EFFECTS OF *MONDIA WHITEI (MUKOMBERO)* ON LUTEINIZING HORMONE, FOLLICLE STIMULATING HORMONE, TESTOSTERONE, SPERM CHARACTERISTICS AND TESTICULAR TISSUE IN MALE WISTER ALBINO RATS

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Effects of *Mondia Whitei (Mukombero)* on Luteinizing Hormone, Follicle Stimulating Hormone, Testosterone, Sperm Characteristics and Testicular Tissue in male Wister Albino Rats

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A thesis submitted in partial fulfilment for the award of the degree of Master of Science in Medical Physiology in the Jomo Kenyatta University of Agriculture and Technology

2020

DECLARATION

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This thesis is my original work and has not been presented for a degree in any other university.

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DEDICATION

I dedicate this work to my family for their unwavering support and encouragement even when I was almost giving up and their sacrifices when I was unable to effectively meet my duties as a son, brother, student, employee, father and husband as I struggled to meet my employment, academic, family and financial responsibilities.

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DEFINITION OF TERMS

Testosterone A sex steroid hormone produced by leydig cells of the testes that is essential for male secondary sexual characteristics and reproduction.

Release–inhibiting hormone A hormone secreted by neuroendocrine cells in the hypothalamus that travels to the anterior pituitary through the hypothalamo – hypophysial portal system and inhibits secretion of a hormone by a specific population of anterior pituitary endocrine cells.

Luteinizing hormone: A hormone produced from the anterior pituitary gland responsible for ovulation in females. In males it is known as the interstitial cell stimulating hormone responsible for production of testosterone

Gonadotropin A hormone that stimulates the gonads (testes) to produce gametes and hormones and also supports and maintains gonadal tissue.

Follicle stimulating hormone A glycoprotein polypeptide hormthat regulates the maturation of germ cells.

Reactive Oxygen Species (ROS) Are highly reactive oxidizing agents which are diffusible molecules that belong to the class of free radicals and peroxides that are generated in cells as by-product of aerobic respiration and metabolism.

ABBREVIATIONS AND ACRONYMS

ANOVA	One way analysis of variance
ART	Artificial reproduction
B .W	Body weight
CASA	Computer-aided sperm/semen analysis
COHES	College of Health Sciences
DNA	Deoxy ribonucleic acid
ECLIA	Electrochemilumin escence immunoassay
ELISA	Enzyme Linked Immunosobent Assay
FSH	Follicle stimulating hormone
GnRH	Gonadotropin releasing hormone
JKUAT	Jomo Kenyatta University of Agriculture and Technology
LH	Luteinizing hormone
KCL	Potassium chloride
NACOSTI	National commission for Science, Technology and Innovation
ICI	Intracytoplasmic injection
SA	Semen analysis
IQR	Interquatile range
LSD	Least significant difference
SPSS	**Stastical package for social science**
IVF	In vitro fertilization
UOE	University of Eldoret
W H O	World Health Organization

ABSTRACT

Infertility affects about 8 to 12% of the world's population and in about half of cases men are either the single cause or contribute to the couple's infertility. Many indigenous plants have been reported to be effective in male fertility regulation. *Mondia whitei* is a widely used medicinal plant across Africa for treatment of sexual dysfunction yet minimal and often conflicting empirical data exists to support its therapeutic value. This study aimed at evaluating the effects of aqueous extract of *Mondia whitei* on reproductive hormone levels, sperm characteristics and gonadal histological changes in male albino rats following oral administration. Thirty six albino male rats weighing between 200mg-400mg were divided into 4 groups, each of nine rats. Group I comprised untreated controls while Groups II, III, and IV were treated with 100, 200 and 400mg/kg body weight respectively, using the aqueous extract of *Mondia whitei* via oral gavage. At the end of experiment, rats were humanely sacrificed using Carbon dioxide narcosis, and blood samples collected through a cardiac puncture and testes and epididymis dissected for sperm collection and histology. Serum levels for testosterone, LH & FSH were determined using immunoanyalzer. Sperm count, total motility, vitality and morphology were determined microscopically using a neubaeur chamber. Data analysis was undertaken using Statistical Package for Social Sciences (SPSS) -Version 21.0). Median (Interquartile range-IQR) and Kruskal Wallis test were employed in the analysis and $p\text{-value} < 0.05$ was considered statistically significant. The serum testosterone, FSH and LH concentration levels progressively decreased with time in all the three groups of rats treated with the extract (exposed) as compared with the controls which remained constant. However there was a rise in testosterone on first 10 days with low dose of 100mg/kg.The Kruskal wallis test indicated that the difference between and within the groups was statistically significant (all $p < 0.05$). The sperm count, motility, vitality concentration levels progressively decreased with time in all the three groups of rats treated with the extract (exposed) while the controls remained constant. The Kruska Wallis test indicated that the difference within and between groups was statistically significant except on the motility and vitality. Normal morphology percentage declined in different test groups as compared to the control groups ($p=0.027$). Trend analysis indicated that within the groups, normal morphology decreased significantly with time (all $p<0.05$) while abnormal head morphology and tail increased with time $p=0.05$. The gonadal tissue from the treated groups exhibited low sertoli cells in the seminiferous tubules and a thin germinal epithelium. There was degeneration of leydig cells within the interstitium and clamping of spermatozoa in the epididymis in relation to increase in extract concentration and duration. This study concludes that *Mondia whitei* may be beneficial only at low dose and short duration but may alter male fertility if taken over a long time and also high doses. This shows that *M whitei* might be cytotoxic and can result in hypogonadotrophic hypogonadism and oligoasthenoteratozoospermia.

CHAPTER ONE
INTRODUCTION

1.1Background

Infertility is an imperative component of reproductive health, and has often been omitted in many reproductive discourses (Cui, W. 2010). Infertility refers to a situation where a couple fails to achieve a pregnancy during the fertile phase of the menstrual cycle even after having regular and unprotected sexual intercourse for one year (Nieschlag *et al.,* 2000; Evers, 2002). According to the report by World Health Organization (WHO, 2015) the annual number of infertile couples globally was found to be 50-80 million. Previous studies had revealed that infertility has affected about 15% of the couples who had sought for clinical treatment in order to have children in Africa (Feng, 2003; Roberts, 1998). In Kenya its estimated that infertility affects 26 % of the couples who attend outpatient services (M'imunya et al 2006). The Male factor contributes to half of infertility cases worldwide (Agarwal *et al.* 2014).

The inability to have children impacts men and women throughout the globe. Infertility can lead to misery and depression, as well as discrimination and ostracism (Chachamovich *et. al* 2010). Human male fertility is a vital issue based totally at the inability of spermatozoa to fertilize and prompt the egg to assist early embryonic existence. However, it's been considered lower in most couples (Jørgensen *et al.,* 2001), with increasing infertility rates in many countries affecting one in six couples (Sharpe *et al.,* 2003; Kamel, 2010).
The issue of male infertility is multi-factorial with some suffering from low fertility in spite of having adequate numbers of sperm with normal morphology and motility. Inadequate knowledge on multi-factorial causes of male infertility poses a challenge on the rational approach towards development of effective therapies (Wu *et al.,* 1989).

Evidence shows that many couples seek medical help to solve infertility issue (Ikechebelu *et al.,* 2003). Medical evidence indicates that around 80 % of Africans

rely on conventional healthcare practitioners however others seek alternative medicinal flowers for their daily healthcare needs (Johnson, ., 2007; McKay and Blumberg, 2007).The natural products have reduced ache, suffering and revolutionized practice of medicine. In regard of this, more than 60% of approved and pre-new drug utility applicants are either natural products or associated with them in view of solving infertility problems (Demain, 1999).
Studies on conventional medicinal plants have suggested that their potential to improve male fertility is partially because of presence of antioxidants, which appear to enhance several processes/stages of steroidogenesis & spermatogenesis (Nantia *et al.,* 2009). A number of plant formulations have been reported to treat idiopathic infertility (Agrawal and Kulkarni, 2003; Rama Devi *et al.,* 2004; Tempest *et al.,* 2005; Xu *et al.,* 2003), and one of the commonest traditional plant formulation in Africa and Kenya that has been used to manage fertility is *Mondia whitei* also known as “Mukombero” among Luyha dialects in Kenya (Gabriel et al 2013).

The genus Mondia of the Apocynaceae family is a woody, robust and vigorous aromatic perennial plant that grows from a large tuberous root stock. It has large heart-shaped opposite leaves and produces reddish, purple flowers borne in branched inflorescences (Aremu *et al.,* 2011). The most popular compound that is isolated from *M. whitei* is 2-hydroxy-4-methoxybenzaldehyde which is a potent tyrosinase inhibitor and an isomer of vanillin (Kubo and Kinst-Hori, 1999b). Other researchers have also isolated this compound from *M. whitei* (Oketch-Rabah, 2012) and Koorbanally *et al.* (2000) isolated isovanillin. Nutritional analysis indicated that Mondia is rich in minerals and vitamins (Iwu, 2014). Phytochemical qualitative analysis of the ethanoic extract of *M. whitei* shows the presence of reducing sugars and triterpenes (Quasie e*t al.*, 2010). It is on the premise of these secondary metabolites that people have used Mondia whitei as an aphrodisiac and a fertility drug.

However, there is not only inadequate research but also conflicting studies that have been done on the effects of *Mondia Whitei* on the hypothalamo-pituitary-gonadal axis to support it’s wide spread use as a fertility drug. The aim of this study was

therefore to determine the effects of *Mondia Whitei* on male fertility using male albino rats.

1.2 Statement of the Problem

About 8% to 12% of the world's population is affected by infertility and in about half of the cases, men are found to be the single cause that contributes to infertility in couples (Cates *et al.,* 1985).It is estimated that 80 million couples in the world suffer from infertility and Africa especially sub Saharan Africa contributes to 20 million (Evers *et al, 2002*). In Kenya the National infertility survey of 2005/2006, showed that infertility is a major reproductive health concern. According to the survey, consultations on matters related to infertility were: 30% (teaching and referral hospital), 27% (provincial hospitals), 15% (district hospitals, 4% (health centres) and 2% (dispensaries), (M'imunya *et al,* 2006). This data clearly shows that infertility is a major issue affecting a considerable number of Kenyans. Earlier studies had similar findings (Gachera *et al* (1984) and Mati *et al* (1987).

The World Health Organization (1999) report shows that male factor contributes to 40% of infertility cases, female factor contributes to 40% while both male and female factors contribute to 15% and 5% are contributed by unknown factors. Infertility has serious social, psychological, economic and medical implications resulting in trauma, stress especially in African society where strong emphasis is placed on childbearing (Otwori CO, 2013).

A lot of potential ways for fertility have been investigated since ancient times, among them being chemical, hormonal and immunological advances. However, there are limited viable options that are effective, less costly and free from negative ramifications.

Anecdotal evidence reports *Mondia whitei* is a widely used medicinal plant across Africa in traditional medicine for treatment of sexual dysfunction, yet very minimal scientific evidence exists to support the therapeutic claims. Furthermore existing studies have reported conflicting results, with some reporting contraceptive activity (Watcho *et al.,* 2001) and others claiming that it is fertility drug and an aphrodisiac (Lampiao *et al* 2010).

1.3 Justification of the Study

Increased cases of male infertility with conventional therapy approaches warrants research on the use of medicinal plants as a viable alternative. Also wide use of mukombero warrants research to establish its effects on male fertility and potential use in the treatment of male fertility.Evidence to provide a biological mechanism of "mukombero" extract on the reproductive functions is needed, by governments, researchers, health professionals, and communities so as to make informed choices between legalizing it for economic reasons, banning it or regulating its consumption due to health concerns.

There is need for greater awareness by health professionals on the use of *Mondia whitei* and the related health problems. This requires health promotion activities which targets communities with high levels of mukombero use in order to help in promoting reduction of harm strategies, increasing the understanding of the potential risks of regular mukombero use and increasing awareness of services available for those experiencing harm (Gerald, 2010). The present research study aimed at filling the information gap existing on the effects of aqueous extract of *Mondia whitei* on male reproductive functions. The findings from this study should be considered in addressing the positive/negative reproductive effects of mukombero use on consumers.

1.4 Research Questions

1. What is the effect of *Mondia whitei* extracts on serum testosterone levels, Luteinizing hormone (LH), and follicle stimulating hormone (FSH) in male albino rats following oral administration of graded doses?
2. What is the effect of Mondia whitei extracts on sperm characteristics in male albino rats following oral administration of graded doses*?*
3. What histological changes would occur in the testicular tissues of male albino rats after oral administration of graded doses of aqueous extracts of *Mondia whitei* ?

1.5 Research Objectives

1.5.1 Broad Objective

To determine on the effects of *Mondia whitei (mukombero),* on the follicle stimulating hormone, luteinizing hormone, testosterone, sperm characteristics and testicular histological tissue changes in male albino rats.

1.5.2 Specific objectives.

1. To determine the acute toxicity of aqueous extract of *Mondia whitei.*
2. To determine serum levels of testosterone, Luteinizing hormone (LH), and follicle stimulating hormone (FSH) in male albino rats following administration of graded doses of aqueous extracts of *Mondia whitei*.
3. To determine the sperm characteristics in male albino rats following oral administration of graded doses of aqueous extract of *Mondia whitei.*
4. To determine testicular histological changes following oral administration of graded doses of aqueous extracts of *Mondia whitei* in male albino rats.
5. To determine the types of phytochemicals found in aqueous extract of Mondia whitei.

1.6 Hypothesis

H0: Aqueous extracts of *Mondia whitei* does not affect the functional integrity of the pituitary-gonadal axis in male albino rats.

H1: Aqueous extract of *Mondia whitei* affects the functional integrity of pituitary-gonadal axis in male albino rats.

CHAPTER TWO
LITERATURE REVIEW

2.1 Infertility

Infertility refers to a situation whereby a couple f ails to achieve a pregnancy during the fertile phase of the menstrual cycle even after having regular and unprotected sexual intercourse for one year (Nieschlag *et al.,* 2000; Evers, 2002). Previous studies had revealed that infertility has affected about 15% of the couples who had sought medical help due childlessness' (Feng, 2003; Roberts, 1998). According to the report by World Health Organization (WHO) (2002) the annual number of infertile couples globally was found to be 50-80 million. The Male factor contributes to half of infertility cases worldwide (Nieschlag *et al.,* 2000).

The majority of cases of male infertility have been attributed to idiopathic causes (Sherins, 1995). The ramification is that, the options of therapy focusing on assisted reproduction (ART), such as intracytoplasmic sperm injection (ICSI) or in vitro fertilization (IVF) is invasive and thus associated with complication (Halliday, 2012). However, if it is a must to do assisted reproduction, investigation of the causes of infertility and understanding of its pathophysiologic mechanisms becomes imperative (Sherins,et al 1995). .

The analysis of semen usually is the first step in the investigation of infertility in males whereby, it entails determination of morphology of sperms (Kruger et al., 1986; Menkveld *et al.,* 1990), its motility (Ron-el *et al.,* 1991; Robinson *et al.,* 1994) and concentration (Calvo *et al.,* 1994). Interestingly, many men have been found to have normal semen parameters despite being infertile (Baker, 1994; Hull *et al.,* 1985; WHO, 1992). Reports by Lewis (2007) shows that when determining infertility in males,WHO criteria's of semen analysis is insufficient without investigating sperm functionality.

In Kenya the National infertility survey of 2005/2006, showed that infertility is a major reproductive health concern. According to the survey, consultations on matters

related to infertility were: 30% (teaching and referral hospital), 27% (provincial hospitals), 15% (district hospitals, 4% (health centres) and 2% (dispensaries), (M'imunya *et al,* 2006). This data clearly shows that infertility is a major issue affecting a considerable number of Kenyans. Earlier studies had similar findings (Gachera *et al* (1984) and Mati *et al* (1987).

2.2 Psychological effects of infertility

Infertility is a painful emotional experience that can lead to several psychological issues including anxiety, reduced self-esteem, lack of sexual satisfaction, depression and diminished quality of life ((Dural, *et al.,* 2016; Causineau TM, et al., 2007, Kamel, 2010; Van & Bos, 2009; Obi *et al,* 2009). Such psychological problems affect males adversely more than their spouses, more so in communities where prejudices exist against infertile men (M' Imunya *et al.,* 2006; Obi *et al.,* 2009 and Farrokh *et al.,* 2014) Childless men are frequently stigmatized, resulting in isolation, neglect, domestic violence and polyandry (Van Balen 2002; Wiersema *et al.,* 2006 and Mar oufizadeh *et al.,* 2015). However, the responses of men to infertility are approximately close to those of women when infertility is caused by male factor (Nachtigall, 1992). This leads to pronounced feelings of defectiveness and incompetence by both men and women and experience a sense of loss of identity (Van Balen 2002).

2.3 Hypothalamo-pituitary-gonadal axis

The hypothalamo-pituitary-gonadal axis is a vital system in the reproductive life and thus it has to be active for puberty to set in (Wistuba *et al.,* 2007). Puberty cycle is initiated through the release of Kisspeptin from the arcuate nuleus of the brain by specialized neurons (kiss-1-neuron) to stimulate gonadotropin releasing hormone (GnRH) release thus, GnRH is released as a result of kiss-1-neuron stimulating GnRH neurons in the hypothalamus leading to expression of GPR54 receptors, which binds kiss-peptin via a G-protein cascade (Smith *et al.,* 2006). In turn, the release of GnRH from the hypothalamus in pulsatile intervals of 60 to 120 minutes, initiates the release of FSH and LH by the anterior pituitary gland. Receptors expressed by Sertoli cells bind FSH, which act to stimulate spermatogenesis whilst

LH stimulates Leydig cells to produce testosterone (Wistuba *et al.,* 2007; Sharpe, 2010).

The initial release of Kiss-peptin initiates pubertal events in both male and female (Smith et al 2006). The hypothalamo-hypophyseal portal blood carries GnRH to the anterior pituitary glands where it binds the gonadotrope cells (receptors of GnRH) on gonadotropes (George *et al.,* 2011). The activation of the GnRH receptors leads to activation of phospholipase C which stimulates release of protein kinase C thus resulting in activation of proteins involved in the synthesis and secretion of LH and FSH (Wistuba *et al.,* 2007). As a result of the stimulation by gonadotropins, steroidal hormones, testosterone, estrogen and progesterone are synthesized and released by target organs (testes in males and ovaries in females). These hormones are important for male and female secondary sexual characteristics which include maintaining, supporting and ensuring reproduction, bone density and muscle mass (Ge *et al.,* 2009; Hall and Guyton, 2011; Cheng and Mruk, 2012). On the contrary increased level of testosterone, inhibin (which is synthesized in the Sertoli cells due to FSH) suppresses the effects of FSH locally in the testis, as well as its secretion by the pituitary (Wistuba *et al.,* 2007), by inhibiting the hypothalamic release of GnRH through a negative feedback. This keeps the HPO axis in equilibrium.

2.4 Gonadotropins (Follicle stimulating hormone and interstitial cell stimulating hormone)

Gonadotropin (LH and FSH) hormones are hetero-dimer glycoprotein hormones secreted from the anterior pituitary gland by gonadotropic cells under the stimulation of GnRH (Pernasetti *et al.,* 2001; Hall and Guyton, 2011). LH and FSH exert their effects on their respective target cells

located chiefly in the testes by activating the cyclic adenosine monophosphate second messenger which activates specific enzymes in the respective target cells (Wistuba *et al.,* 2007).

LH stimulates the Leydig cells in the testis to synthesize and release testosterone hormone; the amount of testosterone is approximately directly proportional to LH levels (Achard *et al.,* 2009). According to Wistuba (2007), the main function of LH

is to determine secondary sexual characteristics by stimulating the secretion of testosterone. Hall and Guyton (2011) in addition gives more insight by observing that the production of mature male gametes is also dependent on LH. Leutinizing hormone (LH) stimulates Leydig cells to produce testosterone (Achard *et al.,* 2009). LH is increased in the perinatal period for a short period (7-13 weeks) before its activity decreases again and remains low until puberty (Kuiri-Hänninen *et al.,* 2014).Its levels begin to rise again at the onset of puberty, whereby Leydig cells are stimulated to produce testosterone. Afterwards, this relationship is maintained through a negative feedback loop during male adulthood (Meccariello *et al.,* 2014).

