EFFECT OF METHANOLIC EXTRACTS OF *Sida cuneifolia* ROOTS ON THE FERTILITY OF ALBINO RATS (*Rattus norvegicus*)

BY

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A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY IN ANIMAL PHYSIOLOGY OF THE UNIVERSITY OF ELDORET

SEPTEMBER, 2014

DECLARATION

Declaration by the candidate

I hereby declare that this thesis is my original work and has not been presented to any other university for an award of a degree of Doctor of Philosophy.

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DEDICATION

This thesis is dedicated to my mother Fulumena, my lovely children Judith, James, Caroline, Lynette and Eleanor, my late father Cosmas and my late husband Japheth.

ABSTRACT

The importance of plant extracts in mammalian reproductive physiology has not been fully studied. This study therefore determined the effects of *Sida cuneifolia* root extracts on the reproductive systems of albino rats (*Rattus norvegicus*). Thirty six female and thirty six male rats weighing an average of 102g and of proven fertility were utilized in the investigation. They were kept in cages, at three per cage, fed with mice pellets and given distilled water as drinking water *ad libitum*. The animals were maintained at 12:12 light/dark cycle. Screening experiments were first carried out using crude water extracts of roots from the plant, given in drinking water to the animals. When the antifertility activity of the plant extract was established, then the investigation proceeded. Nine female and nine male rats were given 10g/L of the root extracts in their drinking water, while nine control females and nine control males rats were given plain distilled water. Vaginal smears from treated and control rats were taken and examined daily for three weeks, in order to establish cyclicity. Vaginal smears taken from treated rats showed a marked decrease in the number of epithelial cells of the superficial type (usually an indicator of fertility) on the day of expected oestrus. Smears from control rats showed vaginal cell morphology characteristic of normal oestrus cycles. Mating experiments were then conducted first between treated females and untreated males, treated males and untreated females and finally between treated males and treated females. As a control, the untreated male and untreated females were also mated. All mating experiments conducted using males and females were executed in triplicates. Pregnancy failed in the treated rats whereas control animals had litter normally Data were analysed by Analysis Of Variance (ANOVA).Weights of ovaries and testes were also taken. Uterine, vaginal and ovarian tissue histology of female rats was examined while in male rats, histology of the testes was examined. In all groups the treated rats were compared with controls. A reduction in the weights of uteri and ovaries was observed in the treated animals unlike the controls. Sections of reproductive organs from treated female rats showed features characteristic of dioestrous and anoestrous, (infertile phases), as compared to controls. Male rats which had been subjected to the extract failed to sire offspring throughout the duration of the research. There was a significant decrease ($P<0.05$) in the weights of ovaries in female rats and testes in male rats. Sections from the gonads showed marked degeneration of germ layers, associated tissues and a significant decrease in spermatozoa in epidydimia. The extracts were characterised by mass spectroscopy. The extracts altered cyclicity, gonadal histology and reduced the weights of the testes.

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DEFINITION OF OPERATIONAL TERMS

Adenohypophysis	Anterior pituitary gland
Diestrus	The period after metestrus
Estrogen	A feminizing hormone such as estradiol. Estrogens may be secreted by the ovary, placenta, testes and adrenal cortex and are responsible for reproduction and secondary sex characteristics
Oestrous cycle	the cycle in which oocytes mature and are ovulated periodically in most female mammals
Estrus	A stage of the estrous cycle around the time of ovulation during which a female uses behaviour to indicate that she is fertile, also called heat.
Gonadotropin	A hormone that stimulates the gonads (ovaries or testes) to produce gametes and hormones and also supports and maintains gonadal tissue
Granulosa cells	Somatic cells surrounding the primary oocyte of an ovarian follicle.
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.
Metestrus	The period following oestrus during which the corpus luteum develops.
Proestrous	The period of follicular development that precedes oestrus

Progesterone	A sex steroid hormone secreted by the corpus luteum of most vertebrates and the placenta of eutherian mammals.
Prolactin	A hormone secreted by cells in the anterior pituitary that stimulates the production of milk in mammals, also performs the variety of functions e.g. water and mineral balance, caring for the young
Testosterone	A sex steroid hormone produced by leydig cells of the testes that is essential for spermatogenesis and for development of male secondary sexual characteristics and reproduction

LIST OFABBREVIATIONS

Dopa	Dihydroxyphenylalanine
E1	Estrone
E_2	Estradiol
FSH	Follicle Stimulating Hormone
GnRH	Gonadotropin Releasing Hormone
HCG	Human Chorionic Gonadotropin
HPLC	High Performance Liquid Chromatography
IRS	Infrared Spectroscopy
LH	Luteinizing hormone
MIS	Mullerian Inhibiting Substance
MRNA	Messenger Ribonucleic Acid
NEC	Nucleated Epithelial Cells
NRC	Non-Nucleated Round Cells
PMN	Polymorphonuclear Cells
PMS	Premenstrual Syndrome
PMT	Premenstrual Tension
po	postovulatory.
RIA	Radioimmunoassay
URF	Uterine Relaxing Factor
VLDL	Very Low Density Lipoprotein
WHO	World Health Organization

ACKNOWLEDGEMENT

I thank my supervisors Dr. Ng'wena and Prof. Ochieng' for their bountiful guidance throughout this study and their useful comments that enhanced the quality of this thesis. I also sincerely thank the University of Eldoret for availing partial funds that facilitated this research and for providing the facility for the research. I thank Dr. Njenga, the Head of the Department of Biological Science and the staff, particularly Mr. Mackambo, Mr. Ngurwe and Mr. Biwott for their continued assistance during the research period. My sincere thanks go to Professor Chepkwony and the Technical Staff of the Department of Chemistry, for assisting in analysing the plant extracts. I sincerely thank Dr. Ndiema and Mr. Kiiru of the Department of Human Anatomy, School of Medicine, Moi University for their assistance in the preparation of the histological specimens in this study. I also thank Apollo for taking care of the animals. I am highly indebted to Janet Kosgey who assisted me immensely in various tasks during the study.

I say thank you to all my friends for the assistance offered, in one way or another, during my entire study period. Finally I wish to thank Ascah Chepchumba for typing the project. To all friends and colleagues thank you and may God bless you all.

CHAPTER ONE

INTRODUCTION

1.1 Background of the study

Regarding the importance of fertility control, the use of fertility-regulating agents of plant origin which are ecofriendly in approach and interfere with the natural patterns of reproduction, becomes necessary (Channing *et al*., 1980). The search for agents of plant origin capable of regulating female and male fertility is as old as the human civilization and reference to them could be found in the ancient texts of Ayurveda and other Indian systems of Medicine (Childs *et al*., 1992).

Efforts are being made to develop antifertility products from plants. Ethno medical literature contains thousands of references to the use of plants for a variety of purposes by the laity since ages. Ethnobotanists and medical Botanists have recorded plants that could be used as contraceptives and abortifacients (Hamma *et al*., 2011).

Several animal studies have revealed anti zygotic, blastocytotoxic, anti-implantation and abortifacient properties of water and organic solvent extracts of many commonly used medicinal plants, sometimes in dose dependent manner (Gebri *et al*., 2005). It has been a common practicethat herbal products are administered over prolonged periods and by persons that have little or no knowledge of science e.g. in Nigeria (Ogbonnia *et al*., 2009). The constituents of these recipes elicit varied physiological activities and there has been a concern over adverse effects on reproduction or systemic toxicity due to prolonged use (Miller and Tainter, 2007).

Many plant extracts have been reported to affect fertility in rodents. Gebri *et al.,* (2005) reported that methanolic extracts of *Rumex steudelii* decreased the number of implantation sites significantly. Nivsarkar *et al*., (2005) showed that extracts of *Hibiscus*

rosasinensis flowers have antifertility and abortifacient effects on female rodents. Kate and Lucky (2010) showed that this extract exhibits antiestrogenic activity, as judged by increase in uterine weight. Kulkarni *et al*., (2003) reported that the alcoholic extract of lemon seeds exerted reversible anti-fertility effect in female mice by virtue of its anti-zygotic action. Thus the present study was an attempt to investigate the effects of *S. cuneifolia* methanolic root extracts on reproductive cyclicity, weights and histology of gonads in female and male testicular histology. Screening experiments showed *Sida cuneifolia* to have a more powerful antifertility effect within a short time than the previously researched plants, prompting the present investigation.

1.2 Statement of the problem

Several potential approaches for infertility have been investigated over long periods, including chemical, hormonal and immunological approaches. In Kenya as well as the rest of the world, there are several medicinal plants associated with antifertility properties (WHO, 2000). Although a number antifertility products have been developed from plant extracts, their potentiality has not been determined accurately. A large number of plant species with anti-fertility effects have been screened in China and India beginning about 50 years ago and were subsequently fortified by national and international agencies (Lohiya, 2000). However, the search for an orally active, and effective plant preparation is yet to be developed. This necessitated the present research on rats with a view to documenting the findings for future contraceptive research.

1.3 Justification of the study

Siphion (Greek) or siphium (Roman) was one of the most valuable plants in the ancient world. Siphium was an herbal morning after pill, readily available to people hundreds of generations ago. The plant made many rich through sale of its antifertility preparations.

The herbalist and pharmacist, Dioscorides, author of *Material medica* recommends siphium for contraceptive and abortive purposes. Siphium has been hard to cultivate and is very expensive. Hence *ferula* species was used, though it was thought to be less effective, since it was cheaper and more abundant (Arwa *et al*., 2010).

The need to venture into herbal contraceptives is of great importance because of their little or no side effects and their high levels of efficacy.

Conventional drugs used as male contraceptives are often inadequate (Ogbuewu *et al*., 2011) therefore; any efforts to explore antifertility effects of any natural product in males carry a great clinical significance, as this can help males also participate significantly in population control programs.A large number of plants have been tested throughout the world for their possible fertility regulating properties; however, very few plants have been studied for their possible male antifertility efficacy (BMJ, 2004).Thus, the current study attempted to investigate the effect of *S. cuneifolia* root extracts on the histology of the testes of albino rats and hence infer the results to their effect on fertility.

1.4 Research Questions

1. What are the effects of *Sida cuneifolia* root extracts on the reproductive cyclicity of female laboratory rats?
2. What are the effects of *Sida cuneifolia* root extracts on the histology of ovaries, uteri and vaginas?
3. What are the effects of *Sida cuneifolia* root extracts on the histology of testes?
4. What are the effects of *Sida cuneifolia* root extracts on the weights of uteri and testes of treated rats?
5. Are*Sida cuneifolia* root extracts toxic to cells?

1.5 Objectives of the study

1.5.1 Main objective

To determine effects of *Sida cuneifolia* methanolic extracts on reproductive cyclicity and gonadal tissues of albino rats (*Rattus norvegicus*).

1.5.2 Specific objective

1. To determine the effects of *Sida cuneifolia* methanolic root extracts on the reproductive cyclicity of the female rats.
2. To determine the effects of *Sida cuneifolia* methanolic root extracts on the histology of ovaries, uteri and vaginas.
3. To determine the effect of *Sida cuneifolia* methanolic root extracts on the histology of the testes.
4. To determine the effect of *Sida cuneifolia* methanolic root extracts on the weights of testes and associated organs.
5. To determine the toxicity of *Sida cuneifolia* root extracts.

1.5.3 HYPOTHESES

H_O: *Sida cunefolia*root extracts do not alter reproductive cyclicity in female laboratory rats.

H_1: *Sida cunefolia*root extracts alter reproductive cyclicity in female laboratory rats.

H_O: *Sida cunefolia* root extracts do not change the morphology of the reproductive organs in rats.

H_1: *Sida cunefolia* root extracts change the morphology of the reproductive organs in rats.

H_O: *Sida cunefolia* root extracts are not toxic to cells.

H_1: *Sida cunefolia* root extracts are toxic to cells.

CHAPTER TWO

LITERATURE REVIEW

2.1 Introduction

Man uses plants in many different ways to meet his basic needs for food, clothing and shelter. Wild plants supply medicine, crafts and cosmetics to rural and urban communities. In addition, wild plants are sources of income and employment to rural areas (Kokwaro, 1976, 1993; Olembo *et al*., 1995; Balick *et al.,* 1996; Karori & Pulu, 2003). World Health Organization (WHO) estimates indicate that 80% of the population mostly in developing countries, still relies on plant-based medicines for primary health care (WHO, 1978). In Kenya, the role of plants as sources of herbal products has been recognized and as scientific knowledge on the medicinal value of indigenous plants increases, so does the need for research on their effects on animals (Rukangira, 2001).

Although many studies concerning the use of medicinal plants in Kenya have been carried out, targeting different communities and localities, there has been little documentation on their effects on various body systems (Bisht *et al*., 2010; Abukutsa-Onyango, 2007; Gachathi, 2007).

Medicinal plants constitute 28 percent of all plants on earth (Farnsworth & Soejarto 1991). In developed countries, they are used as templates for manufacturing modern pharmaceutical drugs whereas, in developing countries they are an important resource for the treatment of various maladies and illnesses, and are a major component of treatment within the primary health care systems (Farnsworth, 1994).

Medicinal plants are the most ancient source of drugs for curing human and animal diseases. Their recognised biological actions led to their cultivation, even in antiquity, in Egypt, Greece, along the Mediterranean and in China (Balick *et al*., 1996). Almost one

quarter of all medicines are derived from the 250,000 flowering plants on the earth's surface. Their use in the crude or refined form is of utmost interest in the efforts aimed at integrating herbal with orthodox medicine (Ekpendu, 1998). Tropical plants have a long history of use in traditional medicine and even today a large proportion of the world's population relies solely on the administration of plant-derived preparations for the treatment of a diversity of ailments (Vlietinck *et al*., 2009).

The African continent has an extraordinary diversity of plant species but also a large number of traditional healers who exploit the vegetable material at their disposal (Jeruto *et al*., 2011). Based on careful observation and judicious choice of plants, it is possible to discover new natural products. Ethnobotanists and medical Botanists have recorded plants that could be used as contraceptives and abortifacients (Sidigia *et al*., 1990; Fratkin, 1996; Hamma *et al*., 2011).

The bioactivity of plant extracts is due to the presence of one or more biologically active principles. Pharmacological assays have shown that the activity is not always due to the main components, but the minor ones, or even to the synergism of all the active principles (Abdel-Kader *et al*., 1997; Piuvezam *et al*., 1999). With modern advances in the techniques for isolation and structure determination of active principles, even minute amounts can be isolated and their structures determined (Jeruto *et al*., 2011).

2.2 Mammalian Reproductive Physiology

2.2.1 Female Reproductive Physiology

Different mammals have different patterns of reproduction. Rats and mice can breed all year round, whereas others have distinct breeding seasons (Bennet *et al*., 2007). The timing of the breeding season is regulated by a biological clock, which is probably adjusted by seasonal changes in day length (Advis *et al*., 2009

In all mammalian females, there is a cycle known as estrous, when the female is most fertile and menstrual cycle**.** The uterine lining in all mammals undergoes a similar pattern of thickening during a reproductive cycle (Blandau & Odor, 1990). However, if fertilization does not occur, the uterine lining of primates breaks down and it is discharged with blood through the vagina, the discharge of blood is called menstruation. The uterine lining of non-menstruating mammals on the other hand, is reabsorbed and bleeding is minimal (Hebel & Stromberg, 1986).

The menstrual cycle in humans lasts approximately 28 days. It is controlled by the interaction of several hormones. The action of one hormone is used to stimulate or inhibit the production of another hormone. The cycle is divided into three phases: follicular phase, ovulatory phase, and the luteal phase. The follicular phase is the first part of the menstrual cycle, where follicle stimulating hormone (FSH) from the anterior pituitary gland causes one or more follicles to start developing into a mature female gamete (Knobil &Neil,1994). The follicle cells surround the oocyte (developing egg cell), and produce oestrogen and progesterone that trigger other responses. The Ovulatory phase is when the oocyte is released from the ovary and passes down the fallopian tube towards the uterus. During the luteal phase**,** most of the follicle cells remain in the ovary after ovulation and continue to develop forming a structure known as the corpus luteum (CL). Under the influence of luteinizing hormone the cells of the corpus luteum rearrange themselves into an outer layer of theca cells that produce oestrogen and an inner group of granulosa cells that produce progesterone (Goodman *et al*., 1994).

The ovaries are organs that are responsible for the development of female gametes. In humans at birth around 400,000 cells have reached prophase of the first meiotic division

and are called primary oocytes or follicles. Each month after puberty, one of these cells completes its development into an ovum. Estrogen, released from the maturing follicles, causes the uterine epithelial lining to proliferate in preparation for a fertilized egg (Rusia and Srivastava, 1998). Progesterone released from the corpus luteum will further mature the uterine lining causing it to enter the secretory phase which will be able to interact with the blastocyst should fertilization occur (Mann *et al.,* 2008*).*

At the start of the estrous cycle, the pituitary gland secretes follicle-stimulating hormone (FSH) which triggers the development of one or more follicles in the ovary. As the follicle grows in size, estrogenis secreted and inhibits further production of FSH (Offiah and Anyanwu, 2008)**.** It stimulates the pituitary gland to secrete luteinizing hormone(LH) (Soede, *et al.*, 2011).It also stimulates growth and repair of the uterine lining. The next phase is the follicular phase**.** As the follicular stage progresses, the developing follicle increases in size and becomes a mature follicle (Raji & Bolarinwa, 1997). Estrogen levels increase rapidly triggering further release of LH causing ovulation, hence this is known as the Ovulatory Phase. The oocyte leaves the ovary and passes into the fallopian tube. Progesterone primes mammary glands to produce milk and development of the uterus in anticipation of pregnancy (Dwivedi *et al*., 2009)**.** High concentrations of progesterone inhibit production of FSH and LH.

