EVALUATION OF EFFECTS OF *ASPARAGUS RACEMOSUS* FROM NAKURU COUNTY, KENYA ON SELECTED FEMALE REPRODUCTIVE PARAMETERS USING WISTAR RAT.

A thesis submitted in partial fulfilment for the award of Master of Science degree of the University of Nairobi in Comparative Animal Physiology (Reproductive Physiology)

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DECLARATION

I hereby declare that this thesis is my original work and has not been submitted to any other University for a degree

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DEDICATION

I dedicate this thesis to my beloved dad and mum, family and friends for the sacrifices, unrelenting love and encouragement to push on to where I am today.

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LIST OF ABBREVIATIONS

ASP- *Asparagus racemosus*

LH- Luteinizing Hormone

FSH- Follicle Stimulating Hormone

PGF2α- Prostaglandin F2 alpha

HD- High Dose

LD- Low Dose

NEG- Negative control

POS- Positive control

NSAIDs- Non-steroidal Anti-inflammatory drugs

OCPs- Oral Contraceptives

IVF- In-vitro Fertilization

DVAP- Department of Veterinary Anatomy and Physiology

OECD- Organization of Economic Co-operation and Development

ABSTRACT

Dysmenorrhea is a major gynecological problem among women of reproductive age globally. *Asparagus racemosus* is traditionally used to manage discomforts related to the female cycle. The effect of the plant on female reproductive parameters namely: mating success, gestation length, litter size, reproductive hormonal profiles and on myometrial quiescence was evaluated using female Wistar rats. Preliminary phytochemistry and acute oral toxicity studies were carried out. Thirty normal cyclic Wistar rats aged between 50-60 days were used for the study. Twenty rats were used to evaluate estrus cyclicity, mating success, gestation length, litter size and reproductive hormonal profiles. Ten rats were used for the *in vitro* studies on myometrial quiescence. Twenty rats were divided into 4 groups (5 animals each). Group I and II received 600 and 300 mg/kg bw *Asparagus racemosus* extract, group III, 20 mg/kg ibuprofen and group IV 0.5 ml physiological saline. The plant extract and drug were administered between 9-10 am through intra-abdominal gavage every other day for 14 days. Estrus cyclicity was monitored daily for two weeks between 9-10 am through vaginal smear microscopy. Approximately 0.5µl of blood was collected from all animals for hormone analysis using Raman spectroscopy. On the 14th day fertile males were introduced into female cages. Female rats were monitored thereafter for mating success. Gestation length and litter size was recorded for each pregnant rat. The effect of *Asparagus racemosus* and ibuprofen on isolated uterine strips was evaluated in non-pregnant animals using serial extract concentrations: (20, 40, 80 and 160 mg/ml). While positive control rats were exposed to 20 mg/ml ibuprofen. Data was analyzed using ANOVA, P values < 0.05 was considered significant.

Asparagus racemosus was non-toxic to rats even at 5000 mg/kg (limit dose). Preliminary phytochemistry revealed the presence of saponins, flavonoids, glycosides and terpenoids.

A*sparagus racemosus* disrupted the estrus cycle with increased appearance frequency of proestrus and estrus phase. There was a significant increase of proestrus ($P < 0.001$) compared to the negative control and a subsequent significant reduction in metestrus ($P < 0.01$) and diestrus ($P < 0.05$). There was no adverse effect on mating and gestation length. However there was a significant difference in gestation length ($P < 0.05$) in treated groups compared to the positive control. The litter size was however non-significant compared to control. *Asparagus racemosus* caused a significant dose dependent disruption of FSH, LH, progesterone and estradiol levels ($P < 0.0001$). The force of uterine contraction was significantly decreased (-0.146%, -5.13%, -7.97%, -19.55 %) respectively ($P < 0.05$ to $P < 0.0001$) in non-pregnant uterine strips exposed to (20, 40, 80, 160 mg/ml) concentrations of *Asparagus racemosus* compared to Ibuprofen that caused a significant ($P < 0.05$) decline in uterine force of contraction (-13.38%). Frequency of uterine contraction was significantly ($P < 0.0001$) reduced (-5.99%, -9.61%, -16.76%, -25.21%) respectively when uterine tissue was exposed to (20, 40, 80, 160 mg/ml) *Asparagus racemosus* extract compared to ibuprofen (-15.78%). In conclusion, 160 mg/ml *Asparagus racemosus* extract caused almost similar reduction in frequency and force of uterine contraction compared to Ibuprofen (positive control). *Asparagus racemosus* extract also has positive effects on other reproductive indices. This study therefore recommends phytochemical isolation using High performance liquid chromatography (HPLC) technique and docking to determine the analgesic component of the plant.