FSH on the other hand stimulates Sertoli cells in the seminiferous tubules of the testes to grow and accelerates spermatogenesis (Wistuba *et al.,* 2007). At the same time, testosterone and dihydrotestosterone diffuse into the seminiferous tubule to enhance spermatogenesis. In addition, evidence has shown that FSH is important in multiplication of spermatogonia supported by testosterone action for proliferation of the germ cell line and plays a major role for the renewal of type-A spermatogonia (Simoni *et al.,* 2008). Thus, both FSH and testosterone contributes to the final maturation of spermatozoa, and protect the germ cell line against apoptosis (Hall and Guyton, 2011).

2.5 Inhibin B Hormone and its function

Inhibin B is a glycoprotein hormone synthesized by Sertoli cell following stimulation by FSH

(Wistuba *et al.*2007). The regulation of inhibin B secretion is strongly associated with gonadotropins whose level decreases in males with age (Ramaswamy and Plant, 2001). The onset of puberty activates production of inhibin B (Meccariello *et al.,* 2014). In early postnatal testis development, an increase of inhibin B levels is seen leading to down-regulation of the hypothalamic-pituitary -gonadal axis which results in low levels of the testosterone, LH and FSH (Wistuba *et al.,* 2007). FSH action and Sertoli cell proliferation are associated with inhibin B activity during pre-pubertal testis formation (Simoni *et al.,* 2008). Afterwards, the level of inhibin B declines

mirroring Sertoli cell density (Wistuba *et al.,* 2007). The main function of inhibin B hormone is to down regulate FSH when is increased (*Simoni et al,* 2008).

The major determinant of inhibin B in adulthood (after completion of spermatogenesis in puberty) is the germ cells (Ramaswamy and Plant, 2001). Rising androgen levels are thought to be the activating factor in germ cell proliferation which secondarily depends on FSH and has been associated with changes in Inhibin B levels (Meccariello *et al.,* 2014). Thus, inhibin B levels are indicated in spermatogenic efficiency since the level directly correlates with sperm count (Wistuba *et al.,* 2007).

The two main functions of heterodimeric gonadotropin FSH (glycoprotein), a major contributing

factor in the regulation of spermatogenesis, in adulthood are: Regulation of inhibin B and

determination of Sertoli cell number in immature testis. FSH influences maturation of Leydig cells (Simoni *et al.,* 2008). All these processes are mediated via G-protein coupled transmembrane FSH receptors (Wistuba *et al.,* 2007).

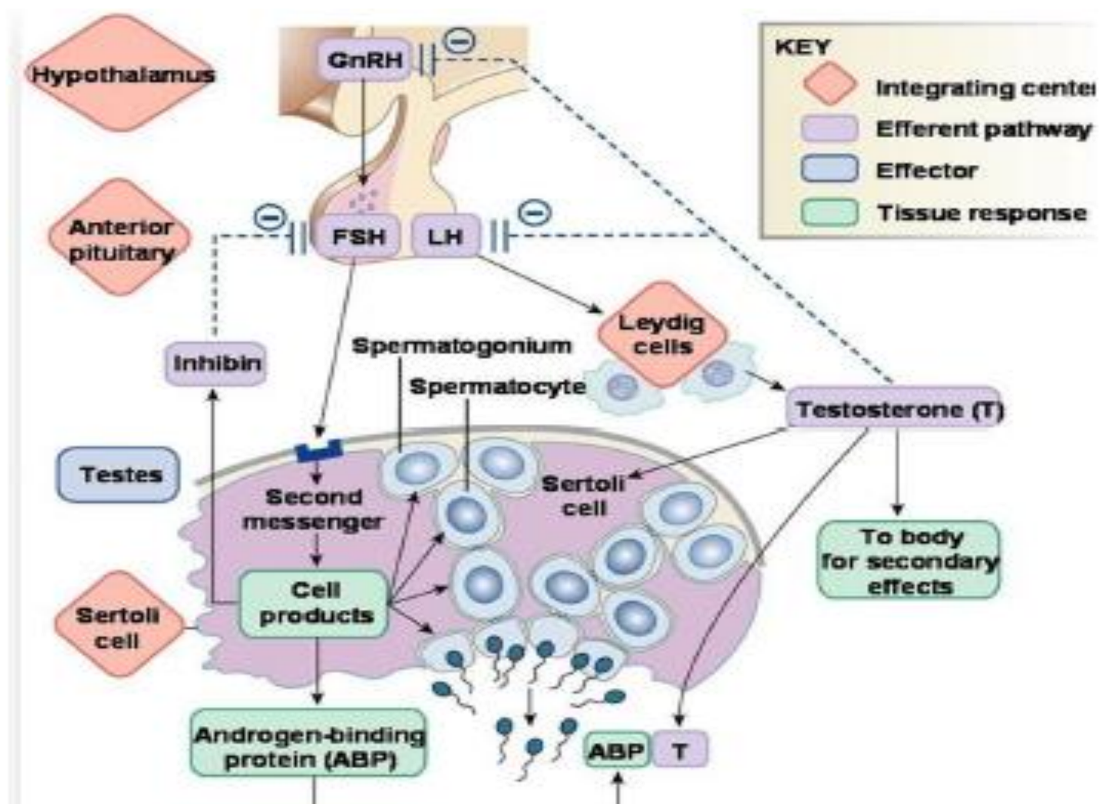


Figure 2.1: Hormones and negative feedback loops that control secretion of testosterone and spermatogenesis.

Source:*http://www.austincc.edu/apreview/PhysText/Reproductive.html#* 6th June 2019

2.6 Testosterone hormone synthesis, secretion and function

Leydig cells commonly referred to as interstitial endocrinocytes are clusters of endocrine cells found between seminiferous tubules, whose function is derived from endocrine and paracrine inter-communication (Ge *et al.,* 2009). These cells mainly synthesize and secret male sex hormones (androgens), the most important of which is testosterone (Wistuba *et al.,* 2007). Endocrine stimulation by LH induces Leydig cells to secret testosterone. In turn, testosterone diffuses into the seminiferous tubule and drives spermatogenesis together with FSH (Ge *et al.,* 2009). Therefore, this makes Leydig cell significant in the process of spermatogenesis (Hall and Guyton, 2011).

The onset of puberty in the human male is strongly characterized by the production of testosterone, which is influenced by stimulation of luteinising hormone (LH) on Leydig cells (Wistuba *et al*., 2007; Knez, 2013). The interstitial connective tissue contains Leydig cells, which releases testosterone (Wistuba *et al.,* 2007). Foetal Leydig cells differentiate and begin to produce testosterone at the end of 4th week of gestation (Quinn and Koopman, 2012).

This differentiation takes place in the interstitial space between the seminiferous cords (Sharpe, 2010). Testosterone produced within the Leydig cells in the developing testis act to stimulate the Sertoli cells proliferation (Quinn and Koopman, 2012). Besides, the number of sperm cells produced in adulthood is determined by the number of Sertoli cells within the testis (Orth *et al.,* 2000; Sharpe *et al.,* 2003). Report by Plant and Marshall (2001), shows that Sertoli cell proliferation and factors that regulate this process during the different periods of development may differ: for instance, during foetal and neonatal period; testosterone seems to play an important role in development of sexual organs. The amount of testosterone produced in male is thought to be approximately directly proportional to LH (Achard *et al.,* 2009).

Both FSH and testosterone contribute to the final maturation of spermatozoa, and protect the germ cell line against apoptosis (Hall and Guyton, 2011). In early postnatal testis development, an increase of inhibin B levels is seen leading to down-regulation of the hypothalamic-pituitary-gonado axis which results in low levels of testosterone, LH and FSH levels (Wistuba *et al*., 2007).

Testosterone is the principle male steroid hormone that belongs to the androgen group (Simoni *et al.,* 2008). In the male, secretion of testosterone is mainly from Leydig cells though small amounts are also secreted by the adrenal glands (Rizzo, 2015). The major function of testosterone in the testes is the maintenance of spermatogenesis via stimulation of Sertoli cells; its actions are mediated through intracellular androgen receptor which acts as a transcription factor (Simoni *et al.,* 2008). Testosterone moves into the Sertoli cells binding to androgen binding hormone receptors within the seminiferous tubules. The normal functioning of Sertoli cells requires the combination of testosterone with the receptors. There is also conversion of testosterone to two other steroids in the Sertoli cells: estrogen and dihydrotestosterone (Hall and Guyton, 2011). In addition, there is secretion of androgen-binding protein by the Sertoli cells into the seminiferous tubules to facilitate spermatogenesis (Rizzo, 2015).

Thereafter, there is binding of testosterone and dihydrotestosterone to androgen-binding proteins which are then carried to the epididymis together with other secretions from seminiferous tubules. According to Hall and Guyton (2011), estradiol and dihydrotestosterone may be the active hormones promoting sperm cell formation. According to Rizzo (2015), other functions of testosterone include: maintenance of normal reproductive function in the adult, differentiation, growth, and function of accessory organs of reproduction, stimulates transport and delivery of sperm to the epididymis and increases sexual drive (libido) in both men and women.

Furthermore, there is association of HPG axis derangements with spermatogenesis abnormalities and dysfunction. Men having azoospermia as a result of secondary testicular failure have small, soft testes which measures less than 10 ml in volume with small, flat epididymis. Primary testicular failure decreases testosterone

production diminishing inhibition of negative feedback and stimulates gonadotropin production (hypergonadotropic hypogonadism) (Kim *et al.,* 2010). Men with this condition often have excessive testosterone conversion by aromatase enzyme to estradiol and have normalization of testosterone levels and improved spermatogenesis when given aromatase inhibitor therapy. However, men having azoospermia condition secondary to obstruction, have their testosterone production become normal with the hormone profile when treated properly. On the contrary, even though spermatogenic failure is consistent with FSH elevation, not all men with spermatogenesis abnormalities have FSH elevation levels. According to Schoor *et al*. (2002), it was observed that 96% of men with azoospermia obstruction had 7.6 mIU/ml and below FSH levels or greater than 4.6 cm testicular long axis while 89% of those with azoospermia non-obstruction had greater than 7.6 mIU/ml FSH levels or less than or equal to 4.6 cm testicular long axis.

In addition, hypogonadotropic hypogonadism (HH) as a result of inadequate production of gonadotropins by pituitary which is required for normal production of adequate testosterone leads to low LH, low testosterone and low FSH. HH may be congenital or acquired. Kallmann's syndrome which is a hypothalamus congenital disorder caused by failure of GnRH secretion, is HH associated with chromosomal abnormalities and less commonly cleft palate and unilateral renal agenesis. Congenital HH may be associated with cryptorchidism and micropenis (Kim *et al.,* 2010).

2.7 Sertoli cells and Spermatogenesis

Sertoli cells were first described in 1865 by Enrico Sertoli who suggested they provide nutrients for the germ cells within the seminal epithelium. A number of studies have identified the role of Sertoli cells in spermatogenesis which include: provision of nutrition for the differentiating sperms, formation of the blood-testes-barrier tight junctions, involvement in selective permeability hindering pathogens that may harm sperm cells and allowing the passing through of beneficial substances like testosterone, secretion of adequate fluid into the lumen of the seminiferous tubule, which helps in sperm maturation towards the epididymis tubule and synthesis

and metabolism of steroids (Dym and Raj, 1977; Wistuba *et al.,* 2007; Sherwood, 2011).

2.8 Spermatogenesis

Spermatogenesis is a series of cellular events where germ cells (spermatogonia) lead to the formation of mature sperm (spermatozoa). The process of spermatogenic normally takes place in the germinal epithelium where tight junctions formed between the Sertoli cells separate it into an apical and basal compartment. According to Kato *et al.* (2009) blood-testes barrier (BTB) formed by these tight junctions offers protection to the developing germ cells against any immunological influences and harmful agents. Bart et al. (2002) reported that their is need for protection since many agents may disrupt meiotic cell division which is a delicate process.

The process of spermatogenesis in humans takes approximately 75 days (Clermont and Antar, 1973; Franca and Godinho, 2003; Clermont and Trott, 1969; Rosiepen *et al.,* 1994, 1997; Andersen Berg et al., 1990; Russell et al., 1990). It is continuous process leading to the production of around 120 million spermatozoa every day from the start of puberty in human males (Clermont and Trott, 1969; Clermont and Antar, 1973).It includes the renewal and proliferation of spermatogonial through the process of mitosis and differentiation which involves progression of cell cycle from spermatogonia of type B to preleptotene spermatocytes in the basal compartment and progression of cell cycle from zygotene and pachytene to diplotene spermatocytes Cheng and Mruk (2012) . This is then followed by meiosis I and II involving round spermatids developing to spermatozoa through the process of spermiogenesis and the release of mature spermatozoa from germinal epithelium into the tubular lumen through the process of spermiation occurring in the apical compartment behind the BTB.

According to Bonomi *et al.,* (2012), the formation of spermatogonia during the foetal period, remain dormant until pubescence and lies next to the basement membrane of the seminiferous tubule. There are two classification of spermatogonia according to Clermont (1966) depending on the shape of nuclei and chromatin patterns and include Type A (Type A pale and Type A dark) and Type B.

The proliferation of Type A and Type B spermatogonia is through mitotic divisions and have different fate where there is reproduction of Type A spermatogonia for the continuous production of a pool of these cells while Type B spermatogonia undergo further changes leading to the development of primary spermatocytes.

Meiotic events lead to the changes in the chromatin condensation in the nucleus leading to the maturation of the spermatocytes. Meiosis involves the occurrence of two divisions whereby spermatogonia enters first meiotic division (prophase) further subdivided into stages including Preleptotene and Leptotene stages where there is condensation of chromatin into visible chromosomes and Zygotene - Pachytene stage where there is pairing of homologous chromosomes into primary spermatocytes. Thereafter, there is crossing over of chromosomes (Diplotene stage) which has a duplicate set of 46 chromosomes comprising of 22 pairs of duplicate autosomal chromosomes, 2 X chromosomes and 2 Y-chromosomes. At the end of first meiotic division, the daughter Type B cells have become the secondary spermatocytes with haploid (23) number of chromosomes. The secondary spermatocytes undergo the second meiotic division resulting in smaller cells called spermatids which is located in the adluminal compartment of the seminiferous tubules (Cheng and Mruk, 2002).

On the other hand, the process of spermiogenesis involves elongation and differentiation of the spermatids produced from spermatogenesis. The spermatids have to undergo 4 phases including Golgi phase, Cap phase, Acrosomal phase and Maturation phase. This involves the appearance of pre- acrosomal granules in the Golgi apparatus and its fusion leads to the formation of a membrane-bound acrosomal vesicle close to the nuclear membrane. Thereafter, there is enclosing of the acrosomal vesicle in the anterior half of the nucleus which then becomes the acrosomal cap (Moore and Persuad, 1998). Thereafter, there is flattening and elongation of the increasingly dense nucleus leading to the migration of the cytoplasm between the acrosomal cap and the anterior cell membrane to the posterior part of the cell. Also, there is ‘pinching off’ of the surplus cytoplasm from the neck and mid-piece regions and are phagocytized by Sertoli cells. The end of spermiogenesis is marked by the disconnection of immature spermatozoa from the Sertoli cell surface and lying freely in the lumen of seminiferous tubule. The process

where spermatozoa are released into the lumen is called spermiation (Moore and Dalley, 1999).

In addition, intratesicular and extratesticular factors control the process of spermatogenesis whereby according to Holstein et al. (2003), intratesticular factors include Leydig cells found in the interstitial space of the testes and Sertoli cells found in the seminiferous tubules. According to Griswold (1995), testosterone and growth factors are secreted by Leydig cells and facilitate the maintenance of the Sertoli cells which function in nursing germ cells.

Furthermore, extratesticular factors originate from the hypothalamus and the pituitary gland where gonadotropin-releasing hormone (GnRH) is secreted from the hypothalamus stimulate anterior lobe of the pituitary gland to release of Luteinizing hormone (LH) and FSH. Thus, both the intratesticular and extratesticular factors forms Hypothalamic–Pituitary–Gonadal axis (Mruk and Cheng, 2010; Kopera *et al.,* 2010).

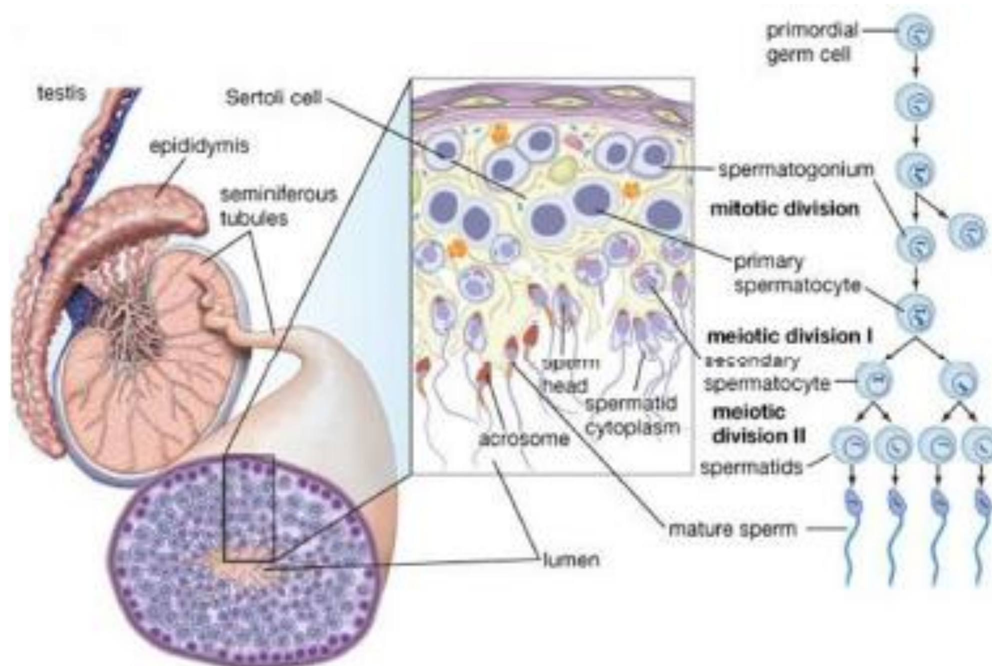


Figure 2.2: Seminiferous tubules and spermatogenesis

Source: http://global.britannica.com/EBchecked/topic/559418/spermatogenesis#6th June 2019

2.9 Sperm parameters analysis

2.9.1 Motility

Sperm motility is an aspect of semen quality which is believed to be important in defining fertility in relation to achievement of pregnancy (Yanagimachi *et al.,* 1976). In general, it gives a measure of integrity of the sperm axoneme and tail structure, metabolic machinery of the mitochondria and morphology (Pacey, 2006). Sperm movement is generated from the flagellum (tail structure) (Paoli *et al.,* 2011) providing the propulsion for swimming, which is critical for sperm motility (Coetzee 18 *et al.,* 1989; Pacey, 2006). Furthermore, sperm with good kinematic motility parameter are able to penetrate cervical mucus better (Aitken *et al.,* 1991).

Studies have shown that sperm motility is a predictor value for pregnancy rates because reduced sperm motility leads to reduction in the chances of fertilization of the ovum, an aspect common in infertile men (Coetzee *et al.,* 1989; Henkel, 2005). Bartoov *et al.* (2002) found that sperm motility significantly correlated with fertilization in vitro and has been clinically used to predict pregnancy (Aboua *et al.,* 2009). Likewise, studies have shown direct correlation of motility to mitochondrial membrane potential (Kasai *et al.,* 2002), plasma membrane integrity (Aitken *et al.,* 1991) and morphology (Kruger *et al.,* 1987). However, knowing the percentage of sperm motility could be convenient for standardizing experimental studies whose interest would be examining sperm movement in isolation (Hirano *et al.,* 2001), but might not be suitable when tests are being performed for clinical purposes (Mortimer *et al.,* 1986).A study by Guzick *et al,.* (2001) strongly support the fact that sperm motility provides useful information for diagnosing male infertility, but not in isolation as diagnostics of infertility.Hirano *et al.,* (2001) and Mortimer (1994) in conjunction with their co-workers, contributed by stating that more detailed evaluation on sperm motility is a potentially valuable contribution in infertility diagnostics. Determining sperm motility parameters has revolutionized over the years (Mortimer *et al.,* 2015).

