EFFECTS OF KENYAN *GREWIA TENAX* (MUKAWA WA GUBA) ROOT EXTRACT ON FEMALE WISTAR ALBINO RATS' ESTRUS CYCLE AND REPRODUCTIVE HORMONES

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Effects of Kenyan *Grewia tenax* (Mukawa Wa Guba) Root Extract on Female Wistar Albino Rats' Estrus Cycle and Reproductive Hormones

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DECLARATION

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

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DEDICATION

I dedicate this thesis to my late aunt, Assumpta Mbinya, who until her death had been there for me, seeing me through education since the demise of my dear parents, thank you for the love and care. I also dedicate this work to my loving wife Floda and our children Kelly and Laura, who have been an inspiration and a rich source of encouragement and support. Finally, to my dear siblings Lillian and Mutiso for being my support pillars all my life, I love you.

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ABBREVIATIONS AND ACRONYMS

JKUAT – Jomo Kenyatta University of Agriculture and Technology

SoMED – School of Medicine

WHO – World Health Organization

GoK – Government of Kenya

NAC - N-acetylcysteine

LH – Luteinizing Hormone

FSH – Follicle Stimulating Hormone

ANOVA – Analysis of variance

DCM – Dichloromethane

LD50 – Lethal dose at 50% of animals

PBS – Phosphate Buffered Saline

GHS – Globally Harmonized System of classification

OECD – Organization for Economic Co-operation and Development

ABSTRACT

Globally, infertility is estimated to be 8-12 percent of couples within the childbearing years, while in Kenya it is at 11.9 per cent. In ethnobotanical studies in Kenya and India, *Grewia tenax*, a shrub (Fam. Teliaceae), has been reported to be used as a pro-fertility herb. The main objective of this research was to determine the effects of Kenyan *Grewia tenax* root extract on female Wistar albino rats' ovarian cycle and reproductive hormones.The specific objectives of the study were to determine the phytochemical constituents of Kenyan *Grewia tenax* root extract and its acute oral toxicity. The study was also determined the extracts' effects on the female Wistar albino rat's estrus cycle and reproductive hormone profile. The study used 30 female Wistar albino rats with established regular estrus weighing 160 – 200 grams. The study was undertaken in 27 consecutive days with the first 10 days being for checking to ensure regular estrous cycle via daily vaginal smears. Regular estrus cycles were disrupted using norethisterone for seven days. In the last 10 days the animals were randomly divided into three groups: Group I; (negative control) administered with normal saline, Group II (test group) divided into three subgroups of 250, 500 and 1000 mg/kg extract administration Group III (positive control), administered with 0.83mg/kg body weight clomiphene. Blood for reproductive hormones analysis was collected daily from each rat tail and put on a slide with silver paste for Raman spectroscopy analysis. Results were analyzed using SPSS, and the means compared using one-way ANOVA followed by Tukey HSD post hoc t-test, p-value <0.05 was considered statistically significant. Data was presented in graphs and charts. Preliminary phytochemistry of the extract revealed the presence of alkaloids, flavonoids, saponins, sterols, terpenes and cardiac glycosides. There were no adverse effects at 5000 mg/kg extract on oral administration. The study showed significant restoration of the estrus cycle at 250, 500 and 1000mg/kg administration of *Grewia tenax* in comparison to the negative control. The frequency of estrus cycle had a significant increase in proestrus phase (P=0.001) and estrus phase (P=<0.001) with subsequent decrease in metestrus (P=<0.001) and diestrus (p=0.001). *Grewia tenax* methanolic root extract caused an increase in reproductive hormone intensity in a dose depended manner. This study concludes that *Grewia tenax* has fertility-enhancing potential due to its ability to regularize disrupted estrus cycles and raise the intensity of reproductive hormones in rats. The study recommends *Grewia tenax* phytochemical compounds isolation and characterization. Subacute and chronic oral toxicity studies should also be undertaken to establish the safety of *Grewia tenax*administration over a more extended period. The species requires further investigation to establish its effects on reproductive hormone receptors.

CHAPTER ONE

INTRODUCTION

1.1 Background information

Infertility is the inability to conceive after a year of regular unprotected intercourse (Ruder, Hartman, Blumberg, & Goldman, 2008). Globally, infertility is estimated to be 15 % of couples within the reproductive age , affecting around 50 to 80 million people (Macaluso *et al*., 2010). In as much as infertility does not confer direct threat to life, it confers mental distress only expressed by the infertile couple (Amudha & Rani, 2016). The use of herbal medicine especially in developing countries like Kenya has excellent popularity with 80% of Kenyans using different kinds of traditional medicine (Gakuya *et al.*, 2020)

There are many plants traditionally used in the management of infertility including Kenyan

Grewia tenax (Kaingu, Oduma, Mbaria, & Kiama, 2013), *Abelmoschus esculentus* roots (Ashidi, Olaosho, & Ayodele, 2013), *Cadaba fruticosa* (Amudha & Rani, 2016) among others. Roots of Kenyan *Grewia tenax* have been reported to be used in the treatment of infertility in an ethnobotanical survey done in Tana River, Kenya (Kaingu *et al*., 2013) and India (Sharma & Patni, 2012). This use has not been validated scientifically, and data on its fertility effects is generally lacking hence the need for emperical research.

Grewia genus is in the family of Teliaceae with many species native to subtropics and tropics in Asia, Africa and Australia (Ahmed, Hamid, Babikir, & Eldor, 2012). *Grewia tenax* grows in areas with hot temperatures and rainfall of between 200 to 1000mm per annum (Sharma & Patni, 2012). Apart from the use in the management of infertility, this plant has also been documented to have antibiotic activity (Kapoor, Raksha, Sanjay, Swati, & Veena, 2013) and is used in the treatment of coughs, intestinal and skin infections and dysentery (Sharma & Patni, 2012). The plant is reported to possess free radical scavenging activities which help in its therapeutic

property against tissue damage. It also has iron and calcium that make it useful for the management of anaemia and strengthening of bones (Sharma & Patni, 2012).

1.2 Statement of the problem

Almost every couple desires to get children, so infertility leads to social and emotional distress (Adegbola & Akindele, 2013). Ovulation failure is the single most common cause of infertility in women with slight deviations in the hypothalamo-pituitary ovarian axis disrupting the cycle and causing unovulation (Rashid, Mahmoud, & Nore, 2013) There is a high social value attached to fertility especially in African culture where in some cultures marriage is not considered consummated unless there is the delivery of a child and survival through infancy (Araoye, 2003). Infertility has been documented as a cause of intimate partner violence (WHO, 2014) hence a major source of social concern. Infertility prevalence differs from region to region and Macaluso (2010) estimates infertility worldwide to be at 15% of couples within reproductive age. In sub-Saharan Africa, infertility is estimated to exceed 30% among couples within reproductive age (Macaluso *et al*., 2010) while in Kenya it is estimated to be at 11.9% in couples within reproductive age (MoH Kenya, 2007). A variety of medical infertility treatment options are available but the majority are within urban centres where personnel and equipment are available. This makes it difficult for the rural population to access these services. The services are generally not affordable to many couples having infertility problem. Infertility medication cost is averaged at $1,182 to as high as $24,373 for in vitro fertilization (IVF) (Katz *et al*., 2011). This cost is especially high in Kenya where per capita income is US$ 1,361 (Kenya Institute for Public Policy Research and Analysis (KIPPRA), 2017). Traditional herbal medicine is gaining increasing worldwide attention in health discussions. In Africa, up to 80% of the population use some form of herbal medication (Tilburt & Kaptchuk, 2008) with these products fetching up to 60 billion US$ in world markets. Use of traditional herbal products in the treatment of infertility would help infertile couples for they are available, accessible and affordable in rural communities. Due to stigma, some of the infertile couples find it hard to seek medical help and would benefit from this traditional medicine which is widely available and does not involve many consultations. Despite the reported traditional use of Kenyan *Grewia tenax* roots in management of infertility in females

(Kaingu, Mbaria, Oduma, & Kiama, 2014), there is no emperical data to show its effects on female fertility.

1.3 Justification

There are several modern medical treatment options for infertility, but these are not available, especially in rural areas. Therefore the need to research on traditional infertility treatment options and scientific validation of their potential to treat infertility. This study would be of great help to rural low-income communities who have difficulties in access or cannot afford other available treatment options.

Results from this study create data on the effects of Kenyan *Grewia tenax* on fertility and inform the public on its use. Results from this study form baseline information for researchers to undertake further research and develop pure molecules in the treatment of infertility. Results from this study form basis for future scientific studies by other researchers for having identified gaps for further studies on Kenyan *Grewia tenax* effects on fertility.

1.4 Research questions

1) What are the phytochemical compounds present in the roots of Kenyan *Grewia tenax* extract?
2) What is the acute oral safety of Kenyan *Grewia tenax* root methanol extract in female wistar albino rats?
3) What are the effects of Kenyan *Grewia tenax* root methanol extract on female Wistar albino rat's estrus cycle?
4) What are the effects of Kenyan *Grewia tenax* root methanol extract on female Wistar albino rat's reproductive hormones?

1.5 Research objectives

1.5.1 General objective

To determine the effects of Kenyan *Grewia tenax* root methanol extract on female Wistar albino rats' ovarian cycle and reproductive hormones

1.5.2 Specific objectives

1) To determine the phytochemical compounds present in Kenyan *Grewia tenax* root extract.
2) To determine the acute oral toxicity of Kenyan *Grewia tenax* root methanol extract in female Wistar albino rats.
3) To evaluate the effects of Kenyan *Grewia tenax* root methanol extract on Wistar albino rat's estrus cycle.
4) To evaluate the effects of Kenyan *Grewia tenax* root methanol extract on female Wistar albino rat's reproductive hormones.

1.6 Hypothesis

1.6.1 Null hypothesis

Kenyan *Grewia tenax* root extract has no effect on female Wistar albino rats' estrus cycle and reproductive hormones

1.6.2 Alternative Hypothesis

Kenyan *Grewia tenax* root extract has effect on female Wistar albino rats' estrus cycle and reproductive hormones

1.7 Ethical consideration

Ethical review was sought from Biosafety, animal use and Ethics committee of the University of Nairobi (UON), Faculty of Veterinary Medicine reference number FVM/ BAUEC/2018/182. All animals were handled humanely and were only used for this research. The plant was harvested observing environmental conservation measures**.**

CHAPTER TWO

LITERATURE REVIEW

2.1 Introduction

Infertility is the inability to conceive after a year of regular unprotected intercourse (Ruder *et al*., 2008). Globally, infertility prevalence is approximated to be 15% of couples within the reproductive age, affecting around 50 to 80 million people (Macaluso *et al*., 2010). In as much as infertility does not confer direct threat to life, it confers mental distress only expressed by the infertile couple (Amudha & Rani, 2016).

Due to increasing knowledge on infertility in the general public and within professionals, there has been an increase in the number of infertile couple referral to infertility expert clinics for both advice and infertility management. This increase may not be attributable to increasing infertility incidence but instead a reflection of an increase in the number of infertile couples seeking to get a solution. General practitioners attend to about 90% of infertile couples and among these approximately three quarters are referred for specialist care (Emslie, Grimshaw, & Templeton, 1993)

2.2 Causes of infertility in females

Causes of infertility are divided into male factors which make up 35% and female factors making up the rest. Female factors include; ovulatory dysfunction at 20%, tubal dysfunction at 30%, abnormal cervical mucus at 5%, and unknown aetiology at 10% (Cook, 2004). Female causes of infertility include:

a) Vaginal disorders
    - ❖ Vaginal aplasia
    - ❖ Imperforate hymen
    - ❖ Congenital vaginal atresia
b) Uterine conditions which include:
    - ❖ Congenital absence of the uterus

- ❖ Endometriosis
- ❖ Pregnancy
- ❖ Post-traumatic uterine synechia
- ❖ Progestational agents

c) Thyroid disorders
- ❖ Hypothyroidism
- ❖ Hyperthyroidism

d) Ovarian disorders
- ❖ XO gonadal dysgenesis
- ❖ XX gonadal dysgenesis
- ❖ XY gonadal dysgenesis

e) Adrenal disorders
- ❖ Congenital adrenal hyperplasia
- ❖ Cushing's syndrome
- ❖ Virilizing adrenal tumours
- ❖ Adrenocortical insufficiency

f) Pituitary hypothalamic disorders
- ❖ Hypopituitarism
- ❖ Constitutional delay in the onset of menses
- ❖ Nutritional disorders
- ❖ Hyperprolactinemia
- ❖ Tumours and infiltrative diseases

g) Central nervous system disorders

h) Congenital absence of the gonad
- ❖ Testicular feminization syndrome
- ❖ 17-hydroxylase deficiency of the ovaries and adrenals
- ❖ Autoimmune oophoritis
- ❖ Resistant ovary syndrome
- ❖ Polycystic ovary syndrome
- ❖ Ovarian tumours
- ❖ Precocious menopause

2.3 Female Reproductive Physiology

2.3.1 Oogenesis, ovulation, and fertilization

Oogenesis is controlled from and outside the ovaries and spans through the embryonic period to the first reproductive cycle (Tingen, Kim, & Woodruff, 2009). Oogonia originate from primordial germ cells (PGCs) formed during early embryonic life from embryonic mesoderm (Tingen *et al*., 2009). If there are mutations to the genes that code for the survival factors of PGCs, the PGCs undergo apoptosis and hence deplete germ cells. After formation, the PGCs migrate to the genital ridge. Simultaneously, sex differentiation occurs with ovary formation following the default pathway, while testes differentiation needs the influence of Y-linked gene *Sry*. Gonads without this gene automatically develop into ovaries. Activin increases the number of PGCs in humans and increases the follicular diameter (Da Silva *et al*., 2004). PGCs undergo mitotic divisions without complete cytokinesis leading to germ cell nests. Still, these divisions stop just before the start of follicle development, and meiosis begins to form primary oocytes that commit to the program of female development (Sánchez & Smitz, 2012). Germ cell nest maintenance processes are not well understood, but studies have shown estrogen hormone maintains them via estrogen receptor (ER)-β (Sánchez & Smitz, 2012)

Meiosis is arrested at prophase 1 until the oocyte is activated by luteinizing hormone (LH) at puberty. Activities of prophase 1 are essential for germ cell survival and meiotic progression, any errors at this phase or interference by chemicals may lead to infertility (Sánchez & Smitz, 2012). During this meiotic arrest period, the oocyte enlarges and synthesizes RNA and proteins as the follicles grow (Küpker, Diedrich, & Edwards, 1998). Around the time of meiotic arrest, germ cell nuclei and the surrounding granulosa cells are packaged to form the primordial germ cell, which forms the initial follicle pool and forms the total germ cell reservoir for the entire female's reproductive life (Tingen *et al*., 2009). Follicle formation happens during the second trimester of fetal life. The majority of oocytes are lost through atresia from fetus to sexual maturity, with approximately 7 million oocytes undergoing atresia in a lifetime, and usually, about 400 to 500 oocytes will ever ovulate (Selvaraj & Sathananthan, 2015).

Primordial follicles are activated and continuously recruited in cohorts to initiate folliculogenesis, which takes approximately six months in humans (Sánchez & Smitz, 2012). PTEN/PI3K signalling pathway is required for primordial follicle activation. Anti-Mullerian hormone (AMH) is needed to strike a balance between the number of primordial follicles activated and remaining in the resting pool. In the absence of AMH, there will be depletion of follicle resting pool (Sánchez & Smitz, 2012).

Primordial follicle develops into the primary follicle where we have the development of granulosa cells into the single-layered cuboidal primary follicle (Woodland, 1958). The primary follicle's development to the secondary follicle is driven by intra-ovarian paracrine factors from oocytes and not FSH. Gonadotropins (FSH and LH) are required for antral development, which starts with antrum formation and granulosa cell differentiation into cumulus and mural cell compartments, making the oocyte able to resume meiosis. FSH leads to the antral formation and induces LH receptors (Lhcgr) mRNA expression in mural cells. The follicles are then able to respond to LH leading to ovulation. Gonadotropin action is by attachment and activation of their receptors (LHR and FSHR); if the gonadotropin receptor is absent, the female becomes infertile because of the arrest of follicle development at the antral stage (Zhang, Poutanen, Wilbertz, & Huhtaniemi, 2001). Through the effect of LH, theca cells produce androgens, which are converted within granulosa cells to estrogen under the influence of FSH (Sánchez & Smitz, 2012)

Once the oocyte reaches 80% of their full-grown size, they acquire the capability to resume meiosis (Durinzi, Saniga, & Lanzendorf, 1995). LH surge happens, leading to the rupture of the follicle wall (ovulation) and the release of the cumulus-oocyte complex. LH surge leads to meiosis resumption and progresses to metaphase II and the first polar body release. Following ovulation, the remaining follicle cells are converted into a corpus luteum, and the theca cells in it become luteal cells that secrete estrogen and progesterone. Expansion of cumulus cells under the influence of LH is essential in the resumption of meiosis; hence low LH concentration disrupts physiological processes in the ovary, consequently leading to infertility (Sánchez & Smitz, 2012).

Once a sperm penetrates the cell membrane (oolemma) of a secondary oocyte in which meiosis had arrested at metaphase II, meiosis is completed with the formation of the mature ovum, which is haploid, and the extrusion of the second polar body(Nielsen, Bahadur, Hinrichsen, Mortimer, & Tesarik, 2001). The union of male and female pronucleus is the culmination of fertilization (Nielsen *et al*., 2001). This creates a single diploid cell (zygote), the first form of human life which develops into the new organism (Georgadaki, Khoury, Spandidos, & Zoumpourlis, 2016). Ejaculated sperms are protected from the acid vaginal media by protective elements that surround them. They swim through the cervical mucus into the uterus, which contracts to propel them into the oviducts. The sperms then swim through the fallopian tubes to the ampulla of the tubes where fertilization takes place (Georgadaki *et al*., 2016). If any of these necessities of sperm movement are interfered with, or there is any anomaly, the sperm can't reach the viable ova; hence fertilization does not occur.

2.3.2 Influence of reproductive hormones on the menstrual cycle

Effective procreation in females requires good coordination of both the nervous system and the peripheral organs. A functional hypothalamic-pituitary-ovarian axis enables this. The central nervous system sends a primary signal via Gonadotrophin Releasing Hormone (GnRH) to the anterior pituitary gland gonadotrope cells, which in turn lead to the regulation and release of both Follicle Stimulating Hormone (FSH) and Luteinizing hormone (LH) (Christensen *et al*., 2012).