CHAPTER ONE

1.0 INTRODUCTION

Female reproductive health problems remain a leading reason worldwide for morbidity and mortality in women of child bearing age (United Nations, 2012). The global prevalence of female reproductive system ailments has been increasing (Prasad *et al*., 2014). The pharmacological agents for treating the ailments are not always affordable particularly in the developing world and many have adverse side effects. The World Health Organization (WHO) therefore continues to advocate for the use of effective locally available medicinal plants as substitutes for synthetic drugs (Zade *et al*., 2013). This has led to increased research on medicinal herbs known to treat various illnesses in both developed and developing countries as alternative remedies. National Policy on Traditional Medicine show that in Kenya alone the number of traditional medicine users is about 27 million (Kaingu *et al*., 2011). Many of the medicinal herbs are used in treatment of female reproductive ailments (Khulbe, 2015). Dysmenorrhea is the major gynecological problem among women of reproductive age irrespective of nationality (Patel *et al*., 2006; Proctor and Farquhar, 2006). Primary dysmenorrhea is a recurrent menstrual pain around the pelvic area just before menstruation and or during menses without any pelvic pathological conditions (Shaha and Bellankimath, 2017). Prevalence of primary dysmenorrhea estimates are about 45 and 95% of menstruating women (Unsal *et al*., 2010). Despite the high prevalence, it is usually under-treated as women regard it as a normal part of the menstrual cycle than an ailment hence few of them seek medical treatment (Wong, 2011; Proctor and Farquhar, 2006). This leaves women prone to other ailments that are linked to chronic pain like insomnia and depression (Iacovides *et al*., 2015; As-sanie *et al*., 2012). Non-Steroidal Anti-inflammatory Drugs (NSAIDs) and Oral contraceptives (OCPs)

are the most common treatment for dysmenorrhea (Zahradnik *et al*., 2010; Proctor *et al*., 2001). These pharmacological remedies have been found to have various side effects like arterial diseases, liver injury, gastrointestinal bleeding, nausea, dizziness and headache (Proctor *et al*., 2001). Women hence have sought alternative remedies including use of herbs. Therefore, knowledge on pharmacological properties, beneficial and adverse effects of traditional remedies is essential as they may offer an effective remedy for dysmenorrhea (Van-andel *et al*., 2014).

1.2 OBJECTIVES

1.2.1 General Objective

To determine the effects of *Asparagus racemosus* root extract on selected female reproductive parameters using the Wistar rat model.

1.2.2 Specific Objectives

- To determine the preliminary phytochemical constituents of *Asparagus racemosus* aqueous root extract
- To evaluate the effect of *A. racemosus* extract on estrous cyclicity, mating success, gestation length and litter size
- To evaluate the effect of *A. racemosus* on plasma estradiol 17β, progesterone, FSH and LH
- To demonstrate the effect of *A. racemosus* extract on isolated uterine tissue

1.3 NULL HYPOTHESIS

Asparagus racemosus root extract does not have any significant effect on reproductive parameters of the female Wistar rats.