If the Oocyte is not fertilized within 36 hours, it loses viability. Without FSH and LH, or if production of these two hormones is inhibited, the cells of the corpus luteum get smaller leading to declining levels of progesterone and estrogen (Handelsman, 1998).At day 28 of the cycle, a lack of progesterone brings about another menstruation. With less estrogen and progesterone, the FSH is no longer inhibited, and the cycle can start again (Susan *et al*., 2004*).*

2.2.2 Male reproductive physiology

Spermatogenesis is controlled by gonadotropins and also requires testosterone. Gonadotropin Releasing Hormone (GnRH) is released from the hypothalamus in a pulsatile form and FSH and LH are secreted from the anterior pituitary gland. Aromatase converts androgens into estrogens and is present in the endoplasmic reticulum of various cells of mammalian testes (PubMed. 2012). In testes, high affinity estrogen receptors, ER α and/or ER β, together with a membrane rapid effect, mediate the effects of estrogens. LH binds to receptors in the cell membranes of the Leydig cells and stimulates them to produce and secrete testosterone (Kerr & Klester, 1975).

Testes

Seminiferous tubules comprise 95% of testicular volume, and are devoted to the production of spermatozoa. Each tubule is 30-70 cm long and 200-300 um in diameter. There are approximately 500 tubules per testis. The tubules are divided by fibrous septae, and surrounded by the tough tunica albuginea. Interstitial tissue located between the seminiferous tubules is comprised of connective tissue, blood vessels, lymphatics, and Leydig cells which produce testosterone.

Sperm produced by the seminiferous tubules pass out of the testes into the ductal system, beginning with the rete testis and on into the epididymis. The epididymis is a single convoluted duct approximately 20m long, and is divided into caput (head), corpus (body), and cauda (tail), which then continues as the vas deferens. Sperm in the vas deferens is joined by seminal vesicle secretions as they pass through the prostate via the ejaculatory ducts into the urethra. (Cheng and Mruk, 1975)

Testicular Function

The testes have two main functions in the adult. They act as exocrine organs, with the production and secretion of sperm. They also act as endocrine organs by producing and secreting testosterone into the blood.

Sperm production occurs within the seminiferous tubules. Under the control of local testosterone production by the Leydig cells, the Sertoli cells within the seminiferous tubules provide an appropriate environment for the development of immature germ cells into mature spermatozoa (Fawcett, 1979).

Sertoli cells

The seminiferous tubules are comprised entirely of Sertoli cells and germ cells. Sertoli cells are tall columnar cells with numerous branches which envelop all the differentiating germ cells from basement membrane to the tubule lumen. Tight junctions between Sertoli cells create a blood-testis barrier, and separate the germinal epithelium into basal and adluminal compartments. Only the most immature germ cells are present in the basal compartment, with more advanced germ cells being found in the micro-environment within the adluminal compartment. A single Sertoli cell may envelop 10-20 developing germ cells.

Sertoli cell functions include: support and nutrition of germ cells; release of mature germ cells into the lumen; translocation of developing germ cells in an adluminal direction; secretion of androgen binding protein, transferrin, inhibin; cell-cell communication via gap junctions to coordinate spermatogenesis; and as a blood-testis barrier. (Fawcett, 1979)

Germ cells begin as spermatogonia, which are the stem cells lining the basement layer of the seminiferous tubule.

Primary spermatocytes go through a series of stages (preleptotene, leptotene, zygotene, pachytene, diplotene) which are identified on the basis of cellular size and increasing nuclear condensation.

Spermatozoa

The morphologically mature spermatozoon is released into the tubule lumen. The head consists of the condensed nucleus, the acrosome, and associated membrane structures. The tail consists of a neck, middle piece containing a sheath of mitochondria, the principal piece, and an end piece. (Lipshultz & Howards, 2011)

Leydig Cells

Leydig cells lie in the testicular interstitium between the seminiferous tubules, and seem primarily involved in the production of testosterone for local and instant purposes. Distant effects of testosterone include masculinization of external and internal reproductive tissues, pubertal changes of deepening voice, facial hair pattern, etc., and CNS actions affecting libido and sexual behavior. Local effects are directed to stimulate and support Sertoli cell function in providing the proper environment for developing germ cells. Testosterone is bound to androgen-binding protein secreted by the Sertoli cell in the testis, and in the circulation is bound to a high affinity plasma globulin (testosterone binding globulin) (Fawcett, 1979).

Semen

Semen is the suspension of spermatozoa in a fluid medium termed the seminal plasma. The seminal plasma is a product of several accessory reproductive organs, primarily the

seminal vesicles and prostate, although contributions are made as well by the bulbourethral glands (Cowper's glands), urethral glands (glands of Littre), the rete testis, the epididymia, and the vasa deferentia and ampullae.

The seminiferous tubules drain into the mediastinum testis posteriorly, and coalesce into a small number of channels termed the tubuli recti, which then form the labyrinthine rete testis. The tubuli recti and rete testis are lined by simple cuboidal epithelium. Ninety percent of testicular fluid secretion is produced by these structures, and this fluid promotes sperm movement from testis to epididymis.

The 8-12 efferent ducts transport sperm and fluid from the rete testis to epididymis.. Fluid absorption rather than secretion occurs her (Wayan, 2012)

Epididymis

The epididymis is a single convoluted duct 20 m in length. It has several functions. First as a sperm conduit, epididymal passage takes about 12 days in man, with sperm propelled by spontaneous rhythmic contractions of the duct. Second, it is a site for fluid resorption. In the ram, about 99% of fluid entering caput is resorbed during epididymal passage, third as a sperm reservoir, for the cauda is a major site of sperm storage and lastly it is a site for sperm maturation. Fertilizing ability and sperm motility improves from caput to corpus to cauda. The acquisition of fertilizing ability may be due to an epididymis-specific maturation process, or may reflect a time requirement for maturation during epididymal transit (Lipshultz & Howards,1997).

Vas deferens

The epididymis continues as the vas deferens. In man the vas is 35-45 cm long, transporting sperm through the upper scrotum and inguinal canal, behind the base of the

bladder to form the dilated ampullae of the vas and then join the seminal vesicles to form the ejaculatory ducts traversing the prostate gland. The vas provides rapid transport of sperm during ejaculation.

Seminal vesicles

Are elongated saccular organs with an irregular branching lumen lined with pseudostratified epithelium and containing seminal vesicle fluid notable for high fructose and prostaglandin content.

Prostate

Weighs about 20 grams and multiple excretory glands which empty into the urethra.
A normal human ejaculate has a volume of 2-5 cc and contains 150-200 million sperm. (Ferris & Shupnik, 2006,)

The hypothalamic pituitary-gonadal axis control of spermatogenesis

The germinal epithelium requires high levels of testosterone, supplied by the Leydig cells as well as stimulation of the Sertoli cells by the pituitary peptide hormone follicle stimulating hormone (FSH). FSH binding to Sertoli cell receptors stimulates cAMP and protein kinases which lead to increased protein synthesis. Testosterone production by the Leydig cells is also under the regulation of another pituitary polypeptide hormone, luteinizing hormone (LH). LH does not seem to have any effects on seminiferous cells directly.

The release of LH and FSH from the pituitary is under regulation of luteinizing hormone releasing hormone (LHRH), also known as gonadotropin releasing hormone (GnRH), a hypothalamic peptide hormone. LHRH is released in pulses averaging every70-90 minutes, and has a short half-life of 2-5 minutes. Variations in pulse frequency may be

responsible for regulating the relative release of FSH or LH from the pituitary (Ferris & Shupnik, 2006; Marshall *et al*., 1993)

Feedback control mechanisms play an important role in male reproductive. In vivo studies in rats and other species confirmed the stimulatory effect of activins and inhibitory actions of inhibins and follistatins on FSH releasephysiology, (Bilezikjian *et al*., 2004). Inhibins which are structurally related to activins are secreted by testicular Sertoli cells and ovarian granulosa cells and act in endocrine fashion to suppress FSH synthesis and secretion. They bind to activin receptors on gonadotropes and prevent activins from producing their stimulatory effects (Lewis KA, *et al*., 2000). Follistatinsare structurally distinct from activinsand inhibins, but bind to activins with high affinity, preventingreceptor binding (Thompson *et al*., 2005). Follistatins also promote internalization and degradation of activins (Cash *et al*., 2009).

Testosterone inhibits the release of LH from the pituitary and LHRH from the hypothalamus. Estradiol is derived from peripheral conversion of testosterone via the enzyme aromatase, and is a more potent inhibitor of both LH and FSH secretion than testosterone. FSH secretion is down-regulated by inhibin. Castration results in a progressive increase of both LH and FSH serum levels since there is no negative feedback (Bilezikjian *et al*., 2004).

Testosterone released into the general circulation provides negative feedback to the anterior pituitary and the hypothalamus. Testosterone from the Leydig cells also influences the function of the Sertoli cells. FSH and testosterone together stimulate the Sertoli cells to secrete paracrine substances that promote proliferation of the spermatogonia and differentiation of the sperm. The Sertoli cells secrete several additional substances, including products that nourish the developing sperm, inhibin,

which inhibits FSH secretion from the anterior pituitary and fluid that fills the lumens of the seminiferous tubules. This fluid contains an androgen-binding protein (ABP) that binds testosterone and keeps it at a high concentration in the lumens. Testosterone and inhibin both exert negative feedback on the anterior pituitary and hypothalamus, keeping FSH and LH secretions relatively low and steady (Ojeda *et al*., 1994).

The constant levels of hormones in most mammalian males permit continuous production of sperm. The sperms are formed in the walls of the seminiferous tubules. Mature sperm have a flagella tail, an enzyme-filled organelle called acrosome and mitochondria stored in the mid-piece. Not yet motile, newly formed sperm enter the lumens of the seminiferous tubules. The fluid continuously secreted by the Sertoli cells moves the sperm along the tubules, out of the testis proper, and into the epididymis, where they undergo further maturation Fluid is reabsorbed from the lumen of the epididymal tubules, so that the sperm become highly concentrated. They are stored, ready for ejaculation, in the last part of the epididymis and in the vas deferens. Just prior to ejaculation, accessory glands secrete seminal fluids that carry the sperm out of the male reproductive tract. Semen consists of the fluid and sperm (Krueger, *et al*., 2006).

2.3 The rat's reproductive cycles

2.3.1 The Oestrous Cycle

Female rats mature in about six to eight weeks. Maturation of the reproductive tract is controlled by exposure to estrogen and progesterone, produced by the ovary in response to pituitary derived gonadotropin hormones, FSH and LH. Changes in the contractibility and development of the reproductive tract are regulated by cyclic changes in patterns of these steroid hormones. The gonadotropin hormones are in turn stimulated by pulses in gonadotropin releasing hormones (GnRH) from the hypothalamus (Shaunfang &

Barbara, 2007). Contractions in uterus and oviduct increase under the influence of estradiol while progesterone decreases them. The endometrium undergoes proliferation in response to rising levels of estradiol. Progesterone causes endometrial glands to become branched and secretory (Gupta & Kachhawa, 2007). Estradiol stimulates synthesis of receptors for progesterone (which inhibits the synthesis of estradiol). The cervix becomes dilated in the follicular phase and constricted with dense consistency in the luteal phase. Each of these changes has relevance to gamete transport (Chattopadhyay, 1998).The structural integrity of the endometrium requires steroidal support and regression of the corpus luteum leads to atrophy necrosis and sloughing off the uterine lining in menstrual animals.

The vagina also undergoes cyclic changes. Epithelial cells (exfoliated) from the vaginal wall can be collected, smeared onto a slide and examined under a microscope. A decline in circulating estradiol, causes sloughing off of the cornified layer and the vaginal mucosa becomes thin. Phagocytic leukocytes can easily migrate into the vaginal lumen.

Oestrus was used first by Heape in 1900 as a Latin adaptation of the Greek word oistros, meaning gadfly, sting, or frenzy to describe the “special period of sexual desire of the female”. He also used: anestrus, non-breeding season when reproductive organs are quiescent; proestrous, animal coming on heat; metestrus, in the absence of conception, when oestrus changes in the reproductive tract subside; and diestrus, reproductive tract prepares for receipt of the ovum (Hubscher *et al*., 2005). Clearly, these “periods,” although accompanied by morphological changes, are not described by them. The morphological appearances of the reproductive organs of the rat have been well characterized under this general behavioural scheme, (Laloraya, 2007). Vaginal smears are also widely used for this purpose (Maeda *et al*., 2000).

The onset of puberty in the female rat results from a cascade of events following establishment of a pulsatile luteinizing hormone (LH) release after the fourth postnatal week (approximately thirty days of age) that leads to ovarian maturation (Andrews & Ojeda, 2009). This change in LH release is apparent eight to nine days before the first proestrus, and this period of change in mode of LH release is considered anestrus (Urbanski & Ojeda, 1985). The first proestrus, oestrus, and diestrus periods then follow (Advis *et al*., 2009).

Ovulation occurs in the young adult laboratory rat every four to five days throughout the year (Ojeda & Urbanski, 1994). Based on vaginal smears, the duration of the individual components of the oestrous cycle for rats with a four- or five-day cycle are proestrus, twelve to 14 hours; oestrus, 25 to 27 hours; metestrus, six to eight hours; and diestrus, 55 to 57 hours (Hartman, 1999). However, as noted above, many authors refer to the day of the cycle, with each period having its own day, and those in a five-day cycle generally showing either an extra day of vaginal cornification (extra day of oestrus) or an extra day of leukocyte infiltration (extra day of diestrus) (Vom Sall *et al*., 1994).

Complete longitudinal sections of the vagina and cervix, transverse sections of the mid portion of both uterine horns, and medial sections of both ovaries are the minimal requirement for an adequate evaluation. Observation of the coordinated morphology of the vagina and uterus is key to consistent staging of the oestrous cycle. The formation, progression, and regression of the corpora lutea are somewhat synchronized, and they can be used as an aid to staging the cycle (Mandl, 2005). Three or more generations of corpora lutea may be present in an individual ovary from the preceding ovulatory cycles, as each corpus luteum persists morphologically for twelve to fourteen days (Yuan and Foley, 2002).

2.3.1.2 Estrous cycle stages

2.3.1.2.1 Diestrous

At the start of diestrus, vaginal epithelium is at its lowest level of approximately three to seven cells thick consisting simply of the stratum germinativum. The stratum germinativum consists of stratum basale as a single layer of columnar epithelial cells and an outer stratum spinosum as multiple layers of polyhedral cells. There is a variable infiltration by leukocytes (Rowen *et al*., 2009). A reduction in the infiltration of leukocytes and a notable epithelial cell proliferation occurs toward the end of the phase with thickening of the epithelium, but with no clear stratum granulosum. The formation of a stratum granulosum is the defining characteristic for the practical staging of the end of diestrus and the beginning of proestrus. The vaginal smear which is characterized by little mucus with some leukocytes, nucleated basophilic cells, and occasional vacuolated cells (Westwood, 2008).

During this phase the uterus is small and inactive, and the horns lack a prominent vasculature and generally show a slit-like lumen. They are lined by a low cuboidal or columnar epithelium showing occasional degenerate cells. There are few mitoses at the start of the stage and the secretory glands are inactive, but there is some increase in activity during the progression of the phase. Also, toward the end of the phase, a slight oedema of the stroma adjacent to the endometrial epithelium can be seen (Westwood, 2008).

By the time diestrus starts, newly formed corpora lutea from the previous ovulation have attained their maximal size in the ovary, and this is the best ovarian marker for diestrus, although degenerate corpora lutea will also be present (Hubscher *et al*., 2005). Vacuoles are commonly present, particularly in the cells in the centre of these large corpora lutea,

indicative of active steroid genesis, and early fibrous tissue formation may be seen in what was previously the central, fluid-filled cavity (Westwood 2008).

2.3.1.2.2 Proestrus

Formation of the stratum granulosum over the stratum germinativum of the vaginal epithelium, consisting of flattened epithelial cells containing many keratohyalin granules in the vagina, marks the start of proestrus (Telang *et al*., 1999). The vaginal epithelium shows mitotic figures throughout the stage, although they are less numerous at the end. Following the early formation of the stratum granulosum, there is a progressive development of the superficial mucoid layer (stratum mucification) according to Yuan and Foley (2002), or rete mucosum according to Hubscher *et al*. (2005), characterized by layers of cuboidal to ovoid cells with mucin-containing cytoplasmic vacuoles, and the formation of a stratum corneum of dense, cornified cells. There is little if any degeneration or desquamation during the early- or mid-proestrus period, and some infiltrates of leukocytes are seen. At the end of the stage, the epithelium is fully cornified and generally shows a superficial mucoid layer exhibiting some desquamation of mucoid cells. The vaginal smear is again consistent with this histological appearance, showing a disappearance of leukocytes and the presence of sheets of, or isolated, nucleated epithelial cells, which become progressively acidophilic with the appearance of cornified cells (Morali & Beyer, 2011).

During this stage, the uterine endometrial lining progresses to large cells, forming a tall cuboidal to columnar epithelial lining. There are frequent mitoses with only limited or no epithelial cell degeneration of the glands and lining epithelium (the appearance of notable endometrial epithelial cell degeneration marks the end of proestrus/start of oestrus) and little inflammatory cell infiltration, although mitotic figures disappear at the

end of the stage (Channing *et al.*, 1980). The endometrial vasculature becomes more prominent, and the stroma shows some oedema, with the lumen generally becoming markedly dilated towards the end of the stage (Westwood, 2008).In the ovaries, ovarian corpora lutea often degenerate, with central fibrous tissue formation, and the cells commonly contain cytoplasmic vacuoles.

2.3.1.2.3 Oestrous

In the vagina, there is a progressive shedding of the superficial mucoid and cornified layers during oestrous, with a reduction in the height of the epithelium, and cell debris is present in the lumen (Rowen *et al.*, 2009). There is also a variable and progressive leukocyte infiltration. The end of the stage is characterized by detachment of the cornified epithelium, although some may persist, particularly adjacent to the vaginal orifice. Virtually complete detachment of the cornified epithelium of the vagina marks the end of oestrous and the start of metestrus. The vaginal smear shows non-nucleated cornified cells, which by late oestrous have diminished when leukocytes appear. Also, large basophilic epithelial cells are present in the smear (Westwood, 2008).

Changes in the endometrial epithelium define the start of oestrus, with the appearance of cellular degeneration/necrosis in the secretory glands first, followed by the lining epithelium, which becomes quite marked. It is accompanied by a loss of mitotic activity and leukocyte infiltration (Channing*et al.*, 1980). Luminal dilatation may persist into late oestrus, although this is generally not the case. Some endometrial epithelial mitotic activity returns by the end of oestrus (Westwood, 2008).