These parameters have now been standardized with the aid of motion analysis systems. WHO (2010) guideline reference values have been adopted as a standard

methodology for laboratories engaged in semen analysis, which recommends the use of motility grading system as follows; Total motility: total spermatozoa moving actively (moving linearly or in a large circle, not considering speed) and all other patterns of motility with an absence of progression.

Progressive motility: spermatozoa moving actively forward in a straight pattern (Hirano *et al.,* 2001):

2.9.2 Sperm Vitality

This parameter is an estimate of membrane integrity of the sperm cell (WHO, 2010). Sperm vitality assay testing is used to determine if the non-motile sperm are alive or dead and is indicated when sperm motility is less than 5% to 10% (Vasan, 2011). This in turn gives a reflection of accuracy of living non-motile spermatozoa. Ideally, the percentage of dead spermatozoa should be less than the percentage of live spermatozoa (Björndahl *et al.,* 2003). Sperm viability testing was introduced in 1984 by Jeyendran and his colleagues; they suggested non-motile viable sperm may be used for intracytoplasmic sperm injection (ICSI) (Hossain *et al.,* 1998). Sperm viability contributes to sperm-oocyte activation and fertilization and further development steps during embryogenesis. Therefore, selection of mature viable sperm for injection into the cytoplasm is very important (Sati *et al.,* 2015).

According to Anerao *et al.,* (2010) the quality of sperm has been found to be more significant than the count. Poor motility has been associated with reduced viability of spermatozoa (Dhar *et al.,* 1968; Savita and Huma, 2010). Moreover, the percentage of viable sperm has been highly correlated with spermatozoa with optimal functional mitochondria (Magistrini *et al.,* 1997; Papaioannou *et al.,* 1997). In addition, a study by De Lamirande and Gagnon (1992), Baumber *et al.* (2000) found that reactive oxygen species (ROS) caused 10% - 20% decline in viability of human and animal spermatozoa.

2.10 Sperm and Hormonal Reference Limits

World Health Organization (2010) semen analysis shows that the determination of reference limits was done through assessment of Semen Analysis data from men who

had a known history of recent paternity. During this analysis, any value above 5th centile was arbitrarily defined as "normal" parameter while those below the 5th centile were labeled as "abnormal." However, the 5th edition of WHO only included SA values from men who had fathered at least one child after ≤12 months of trying (Chiles & Schlegel, 2015). In male albino rats, the normal levels of hormones have been established to be 1.93(±0.76) ng/ml for LH, 4.4(0.89) ng/ml for FSH and 7.57 (±1.13) ng/ml for testosterone (Moghny & Ashour, 2016).

2.11 Ethno-medicine approaches in management of Infertility

Infertility has been an issue of concern for many years and communities would consider one worthless if they could not get children. Therefore, there are traditional methods that have been used overtime to manage infertility and mostly involve use of herbal medicine. Herbal medicines may enhance fertility by supporting the natural functions of the spermatogenesis and fertility process.

The table below illustrates traditional herbal medicines that are used in Kenya;

Table 1: Medicinal plants that are used to manage infertility in Kenya

FAMILY	PLANT SPECIES	LOCAL NAME	METHOD OF PREPARATION, ROUTE OF ADMINISTRATION AND DOSE.
Periploceae Aloea ceae	Mondia whitei Aloe volkensii Engl. CK 027	Mukombero (Luyha) Hargeis, D'aar (ORMA)	Roots washed and taken orally that are used to enhance fertility and libido Leaves squashed in water, decoction used to wash genital organ 3 times daily until effective
Copparaceae	Cadaba grandulosa Gilgi. CK 037	Alakai (ORMA)	Roots boiled in water and decoction taken orally, half a glass daily for 3 days.
Capporaceae	Cadaba farinose Ck 038	Kumis (ORMA)	Roots boiled in water and decoction taken orally, half a glass daily for 2 days.
Labiatae	Hoslundia opposite rah) CK 045	Mtserere(pokomo)	Roots boiled in water and decoction taken orally half glass 2-3 times daily for 3 days.
Passifloraceae	Adenia summifera (Harv) Harms. CK 019	Mujoka (pokomo)	Roots/stems boiled in water and decoction taken orally half a glass daily for 3 days.
Rufaceae	Citrus sinensis(L) Osbeck CK -012	Mudimu (Giryama)	Roots/stem bark boiled in water and decoction taken orally.

Papidaceae	Allophylus pervilleria (A. Rich) engl. CK 047	Mnyanga Kiswa(Pokomo)	Roots boiled in water and decoction taken orally one glass daily for 3 days.

Patients in developing countries have been forced to seek traditional medical attentions since they are unable to afford modern medical healthcare facilities Watcho *et al.,* (2007). The estimation by World Health Organization (2010) shows that almost 80% of the world population relies mostly on traditional medicines. Moreover, Farnsworth and Soejarto (1985) discovered that from the 119 plants listed by WHO for deriving drugs, 74% was used in isolating active compounds through chemical studies for use in traditional medicine requiring the use of original plant. According to the studies by Nwude and Ibrahim (1980), traditionally, most plants in Nigeria have medicinal value in the treatment of various illnesses in both animals and humans. However, according to Nwosu *et al.,* (2011), the safety and efficacy of these plants are still doubtful since few of them have proper identification and documentation. Particularly, *Mondia Whitei* will be of interest in this study which is also called tonic root or white's ginger. Watcho *et al.,* (2006) describes it to belong to Periploceae family and as an aromatic plant which among the Yoruba people in Nigeria is commonly called Isirigun. *Mondia Whitei* is distributed in tropical Africa regions and is believed to have great medicinal value (Aremu*et et al,.* (2011). According to Burkill *et al.,* (1997), it is well pronounced that *Mondia Whitei* has roots and root bark with vanilla-like oduor and ginger and Licorice taste of mixture.

2.12 Mondia whitei and its medicinal value

Commercialization of plants currently in the global world is becoming to gain more interest and popularity as many industries such as nutraceutical, pharmaceutical and cosmeceutical are using them. In Africa, *Mondia Whitei* is a popular endemic medicinal plant used in the treatment of various ailments since antiquity. For instance, roots were used for treating stress, anorexia, sexual dysfunction, bilharzias and general pains and aches. Aremu *et al.,* (2011) reported that there has been evaluation of the efficacy of these claims by various researchers through screening

for biological activities of *Mondia Whitei* including anti-inflammatory, antihelmintic efficacy and antimicrobial.

Mondia whitei root extracts contains phenolic and flavonoid compounds (Bouba *et al.,* 2010; Gakunga *et al*., 2013), as well as vitamin E (Sharma and Agarwal, 1996; Van Wyk, 2011), all of which possess radical scavenging activities (Moyo *et al.,* 2010), hence, generally protecting spermatozoa from oxidative stress caused by free radicals which may lead to protein oxidation, DNA damage, and lipid peroxidation (Halliwell, 1996).

A short-term androgenic and a long-term reversible antispermatogenic and antifertility effects of the aqueous extract of M. whitei (400 mg/kg) in adult male rats has shown that *M. whitei* extracts antagonizes the contractile responses to potassium chloride and Adrenaline in isolated rat vas deferens, which could be due to the blockade of voltage gated calcium channels (Watcho *et al.,* 2006).

Moreover, the results from an investigation by *Watcho et al* (2006) has shown that a fraction of *Mondia Whitei* extracts with hexane has molecules that are bioactive hence blocking both calcium channels including voltage-operated and receptor-operated which prevents calcium entry as vas deferens are being depolarized by Adrenaline and KCI .

Also, there is increment of the testicular weight which concurs with the studies by Watcho *et al.,* (2004) hence supporting the significance of male fertility and androgenic activity of *Mondia whitei* (Lampiao, 2009). Furthermore, it has been observed that there is association between the increment in serum testosterone or androgens in rats treated with M whitei extracts increases organ weight and secretory activity (Venter *et al.,* 2009).

2.12.1 Morphology and taxonomy of Mondia whitei

M. whitei with a height of 10-20 ft is a climbing perennial woody plant with twining stems which when cut exudes white latex as shown in Fig 2.3 A. Its leaves as seen in Fig 2.3 B it is large and oppositely positioned heart-shaped leaves. *Ross et al* (1978) described the surface of the leaves to have distinctive star-shaped stipules and endowed with soft hairs. It has attractive reddish purple flowers that are borne in branched inflorescences as seen in Fig 2.3 C. Flies are assumed to pollinate flowers

and has short survival for about 3-4 days. Venter *et al.,* (2009) observed that in the southern equator, the flowering season of *Mondia whitei* is between October to March with peak season between November and January while in the northern equator is between May to August with peak season between June and July. Moreover, the growth of M. *whitei* is from a large, tuberous rootstock and is characterized with very distinct vanilla aroma roots as a result of 2-hydroxy-4-methoxybenzaldehyde as shown in Fig 2.3 D. It has been observed that the roots has lateral growth spreading out beneath the surface of the soil and covers large areas hence harvesting becomes easier (Ross, 1978; Van Wyk and Gericke, 2000). Furthermore, the dehiscing of the ovoid follicle fruit releases about 180-320 comose seeds whose dispersion is by wind (Ross, 1978).

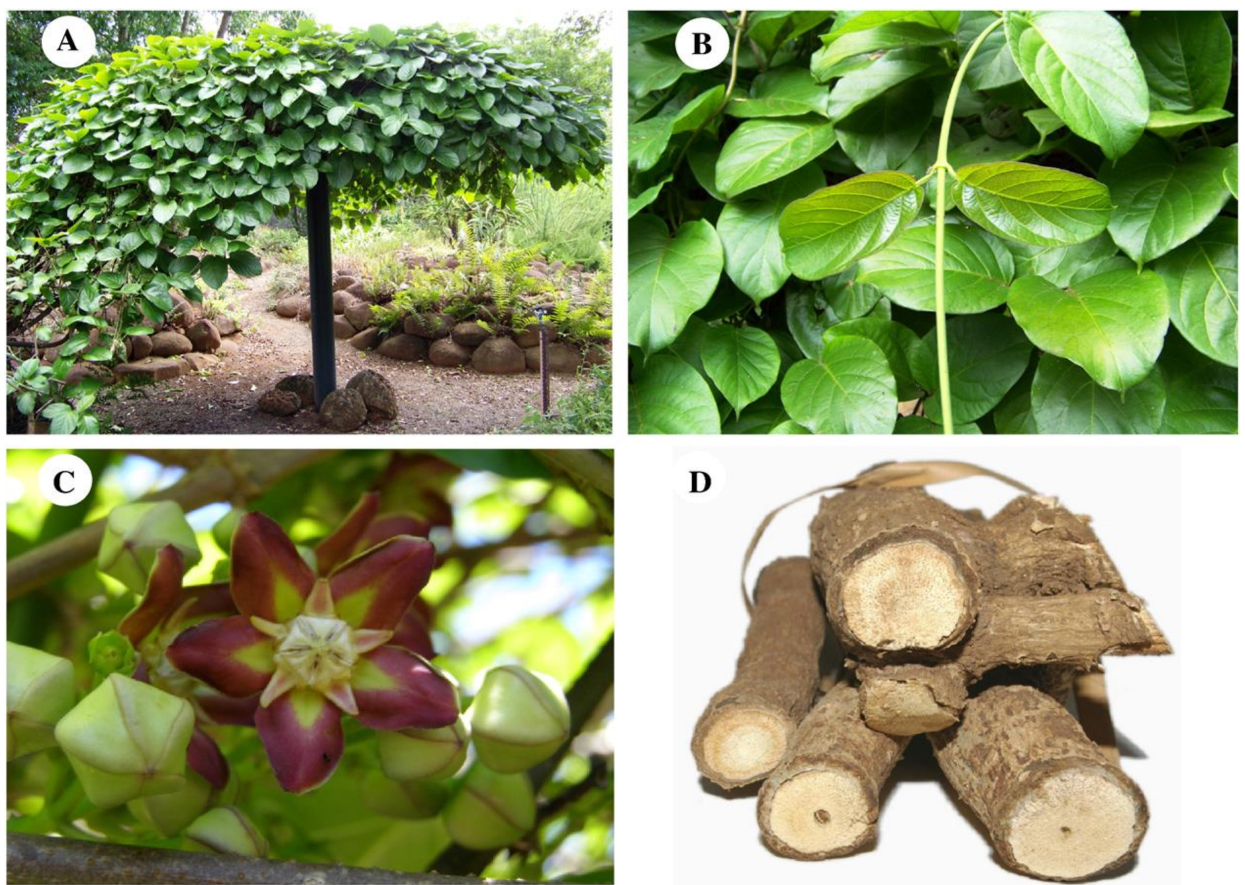


Figure 2.3: Morphology of M.whitei(Aremuet al., 2011)

2.13 *Mondia whitei* and reproductive hormones

In a study by Watcho *et al.,* (2006) on the long term investigation of a hexane extract of Mondia on doses of 500 and 1000mg/Kg on body organ weight, it was found that the relative weight of caput epididymis, ventral prostate and seminal vesicle were increased. More studies using both hexane extract of Mondia in spinal rat showed effect of fictive ejaculation, which appeared not to be mediated through dopamine pathway, hence justifying ethno medicinal use of the plant (Watcho *et al.,* 2013). Another report by Watcho *et al.* (2004) showed that administration of *Mondia whitei* over a period of 10 days caused an increase in serum and intratesticular testosterone levels after treatment, suggesting an androgenic effect of the *Mondia whitei* aqueous extract.Additionally Kamtchouing *et al,* (2002) reported an increase in testosterone concentration by *Zingiberofficinale,Pentadiplandrabrazzeana, Hibiscus macranthus* and *Basellaalba* treatment in rats. However, in another study by Watcho *et al.* (2001) after treatment of rats for over 55 days with the same dose of *Mondia whitei* extract yielded no change in testosterone concentration. It could then be assumed that as the duration of the treatment is prolonged, the sensitivity of the steroidogenic mechanism to the bioactive molecules present in the plant extract decreases (Watcho *et al.* 2004).

The decreased level of FSH reveals that *M. whitei* aqueous extract may have less potential to influence the release of gonadotrophic hormones from the pituitary since FSH by itself is of critical importance in the initiation and expansion of spermatogenesis in mammals (Sharpe, 1989). The decrease in levels of FSH may probably be due to failure to suppress negative feed-back inhibition of anterior Pituitary (Krishnakumari *et al.,* 2012; Anitha *et al.,* 2013, Woode *et al.,* (2012).

It is well-known that testosterone production by Leydig cell is primarily under the control of LH and stimulation of LH is usually followed by stimulation of testosterone, (Huthaniemi and Toppari, 1995). Leydig cells secrete testosterone by the stimulatory effect of LH (O'Donnell *et al*., 1999; Woode *et al*., 2012) in males; reduction of testosterone level may impair spermatogenesis and cause male infertility (Anitha *et al*., 2013). Thus the extract shows the inhibitory activity on the proliferation of spermatogonia.

2.14 *Mondia whitei* (mukombero) and sperm characteristics

According to Martey and He (2010), the roots of Mondia are often chewed by men as a tonic and aphrodisiac, and for treatment of sexual asthenia, erectile dysfunction and increase sperm production. A study done by Lampiao *et al.,* (2008) on the effects of aqueous root extract of M. whitei on total human sperm count and progressive motility indicated that it significantly enhanced total motility as well as progressive motility in a time-dependent manner. In another study, treatment of sperm with aqueous root extract of *M. whitei* significantly increased total and progressive motility in patients' spermatozoa with a positive trend towards higher percentages of total motile spermatozoa. Moreover, it can be deduced that aqueous root extract of *Mondia whitei* showed physiological effects on total and progressive motility especially in the patient group.

Ruiz-Pesini *et al.,* (1998) reported that various studies on plant extracts have shown different outcome on sperm motility, which is said to be predominately dependent on energy produced from the mitochondrial compartment of the sperm mid-piece. For instance, a study by Shalaweh *et al.,* (2015) reported that different concentration of aqueous root extract of Cissampelos capensis (dawidjies) showed no effect on the sperm total and progressive motility. However, hyperactivation and beat cross frequency increased with increase in concentration on the effect of aqueous extract of M. whitei on the motility and progressive motility of spermatozoa on a time dependant manner, showed no significant difference between the control and the treated group at 30, 60 and 90 minutes after incubation (Lampiao *et al.,* 2008). A statistical difference was seen after 120 minutes of incubation with highest concentration being significantly higher than the control group.

2.15 Mondia whitei (mukombero) on histological tissues

Neaves W.B (2014) in his study noticed that leydig cells of non-breeding rock hyraxes were crowded with lipid droplets and a reduction in serum testosterone levels. Administration of exogenous testosterone resulted in the disappearance of the droplets and a marked increase in the levels of serum testosterone. Okon *et al.,* (2012) reported that Leydig cell nuclear area and mature Leydig cell numbers were

significantly reduced on oral administration of aqueous extract of *Mondia whitei* to male rats at the dose level of 400 mg/kg body weight per rat per day for 30 days.

According to Ogbuewu *et al.,* (2011) numerous screened plants have been reported to boost male reproductive functions, yet it may also lead to deterioration of testicular functions. Kamal *et al.*, (2003), D'Cruz *et al.,* (2010) and Ogbuewu *et al.,* (2011) in their studies comprehensively examined several plants with known effects of anti-fertility effects which included: Embeliaribes berries which showed spermicidal activity through inhibition of sperm count and enzymes activity of energy metabolism and alteration of testicular histology (Purandare *et al.,* 1979; Agarwal *et al.,* 1986); Azadirachtaindica (neem) which affects testis structure and function by damaging the seminiferous tubules hence leading to the loosening of germinal epithelium, germ cells degeneration and the derangement of germ cell types (Choudhary *et al.,* 1990; Shaikh *et al.,* 1993; Joshi *et al*., 1996; D'Cruz *et al.,* 2010).

Dikibo *et al.,* (2012) found that *Mondia whitei* had cellular pyknosis, necrosis, degenerative vacoulations, infarctions and parenchymal erosion in the brain tissue following administration of *Mondia whitei*. In another study by (Okon *et al.,* 2012), revealed that *Mondia whitei* caused severe fibrillilytic changes, myocardial necrosis, inflammatory cell infiltration and Oedema in a dose dependent manner.

CHAPTER THREE:

3.0 MATERIALS AND METHODS

3.1 Study Site

The study adopted a laboratory experimental design that was carried out at University of Eldoret, Moi University and Moi Teaching and Referral Hospital in Eldoret (MTRH). The experimental animals were kept, maintained and treated at the animal house of University of Eldoret. They were humanely sacrificed at the same institution and samples collected at the Zoology laboratory of University of Eldoret. The sperm analysis was done at the Zoology laboratory while histological studies and hormonal assays were done at Moi University School of Medicine histology laboratory and MTRH Immunology laboratory respectively.

3.2 Study design

The study design adopted was a true experimental design.

3.2.1 Sample size determination

The minimum Sample Size determination for one way ANOVA Design was employed as follows (Arifin W N *et al.,* 2017)

n= (DF/K) + 1, Where DF= the within-subject degrees of freedom (minimum-10, K= number of groups (4), and n= number of subjects per group.

On substitution

n= (10/4) + 1 = 3 rats per group

There are 4 groups that received 100mg/kg b w t, 200mg/kg, and 400mg/kg of the aqueous extract and control group that received water only and they were sacrificed at three time points of (10th , 15th and 30th day) .Total number of animals used is 9x4=36

3.3 Experimental Animals

Thirty-six (36) male albino rats weighing between 200-300g aged between six to seven weeks were bought from University of Nairobi Chiromo Campus and transported in well ventilated wooden cages to the University of Eldoret that has a well-equipped animal house. This is the age at which rats have reached sexual maturity. The animals were kept at room temperature (22-23°C), with a reverse natural light-dark cycle and were used for research that lasted for 60 days. Thirty days for the study was also adequate for absorption of *Mondia whitei* phytochemicals. The rats were kept in a conducive environment and allowed to acclimatize for three weeks with close monitoring of their health status before and during the experiment. They were fed with normal rat feed and portable water *ad libitum*.