1.4 JUSTIFICATION OF THE STUDY

Dysmenorrhea is the major gynecological issue amongst women of reproductive age with a prevalence of 45-95% (Proctor and Farquhar, 2006; Unsal *et al*., 2010). Schoep *et al*., (2019) reported unproductivity of women due to dysmenorrhea at about 80 % with low performance at work place, delay to work, absenteeism and decreased daily activities being the common reasons. Despite this, its poorly managed and disregarded by health professionals and women accepting it as a normal part of menstrual cycle (Wong, 2011). Conventional treatments have been used especially analgesics like ibuprofen but their continued use is accompanied with unpleasant side effects. This has led to continued search for alternative treatments including herbal remedies. Medicinal plants have been used widely to curb the various gynaecological health issues due to their affordability and accessibility (Bafor, 2017; Prasad *et al*., 2014). *Asparagus racemosus* has been reported to alleviate the pain of dysmenorrhea, nonetheless, the specific effects of *Asparagus racemosus* on dysmenorrhea have not been studied. This research is aimed at studying the specific effects of *Asparagus racemosus* on estradiol17β, progesterone, FSH and LH levels, related estrous cyclicity, pregnancy outcome and its effect on isolated myometrial quiescence. This will help give scientific credence to folklore use of this plant in the management of dysmenorrhea. The findings will also be used in drug discovery efforts to find novel therapies for dysmenorrhea.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Overview of Mammalian Reproductive Physiology

Mammalian female reproductive system consists of a pair of ovaries, oviduct, uterine horns, cervix, vagina, vestibule and external genitalia. Oogenesis, folliculogenesis and steroidogenesis occurs in the ovary. The fallopian tubes provide passage for the fertilized ova to the uterus where implantation takes place and also passage of unfertilized egg which eventually leads to menses. Ovulation is the release of ova from dominant graafian follicle under the influence of surge in plasma luteinizing hormone (LH). The theca cells in the ovary have receptors for LH, thus during folliculogenesis LH will bind to these receptors and stimulate release of testosterone hormone. Testosterone then diffuse to the granulosa cells which have receptors for follicle stimulating hormone (FSH) and on binding, granulosa cell receptors lead into aromatase P_{450} expression that converts testosterone into estradiol (Kadioglu *et al*., 2008). Estradiol (E2) promotes growth of granulosa cells, induces pre-ovulatory LH surge and prevents early luteinization of the granulosa cells. Once ovulation has taken place the granulosa cells become luteinized and start secreting hormone progesterone (P4) which prepares the endometrium for implantation by increased growth and blood supply. The ovarian cycle and the uterine cycle are dependent on steroid hormones from the ovary and function via feedback mechanisms of the hypothalamic pituitary gonadotropin axis (Speroff and Fritz, 2005).

2.1.1 Mammalian oestrus cycle

Mammalian estrous cycle consists of the follicular phase and luteal phase. The cycle is regulated by the negative and positive feedback mechanisms driven by the reproductive hormones. These hormones are gonadotropin releasing hormone (GnRH) released from the hypothalamus, FSH

and LH) from anterior pituitary gland and (estradiol and progesterone) from ovaries (Soede *et al*., 2011; Smith *et al*., 2005).

The follicular phase starts with regression of corpus luteum (luteolysis). Luteolysis causes a decrease in the levels of progesterone hormone hence a negative feedback on the pituitary LH secretion. Continuous decrease in circulating progesterone causes LH pulse frequency to increase hence follicular estradiol is secreted (Johnson, 2002). Follicular estradiol production is due to influence of LH and FSH on the theca and granulosa cells of the follicular wall (Aritonang *et al*., 2017; Johnson, 2002). FSH binds to membrane receptors on granulosa cells increasing aromatase activity that converts androgens to estradiol. Estrogen production by granulosa cells of the preovulatory follicle induce overt signs of estrus in mammals leading to receptivity to the male (Satue and Gardon, 2013). Increasing levels of estradiol then induce preovulatory gonadotropin surge necessary for ovulation. FSH also stimulates growth and development of the ovarian follicles with development of the ovary. LH promotes completion of follicular maturation and enlargement of the preovulatory follicle thus inducing ovulation (Satue and Gardon, 2013).

The luteal phase begins after ovulation with formation of corpus luteum and ends at luteolysis. The granulosa cells after ovulation become luteinized and starts producing progesterone instead of estradiol. Progesterone gradually increases after ovulation and will play a role in implantation, pregnancy support and inhibit myometrial contraction (Aritonang *et al*., 2017). At this stage, high levels of progesterone prevent secretion of FSH and LH. If no pregnancy occurs, PGF2α secreted from the endometrium leads to regression of corpus luteum and decline in progesterone levels (Ginther *et al*., 2008). Rapid withdrawal of estradiol and progesterone leads to normal uterine bleeding (menstruation) which may be accompanied by cramping at the abdominal area due to production of prostaglandins (dysmenorrhea). Once the pituitary gland increases

production of FSH and LH, another cycle is initiated with resumption of follicular growth. If pregnancy occurs the ratio of estradiol to progesterone is maintained to support pregnancy.