LH and FSH exert their effects on the ovaries, where FSH leads to recruitment and differentiation of follicles in each cycle. As the follicles develop under FSH's influence, they start to release estradiol from granulosa cells, which negatively regulates GnRH and FSH, leading to their reduction. FSH and activins up-regulate LH receptors in the follicles. In the mid-follicular phase, estradiol's FSH negative feedback leads to a shift from FSH to LH dependent follicle stimulation. This shift rescues the dominant follicle that will respond to the LH surge at ovulation. The rest of the follicles which had been recruited under FSH undergo atresia. (Rojas *et al*., 2015)

The dominant follicle increases in size, resulting in increased estrogen synthesis. Activin levels decrease as inhibin synthesis from granulosa cells rises. Inhibin enhances androgen production from the theca cells and interferes with the growth of all non-dominant follicles. Estrogen induces endometrial proliferation with the increased stromal and epithelial mitotic division leading to endometrial thickening with accompanying elongation and coiling of spiral arteries. At its peak, estradiol production triggers GnRH surge, which leads to a rise in LH release from the anterior pituitary (Christensen *et al*., 2012) and the ultimate rise in ovarian estrogen, completing the positive feedback circuit. LH surge promotes progesterone secretion and augments plasminogen activator in granulosa cells hence an increase in tissue plasmin. This activates collagenases and stimulates tumour necrosis factor release by theca cells, further enhancing collagenolysis weakening the follicular wall, and eventually leading to ovulation (Rojas *et al*., 2015).

Following ovulation, the follicle luteinizes, and there is complete reorganization forming corpus luteum to support the ovulated oocyte. Progesterone production continues through the luteal phase from the corpus luteum and after fertilization before formation of the placenta, which continues progesterone production in pregnancy. Progesterone continues with thickening of the uterus for optimal implantation and maintenance of pregnancy after fertilization (Christensen *et al*., 2012)

2.3.3 Implantation

The fertilized ova travels through the oviduct for approximately three days into the uterus. At this point, it has divided mitotically into a 12 cell embryo called morula (Lopata, 2012). By day six, the morula has divided further and acquires a fluid-filled cavity and is referred to as a blastocyst, with 186 cells that undergo more division. Implantation begins on day 7 or 8 after fertilization (Lopata, 2012). Implantation is key to a successful pregnancy, with 75% of all lost pregnancies due to implantation failure (Norwitz, Schust, & Fisher, 2001). The blastocyst adheres to the upper posterior uterine wall (fundus) where the microvilli of the syncytiotrophoblast interdigitate with microprotrusions (pinopodes) of the uterine epithelium. More invasion of the uterine wall by the blastocyst happens, and then invasion as the

syncytiotrophoblast penetrates the uterine epithelium. The blastocyst embeds into the uterus's surface by day ten after fertilization (Cha, Sun, & Dey, 2012).

2.4 Rat oestrus cycle

Estrus is from the Greek word estrus, which means sting, gadfly, or frenzy to describe a particular female period of sexual desire (Westwood, 2008). It is characterized by epithelial morphological changes in the vaginal lining. The rat has four phases, referred to as proestrus, estrus, metestrus, and diestrus (Paccola, Resende, Stumpp, Miraglia, & Cipriano, 2013). Different vaginal histology characteristics typically characterize these phases of the estrus cycle. The proestrus phase is demonstrated by nucleated epithelial cells, estrus phase by epithelial cells, which lack the nucleus. The metestrus phase is shown by leukocyte cells, cornified cells, and nucleated cells of equal proportions. The diestrus phase is mainly characterized by leukocytes (Catherine, 2016).

The duration of proestrus and estrus phases is 12 hours for each of the phases then transitions to the next phase; metestrus lasts typically for 21 hours. The diestrus phase is the longest, lasting for approximately 57 hours. In literature, these phases of the estrus cycle are put in five phases: proestrus, estrus, metestrus I, metestrus II, and diestrus. Metestrus I, also called early metestrus, lasts for 15-18 hours, and metestrus II also called late metestrus and lasts for six hours. The cycle is also divided into proestrus, estrus, diestrus I (or metestrus), and diestrus II. This cycle has also been categorized in a 4-day cycle where diestrus lasts two days and is divided into diestrus I and II. Its also described in a 5-day cycle where diestrus is prolonged to last for three days hence has diestrus I, I, and III. Other authors have described the existence of an additional phase called anestrus, which they say is denoted by lack of ovarian activity and is marked in quiescent reproductive life (Westwood, 2008)

A female rat comes on heat every 4 to 5 days. Sexual activity happens in the dark during the estrus phase. It has also been documented that female rats can accept males towards the end of the proestrus phase. Estrogen and progesterone hormones are responsible for the structural changes seen in female rats' vaginal epithelium during the estrus. This makes the rat's vagina a reflection of ovarian function that reflects the role of sex hormones (Paccola *et al*., 2013)

Estrogen levels are elevated, and ovarian follicles grow fast during the proestrus phase, while ovulation occurs on the night of estrus 10-12 hours after the luteinizing hormone (LH) surge. If the rats do not mate while ovulating, the corpora lutea are transiently functional and secrete small amounts of progesterone. If the rats breed during ovulation, about 90% of ova are fertilized during the third hour following ovulation. The luteal life is then extended throughout the first half of pregnancy to maintain progesterone production. In the second half of pregnancy, progesterone is synthesized by the placenta (Paccola *et al*., 2013)

2.5 Effects of oxidative stress on the female reproductive system

In oxidative stress, reactive oxygen species (ROS), which are oxygen free radicals, causes increased cell damage by altering enzyme systems and cell signalling pathways (Tvrda, Knazicka, & Lukac, 2011). A functional female reproductive system requires an intact pituitary and ovaries, leading to ovulation, effective fertilization, embryo development, and implantation. Oxidative stress affects the effectiveness of these fertility success processes (Tvrda *et al*., 2011)

ROS are produced in aerobic metabolism and from exogenous sources like cigarettes, alcohol, and environmental pollutants like heavy metals. These ROS react with any molecule modifying them oxidatively. ROS causes impaired angiogenesis in ovarian follicles, which leads to atresia of the follicles (Ruder *et al*., 2008). Further compounding ROS effect on fertility is their association with endometriosis, hydrosalpinges, polycystic ovary syndrome (PCOS) (Smits, Mackenzie-Proctor, Fleischer, & Showell, 2018).

Cohort studies have shown intake of antioxidants may increase fertility, having been associated with increased rates of pregnancy and increased live births (Smits *et al*., 2018)

2.6 Management of infertility using medicinal plants

There are various conventional ways for infertility management. However, these methods are not affordable to many and are not readily accessible, especially for the rural population in developing countries (Oladimeji & Aroyehun, 2015). Traditional

herbal remedies have been used over the years in the management of infertility in different communities. In a study by Oladimeji (2015), leaves of *Newbouldia laevis* were shown to decrease prolactin levels and subsequently increase LH and estrogen levels. Since prolactin is known to suppress ovulation and loss of menses, its decrease increases the chances of ovulation with an increase of LH and estrogen promoting estrogen hence validating the plant's use in treating hypothalamic pituitary ovarian system-related infertility (Oladimeji & Aroyehun, 2015). Aqueous extract of *Ficus asperifolia* is reported to increase the number of implantation sites in rats. This increase was attributed to the uterotrophic effects of its phytochemical compounds (Watcho, Alango, Benoît, & Kamanyi, 2009). Ethanolic extract of *Anthocleista vogelii* decreases the levels of CD4+ and CD8+ hence creating a suitable immunological environment required for a successful pregnancy and also increases plasma highest of estrogen, therefore, enhancing fertility (Oladimeji, Igbalaye, & Coleshowers, 2014). Increase in steroid hormones during the menstrual cycle is associated with lower cell-mediated immunity.

2.7 *Grewia tenax* distribution and uses

Grewia tenax belongs to the family *Tiliaceae*, genus *Grewia*, and species *tenax.* It is a multipurpose plant species that is a source of food, fodder, fiber, fuelwood, timber, and a range of traditional medicines that cure various diseases.

Figure 2.1: *Grewia tenax* plant with fruits

Grewia tenax is highly drought resistant plant commonly found in arid and semi-arid areas. It is adopted to hi temperatures and dry conditions, its deep roots stabilize sand dunes. *G. tenax* is wide spread in Africa from transvall and South-West Africa to Ethiopia and Arabia in the North – East and through West Africa to Senegal. It is only found in the driest types of woodland or semi-desert scrub. Geographically distributed in Algeria, Botswana, Chad, Djibouti, Ethiopia, Iran, Kenya, Mali, Mauritania, Morocco, Namibia, Niger, Nigeria, Saudi Arabia, Senegal, South Africa, Sudan, Tanzania, Uganda, Zimbabwe, India and Pakistan (Sharma & Patni, 2012)

The plant preparations are used for the treatment of bone fracture and bone strengthening and tissue healing. The fruits are used for promoting fertility in females and are incorporated into special diets for pregnant women and anaemic children (Sharma & Patni, 2012). *Grewia tenax* extracts and preparations have shown many biological effects, including analgesia, antimicrobial, and antioxidant effects (Goyal, 2012). This plant has free radical scavenging activities which aid in its therapeutic action against tissue damage (Sharma & Patni, 2012)

2.8 Plant phytochemical screening

The use of different medicinal plants to cure/manage other medical conditions has been a practice world over, with 80% of the world's population using some form of traditional medicine (Wambugu *et al*., 2011). This is due to different bioactive chemicals (phytochemical compounds) found in these plants, which lead to physiological action in the body. Some of these phytochemical compounds include alkaloids, sterols and steroids, flavonoids, saponins, tannins, terpenes, and glycosides.

2.9 Acute oral toxicity studies

Herbal products are a source of novel pharmacological effects. Therefore, safety must be ensured before these products are used (Kasthuri & Ramesh, 2018). The assumption that natural products are just safe without testing can be lethal. Many deaths reported in animal studies lead to these product safety being questioned, hence determining their safety is paramount (Kabubii, Mbaria, & Mbaabu, 2015). Acute oral toxicity studies are the adverse effects that occur upon the acute exposure of a single dose of a substance (Walum, 1998). The evaluation of a plant extracts systemic toxicity is done by giving a single dose of the plant extract to a rat to determine the nature and duration of any acute toxic response involving also delayed toxicity. It helps in establishing the highest non-lethal dose and inform on single-dose exposure in humans. LD_{50} is a statistically derived dose that, when acutely administered, is expected to cause 50% death of treated animals within a given period (Walum, 1998). Acute toxicity causes adverse effects resulting in physiological impairment, biochemical lesions and affects whole-organism performance (Kabubii *et al*., 2015). Toxicity studies help determine the dose level that can be used safely and determine the product's therapeutic index (Kasthuri & Ramesh, 2018).

2.10 Raman Spectroscopy

The physical phenomenon of Raman scattering has been in use since 1928 when it was discovered by an Indian physicist C. V Raman (Ramos, Malkin, & Lyng, 2015). Spectroscopy uses the absorption, emission, or scattering of electromagnetic

radiation by matter to quantitatively and qualitatively study the matter (Kalantri, Somani, & Makhija, 2010). Surface-enhanced Raman spectroscopy is an analytical skill that is highly sensitive and enables one to detect single molecules and is widely used to determine biological and chemical molecules in a real-time and automated manner (Liu *et al*., 2018). Raman spectroscopy provides information on whole blood's molecular components; it has found use in biomedical applications (Ullah *et al*., 2019). Raman spectroscopy detects a single molecule, making it see minute changes in the chemical and biological sample (Han, Pienpinijtham, Zhao, & Ozaki, 2011). Raman spectrometer has been used in the diagnosis of cancer (Auner *et al*., 2018), measuring glucose levels (Birech, Mwangi, Bukachi, & Mandela, 2017), and in hormonal studies (Liu *et al*., 2018). Other available hormonal assays like Enzyme Liked Immunosorbent Assay (ELISA), radioimmunoassay, and high-pressure liquid chromatography though highly sensitive require sample pre-treatment which is costly. Raman spectroscopy is simple, rapid, highly sensitive and less costly to use especially in research setting and avoids the large quantity of sample required by other methods hence less number of animals used in studies.

Raman spectroscopy entails directing a focused laser onto a sample and then recording the light's energy profile scattered with every compound giving a unique spectrum arising from the excitation of the molecule's vibrational modes. In contrast, mixed samples spectra are a superposition of each constituent's signals, allowing relative band intensities to be used for quantitative analysis (Kalantri *et al*., 2010). Raman spectroscopy obtains spectral profiles that depict peaks and valleys that indicate the sample's rotational and vibrational transitions under study. The spectra displayed gives the fingerprint spectra unique to each specific compound, making it easy to identify substances (O'Brien *et al*., 2017). Every compound provides a unique spectrum arising from the excitation of the vibrational modes of the molecule. The Raman spectrometer is fed with either 532nm or 785nm monochromatic laser, which uses a green or a red laser beam of light delivered through an optic fiber. The beam is delivered to the bandpass filter via a shutter, which varies as per the laser used. The light is directed to the beam splitter from the bandpass filter, which splits the beam into two equal portions, of which 50% is reflected.

In contrast, the remaining 50% is transmitted to the sample to enable shattering, measured by the imaging spectrometer and a CCD camera. The spectrometer has 1800, 1200, and 600 BLZ gratings. The measurements must be taken in a dark room to reduce fluorescence produced by the background light. A motorized stage is used to focus the sample on a microscope with x10, x20, x50, and x100 objectives, and a computer with STR software is used to visualize the sample.

2.11 Norethisterone

Norethisterone is an effective oral contraceptive composed of potent progesterone with a high affinity for progesterone receptors in the pituitary and reproductive system (Kuhl, 2005). 5α-dihydronothisterone (5α-DHNET) is its main metabolite and inhibits steroidogenesis by its 5α-reductase inhibition activity. These antiadrogenic effects of 5α-DHNET are prolonged in rodents than humans. It has antigonadotropic effects causing a suppression of hypothalamic-pituitary-ovarian axisresulting in unovulation, changes in uterine lining preventing implantation and also thickening cervical mucus hence impeding fertilization (Ahrendt & K.J. Buhling, 2006; Kuhl, 2005).

2.12 Clomiphene

Clomiphene is a drug used in treatment of infertility in women especially due to unovulatory cycles including polycystic ovarian syndrome (Kousta, White, & Franks, 1997). It binds to estrogen receptors in the brain and causes pituitary follicle Stimulating Hormone (FSH) hence promoting follicullogenesis and as a result promoting steroidogenesis. It causes progesterone raise rise addressing luteal phase defect during the second half of the cycle making the length of the cycle more predictable (Dickey & Holtkamp, 1996)

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study site/area

The plant was harvested at Kisamis; the study site. Kisamis is found in Keekonyokie Ward, Kajiado West constituency, Kajiado County. Keekonyokie ward comprises of Esonorua, Oltepesi, Olteyani, Naserian, kisamis, kisaju, Iloodoariak, kipeto and oloyiankalani sub–Locations of Kajiado County. This ward is county assembly ward number 0926 and has a population of approximately 36,562 persons and covers an area of approximately 807.6 Sq. Km. Its a sub- arid hilly area with sandy rocky soil.

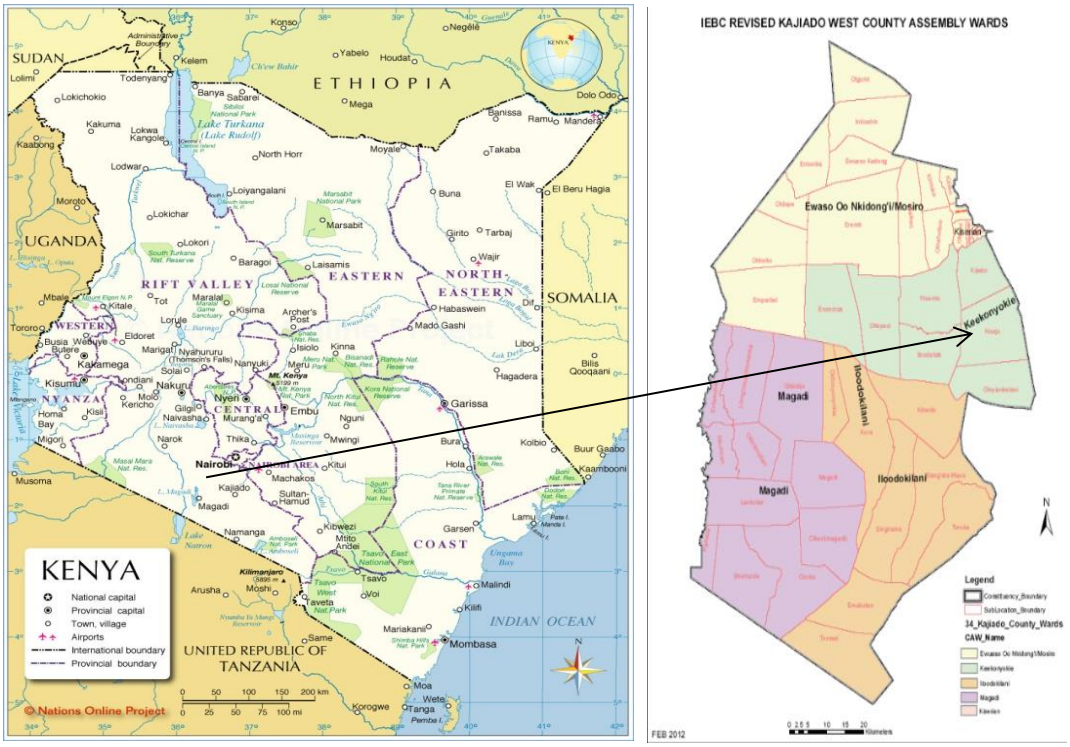


Figure 3.1: Administrative map of Kenya and Kajiado west constituency administrative wards

Plant extraction and phytochemical screening was done at JKUAT botany laboratory. All animal studies were done at the University of Nairobi (UON), department of

Veterinary Anatomy and Physiology animal house. Raman spectroscopy hormonal analysis was done at Laser Physics laboratory, Department of Physics, UON.

3.2 Study design

An experimental laboratory-based study design was used.