3.4 Experimental procedures.

Mondia whitei was obtained from Kakamega forest with the help of a local botanist. The WHO 2003 guidelines on collection of medicinal plants were observed and

transported as freshly packed roots in foil papers to maintain its moisture content and viability of the chemical composition. The specimen voucher no. CM/11/8/18/001 was deposited for identification and verification of the plant using taxonomic key at the natural herbarium of University of Eldoret. Then roots were washed, air dried (shade) for a period of 30 days, sliced into smaller pieces and grounded using a laboratory mill into a fine uniform powder. Thereafter 200 g of the powdered roots was dissolved in 1.3 L of distilled water, then in 250 ml of 70% ethanol and kept for 72 h at 4° C, and occasionally stirred. Filtration of the extract was done by use of Whatman No.1 filter paper (model number 1001,150 mm) to get fine extract. It was repeated twice to ensure finer extract. Then complete evaporation was done using a rotavac control evaporator (Heidoph, Germany) at 65,100 r.p.m & 240 pascal pressure, for 30min to give 150 g of brown residue. The aqueous extract used was prepared by dissolving 1 g of the brown residue in 10 mL of distilled water and was refrigerated at -20 degrees for the entire research period (Gundidza et al 2009). The doses used in our study were a range of 100 mg/kg b.w (0.2ml), 200mg/kg (0.4ml) and 400 mg/kg b.w (0.8ml) of the extract.

3.5 Acute Toxicity Assay

The acute toxicity test on aqueous extracts of Mondia whitei was determined by Lorke's method. A total of twelve rats were used in two different phases lasting 24 hours each. The 1st phase comprised of three groups named Group A, B and C. Each group had three rats. Group A received 10 mg/Kg b.w of the extract while group B received 100mg/Kg b.w and Group C received 1000 Mg/Kg B.W. The animals were monitored for a period of 24 hours to detect changes in behaviour, feeding, dullness and mortality. All the rats were monitored closely for signs of toxicity which are mortality, changes in gross appearance of the skin (piloerection) and fur, mucous membrane of the eye, respiratory distress, somatomotor activity, behaviour, and special attention was given to observation of tremors, salivation, diarrhoea, coma and convulsions, changes during the first 48 hours post dosing. The observation schedule was as follows; immediately, ½ an hour, 1 hour, 4 hour, 24 and 48 hours, the monitoring for signs of toxicity continued daily for 3 days. The body

weights were monitored as follows; day 0 (initial weight), day 3 and day4 (terminal weight).

Terminal sacrifice of all surviving rats done on 4th day (after 72hrs) following fasting them overnight and euthanizing them with carbon dioxide and gross necropsies were performed. All Organs and tissue were harvested, grossly examined.

Phase two comprised of three groups named Group D, E and F each group had one rat. Group D received 1600 mg/Kg B.W, group E received 2900 mg/Kg B.W and group F received 5,000 mg/Kg B.W. The animals were also monitored for dullness and mortality over a period of 24 hours.

Thereafter the animals were sacrificed for post mortem examination on major organs. Lethal dose (LD50) was calculated as per Lorke's method**.**

LD 50 = $\sqrt{D0 \ x \ D100}$

Where

(i) LD 50 - lethal dose at which 50% of the animals died

(ii) D0 – The highest dose administered to the animals without mortality witnessed

(iii)D100 - The lowest dose administered to the animals with mortality witnessed.

3.5.1 Administration of *Mondia whitei* extract on the test groups

The grouping of the thirty-six male albino rats into four of 9 rats each where those in Group I (control) were fed with normal rat feed and water *ad libitum* for 30 days. Test groups II, III, and IV were treated with 100mg, 200mg and 400mg per kilogram per day of the extract respectively and the normal rat feed and water *ad libitum* for 10 days, 15 days and 30 days respectfully as per the test group. The administration of the extract was done orally and on daily basis using gavage syringes between the hours of 8.00am and 9.00 am.

3.6 Phytochemical analysis

In the determination of phytochemical constituents of the extract of the *M. Whitei* the study adopted the procedure described by Tiwari *et al.*, (2011). The criteria for

grading were done on the basis of the intensity of colour produced from reactions observed in the test tubes and the amount of froathing. The reaction were denoted as follows; +++ for strong positive reaction, ++ for a positive reaction, + weak positive reaction and – for represented no observable reaction (Savithramma, Rao and Ankanna, 2015)

1. Test for Alkaloids (wagner's Reagent)

A fraction of the extract was treated with 3 -5 drops of Wagner's reagent (1.22 g of iodide and 2g of potassium iodide in 100 mls of water and observed for formation of brown residue (coloration).

2. Test for Flavonoids (Alkaline reagent test)

Two mls of extract was treated with few drops of 20% sodium hydroxide solution formation of intense yellow color, which then became colourless in addition of dilute HCL was observed.

3. Test for phenol (Ferric Chloride)

A fraction of the extract was treated with aqueous 5% ferric chloride and observed for a formation of deep blue or black color.

4. Test for Saponins (Foam test)

Two mls of the extract was added to 6 mls of water in a test tube. The mixture was shaken vigo rously and observed for the formations of persistent foam that confirms the presence of saponins.

5. Test for Tannins (Braymer's test)

Two mls of extract was treated with 10% alcoholic ferric chloride solution and observed for formation of blue or greenish color.

The detection of the active compounds in the plant extract was done as it was described by Harborne *et al* (1984)

3.7 Sample collection and Hormonal Assay

Blood samples were collected at the University of Eldoret Zoology lab on 10th, 15th and 30th day after treatment by cardiac puncture after anaesthetizing the rats with carbon dioxide narcosis and sacrificing them. Two ml of blood was collected from each rat. The samples were carefully introduced into plain vacutainers free from anticoagulant, properly labeled and transported immediately under room temperature to Moi Teaching and Referral Hospital Immunology laboratory where hormonal assay was done. The blood samples were allowed to clot, retract and then centrifuged for 5minutes at a speed of 3000 revolutions per minute. The serum was then aliquoted and, refrigerated at -20°C waiting assaying of the hormones.

3.7.1 Determination of Testosterone levels

1st Incubation (9 minutes):

This involved the incubation of 20 µL of the sample with a biotinylated monoclonal testosterone specific antibody and 2-bromoestradiol (to release testosterone) with the amount of antibody binding sites which was subsequently occupied depending on the concentration of testosterone in the sample.

2nd Incubation (9 minutes):

Addition of streptavidin-coated micro particles and a ruthenylated testosterone derivative were added to the reaction mixture and the binding of the complexes to the solid phase via biotin streptavidin interactions.

Measurement:

This involved transfer of the reaction mixture to a measuring cell and the magnetic capturing of the micro particles onto the surface of an electrode and any unbound sample was washed away before the induction of the chemiluminescent reaction through application of a voltage to the electrode. The measurement of chemiluminescence by a photomultiplier and the concentration of Testosterone within the sample were then calculated using a calibration curve.

3.7.2 Follicle stimulating hormone

1st Incubation (9 minutes):

This involved the incubation of 40 µL of the sample with both a biotinylated, monoclonal FSH-specific antibody and a ruthenylated, monoclonal FSH-specific antibody to form a sandwich complex.

2nd Incubation (9 minutes):

Then addition of streptavidin-coated micro particles to the reaction mixture binding the complex to the solid phase via biotin-streptavidin interactions.

Measurement

This involved transfer of the reaction mixture to a measuring cell and the magnetic capturing of the micro particles onto the surface of an electrode where unbound sample was washed away before induction of chemiluminescent reaction by applying a voltage to the electrode. The measurement of chemiluminescence was by a photomultiplier and the concentration of FSH within the sample was calculated using a calibration curve.

3.7.3 Luteinizing hormone

1st incubation (9 minutes)

This involved incubating20 µL of sample, a biotinylated monoclonal LH specific antibody, and a monoclonal LH specific antibody with a ruthenium complex that formed a sandwich complex.

2nd incubation (9 minutes)

Then addition of streptavidin-coated micro particles leads to the binding of the complex to the solid phase through interaction of biotin and streptavidin

Measurements

This involved the aspiration of the reaction mixture into the measuring cell where there was magnetic capturing of the micro particles onto the surface of the electrode. Unbound substances were then removed with Pro Cell/Pro Cell M. The application of a voltage to the electrode then leads to the induction of chemiluminescent emission which was measured by a photomultiplier.

Results

The determination of the results was through a calibration curve which was an instrument specifically generated by a 2 point calibration and a master curve provided through the reagent barcode or e barcode.

3.8 Epididymal sperm count, motility, vitality and morphology

This involved the removal of the caudal part of the epididymis and placing it in a beaker containing 1 ml physiological saline solution and allowing it to stand for few minutes to allow for the swimming of spermatozoa out of the solution. Sperm count was done under the microscope. The determination of the sperm count was done using the Neubauer's counting chamber as discussed by Saalu *et al.,* (2012). This involves the placement of few drops of semen on a slide followed by addition of two drops of eosin Y and the covering of the slide with cover slip and examination under the microscope using X40 objective for sperm morphology. The sperm concentration was then calculated.

The magnification of light microscope at X400 was used in the evaluation of sperm morphology. Five hundred sperm from the sample was scored for morphological abnormalities according to Ilbey *et al.,* (2009). Abnormal sperm morphology was considered to have the following features including detached head, round head and rudimentary tail and expressed as a percentage of normal sperm morphology.

Vitality characteristics of the isolated sperms were analyzed as per WHO laboratory manual in examining human semen (1999), this is a modified Blom's technique that uses a 2-step eosin-nigrosin technique in obtaining the dark background for contrast and yields reliable evaluation using ordinary microscope optics. The non-motile sperms were identified from other objects such as erythrocytes, dirt, spermatids or leukocytes, erythrocytes by their intensity and size. High and low gates for these characteristics were defined as factors of the mean size or intensity of the motile sperm.

3.9 Histological preparations of the epididymis and the testes

The preparation of tissues for histological studies was done at Moi University School of Medicine in the Anatomy/Histology lab as follows. It involved eight main stages: Preparation of tissues for histological studies involved Fixation, Dehydration,

Clearing, and Impregnation with paraffin wax, Sectioning, Staining and Mounting. Tissues were fixed by immersing in 10% Formalin for 24 hours. This was done to preserve the tissues and prevent autolysis. After fixation tissues were treated with increasing strengths of alcohol from 70%, 80%, 90% and two changes of absolute alcohol to dehydrate them. The dehydrated tissues were then treated with two changes of xylene for two hours in each, in order to remove alcohol and prepare them for infiltration with molten paraffin wax at 60^0C. Infiltration with molten paraffin wax was done by immersing tissues in molten paraffin wax for two hours, to increase the consistency of the tissues and to facilitate sectioning of thin slices for microscopy. The tissues were fitted into suitable cassettes which were attached to a rotary microtome and one cell thick sections made (approximately 3-5μm thick). The tissue sections were attached on to glass slides and incubated in an oven for one hour at $65\text{-}70^0$C to remove all the paraffin wax. The sections were then stained with Haematoxylin for twenty minutes and counterstained with Eosin for three to five minutes. The stained slides were mounted using DPX and studied under the microscope. Tissues were prepared for histological observation as previously described by (Cardiff *et al.*, 2014).

The sections were examined at low magnification (x400) and at high magnification (x1000) using an Olympus microscope (Japan) fitted with a Kodak camera. Several photomicrographs were taken in bright field.

3.10 Ethical considerations

Ethical clearance was obtained from the University of Eastern Africa Baraton Animal Ethical Committee (UEAB/10/11/2018) and National Commission for Science, Technology and Innovation (NACOSTI/P/19/81106/27253). The protocol followed Guidelines for Care and Use of Laboratory Rats in Biomedical Research, and the rats were only used once in the experiment. They were all sacrificed using humane end points at the end of the study (Leary *et al.*, 2013)**.**

3.11 Statistical analysis

Data entry was done using Microsoft excel and later exported to SPSS V.21 for analysis. Normality test was performed using the Shapiro wilks test with Ho being

that data follows the normal distribution. Since the data failed the normality test (skewed), it was summarized using median (IQR) and variance among the groups and across time was tested using the non-parametric alternative to Anova (Kruskal Wallis test). Trend analysis was done to establish significant changes in estimates with increase in time. Significance was set at $p<0.05$. Results were presented in terms of bar graphs and tables.

CHAPTER FOUR

4.0 RESULTS

4.1 Results on Acute Toxicity

The Lethal dose LD 50 of aqueous extracts of *Mondia Whitei* was above 5000mg/Kg and thus generally safe. There was no mortality experienced but dullness was witnessed in group E and F and not feeding well. There were gross pathological changes observed like liver congestion and lung hyperaemia but no bleeding or swelling on the brain, heart, during autopsy.

4.2 Mortality rate and behaviour observations in acute oral toxicity determination of *M. Whitei*

General behaviour of each animal was observed for the first 30 minutes, 1 hour, 4 hours, 24 hours, and 48 hour of acute toxicity were normal. They included pilo-erection, alertness, and muscle tone on hind limbs, pain and feeding activity. However respiratory system signs of distress were noted at >5000 mg in the first 24 hours of administration and subsided thereafter.

Table 4.1. Shows observations for signs of acute toxicity and mortality rate after administration of Mukombero

Experiment	**Doses (mg)**	**Observation in hours**							
		Immediate	0.5	1	4	24	48	**Mortality**	**Mortality rate (%)**
Phase I (Aqueous extract of M. whitei)	10	Normal activity	Normal activity	Normal Activity	Normal activity	Normal Activity	Normal activity	0/3	0
	100	Normal activity	Normal activity	Normal Activity	Normal activity	Normal Activity	Normal activity	0/3	0
	1000	Normal activity	Normal activity	Normal Activity	Normal activity	Normal Activity	Normal activity	0/3	0
Control	0	Normal	Normal	Normal	Normal	Normal	Normal	0/3	0
Phase II (Extracts of M. Whitei)	1600	Normal activity	Normal activity	Normal Activity	Normal activity	Normal Activity	Normal activity	0/1	0
	2900	N	N	N	N	N	Reduced activity	0/1	0
	5000	RD	RD	RD	RD	Normal activity	Reduced activity	0/1	0

Key: M-Mondia

N-Normal

RD- Respiratory distress

4.3 Post-mortem results of gross pathology findings on the rats organs during acute toxicity study

The following post-mortem results of gross pathology on the organ of the rats were observed after administration of extracts of *M. Whitei*, liver congestion and lung hyperaemia was revealed in animal administered with 5000 mg, While the other organs which includes the kidney, spleen, brain, prostate and testis there were no obvious gross pathology observed (Table 4.3.).

Table 4.2: Shows post mortem results of gross pathology findings on the rats organs against the various doses of Mukombero during acute toxicity study.

	Gross pathology results					
Organ	**Dose (mg/ kg body weight)**					
	10	**100**	**1000**	**1600***	**2900***	**5000***
Kidney	None	None	None	None	None	None
Lungs	None	None	None	None	None	Hyperaemia
Liver	None	None	None	None	None	Liver congestion
Spleen	None	None	None	None	None	None
Brain	None	None	None	None	None	None
Prostate	None	None	None	None	None	None
Testis	None	None	None	None	None	None

Key: n=3

**Dose groups with single rat per group*

4.4 Effect of *M. Whitei* aqueous extracts on testosterone serum concentration after 10, 15 and 30 days and between treatments of 100(mg/kg), 200(mg/kg) and 400(mg/kg) and negative controls

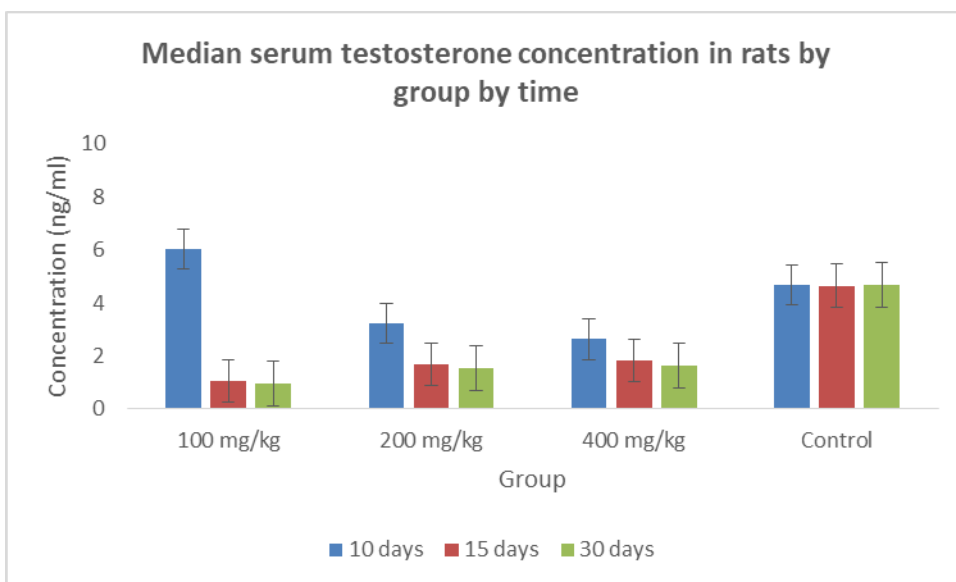


Figure 4.1: Effect of *M. whitei* aqueous extracts (100,200 and 400 mg/kg) for 10, 15 and 30 days on serum testosterone (ng/ml) concentration in albino rats.

Table 4.3 shows comparison in serum testosterone concentrations in rats treated with *M. whitei* extract and negative control rats after 10, 15 and 30 days and between treatments of 100(mg/kg), 200(mg/kg) and 400(mg/kg) and negative controls.

Table4.3: Effect of M. whitei aqueous extracts (100,200 and 400 mg/kg) for 10, 15 and 30 days on serum testosterone concentration in rats

Treatment	**Testosterone concentration ng/ml Median (IQR)**			**Chi-value**	**p-value**
Rat Groups	**10 days**	**15 days**	**30 days**		
100 (mg/kg)	6.02(6.01, 6.05)	2.06(1.04, 1.09)	0.96(0.95, 0.99)	6.231	0.024
200 (mg/kg)	3.22(3.21, 3.23)	1.67(1.65, 1.72)	1.52(1.49, 1.56)	4.220	0.037
400 (mg/kg)	2.63(2.60, 2.68)	1.83(1.80, 1.84)	1.63(1.60, 1.64)	7.200	0.019
Control	4.68(4.67, 4.69)	4.65(4.64, 4.70)	4.67(4.66, 4.67)	1.747	0.417
Chi-value	10.385	9.335	10.221		
P-value	0.016	0.026	0.015		

*IQR= Interquartile range

After 10 days of treatment, the median (IQR) serum testosterone in *M. whitei* treated rats with 100, 200, 400 mg/kg and controls were 6.02 (6.01, 6.05), 3.22 (3.21, 3.23), 2.63 (2.60, 2.68) and 4.68 (4.67, 4.69) ng/ml respectively. The Kruskal Wallis test indicated that the difference in serum testosterone concentration (ng/ml) between the groups was statistically significant (chi=10.385, p=0.016), (chi=9.335, p=0.026) and (chi=10.221, p=0.015) at 10days, 15 days and 30 days respectively. Post hoc test (LSD) indicated that the difference existed between the rats administered with 100 mg/kg and 200mg/kg, 400 mg/kg as well as the control

($p<0.001$), between 200 mg/kg and 400 mg/kg ($p=0.001$), 200 mg/kg and the controls ($p<0.001$), between 400 mg/kg and control ($p<0.001$). A similar trend was also observed after 15 days as wells 30 days.