2.1.2 Fertilization

Successful fertilization involves interaction between the spermatozoa and the oocyte (s). The sperm travels through the female reproductive system until it reaches the ampulla of the oviduct for fertilization and here the oviduct optimizes the environment by contracting to aid in sperm movement. Oviduct smooth muscle contraction is regulated by prostaglandins modulated by estradiol and progesterone through their receptors (Huang *et al*., 2015). Once the oocyte and the spermatozoa reach the ampulla, the sperm penetrates into the oocyte and oocyte cortical granules release ovastacin that cleaves to the zona pellucida 2 protein causing zona to harden so as to prevent polyspermy (Burkart *et al*., 2012). After sperm oocyte recognition they fuse and the pronuclei form beginning the process of embryogenesis. Any compromise on the oocyte, the spermatozoa or the internal environment may lead to fertilization failure.

2.1.3 Implantation

Implantation requires active participation of both the embryo and the mother. The process involves molecular signaling by the embryo, apposition, nidation (endometrial attachment) followed by endometrial invasion by the embryo. (Hoozemas *et al.*, 2004). Successful implantation and pregnancy is determined by embryonic factors. Endometrial implantation of the embryo occurs only during the implantation window period (Lessey, 2000) which in humans for instance, is only 7-10 days after fertilization (Acosta *et al*., 2000). Progesterone and estrogen are the main hormones regulating implantation by initiating a cascade of paracrine and autocrine signal transductions leading to attachment and invasion of the blastocyst into the endometrium. Imbalance in estrogen and progesterone interaction could cause delayed secretory endometrial

maturation, a luteal phase defect known to largely cause implantation failure as a result of dys-synchronicity of the embryo and endometrium (Hoozemas *et al*., 2004). Progesterone is particularly implicated during the window period of implantation as it influences endometrium receptivity indirectly by promoting or suppressing expression of apposition and attachment molecules, growth factors and cytokines (Hoozemas *et al*., 2004). It also up regulates prostaglandins. Thus low levels of progesterone hormone will cause implantation failure due to unreceptivity of the uterus.

2.2 Rat estrus cycle

The estrus cycle in rats like in other mammals is influenced by pituitary gonadotropins and hormones produced by the ovary. The rat estrus cycle is divided into proestrus, estrus, metestrus and diestrus stages/phases characterized by different vaginal epithelial cell types. The morphology of vaginal cells at different stages shows functional status of the hypothalamic-pituitary- ovarian axis. The rat estrus cycle averages 4-5 days (Cora *et al*., 2015). Proestrus is dominated by round nucleated epithelial cells, estrus by anucleated epithelial cells, metestrus by leukocytes, few nucleated epithelial cells and/or cornified cells, diestrus by leukocytes (Mohammed *et al*., 2018; Cora *et al*., 2015; Paccola *et al*., 2013). The cycle is recurrent and describes the changes in reproductive hormone levels due to ovarian activity influenced by the pituitary hormones. Estrogen and progesterone are the primary contributor to the morphology of vaginal epithelial cells during the estrus cycle (Paccola *et al*., 2013). There is an increased level of estrogen hormone during proestrus phase which triggers release of FSH for the growth and maturation of the ovarian follicles (Maeda *et al*., 2000). Estrus behavior is brought about by increased levels of estradiol which also induce the preovulatory gonadotropin surge which is necessary for ovulation. The female rat is normally receptive to the male during the estrus phase

(heat period), but can also be receptive during the end of proestrus (Westwood, 2008). It is during the estrus phase that LH surge and ovulation normally occurs at the night of estrus (Paccola *et al*., 2013). After ovulation the levels of LH decline and progesterone peaks until the end of diestrus. Any alterations in the hormones thus compromises the regular cycle affecting fertilization and implantation. The litter size of the rat is an average of 9±3 and the gestation length is short (21 ± 2 days) making it an ideal model for studying any chemical factors affecting reproduction (Paccola *et al*., 2013).