3.3 Plant collection

Kenyan *Grewia tenax* roots were collected in Kisamis, Keekonyokie Ward, Kajiado West constituency, Kajiado County, Kenya, in March 2019 with the help of a plant taxonomist and brought to JKUAT botany department. A voucher specimen was deposited in the UON botany herbarium voucher number PJK 2019/001 for future reference. The roots were cut into small pieces using a knife. The small pieces of the roots were then air-dried under a shade at room temperature for three weeks. They were then ground into a coarse powder using an electric grinder made at the Mechanical Engineering Department at JKUAT. The powder was then packaged in a khaki bag and stored in a cool, dry cupboard away from direct sunlight.

3.4 Extraction

Extraction was done with distilled water, ethanol, methanol, dichloromethane (DCM), and ethyl acetate.

3.4.1 Aqueous extraction

Aqueous extraction was done by hot maceration. 50g of the *Grewia tenax* root powder was added to 500ml of distilled water in a 1litre flask then boiled for 15 minutes. The boiled mixture was then filtered using Whatman No. 1 filter paper, and the extract evaporated to a powder by use of a freeze dryer (BUCHI Lyovapor™ L-300). The lyophilized sample was kept at 4^{o}C as described by Wangia *et al.*, 2016.

3.4.2 Organic extraction

Organic extraction was done by cold maceration. 500ml of Ethanol, Methanol, Dichloromethane (DCM), and ethyl acetate respectively were added to 50mg of the

Grewia tenax root powder for 72 hours then the extract was concentrated by use of rotary evaporator (BUCHI Vac® V-500) at 45^0c then stored at 4^0C till the point of use as described by Wangia *et al.*, 2017.

Figure 3.2: Rotary evaporator (BUCHI Vac® V-500)

3.5 Phytochemical screening of Kenyan *Grewia tenax*

Kenyan *Grewia tenax* extract's phytochemical compounds screening was done to test for the presence of alkaloids, sterols, and steroids, flavonoids, saponins, tannins, terpenes, and glycosides (Harborne, 1998). *Grewia tenax root* extracts from the five different solvents (Methanol, petroleum ether, DCM, ethyl acetate, and water) were screened independently, and results were recorded. Phytochemical screening was done by observing precipitate formation and colour change (Banu & Cathrine, 2015)

The percentage *Grewia tenax* extract yield was calculated as shown below

$$\text{Percentage yield} = \frac{\text{Weight of the plant after extraction}}{\text{Weight of the plant before extraction}} \times 100$$

3.5.1 Saponins

A quantity of 20 ml of distilled water was added to 5 mg of the *G. tenax* extract in a graduated cylinder then shaken vigorously for 15 minutes. The formation of foam that persists for 15 minutes after shaking was indicative of the presence of saponins (Savithramma, Rao, & Suhrulatha, 2011)

3.5.2 Terpenes –Liebermann-Burchard Test

Chloroform was added to the extract then the solution was filtered. A few drops of acetic anhydride was added to the filtrate. The solution was boiled and then cooled. Concentrated Sulfuric acid was slowly added along the sides of the test tube. The appearance of a brown ring at the junction indicated the presence of terpenes (Tiwari, Kumar, Kaur, Kaur, & Kaur, 2011).

3.5.3 Alkaloids

To 1 g of the extract in a test tube, two drops of Mayers reagent were added along the test tube's sides. A white creamy precipitate appearance indicated the presence of alkaloids (Banu & Cathrine, 2015).

3.5.4 Tannins

To 5ml of distilled water, 50 mg of the extract was added and allowed to dissolve, after which a few drops of neutral 5% ferric chloride solution was added to the mixture. The presence of phenolic compound was indicated by the appearance of a dark green colour (Banu & Cathrine, 2015)

3.5.5 Sterols

The Salkowaski method was used. To 1 g of extract in a test tube, 0.5 ml acetic anhydride and 0.5 ml chloroform were added. Concentrated sulfuric acid was slowly added along the sides of the test tube. A red colouration was an indication of the presence of sterols (Catherine, 2016)

3.5.6 Flavonoids

5 ml of 10% ammonium hydroxide solution was added to *Grewia tenax* extract. The appearance of a yellow fluorescence was indicated the presence of flavonoids (Banu & Cathrine, 2015)

3.5.7 Glycosides - Keller – Killian test

To 1 ml of 3.5% ferric chloride in acetic acid, 1mg of the extract was added, followed by a careful dropwise addition of 1.5 ml concentrated sulfuric acid by the sides of the test tube to form a separate bottom layer. A brown ring at the interface because of the presence of de-oxy sugar was characteristic of cardenolides, and pale green colour in the upper layer due to the steroid nucleus indicated presence of cardiac glycosides as described by Wangia *et al.*, 2017.

3.6 Experimental animals and their welfare

Female Wistar albino rats weighing 160 - 200 g were used in the study. This was because: (i) they reach sexual maturity early i.e. 30 – 50 days (ii) they have short (4-5 days) regular estrus cycle. The animals were purchased from Medical Physiology Department animal house, UON, and were housed in the Veterinary Anatomy and Physiology animal house, UON. Animals were housed in standard cages, 17*13 Inches size. There where six animals per cage which were provided with *ad libitum* commercial rat pellets from Belmil feeds limited (Kenya) and water. The temperature of the animal house was maintained at $22^{o}C \pm 3$ and relative humidity of 30 – 70%. The animals were exposed to approximately 12 hours of light and 12 hours of darkness daily. Wood shavings were used for beddings and were changed every other day. The animals were allowed to acclimatise to laboratory conditions for a period of 7 days before commencement of the experiment.

3.6.1 Acute oral toxicity studies

Acute oral toxicity studies were done using OECD (organization of economic corporation and development's) guideline number 423 (OECD, 2001). A total of 12 rats were used for the whole oral toxicity study. Three female, nulliparous non-

pregnant rats weighing 160 – 200 grams were used for each dose of the extract. The animals were randomly selected, marked with picric acid for identification and put in their cages for seven days to acclimatize to laboratory conditions. All the rats were fasted overnight before administration of the extract via gastric lavage but *ad libitum* access to water was allowed. Food was withheld 4 hours after administration of the extract. The extract was administered in 0.5ml of normal saline to all rats.

Individual rats were put in the observation chamber. They were observed intensively during the initial 30 minutes upto 4 hours after extract administration. Observation was carried out again after 24 hours then daily for 14 days. The observations included: changes in skin and fur, eyes and mucous membranes, respiratory, circulatory, and autonomic and central nervous systems. They were monitored for tremors, convulsions, salivation, diarrhea, lethargy and sleep pattern..

The starting dose of the plant extract was 300mg/kg body weight using three rats. None of the animals died at 300mg/kg do so the procedure was repeated using 2000 mg/kg body weight of the extract. There was no mortality observed at 2000 mg/kg body weight dose and the procedure was repeated using 5000 mg/kg body weight. A control group composed of three rats were fasted overnight and given only 0.5ml of normal saline then observed and weighed like the rest of the test groups. All animals were weighed before fasting, at day zero, day one, day two, day seven and day fourteen.

At the end of the study, all the rats were humanely sacrificed by inducing hypoxia by use of CO_2 from a compressed CO_2 cylinder. The rats would be introduced to a clear chamber then CO_2 introduced for 2 – 3 minutes, death was confirmed by ascertaining cardiac and respiratory arrest, fixed and dilated pupils. After euthanasia, the carcasses were incinerated.

3.6.2 Disruption of estrus cycle in Wistar albino rats

Rats for this study were monitored daily for ten days to ensure regular cycles. This was done through daily vaginal wash of each rat between 9-10 am. Once it was established that they had regular estrus cycles, the thirty female nulliparous non-gravid albino rats were fasted for 12 hours. Then norethisterone (Micronor) 20ug/kg

body weight in 1ml of normal saline was administered daily for 7 days to disrupt the estrus cycle (Oladimeji & Aroyehun, 2015). After the seven days of estrus cycle disruption, the rats were divided into their respective groups and administered with varied doses of *Grewia tenax* in 0.5ml phosphate buffered saline (PBS).

3.6.3 Effects of Kenyan *Grewia tenax* on estrus cycle cyclicity

Each rat from section 3.6.2 was held gently but firmly by placing a free hand over the shoulders and quickly grasping the scuff of the neck close to the base of the skull between the thumb and forefinger. The tail was restrained and retracted backwards by use of the little finger. Specimen collection from the vaginal orifice was via gentle but quick insertion of the tip of a pipette and depositing 0.5ml of physiological saline which was then sucked out. The specimen was then put on a clean glass slide and observed under light microscope 10x (Kaingu, Oduma, Mbaria, & Kiama, 2017). Cytological features that distinguish the four stages of estrus cycle, proestrous, estrous, metestrous and diestrous (Westwood, 2008) were observed and recorded.

The epithelial cell change in the different phases are as follows: proestrous phase - nucleated epithelial cells, estrous anucleated epithelial cells, metestrous - leukocytes, cornified and nucleated epithelial cells in equal proportions, diestrous - mainly leukocyte cells (Westwood, 2008). The effect of extract on cyclicity was monitored daily during the entire period of the study.

3.6.4 Effect of *Grewia tenax* extract on reproductive hormones

Conductive silver paste (SPI supplies, USA) was smeared on the microscope glass slides using a brush, and allowed to dry for 20 minutes. A blood sample approximately 50µl was collected from each rat tail daily after cleaning with cotton soaked in ethanol, by cutting the tail vein and squeezing gently. The blood was smeared on the silver pasted glass slide for each rat, each group on one slide and allowed to dry for 30 minutes. The samples were taken for spectral data collection using Raman spectroscopy. The analysis entailed the use of Raman spectroscopy system (STR, Seki Technotron Corp) using a 785 nm laser and a spectrometer with 600 lines grating with 1000 cm^{-1} centre wavenumber (Princeton), X10 microscope lens with 0.3 numerical aperture the excitation power of 50% and five accumulations

per spot and exposure time of 10 seconds. Before sample analysis, calibration of the spectrometer was done using a silicon wafer with a peak of 520.86nm. Vancouver Raman Algorithm was used to remove fluorescence. Further analysis of the spectra obtained was done then in comparison with the standard hormone spectra, the intensity of the hormones for the different groups was determined.

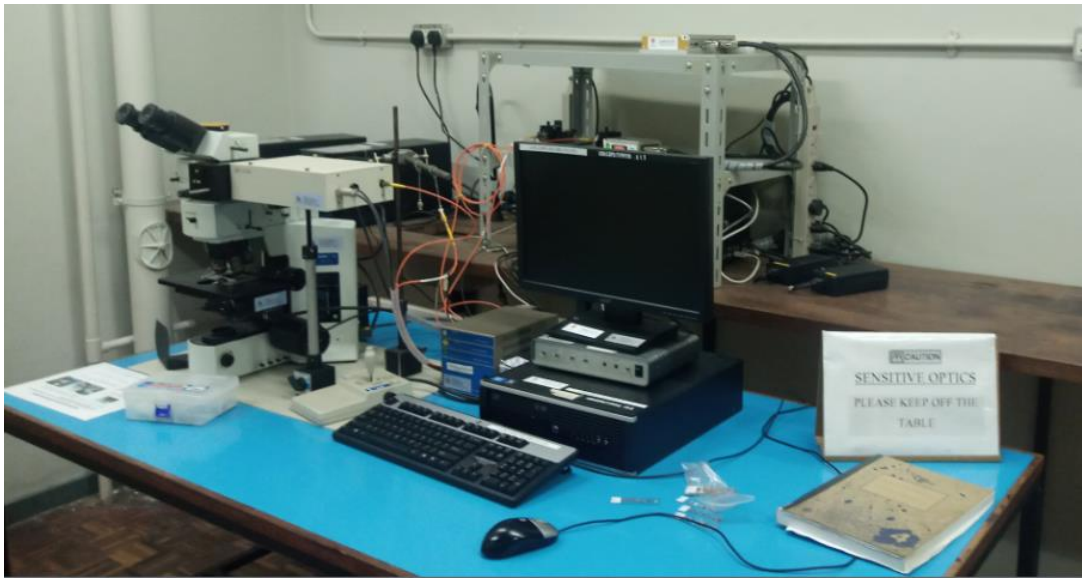


Figure 3.3: Raman spectroscope

3.6.5 Procedure for anaesthetizing the animals

On the last treatment day, all the animals were sacrificed humanely by inducing hypoxia using CO_2 from a compressed CO_2 cylinder. The animal was introduced to a clear chamber then the gas introduced for 2 – 3 minutes, death was confirmed by ascertaining cardiac and respiratory arrest, fixed and dilated pupils.

3.7 Sample size determination and animal grouping

3.7.1 Sample size determination

"Resource equation" was used to determine the sample size (Charan & Kantharia, 2013)

- E = Total number of animals – Total number of groups, 10<E>20
- There are 5 groups of 5 animals per group ($5\times 5 = 25$)

- E = 25 – 5 = 20
- 20% of animals were added to cater for attrition, 20% of 25 = 5 adding 5 animals making 6 animals per group
- The sample size was 30 animals

For acute toxicity studies, there was a separate group of animals whose sample size was determined as per OECD guideline number 423 described above. A total of 12 rats were used.

3.7.2 Animal groupings

The animals were grouped as follows:

Group I – Negative control – Norethisterone induced female Wistar albino rats administered with 0.5ml normal saline + PBS

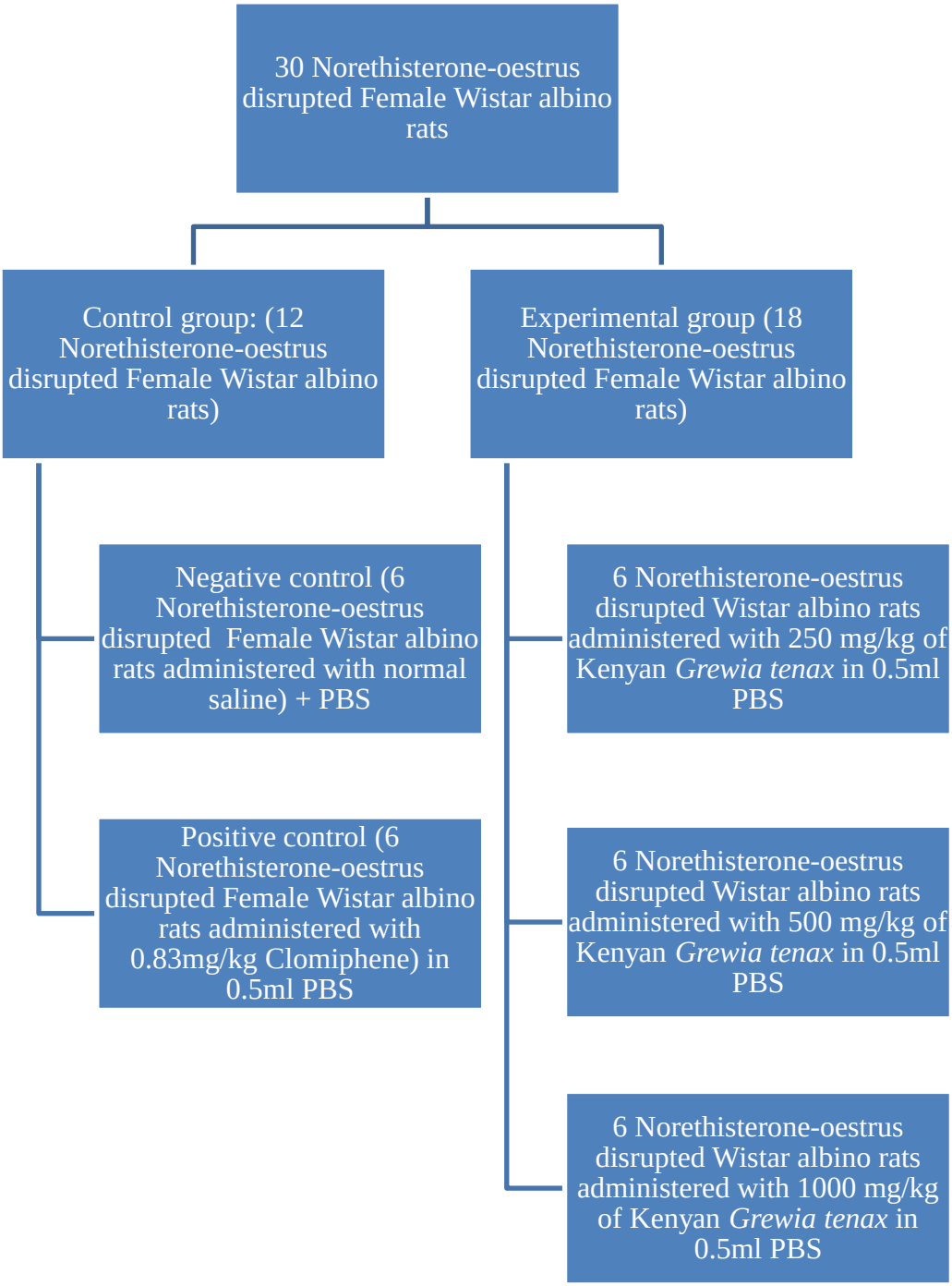
Group II – Interventional groups

Sub-group 1 – Norethisterone-oestrus disrupted female Wistar albino rats administered with 250 mg/kg body weight of the *Grewia tenax* root methanol extract in 0.5ml PBS

Sub-group 2 - Norethisterone-oestrus disrupted female Wistar albino rats administered with 500 mg/kg body weight of the *Grewia tenax* root methanol extract in 0.5ml PBS

Sub-group 3 - Norethisterone-oestrus disrupted female Wistar albino rats administered 1000 mg/kg body weight of the *Grewia tenax* root methanol extract in 0.5ml PBS.

Group III – Positive control - Norethisterone-oestrus disrupted female Wistar albino rats administered with 0.83mg/kg body weight of clomiphene in 0.5ml PBS.



KEY: PBS – Phosphate Buffered Saline

Figure 3.4: Animal grouping

3.8 Personnel Occupational safety precautions

All personnel at all times wore white laboratory coats and gloves while working in the laboratory and handling animals.

3.9 Data Management, analysis and presentation

Data analysis was done using statistical package for social sciences (SPSS) version 21 and GraphPad prism 8. Data comparison and statistic testing were made using one-way analysis of variance (ANOVA) followed by Tukey HSD post hoc t-test. *P* value <0.05 was considered statistically significant Data presentation is done using tables, figures and graphs.