Degenerate ovarian corpora lutea are often present in ovaries at oestrus, but the newly formed corpora lutea are small, with cells showing a basophilic cytoplasm and occasionally a central fluid-filled cavity retained from the follicular stage. Central fibrous

tissue is generally not present (Channing *et al.*, 1980; Westwood, 2008). The start of metestrus is marked by the mid region of the vagina showing a complete detachment of the cornified epithelium, generally with residual squames present in the lumen. Somecornified epithelial cells may persist, particularly adjacent to the vaginal orifice (Rowen *et al.,* 2009).

There is a continued desquamation of epithelial cells throughout the stage, with a progressive loss of the stratum granulosum and upper germinativum (stratum spinosum). There is an accompanying variable leukocyte infiltration. As noted under diestrus, the end of metestrus/start of diestrus is marked by the epithelium reaching its lowest level. The smear during this stage shows leukocytes, a few cornified cells, and basophilic cells (Westwood, 2008).During metestrus, the uterine endometrial epithelium shows continued vacuolar degeneration and also a marked return of mitotic activity. There is also a variable leukocyte infiltration (Rowen *et al.*, 2009).

Ovarian corpora lutea may still contain a fluid-filled central cavity generally devoid of fibrous tissue, and the cytoplasm of the new corpora lutea are less basophilic than at oestrus but smaller than that seen at diestrus (Gosden *et al*., 2007).

2.3.1.2.4 Metestrus

The start of metestrus is marked by the mid region of the vagina showing a complete detachment of the cornified epithelium, generally with residual squames present in the lumen. Somecornified epithelial cells may persist, particularly adjacent to the vaginal opening. There is a continued desquamation of epithelial cells throughout the stage, with a progressive loss of the stratum granulosum and upper germinativum (stratum spinosum) (Hess &de Franca, 2013). There is an accompanying variable leukocyte infiltration. As noted under diestrus, the end of metestrus/start of diestrus is marked by

the epithelium reaching its lowest level. The smear during this stage shows leukocytes, a few cornified cells, and basophilic cells (Westwood, 2008).

During metestrus, the uterine endometrial epithelium shows continued vacuolar degeneration, but also a return of mitotic activity, so both are seen together. There is also a variable leukocyte infiltration (Raji & Bolarinwa, 2010).Ovarian corpora lutea may still contain a fluid-filled central cavity generally devoid of fibrous tissue, and the cytoplasm of the new corpora lutea are some are lessbasophilic than at oestrous but smaller than that seen at diestrus (D'Cruz *et al*., 2010).

2.4 Effects of herbal extracts on the female rat fertility

During preclinical investigations into the safety of drugs and chemicals, many are found to interfere with the reproductive functions of female rats. This interference is commonly expressed as a disturbance in the duration of particular phases of the oestrous cycle or a change in normal morphology of the reproductive tract (Yuan & Foley, 2002).Oral administration of aqueous *Mangifera indica* (MILE) at a dose of 500 mg/kg b.w. /day for a period of 30 days, significantly ($p<0.05$) alters the normal oestrous cycling in pubertal female rats. Oestrous phase occurrence in extract treated rats was reduced to 1.80 - 0.36 days while that control was 6.40 - 0.36 days (Awobajo *et al*., 2013)

2.4.1 Effect of the extracts on the oestrus cycle

Ethanol and aqueous extracts of *Calotropis procera* roots have been documented to interrupt the normal oestrous cycle in 60 and 80% of treated rats respectively (Circosta *et al*,.2001).Estrous cycles of rats became irregular with prolonged estrus and metestrus phases, and reduced diestrus and proestrus phases after the oral administration of petroleum ether, benzene, chloroform and alcohol extracts of the seeds of *Momordica charantia* at a dose level of 25mg/100g body weight. The results showed reduced

ovarian weight, number of developing follicles, Graffian follicles and corpora lutea and an increased number of atretic follicles in histological sections of the ovary (Shivalingappa *et al*., 2002).The presence of saponins, tannin, steroids, alkaloids, glycosides and terpenes in *Aspilia africana* have been found to possess antiovulatory and anti-fertility properties(Mukherjee*et al.*, (2006).

Administration of *Garcinia kola* seed extract partially blocks ovulation, alters oestrous cycle with a prolonged diestrus and may cause a dose dependent adverse effect on foetal development in Sprague-Dawley rats (Akpantah*et al.,* 2005). Ethanol and aqueous extracts of *Calotropis procera* roots have been documented to interrupt the normal oestrous cycle in 60 and 80% of treated rats respectively. (Circosta *et al.,* 2001).

2.4.2 Anti-implantation effects of plant extracts

The number of corpora lutea, as well as number and weights of live births were significantly reduced in rats treated with aqueous extracts from leaves of *Hymenocardia acida* (Adakole & Uchendu, 2011). Gebrie *et al*., (2005) have suggested inhibition of implantation reduction of estrogen level and increment of progesterone level as the possible mechanism of antifertility effect of the methanolic extracts of *Rumex steudelii* (300 g/kg b.wt.).Pretreatment with ethanol extracts of *Allium cepa* in ratsshowed significant inhibition of the number of implant sites at a dose of 300 mg/kg (Thakare *et al*., (2009). Administration of *Physalis alkekengi* alcoholic extracts in female rats at a dose of 150 mg/kg b.wt. on days 1-5 of pregnancy, significantly decreased the number of implantation sites, number and weight of neonates. (Montaserti *et al*., 2007) mentioned that the *Plumbago indica* ethanol extract of roots and *Aerva lanata* ethanol extract of aerial parts have shown 50% and 30% anti-implantation activity at 400 mg/kg b/w, respectively in albino rats (Savadi & Alagawadi, 2009). Ethanol extracts of *Sida acuta*

leaves, at a dose of 50mg/kg body weight, were found to have an antiimplantation activity and inhibited pregnancy in 6/6 rats (Ramesh *et al*., 2009).

2.4.3 Abortifacient effect of plant extracts

The extract of *Momordica cymbalaria* (500 mg/kg b.wt.) showed 100% abortifacient activity in female albino rats (Koneri *et al*., 2007).The methanolic leaf extract of *Achyranthes aspera* (5.5g/kg b.wt.) induced abortifacient activity and increased uterine weights in female rats (Shibeshi *et al*., 2006).

Ethanol extracts of the roots of *Derris brevipes* at a dose of 600 mg/kg body weight exhibited 40% anti-implantation activity and none of these rats delivered any litters and showed 100 % antifertility (anti-implantation as well as abortifacient activity (Govindaraj *et al*., 2009).

On binding with estrogen receptors in cells, some Phytoestrogens translocate to the nucleus and stimulate cell growth in a manner similar to estradiol. Phytoestrogens affect the two estrogen receptor (ER) sites on DNA — ER-α and ER-β. They differ not only in their binding affinities for the ER, but also in their potential to increase the rate of receptor binding too (Kostelac *et al.*, 2003).Extracts of *S. Cordifolia* at a dose of 500mg/kg body weight produced an abortifacient and antiimplantation activity in albino mice (Swati & Kala, 2012).

2.5 Effects of plant extracts on female gonadal histology

Sanger and Bell (1961) investigated the effect of bluegrass (*Poa pratense*) pasturage on fertilization of ova in sheep. There was 75 per cent cleavage of the ova from 22 sheep pastured on bluegrass. Three possible explanations are given for this: (Adams N.R, 1995)

inhibition of follicle formation, interference with sperm activity and swimming of sperm, and failure of follicles to rupture (Adams, 1977).

Largedoses of oestrogen can inhibit follicular growth by suppressing the secretion of pituitary gonadotrophins, whereas small doses may enhance follicular development (Smith and Bradbury (2003).A decrease in the number of Graffian follicles has been reported to occur in albino mice orally treated with nicotine at 0.3 mg/kg for 15 days (Patil *et al.,* 1998). It was reported by Lamartiniere *et al.,* (1998a, b) that ovarian follicular development was adversely effected with the genistein administration to rats during neonatal period.

Similar observations on antifertility, anti-implantation or pregnancy interceptory properties suggestive of an ovulatory, antiprogesterogenic or estrogenic effects have been made on extracts of *Calotropis gigantea* (Srivastava *et al*., 2007) and *Morinda citrifolia* (Muller *et al*., 2009). Sensitivity of experimental animals, dose of extract used, period and route of administration as well as physiological or pharmacological mechanisms are some of the factors affecting the implantation process (Costa-Silva *et al*., 2008).Yamada *et al*.,(2012) reported a decrease in primordial follicles in the ovaries of female gerbils treated with *Cannabis* extract at 2.5 mg/day for 60 days and (Sinha *et al*., 2009) also reported a decrease in follicles in rats treated with an aqueous suspension of the dried seed powder of *Sapindus trifoliatus* at 50, 100 and 150 mg/kg for 31 days.

2.5.1 Effects of plant extracts on male gonadal histology

Leydig cell nuclear area and mature Leydig cell numbers were significantly reduced on oral administration of methanolic extract of *Tinospora cordifolia* stem to male rats at the dose level of 100 mg per rat per day for 60 days (Austin and Short, 1972). Ethanol extracts of *Colebrookea oppositifolia* when administered orally for 8–10 weeks was

reported to cause a decrease in the nuclear and cytoplasmic surface area of Leydig cells (Blake, 1976).Significant decreases in the weights of testes, epididymia, seminal vesicle and ventral prostatewere observed in ethanol extract of *Martynia annua* root treated animals (Mali *et al.,* 2002).Histological studies revealed that piperine from *Piper longum*at a 10 mg dose, caused severe damage to the seminiferous tubule, decrease in seminiferous tubular and leydig cell nuclear diameter and desquamation of spermatocytes and spermatids (Mishra & Singh, 1999).

2.5.2 Effects of plant extracts on weights of male gonads

The weights of the testes are one of the markers of a possible alteration in androgen status. A decrease in testicular weight and gonadal-somatic index (GSI) (a better way to access the damage to the testes in relation to the body in experimental rats, is most likely due to decreased levels of serum testosterone as it exerts its major effect on weights of gonads. In other cases reduction in GSI may be due to poor nutrition).

Gonadal- somatic Index is obtained by the formula.

Gonadal- somatic index (G.S.I) = Gonad weight/ total body weight x 100

$$GSI = \frac{\text{Gonad weight}}{\text{Total body weight}} \times 100$$

Where gonad weight is weight of right testis + weight of left testis/2

Another cause in the decrease in the weights of the testis and hence GSI may be due to decreased spermatogenesis. There is a high correlation between testis weight and spermatogenesis in mice (Rabia Latf, *et al*., 2008).

A decline in GSI may also be due to inhibition of potential gonadotropin secretion as pituitary FSH has been shown to increase the testicular size. Lack of FSH therefore can serve as a reason for decrease in absolute testicular weight (Almaida, 2000).

Rats treated with extract from stems, leaves and roots of *Ximenia americana* showed a significant reduction in testicular weight and sperm count with roots showing the most deleterious effect. The extracts also increased the incidence of sperm head and tail abnormalities (Adeiza *et al*., 2011).

2.5.3 Effects of plant extracts on hormonal fluxes

Reduction in the levels of testosterone, luteinizing hormone (LH) and follicle-stimulating hormone (FSH) was reported when the crude methanol extract of medicinal plants was administered to male albino rats (Adams, 1997)Tests on twenty male participants given neem extracts, resulted in sterility during the full period of the study. Fertile sperm count returned to normal 5-6 weeks once the ingestion of Neem capsules ceased. *Mentha piperita* (20 g L^{-1}) and *Mentha spicata* (20 g per litre) herbal teas when fed to Wistar rats increased the FSH and LH levels and decreased total testosterone levels (Biwott, 2007).*Cissampelos pareria* leaf extract, when administered orally altered gonadotropin release (LH, FSH and prolactin) and estradiol secretion. The oral LD 50 of the extract was found to be7.3 g/kg in mice. (Ganguly *et al*., 2007).

2.6 Phytoestrogens

Phytoestrogens are plant derived xenoestrogens (foreign oestrogens), acting as the primary female sex hormones not generated within the endocrine system, but consumed by eating phytoestrogenic plants. They are a diverse group of naturally occurring non-steroidal plant compounds that, because of their similarity to estradiol have the ability to cause estrogenic or antiestrogenic effects in animals (Yildiz, 2005). It has been proposed that plants use phytoestrogens as a natural defence against the overpopulation of the herbivore animals by controlling their fertility (Hughes, 1988).

Phytoestrogens belong to a large group of natural phenolic compounds; coumestans (coumestrol), flavonoids (quercertin, kaempferal) and isoflavones (genistein, daidzen, glycitein, equal and biochanin). The most studied are isoflavones commonly found in soy and red clover (Moutsatou, 2012).If exogenous estrogens, either synthetic or from plant sources, are present in the circulation they may bind to the intracellular estrogen receptors more rapidly than endogenous estrogens because they are not bound as strongly to plasma proteins (Arnold *et al*., 1996).

Phytoestrogens exert their effects through binding to oestrogen receptors (ER). These occur as ER alpha and ER beta and many phytoestrogens have higher affinity for ER-β than for ER-α (Turner *et al*., 2007). Environmental oestrogens exert their effects through classical, genomic, or nongenomic pathways. Due to their similarity with the endogenous hormones, these compounds can bind to nuclear receptors. Their affinities for ERα and ERβ are relatively weak compared to endogenous E2; thus, they can have agonist or antagonist activity depending on the presence of E2. (Shanle & Xu, 2011).

The key structural elements that enable phytoestrogens to bind to oestrogen receptors and display oestradiol-like effects are the phenolic ring that is indispensable in binding to oestrogen receptors, the ring of isoflavones mimicking a ring of oestrogen receptors, low molecular weight similar to oestrogens (MW=272) and distance between two hydroxyl groups at the isoflavone nucleus similar to that occurring in oestradiol as well as optimal hydroxylation pattern.

Phytoestrogens may also modulate the concentration of endogenous estrogens by binding or inactivating some enzymes, and may affect the bioavailability of sex hormones by binding or stimulating the synthesis of sex hormone binding globulin (Johnston, 2003,).

2.7 Taxonomy of *Sida cuneifolia* A. gray

Kingdom:	Plantae
Subkingdom:	Tracheobionta
Superdivision:	Spermatophyta
Division:	Magnoliophyta
Class:	Magnoliopsida
Subclass:	Dilleniidae
Order:	Malvales
Family:	Malvaceae

Accepted name:*Billieturnera helleri* (Rose ex A. Heller) Fryxell

Synonyms:

= *Sida grayana* I. Clem.

= *Sida helleri* Rose ex A. Heller (USDA, NRCS. 2009).

2.8 Ecology, description and Medicinal Values of *Sida cuneifolia*

2.8.1 Ecological distribution

The plant grows in the southern counties of the state of Texas in the united states (USDA, NRCS. 2009). In Uganda it grows abundantly in the western and north western regions of the country (Namukobe *et al*., 2011). In Kenya it is widely distributed, growing at all altitudes and being found in a number of ecological regions. The plant also grows in different regions of Ethiopia and South Africa (*www.ngkenya.com › Plants*, 2012).

2.8.2 Description of Genus *Sida*

Plants in this Genus are perennial or annual, subshrubs or shrubs growing up to 2 m high. Most parts have stellate, simple and/or glandular hairs. Leaves are simple, stipules

threadlike to narrowly lanceolate, leaf blades entire (sometimes lobed), usually dentate and without foliar nectaries. Flowers are solitary or paired, axillary or subterminal, often in axillary or terminal racemes or panicles, rarely in umbels or glomerules. The epicalyx is absent and the Calyx is campanulate or cup-shaped, 5-lobed, often 10-ribbed basally and plicate in bud. The Corolla is mostly yellow, rarely white or orange [rose or purplish], and sometimes with a dark center. Petals are 5, free; basally connate. The filament is a tube that is either pubescent or glabrous, with many anthers at apex. The ovary is 5-10-loculed with one ovule per locule. There is a that branches into many as carpels. The stigma is capitate. The schizocarp is disk-shaped or globose with (4-) 5-10(-14) mericarps, that are sculptured or smooth, sometimes partly membranous, mostly beaked, often with 1 or 2 apical awns , and often minutely stellate puberulent, dehiscent or indehiscent. There is one seed per mericarp. Seeds are smooth and glabrous except sometimes for minute hairs around the hilum (Brands, 1989).

2.8.3 Description of *Sida cuneifolia*

Plate 2. 1: S*ida cuneifolia* image and description of the plant (Source: Author, 2014)

Sida cuneifolia is a shrub with very tough woody stems and very tough roots. Stems can also grow a meter or more tall if left ungrazed. Flowers are yellow with five distinct petals and five sepals. Numerous stamens arise from a fleshy column derived from the fused filaments. Leaves are elliptic, notched at the tip, without spines but hairs on leaves and stems are coarse and unpleasant to touch. There can also be small teeth in some populations, but the specimen used in this investigation, had smooth margins. Fruits are dry capsules which break into five or so segments (Vollesen, 1986).

2.8.4 Medicinal uses of *Sida cuneifolia*

The plant has been used to relieve chest and muscle pains. Freshleaves of *S.cuneifolia* are pounded added to cow ghee boiled and applied twice daily for three days (Namukobe

et al., 2011). Roots of *S.cuneifolia* are chewed to treat sore throat (Okello *et al*.,2010). To treat stomachache roots of *S.cuneifolia* are chewed (Okello *et al*., 2010.A hot water extract of the dried entire plant is administered orally in India as a febrifuge, an abortifacient and a diuretic (Kholkute *et al*., 2007). The fresh root is chewed for the treatment of dysentery (Holdsworth, 1998). The leaf juice is also used for vomiting and gastric disorders (Ramachandran & Nair, 2001). In some parts of Kenya, the decoction of the entire plant is taken orally for asthma, fever, aches and pains, ulcers and as an anti-worm medication; while a decoction of the dried entire plant is taken orally for venereal diseases (Biwott, 2007).