2.3 Female Reproductive Ailments and Treatment

2.3.1 Dysmenorrhea

Dysmenorrhea is a painful uterine contraction due to endometrial laceration (Sharghi *et al*., 2019) and it is a common ailment in adolescent and women of reproductive age. It is highly prevalent ranging between 45- 97% among groups of different ages and nationalities (Kim *et al*., 2017; Lee *et al*., 2016). Theories have been postulated on the causes for dysmenorrhea including hormonal imbalance, abnormal myometrial activity and effects of prostaglandins among others (Iacovides *et al*., 2015). Dysmenorrhea can be either primary dysmenorrhea which is not linked to any pathological conditions or secondary dysmenorrhea that may be associated with organic pathological conditions (physiological change to some tissues/organs) like endometriosis, ovarian cysts, fibroids, adenomyosis and pelvic infections (Marzouk *et al*., 2013).The major cause of primary dysmenorrhea to date is not clear but scientists have explanations based on increased production of prostaglandins especially prostaglandins E2 and F2α. As endometrial cells begin to disintegrate toward the onset of menstruation they release PGF2α which stimulate myometrial contraction and vasoconstriction leading to uterine contractions (Wallace *et al*., 2010). The uterine contractions cause increased intrauterine pressure and reduced blood flow to

the uterus, causing myometrial ischemia and hypoxia hence the pain and cramping (Xu *et al*., 2017; Altunyurt *et al*. 2005; Howard, 2000). The other explanation for dysmenorrhea is the hormonal imbalance theory. Levels of prostaglandins are found to be low during follicular phase until early luteal phase but increased during menstruation due to withdrawal of progesterone (Iacovides *et al*., 2015). This suggests that prostaglandin action on the uterus is dependent on progesterone levels in the blood. Increased levels of progesterone renders the uterus resistant to prostaglandin stimulation but as menstruation approaches which involves withdrawal of progesterone there is excess production of prostaglandins leading to dysmenorrhea.

Several remedies exist for alleviating dysmenorrhea. NSAIDs are used as a pharmacological remedy to dysmenorrhea by blocking cyclooxygenase-2 enzyme thus inhibiting the production and release of prostaglandins (Zahradnik *et al*., 2010). Oral contraceptives (OCPs) containing estrogen and progesterone that prevent ovulation and endometrial thickening have also been used. These reduce volume of menstrual fluid hence less production of prostaglandins (Strowitzki *et al*., 2012). Other remedies are electric nerve stimulation leading to alteration of the body's ability to perceive pain, transdermal nitroglycerin patches that prevent uterine contractions and surgical methods which have been shown to be less effective (Gharloghi *et al*., 2012; Cho and Hwang, 2010). Some people use non-pharmacological methods including more sleep time, physical exercise, heating pads, increased intake of certain foods like beans and aromatic oils (Lasco *et al*., 2012). NSAIDs and OCPs are the most commonly used remedies (Sharghi *et al*., 2019). Continued use of NSAIDs and OCPs has been linked to adverse side effects like nausea, gastrointestinal bleeding, headache, vomiting, dizziness, and also effects on reproductive processes like ovulation, fertilization and implantation (Sharghi *et al* ., 2019). This has led to use of alternative remedies including herbal medicinal plants.

2.3.2 Premenstrual syndrome

Premenstrual syndrome (PMS) are cyclic appearance of various symptoms including: abdominal bloating, depression, fatigue, breast tenderness, lack of energy, anxiety, social withdrawal, poor concentration, change of appetite and mood swings during 7-10 days prior to menstruation but relieved after menses flow begins (Zaka and Mahmood, 2012; Henshaw, 2007). Previous studies have reported about 90% of women of reproductive age experience PMS. Four in every ten women suffer from PMS symptoms of which 5-8% experience severe PMS disrupting the woman's life (Pearlstein, 2007). The aetiology of PMS is not clear but it is thought to involve the ovarian hormonal cycle. The theories explain it as due to low progesterone and high estrogen levels and the changing E2/P4 ratio and neurotransmitters serotonin and γ-aminobutyric acid (GABA) being involved (Zaka and Mahmood, 2012). Various therapies have been adopted including: healthy diet, use of oral contraceptives, monoamine oxidase inhibitors, vitamins, exercise, surgery but the most commonly used are the pharmacological therapies that have greater health risks and mainly are given to those with severe PMS (Zaka and Mahmood, 2012). Women therefore have sought for herbal remedies that are more affordable and have less or no health risks.