A significant difference in serum testosterone concentration (ng/ml) was observed within the groups with respect to time interval (all $p<0.05$). Within the group administered with 100 mg/kg, the significant difference in serum testosterone was observed between 10th day and 15th day, 10thday and 30th day as well as 15th day and

30th day (all $p<0.001$). Trend analysis indicated that within the groups, testosterone concentration decreased significantly with in time (all $p<0.05$)

4.5 Effect of *M. Whitei* aqueous extracts on LH concentration after 10, 15 and 30 days and between treatments of 100(mg/kg), 200(mg/kg) and 400(mg/kg) and negative controls

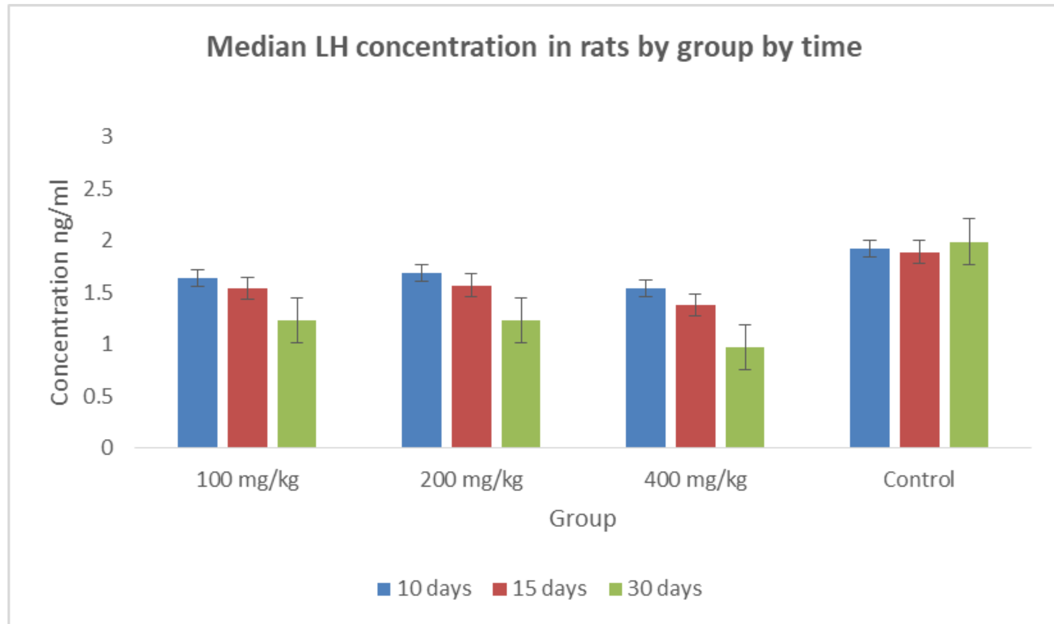


Figure 4.2: Effect of M. whitei aqueous extracts (100,200 and 400 mg/kg) for 10, 15 and 30 days on LH (ng/ml) concentration in albino rats.

Table 4.2 indicates comparison in LH concentrations in rats treated with *M. whitei* extract and negative control rats after 10, 15 and 30 days and between treatments of 100 (mg/kg), 200(mg/kg) and 400(mg/kg) and negative controls.

Table4.4: Effect of M. whitei aqueous extracts (100,200 and 400 mg/kg) for 10, 15 and 30 days on LH concentration in rats

Treatment	**LH concentration ng/ml Median (IQR)**			**Chi-**	**p-**
Rat Groups	**10 days**	**15 days**	**30 days**	**value**	**value**
100 (mg/kg)	1.64(1.63,1.66)	1.54(1.52,1.57)	1.23(1.22,1.27)	7.200	0.027
200 (mg/kg)	1.69(1.67,1.71)	1.57(1.55,1.58)	1.23(1.17, 1.28)	6.453	0.042
400 (mg/kg)	1.54(1.53, 1.58)	1.38(1.35, 1.42)	0.97(0.97, 0.99)	9.234	0.029
Control	1.92(1.89, 1.93)	1.89(1.89, 1.90)	1.99(1.88, 2.06)	1.195	0.550
Chi-value	10.385	9.877	9.412		
P-value	0.016	0.020	0.024		

*IQR= Interquartile range

After 10 days of treatment, the median (IQR) LH in *M. whitei* treated rats with 100, 200, 400 mg/kg and controls were 1.64 (1.63, 1.66), 1.69 (1.67, 1.71), 1.54 (1.53, 1.58) and 1.92 (1.89, 1.93) ng/ml respectively. The Kruskal Wallis test indicated that the difference was statistically significant (chi=10.385, p=0.016), (chi=9.877, p=0.020) and (chi=9.412, p=0.024) at 10 days, 15 days and 30 days respectively. Post hoc test (LSD) indicated that the difference existed between the rats administered with 100 mg/kg, 200mg/kg, 400 mg/kg and the control (p<0.001), between 100 mg/kg and 400 mg/kg (p=0.041). A similar trend was observed after 15 days as wells 30 days.

A significant difference in LH levels was observed within the groups with respect to time interval (all p<0.05). Within the group administered with 100 mg/kg, the significant difference in LH was observed between 10th day and 15th day, 10th day and 30th day as well as 15th day and 30th day (all p<0.05). Trend analysis indicated that, within the groups, LH decreased significantly with time (all p<0.05)

4.6 Effect of *M. Whitei* aqueous extracts on FSH concentration after 10, 15 and 30 days and between treatments of 100(mg/kg), 200(mg/kg) and 400(mg/kg) and negative controls

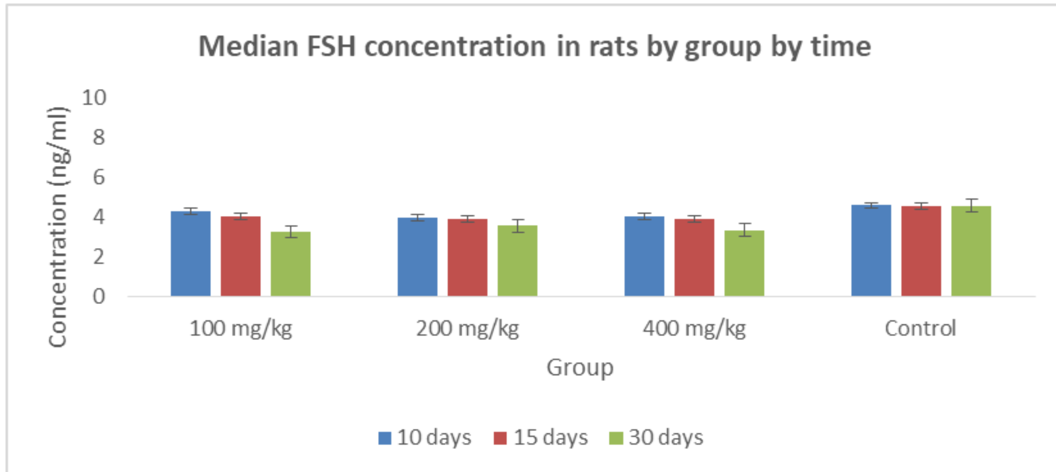


Figure 4.3: Effect of M. whitei aqueous extracts (100,200 and 400 mg/kg) for 10, 15 and 30 days on FSH (ng/ml) concentration in albino rats.

Table 4.3 indicates comparison in FSH concentrations in rats treated with *M. whitei* extract and negative control rats after 10, 15 and 30 days and between treatments of 100(mg/kg), 200(mg/kg) and 400(mg/kg) and negative controls.

Table 4.5: Effect of M. whitei aqueous extracts (100,200 and 400 mg/kg) for 10, 15 and 30 days on FSH concentration in rats

Treatment	**FSH concentration ng/ml Median (IQR)**			**Chi-value**	**p-value**
Rat Groups	**10 days**	**15 days**	**30 days**		
100 (mg/kg)	4.3(4.28, 4.31)	4.03(4.0, 4.04)	3.28(3.27, 3.29)	7.238	0.037
200 (mg/kg)	3.96(3.95, 3.99)	3.89(3.86, 3.92)	3.56(3.54, 3.59)	8.310	0.016
400 (mg/kg)	4.02(4.0, 4.04)	3.92(3.89, 3.94)	3.35(3.31, 3.37)	8.500	0.028
Control	4.59(4.55, 4.59)	4.57(4.55, 4.60)	4.58(4.58, 4.70)	0.707	0.702
Chi-value	10.421	9.735	9.422		
P-value	0.015	0.021	0.025		

After 10 days of treatment, the median (IQR) FSH in *M. whitei* treated rats with 100, 200, 400 mg/kg and controls were 4.3 (4.28, 4.31), 3.96 (3.95, 3.99), 4.02 (4.0, 4.04) and 4.59 (4.55, 4.59) ng/ml respectively. The Kruskal wallis test indicated that the difference was statistically significant (chi=10.421, p=0.015), (chi=9.735, p=0.021) and (chi=9.422, p=0.025) at 10 days, 15 days and 30 days respectively. Post hoc test (LSD) indicated that the difference existed between the rats administered with 100 mg/kg, 200 mg/kg, 400 mg/kg and the control (p<0.001), between 100 mg/kg and 400 mg/kg (p<0.001). A similar trend was observed after 15 days as wells 30 days.

A significant difference in FSH was observed within the groups with respect to time interval (all p<0.05). Within the group administered with 100 mg/kg, the significant difference in FSH was observed between 10th day and 15th day, 10th day and 30th day as well as 15th day and 30th day (all p<0.05). Trend analysis indicated that within the groups, FSH decreased significantly with increase in time (all p<0.05)

4.7 Effect of *M. Whitei* aqueous extracts on sperm count concentration after 10, 15 and 30 days and between treatments of 100(mg/kg), 200(mg/kg) and 400(mg/kg) and negative controls

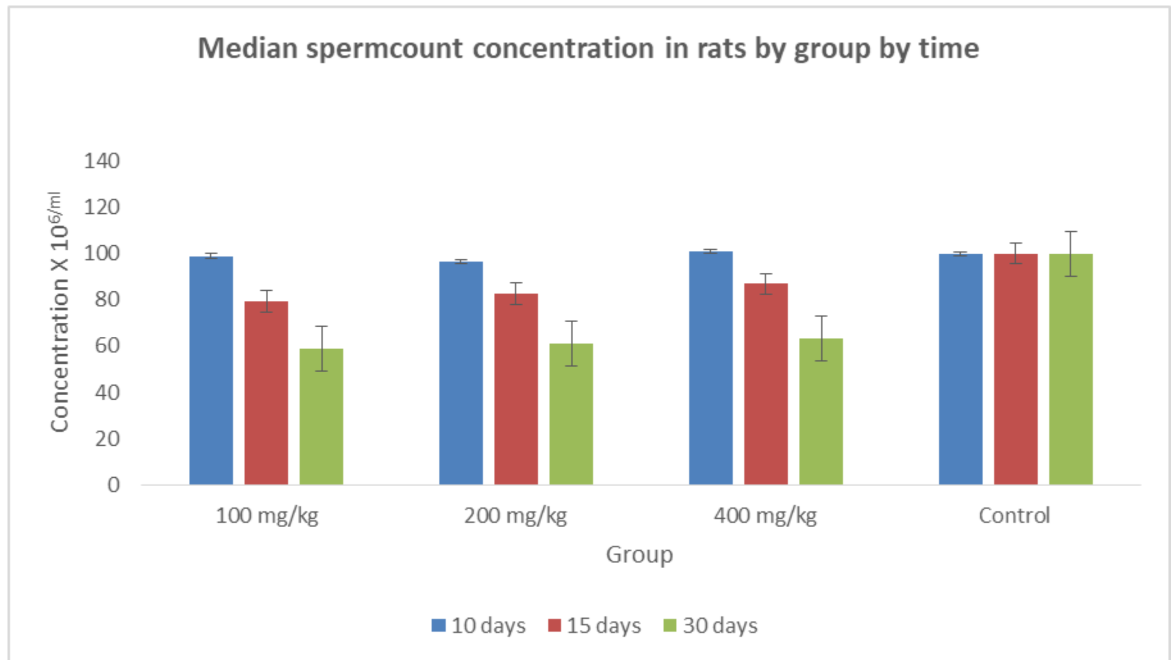


Figure 4.4: Effect of M. whitei aqueous extracts (100,200 and 400 mg/kg) for 10, 15 and 30 days on sperm count (x1000/ml) concentration in albino rats.

Table 4.4 indicates comparison in sperm count concentrations in rats treated with *M. whitei* extract and negative control rats after 10, 15 and 30 days and between treatments of 100(mg/kg), 200(mg/kg) and 400(mg/kg) and negative controls.

Table 4.6: Effect of M. whitei aqueous extracts (100,200 and 400 mg/kg) for 10, 15 and 30 days on sperm count concentration in rats

Treatment	**Sperm count concentration x10^6/ml Median (IQR)**			**Chi-**	**p-**
Rat Groups	**10 days**	**15 days**	**30 days**	**value**	**value**
100 (mg/kg)	99(98, 101)	79.29(79.18, 79.51)	58.88(58.80, 58.93)	10.211	0.011
200 (mg/kg)	96.66(96.65, 96.68)	82.69(82.48, 82.83)	60.90(60.79, 60.98)	7.242	0.026
400 (mg/kg)	100.98(100.88, 101.47)	87(86, 91)	63.16(63.01, 63.20)	9.210	0.029
Control	100.03(100.03,100.04)	100.02(100.02, 100.05)	100.03(100.02, 100.03)	1.208	0.547
Chi-value	8.157	11.455	10.421		
P-value	0.043	0.010	0.015		

* IQR= Interquartile range

After 10 days of treatment, the median (IQR) sperm count in *M. whitei* treated rats with 100, 200, 400 mg/kg and controls were 98 (98, 101), 96.66 (96.65, 96.68), 100.98 (100.88, 101.47) and 100.03 (100.03, 100.04) concentration x 10^6/ml respectively. The Kruskal wallis test indicated that the difference was statistically significant (chi=8.157, p=0.043), (chi=8.157, p=0.010), (chi=10.421, p=0.015) at 10 days, 15 days and 30 days respectively. Post hoc test (LSD) indicated that the difference existed between the rats administered with 100 mg/kg, 200 mg/kg and 400 mg/kg (p<0.05), between 200 mg/kg and 400 mg/kg (p<0.001). A similar trend was observed after 15 days as wells 30 days.

A significant difference in sperm count was also observed within the groups with respect to time interval (all p<0.05). Among the group administered with 100 mg/kg, the significant difference in sperm count was observed between 10th day and 15th day, 10th day and 30th day as well as 15thday and 30th day (all p<0.001). Trend analysis indicated that within the groups, sperm count decreased significantly with increase in time (all p<0.05).

4.8 Effect of *M. Whitei* aqueous extracts on percentage motility concentration after 10, 15 and 30 days and between treatments of 100 (mg/kg), 200 (mg/kg) and 400 (mg/kg) and negative controls

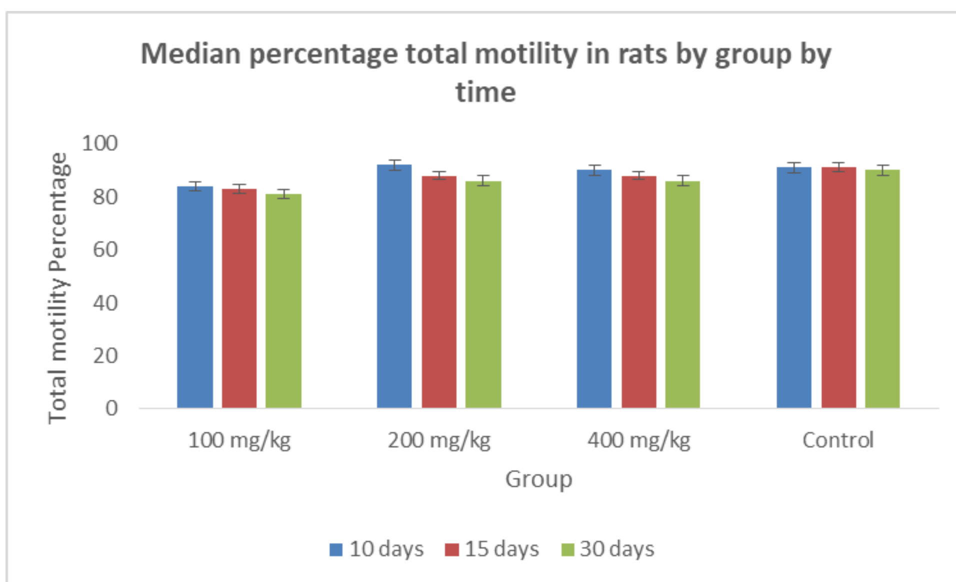


Figure 4.5: Effect of M. whitei aqueous extracts (100,200 and 400 mg/kg) for 10, 15 and 30 days on total motility (%) in albino rats.

Table 4.5 indicates comparison in percentage motility concentrations in rats treated with *M. whitei* extract and negative control rats after 10, 15 and 30 days and between treatments of 100(mg/kg), 200(mg/kg) and 400(mg/kg) and negative controls.

Table 4.7: Effect of M. whitei aqueous extracts (100,200 and 400 mg/kg) for 10, 15 and 30 days on total motility (percentage) in rats

Treatment	Total Motility(percentage) Median (IQR)			Chi-value	p-value
Rat Groups	**10 days**	**15 days**	**30 days**		
100 (mg/kg)	84(81, 85)	83(81, 84)	81(80, 82)	2.713	0.258
200 (mg/kg)	92(91, 93)	88(87, 93)	86(84, 88)	4.497	0.106
400 (mg/kg)	90(88, 90)	88(84, 89)	86(81, 88)	3.988	0.136
Control	91(90, 92)	91(90, 91)	90(90, 91)	1.147	0.564
Chi-value	7.868	9.787	8.046		
P-value	0.049	0.02	0.045		

After 10 days of treatment, the median (IQR) percentage motility concentration in *M. whitei* treated rats with 100, 200, 400 mg/kg and controls were 84(81, 85), 92(91, 93), 90(88, 90) and 91(90, 92) respectively. The Kruskal wallis test indicated that the difference was statistically significant (chi=7.686, p=0.049), (ch=9.787, p=0.02) and (chi=8.046, p=0.045) at 10 days, 15 days and 30 days respectively. Post hoc test (LSD) indicated that the difference existed between the rats administered with 100 mg/kg, 200 mg/kg, 400 mg/kg and the control (p<0.001), between 100 mg/kg and 400 mg/kg (p=0.041). A similar trend was observed after 15 days as wells 30 days.

A significant difference in total percentage motility was observed within the groups with respect to time interval (all p<0.05). Within the group administered with 100 mg/kg, the significant difference in motility was observed between 10th day and 15th day, 10th day and 30th day as well as 15th day and 30th day (all p<0.001). Within the groups, even though percentage motility decreased with increase in time, trend analysis indicated that it was not statistically significant (all p>0.05)

4.9 Effect of *M. Whitei* aqueous extracts on Vitality concentration after 10, 15 and 30 days and between treatments of 100(mg/kg), 200(mg/kg) and 400(mg/kg) and negative controls.

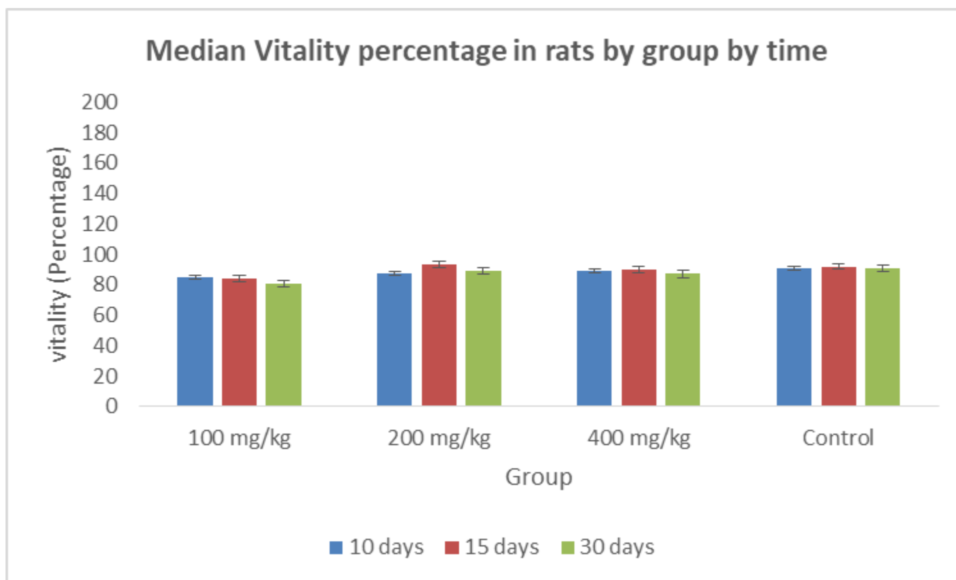


Figure 4.6: Effect of M. whitei aqueous extracts (100,200 and 400 mg/kg) for 10, 15 and 30 days on Vitality (percentage) in albino rats.