CHAPTER FOUR

RESULTS

4.1 The percentage yield of Kenyan *Grewia tenax* root extract in different solvents

Methanol % yield

$2.482 = \frac{2.482}{50} \times 100 = 4.964$

Petroleum ether % yield

$0.894 = \frac{0.894}{50} \times 100 = 1.788$

DCM % yield

$1.007 = \frac{1.007}{50} \times 100 = 2.014$

Ethyl acetate % yield

$1.36 = \frac{1.36}{50} \times 100 = 2.72$

Aqueous % yield

$1.47 = \frac{1.47}{50} \times 100 = 2.94$

4.2 Phytochemical compounds of Kenyan *Grewia tenax* root extract

Phytochemical compounds of Kenyan *Grewia tenax* root extract of the different solvents (water, ethanol, methanol, Ethyl acetate, DCM) were screened. Presence of alkaloids, flavonoids, saponins, sterols, steroids, tannins, terpenes and cardiac glycosides was detected as shown in Table 4.1.

Table 4.1: Phytochemical screening of Kenyan Grewia tenax roots

Solvents	Alkaloids	Flavonoids	Saponins	Sterols	Steroids	Tannins	Terpenes	Cardiac glycosides
Aqueous	++	-	++	-	-	-	-	+
Methanol	++	+	++	+	-	-	+	++
Ethyl acetate	++	-	-	-	-	-	-	+
Petroleum ether	++	-	-	-	-	-	-	++
Dichloromethane (DCM)	++	-	++	-	-	-	-	+

Key '+' Mild presence, '++' Moderate presence, '-'absent

The aqueous extract screening revealed presence of alkaloids, saponins and cardiac glycosides but flavonoids, steroids, tannins and terpenes. Methanol extraction yielded the highest number of different phytochemical compounds; alkaloids, saponins cardiac glycosides, flavonoids, sterols and terpenes. The ethyl acetate extract revealed presence of alkaloids and cardiac glycosides. Petroleum ether extract revealed presence of alkaloids and cardiac glycosides. Dichloromethane extract revealed the presence of alkaloids, saponins and cardiac glycosides.

All solvents extract revealed presence of alkaloids and cardiac glycosides and a total lack of steroids and tannins with only methanol extract demonstrating presence of terpenes and flavonoids while saponins were present in all solvent extracts apart from ethyl acetate and ethanol extract. The methanol extract had the highest number of various phytochemical compounds.

The Kenyan *Grewia tenax* root methanol extract was therefore chosen as the ideal solvent for use in the rest of the study.

4.3 Acute oral toxicity studies of the methanol Kenyan *Grewia tenax* root extract

A starting dose of 300 mg/kg was administered to 3 female rats. All rats had normal breathing and were active following administration up to 24 hours and the entire 14 days of study. No mortality occurred at 300 mg/kg so three other rats were administered with 2000 mg/kg of the extract. All animals had normal breathing and

were active following the extract administration up to 24 hours and the entire 14 days. No mortality occurred at 2000 mg/kg hence 3 other rats were administered with 5000 mg/kg body weight. All the rats developed rapid breathing and were restless within the first thirty minutes and resumed normal breathing and body grooming in an hour time. A control group of three female rats were administered with 0.5 ml normal saline to serve as negative control. This group demonstrated normal breathing and normal activity throughout the fourteen days.

Oral administration of *Kenyan Grewia tenax* at even 5000mg/kg did not cause any mortality or any clinical symptom of toxicity. No gross pathology was observed from all organs in postmortem of the rats at the end of the study.

Table 4.2: Clinical symptom observation of rats treated with Kenyan *Grewia tenax* root methanol extract

Doses (Mg)	**Observation in time**								
	Immediate	½ hour	1 hour	4 hours	24 hour	Day 7	Day 14	**Mortality**	**Mortality rate (%)**
300	Normal activity	Normal activity	Normal activity	Normal activity	Normal activity	Normal activity	Normal activity	0/3	0
2000	Normal activity	Normal activity	Normal activity	Normal activity	Normal activity	Normal activity	Normal activity	0/3	0
5000	Fast breathing,	Fast breathing	Normal activity	Normal activity	Normal activity	Normal activity	Normal activity	0/3	0
Control (Normal saline)	Normal activity	Normal activity	Normal activity	Normal activity	Normal activity	Normal activity	Normal activity	0/3	0

Table 4.3: Weights change of treatment and control rats during the acute toxicity studies

Animals	Varying doses in mg/kg	Different animal weights in grams				
		Fasting day	Day 0	Day 1	Day 7	Day 14
Rat 1	300	182.72	170.26	180.57	184.55	192.20
Rat 2	300	186.75	161.77	184.55	195.70	202.30
Rat 3	300	181.76	172.61	182.41	185.64	190.40
Rat 4	2000	183.81	163.62	177.58	185.62	190.99
Rat 5	2000	169.68	156.68	164.24	172.08	174.76
Rat 6	2000	177.36	164.77	169.06	183.11	184.28
Rat 7	5000	175.33	162.15	183.55	186.40	188.23
Rat 8	5000	178.28	175.13	183.31	194.14	196.83
Rat 9	5000	177.58	161.37	169.22	181.65	182.87
Rat 10	Saline 0.5ml	182.99	174.76	180.65	187.46	192.99
Rat 11	Saline 0.5ml	173.65	168.72	177.46	183.06	188.41
Rat 12	Saline 0.5ml	176.75	172.22	177.90	183.82	185.45
	Mean	178.89	167.84	178.38	185.27	189.14

Table 4.:4 Mean body weights for *Grewia tenax* treated and control group for the acute oral toxicity study

***Grewia tenax* (mg/kg)**	**Fasting Day**	**Day 0**	**Day 1**	**Day 7**	**Day 14**
Control	177.80±2.747	171.90±1.751	178.67±0.998	184.78±1.358	188.95±2.193
300	183.74±1.529	168.21±3.292	182.51±1.150	188.63±3.549	194.97±3.703
2000	176.95±4.084	165.02±5.269	173.63±4.713	180.27±4.159	183.34±4.709
5000	177.06±0.890	166.22±4.462	178.69±4.737	187.40±3.640	189.31±4.066
***P*-value**	0.275	0.639	0.393	0.365	0.270

P-value set at ≤0.05

Table 4.3 and 4.4 show the mean body weights ±SEM of *Grewia tenax* extract treated rats compared to the negative control at fasting day, days 0, 1, 7 and day14. There was no significant difference between treated groups and the control group. There was no mortality caused by *Grewia tenax* methanol extract even at 5000mg/kg body weight.

4.4 Effects of *Grewia tenax* methanol extract on female Wistar albino rats cyclicity

Results on effects of the extract on estrus cyclicity were presented as per each dosage i.e (250,500 and 1000), the negative and positive control by use of tables and figures below

4.4.1 A representative photomicrograph of oestrus cycle cytology features at x10 magnification

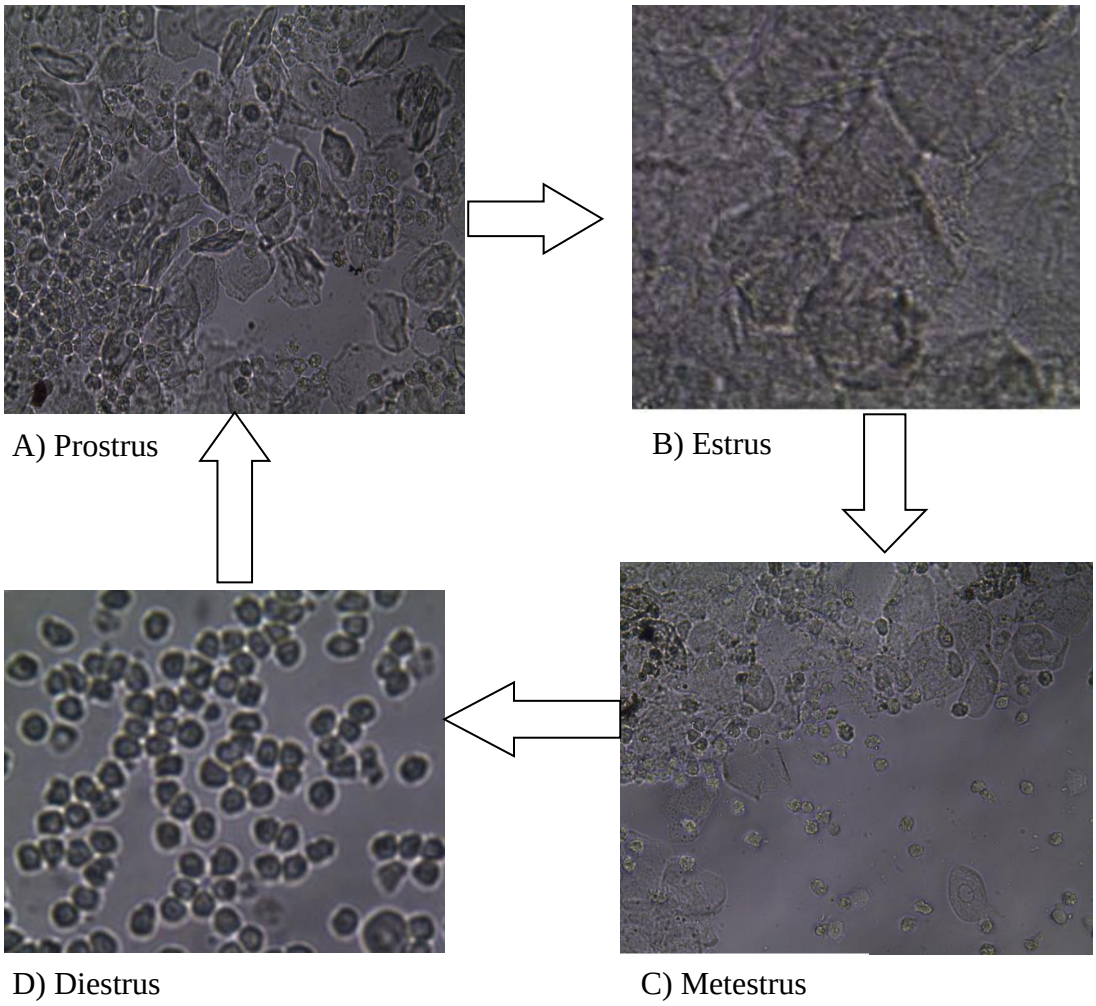
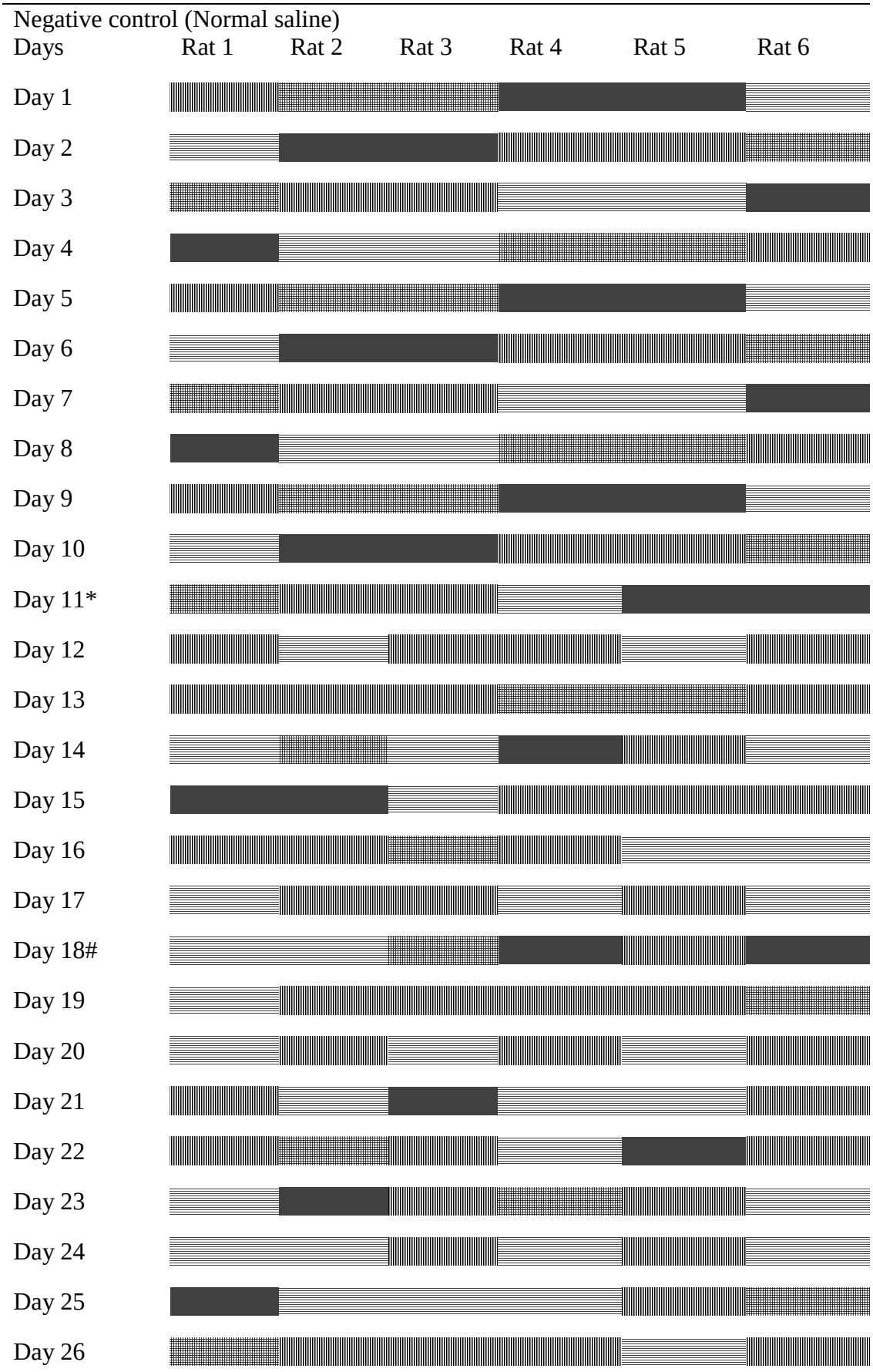


Figure 4.1: Photomicrograph of estrus cycle cytology features

4.1.1Negative control group female Wistar albino rats estrus cycle

The negative control group composed of rats whose estrus cyclicity was disrupted using norethisterone for 7 days and then were administered with normal saline for 10 days. The irregular estrus cycle pattern was subsequently observed during the 10 days normal saline administration. The rats showed an irregular estrus cycle pattern with metestrus and diestrus phases being more frequent and estrus and proestrus phases being less frequent.

Negative control (Normal saline)



KEY:

Proestrus phase

Estrus Phase

Metestrus phase

Diestrus phase

*Norethisterone adm #Normal saline adm

Figure 4.2: The effect of norethisterone on estrus cycle

Table 4.5: The frequency of appearance of each estrus phase in norethisterone estrus disrupted rats administered with normal saline (negative control)

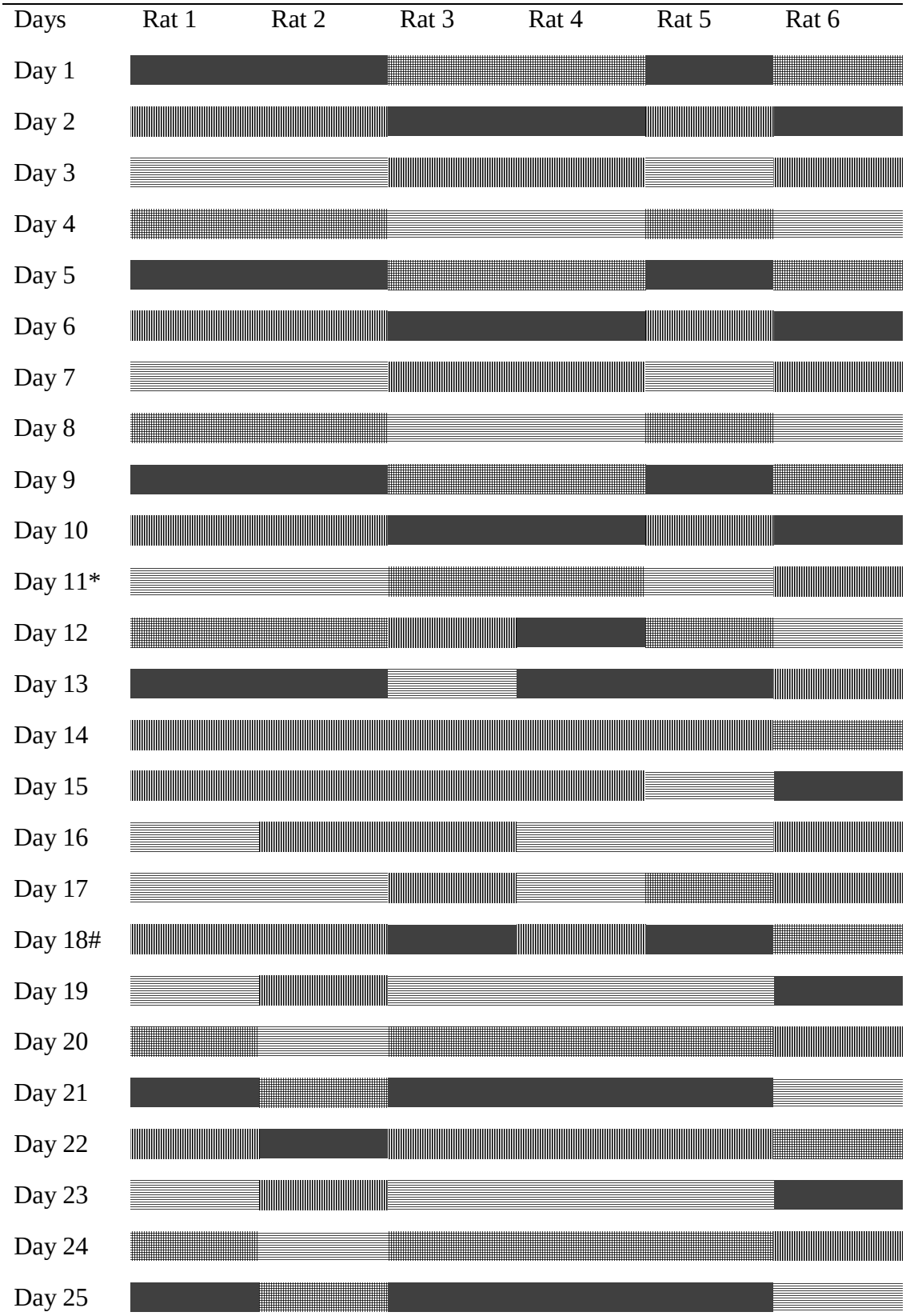
	Negative control estrus cyclicity						
Estrus Phase	Rat 1	Rat 2	Rat 3	Rat 4	Rat 5	Rat 6	Mean no of days
Proestrus	1	1	1	1	0	2	1
Estrus	1	1	1	1	1	1	1
Met-estrus	3	4	5	4	5	5	4.33
Di-estrus	5	4	3	4	4	2	3.67

The table shows the frequency of estrus cycle phases observed on a 10 day normal saline treatment period. Metestrus and diestrus phases were more frequent with subsequent reduction in frequency of appearance of proestrus and estrus phases. There was a significant difference in the estrus cycle phases compared to the positive control.