2.9 Cytotoxicity

Cytotoxicity is the quality of being toxic to cells. Examples of toxic agents are a chemical substance, an immune cell or some types of venom (Niles *et al*., 2009). Cytotoxicity assays are widely used by the pharmaceutical industry to screen for cytotoxicity in compound libraries. In research cytotoxic compounds are investigated, when one is interested in developing a therapeutic agent that targets rapidly dividing cancer cells, for instance; or one can screen "hits" from initial high-throughput drug screens for unwanted cytotoxic effects before investing in their development as a pharmaceutical (Decker, 1988). Cytotoxicity can also be monitored using the MTT or MTS assay. This assay measures the reducing potential of the cell using a colorimetric reaction. Viable cells will reduce the MTS reagent to a colored formazan product. A similar redox-based assay has also been developed using the fluorescent dye, resazurin. Cytotoxicity can also be measured by the sulforhodamine B (SRB) assay, WST assay and clonogenic assay. A label-free approach to follow the cytotoxic response of adherent animal cells in real-time is based on electric impedance measurements when the cells are grown on gold-film electrodes. This technology is referred to as electric cell-substrate

impedance sensing. Label-free real-time techniques provide the kinetics of the cytotoxic response rather than just a snapshot like many colorimetric endpoint assays (Niles *et al.* 2007). The dichloromethane extracts of *Strychnos heningsii* had an IC 50 ranging from 35.2 to.4 µg/ml has reported by Muthaura *et al.* (2007) who was investigating antimalarial properties of medicinal plants. Some plants extracts like that of *Pittosporum viridiflorum* which is used traditionally in Kenya as an antimalarial has been shown to exhibit the highest cell cytotoxicity on Vero E6 cells. Methanol and water extracts presented CC_{50} of 18.08 and 69.21g/mL, respectively, as reported by Muthaura *et al*., (2007). The CC_{50} of this plant was below 50% thus it was reported to be very toxic to the cells. In East Africa a decoction or infusion of the stem bark of *P. viridiflorum* is used for malaria and fevers (Gakunju *et al*., 1995).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study area

The plant was collected from the field in the University of Eldoret. The preparation of extracts was carried out in the laboratories at the University. The University of Eldoret is situated about 9 Kilometres from Eldoret town on Ziwa road and lies between latitude 0 35' N and 0 35' S and longitude 0 37' E and 0 50' E at an altitude of 2180 m above sea level. The rainfall distribution is bimodal with an annual mean of 986 mm in two distinct seasons; March to July with a peak in May (long rains) and October to December (short rains). The daily mean maximum and minimum temperature recorded in the area is 17.6º C and 10º C respectively.

3.2 Collection and processing of plant materials

The plants were identified in the field, collected and authenticated in the herbarium by University taxonomist of the University of Eldoret. A card reference number ANN/KYO/06/10/001 was deposited in the herbarium. Roots were obtained from the plant and dried under sterile condition to constant weight at room temperature (25^{o}C) and eventually ground to powder.

3.3 Preparation of plant extracts

3.3.1 Preparation of crude extracts

Extraction of the plant material was prepared by mixing with methanol in the ratio of 1:100 (plant material/solvents). The mixture was then boiled for five minutes cooled and then filtered using Whatman Filter paper No. 1 filter paper. The ground material was soaked in methanol overnight, it was then filtered and the filtrate concentrated by rotary evaporator using the vacuum pump to facilitate. The extracts were kept in stoppered

sample vials at room temperature until they were used (Ana *et al*., 2005). Total water extract of plant material was done by soaking 50g dry powder in 5 litres of distilled and shaking for 2 hours with an electric shaker. The suspension was filtered and evaporated to dryness. The dry powder was kept in stoppered vials in a desiccator to avoid absorption of water. 1 g of the powder was dissolved in 100 ml of water boiled for few minutes and cooled before administering to 3 rats. Screening experiments to determine effects of the plant were done using crude extracts.

3.3.2 Extraction of alkaloids

Alkaloids were obtained from the extracts using the acid separation method. 50 ml of the crude extract was mixed with 50g of silica gel in a pestle and mortar. It was air dried and the extract together with the silica gel was then placed on top of silica in a column. Slurry of silica gel was made in hexane, stirred and poured in the column. A 100 ml of Hexane was poured in the column and allowed to drip into test tubes. This was repeated each time leaving the sample to drip to dryness. The polarity of hexane was then gradually changed using methanol in increasing strengths. The procedure involved mixing 80ml hexane with 20ml methanol, 60ml hexane with 40ml methanol, 40ml hexane with 60ml methanol, 20ml hexane with 80ml methanol and finally with 100ml methanol. Thin Layer chromatography was then carried out on the fractions to identify compounds in different tubes. The fractions containing the alkaloids were further concentrated by a rotary evaporator to remove the solvent. A thick paste containing the alkaloids was obtained and kept for later use.

3.4 Experimental Design

Thirty six albino rats of the species *Rattus norvegicus* of the Wistar strain, comprising of 18 males and 18 females were randomly selected from a colony maintained in the animal house of the University of Eldoret. The rats weighing 102 ± 2.4 g and of proven fertility were used in the investigation. The two groups were each divided into nine controls and nine experimental and housed in cages 3 per cage. Rats in all groups were fed with mice pellet *ad libitum* from Maraba Millers in Eldoret town. All were also given distilled water as drinking water. Their body temperatures and weights were recorded before, during and after the investigation. This was done by inserting a thermometer into the anus. The weights of the rats were taken using a perforated container whose weight was known and weighed by a ZERO balance manufactured by Denver Instrument Company Serial No.N0091691.U.S.A 3100gs. The weight of each rat was obtained by subtracting the weight of the container from the weight of the container and the rat. The cages were kept at room temperature 25°C and exposed to twelve to twelve cycle of darkness and light and in a quiet environment.

To synchronize their oestrous cycles before commencing the investigation, each female rat was injected intraperitoneouly with 0.1 ml oestrogen (Gonabreed) and left for seven days. On the seventh day the procedure was repeated with 0.1 ml progesterone and the rats left for two days. On the ninth day, 0.1 ml oestrogen and 0.1 progesterone was given. Vaginal cytology was studied by vaginal lavage using mammalian physiological saline (0.9% NaCl solution). The material obtained was placed on clean slides and smears made. These were observed under high power magnification. The smears were taken every day at 9 a.m. for the next eight days corresponding to four two cycles. They were stained with Giemsia stain and viewed under (x4) and (x10) high power (×100) objectives (using an OLYMPUS microscope manufactured by Mic Labomed. LABO

America Inc. U.S.A Ser. NO 080121175) and were photographed digitally by a Kodak camera attached to it. Numbers of different cells were recorded for each day of the cycle for the next eight days corresponding to two oestrus cycles. When normal cyclicity was observed the rats were divided into two groups. Experimental rats were given extracts from *Sida cuneifolia* roots at a concentration of 1g in 100ml distilled water making a 1% solution. This concentration was adopted as it gave the best results on cyclicity. 2 ml of the solution was given to each rat by gavage (Davis, 1987) at 3 hour intervals every day for 2 days, thereafter they continued to take distilled water throughout the investigation. Control rats were given distilled water throughout the investigation. The duration of administration of the extract corresponded to the fertile phases of the oestrus cycle.

On the third day at 9 am vaginal smears were obtained from both control and treated groups. This was repeated everyday for the next 3 weeks. The cytology of the smears was studied in terms of types and numbers of different cells and general appearance of the smears. Treated and control female rats were then mated with untreated males in a ratio of 1:1

3.5 Histological studies

3.5.1 Female rats

After three weeks on day 2-3 of the cycle (corresponding to proestrous and oestrus respectively) some rats from each group were anaesthetized under ether and sacrificed. Ovaries, uteri and vaginas were obtained from both control and treated rats. For female rats, this was done on third day which is the proestrous to oestrous phase (Westwood, 2008). The phase was preferred because prominent changes occur in the reproductive tracts of the animals, in response to marked changes in the levels of reproductive

hormones. The organs were washed in physiological saline, blotted dry with blotting paper, weighed with an analytical balance and preserved in 10% formalin for later use.

Three longitudinal sections of ovaries and longitudinal sections of the mid portions of the uterus were obtained for histological examination. The procedure was done at the Anatomy Department of Moi University. Preparation of tissues for histological studies involved Fixed in 10%, Dehydrated with increasing concentration of ethanol, Cleared with xylene, and Impregnation with paraffin wax for 24 hrs, were embedded in wax before sectioning, they were then dewaxed with xylene, hydrated decreasing of ethanol then Staining with haematoxylin and counter stain with Eosin, the tissues were dehydrated with increasing concentration of ethanol, cleared with xylene and Mounting using DPX. Tissues were prepared for histological observation as previously described by (Wallington, 1980).

3.5.2 Male rat fertility

The setup was repeated for male rats i.e. 9 controls and 9 treated groups. Administration of the extracts was similar to the procedure adopted for female rats. However in synchronization was not necessary for male rats spermatogenesis is a continuous process. Some of the male rats were sacrificed according to the procedure described earlier. Testis were obtained from the rats and subjected to the tissue processing procedure already described. Testis, prostate gland, epididymia and seminal vesicles were removed and weighed. Cross sections of the testis and epididymia were obtained and processed according to the procedure described and then mounted. Also photographs of testis epididymia were photographed.

3.6 Light Microscopy

The sections were examined at (x400) and at (x1000) magnifications using an fitted with a Japan made, Canon digital camera, model 400D, Several photomicrographs were taken in bright field.

3.7 Chemical analysis of extracts

The chemical components of the extracts were analysed by gas chromatography and mass spectroscopy.1 mg of the paste was dissolved in 1 mL of Dichloromethane and vortexed for 5 minutes. The homogenate was then centrifuged at 13,000 rpm.1µl of the supernatant was injected in the GC-MS and readings obtained (Zhang & Zuo, 2004).

3.8 Cytotoxicity tests

The extracts of the *S. cuneifolia* roots were tested for *in vitro* cytotoxicity, using human embryonic lung fibroblast (HELF) Vero-199 cells using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (Mossman 1983). The HELF cells were cultured and maintained in minimum essential medium (MEM) supplemented with10% Foetal calf bovine serum (FBS). The cells were cultured at 37^{o} C in 5% CO_2 and harvested by trypsinization, pooled in a 50ml vial. Approximately 100ul cell suspension (1×10^{5} cells/ml) was added to each well in a 96-well micro-titer plate. Each sample was replicated 3 times and the cells incubated at 37^{o}in 5% CO_2 for 24 hrs for attachment. 150ul of the highest concentration (1000 mg/ml) of each of the test samples (a serial dilution, prepared in MEM) was added into the same row and a serial dilution done. The experimental plates were incubated further at 37^{o}C for 48hrs. The cells in media without drugs were used as controls. After 48hrs of incubation visualization dye was added into each well. The cells were incubated for 4 hrs or until a purple precipitate was clearly visible under a microscope. The medium together with the dye was aspirated off from the

wells and dimethlysulfoxide (DMSO) (100µl) was added and the plates shaken for 5 min. This method was described by Kurokawa *et al*.,(2001). The absorbance for each well was measured at 562nm in a micro-titre plate reader (Mossman, 1983) and percentage cell viability (CV) calculated via an excel program with the formula;

$$\%CV = \frac{\text{Average abs of duplicate drug wells} - \text{Average abs of blank wells}}{\text{Average abs of control wells}} \times 100\%$$

Key: abs average absorbance

A dose–response curve was plotted which shows the % CV (of the Vero-199 cells) (Kigondu, 2009).

3.9 Data analysis

All statistical analyses were performed with STATISTICA 10.0 statistical package. Data was analysed using One-Way ANOVA. All analysed results were declared significant at $P<0.05$. Normality of data distributions were checked by means of the skewness and kurtosis to determine any need for applying appropriate data transformation procedures as described in Zar (2001) and t-test was used to compare the various measured reproductive parameters.

CHAPTER FOUR

RESULTS

4.1 Effect of *Sida cuneifolia* methanolic root extracts on the reproductive cyclicity of female albino rats

A normal oestrous cycle in laboratory rats lasts four to five days. Plates 4.1 to 4.15show changes in cells observed in vaginal smears in both control and treated rats. Figures 4.1-4.3 are graphs showing numbers and types of cells of normal cyclicity up to day 8 and those obtained after treatment with the root extracts.

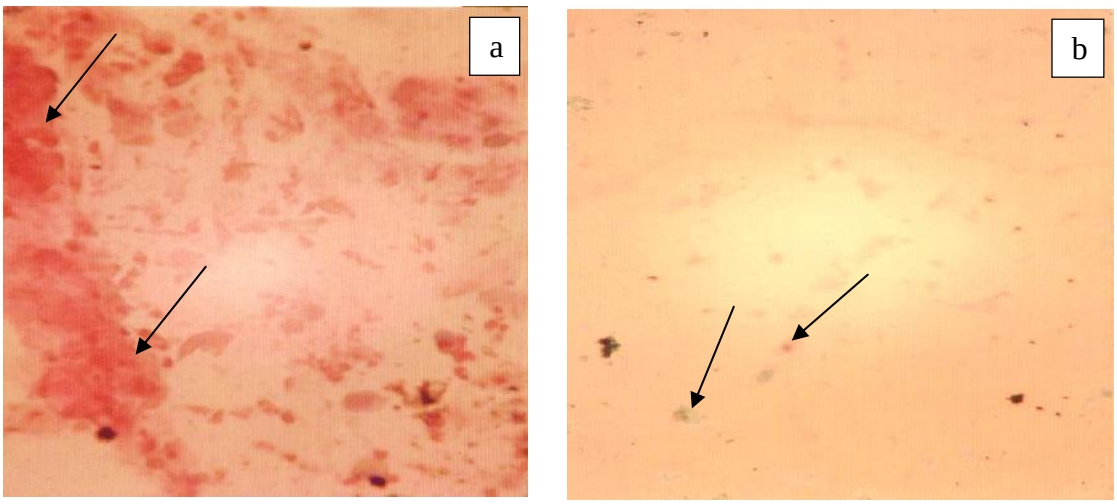

Plate 4.1(a) and (b): Histological examination of vaginal smears taken on the first day of the oestrous cycle (Proestrous/Oestrous) (H & E x400) (Source: Author, 2014)

Smears from control rats (a) showed cell debris, mucus and a bloody looking discharge (arrows) while those from treated rats showed scattered parabasal cells and lacked mucus (arrows).

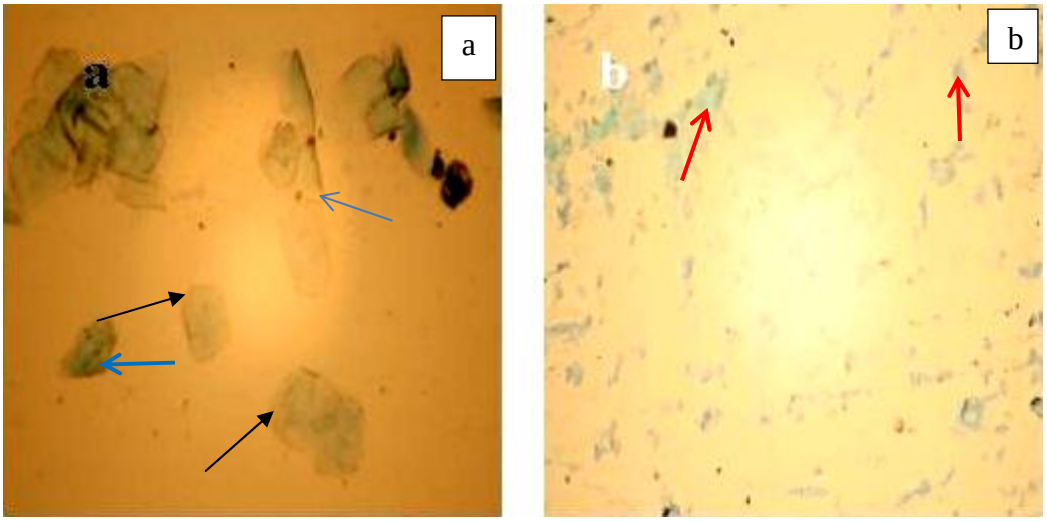

Plate 4.2(a) and (b): Histological examination of vaginal smears taken on the second day of the cycle (Oestrous) (H & E x 400) (Source: Author, 2014)

In smears from control rats, large cells, with irregular outlines and piknotic nuclei were predominant (blue arrows). These are epithelial cells of the superficial type, which appear only at estrous (fertile) phase of normally cycling rats. The slides contained large amounts of watery mucus and cornified cells were also observed in the smears (black arrows). Few leucocytes were observed in the smears. Variation in cell numbers started to occur immediately after treatment. Superficial cells completely disappeared in the treated rats and were replaced by non-nucleated cornified cells (red arrows) (b).

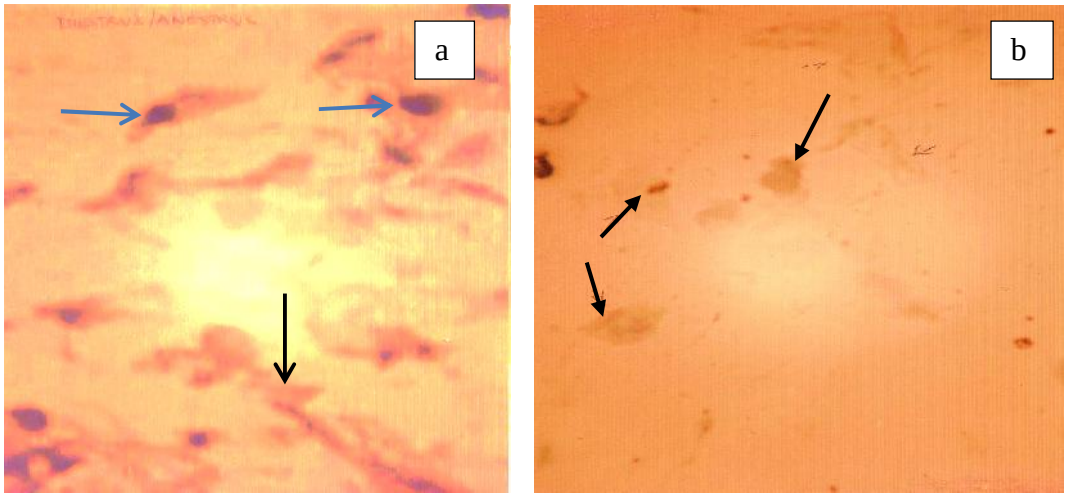

Plate 4.3 (a) and (b): Histological examination of vaginal smears taken on the third day of cycle (Oestrous/Metestrous) (H&E x400) (Source: Author, 2014)

In control rats (a), superficial cells had disappeared from the smear and had been replaced by a high number of leukocytes (blue arrows) and thick stringy mucus(black arrow). In treated rats (b) the smear showed few scatterednon-nucleated parabasal cells (arrows).