Table 4.6 indicates comparison in percentage vitality in rats treated with *M. whitei* extract and negative control rats after 10, 15 and 30 days and between treatments of 100(mg/kg), 200(mg/kg) and 400(mg/kg) and negative controls.

Table 4.8: Effect of M. whitei aqueous extracts (100,200 and 400 mg/kg) for 10, 15 and 30 days on percentage Vitality in rats

Treatment	**Vitality Percentage Median (IQR)**			**Chi-value**	**p-value**
Rat Groups	**10 days**	**15 days**	**30 days**		
100 (mg/kg)	85(82, 86)	84(80, 85)	81(80, 82)	3.282	0.194
200 (mg/kg)	87(85, 89)	93(92, 94)	89(87, 94)	4.171	0.124
400 (mg/kg)	89(86, 90)	90(88, 92)	87(82, 89)	2.734	0.255
Control	91(90, 91)	92(90, 92)	91(91, 91)	1.333	0.513
Chi-value	8.286	9.000	8.372		
P-value	0.040	0.029	0.033		

After 10 days of treatment, the median (IQR) percentage vitality in *M. whitei* treated rats with 100, 200, 400 mg/kg and controls were 85 (82, 86), 87 (85, 89), 89 (86, 90) and 91 (90, 91) percentage respectively. The Kruskal Wallis test indicated that the difference was statistically significant (chi=8.286, p=0.040), (chi=9.000, p=0.029) and (chi=8.372, p=0.033) at 10 days, 15 days and 30 days respectively. Post hoc test (LSD) indicated that the difference existed between the rats administered with 100 mg/kg, 200mg/kg, 400 mg/kg and the control ($p<0.001$), between 100 mg/kg and 400 mg/kg ($p<0.001$). A similar trend was observed after 15 days as wells 30 days.

A significant difference in percentage vitality was observed within the groups with respect to time interval (all $p<0.05$). Within the group administered with 100 mg/kg, the significant difference in percentage vitality was observed between 10th day and 15th day, 10th day and 30thday as well as 15th day and 30th day (all $p<0.05$). Trend analysis indicated that within the groups, vitality did not vary significantly with time (all $p>0.05$)

4.10 Effect of *M. whitei* aqueous extracts (100,200 and 400 mg/kg) for 10, 15 and 30 days on normal morphology percentage in rats

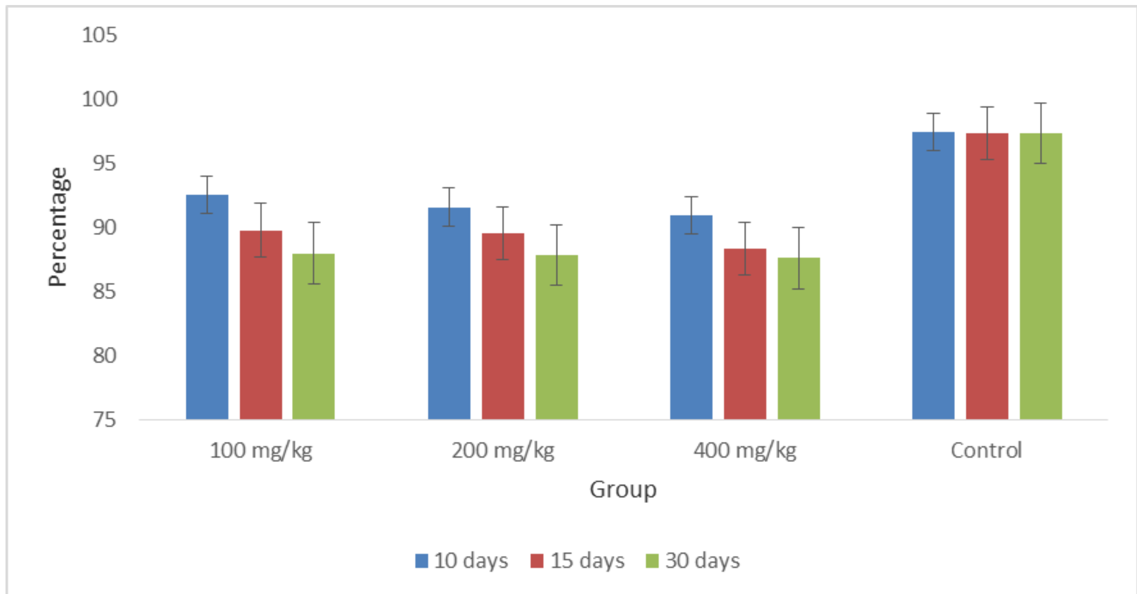


Fig 4.7: Effect of *M. whitei* aqueous extracts (100,200 and 400 mg/kg) for 10, 15 and 30 days on normal morphology percentage in rats

Table 4.9: Effect of M. whitei aqueous extracts (100,200 and 400 mg/kg) for 10, 15 and 30 days on normal morphology percentage in rats

Treatment Rat Groups	Normal morphology percentage Median (IQR) 10 days	15 days	30 days	Chi-value	p-value
100 (mg/kg)	92.54(92.48, 92.59)	89.81(89.76, 89.83)	88.01(88.0, 88.06)	6.212	0.022
200 (mg/kg)	91.61(91.56, 91.62)	89.57(89.52, 89.58)	87.87(87.85, 87.94)	7.200	0.027
400 (mg/kg)	90.99(90.98, 90.99)	88.34(88.32, 88.37)	87.62(87.60, 87.64)	7.261	0.033
Control	97.45(97.42, 97.46)	97.40(97.32, 97.47)	97.39(97.01, 97.42)	2.711	0.258
Chi-value	10.421	9.385	7.325		
P-value	0.015	0.016	0.020		

After 10 days of treatment, the median (IQR) normal morphology in *M. whitei* treated rats with 100, 200, 400 mg/kg and controls were 92.54 (92.48, 92.59), 91.61 (91.56, 91.62), 90.99 (90.98, 90.99) and 97.45 (97.42, 97.46) in percentage respectively. The Kruskal wallis test indicated that the difference was statistically significant (chi=10.421, p=0.015), (chi=9.385, p=0.016) and (chi=7.325, p=0.020) at 10 days, 15 days and 30 days respectively. Post hoc test (LSD) indicated that the difference existed between the rats administered with 100 mg/kg, 200 mg/kg, 400 mg/kg and the control (p<0.001), between 100 mg/kg and 400 mg/kg (p<0.001). A similar trend was observed after 15 days as wells 30 days.

A significant difference in normal morphology was observed within the groups with respect to time interval (all p<0.05) except for the controls (p=0.258). Within the

group administered with 100 mg/kg, the significant difference was observed between 10th day and 15th day, 10th day and 30th day as well as 15th day and 30th day (all $p<0.05$). Trend analysis indicated that within the groups, normal morphology decreased significantly with increase in time (all $p<0.05$)

4.11 Effect of *M. whitei* aqueous extracts (100,200 and 400 mg/kg) for 10, 15 and 30 days on abnormal head morphology percentage in rats

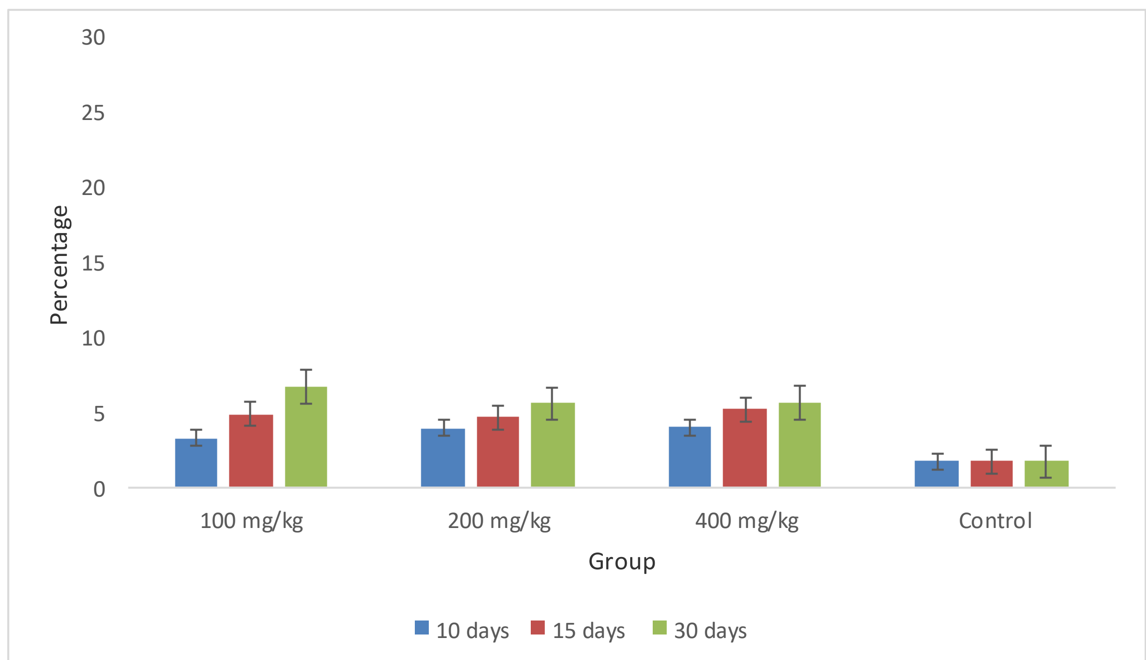


Fig 4.8: Effect of *M. whitei* aqueous extracts (100,200 and 400 mg/kg) for 10, 15 and 30 days on abnormal head morphology percentage in rats

Table 4.10: Effect of M. whitei aqueous extracts (100,200 and 400 mg/kg) for 10, 15 and 30 days on abnormal head morphology percentage in rats

Treatment	Head morphology concentration ng/ml Median (IQR)			Chi-	p-
Rat Groups	**10 days**	**15 days**	**30 days**	**value**	**value**
100 (mg/kg)	3.27(3.23, 3.9)	4.9(4.86, 4.92)	6.74(5.73, 5.80)	9.222	0.027
200 (mg/kg)	3.93(3.91, 3.95)	4.71(4.69, 4.73)	5.61(5.59, 5.63)	5.200	0.021
400 (mg/kg)	4.02(3.98, 4.04)	5.22(5.15, 5.24)	5.65(5.62, 5.68)	5.220	0.023
Control	1.73(1.70, 5.60)	1.74(1.70, 5.62)	1.74(1.73, 1.76)	0.274	0.872
Chi-value	4.446	4.866	9.974		
P-value	0.183	0.191	0.019		

After 10 days of treatment, the median (IQR) abnormal head morphology in *M. whitei* treated rats with 100, 200, 400 mg/kg and controls were 3.27(3.23, 3.29), 3.93(3.91, 3.95), 4.02(3.98, 4.04) and 1.73(1.70, 5.60) percentage respectively. The Kruskal wallis test indicated that the difference was not statistically significant (chi=4.846, p=0.183), (chi=4.866, p=0.191) and (chi=9.974, p=0.019) at 10 days, 15 days and 30 days respectively. However, a significant difference in head morphology was observed within the groups with respect to time interval (all $p<0.05$) except for the controls (p=0.872). Among the group administered with 100 mg/kg, the significant difference was observed between 10th day and 15th day, 10th day and 30th day as well as 15th day and 30th day (all $p<0.05$). Trend analysis indicated that within the groups, abnormal head morphology increased significantly with increase in time (all $p<0.05$)

4.12 Effect of *M. whitei* aqueous extracts (100,200 and 400 mg/kg) for 10, 15 and 30 days on abnormal tail morphology percentage in rats

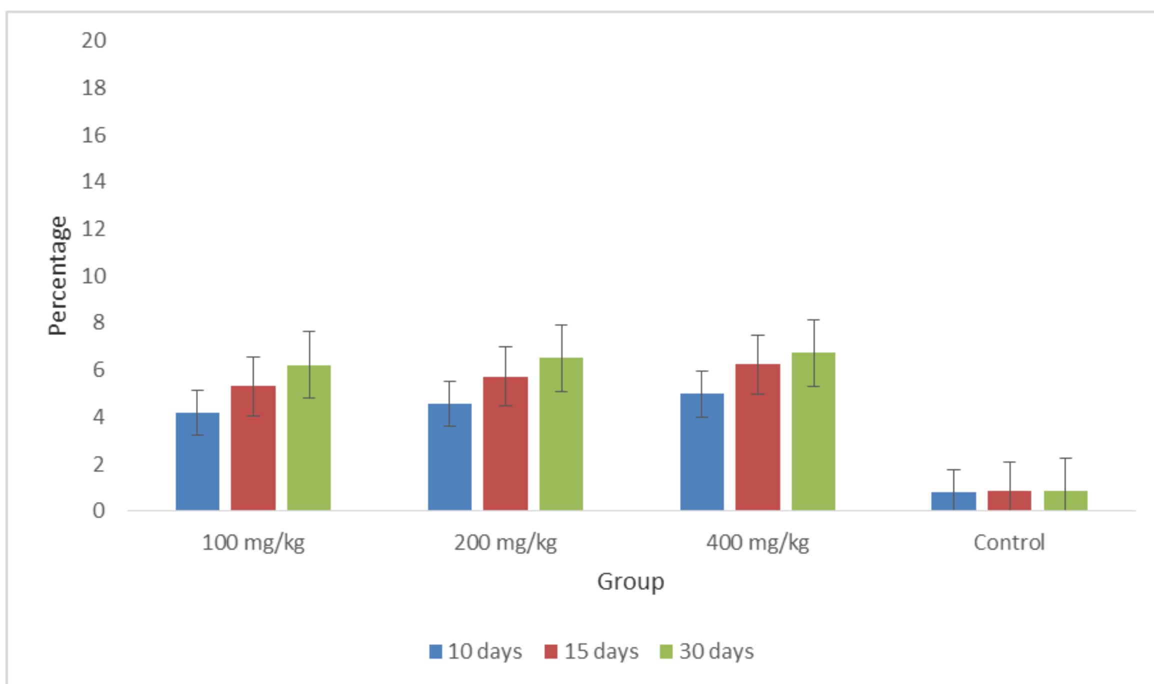


Fig 4.9: Effect of *M. whitei* aqueous extracts (100,200 and 400 mg/kg) for 10, 15 and 30 days on abnormal tail morphology percentage in rats.

Table 4.11: Effect of M. whitei aqueous extracts (100,200 and 400 mg/kg) for 10, 15 and 30 days on abnormal tail morphology percentage in rats

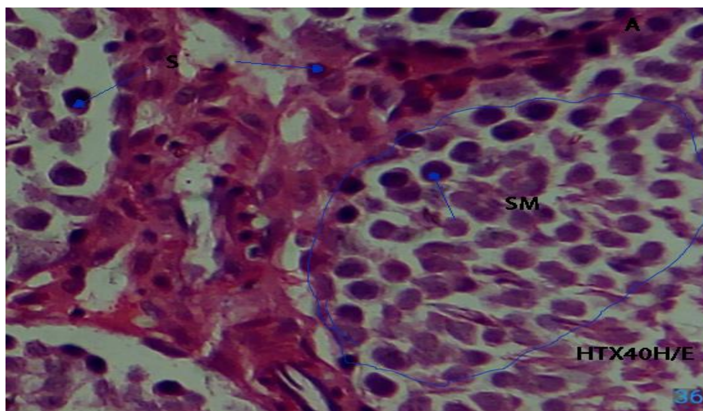
Treatment	Abnormal tail morphology in percentage Median (IQR)			Chi-	p-
Rat Groups	**10 days**	**15 days**	**30 days**	**value**	**value**
100 (mg/kg)	4.19(4.16, 4.24)	5.30(5.28, 5.34)	6.22(6.18, 6.25)	9.201	0.002
200 (mg/kg)	4.57(4.53, 4.59)	5.73(5.70, 5.76)	6.50(6.48, 6.52)	7.200	0.025
400 (mg/kg)	4.97(4.96, 5.06)	6.28(6.2s7, 6.30)	6.73(6.72, 6.74)	7.261	0.027
Control	0.80(0.77, 0.86)	0.83(0.78, 0.84)	0.83(0.78, 0.86)	0.205	0.903
Chi-value	11.385	12.300	7.315		
P-value	0.016	0.010	0.042		

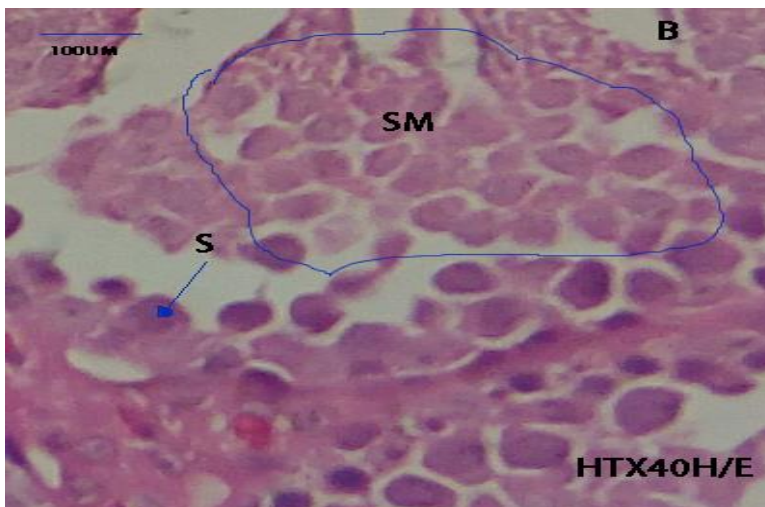
After 10 days of treatment, the median (IQR) abnormal tail morphology in *M. whitei* treated rats with 100, 200, 400 mg/kg and controls were 4.19 (4.16, 4.24), 4.57 (4.53, 4.59), 4.97 (4.96, 5.06) and 0.8 (0.77, 0.86) percentage respectively. The Kruskal wallis test indicated that the difference was statistically significant (chi=10.385, p=0.016), (chi=12.300, p=0.010) and (chi=7.315, p=0.042) at 10 days, 15 days and 30 days respectively. Post hoc test (LSD) indicated that the difference existed between the rats administered with 100 mg/kg, 200 mg/kg, 400 mg/kg and the control (p<0.001), between 100 mg/kg and 400 mg/kg (p<0.001). A similar trend was observed after 15 days as wells 30 days.

A significant difference in abnormal tail morphology was observed within the groups with respect to time interval (all p<0.05) except for the controls (p=0.903). Among the group administered with 100 mg/kg, the significant difference was observed between 10th day and 15thday, 10th day and 30th day as well as 15th day and 30th day (all p<0.05). Trend analysis indicated that within the groups, abnormal tail morphology increased significantly with increase in time (all p<0.05)

4.13 Effects of *Mondia whitei* "mukombero" aqueous extracts on the male albino testicular histology

The Changes in the male gonadal histology following treatments with aqueous extracts of *mondia whitei* are shown in plate's 4.1 i-4.1vi. Sections of testes from the control rats (Plate 4.1i), demonstrated seminiferous tubules of normal sizes with clear outlines. Spermatogonia were also clearly evident in them (arrow). Those from treated rats (Plate 4.1ii arrow); seminiferous tubules that had thin distorted outlines and many intercellular spaces within them. Although spermatogonia were observed in both treated and untreated sections, the latter clearly showed few spermatozoa that were even clamped together and an arrest in further development as evidenced by a reduction in the number of cell layers and distortion in the cells.