4.4.2 Effect of 250mg/kg body weight *Grewia tenax* methanolic extract on estrus cycle

Figure 4.3 shows the frequency of each estrus cycle phase over a 10 day period of administration of 250 mg/kg *Grewia tenax* methanol extract. Regular patterns of the estrus cycle was established; with the proestrus and estrus phases being slightly more frequent compared to metestrus and diestrus phases. *Grewia tenax* methanol extract reverted the irregular estrus cycle caused by Norethisterone back to normal. There

was no significant difference in the estrus cycle phases compared to the positive control.



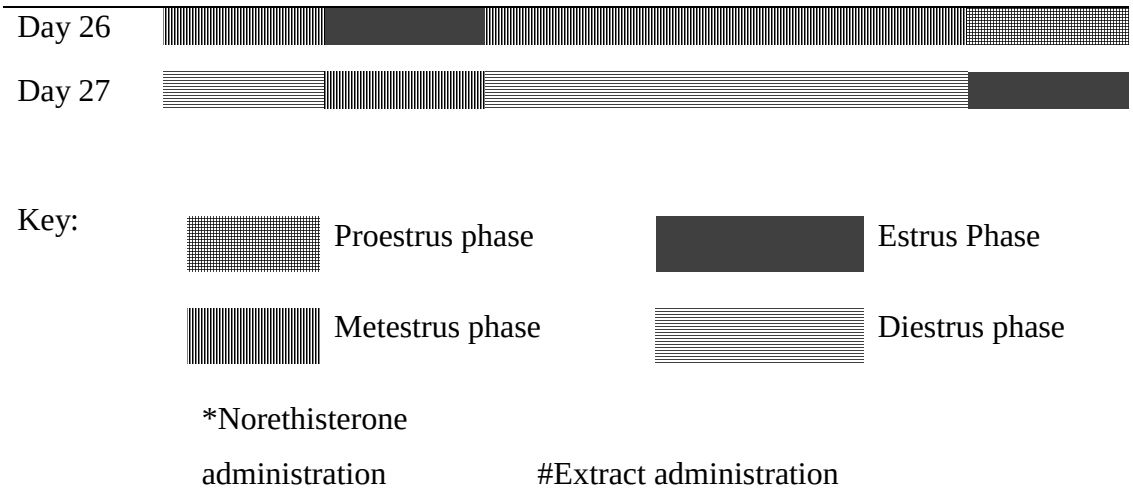


Figure 4.3: The effect of 250 mg/kg *Grewia tenax* methanol extract on norethisterone induced disrupted estrus cycles

All the rats had normal estrus cycles during the 10 day acclimatization period. The cycle was however disrupted during the 7 day norethisterone treatment period. The estrus cycles later regularized following administration of *Grewia tenax* 250 mg/kg body weight.

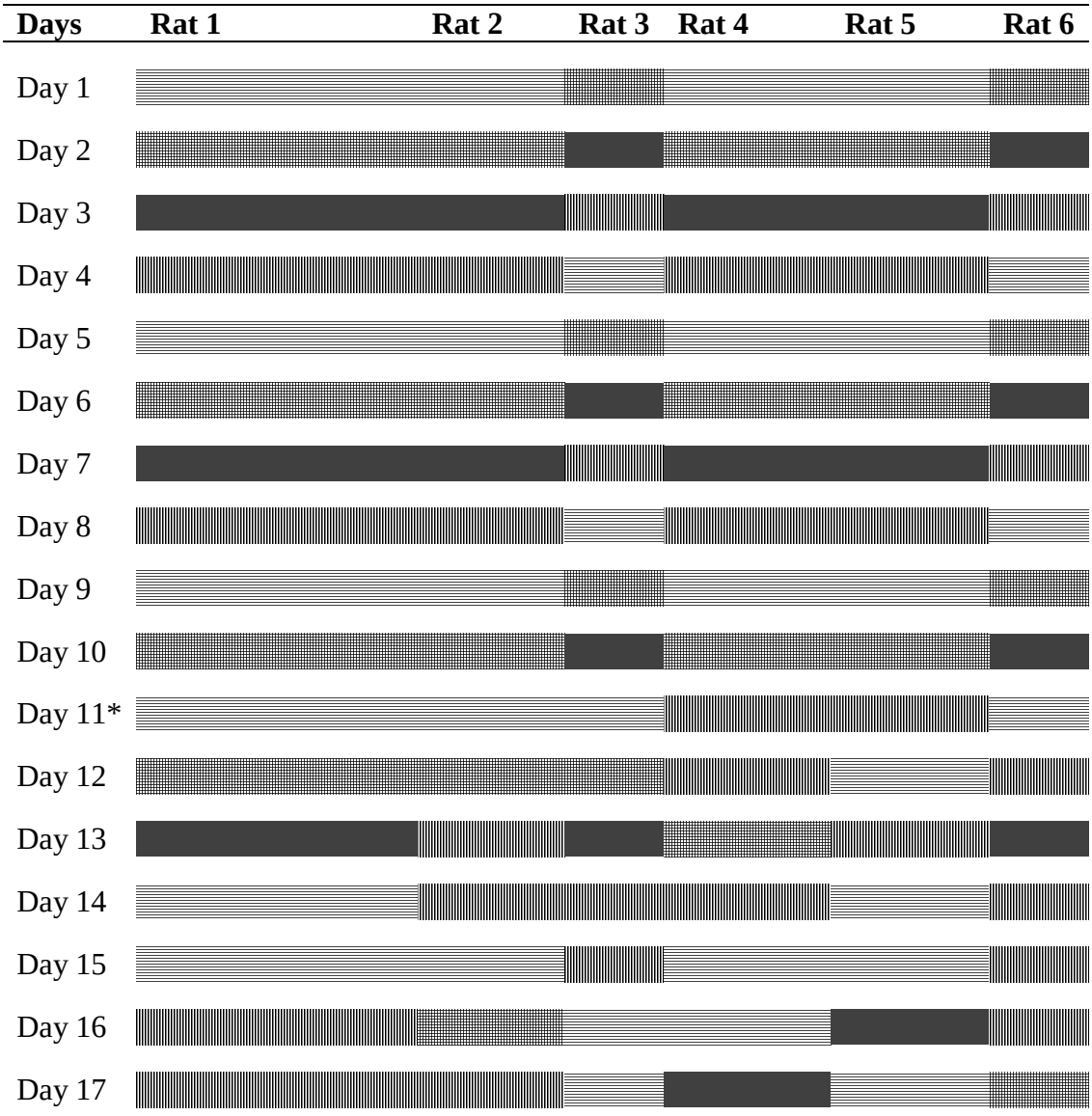
Table 4.6: Frequency of estrus cycle phases in animals treated with 250 mg/kg *Grewia tenax* methanol extract.

	250 mg/kg *Grewia tenax* estrus cyclicity						
Estrus Phase	Rat 1	Rat 2	Rat 3	Rat 4	Rat 5	R1at 6	Mean no of days
Proestrus	2	2	2	2	2	3	2.17
Estrus	2	2	3	2	3	3	2.5
Metestrus	3	4	2	3	2	2	2.67
Diestrus	3	2	3	3	3	2	2.67

The table shows the frequency of estrus cycle phases observed in a 10 day 250 mg/kg *Grewia tenax* treatment period. The estrus cycle phases were evenly distributed as per a normal estrus cycle pattern.

4.4.3 Effect of 500mg/kg body weight *Grewia tenax* methanolic extract on estrus cycles

All the rats had a normal estrus cycle during the 10 day acclimatization period. A seven day treatment with Norethisterone disrupted the regular estrus cycle phases. Administration of 500 mg/kg *Grewia tenax* reverted the disruption. Figure 4.4 shows the frequency of each estrus phase over a 10 day treatment period 500 mg/kg *Grewia tenax* methanol extract. Regular patterns of the estrus cycle were established with a non significant increased frequency of proestrus and estrus compared to metestrus and diestrus phases. *Grewia tenax* methanol extract regularized the disrupted estrus cycles back to normal.



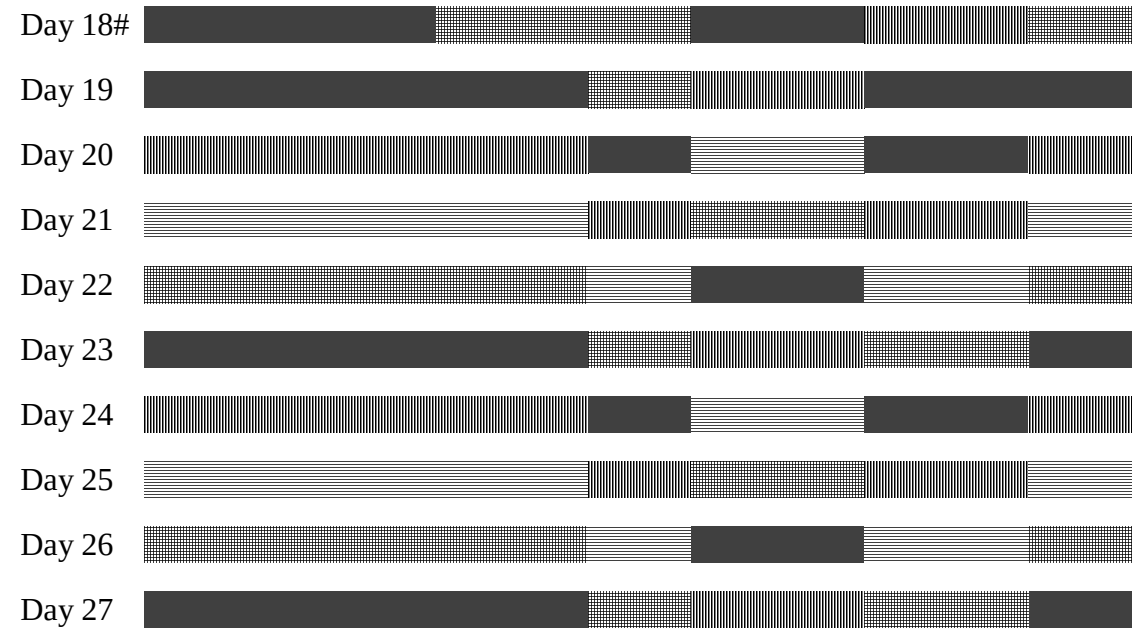


Figure 4.4: Effect of 500 mg/kg *Grewia tenax* methanol extract on norethisterone induced disrupted estus cycles

There was no significant difference in the estrus cycle phases compared to the positive control.

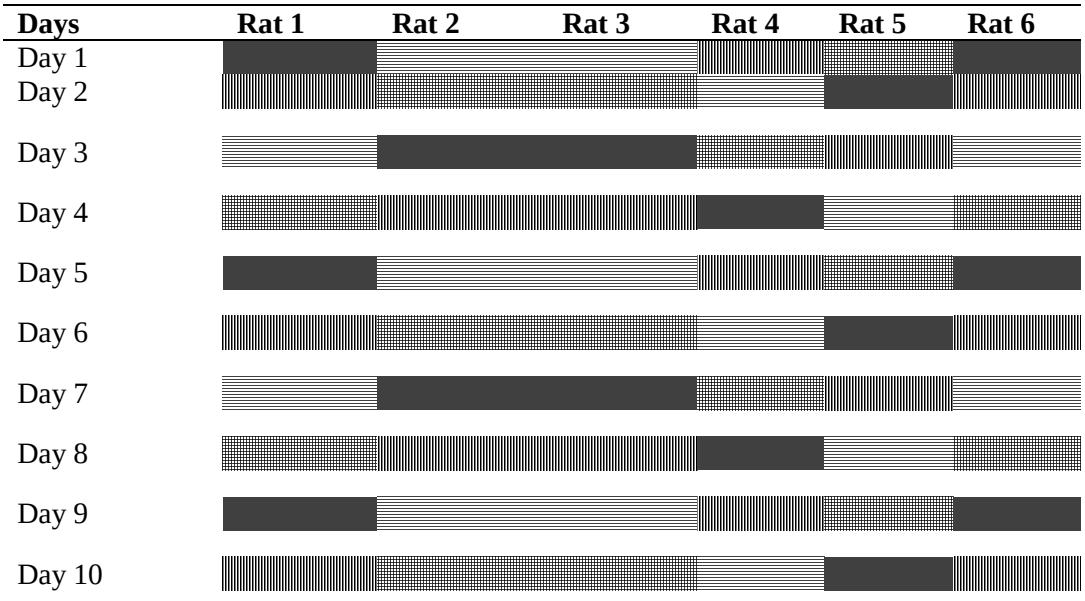
Table 4.7: Frequency of appearance of estrus cycle phases in rats treated with 500 mg/kg *Grewia tenax* methanol extract.

	500 mg/kg *Grewia tenax* estrus cyclicity						
Estrus Phase	Rat 1	Rat 2	Rat 3	Rat 4	Rat 5	Rat 6	Mean no of days
Proestrus	2	3	4	2	2	3	2.67
Estrus	4	3	2	3	3	3	3
Metestrus	2	2	2	3	3	2	2.33
Diestus	2	2	2	2	2	2	2

The table shows the frequency of estrus cycle phases observed over a 10 day 500 mg/kg *Grewia tenax* treatment period. There was no significant difference in the estrus cycle phases compared to the positive control.

4.4.4 Effect of 1000mg/kg body weight *Grewia tenax* methanolic extract on estrus cycles

1000 mg/kg body weight of *Grewia tenax* methanol extract in rats reverted the norethisterone induced disrupted cycles back to regular patterns. There was a non significant increase in frequency of proestrus and estrus phases compared to metestrus and diestrus phases. At this dose; *Grewia tenax* methanol extract regularized the norethisterone dysrupted cycles.



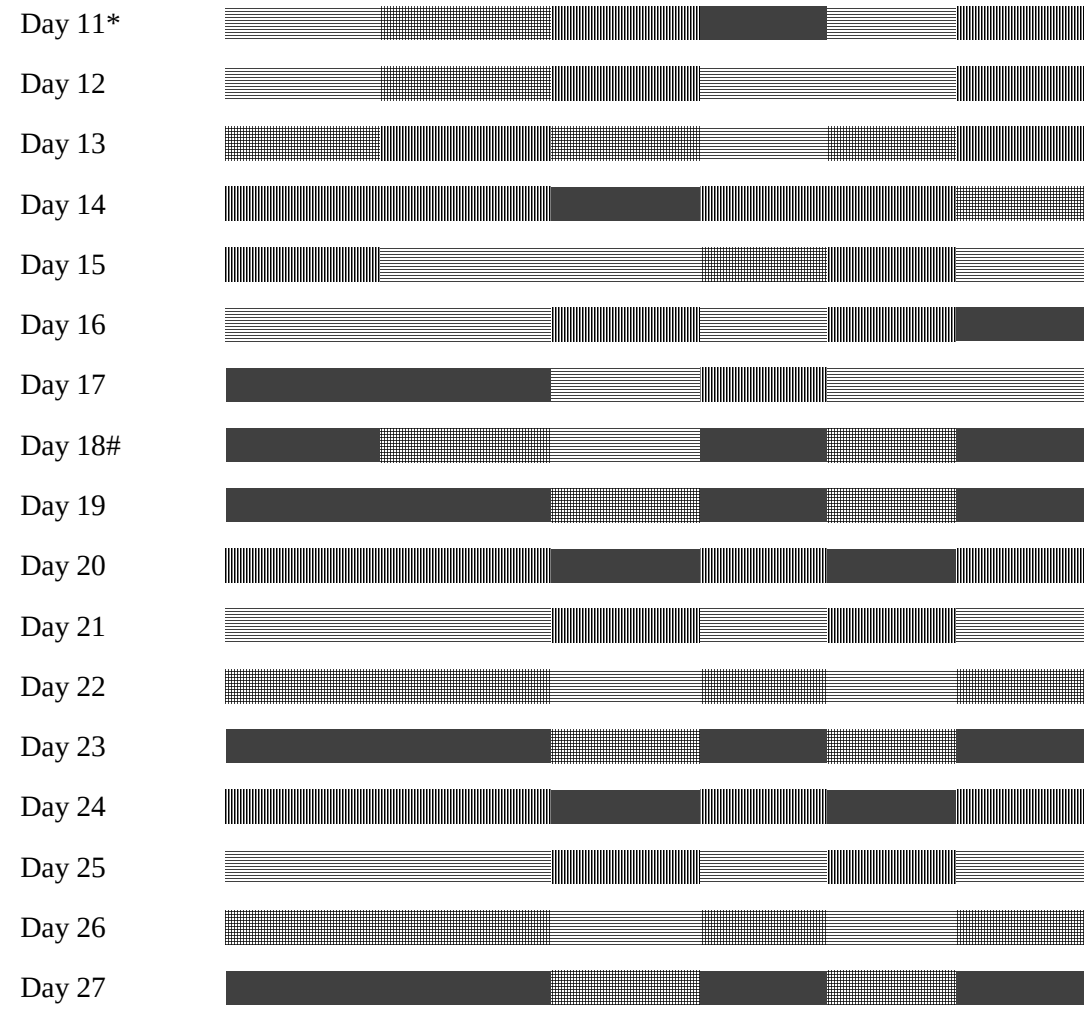


Figure 4.5: Effect of 1000mg/kg *Grewia tenax* methanol extract on estrus phases appearance

All animals had a normal estrus cycle during the ten-day acclimatization period. These cycles were subsequently disrupted by a seven day treatment with norethisterone. 1000 mg/kg body weight *Grewia tenax* regularized the estrus cycle.