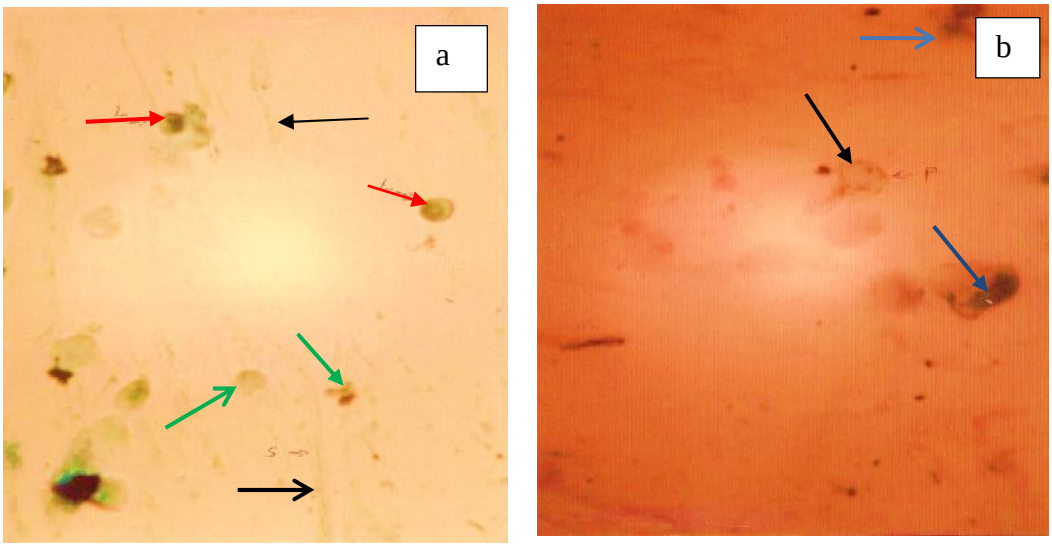

Plate 4.4 (a) and (b): Histological examination of vaginal smears taken on the fourth day of cycle (Metestrous/Diestrous)(H & E x400) (Source: Author, 2014)

There were many Leucocytes (red arrows), non-nucleated parabasal cells (green arrows) and a lot of mucus (black arrows) in smears from control rats (a). Smears from treated rats (b) showed presence of non-nucleated parabasal cells (black arrows) and a few leucocytes (blue arrows).

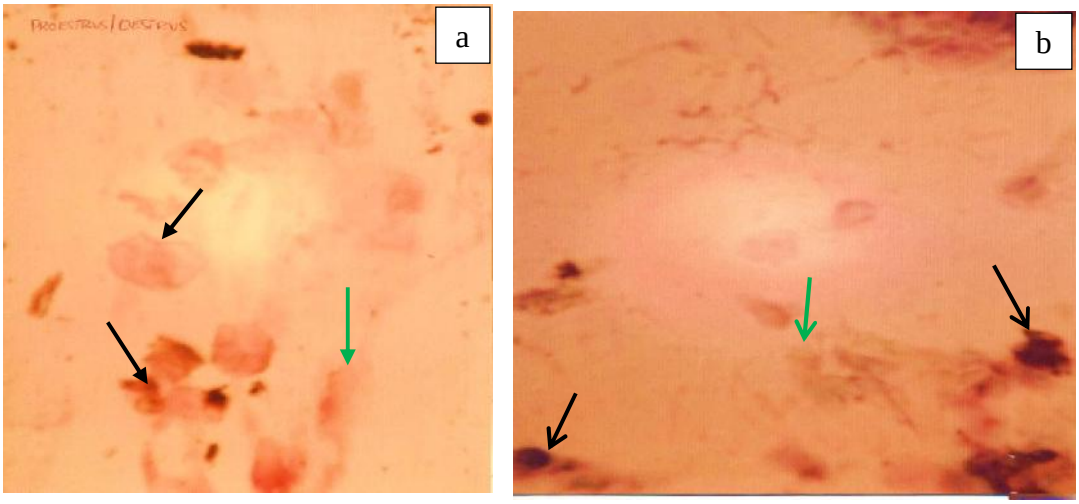

Plate 4.5 (a) and (b): Histological examination of vaginal smears taken on the fifth day of cycle (Proestrous/Oestrous)(H&E x400) (Source: Author, 2014)

Smears from control rats were dominated by epithelial cells of the superficial type, (black arrows) indicating a return to cyclicity and the mucus (green arrow) was light in texture. Smears from treated rats (b) showed a high number of leucocytes (black arrows) and thick stringy mucus (green arrows).

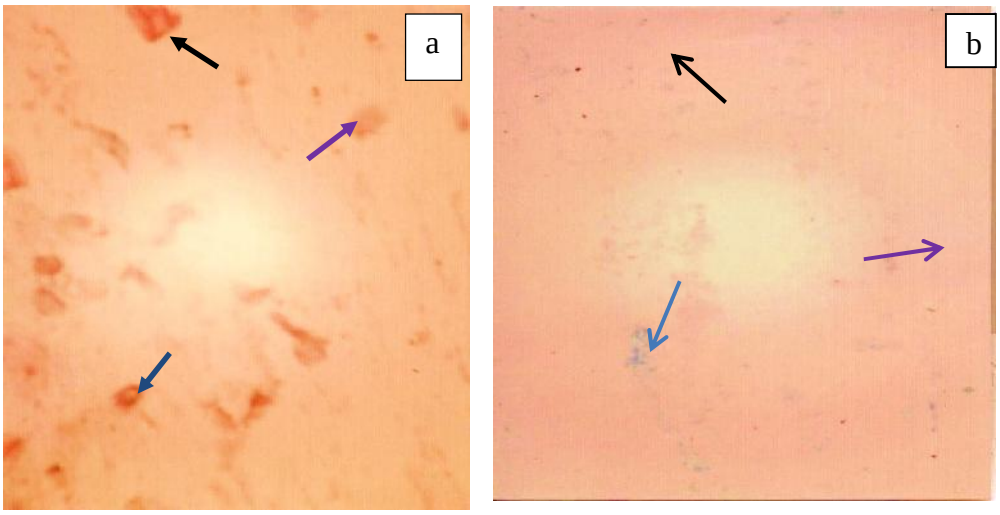

Plate 4.6 (a) and (b): Histological examination of vaginal smears taken on the sixth day of cycle (Oestrous/Metestrous)(H&Ex400) (Source: Author, 2014)

Smears from control rats (a) showed diminishing numbers of epithelial cells of the superficial type (black arrow) and leucocytes (blue arrow). Light textured mucus was also observable (purple arrow). Epithelial cells had completely disappeared in smears taken from treated rats (b) and only non-nucleated parabasal cells were observed (arrows). These features are characteristic of infertile phases (metestrus/diestrus) of cycling rats.

The appearance of the smears from extract treated rats, continued that way for the rest of the study eventually showing very scanty numbers of cells at the end of the investigation.

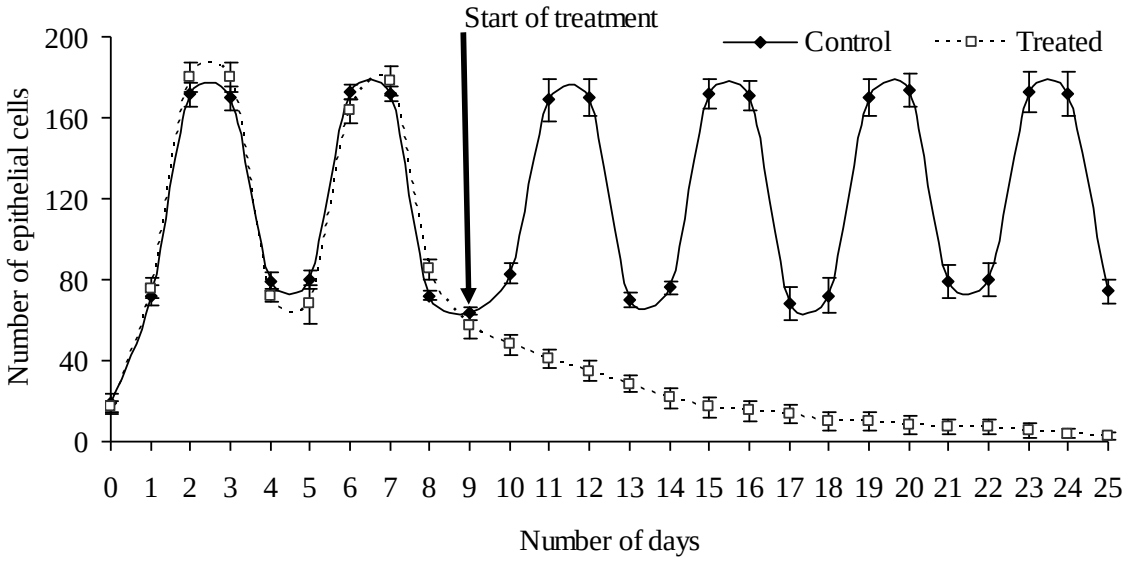

Figure 4.1: Number of epithelial cells in vaginal smears

The number of epithelial cells during the reproductive cycle of control (continuous lines) and of rats treated with root extracts from *Sida cuneifolia* (Dotted lines) is shown in fig.4.1

There was a clear inhibition of epithelial cells over the 4-5 days corresponding to one estrous cycle after treatment with extracts and continued in the same manner over subsequent cycles.

From the 9^{th} day, rats that had been given the extracts, started to show a continuous decline of epithelial cells which finally disappeared from the smears by the end of the investigation. Smears from control rats on the other hand, continued to show cyclic changes of epithelial cells characteristic of normal oestrus cycles. After treatment there was a considerable increase in the number of leucocytes which reached a peak of an average of 275 cells/field of view and remained high for the rest of the investigation. The number of leucocytes in the control rats showed normal cyclicity.

However, smears from control rats showed normal cyclicity with a rise and fall in numbers of these cells, where each peak in superficial cells corresponded to the fertile phase (proestrous/oestrous on days 1-2) and trough to the infertile phase (metestrous/diestrous on days 3 to 5). Such peaks were completely missing in treated rats. The rise and fall in numbers of cornified cells closely followed those of epithelial cells as seen in normal cycling rats, in the vagina. This was observed to be the case in control rats.

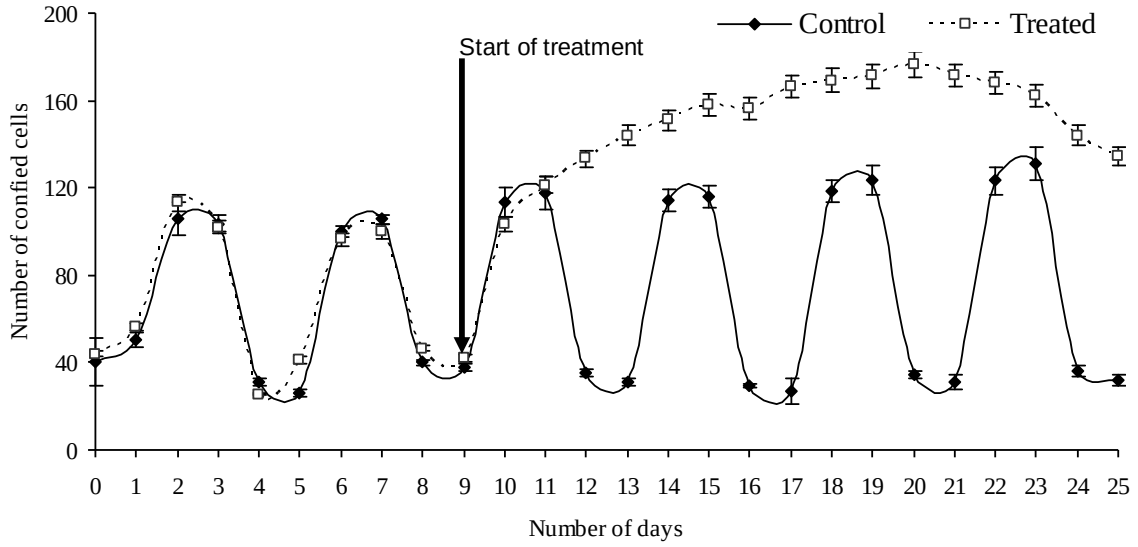

Figure 4. 2: Number of cornified cells in vaginal smears.

The number of cornified cells during the reproductive cycle of control (continuous lines) and of rats treated with extracts from *Sida cuneifolia* (dotted lines) is shown in fig. 4.2.

Smears from treated rats showed a continuous increase in cornified cells reaching an average of 190 cells/field of view on the 11th day after treatment. Thereafter the number of cells started to decrease. The trend was maintained over subsequent cycles. In contrast, smears from extract treated rats did not show such a trend. Figure 4.2 also showed a similar pattern of changes in cornified cells with the cornified cells increasing

to 115 cells/field of view and then declining to an average of 20 cells/field of view in control rats.

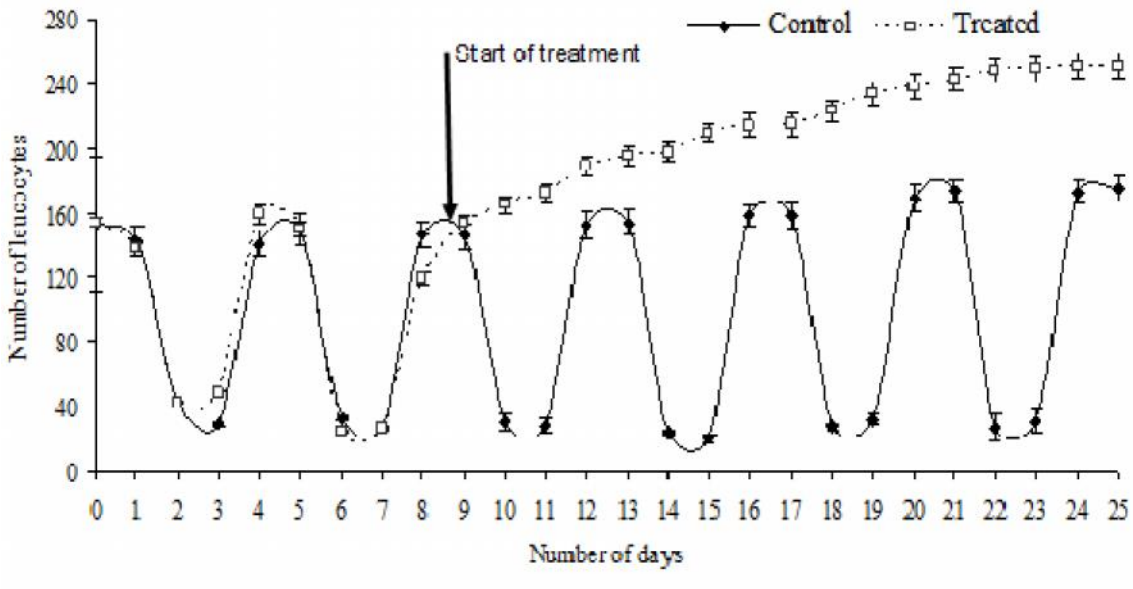

Figure 4.3: Number of leucocytes in vaginal smears

The number of leucocytes in vaginal smears during the reproductive cycle of control (Entire lines) and of rats treated with extracts from *Sida cuneifolia* (Dotted lines) is shown in fig.4.3

In control rats, leucocytes showed a trend of decline during days 2-3 corresponding to the fertile period, later rising in numbers during days 4-5 which corresponds to the infertile period (Metestrous/Dioestrous).

Numbers of leucocytes showed a cyclic change in control rats while in treated rats the numbers rose and remained high throughout the investigation. The number of cells was lowest on the first to second day (proestrus/oestrus) at an average of 10 cells/field of view and started to increase reaching a maximum of 165 cells/field of view on the third to fourth day (oestrus/metestrus). This pattern was repeated over the next three cycles.

4.2 Effects of *Sida cuneifolia* methanolic root extracts on the histology of ovaries, uteri and vaginas of treated rats

Changes in the gonadal histology in female rats, following treatments with root extracts of *S. cuneifolia* are shown in plates 4.7- 4.10

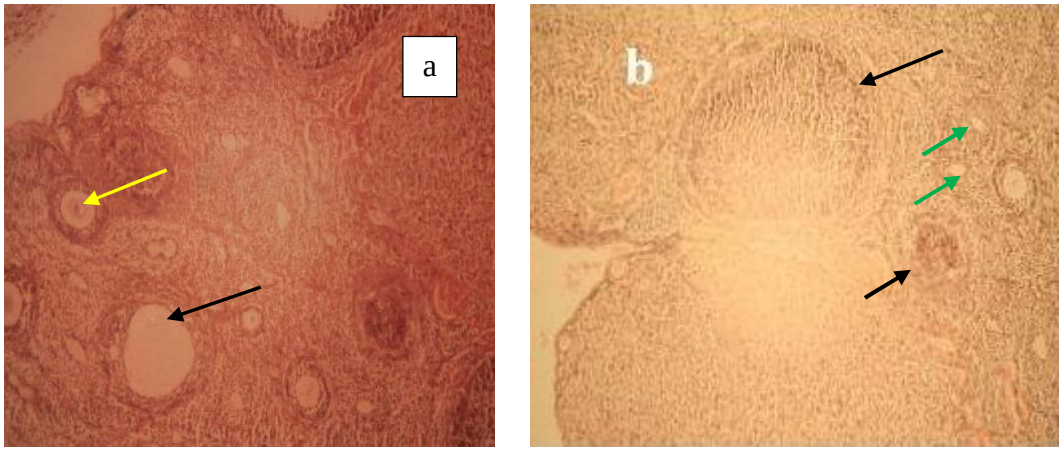

Plate 4.7: Longitudinal sections of ovaries in the untreated control (a) and in the treated animals (b) (H and E x 400) (Source: Author, 2014)

Note many follicles in various stages of development in the control black arrows, yellow arrow shows follicle with ovum (a) and degenerate follicles, green arrows and Corpora lutea filled with a lot of fibrous material in the treated, blue arrows (b).