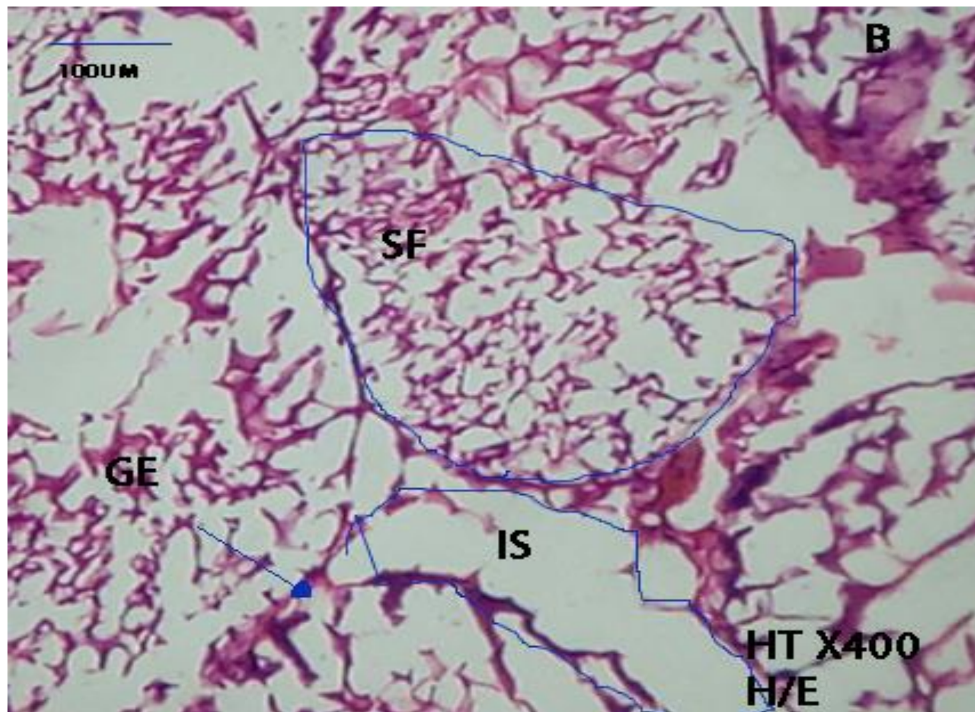


Plate 4.1 (i) and 4.1 (ii) - Histological examination of seminiferous tubules in the untreated control (a) and treated (b) . Note regularly shaped seminiferous tubules with clear spermatogonal layer, few intercellular spaces and narrow lumens with spermatozoa, (arrow) in control (a). Enlarged seminiferous tubules with large lumens having scanty spermatozoa and many intercellular spaces, (arrow) are visible in the treated (b).
GE-germinal epithelium, IS-intersitial space,SM-spermatogenesis,

Leydig cells of control plate (4.1iii) rats (showed normal sizes typical of normal male rats while those of treated rats, were enlarged, ruptured and contained prominent lipid droplets and with many intercellular spaces (4.1 iv). Spermatogenesis in adjoining seminiferous tubules showed arrested development. This was evident in the number of cell layers characteristic of normal spermatogenesis, in that instead of the normal four to five cell thickness, these showed one to two cell thicknesses. Some of the leydig cells appeared to be clumping together (plate 4.1iv).

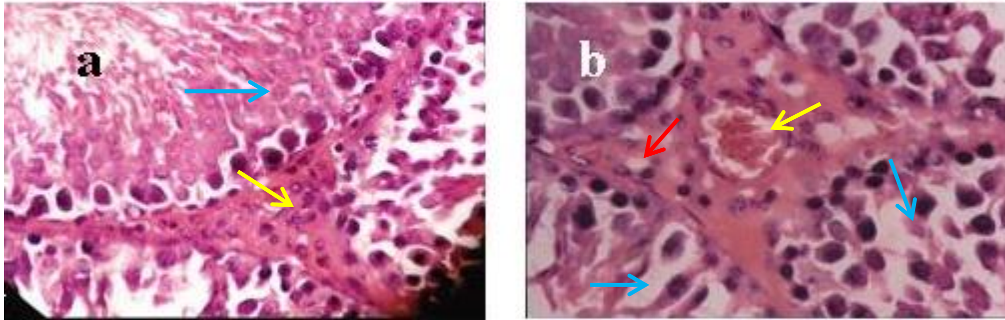
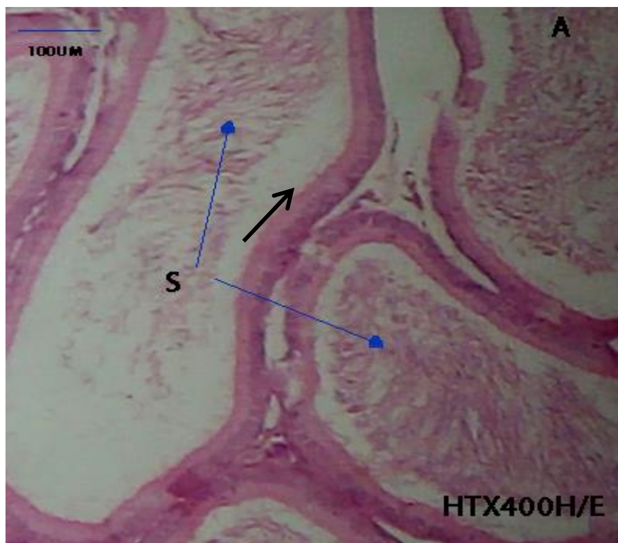


Plate 4.1 (iv): Histological examination of leydig cells in the untreated control (a) and in the treated cells (b) (H and E x1000).Leydig cells lacking fat deposits visible in control yellow arrow and with nomal sperm development (light blue arrow) (a) whereas clumping of cells many intercellular spaces, (red arrow) and fat deposits (yellow arrow) were visible in the treated (b). Also note large intercellular spaces and arrested development of sperms in neighbouring seminiferous tubules (light blue arrows),

Epididymis of testes from control rats showed clear normal outlines(black arrow) and their lumens were filled with sperm (blue arrow), (plate 4.1v) while those of testes from treated rats, showed large empty cells (blue arrows) with thin broken outlines, and detachment from adjoining cells (blue arrows) (4.1vi).



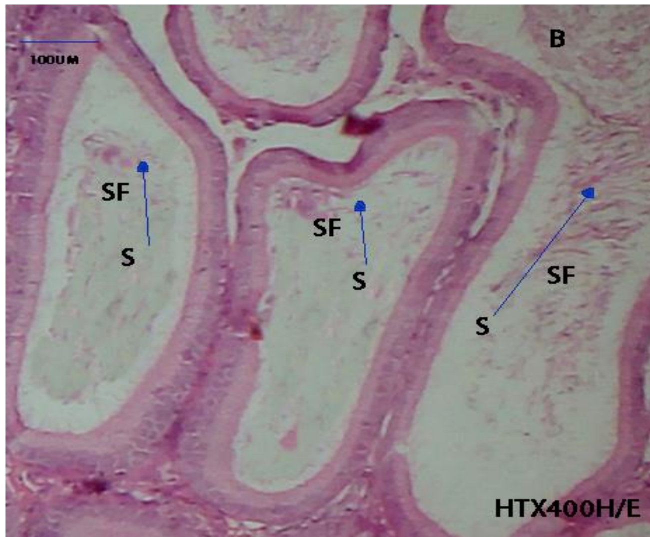


Plate 4.1(v)&(vi) : Histological examination of cross sections of epididymis in the untreated control (a) and in the treated rats (b) (H and E x400). SF(seminiferous), S(spermatozoa)
Note clear outlines of epididymal cells (black arrows), and lumens filled with spermatozoa(blue arrow) in the control (a) and enlarged cells with distorted outline(blue arrows) and large empty lumens in the treated (blue arrows) (b).

4.13.1Correlation Analysis

Relationship between's factors is a proportion of how factors are connected. The most widely recognized proportion of connection in insights is the Pearson Correlation (actually called the Pearson Product Moment Correlation or PPMC), which demonstrates the direct connection between two factors. Results are between - 1 and 1, if the value of R is close to one, then it shows there is a strong correlation between the variables. If the value of R is close to zero, then the correlation is weak. A result of - 1 implies that there is a perfect negative linear relationship between's the two qualities while r value of 1 implies that there is a perfect linear positive correlation between's the two factors. A value 0 implies that there is no relationship between's the two factors (Gujarat, 2004).

Table 4.12: Correlation between sperm count, vitality and morphology and the respective hormones.

	Testosterone(ng/ml)	FSH(ng/ml)	LH(ng/ml)
Sperm count ($\times 10^3$)	0.764	0.643	0.459
Sperm vitality (%)	0.876	0.597	0.403
Morphology of spermatozoa (%)	0.634	0.407	0.518

Table 4.7 shows positive correlation (0.764) between Sperm count ($\times 10^3$) and Testosterone hormone, there was a strong positive correlation (0.876) between sperm vitality (%) and Testosterone hormone, a positive correlation between Morphology of spermatozoa (%) and Testosterone hormone.

The relationship between sperm parameters and FSH hormone was also recorded. Positive correlation (0.643) was observed between Sperm count ($\times 10^6$) and FSH, a positive correlation (0.597) was observed between sperm vitality (%) and FSH. Also, positive correlation (0.407) was observed between Morphology of spermatozoa (%) and FSH. The relationship between sperm parameters and LH was investigated. Positive correlation (0.459) was observed between Sperm count ($\times 10^6$) and LH, a positive correlation (0.403) was observed between sperm vitality (%) and FH. Also, positive correlation (0.518) was observed between Morphology of spermatozoa (%) and LH.

4.14 Active compounds detected in *Mondia whitei* extracts.

The table below shows bioactive compounds of aqueous extract of *Mondia whitei* that were detected.

Table 4:13 phytochemical detected in aqueous extract of Mondia Whitei

Active Compounds	Reagents	Indication	Results
Alkaloids	Wagner’s reagent	Brown residue	Positive
Flavonoids	Alkaline reagent	Yellow color changes to colourless	Positive
Phenols	Ferric chloride test	Deep blue color	Positive
Saponins	Form test	Persistent foam	Positive
Tannins	Braymer’s test	Formation of blue or greenish color	Negative

CHAPTER FIVE

DISCUSSION, CONCLUSION AND RECOMMENDATION

5.0 DISCUSSION

5.1 The Acute Oral Toxicity of the M. Whitei

The acute oral toxicity of the Mukombero study showed no mortality for the entire period of 4 days even after administration of the highest dose of *M.Whitei* extracts which was 5000 mg/kg body weight. This concurs with the findings of study done by Aremu *et al.,* (2011) which showed no mortality during the entire period of fourteen days. In addition, the study also found that there was respiratory distress at a dose of 5000 mg/kg bwt in the first 24 hour which disagree with a study done by Aremu *et al* (2011), the respiratory distress observed during the study could be attributed to high level alkaloids which would have led to haemolysis and consequently leading to transient respiratory distress.

In addition, the study also found the following gross pathology after administration of aqueous extracts of *M. Whitei* at a dose of 5000 mg/kg bwt, the lungs were hyperaemia and the liver was congested. This was contrary to study findings by Nwosu *et al.,* (2011) which showed no pathologies at highest dose of 5000 mg/kg body weight. The gross pathologies findings in liver and lungs in this study could be attributed to alkaloids in the extract. It is also possible that with the increase in dose of alkaloids the toxicity to the lung increased. In addition, studies by *Kamal et al (2013*) established that alkaloids have detrimental effects on the cells. Further, the liver congestion can also be attributed, to one of its roles which is biotransformation of xenobiotic (Abass *et al.,.*, 2012). The lung congestion observed in one of the rats at a dose of 5000 mg/kg body weight could be as result of accidental aspiration of extract in the lung.

The 5000 mg/kg body weight was found to be safe as proposed by Lorke, 1984. This is also supported by lack of mortality among rats in all the dose groups during the entire 72hrs of acute toxicity experimentation.

5.2 Effects of *Mondia whitei* "mukombero" on serum Testosterone, Follicle stimulating hormone and Luteinizing hormone levels.

FSH is synthesized in the anterior lobe of the pituitary gland and stimulates Sertoli cells to release inhibin B. LH is additionally synthetized within the anterior lobe of the hypophysis gland and increases intratesticular testosterone levels by acting on Leydig cells. Intratesticular testosterone hormone stimulates the Sertoli cells in a paracrine manner and increases spermatogenesis (Mehmet et al 2015). In this study, the level of serum testosterone declined in rats treated with the extract as compared with the control group in relation with time p=0.27. The decline in serum testosterone level could be ascribed to deleterious effects on Leydig cells. Results indicated significant differences ($P<0.05$) between serum testosterone concentrations after 10, 15 and 30 days in rats treated with M. whitei extract and negative control rats. However in the first 10 days rats treated with 100mg/kg body weight of the extract recorded high levels of testosterone, this could be ascribed to either the plant extract contained active compound such as flavonoids (Yang, 2008) or it was due to immobilization that led to production of catecholamines thus increase in testosterone, (Wheeler G. *et al.,* 1994).It appears that at low doses the plant acted as a pro-androgen while at high doses it had negative effects.

The decrease in the level of testosterone is caused by the effect of steroidogenic enzymes in testes or the properties of inactivation on adrenergic systems that are involved in steroidogenesis. This effect is associated with the anti-androgenic action and the stimulation of steroidal anti-androgen to the negative feedback inhibition of the hypothalamus resulting to low plasma testosterone concentration (Mocktary, 2007). The decline in testosterone levels can also be attributed to the fact that secondary metabolites found within the extracts induces necrosis in the seminiferous tubule leading to significant decrease in interstitial cells number hence decline in the secretion of testosterone from interstitial cells (Hibi, H.; Yamamoto, M. and Miyake, K .1995). Indeed alkaloids like endocrine disrupters affects the production of testosterone directly or through the influence of gonadotropin production control since the endocrine system of the male reproduction contains components of the

hypothalamus, pituitary and the testes. This influences the synthesis or release of FSH, LH including the chemicals that leads to the interference of the synthesis of the hormone receptor or function (Campion, 2012).

This present study findings shows that the median (IQR) FSH levels of the albino male rats were lower than the control ($p<0.05$). The decline of FSH levels could be attributed to the effect of M. whitei aqueous extract on pituitary gland which inhibited the secretion of FSH. The decreased level of FSH reveals that M. whitei aqueous extract may have less potential to influence gonadotrophic hormones from being released from the pituitary as FSH is of great importance in initiating and expanding spermatogenesis process in mammals (Sharpe, 1989). The decreased levels on FSH may probably be due to failure to suppress negative feed-back inhibition of anterior Pituitary (Krishnakumari *et al.,* 2012; Anitha *et al.,* 2013).

This study also demonstrates that M. whitei aqueous extract lowers the median (IQR) serum LH in male rats as compared to the control group. The mean LH levels of the albino male rats were lower than the control ($p<0.05$) hence statistical significant differences. The decreased serum LH and FSH levels observed may probably be due to failure to suppress the negative feed-back inhibition of anterior pituitary (Woode *et al.,* 2012).

Therefore, the decreased levels of serum testosterone of rats that were treated with the M. white aqueous extract was caused by the decreased in LH which shows that etiology is caused by pituitary disorder and testicular effect associated with low levels of serum testosterone caused by low serum levels of LH. This could explain that the plant extract effect on the hypothalamus which produces the Gonadotropin-releasing hormone (GnRH) which acts on the anterior pituitary gland leading to the release of LH and FSH. As FSH stimulates the sperm production in the sertoli cells and LH stimulates testosterone production by the leydic cells, then the whole reproductive process is impaired by the treatment.

It is common that the production of testosterone by Leydig cells is primarily under LH control and LH stimulation is usually followed by testosterone stimulation (Huthaniemi and Toppari, 1995). The secretion of testosterone by leydig cells is under the stimulatory effect of LH (O'Donnell etal., 1999; Woode et al., 2012) in

male as reduced level of testosterone leads to impairment of spermatogenesis and causes infertility in male (Anitha *et al.,* 2013). Thus it is likely that the extract used in this study exerted inhibitory activity on the proliferation of spermatogonia.

Findings from the present study are similar to the earlier findings (Udoh and Kehinde, 1999; Udoh and Ekipeyong, 2001 & Udoh *et al.,* 2005a; Shajeela *et al.,* 2011; Krishnakumari *et al.,* 2012; Anitha *et al.,* 2013). However, the present findings differ with other studies in some aspects. For example, Watcho *et al.* (2001) originally recommended that the aqueous root extract of M. whitei has contraceptive properties. This finding was founded on the experimental findings of in vivo spermatogenic inhibition and fertility reduction. The researchers further assessed the in vivo androgenic activity of the same extract and found increased levels of serum and intra-testicular testosterone after chronic exposure to M. whitei aqueous root extract (Watcho *et al.,* 2004).

Watcho *et al.,* (2006) used three different extraction solvents; hexane, methylene chloride and methanol for the extraction of M. whitei roots. The researchers conducted both in vivo and in vitro androgenic activity. Findings indicated that the methanolic extract showed revocable androgenic properties. On the other hand, the hexane fraction had important inhibitory effects against KCl-and adrenaline-induced contractions in isolated deferent ducts in vitro. On the other hand Watcho *et al.,* (2007) found that the extracts increased the number of intromissions and erectile occurrences of inexperienced male rats. From the above mentioned studies, it was presumed that the extract demonstrated both contraceptive properties as well as properties associated with enhancing male fertility. The difference in their results and our results could be attributed to the extraction solvent and the duration of treatment. Their study exposed rats to *M. whitei* hexane, methylene chloride and methanol root extract for a longer period of time than the present study did.

5.3 Effect of *Mondia whitei* (mukombero) on sperm parameters

Male infertility is generally attributed to insufficiencies in the semen which are mainly considered by low motility and viability of sperm (Banihani *et al.,* 2012). Therefore, low production of sperm (oligoozoospermia), poor motility of sperm (asthenozoospermia) or abnormal morphology of sperm (teratozoospermia) or

combination of the three factors (oligoasthenoteratozoospermia) (Guzick *et al.,* 2001) leads to infertility in males.

Findings from the present study shows that sperm concentration had significant differences ($p<0.05$) after 30 days between rats treated with 100, 200 and 400 mg/kg of *Mondia whitei* and the control group. These findings could be attributed to the fact that certain alkaloids found in some plant extract have been implicated in reduced sperm viability. The postulated mechanism of action of such alkaloids involves metabolites release binding cell molecules and cross linking DNA causing cytotoxicity (Saalu *et al.,* 2010).

Previous studies done showed that decrease in sperm count and vitality is correlated with testosterone levels decline and oxidative damage as evident from suppressed antioxidant enzyme activities (Pandya *et al.,* 2012). Further, studies have revealed that spermicidal properties of plant extracts can lead to reduced human sperm motility (Harat *et al.,* 2008).

Assessments of the viability of spermatozoa is one of the most important techniques of semen analysis where there is establishment of the amount of dead/ live cells and this method is employed as a cytotoxic marker. Eosin-Nigrosin techniques were employed in the present study since spermatozoa whose cell membrane was structurally intact did not take up the stain as explained by Bjorndahl *et al.,* (2004). Therefore, this method provided insights to the effects of *M. whitei* on the viability of spermatozoa in vivo.

It was also found out that the effect of *M. whitei* on the viability of spermatozoa leads to the decrease in sperm viability as recorded. In addition, viable cells decreased with increase in treatment time. These findings agree with the findings from other studies where plant extracts were shown to decrease sperm cell viability at higher levels. Studies have shown that plant extracts might increase, have no effect or decrease cell viability depending on the plant's chemical composition (Cowan *et al.,* 1999). On the other hand, certain alkaloids found in some plant extract have been associated with reduced sperm viability. The assumed mechanism of action of such alkaloids is said

to involve the release of metabolites which end up binding to cell molecules and cross link DNA initiating cytotoxicity (Saalu *et al.,* 2010).