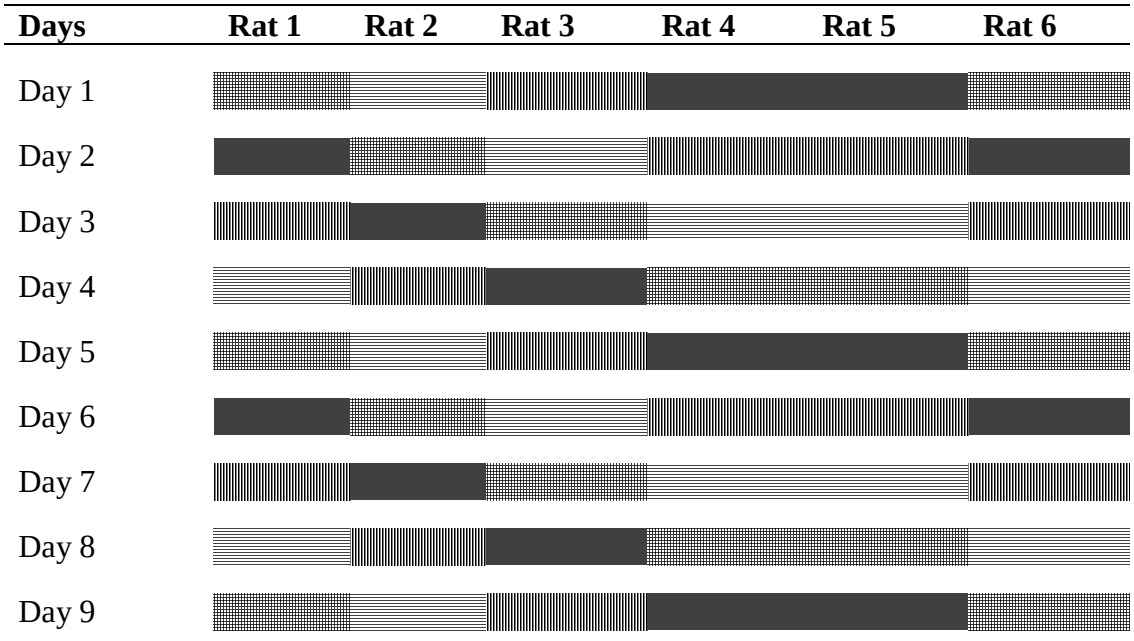
Table 4.8: Frequency of estrus phases in animals treated with 1000 mg/kg *Grewia tenax* methanol extract

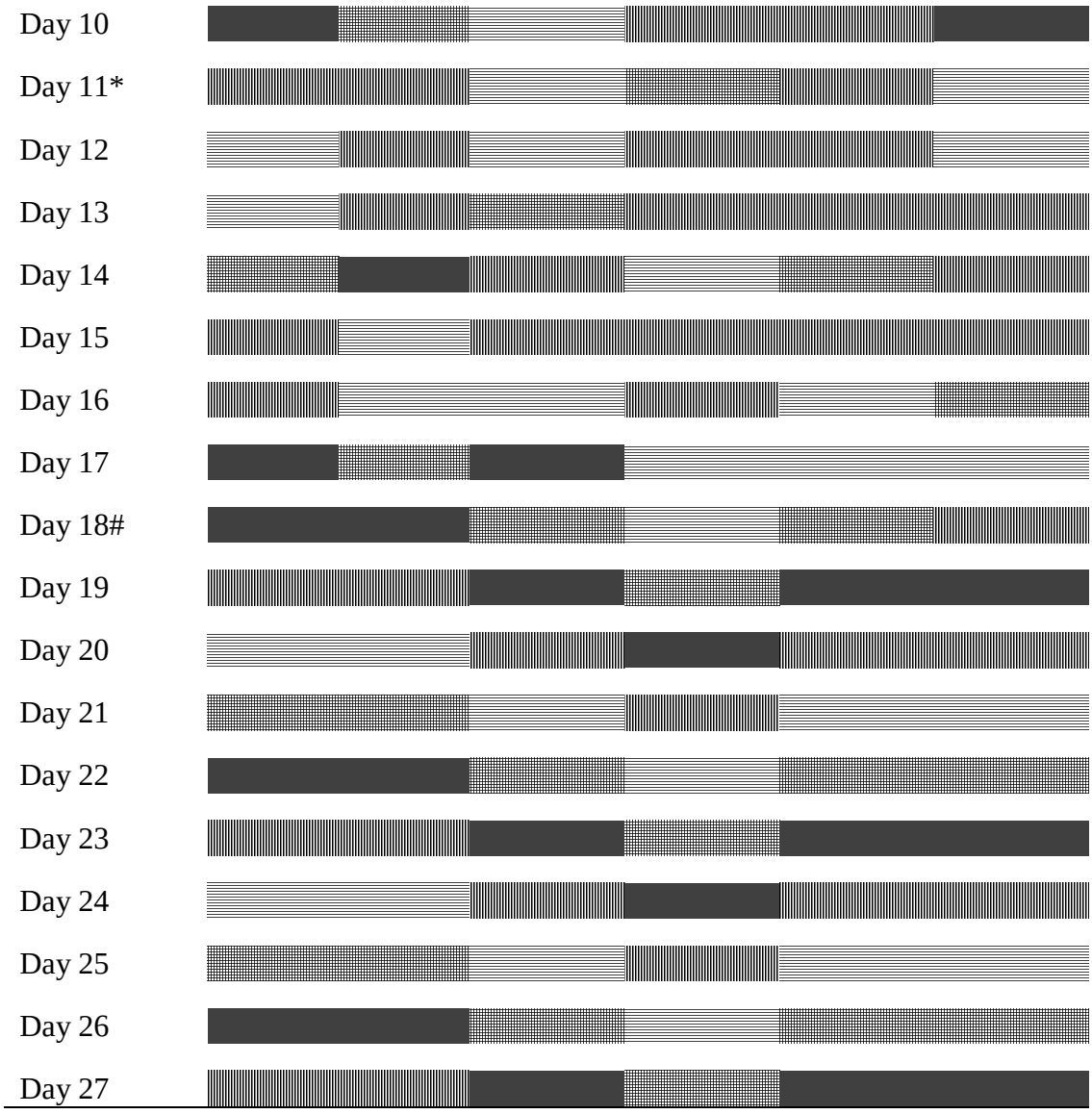
1000 mg/kg *Grewia tenax* estrus cyclicity							
Estrus Phase	Rat 1	Rat 2	Rat 3	Rat 4	Rat 5	Rat 6	Mean no of days
Proestrus	2	3	3	4	4	2	3
Estrus	4	3	2	2	2	4	2.83
Metestrus	2	2	2	2	2	2	2
Diestus	2	2	3	2	2	2	2.17

The table shows the frequency of estrus cycle phases observed in a 10 day 1000 mg/kg *Grewia tenax* treatment period. The extract caused a non significant increase in proestrus, estrus and diestrus phases with a subsequent non significant decrease of metestrus. There was no significant difference in the estrus cycle phases compared to the positive control.

4.4.5 Positive control estrus cyclicity

Figure 4.6 shows the frequency of estrus cycle phases over a 10 day 0.83mg/kg clomiphene administration period. The figure shows regular estrus cycle patterns with a non significant increased frequency of proestrus and estrus phases compared to metestrus and diestrus phases. 0.83mg/kg clomiphene regularized the irregular norethisterone induced estrus cycles.




Day 10
Day 11*
Day 12
Day 13
Day 14
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Day 25
Day 26
Day 27

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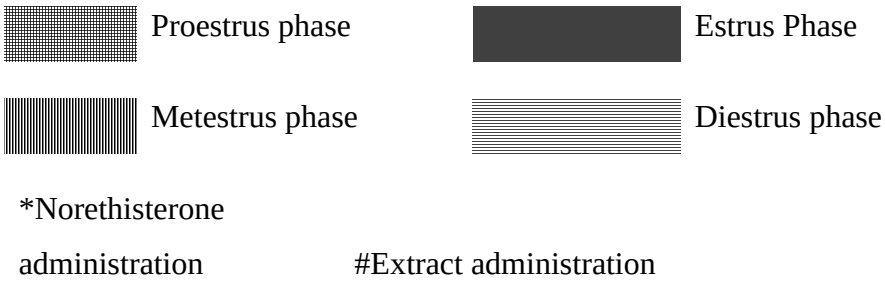


Figure 4.6: The effects of Clomiphene (Positive control) on estrus phases appearance

Table 4.9: The frequency of estrus cycle phases in animals treated with clomiphene (positive control).

	Positive control estrus cyclicity						
Estrus Phase	Rat 1	Rat 2	Rat 3	Rat 4	Rat 5	Rat 6	**Mean no of days**
Proestrus	2	2	3	3	3	2	**2.50**
Estrus	3	3	3	2	3	3	**2.83**
Metestrus	3	3	2	2	2	3	**2.50**
Diestus	2	2	2	3	2	2	**2.17**

Table 4.9 shows the frequency of appearance of estrus cycle phases observed in a 10 day 0.83mg/kg clomiphene (positive control) treatment period. Clomiphene regularized the irregular norethisterone induced estrus cycles.

4.4.6 Comparison of the mean number of days each estrus cycle phase lasted in each treatment group compared to the negative and positive control.

There was a significant difference (<0.01) in the frequency of occurrence of all estrus cycle phases in 500mg/kg and1000mg/kg *Grewia tenax* extract treated groups compared to the negative control. There was a significant difference (<0.05) in the

frequency of occurrence of all estrus phases in the group treated with 250mg/kg *Grewia tenax* extract compared with the negative control group

Table 4.10: Mean number of days each estrus cycle phase lasted per treatment group and positive control compared to the negative control

Estrus Phase	Negative control	250mg/kg	500mg/kg	1000mg/kg	*P*-Value
Proestrus	1±0.258	2.17±0.167[a]	2.67±0.333 [a]	3±0.365 [b]	0.001
Estrus	1±0.000	2.5±0.548[a]	3±0.632[b]	2.83±0.938[b]	<0.001
Metestrus	4.33±0.333	2.67±0.333[a]	2.33±0.210[b]	2±0.000[b]	<0.001
Diestus	3.67±1.033	2.67±0.516[a]	2±0.000[b]	2.17±0.408[b]	0.001

The test of significance was performed in rows for treatment groups for each estrus phase and columns for estrus phases for each treatment group

Key: [a] *Indicates values that where significantly different (P < 0.05) from the negative control using Anova Tukey's HSD post hoc t test*

[b] *Indicates values that where significantly different (P < 0.01) from the negative control using Anova Tukey's HSD post hoc t test*

The table shows the mean frequency of each estrus cycle phase per treatment group in the 10 day treatment period. The mean appearance of all estrus cycle phases at 500 mg/kg and 1000 mg/kg were significantly (P<0.01) different compared to the negative control. The mean appearance of all estrus phases of the 250mg/kg treatment group was significantly (<0.05) different when compared with the negative control group. The values are expressed as mean ± standard error mean (n=6)

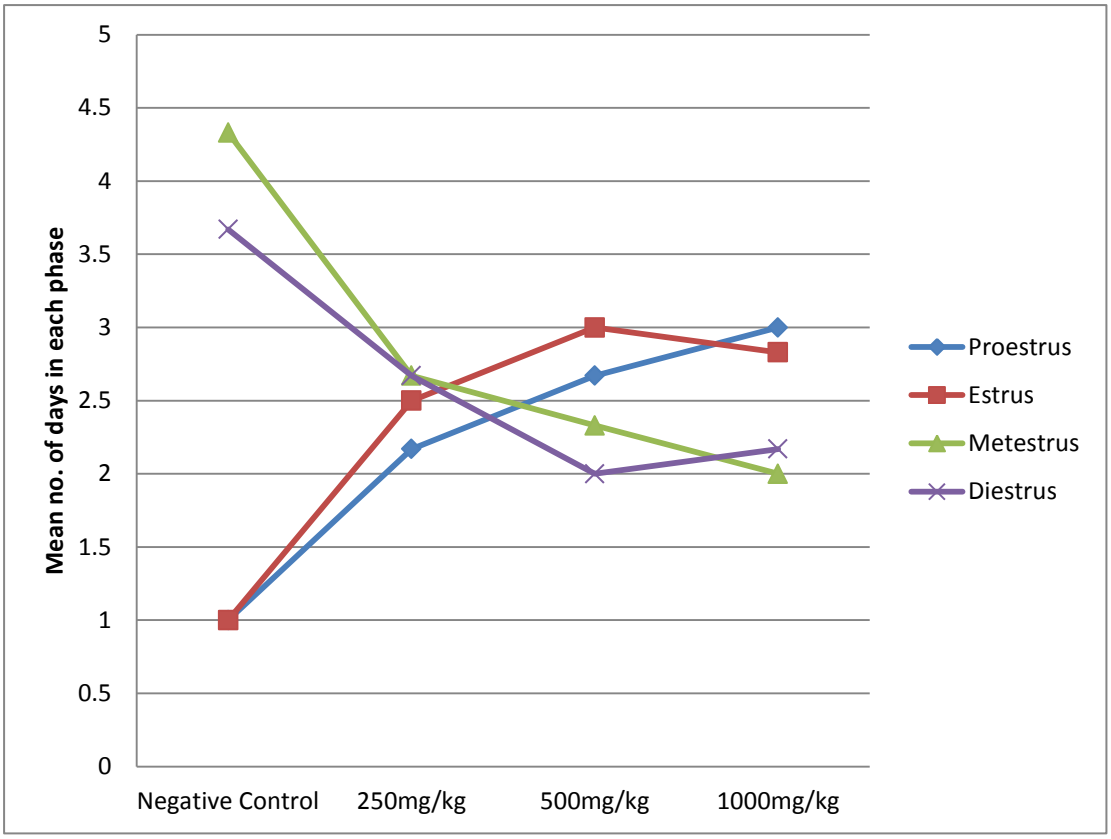
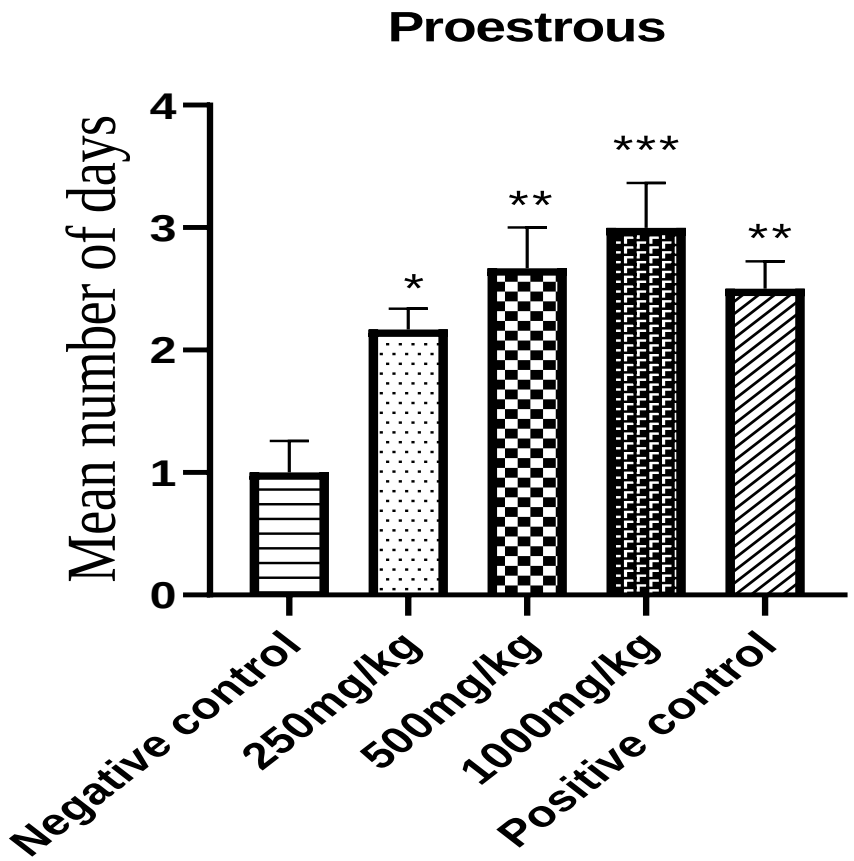
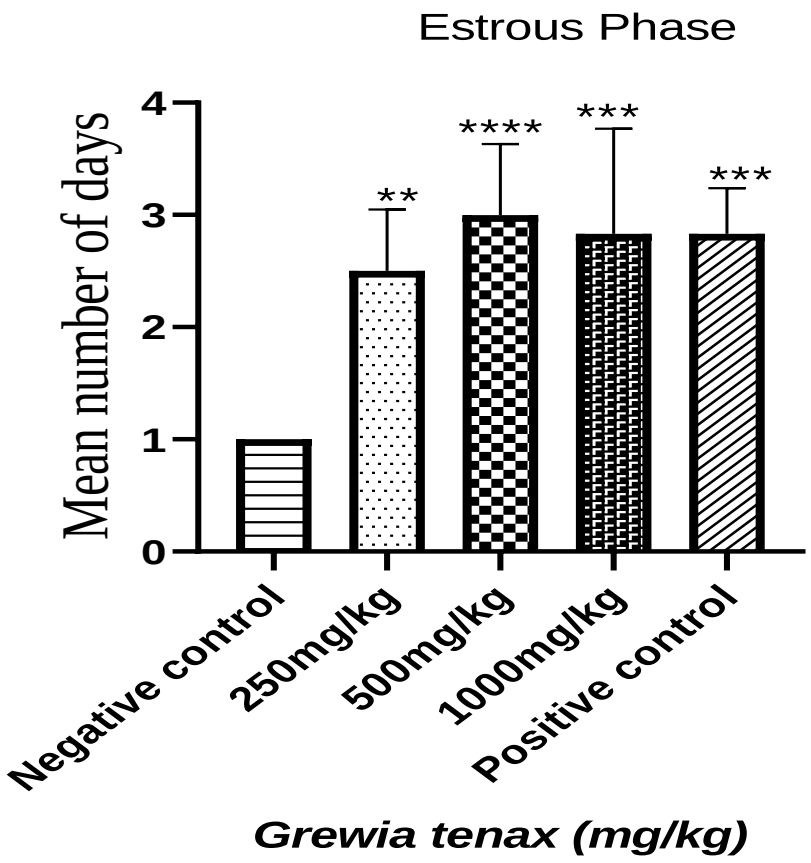


Figure 4.7: Graph representation of the mean number of days of each estrus cycle phase per treatment group



