented as mean ± SEM. n=5. n=5. *P<0.05 as compared to control. $^{\#}$P<0.05 compared to 1.0 mL/kg/day. $^{\alpha}$P<0.05 compared to 3.2 mL/kg/day.

8. Effect of *Saccharum officinarum* juice on sperm viability.

Group	**Sperm viability (%)**
Control	69.67±4.738
1 mL/kg/day	63.17±5.382
3.2 mL/kg/day	57.17±5.425
10 mL/kg/day	38.5±4.507$^{*\#\alpha}$

Data presented as mean ± SEM. n=5. *P<0.05 compared with the control. $^{\#}$P<0.05 compared with 1 mL/kg/day. $^{\alpha}$P<0.05 compared with 3.2 mL/kg/day.

9. Effect of *Saccharum officinarum* juice on sperm motility.

Group	Sperm motility (%)
Control	58.5±10.36
1 mL/kg/day	40.17±6.295*#
3.2 mL/kg/day	44.5±4.357*#
10 mL/kg/day	61.5±4.507

Data presented as mean ± SEM. n=5. *P<0.05 compared with control. #P<0.05 compared with 1.0 mL/kg/day.

10. Retention time and percentage of total constituents in *Saccharum officinarum* molasses aqueous fraction.

Peak #	R.T. min	first scan	max scan	last scan	PK TY	Peak height	corr. area	corr. % max	% of total
1	13.249	1747	1776	1780	BV 3	295284	1588845	3.52%	1.671%
2	13.445	1780	1810	1932	VV	5365329	451059651	100%	47.428%
3	42.142	6756	6825	6827	PV	23392	1419071	0.31%	0.149%
4	42.818	6928	6944	6945	VV	15788	916853	0.20%	0.096%
5	42.904	6945	6959	6960	PV	38815	621260	0.14%	0.065%
6	42.925	6960	6962	6964	VV 10	28852	245213	0.05%	0.026%
7	43.552	7052	7072	7087	VV 4	127127	7071804	1.57%	0.744%
8	44.005	7132	7151	7183	VV 2	879356	56747969	12.58%	5.976%
9	44.663	7231	7266	7306	VV 6	5867465	417061520	92.46%	43.854%

Sum of corrected areas: 951031684

11. Effect of *Saccharum officinarum* molasses methanol fraction on fasting blood glucose level.

Group	Fasting blood glucose (mmol/l)
Control	113±1.37
1.0 mL/Kg SOMMF	110.2±4.17
3.2 mL/Kg SOMMF	114.2±3.71
10.0 mL/Kg SOMMF	120.8±1.80

Data presented as mean ± SEM. n=5

Saccharum officinarum molasses methanol fraction (SOMMF)

12. Effect of *Saccharum officinarum* molasses methanol fraction on percentage body weight change.

Group	Percentage body weight change (%)
Control	4.32294
1.0 mL/Kg SOMMF	3.45863
3.2 mL/Kg SOMMF	3.96381
10.0 mL/Kg SOMMF	4.32981

Data presented as mean ± SEM. n=5

Saccharum officinarum molasses methanol fraction (SOMMF)

13. Effect of *Saccharum officinarum* molasses methanol fraction on sperm concentration.

Group	Sperm concentration (million/mL)
Control	125.30±7.00
1.0 mL/Kg SOMMF	109.30±5.16
3.2 mL/Kg SOMMF	103.20±9.41*
10.0 mL/Kg SOMMF	92.17±10.22*

Data presented as mean ± SEM. n=5. *P<0.05 relative to control.
Saccharum officinarum molasses methanol fraction (SOMMF)

14. Effect of *Saccharum officinarum* molasses methanol fraction on sperm viability.

Group	**Sperm viability (%)**
Control	91.67±2.01
1.0 mL/Kg SOMMF	86.67±1.67
3.2 mL/Kg SOMMF	79.17±2.39*
10.0 mL/Kg SOMMF	71.33±5.04*$^{\#}$

Data presented as mean ± SEM. n=5. *P<0.05 relative to control. $^{\#}$P<0.05 relative to 1.0 mL/kg/day SOMMF.