Histological sections of ovaries from control rats showed normal ovarian morphology. The covering epithelium (tunica albuginea), cortex and medulla were clearly visible. The stroma was made up of reticular fibres and fusiform cells with follicular cells at different stages of development. Some follicles had large antra, with ova in others and with thick follicular walls showing normal development (plate.4.7a). Sections of ovaries from treated rats showed thin ovary outlines (tunica albuginea), contained corpora lutea with a

lot of fibrous material, small atretic follicles with thin follicular walls and reduced antra lacking ova in some (plate 4.7b).

Whole uteri from control rats were large with little fat deposit around them and with wide lumens. Sections from the uteri of control rats revealed normal features of uterine morphology. The myometrium was surrounded by the serosa and below it a rich network of blood vessels.

In vaginal sections of control rats, different layers were clearly visible namely stratum mucosum, stratum corneum, and stratum germinativum and vaginal lumens were wide, which are features normally observed at oestrus of normal cycling rats (Plate 4.4a). Lumen contents contained mucus, since this was plentiful in freshly prepared slides. Secretory glands were clearly visible in the underlying layers of the vaginal walls. Sections from treated rats showed drastic changes in the vaginal morphology. The section showed a degeneration of layers and clearly visible spaces within the vaginal walls. No secretory glands were visible and the vaginal lumen was full of cell debris.

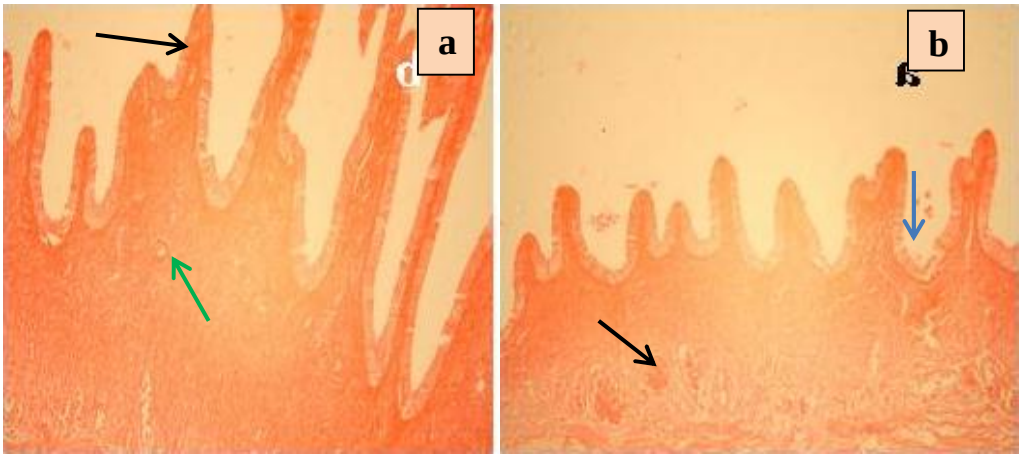

Plate 4.8: Histological examination of uteri of the untreated control (a) and treated rats (b) (H and E x400) (Source: Author, 2014)

Note the thick uterine lining, tall columnar cells (black arrow) and glands (green arrow) in control (a) and degenerating uterine wall (blue arrow) and glands (black arrow) in treated (b)

The sections also showed an enlarged endometrium made up of a single layer of columnar epithelium. Deep within it, was the lamina propria which connected it with the compactly arranged smooth muscle layer that had normal uterine glands (plate 4.8a). On the other hand, whole uteri of treated rats were shrunk with narrow lumens and considerable fat deposits around them. Sections of uteri showed sloughing off, of the uterine lining into the lumen. There were large epithelial cells with vacuolations and a high infiltration of polymorphonuclear cells extending into the endometrium. Few endometrial glands showing disruption of glandular epithelia and an increase in fibrous interstitial tissue was also evident. Instead of tall columnar cells characteristic of the oestrus phase, these were degenerate and in some places sloughing off the basement membrane. There were no clear demarcations of layers characteristic of normal uteri at oestrous.

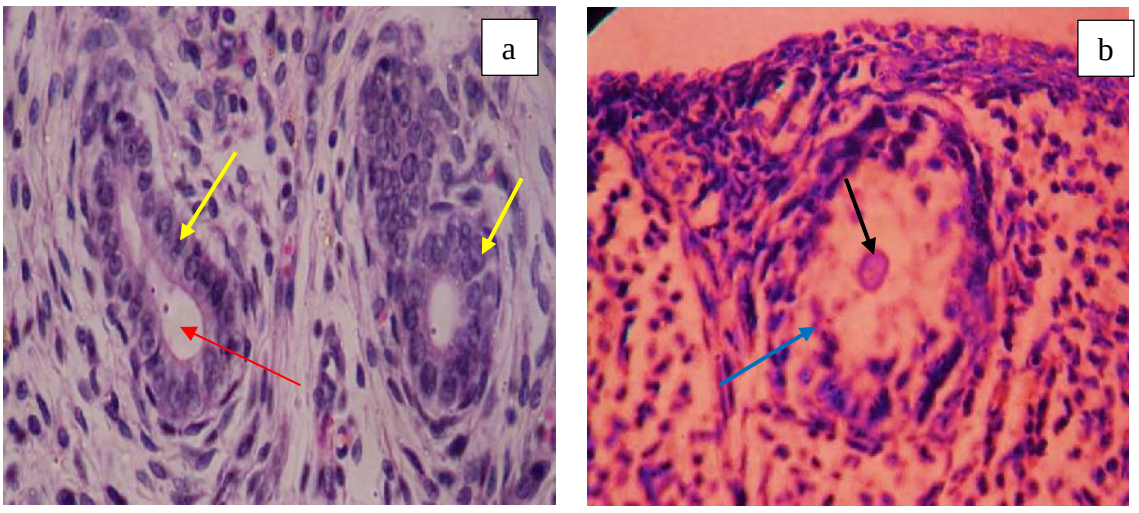

Plate 4.9: Histological examination of secretory glands in uteri (GIEMSIAx400)
(Source: Author, 2014)

Glands in untreated control (a). Note prominent cuboidal cells in walls of glands (yellow arrows) and lack of lipid droplets (red arrow). In treated rats (b) Note disintegrating cells in walls of glands(blue arrow) and accumulation of lipids (black arrow) in lumens of the glands.

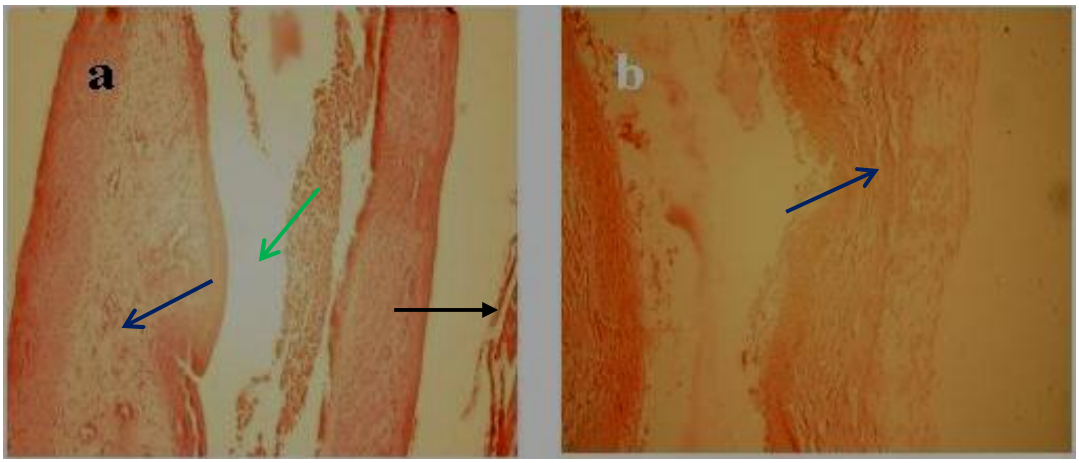

Plate 4.10: Histological examination of vaginal walls in the untreated control (a) and in the treated rats (b) (H & E x400) (Source: Author, 2014)

Note dilated lumen, (green arrow), clear layers and glands, (blue arrow) in control (a) and disappearance of layers and loss of glands (blue arrow) and narrow lumen with cell debris (black arrow) in treated (b).

4.3 Effects of *Sida cuneifolia*methanolic root extracts on the testicular histology of male rats

Changes in the male gonadal histology following treatments with extracted *S. cuneifolia* are shown in plates 4.11-4.15.

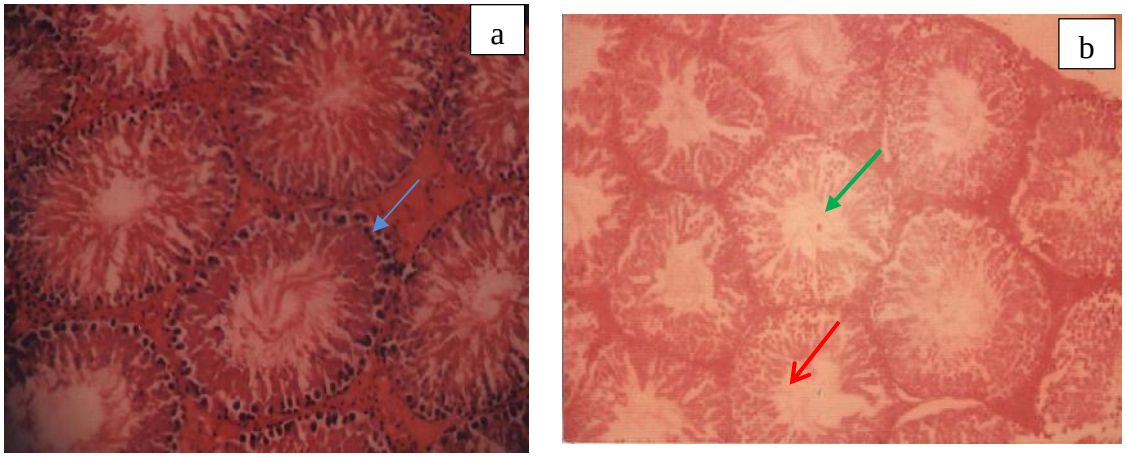

Plate 4.11: Histological examination of seminiferous tubules (H&E x400). In the untreated control (a) and in the treated rats (b) (Source: Author, 2014)

Note prominent spermatogonia (green arrow) and sperm filled lumens in control (red arrow) and in (b) lack of spermatogonia (black arrows) and lumens devoid of sperm (blue arrow).

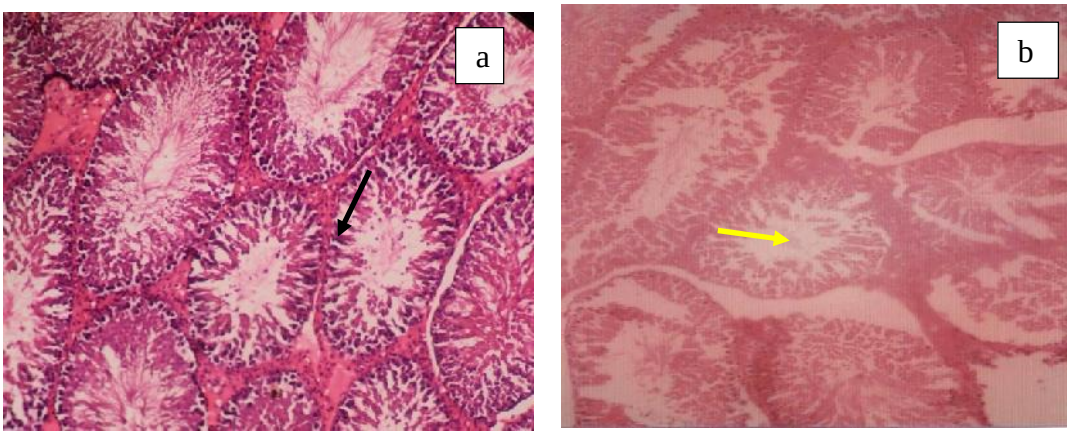

Plate 4.12: Histological examination of testes from controls (a) and treated (b) taken after twenty days (Giemsia x40) (Source: Author, 2014)

Seminiferous tubules in controls appear normal with prominent spermatogonia (black arrow) whereas in treated rats the tubules continue to be disrupted (black arrow) and lack spermatogonia (green arrow).

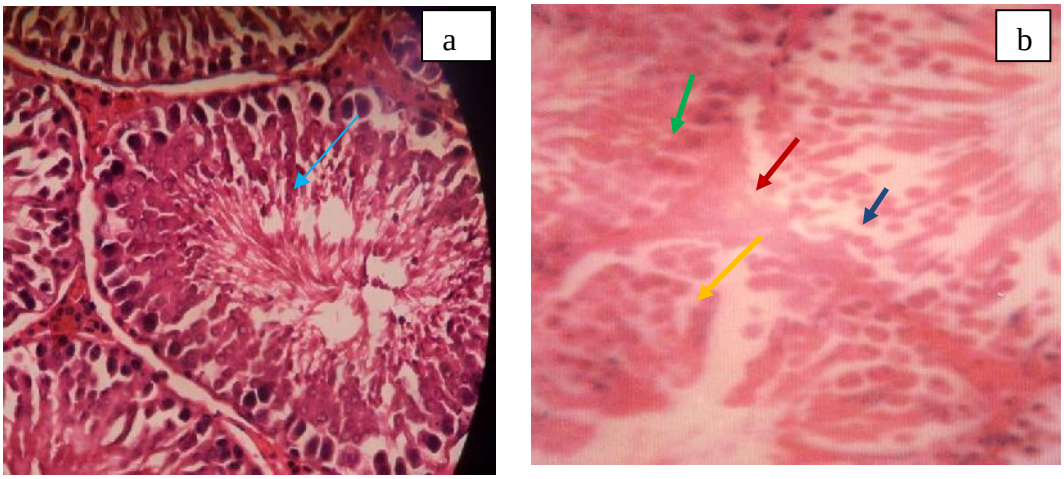

Plate 4.13: Histological examination of testes in controls (a) and treated (b) after twenty days (Giemsia x100) (Source: Author, 2014)

The slides from control rats still showed prominent spermatogonia (yellow arrow) leydig cells (green arrow) and sperm in lumens (blue arrow). In treated rats many intercellular spaces (blue arrows) and disintegrating leydig cell (red arrow) were observed

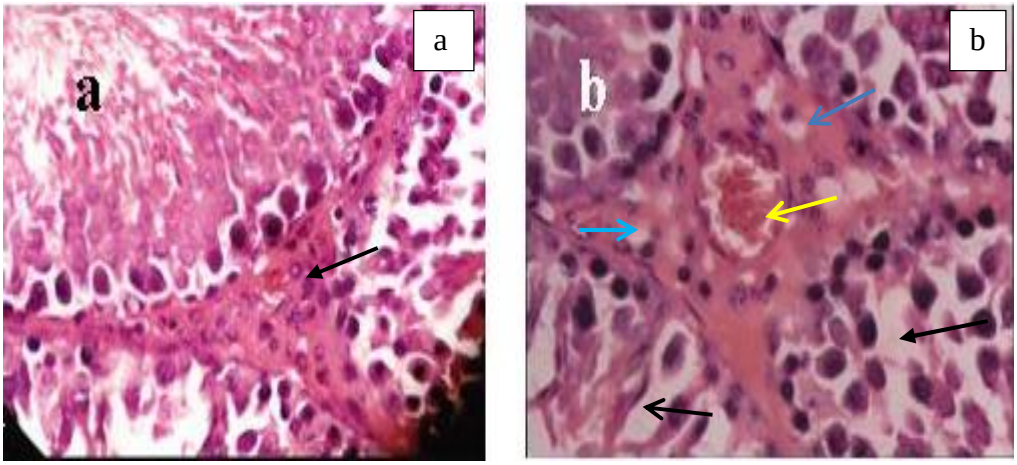

Plate 4.14: Histological examination of leydig cells in the untreated control (a) and in the treated rats (b) (H & E x1000) (Source: Author, 2014)

Leydig cells of control rats showed sizes typical of normal rats and lacked fat deposits (black arrow) while those of treated rats, were enlarged, contained prominent lipid droplets that appeared clumped(yellow arrow).Many intercellular spaces were also observed (blue arrows). 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. There were many intercellular spaces (black arrow) probably due to destruction of Sertoli cells.

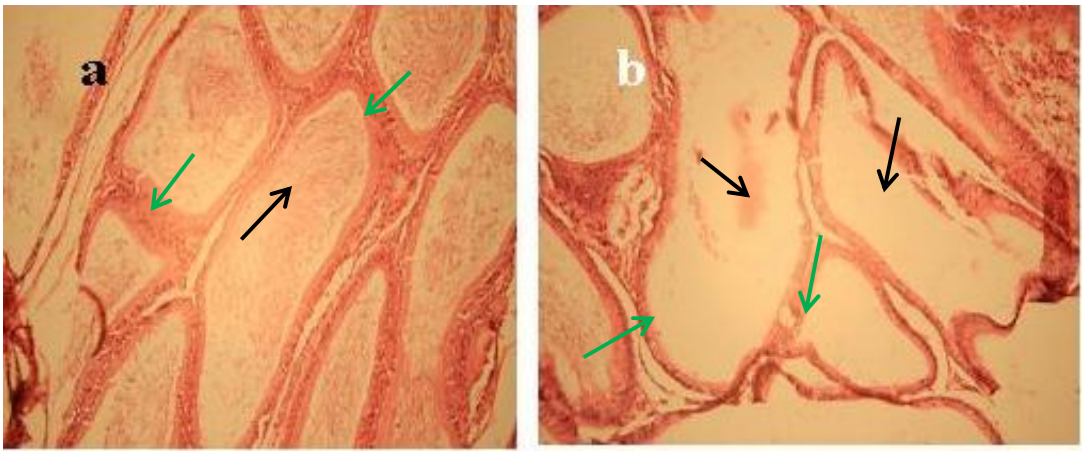

Plate 4.15: Histological examination of cross sections of epididymia in the untreated control (a) and in the treated rats (b) (H & E x1000) (Source: Author, 2014)

Note clear outlines of epididymal cells (green arrows), and lumens filled with spermatozoa(black arrow) in the control (a) and enlarged cells with distorted outline (black arrows) and large empty lumens in the treated (green arrows) (b).Epididymia of testes from control rats showed clear normal outlines and their lumens were filled with sperm, whereas those of testes from treated rats, showed large empty cells with thin broken outlines, and detachment from adjoining cells.