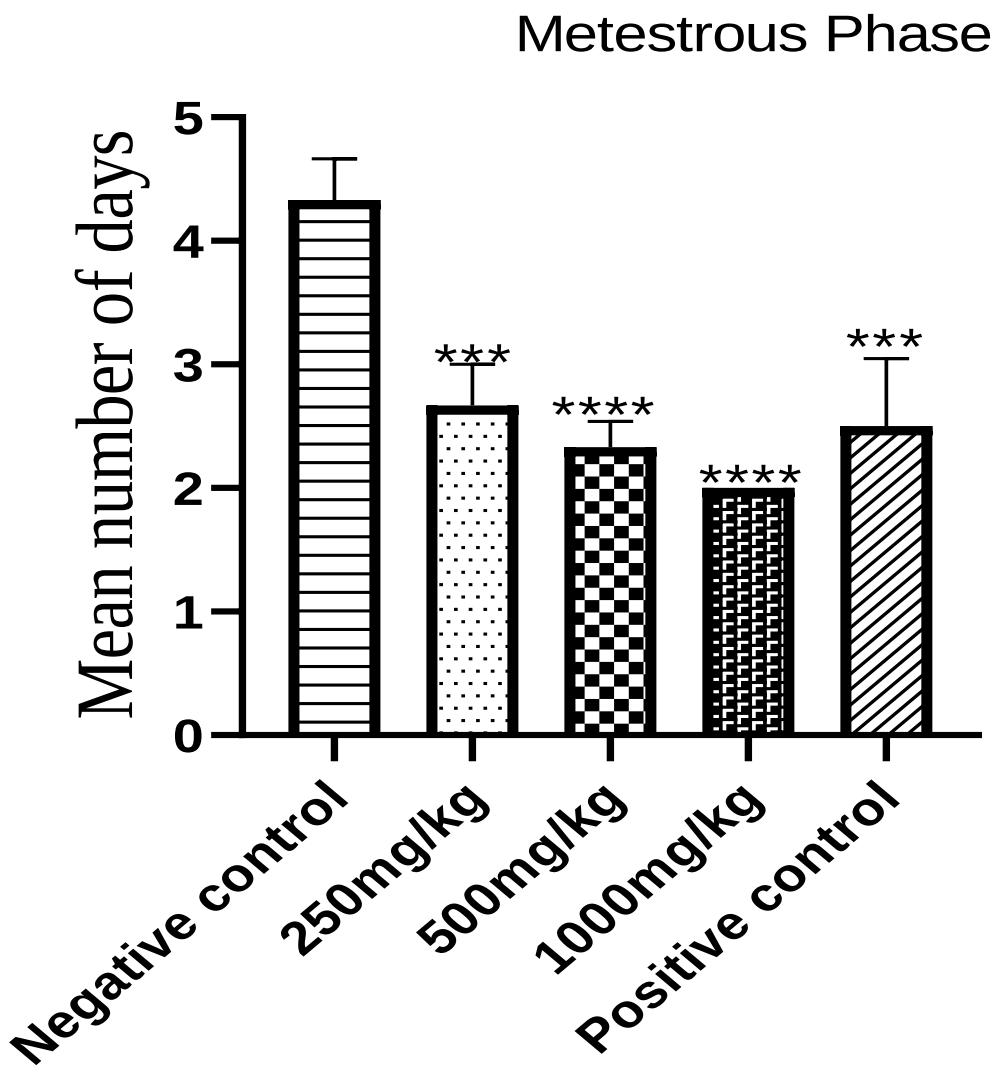
Key: *p<0.05, **p<0.01, ***p<0.001

Figure 4.8: Mean number of days of proestrus phase at 250, 500 and 1000 mg/kg *Grewia tenax* methanol extract respectively compared to the positive and negative control..



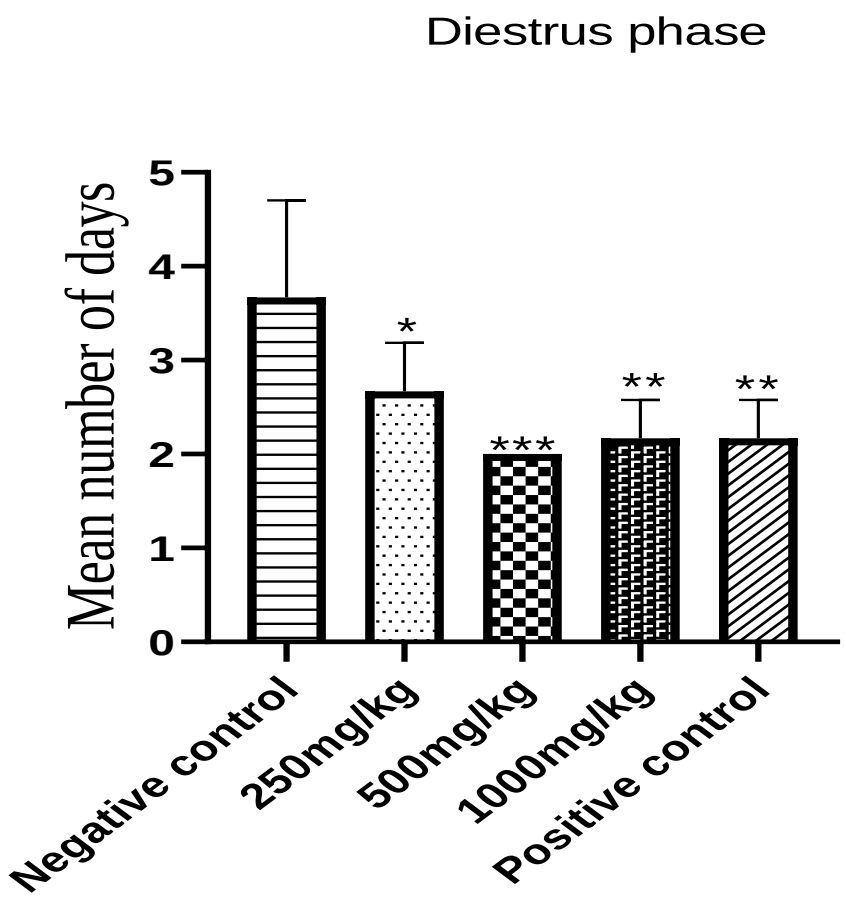
Key: $p<0.05$, $p<0.01$, $p<0.001$, $p<0.0001$

Figure 4.9: Mean number of days of estrus phase at 250, 500 and 1000 mg/kg *Grewia tenax* methanol extract respectively compared to the positive and negative control.



Key: $p<0.05$, $p<0.01$, $p<0.001$, $p<0.0001$

Figure 4.10: Mean number of days of metestrus phase at 250, 500 and 1000 mg/kg *Grewia tenax* methanol extract respectively compared to the positive and negative control.

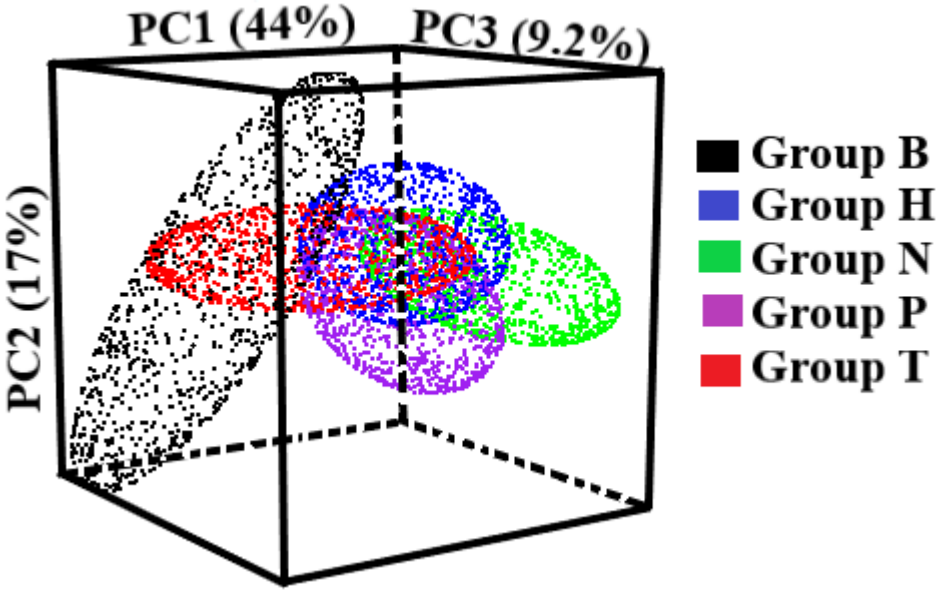


Key: *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001

Figure 4.11: Mean number of days of diestrus phase at 250, 500 and 1000 mg/kg *Grewia tenax* methanol extract respectively compared to the positive and negative control.

4.5 Effect of *Grewia tenax* methanol extract on reproductive hormones in female Wistar albino rats.

After seven days of estrus cycle disruption, blood was taken from each rat tail daily, i.e. from day eight and smeared on a glass slide with silver paste and analyzed using Raman spectroscopy up to the morning of day 18 when the last blood sample was collected before the rats were humanely sacrificed.



Key: Group B – Negative control, Group H – 250mg/kg group, Group N – 500mg/kg group, Group P – Positive control, Group T – 1000mg/kg group

Figure 4.12: 3D score plot demonstrating how different treatment groups were differentiated.

A 3D score plot of data obtained from all five groups (negative control, positive control, 250, 500 and 1000 mg/kg group respectively enabled differentiation. Clustering of the data showed that the obtained Raman spectra for each group was different. None overlapped and the negative control (Group B) was distingtively different from the rest of the treatment groups.

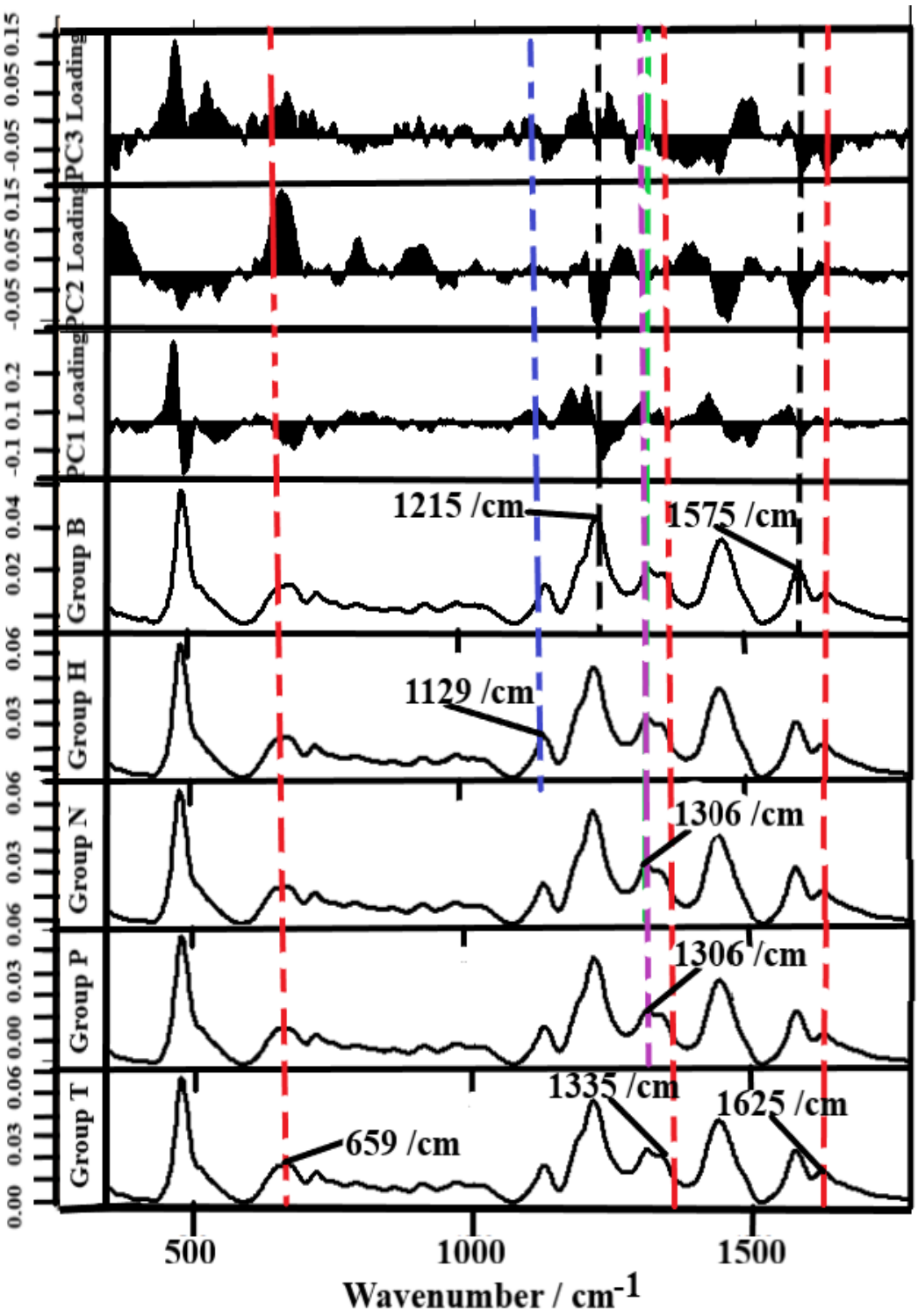
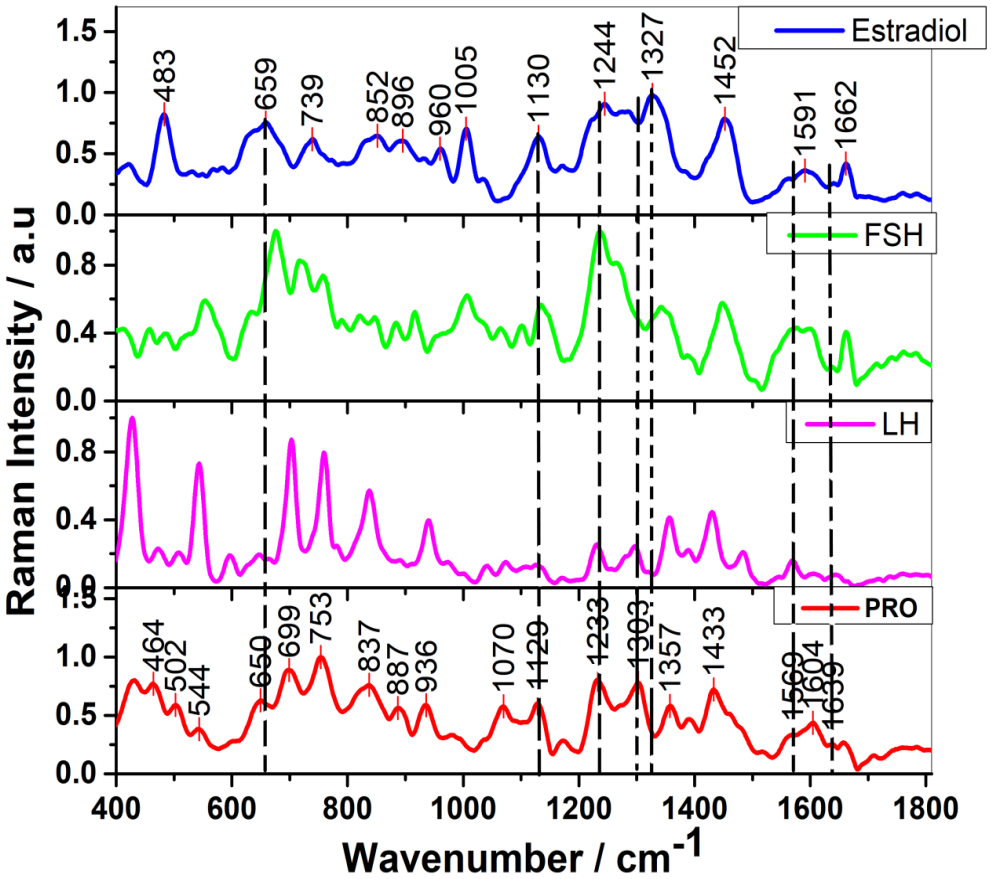


Figure 4.13: Loading plot showing spectral bands that led to the spectral patterns across the groups

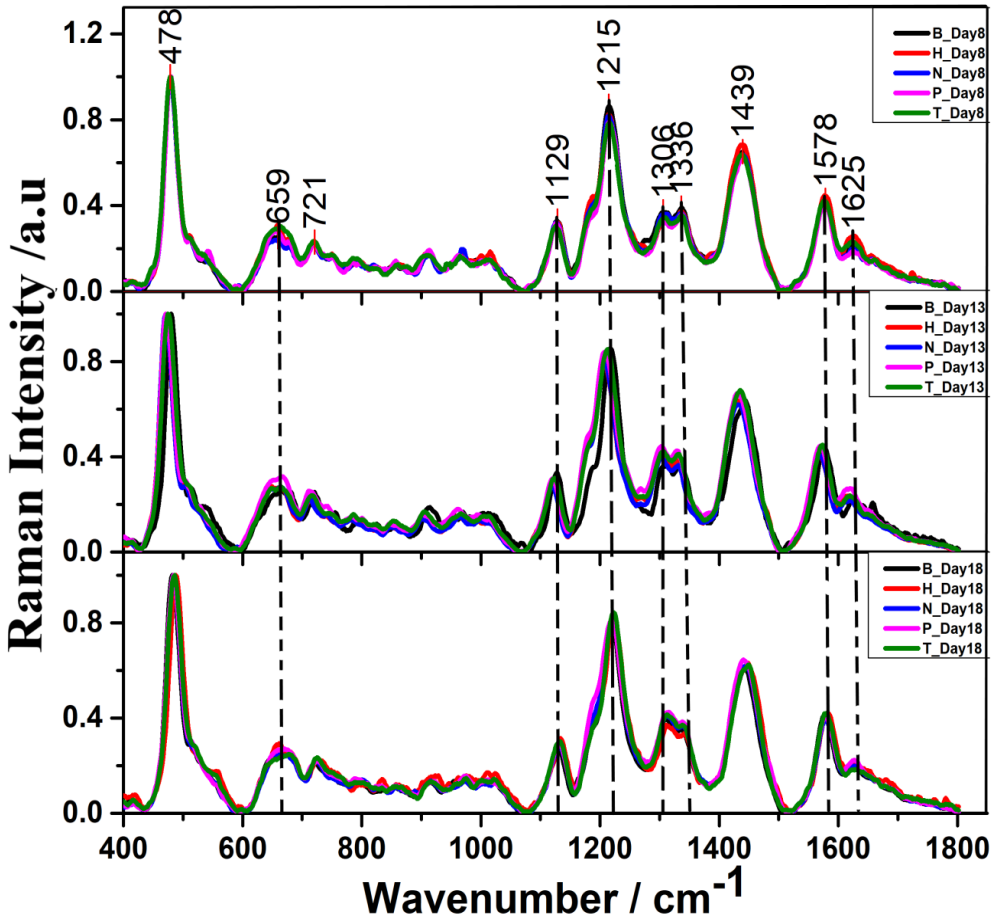
Loading plot showing the spectral bands that led to the spectral patterns across the groups.