Saccharum officinarum molasses methanol fraction (SOMMF)

15. Effect of *Saccharum officinarum* molasses methanol fraction on sperm motility.

Group	**Sperm motility (%)**
Control	89.17±2.39
1.0 mL/Kg SOMMF	90.00±2.58
3.2 mL/Kg SOMMF	80.00±3.42
10.0 mL/Kg SOMMF	69.67±4.6*

Data presented as mean ± SEM. n=5. *P<0.05 as related to control.
Saccharum officinarum molasses methanol fraction (SOMMF)

16. Effect of *Saccharum officinarum* molasses methanol fraction on epididymal volume.

Group	**Epididymal volume (mL)**
Control	0.25±0.02
1.0 mL/Kg SOMMF	0.23±0.04
3.2 mL/Kg SOMMF	0.27±0.02
10.0 mL/Kg SOMMF	0.35±0.03*[#]

Data presented as mean ± SEM. n=5. *P<0.05 relative to the control. [#]P<0.05 relative to 1.0 mL/kg/day SOMMF.

Saccharum officinarum molasses methanol fraction (SOMMF)

17. Effect of *Saccharum officinarum* molasses methanol fraction on abnormal sperm morphology.

Group	**Abnormal sperm morphology (%)**
Control	9±0.73
1.0 mL/Kg SOMMF	9.5±-.43
3.2 mL/Kg SOMMF	10.33±1.05
10.0 mL/Kg SOMMF	11.83±0.79

Data presented as mean ± SEM. n=5.

Saccharum officinarum molasses methanol fraction (SOMMF)

18. Effect of *Saccharum officinarum* molasses methanol fraction on serum follicle stimulating hormone (FSH) and luteinizing hormone (LH) level.

Group	FSH (mIU/mL)	LH (mIU/mL)
Control	8.422±0.76	4.213±0.75
1 ml/kg/day SOMMF	6.644±0.99	3.067±0.72
3.2 mL/kg/day SOMMF	8.474±1.63	3.32±0.71
10.0 ml/kg/day SOMMF	8.929±0.77	3.867±0.77

Data presented as mean ± SEM. n=5

Saccharum officinarum molasses methanol fraction (SOMMF)

19. Effect of *Saccharum officinarum* molasses methanol fraction on serum testosterone level.

Group	Serum testosterone (ng/mL)
Control	1.909±0.04
1.0 ml/kg/day SOMMF	1.722±0.16
3.2 mL/kg/day SOMMF	1.936±0.02
10.0 ml/kg/day SOMMF	1.784±0.08

Data presented as mean ± SEM. n=5

Saccharum officinarum molasses methanol fraction (SOMMF)

20. Effect of *Saccharum officinarum* molasses aqueous fraction on fasting blood glucose level.

Group	Fasting Blood Glucose Level (mmol/l)
Control	113±1.37
1.0 mL/Kg SOMAqF	114.7±2.20
3.2 mL/Kg SOMAqF	114.7±3.33
10.0 mL/Kg SOMAqF	114.7±2.46

Data presented as mean ± SEM. n=5

Saccharum officinarum molasses aqueous fraction (SOMAqF)

21. Effect of *Saccharum officinarum* molasses aqueous fraction on percentage body weight change.

Group	Percentage body weight change (%)
Control	4.32±0.23
1.0 mL/Kg SOMAqF	3.64±0.25
3.2 mL/Kg SOMAqF	0.37±0.19 $^{*\#\alpha}$
10.0 mL/Kg SOMAqF	2.69±0.13

Data presented as mean ± SEM. n=5. *P<0.05 compared with control. $^{\#}$P<0.05 compared with 1.0 mL/kg/day SOMAqF. $^{\alpha}$P<0.05 compared 10.0 mL/kg/day.
Saccharum officinarum molasses aqueous fraction (SOMAqF)

22. Effect of *Saccharum officinarum* molasses aqueous fraction on sperm concentration.

Group	Sperm concentration (million/mL)
Control	125.3±6.70
0.6 g/Kg SOMAqF	107.0±7.49
2.0 g/Kg SOMAqF	97.7±5.56*
6.4 g/Kg SOMAqF	86.5±3.03*

Data presented as mean ± SEM. n=5. *P<0.05 relative to control.
Saccharum officinarum molasses aqueous fraction (SOMAqF)

23. Effect of *Saccharum officinarum* molasses aqueous fraction on sperm viability.

Group	Sperm viability (%)
Control	91.67±2.01
0.6 g/Kg SOMAqF	85.83±2.01
2.0 g/Kg SOMAqF	78.33±2.11*
6.4 g/Kg SOMAqF	74.17±3.01*

Data presented as mean ± SEM. n=5. *P<0.05 as related to the control.
Saccharum officinarum molasses fraction (SOMAqF)

24. Effect of *Saccharum officinarum* molasses aqueous fraction on sperm motility.

Group	Sperm motility (%)
Control	89.2±2.40
0.6 g/Kg SOMAqF	87.5±2.50
2.0 g/Kg SOMAqF	86.5±2.75
6.4 g/Kg SOMAqF	80.0±2.89

Data presented as mean ± SEM. n=5.