4.4 Effect of *Sida cuneifolia* methanolic root extracts on weights of testis and associated organs of rats

Results showing the effects of *S. Cuneifolia* methanolic root extracts on body weights and gonadal parameters for the male rats are shown in Table 4.1. The extracts significantly ($p<0.05$) reduced the weights of testes and associated organs in treated rats.

Table 4.1: Effects of *Sida cuneifolia* extracts at a dose of 100 mg/100 g body weight in different parameters of the body in male *R. norvegicus* after 7 and 14 days treatment with plant extracts

Group	Initial body weight (IBW)	Final body weight (FBW)	Weight of testis (g)	Weight of prostate gland	Weight of epidydimia (g)	Testiculo-somatic index (g%)	Prostrate-somatic index (g%)	Epididymal-somatic index (g%)
7 day control	101.2 ± 1.3	122.5 ± 4.5	3.79 ± 0.5[a]	0.52 ± 0.04[a]	0.91 ± 0.06[a]	3.09 ± 0.35[a]	0.39 ± 0.03[a]	0.74 ± 0.08[a]
7 day treated	102.3 ± 1.7	124.5 ± 5.2	3.51 ± 0.4[a]	0.46 ± 0.05[a]	0.86 ± 0.05[a]	2.82 ± 0.28[a]	0.37 ± 0.04[a]	0.69 ± 0.07[a]
14 day control	102.3 ± 1.3	157.3 ± 6.7	3.84 ± 0.5[a]	0.48 ± 0.04[b]	0.82 ± 0.03[a]	2.43 ± 0.23[b]	0.31 ± 0.06[b]	0.52 ± 0.05[b]
14 day treated	100.9 ± 1.4	157.9 ± 6.1	2.54 ± 0.7[b]	0.32 ± 0.02[a]	0.65 ± 0.04[a]	1.61 ± 0.22[a]	0.20 ± 0.05[a]	0.41 ± 0.06[a]

Data are expressed as mean ± SEM of five animals. Values with different superscripts (a,b) differ from each other significantly; $p < 0.05$ (two tail *t*-test) on each sampling date.

After 14 days of exposure to the plant extracts, the relative weights of testes, prostrate and epididymia were significantly (two tail t-test; $p < 0.05$) reduced. In contrast, no significant change in value of these parameters was observed 7 days after treatment in respect to the controls. The weights of the same organs in the control showed little change (Table. 4.1)

4.5 In vitro toxicity of *S. cuneifolia* plant extract

Hexane extracts of *S. cuneifolia* had a CV of 100% at both concentration 1000 mg/ml and100 mg/ml. The methanol extracts had a CV of 99.34% at concentration 1000 mg/ml and 100% at 100 mg/ml concentration. Methanol and water extract of *S. Cuneifolia* had a CC_{50} of 100%. The water extracts recorded CV of 23.75% and 31.56% at 1000 mg/ml and 100 mg/ml concentration, respectively. The DCM extract of *S. cuneifolia* had a CV of 11.28% and 28.57% at 1000 mg/ml and 100 mg/ml, respectively. The methanol extracts recorded a CV of 99.34% and 100% at 1000 mg/ml and 100 mg/ml concentration, respectively. Dimethylsulfoxide and sterile distilled were used as negative control recorded a CV of 100 at both concentrations. Chloroquine (CQ) and TDR (22651/1) was used as positive controls. Chloroquine recorded CV of 25.28% and 51.94% at concentration 1000 mg/ml and 100 mg/ml, respectively. The other control TDR 22651/1 recorded a CV of 7.89% and 11.28% at concentration 1000 mg/ml and 100 mg/ml respectively. Cell toxicity was done on microtitre plates.

4.6 GC-MS Analysis of *Sida cuneifolia* methanolic root extracts.

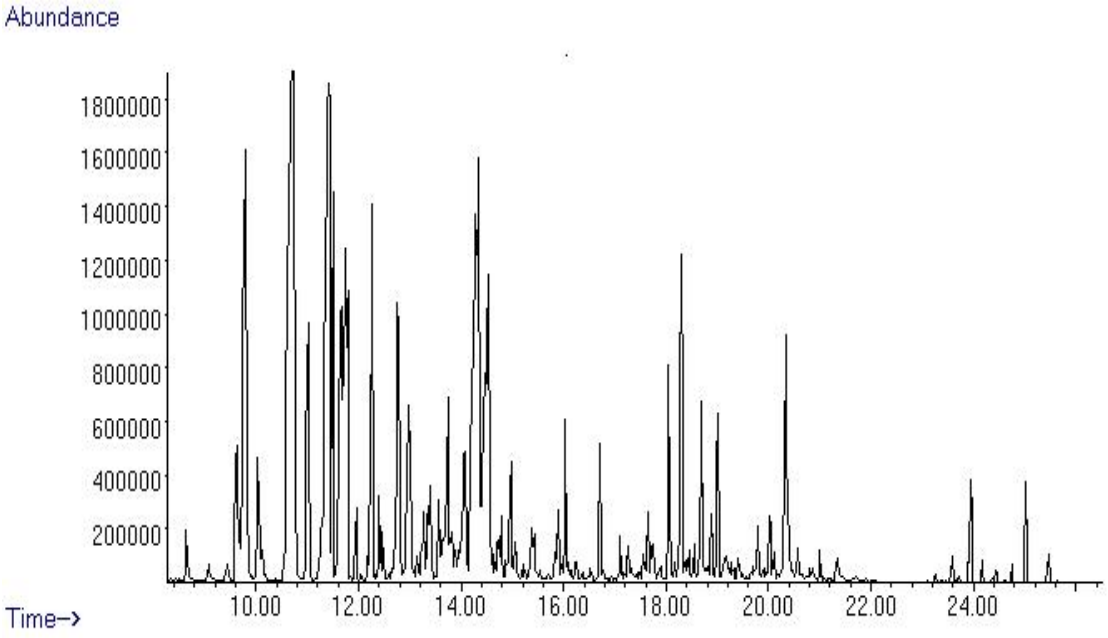

Figure 4.4: Histogram printout for MS of biocompounds in *S. cuneifolia* root extracts (Source: Author, 2014)

Results of the GC-MS analysis of the methanolic root extracts of *S. cuneifolia*, showing the compounds and their relative abundance are given in Table 4.4.

Table 4. 2: Biocompounds in *S.cuneifolia* methanolic root extracts

	Compound name	RT (min)	% of total
1	Thujene<alpha->	9.649	1.449
2	Pinene<alpha->	9.784	5.905
3	Pinene<alpha->	9.784	5.905
4	Fenchene<alpha->	10.030	1.363
5	Sabinene	10.702	12.092
6	Myrcene	11.015	2.704
7	Carene<delta-3->	11.396	9.188
8	Mentha-2,4(8)-diene<para->	11.508	2.127
9	Cymene<para->	11.665	3.083
10	Sylvestrene	11.732	4.872
11	Terpinene<gamma->	11.755	4.782
12	Cineole<1,8->	11.799	4.782
13	Terpinene<gamma->	12.270	2.946
14	Sabinene hydrate<trans->(IPP vs OH)	12.404	0.482
15	Linalool	12.986	2.390
16	Bicyclo[2.2.1]heptan-2-one, 1,7,7-trimethyl-, (1R)-	13.748	2.496
17	Mentha-1,5-dien-8-ol<para->	14.084	1.839
18	Terpinen-4-ol	14.353	9.292
19	Terpineol<alpha->	14.532	5.058
20	2-Methylenebornane	14.778	0.769
21	Citronellol	14.980	1.117
22	Bornyl acetate	15.853	0.800
23	Terpinolene	16.032	0.992
24	trans-b- Ocimene	16.032	0.992
25	Muurola-4(14),5-diene<trans->	18.048	1.276
26	Muurola-4(14),5-diene<trans->	18.339	0.204
27	Epizonarene	18.698	1.528
28	Alaskene<beta->	18.877	0.469
29	Calamenene<cis->	19.011	1.147
30	Cuprenene<alpha->	19.773	0.648
31	Italicene	20.333	2.408
32	Naphthalene, 1,2,3,4,4a,5,6,8a-octahydro-7-methyl-4-methylene-1-(1-methylethyl)-, (1.alpha.,4a.alpha.,8a.alpha.)-	20.579	0.316
33	Isophyllocladene	23.939	0.758
34	Abietadiene	25.014	0.494
35	Nezukol	25.462	0.514

From the results in table 4.4 the most abundant compounds (when % abundance of the compounds was measured against retention time) in the extracts were Sabenene,Terpinen-4-ol, Pinene<alpha->, Thujene<alpha->, Sylvestrene

Terpineol<alpha->, Terpineol<alpha->, Terpineol<alpha->, and Cineole<1,8->. These have ring structures mimicking the structure of phytoestrogens. They may either be the ones responsible for suppressing fertility or may be working synergistically with other compounds.Structures of some the compounds found in the extracts are represented in figures 4.5 (a) to (j) below.

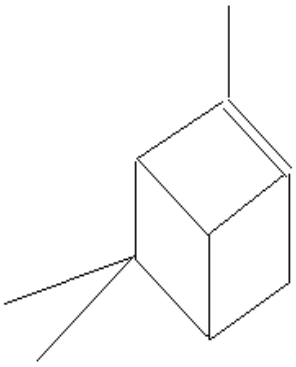

Fig.4.5 (a) Pinene alpha

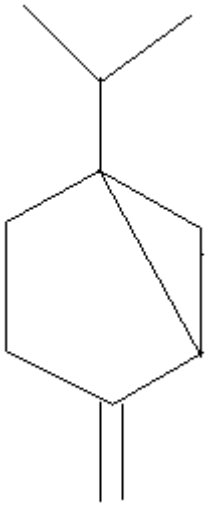

Fig. 4.5 (b) Sabinene

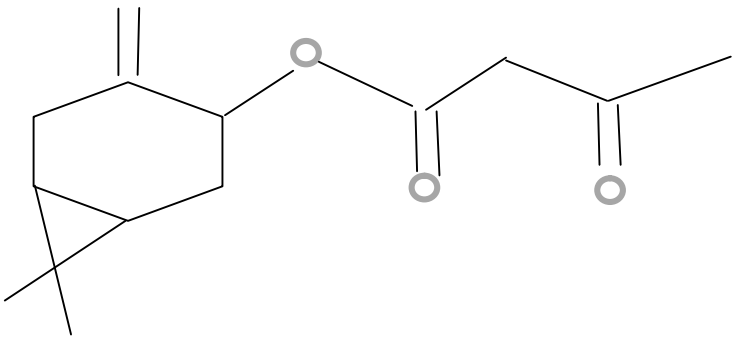

Fig.4.5 (c)Caren-4-ol, acetoacetic acid ester

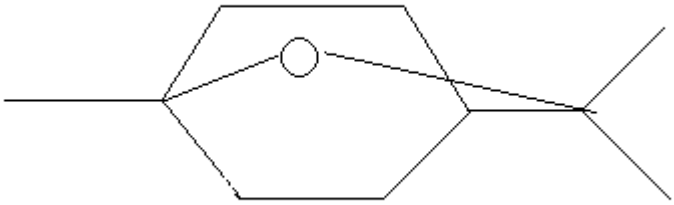

Fig.4.5 (d)8.76 Cineole<1,8->

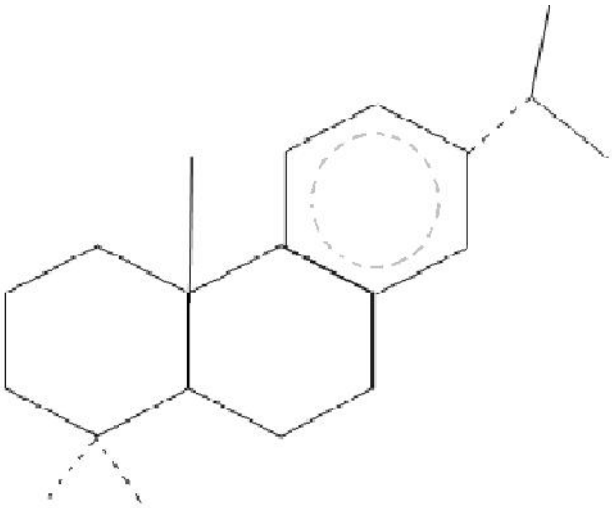

Fig.4.5 (e) Bornyl acetate

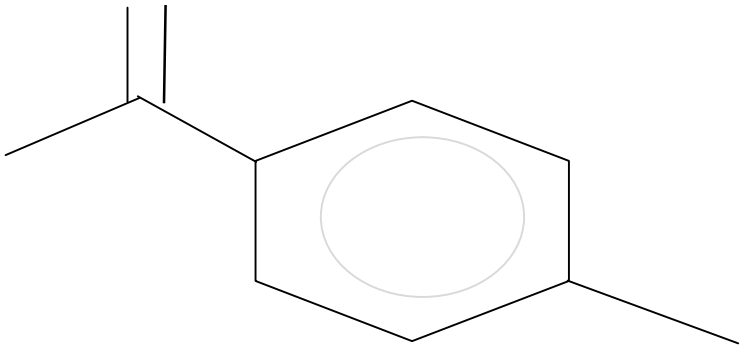

Fig.4.5 (f)Cymenene<para->

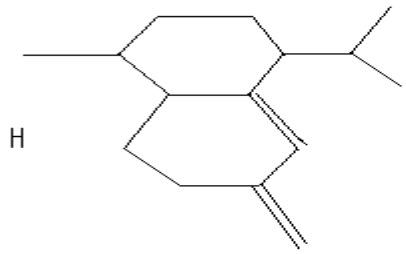

Fig.4.5 (g) Muurola-4(14),5-diene^ trans^

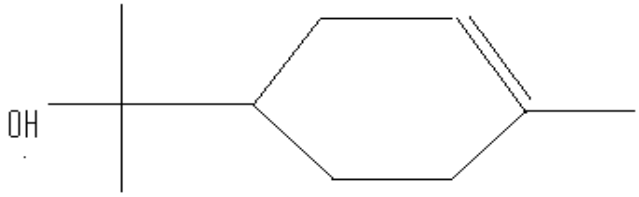

Fig.4.5 (i) 15.21 Terpenol<alpha>

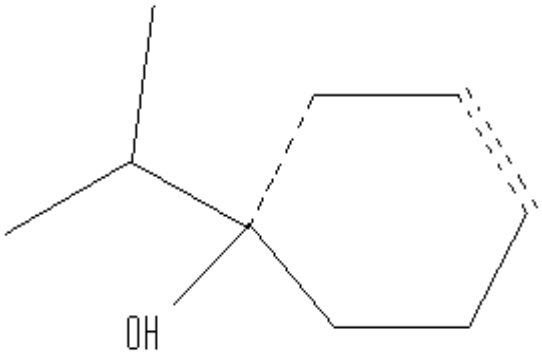

Fig.4.5 (j) 14.66 Terpinen-4-ol

The compounds belonged to a group of alkaloids, with antifertility effects, known as tarpenes ortarpenoids. Terpenes are among the most widespread and chemically diverse groups of naturalproducts. They are flammable unsaturated hydrocarbons, existing in

liquid form commonly found in essential oils, resins or oleoresins (Firn, 2010). Terpenoids includes hydrocarbons ofplant origin of general formula (C5H8) n and are classified as mono-, di-, tri- and sesquiterpenoids depending on the number of carbon atoms. Examples of commonly important monterp4.5 In vitro toxicity of *S. cuneifolia* plant extract.

Tarpenes include terpinen-4-ol, thujone, camphor, eugenol and menthol(Martinez *et al.,* 2008).They exhibit antioestrogenic and oestrogenic activity in rats resulting in altered cyclicity in female rats and histology of gonads in both female and male rats.

4.7 Results of the in vitro toxicity tests of the extracts.

Hexane extracts of *S. cuneifolia* had a CV of 100% at both concentration 1000 mg/ml and100 mg/ml. The methanol extracts had a CV of 99.34% at concentration 1000 mg/ml and 100% at 100 mg/ml concentration. Methanol and water extract of *S. Cuneifolia* had a CC_{50} of 100%. The water extracts recorded CV of 23.75% and 31.56% at 1000 mg/ml and 100 mg/ml concentration, respectively. The DCM extract of *S. cuneifolia* had a CV of 11.28% and 28.57% at 1000 mg/ml and 100 mg/ml, respectively. The methanol extracts recorded a CV of 99.34% and 100% at 1000 mg/ml and 100 mg/ml concentration, respectively. Dimethylsulfoxide and sterile distilled were used as negative control recorded a CV of 100 at both concentrations. Chloroquine (CQ) and TDR (22651/1) was used as positive controls. Chloroquine recorded CV of 25.28% and 51.94% at concentration 1000 mg/ml and 100 mg/ml, respectively. The other control TDR 22651/1 recorded a CV of 7.89% and 11.28% at concentration 1000 mg/ml and 100 mg/ml respectively. Cell toxicity was done on microtitre plates.

CHAPTER FIVE

DISCUSSION

5.1 Effect of *Sida cuneifolia* methanolic root extracts on the reproductive cyclicity of female rats.

The root extracts of *Sida cuneifolia* exhibited an antifertility and sterility effect on both female and male albino rats. This was evidenced by the reduction in the number of epithelial cells of superficial type as early as the second day of the cycle. These cells are usually abundant during the first and second day of the cycle and therefore are an indicator of fertility. Their absence meant that the rats had become infertile. The epithelial cells observed in the smears, were mostly those of the parabasal and intermediate type which characterise infertile phases showing a continued phase of infertility. The replacement of the light watery mucus with thick stringy mucus, characteristic of the infertile phase, also indicated a disruption in cyclicity. Production of light watery mucus is influenced by oestrogen and occurs prior to ovulation. Thick stringy, mucus does not favour swimming of sperm and is associated with low levels of oestrogen and occurs when ovulation is inhibited during the infertile phases of the cycle (http://www.gumbodesign.co.n.z).