Key: Pro – Progesterone, LH – Luteinizing hormone, FSH – Follicle Stimulating Hormone

Figure 4.14: Standard hormone spectra

A plot of standard hormone spectra to enable comparison between the loading plot and standard hormones to determine spectral patterns for standard hormones in the various study groups. The peak at 659/cm was influenced by estradiol and progesterone while the peak at 1130/cm was influenced by estradiol, FSH and progesterone. At peak 1233/cm all the four hormones influenced it. At peak 1303, progesterone and LH had an influence. Estradiol influenced the peak at 1327/cm while both LH and FSH influenced at 1569/cm.



Key: Group B – Negative control, Group H – 250mg/kg group, Group N – 500mg/kg group, Group P – Positive control, Group T – 1000mg/kg group

Figure 4.15: Comparison of Raman spectra within treatment groups for days 8, 13 and 18 of the study

The spectra were analysed for days 8, 13 and 18. Shown are the prominent peaks. On day 8, when extract was adminstered, the Raman spectra intensity between the various treatment and control groups had no variations in hormonal intensity peaks.

On day 13, the Raman spectra intensity peaks were different in the treatment groups compared to the negative control. Hormonal influence in the peaks (Figure 4.13) was as follows:

Peak at 659/cm (Figure 4.14) was influenced by estradiol and progesterone with the highest intensity being that of positive control followed by 1000 mg/kg group and of least intensity being that of the negative control.

The peak at 1129/cm (Figure 4.14) was influenced by estradiol, FSH and progesterone. The highest intensity was caused by 1000 mg/kg treatment group and the lowest intensity was caused by the negative control.

Peak 1215/cm (Figure 4.14) was influenced by all the four hormones. 1000mg/kg treatment group had the highest intensity compared to the negative control that had the lowest intensity.

Peak 1439/cm (Figure 4.14) was influenced by estradiol. The highest intensity seen at 1000 mg/kg treatment group compared to the negative control that had the lowest intensity.

The peak at 1578/cm (Figure 4.14) was influenced by LH and FSH. The highest intensity seen at 1000 mg/kg treatment group compared to the negative control that had the lowest intensity.

On day 18, the raman spectra peaks were influenced as follows:

Peak at 659/cm (Figure 4.14) was influenced by estradiol and progesterone. The highest intensity seen at 250 mg/kg treatment group and the positive control. The least intensity wa seen at 1000 mg/kg treatment group.

The peak at 1129/cm (Figure 4.14) was influenced by estradiol, FSH and progesterone with high intensity at 250 mg/kg and 1000 mg/kg treatment groups compared to the negative control that had the lowest intensity.

Peak at 1215/cm (Figure 4.14) was influenced by all the four hormones. The highest peak seen at 1000 mg/kg treatment group and the positive control compared to the negative control that had the lowest intensity.

Peak at 1439/cm (Figure 4.14) was influenced by estradiol. The highest intensity caused by the positive control group and 1000 mg/kg treatment group compared to the negative control that had the lowest intensity.

The peak at 1578/cm (Figure 4.14) was influenced by LH and FSH. The highest intensity seen at 1000 mg/kg treatment group compared to the negative control that had the lowest intensity.

The negative control group had the least hormonal intensity peaks when compared to the treatment groups at day 13 and 18 but there was no hormonal intensity peak variations between treatment groups and negative control at day 8 (start of extract administration).

CHAPTER FIVE

DISCUSSION

5.1 Phytochemical compounds of Kenyan *Grewia tenax* root extract

Grewia tenax has been used as a fertility-enhancing herb by traditional healers. The plant treats women with fertility problems among the Pokomo community of Kenya (Kaingu *et al*., 2014). In this study, preliminary phytochemical studies indicated the presence of flavonoids, alkaloids, terpenes, sterols, saponins and cardiac glycosides with the use of various solvents. These phytochemicals especially flavonoids, saponins, glycosides and alkaloids could be probably responsible for the fertility-enhancing effect of *Grewia tenax* reported by traditional medicine healers. Flavonoids, alkaloids and terpenes (Oke & Hamburger, 2002; Oladimeji, Lawal, & Ogundimu, 2014; Oladimeji *et al*., 2014) are phenolic compounds that have been reported to be fertility-enhancing due to their antioxidant and radical scavenging properties. Oxidative stress affects ovulation, fertilization, embryo development and implantation hence negatively impacting female fertility (Tvrda *et al*., 2011). Elevated levels of reactive oxygen species (ROS) can damage the ovum after release and also damage sperm within the female reproductive tract since the sperm is very sensitive to oxidative stress hence affecting fertilization in females with no tubal problems (Tvrda *et al*., 2011). Antioxidants have been shown to improve fertility in by improving blood supply to the endometrium, decrease insulin resistance within reproductive system cells, improve cervical mucus to ease fertilization, and has a positive influence on steroidogenesis (Smits *et al*., 2018). *Grewia tenax* methanol extract has been shown to have strong antioxidant and radical scavenging activity have also been reported in a study "Evaluation of antioxidant activities by use of various extracts from *Abutilon pannosum* and *Grewia tenax* in the kachchh region"(Dave, 2017). Similar studies (Amudha & Rani, 2016; Ashidi *et al*., 2013) on fertility enhancing plants have shown presence of flavonoids and alkaoids was thought to cause their fertility enhancing properties for they are anti-oxidants hence reduce stress within reproductive cells leading to enhanced fertility. There exists a relationship between total flavonoid content with the anti-oxidant activity emphasizing the use of flavonoids as anti-oxidants (Pourmorad, Hosseinimehr, &

Shahabimajd, 2006). Another similar study on *Justicia insularis* fertility effects attributed its ability to induce steroidogenesis and folliculogenesis in rats to the presence of flavonoids, alkaloids and glycosides in the extract (Telefo, Tagne, Elodie, Koona, & Didiane, 2012). Alkaloids and flavanoids reduce fertility (Yakubu, 2008) which contrasts this study in that they are reported to reduce LH, estradiol and FSH levels therefore leading to alterations of circulating hormones seen on *Cnidoscolous aconitifolius*.

Saponins also present in *Grewia tenax* have been reported to be responsible for regularization of estrous cycle in mice which had an irregular cycle and were treated with a combined extract of *Turraeanthus africanus* and *Lepidium meyenii* (Amudha & Rani, 2016). Saponins, flavonoids and glycosides are phytoesteroidal compounds which have been reported to cause estrogen hormone balance hence regularizing fertility (Kaaria, Oduma, & Kaingu, 2019). *Ficus Platyphylla* and *Anthocleista vogelii* promotion of fertility was associated with the presence of phytosteroids (Ugwah-oguejiofor *et al*., 2011, Oladimeji *et al*., 2014).

5.2 Acute oral toxicity studies

The use of herbal medicine has gained popularity around the globe and especially in developing countries but this popularity has come with the assumption that plant products are safe. This grave assumption negates the ill health effects that the bioactive compounds in these plant products may have without proper toxicological data profile given the fact that they are used as self-medication (Prasanth, Suba, Ramireddy, & P, 2014). The lack of toxicological data necessitates the need for acute toxicological studies and evaluates effects of different dosages in animal studies. This study undertook to establish the acute oral toxicity of Kenyan *Grewia tenax* methanol root extract in female albino rats.

In the fourteen-day observation period, there was no mortality or morbidity observed. There were no adverse reactions noted even at 5000 mg/kg indicating the LD50 was above 5000 mg/kg. There was no weight gain significant differences in treated animals and the negative control indicating Kenyan *Grewia tenax* is none toxic. The safety of the plant was classified as GHS category 5.

5.3 Effect of *Grewia tenax* methanol extract on female rat cyclicity.

There was a significant restoration of the estrus cycle at 250, 500 and 1000 mg/kg *Grewia tenax* doses respectively compared to the negative control. The restoration was similar to the one observed in rats treated with clomiphene. The plant extract at 500 and 1000 mg/kg caused a significant increase in the frequency of proestrus and estrus ($p<0.01$) compared to the negative control (Table 4.10). These were accompanied by a subsequent significant decrease in the frequency of metestrus and diestrus phases ($p<0.01$) compared to the negative control (Table 4.10). At 250 mg/kg, there was a significant increase in frequency of proestrus and estrus phases ($p<0.05$) and a subsequent significant decrease in the frequency of metestrus and diestrus phases ($p<0.05$) compared to the negative control (Table 4.10). Ovarian steroids under the control of the hypothalamus and pituitary hormones regulate estrus cyclicity. A balanced ratio of ovarian and extra-ovarian steroid hormones influences growth of follicles (Amudha & Rani, 2016). LH, FSH and prolactin concentrations remain relatively low in the course of the estrus cycle. Levels however increase during proestrus phase. Estrogen levels are lowest during estrus phase and increase gradually from the metestrus phase and peaks during proestrus phase. Progesterone concentrations are low during early proestrus; peaks at the end of this phase then decline and start increasing during metestrus and diestrus phases (Marcondes, Bianchi, Tanno, 2002).

The reduction in frequency of metestrus and diestrus phases and the increase in estrus and proestrus phases in all treatment groups could be due to estrogenic properties of *Grewia tenax*. These increase in frequency of the proestrus and estrus indicates an increase in sexual receptivity of the animal hence enhanced fertility. The reduction in frequency of the diestrus is similar to the findings of Daramola et.al (2017) though in a different plant species whereby estrogenic properties the extract reduced frequency of the diestrus phase which was suggestive of development and maturation of follicles. This might be due to the predominant event of diestrus phase in raising estrogen levels (Daramola, Dogru *et al*., 2017). This gives way to the proestrus phase when follicles recruitment occurs and the dominant follicle grows to full size and is ready for ovulation (Marcondes, F. K; Bianchi, F. J; Tanno, 2002).

Estradiol is also responsible for the growth and proliferation of the endometrium in preparation for implantation.

In this study, the extract reverted disrupted estrus cycles back to normal with significantly increased proestrus and estrus phases and decreased diestrus and metestrus phases compared to the negative control. This could probably be due to a direct and indirect effect of *Grewia tenax* on hypothalamic, pituitary and ovarian hormones. *Grewia tenax* may regularize the estrus cycle by probably increasing levels of gonadotropins. It may also probably be due to the presence of antioxidant compounds in the methanol root extract of *Grewia tenax*.

Similar studies on estrus cyclicity but on different plant species have reported altered estrus cycles in rats due to medicinal plant administration. *Cleome gynanda* disrupted the cycle in rats and reduced number of primary, secondary and graffian follicles significantly (Ann Monima *et al*., 2019). *Cissus rotundifolia* caused a dose dependent significant increase of proestrus and metestrus with a subsequent reduction in frequency of appearance of estrus and diestrus (Mziray, Maina, & Kaingu, 2020). Leaf aqueous extract of *Boerhavia diffusa* caused irregular patterns of the estrus cycle in rats (Adebajo, Isah, & Ajayi, 2018) while *Asparagus racemosus* (Kaaria *et al*., 2019) disrupted the estrus cycle with increased appearance frequency of proestrus and estrus phases compared to the negative control.

5.4 Effects of *Grewia* tenax methanol root extract on female albino rat reproductive hormones

This study showed increased levels of gonadotropins (FSH and LH) and sex steroids (estradiol and progesterone) in all three doses of *Grewia tenax* methanol root extract-treated animals compared to the negative control. Optimal female reproduction function requires an effective, well regulated hormonal balance between the hypothalamic Gonadotropin-Releasing Hormone (GnRH), pituitary gonadotropins (FSH & LH) and ovarian steroids (Estrogen and progesterone) (Christensen *et al*., 2012).

Increased levels of FSH and LH as seen in this study may indicate the positive effect of methanolic root extract of *Grewia tenax* on the pituitary and also the

hypothalamus probably leading to a reduction of follicle attrition and increased levels of estradiol. FSH is responsible for follicle growth, from the primary to the antral stage and leading to the release of estradiol from the antral follicles. LH is essential in folliculogenesis, ovulation of the dominant follicle and also plays a role in the synthesis of progesterone and estradiol. Estrogen through a negative feedback mechanism influences production of FSH. In decreased gonadotropin secretion states, there is ovarian malfunction and cessation of reproductive cycles in females. An increase in LH levels may indicate the ability of *Grewia tenax* methanolic root extract to trigger ovulation and probably ensures no anovulatory cycles, hence enhancing fertility. An increase of FSH in this study may indicate the ability of the plant extract to enhance folliculogenesis and steroidogenesis leading to better fertility outcomes. This study finding corroborates findings of (Mokhtar Mokhtari, Esfandiar Sharifi, 2009) though in a different pro-fertility plant species extract where the study reported an increase of FSH and LH leading to enhanced folliculogenesis and ovulation hence enhancing fertility.

An increase in the levels of estradiol in this study may indicate effect of *Grewia tenax* on ovarian function. Estradiol has significant roles in female reproductive function. It is responsible for the formation of cilia and differentiation of the fallopian tube epithelium which enhances fertilization by ensuring fast transit of spermatozoa. In the fallopian tubes, estrogen is responsible for production and synthesis of glycoproteins that nourish the fertilized ova on transit to the uterus for implantation. So in this study increased intensity of estradiol would enhance the integrity of the fallopian tube hence promote fertility. At its peak levels, estrogen probably triggered GnRH surge which in turn led to LH surge causing ovulation and promoting fertility. High levels of estradiol in this study may also enhance fertility by inducing endometrial proliferation leading to effective implantation window. The findings of this study corroborate Amudha et.al (2016) in which they suggested that the increased levels of estradiol in animals treated with different pro-fertility plant might be due to the plant enhancing the pituitary ovarian axis and hence its use in the treatment of infertility. In another study on a different plant species by Kaingu et.al (2017), reduced estradiol levels were reported to cause ant-fertility effects and interrupting the pituitary ovarian axis thereby reducing fertility. Increased levels of

estradiol in another pro-fertility plant species (Jha, Asad, Asdaq, Das, & Prasad, 2010) were reported to induce fertility in rats that had hyperprolactinemia induced infertility.

Increased progesterone levels in this study may indicate the effect of the plant extract on ovaries and subsequently on the endometrium. Progesterone thickens the functional layer of the uterus facilitating implantation and also maintains pregnancy. These findings are in agreement with those of Jha *et al*., 2010 who reported fertility enhancing effect of a different pro-fertility plant species in female rats following increased levels of progesterone. This finding is in contrast to Kaingu *et.al* (2017) who reported that increased levels of progesterone reduced fertility.

According to the results from this study, the methanol root extract of *Grewia tenax* may enhance fertility by enhancing the pituitary ovarian axis with subsequent increase in FSH, LH Estradiol and progesterone. This is further evidenced by regularization of the estrus cycle as shown in figures 4.3, 4.4 and 4.5.

CHAPTER SIX

CONCLUSION AND RECOMMENDATIONS

6.1 Conclusion

This study found the roots of Kenyan *Grewia tenax* extract to contain alkaloids, flavonoids, saponins, sterols, terpenes and cardiac glycosides which are reported to have anti-oxidant and radical scavenging properties which probably reduce oxidative stress within the female reproductive system leading to improved fertility.

Kenyan *Grewia tenax* methanol root extract was found to have high margin of safety and was classified as GHS category 5.

Kenyan *Grewia tenax* methanol root extract was found to significantly restore disrupted estrus cycle in a dose dependent manner. The increase frequency of proestrus and estrus phases probably increase sexual receptivity of the animals hence increased fertility

The study found *Grewia tenax* extract methanol extract having ability to raise the intensity of both gonadotropins and sex steroids. This might be due to positive effect of the extract on the folliculogenesis and steroidogenesis hence increased fertility .

The study concludes that *Grewia tenax* methanol root extract has pro-fertility effects.

6.2 Recommendations

This study made the following recommendations:

1. Quantitative analysis of phytochemical components of *Grewia tenax* methanolic root extract should be established.
2. Further studies on sub-acute and chronic oral toxicity of *Grewia tenax* should be conducted
3. *Grewia tenax* has pro-fertlity potential and the species requires evaluation of its mechanism of action

4. Further studies on the effects of *Grewia tenax* root methanol extract on reproductive hormone receptors

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APPENDICES

Appendix I: Biosafety, animal use and ethics certificate

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Mr Peter Joseph Kasyoki
C/o Dr Catherine Kaluwa
Dept. of Vet. Anatomy & Physiology

REF: FVM BAUEC/2018/182
14/12/2018

Dear Mr Kasyoki,

RE: Approval of Proposal by Biosafety, Animal use and Ethics committee

Fertility effects of Kenyan Grewia tenax (Mukawa wa Guba) root extract in female Albino Rats

By Peter Joseph Kasyoki (Ref:HSM 302-4017/2016)

We refer to the above MSc proposal that you re-submitted to our committee for review and approval. We have now reviewed the proposal and are satisfied that you have satisfactorily addressed the issues we had raised in out letter to you dated 7/11/2018. These included issues related to: numbers of animals to be used, animal husbandry and handling and monitoring of animals following administration of extracts. Furthermore, you have also addressed occupational safety and euthanasia of the animals.

We hereby approve your work as per your revised proposal

Rodi O. Ojoo BVM M.Sc Ph.D
Chairman, Biosafety, Animal Use and Ethics Committee,
Faculty of Veterinary Medicine

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Appendix II: Safety and fertility potential of the plants secondary metabolites

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Safety and Fertility Potential of Kenyan *Grewia tenax* (*Mukawa Wa Guba*) Root Extract Secondary Metabolites

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Authors' contributions

This work was carried out in collaboration among all authors. Author PJK designed the study, wrote the protocol, performed laboratory work, did the statistical analysis and wrote the first draft of the manuscript. Author CKK worked on the acute oral toxicity studies and author COW developed extraction procedures and phytochemical screening and developed the manuscript. Author FOA managed literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Aim: To screen Kenyan *Grewia tenax* root extract phytochemicals and correlate the attribute fertility enhancing effects and safety in female albino rats.
Study Design: An experimental study design was used.
Place and Duration of Study: The phytochemical studies were done at Jomo Kenyatta University of Agriculture and Technology (JKUAT), Department of Botany Laboratory, while acute oral toxicity studies were done at the Department of Veterinary Anatomy and Physiology, University of Nairobi (UON) animal house. The study was done during the month of March to June 2019.

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