Saccharum officinarum molasses aqueous fraction (SOMAqF)

25. Effect of *Saccharum officinarum* molasses on epididymal volume

Group	Epididymal volume (%)
Control	0.25±0.02
0.6 g/Kg SOMAqF	0.30±0.04
2.0 g/Kg SOMAqF	0.33±0.03
6.4 g/Kg SOMAqF	0.38±0.02*

Data presented as Mean±SEM. n=5. *P<0.05 relative to the control.
Saccharum officinarum molasses aqueous fraction (SOMAqF)

26. Effect of *Saccharum officinarum* molasses on abnormal sperm morphology.

Group	Abnormal sperm morphology (%)
Control	9.00±0.73
0.6 g/Kg SOMAqF	9.83±0.7
2.0 g/Kg SOMAqF	13.08±1.34 *
6.4 g/Kg SOMAqF	14.17±1.52 *$^{\alpha}$

Data presented as mean ± SEM. n=5. *P<0.05 relative to the control

$^{\alpha}$P<0.05 relative to 0.6 g/kg/day SOMAqF.

Saccharum officinarum molasses aqueous fraction (SOMAqF)

27. Effect of *Saccharum officinarum* molasses aqueous fraction on serum follicle stimulating hormone and Luteinizing level.

	Serum hormone concentration (mIU/ml)	
Group	**Follicle stimulating hormone**	**Luteinizing level**
Control	8.422±0.76	4.213±0.75
0.6 g/Kg SOMAqF	6.616±0.52	2.773±0.82
2.0 g/Kg SOMAqF	8.454±0.61	4.153±0.60
6.4 g/Kg SOMAqF	8.16±1.11	3.747±0.60

Data presented as mean ± SEM. n=5. *P<0.05 relative to the control.
Saccharum officinarum molasses aqueous fraction (SOMAqF)

28. Effect of *Saccharum officinarum* molasses aqueous fraction on serum Testosterone level.

Group	**Testosterone (ng/ml)**
Control	1.91±0.04
0.6 g/Kg SOMAqF	1.90±0.01
2.0 g/Kg SOMAqF	1.86±0.04
6.4 g/Kg SOMAqF	1.66±0.10*[#]

Data presented as mean ± SEM. n=5. *P<0.05 relative to the control.

[#]P<0.05 relative to 0.6 g/kg/day SOMAqF.

Saccharum officinarum molasses aqueous fraction (SOMAqF)

29. Determination of viable and proliferating cells using MTT cell Proliferation assay.

Cells/well	**Absorbance**
0	0.1475±0.0091
113000	0.2848±0.0140*
125600	0.3545±0.0103*
141250	0.4265±0.0108*
161400	0.543±0.01998*
188300	0.5456±0.0107*
226000	0.5696±0.0089*
285000	0.7721±0.0070*
376700	0.8715±0.0115*
565000	1.377±0.0234*
1130000	2.306±0.0131*

Data presented as mean ± SEM. Number of replicates (N) = 16, *P<0.05

30. Effects of sample administration at 250ug/mL on viable and proliferating cells seeded at 1.26 X 10^6 cells/mL after 24 hours of incubation.

Sample	Absorbance
Medium	0.275±0.0275
Cells	1.89±0.1801
Cells+SOMMF	2.858±0.2664*
Cells+SOMAqF	1.828±0.2125
Cells+0.5% DMSO	2.205±0.06513
Cells+Diethyl Phthalate	1.113±0.1711*
Cells+1% Alcohol+0.5% DMSO	2.125±0.04252
Cells+Lupeol	1.063±0.1069*

Data presented as mean ± SEM. Number of replicates (N) = 8 *P<0.05

31. Effects of sample administration at 62.5ug/mL on viable and proliferating cells seeded at 315 X 10^3 cells/mL after 24 hours of incubation.

Sample	**Absorbance**
Medium	0.3±0.0196
Cells	0.6025±0.0189
Cells+SOMMF	0.7958±0.0400*
Cells+SOMAqF	0.5575±0.0048
Cells+0.5% DMSO	0.575±0.0096
Cells+Diethyl Phthalate	0.6325±0.0298
Cells+1% Alcohol+0.5% DMSO	0.575±0.0029
Cells+Lupeol	0.615±0.0312

Data presented as mean ± SEM. Number of replicates (N) = 8 *P<0.05

32. Effects of varying dilutions of *Saccharum officinarum* molasses methanol fraction on *in vitro* testosterone biosynthesis of testicular cells seeded at 5.8 X 10^6 cells/mL after 4 hours of incubation.