Katz *et al.* (1982) reported that analysis of sperms morphology is a significant aspect in the assessment of sperm functions. A significant increase in incident of sperms with abnormal head and tail was detected in all the test groups. This indicates that the plant extract effects on morphological abnormalities of sperms is dose-dependent. This could be attributed to the fact that, significant effects of the normal sperm morphology was observed to be caused by the increased in percentage of sperms whose head are detached and increased sperms with abnormal tail morphology. These findings are the same with those observed from both animal studies (El-Demerdash *et al.,* 2004) and humans studies (Benoff *et al.,* 2009) and (Wang *et al,* 2016).

Moreover, general improvement of observer accuracy according to Menkveld *et al.,* (2011) after introduction of stricter criteria by WHO manual leads to the observed decrease in normal sperm morphology. On the other hand, abnormal forms according to Prisant *et al.,* (2011) have been observed over time resulting to the decrease in the percentage of normal forms over time. These results have been further documented and confirmed through reanalysis of old smears and have been ruled out as being the main reason of the observed decrease (Menkveld *et al.,* 2010).

5.4 Effect of *Mondia whitei (mukombero) aqueous* root extracts on the male rat's testicular histological changes.

In this study, the seminiferous tubules had distorted outlines and few spermatogonial cells in treated male rats, which suggest diminished serum levels of Follicle Stimulating Hormone and Interstitial Cell Stimulating Hormone (ICSH). Luteinizing hormone, also known as ICSH in males, is responsible for initiation of spermatogenesis by causing growth and enlargement of spermatogonia. It also stimulates Leydig cells to produce Testosterone which together with FSH cause maturation of sperm. Degeneration of seminiferous tubules meant that sertoli cells responsible for spermatogenesis, were being affected. The many intercellular spaces observed in seminiferous tubules pointed to the destruction of Sertoli cells. These

cells produce oestrogen, Androgen Binding Protein (ABP) after priming with FSH and also provide nutrition for developing sperm. Mature sperm are transported out of the tubules by Androgen Binding Protein. Degenerated tubule outlines also meant that the germinal epithelium, that gives rise to spermatogonia, was not properly maintained, probably due to lack of FSH. Sections from control rats showed normal histological picture of tubules with prominent spermatogonia and sperm in various stages of development. Absence of large intercellular spaces in the controls suggested normal Sertoli cells. The tubules were also filled with spermatozoa, indicating normal process of spermatogenesis.

Accumulation of lipid droplets within the interstitial spaces suggested that leydig cells had also been affected. There was detachment of cells from neighbouring ones, hence a lot of vacoulation (Plate 4.1 iii). Neaves (2014) noticed that leydig cells of non-breeding rock hyraxes were crowded with lipid droplets and a reduction in serum testosterone levels. Administration of exogenous testosterone resulted in the disappearance of the droplets and a marked increase in the levels of serum testosterone. This may have been the case in this investigation. The presence of lipid droplets suggested inhibition of testosterone production by the cells. Lipids are precursors for steroidogenesis and impairment of the process causes them to accumulate in interstitial cells. Their presence in the cells therefore meant that androgens, including testosterone were not being synthesized, leading to low levels of both testosterone and gonadotrophs. Those from control rats showed normal morphology and lacked fat deposits and vacoulation (Plate 4.1 i). There was significant reduction of the leydig cell nuclear area and mature Leydig cell numbers during oral administration of aqueous extract of *Mondia whitei* to male rats at the dose level of 400 mg/kg body weight per rat per day for 30 days. These findings are smilar with those of (Okon *et al.,* 2012) on degenerative changes of the heart muscles following oral administration of *Mondia whitei*.

Sections from epididymis demonstrated greatly distended cells, broken outlines and scantly filled lumens (Plate4 .1 vi). This demonstrates low level of sperm concentration in the treated animals. The extract shows possible anti-androgenic activity as this was observed in sections of epididymis from treated rats. The extract

may thus be inhibiting Follicle Stimulating Hormone, (FSH) which is responsible for the development and maturation of spermatozoa. By extension, the extract may very well be interfering with the Hypothalamic-pituitary and gonadal axis through inhibiting the tropic hormone Follicle Stimulating Hormone Releasing Hormone (FSHRH) from the Hypothalamus. This hormone causes the release of FSH that in turn maintains proper functioning of testes. *Mondia whitei* (mukombero) had negative effects on the testes, may be through suppression of Testosterone, or the tropic hormone Interstitial Cell Stimulating Hormone (ICSH), responsible for its production. The hormone is responsible for maturation of sperm and its absence, as evidenced by degeneration of interstitial cells which produce it, has a drastic effect on maturation on the process. In normal spermatogenesis mature sperm are transported out of seminiferous and temporally stored in the epididymis. The low levels of spermatozoa and clamping of the spermatozoa, indicates that spermatogenesis had been greatly interfered with by the extract resulting in possible anti-fertility changes.

According to Ogbuewu *et al.* (2011) numerous screened plants have been reported to boost male reproductive functions, yet they may also lead to deterring of testicular functions. Kamal *et al.,* (2003), D'Cruz *et al.,* (2010) and Ogbuewu *et al.,* (2011) in their studies, they examined several plants with known effects of anti-fertility effects which includes: Embeliaribes berries which showed spermicidal activity by inhibition of sperm count and enzymes activity of energy metabolism and alteration of testicular histology (Purandare *et al.,* 1979; Agarwal *et al.,* 1986);and Azadirachtaindica (neem) affecting testis structure and function by damaging the seminiferous tubules hence leading to the loosening of germinal epithelium, germ cells degeneration and the derangement of germ cell types (Choudhary *et al.,* 1990; Shaikh *et al.,* 1993;Joshi *et al.,* 1996; D'Cruz *et al.,* 2010).

In addition, Crude ripe paw paw (Carica papaya) seeds leads to the degeneration of the germinal epithelium and germ cells (Udoh and Kehinde, 1999); Abrusprecatorious causes degeneration of testicular tissue which is characterized by reduction of the number of cells in the epithelium along with reduced number of sperm cells (Adedapo *et al.,* 2007); Ocimum sanctum demonstrated detrimental

effects on the ultra-structure of the testis (Lohiya *et al.,* 2008; Manivannan et al., 2009); The spesiapopulnea cause enlargement of the Sertoli cells and reduction of germ cell attachment (Krishnamoorthy and Vaithinathan, 2003). In this study, degeneration of the cells was observed in higher doses indicating that the substance could have had necrotic changes in the cytoarchitecture of the testis as have been demonstrated earlier with brain tissues (Dikibo *et al.*, 2012). This study is in agreement with (Dikibo *et al.,* 2012) who found that *Mondia whitei* had cellular pyknosis, necrosis, degenerative vacoulations, infarctions and parenchymal erosion in the brain tissue following administration of *Mondia Whitei*. In another study by (Okon *et al*., 2012), revealed that *Mondia Whitei* caused severe fibrillolytic changes, myocardial necrosis, inflammatory cell infiltration and Oedema in a dosage dependent manner on heart tissues.

The mechanism of actions may not be clearly understood but known mechanism like lipid peroxidation (Myers *et al.,* 1977), damaging of mitochondria (Bier and Jaenke, 1976), and generation of reactive oxygen species like super oxide anion and nitrogen peroxide have been reported to impare cell functions (Daoud, 1992). It is also imperative to note that testosterone hormone and follicle stimulating hormone have inhibitory effects on programmed cell deaths, therefore in the current study, decrease in testosterone and FSH could have led to decrease in mitochondrial capacity for oxidative phosphorylation while simultaneously increasing reactive oxygen species production (Olanlokun *et al.,* 2018).

5.5 Conclusion

1. The study recorded significant effect of the *M. whitei* extract on serum testosterone concentrations after 30 days of treatment. An increase in testosterone after 10 days and a decline in the concentrations of testosterone levels, Luteinizing hormone (LH) and follicle stimulating hormone (FSH) after 15 and 30 days. Therefore, the current study suggets that extract of *M. whitei* has a significant effect on the levels of serum testosterone, LH and FSH.

2. The investigation on the effect of the aqueous extract of *Mondia whitei* on sperm parameters showed significant decrease in sperm cells, total motility, viability and increase in head and tail abnormalities.

3. The study demonstrated histopathological changes in testes due to effect of *M. whitei* in treated rats including a decrease in leydig cells. Therefore, the study suggest that in addition to *M. whitei* negatively affecting fertility in male rats, it may also cause certain toxicities if used at high dose for a long period of time like hypogonadotrophic hypogonadism and oligoteratoasthenozoospermia.

5.6 Recommendation

- This study recommends that *Mondia whitei* should only be used at low doses and short duration since high doses and long duration have deleterious effects.
- Further scientific validation is encouraged and more specific investigation should be done on *Mondia* extract for a clear understanding on its mechanism of action.
- Further studies on the prevalence of 'mukombero' use among males would advise need for a study on their reproductive hormones and sperm functionality following documented usage.
- Further studies on histostereological effects of *Mondia whitei* on the testicular tissues is advised.

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APPENDICES

Appendix 1: Histology procedure

Fixation: Small testicular and the epidydimal tissue sections was put inside labelled universal bottles containing 10% formalin pH 7.0 allowing a volume ratio of fixative to tissue as 10:1. This is to preserve the shape and the chemical constituents of the tissue.

Dehydration: The dehydrating agent analytical ethanol was used to remove water from the testicular and epidydimal tissues. This is water which either occurs naturally, water from the fixative, and the water used to wash the tissue. Dehydration was carried out by putting the tissue straight into ethanol as follows: 70% for 30 minutes, 90% ethanol for 30minutes, then in absolute alcohol for 30mins (x3) for removal of water. The gradual removal of water from the tissues is to avoid sudden shrinkage of the cells.

Clearing (de-alcoholization):

The tissues were then put in xylene to remove the alcohol that permeates the tissues during the dehydration process. This is because alcohol is immiscible with the molten paraffin wax which is to be used later. The clearing agent raises refractive index and also makes the tissues translucent. Xylene was used as the clearing agent and tissue samples were inserted into the agent as follows: Xylene/ethanol (1:10 for 20 minutes (x2), then 100% Xylene for 30 minutes(x3).

Impregnation/infiltration:

The tissues were then treated with molten paraffin wax to remove (displace) the clearing agent and provide internal support to the tissue. The embedding medium used was paraffin wax melting point of 56^0C.The molten paraffin wax used for infiltration was kept in electrically heated, thermostatically controlled oven (J P Selecta S. No. 324596, Spain) at a temperature of 2^0C above the melting point ensured that the paraffin wax is in molten state all the time. The temperature above the melting point is to ensure the paraplast remains in molten state at all times. The infiltration of the tissues was carried out by placing the tissues into the molten paraffin wax as follows: After clearing, the tissues were first transferred into the

mixture of xylene/paraffin wax (1:1). This was then be placed inside the oven, in a warm spot at 35^0C, for 15minutes. The tissues were then placed into molten paraffin wax for 30 minutes two times, then in paraffin wax for 60 minutes. Impregnated tissue is to be held in the desired orientation in order to view the structure of the tissue as desired for research requirement of this study

Embedding:

After infiltration of tissues with molten paraffin wax, a solid block of wax containing the tissue was obtained. This was done by putting a drop of molten paraffin wax in the embedding mold. The tissue section was placed in the mold before the initial wax solidifies to create continuity of the block. The mold was filled with molten wax. Care was taken to make sure there are no air bubbles in the wax during blocking since on solidifying, air bubbles leave air spaces. The wax was left to cool at room temperatures in the mould to solidification. After the block of wax is cooled it was attached to a wooden block to allow attachment to the microtome block holder. The block was then pressed onto this hardened wax and held on to it.

Sectioning (Microtomy):

The rotary microtome (Leiza, Sakura Company, Germany) was used to cut paraffin wax embedded tissues to thin sections (3 μ). The vertical sectioning perpendicular to the surface of the tissues was carried out and tissue sections cut into 3 micrometers (3 μ) thick. The thickness of the sections was set at 3 micrometers and when the knife is securely in place the microtome was operated in a steady continuous rhythmic movement to obtain the ribbon sections. Rotary microtome is recommended for paraffin embedded tissues. A camel brush and a blade will then be used to disconnect the ribbons which will then put in the section box.

Floating of sections and mounting on glass slides.

This is the process by which the sections were floated on water and then transferred the tissue onto the labelled glass slides. Floating involves the lifting of the sectioned tissue from the box with a camel brush or forceps and floating them on the surface of 37^0C water bath. The sections was floated and oriented into position in the centre of ¾ of the glass slides and allowed to drip for 15 minutes in a slanting position for it to dry. The slides were then stored overnight at room temperature ready for staining.

The preparation of Haematoxylin and Eosin was then done as follows:-

Preparation of Ehrich's Haematoxylin is to be carried out by mixing the following: Haematoxylin 2g, absolute ethanol 100 ml, glycerol 100 ml, distilled water 100ml, glacial acetic acid 10 ml, potassium aluminium sulphate 3g. Eosin is prepared by 1g of eosin and 100ml of water.

Dewaxing and staining:

Tissue sections was stained using haematoxylin and eosin (H&E) method twice.

Dewaxing was followed by putting it in xylene for 20 minutes twice. (x2) Hydration is done by rinsing it in absolute ethanol for 5minutes twice (x2), then in 90% ethanol for another 5 minutes and finally in 80% Ethanol.

Staining was done by Haematoxylin 20 minutes; hydration to be done with 50% Ethanol for 5 minutes, then 30% Ethanol for another 5 minutes, then hydration with tap water for 20 minutes. It was counter stained in eosin for 1second.

Dehydration was done in 30% ethanol for 5 minutes, then in 50% ethanol for 5 minutes, in 80% ethanol for 5 minutes, in 90% ethanol for 5 minutes, and in absolute ethanol for 5 minutes (x2) .Clearing was done by use of xylene for five minutes (x2).Excess Xylene wasremoved from around the section taking care not to dry the section. A drop of DPX (Dextrine plasticizer xylene) mountant was placed on the wet section of the slide and cover slip lowered gently to cover the section and was left to settle on its own weight. Care was taken not to trap air bubbles under the cover slip. After ascertaining that the sections are stained, the edges of the cover slips were sealed with clear nail vanish. The slides will then be labelled with the date (date the animals were sacrificed), tissue type, and whether they are control or experimental.

Photomicrography

The stained mounted slide was placed under a microscope. The field of interest was identified using a magnification of x400. The images of these tissues were projected into digital camera (Sony Japan) fitted onto the microscope eye piece. The camera captured digitalized field images that were fed into a computer for qualitative image

assessing and printing. The photomicrographs were assessed for comparative differences in tissues of “mukombero” extract treated animals from control animals.

Appendix II: Ethics Clearance

OFFICE OF THE DIRECTOR OF GRADUATE STUDIES AND RESEARCH

UNIVERSITY OF EASTERN AFRICA, BARATON

P. O. Box 2500-30100, Eldoret, Kenya, East Africa

November 22, 2018

REG NO: HSM 302/4025/16

Cyprian Mabonga
School of Medicine
Department of Medical Physiology
Jomo Kenyatta University of Agriculture and Technology

Dear Cyprian,

Re: ETHICS CLEARANCE FOR RESEARCH PROPOSAL (REC: UEAB/10/11/2018)

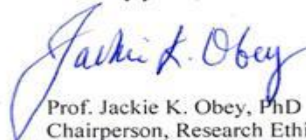
Your masters thesis proposal entitled "***Effects of Mondia White on Hypothalamic, Pituitary Gonadol Axis Male Albino Rats***" was discussed by the Research Ethics Committee (REC) of the University and your request for ethics clearance was granted approval.

This approval is for one year effective November 22, 2018 until October 21 2019. For any extension beyond this time period, you will need to apply to this committee one month prior to expiry date.

Note that you will need a research permit from the National Commission for Science, Technology, and Innovation (NACOSTI) and clearance from the study site before you start gathering your data.

We wish you success in your research.

Sincerely yours,



Prof. Jackie K. Obey, PhD
Chairperson, Research Ethics Committee

A SEVENTH-DAY ADVENTIST INSTITUTION OF HIGHER LEARNING
CHARTERED 1991

Appendix III: Research Permit

THIS IS TO CERTIFY THAT:
MR. CYPRIAN MABONGA WAPUKHA
of JOMO KENYATTA UNIVERSITY OF AGRICULTURE AND TECHNOLOGY, 6706-30100 ELDORET, has been permitted to conduct research in *Uasin-Gishu County*

on the topic: *EFFECTS OF MONDIA WHITEI ON HYPOTHALAMIC,PITUITARY &GONADOL AXIS IN MALE ALBINO RATS*

for the period ending:
23rd January,2020

Permit No : NACOSTI/P/19/81106/27253
Date Of Issue : 24th January,2019
Fee Recieved :Ksh 1000

....
Applicant's Signature

....
Director General
National Commission for Science, Technology & Innovation

Appendix IV: Research Authorization

NATIONAL COMMISSION FOR SCIENCE, TECHNOLOGY AND INNOVATION

Telephone:+254-20-2213471,
2241349,3310571,2219420
Fax:+254-20-318245,318249
Email: dg@nacosti.go.ke
Website : www.nacosti.go.ke
When replying please quote

NACOSTI, Upper Kabete
Off Waiyaki Way
P.O. Box 30623-00100
NAIROBI-KENYA

Ref: No. **NACOSTI/P/19/81106/27253**

Date: **24th January, 2019**

Cyprian Mabonga Wapukha
Jomo Kenyatta University of
Agriculture and Technology
P.O. Box 62000-00200
NAIROBI.

RE: RESEARCH AUTHORIZATION

Following your application for authority to carry out research on ***"Effects of Mondia Whitei on Hypothalamic, Pituitary & Gonadol Axis in male Albino Rats"*** I am pleased to inform you that you have been authorized to undertake research in **Uasin Gishu County** for the period ending **23rd January, 2020.**

You are advised to report to **the County Commissioner and the County Director of Education, Uasin Gishu County** before embarking on the research project.

Kindly note that, as an applicant who has been licensed under the Science, Technology and Innovation Act, 2013 to conduct research in Kenya, you shall deposit **a copy** of the final research report to the Commission within **one year** of completion. The soft copy of the same should be submitted through the Online Research Information System.

GODFREY P. KALERWA MSc., MBA, MKIM
FOR: DIRECTOR-GENERAL/CEO

Copy to:

The County Commissioner
Uasin Gishu County.

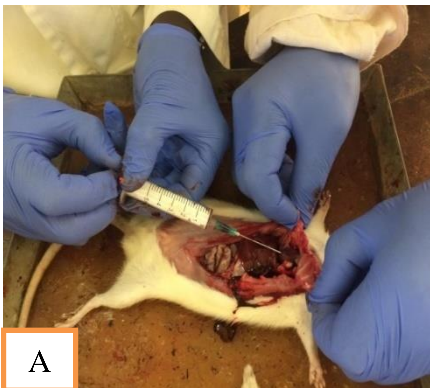
The County Director of Education
Uasin Gishu County.

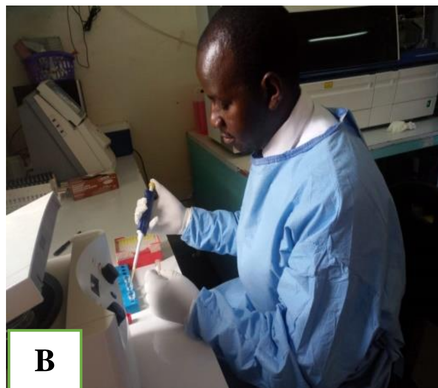
Appendix V: Plates and Figures

Experimental animal

a b

(a) Roots of Mondia whitei, (b) rotavac control evaporator





A: Blood collection from the cardiac chamber B: A liquoting the serum C: Centrifuging D,E,and F: Immunoanalyzer (cobas e 411)

(A)Automatic tissue processor machine, MTRH, (B) Microtome and a float bath, (C) Embedded blocks, () Automatic tissue processor machine.

Administering the extract to the rats

The researcher using a microscope to study sperm analysis.