Numbers of cornified cells and leucocytes in treated rats kept on increasing and remained high over the period of study. The numbers of superficial epithelial cells on the other hand, declined and remained low throughout the investigation. Both observations indicated interference with cyclicity and therefore inhibition of fertility in the rats. These findings agree with those recorded by Shaunfang and Barbara (2007) who recorded similar observations in normally cycling rats at the end of Metestrus.

The increase in cornifield cells in the treated animals was due to the breakdown of the stratum corneum. In normally cycling rats, cornified cells closely follow the pattern of superficial cells since they are known to assist in the swimming of sperm in the vagina. The mucus also aids sperm in swimming toward the ovum. The persistent high numbers of these cells in treated rats, indicated that mitosis had been interrupted and proliferation of the uterine lining did not occur, due to absence of oestrogen, a hormone responsible for the same. In the absence of the oestrogen therefore there was sloughing off of the stratum corneum. This explains the presence of a lot of cell debris observed in vaginal smears obtained from treated rats. All groups of female rats given the 1% root extract failed to conceive even after being kept with untreated males continuously in excess of seven months, after termination of the investigation. These observations show that cyclicity was completely interrupted in rats subjected to *Sida cuneifolia* root extracts at 1% concentration leading to a complete absence of proestrus.

The results also agree with those obtained by Mitak *et al*., (2001) who observed a complete absence of proestrus and oestrus, hence disruption of cyclicity, in animals treated with atrazine. The antifertility effect of the *S. cuneifolia* extract was immediate, as evidenced by changes in the cytology of the vaginal smears taken at the start of the cycle corresponding to diestrus/proestrus. Smears from control rats showed cells with normal cytology characteristic of the fertile phase, predominantly epithelial cells, of the superficial type i.e. large cells with irregular outlines and piknotic nuclei.

The variation of the numbers of cells in this investigation differ from those recorded by Murthy *et al*., (2008) who recorded a moderate change in the number of these cells at each phase compared to the those obtained in this investigation. This may due to a more potent effect of the *Sida cuneifolia* extracts than the one used by the researcher. Smears

from treated rats indicated a shift towards sterility as early as the second day of the cycle. The rats had ceased to cycle and had become sterile, therefore the smears continued to show similar morphology for the rest of the investigation. This necessitated stopping further taking of smears.

The rapid increase in the number of cornified cells and Leukocytes and marked disappearance of superficial epithelial cells in treated rats indicated complete sterility in these animals. There was a complete failure to reverse the infertility and this was in contrast to results obtained by Wiest *et al*., (1970) who observed a resumption of cyclicity on withdrawal of treatment, in rats that had been subjected to intraperitoneal injections of Winter cherry (*Physalis ekegengi*). Very high numbers of cornified cells are associated with pseudopregnancy and leucocytes are known to phagocytose sperm, hence preventing fertilization. On the other hand an increase in epithelial cells indicates mitosis and a return to cyclicity (Whitten *et al*., 1992).

The results in the present investigation, however agreed with those obtained by Chatterjee, (2011), who studied the antifertility effect of *Hymenocardia acida* in rats. The researcher observed a significant reduction ($p>0.05$) in the number of implantations in rats treated with stem bark extract of the plant. In the present investigation, this was supported by the presence of form cells (cells that had started cleaving) observed by the researcher in the vaginal smears of extract treated rats. Hence, it may be the anti-implantation action of the extract that is responsible for the antifertility effect of *S. cuneifolia*.

5.2 Effects of *Sida cuneifolia* methanolic root extracts on the female rat gonadal histology

The histological features of the ovary of the treated rats exhibited structural changes in comparison to the control rats. Sections from the ovaries of control rats showed all the histological features typical of healthy rats. The methanolic root extracts on the other hand impaired the normal histoarchitecture of the ovary. Whole ovaries were considerably reduced with fat deposits around them. Their outlines were thin and broken, indicating a degenerate germinal epithelium. Few follicles were observed in their sections and most of them had missing ova. These results agreed with those of Nelson *et al*., (1984) who observed a reduction in the number of follicles in sheep treated with Ladino clover. There was a lot of fibrous tissue in corpora lutea with few granulosa cells. These were corpora lutea from previous cycles as new ones had been inhibited from forming, by the extract. There were few follicles in primary stage of development indicating arrested oogenesis. Amabe *et al.,* (2010) noticed no observable changes in the ovaries of rats treated with *Azadirachta indica.* Results in this study are in contrast with theirs, probably due to a more powerful antifertility effect of *S. cuneifolia*.

The results in this investigation closely resembled those obtained by Westwood (2008), in which the ovary at metestrus shows vacuolation and a lot of fibrous material. Sections from ovaries of control rats showed follicles in various stages of development and some with clearly visible ova. When benzene extracts of the flowers of *Hibiscus rosa* were administered intraperitoneally at the doses of 125 mg or 250 mg/kg b.w. to adult mice, it resulted in an irregular estrous cycle with prolonged estrus and metestrus phases. An increase in atretic follicles and the absence of corpora lutea was an indication of the antiovulatory effect of the extracts (Akpoviroro & Fotherby., 1984).Abnormalities in ovulation may be due to direct ovarian actions since administration of another plant

hormone β-sitosterol in ewes inhibited follicular development and altered the size and distribution of the follicles (Lamartiniere *et al*.,1998a, b). Lamartiniere *et al*., (1998b) reported that genistein (phytoestrogen from soya) treatment in neonates, reduced the number of corpora lutea but increased the number of antral atretic and growing atretic follicles**.** These also appeared degenerate or atretic with intrafollicular hemorrhage. Gonadotrophic hormones (LH and FSH), especially FSH accelerates the growth of immature ovarian follicles whose walls produce oestrogen. The oestrogen in turn stimulates production of LH that is responsible for ovulation, formation and maintenance of the corpus luteum. The corpus luteum then produces oestrogen and progesterone. The former stimulates the production of FSH and a restart of the cycle. Normal cyclicity therefore depends on the ovarian hormone estrogen, therefore inhibition of its production leads to acyclicity and eventual infertility.

This resulted in a decrease in circulating progesterone concentrations. Phytoestrogens present in clover were responsible for the depression of fertility observed in sheep grazing on clover pastures, decreasing serum progesterone or pituitary LH. From the results of the present investigation, it has been confirmed that the root extracts influenced the ovarian physiology in albino rats preventing the normal fertile condition. Since ovarian growth and maturation is under the influence of FSH, these observations point to the inhibition of either the activity or the production of the hormone.

The results of this study revealed a disrupted histology of ovaries and uteri of rats treated with *S. Cuneifolia* methanolic root extracts. This is in line with Upadhayay *et al.,* (2007) who reported abnormal uterine and ovarian morphologies, and functions with the seed oil extract of *S. cuneifolia* which inhibited oestrogen. Observations in this study also resembled those recorded by Westood (2008) who observed necrosis of the uterine wall,

diminished glands and unclear demarcation of uterine layers at metoestrus and dioestrus. This also corroborates the findings of Prakasha *et al*., (2008) who earlier reported abnormal histo-architecture of the uteri of rats treated with neem oil extract.

5.3 Effect of *Sida cuneifolia* methanolic root extracts on the male rat testicular histology.

The enlarged seminiferous tubules, with thin distorted outlines and missing spermatogonial cells in treated male rats, suggested diminished serum levels of FSH and LH, also known as ICSH in males, 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. Enlargement of tubules meant that fluid responsible for transporting sperm out of them, was accumulating in the lumens and distending them. This occurs when oestrogen, which is responsible for reabsorption of the fluid, is lacking. The many intercellular spaces observed in seminiferous tubules pointed to the destruction of Sertoli cells. These cells produce oestrogen and androgen binding protein (ABP) after priming with FSH and also nourish developing sperm. Mature sperm are transported out of the tubules by ABP. This scenario may have been responsible for the distended tubules and the noticeably few, underdeveloped and distorted sperms observed in them. Degenerate tubule outlines also meant that the germinal epithelium, that gives rise to spermatogonia, was not properly maintained, probably due to lack of FSH. Sections of semeniferous from control rats showed normal histoarchitecture of tubules with prominent spermatogonia and sperm in various stages of development. The tubules were also filled with spermatozoa, a fact that pointed to normal functioning of the hormones.

Interstitial cells were either degenerated with deposits of fat droplets or altogether missing, in some areas. There was a lot of vacuolation (Plate 4.7b) pointing to degenerating leydig cells. This is typical of leydig cells of nonbreeding male animals and the results are in line with Neaves (1976) who noticed that leydig cells of non-breeding rock hyraxes were crowded with lipid droplets, along with 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. Lipids are precursors for steroid genesis 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 infertility observed in the male rats. Those from control rats showed normal morphology and lacked fat deposits and vacoulation. Leydig cell nuclear area and mature Leydig cell numbers were significantly reduced on oral administration of 70% methanolic extract of *Tinospora cordifolia* to male rats (Austin & Short, 1972). Observations in this investigation agree with their findings.

Sections from epididymia showed greatly distended cells, broken outlines and with scanty spermatozoa in the lumens. This was indicative of aspermia in the treated animals. The decrease in the weights of the testes themselves is an indication of arrested spermatogenesis. Excessive accumulation of fluid in epididymia, resulting in distension of the cells happens in the absence of oestradiol, a hormone responsible for fluid reabsorption and transport of spermatozoa (Steinberger, 1975). These observations indicated an antioestrogenic activity by the extract as observed in sections of epididymia from treated rats. The extract may also inhibit FSH, which is responsible for the development and maturation of spermatozoa. By extension, the extract may be interfering with the hypothalamo-hypophysial-gonadal pathway 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.

The effect of*Sida cuneifolia*methanolic root extracts on the testes may have been through suppression of testosterone, or the tropic hormone interstitial cell stimulating hormone (ICSH), responsible for its production. Testosterone is responsible for maturation of sperm and its absence, as evidenced by degeneration of interstitial cells, had a drastic effect on the sperm maturation process. In normal spermatogenesis mature sperm are transported out of seminiferous tubule and temporally stored in the epididymia. The lack of sperm, in these structures, indicated that spermatogenesis had been significantly inhibited by the extract.

5.4 Effect of *Sida cuneifolia* methanolic root extracts on body weights and weights of gonadal organs in rats

There was no change in the body weights of rats treated with *S. cuneifolia* methanolic root extract, which showed that the dose selected did not exert any harmful effect and the metabolic processes of the treated animals. Diminution in sex-organ somatic indices in extract treated animals is an indication of the effects of these extracts on reduction of steroid biosynthesis (Debanka *et al*., 2005). Results in this study suggest a similar effect since there was a significant reduction ($p<0.05$) in testicular weights in all rats given the extract. Testicular growth is an indicator of levels of gonadotrophins and testicular steroid biosynthesis an indicator of plasma levels of testosterone and growth of accessory sex organs (Debanka *et al*., 2005). Decrease in sperm density due to treatment by *Sida cuneifolia* root extracts strongly suggested low levels of FSH leading to suppressed spermatogenesis and the decrease in the Gonado- Somatic Index.

In an investigation of antifertility property of a triterpenoid glycoside isolated from *Dalbergia saxatilis* in female rats, a decrease in maternal body weights and inhibition of conception were observed (Khanna & Chaudhari, 2007). In this study, there was no change in body weights of controls and treated rats. However, there was a significant reduction ($P<0.05$) in the weights of ovaries and uteri of extract treated animals. The uterus exhibits signs of increased blood flow following estrogen administration in ovariectomized rats and rabbits and may increase in weight due to the accumulation of extracellular fluid (Kholkute *et al*., 2007).

Root extracts of *S. cuneifolia* at a dose of 100 mg/100g body weight significantly reduced the weights of reproductive organs of the male rats, a fact that supports the inhibition in testicular androgenesis by the extract. Reduction in the weight of testes and other accessory sex organs might be due to low levels of androgens especially a decrease in serum testosterone (Koneri *et al*., 2010). Ravindranath *et al.,* (2011) observed a reduction in weights of testes and accessory organs after treating male albino rats with 1.5 mg/kg bw of azadirachtin A, from neem plants. They concluded that the observation may have been due to the resulting low levels of serum testosterone, which were not enough to maintain the tissues of the gonads and accessory organs (Sharma & Jacob, 2011). It is an established fact that the accessory sex organs i.e. epididymia and vas deferens are androgen dependent target organs and manifest differential sensitivity to androgens for maintenance of their structure and function. It is also a fact that, any change in circulating androgens affects the internal microenvironment of epididymia and thereby leads to alteration in sperm motility and metabolism (Khan & Awasthy, 2003). Previous studies have documented that testicular inhibitory action resulted in elevation of testicular cholesterol level which may be due to the inhibition in the testicular androgenesis (Morris and Chaikoff, 1999). The *S. cuneifolia*root extract treatment for 14

days resulted in a significant diminution in testiculo-somatic, prostato-somatic and epididymal-somatic indices which may also be due to the inhibition in the testicular testosterone synthesis as testosterone is one of the prime regulators of sex organ growth and development (Patil *et al*., 1998). Two possible hypotheses may be proposed to explain the antigonadal activities of the extract. Decrease in sperm density by use of the *Sida cuneifolia* extracts strongly favored the suppression of spermatogenesis and that caused a decrease in GSI. One hypothesis is that the active ingredient(s) in the extract may alter the pituitary-testicular hormonal milieu. The alternative hypothesis is that the effective ingredient(s) may induce oxidative stress in testicular tissue leading to generation of free radical which may result in low testicular growth and function. This may explain the failure to sire offspring in male treated rats.

5.5 Cytotoxicity of the extracts

The results of the cytotoxicity tests showed the extracts to be nontoxic to cells at the concentration used. All cells exposed to the extracts showed no adverse effects, indicating that the extracts were 100% non-toxic to the cells.

CHAPTER SIX

CONCLUSION AND RECOMMENDATIONS

6.1 Conclusions

In conclusion this study found that:

Methanolic root extracts from the plant *Sida cuneifolia* have a contraceptive effect on both male and female rats. In female rats this was evidenced by the disruption of cyclicity, noticeable by cells characteristic of infertile phases in vaginal smears, during oestrus. Treated rats also failed to produce any young because they had become sterile

Methanol extracts of roots of the *S. Cuneifolia* plant have a disruptive effect on the histo-morphologies of the ovaries, uteri and vaginal walls of the treated rats. The extract also altered the histoarchitecture of testes, Leydig cells and Epididymia may be due to inhibition of GnRH and subsequently FSH and LH. *Sida cuneifolia* greatly reduced the weights of testes and associated organs.

6.2 Recommendation

The potential of this medicinal plant as a useful source of an antifertility agent in the species warrants further investigation, on the central mechanisms by which it acts. Further investigations are recommended, to be carried out on isolation and characterization of the components responsible for the effects, with a view to developing a future antifertility option for humans. The plant grows in the wild therefore there is need for conservation. It is also recommended that the effects of root extracts be investigated on primates.

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APPENDICES

Appendix I: Mean Epithelial cell counts

Day	Control	Treated	Mean	SD
0	19	17	5.1156	2.4338
1	72	76	4.9263	4.6614
2	172	180	5.7422	7.4547
3	170	180	6.0994	7.4547
4	79	72	4.1192	2.9819
5	80	68	4.9683	9.6220
6	173	163	3.6318	5.7351
7	172	178	3.6191	7.3719
8	72	85	2.3115	4.9653
9	63	57	3.3034	5.9366
10	83	48	5.1546	4.9992
11	169	41	10.4956	4.2702
12	170	35	9.1218	4.9242
13	70	29	3.6473	4.0469
14	76	21	3.1999	5.1681
15	172	17	7.2335	5.0531
16	171	15	7.1956	5.2311
17	68	13	8.2308	4.7492
18	72	10	8.7150	4.3267
19	170	10	8.8577	4.3267
20	174	8	8.5429	4.3532
21	79	7	8.2487	3.8635
22	80	7	8.3320	3.8635
23	173	5	9.8745	3.7554
24	172	4	11.0017	2.6177
25	74	3	6.3141	2.0369

Appendix II: Cornified cell counts

	Control	Treated	Means	SD
0	40	44	10.8842	1.3823
1	51	56	3.4553	1.7592
2	106	113	7.3287	3.5562
3	104	102	3.7214	3.2043
4	31	26	1.6071	0.8042
5	26	41	1.6209	1.2943
6	100	96	2.1042	3.0253
7	106	100	2.2241	3.1478
8	40	47	1.2842	1.4608
9	38	42	1.9695	1.3194
10	113	103	7.0426	3.2483
11	118	121	7.3034	3.8106
12	35	133	1.8964	4.1845
13	31	144	1.6100	4.5238
14	114	151	4.8125	4.7468
15	116	158	4.8841	4.9699
16	29	156	1.2379	4.9070
17	27	166	5.6602	5.2212
18	119	169	4.9893	5.3154
19	124	171	6.4349	5.3782
20	35	177	1.6978	5.5447
21	31	171	3.2182	5.3845
22	124	168	6.3036	5.2777
23	131	162	7.5003	5.0955
24	36	144	2.3158	4.5238
25	32	135	2.7267	4.2253

Appendix III: Leucocyte cell counts

Day	Control	Treated	Mean	SD
0	153	153	41.6319	4.6520
1	143	138	9.7842	4.1959
2	42	42	1.4062	1.2770
3	30	48	1.0776	1.4595
4	141	159	7.3335	4.8345
5	150	150	9.3156	4.5608
6	33	25	0.6944	0.7601
7	27	27	0.5681	0.8209
8	147	119	7.6577	3.6182
9	146	154	7.6072	4.6824
10	31	165	5.0252	5.0169
11	28	172	4.5389	5.2297
12	152	189	8.1656	5.7466
13	154	195	8.0240	5.9291
14	24	197	2.2610	5.9899
15	20	209	1.8842	6.3547
16	158	214	6.6525	6.5068
17	158	215	8.2325	6.5372
18	28	223	1.4589	6.7804
19	32	234	3.0467	7.1149
20	169	238	8.3166	7.2365
21	174	242	7.1412	7.3581
22	27	248	8.2121	7.5405
23	31	249	7.3074	7.5709
24	173	251	6.2998	7.6318
25	175	251	7.6741	7.6318

Appendix IV: Chromatography column

Appendix V: Rotary evaporator