Sample	**Unstimulated cells**	**LH-Stiumulated cells**
Cells alone	1.207±0.0	1.232±0.0
15.625ug/mL	1.204±0.0026	1.20±0.0063
31.25ug/mL	1.212±0.0009	1.197±0.004
62.5ug/mL	1.195±0.001*	1.17±0.0004*
125ug/mL	1.166±0.0024*	1.66±0.0003*
250ug/mL	1.163±0.0008*	1.166±0.0038*

Data presented as mean ± SEM. Number of replicates (N) = 4 *P<0.05

33. Effects of varying dilutions of Lupeol on *in vitro* testosterone biosynthesis of testicular cells seeded at 5.8 X 10^6 cells/mL after 4 hours of incubation.

Sample	**Unstimulated cells**	**LH-Stiumulated cells**
1%alcohol+0.5% DMSO	1.1961±0.009	1.205±0.009
Cells alone	1.2069±0.0	1.232±0.0
15.625ug/mL	1.207±0.0079	1.195±0.0153
31.25ug/mL	1.225±0.0026	1.226±0.0100
62.5ug/mL	1.219±0.0002	1.181±0.0037*
125ug/mL	1.18±0.0001	1.177±0.0011*
250ug/mL	1.199±0.0058	1.195±0.0066

Data presented as mean ± SEM. Number of replicates (N) = 4 *P<0.05

34. Effects of varying dilutions of SOMAqF on *in vitro* testosterone biosynthesis of testicular cells seeded at 5.8 X 10^6 cells/mL after 4 hours of incubation.

Sample	**Unstimulated cells**	**LH-Stiumulated cells**
Cells alone	1.2069±0.0	1.232±0.005
15.625ug/mL	1.208±0.0017	1.205±0.009
31.25ug/mL	1.216±0.0029	1.189±0.008
62.5ug/mL	1.208±0.0022	1.16±0.0003*
125ug/mL	1.18±0.0047	1.158±0.003*
250ug/mL	1.202±0.0119	1.174±0.003*

Data presented as mean ± SEM. Number of replicates (N) = 4 *P<0.05

35. Effects of varying dilutions of Diethyl phthalate on *in vitro* testosterone biosynthesis of testicular cells seeded at 5.8 X 10^6 cells/mL after 4 hours of incubation.

Sample	**Unstimulated cells**	**LH-Stiumulated cells**
0.5 DMSO	1.1949±0.0028	1.2032±0.0018
Cells alone	1.207±0.0018	1.232±0.0038
15.625ug/mL	1.205±0.0033	1.201±0.0087
31.25ug/mL	1.217±0.0046	1.193±0.0067
62.5ug/mL	1.208±0.0009	1.163±0.0081*
125ug/mL	1.167±0.0141*	1.154±0.0038*
250ug/mL	1.186±0.0194	1.177±0.0038*

Data presented as mean ± SEM. Number of replicates (N) = 4 *P<0.05

36. Effects of sample administration (62.5ug/mL) on *in vitro* testosterone biosynthesis level of unstimulated cells seeded at 6.78 X 10^6 cells/mL after 4 hours and 24 hours of incubation.

Sample	**4 hours**	**24 hours**
Cells alone	1.534±0.0183	1.066±0.0552
SOMMF	1.575±0.0352	1.035±0.0228
SOMAqF	1.376±0.0335*	0.9286±0.0303
0.5% DMSO	1.531±0.0110	1.085±0.1057
Diethyl Phthalate	1.468±0.0400	1.058±0.0921
0.5%+DMSO+1% Alcohol	1.493±0.0217	1.018±0.0079
Lupeol	1.382±0.02577*	0.9977±0.0350

Data presented as mean ± SEM. Number of replicates (N) = 4 *P<0.05.

37. Effect of sample administration on *in vitro* testosterone biosynthesis of LH-stimulated cells seeded at 6.78 X 10^6 cells/mL after 4 and 24 hours of incubation.

Sample	4 hours	24 hours
Cells alone	1.55±0.0976	1.15±0.0117
SOMMF	1.583±0.0191	1.061±0.0828
SOMAqF	1.452±0.0295	1.014±0.0286
0.5% DMSO	1.533±0.1058	1.085±0.0087
Diethyl Phthalate	1.378±0.0243	0.947±0.0292
0.5%+DMSO+1% Alcohol	1.498±0.0079	1.018±0.0217
Lupeol	1.504±0.0362	0.8445±0.0726*

Data presented as mean ± SEM. Number of replicates (N) = 4 *P<0.05