2.3.3 Amenorrhea

Amenorrhea is lack or abnormal halting of the menses (Tarannum, 2006). The major causes of amenorrhea are functional disorders of the hypothalamus, polycystic ovarian syndrome, hyperprolactinemia (associated with low levels of estradiol) and ovarian failure.

2.3.4 Postpartum haemorrhage (PPH)

Postpartum haemorrhage (PPH) accounts for about 35% of maternal deaths, and it is the major contributor to maternal deaths in the developing countries (Haeri and Dildy, 2012). About 99%

of deaths due to PPH occur in developing countries compared to the 1% in the developed countries (Haeri and Dildy, 2012). Inefficient uterine contraction is the major cause of PPH, but may also be caused by retained placenta parts, tears along the vagina and the cervix, uterine rapture and inversion (Haeri and Dildy, 2012). In the developing countries inaccessibility to treatment and inadequate intensive care and blood facilities are the major causes of high mortality rates (WHO, 2010; Khan *et al*., 2006). Management of PPH has been through uterotonics such as oxytocin, prostaglandins, haemostatic drugs, and surgery (Haeri and Dildy, 2012). The treatments are expensive and reported to have side effects like headache, nausea, diarrhea, abdominal pains and hypotension (Peitsidis and Kadir, 2011).

2.4 Medicinal plants use in reproduction

2.4.1 Influence of medicinal plants on reproductive hormones

Reproductive hormonal balance plays a major role in healthy and successful reproductive processes. To enhance fertility, researchers have investigated the role of medicinal plants and their products in regulating reproductive hormones (Farahbod and Soureshjani, 2018). Medicinal plants compounds work by mimicking the action of endogenous hormones by acting as either agonists or antagonists thus altering their synthesis, metabolism or modifying their receptors (Khulbe, 2015). Therefore this study will evaluate the effect of *Asparagus racemosus* extract on reproductive hormones.

2.4.2 Medicinal plants used in management of female reproductive ailments

2.4.2.1 Use on diverse reproductive ailments

Today the basis of many pharmaceuticals is plant and plant based medications to treat various ailments (Katiyar *et al*., 2012). Primary health care using traditional medicine in developing countries is about 80%, most of which involve plant extracts (Sandhya *et al*., 2006). Some of the

female reproductive ailments managed using the traditional herbs include: infertility, problems related to menstruation (dysmenorrhea, amenorrhea, oligomenorhea, metrorraghia, polymenorhea), postpartum hemorrhage, abortion, uterine infections among others (Prasad *et al*., 2014). A study by Prasad *et al*., (2014) on medicinal plants used in Wayanad district in India found that 28 out of the 35 plants studied were used for treatment of reproductive ailments, most of which were for dysmenorrhea (Prasad *et al*., 2014).

The bark extract of *Saraca indica* is reported to treat menorrhagia, leucorrhea and uterine disorders and a drug (U-3107) synthesized as an analogue from this plant is said to treat menorrhagia, dysmenorrhea, PMS and threatened abortion (Mishra *et al*., 2013). *Zingiber officinale*, the ginger rhizome, treats nausea and vomiting during pregnancy, dysmenorrhea and menstrual irregularities (Rahnama *et al*., 2012). A study by Awed *et al* (2013) reported that it confers both anti-inflammatory and anti-carcinogen properties which inhibit release of prostaglandins in the uterine muscle that would result in pain owing to myometrial contractions.

Leonurus japonicas stimulate uterine contraction and reduce postpartum hemorrhage hence its use in expulsion of the placenta after child birth (Van *et al*., 2014).

2.4.2.2 Medicinal plants used in management of dysmenorrhea

Medicinal plants have been used to treat dysmenorrhea and its associated symptoms very effectively, with some plants having prostaglandin inhibitory effect and anti- spasmodic activity (Mirabi *et al*., 2014). *Foeniculum vulgare* of Apiaceae family is one such plant with anti-inflammatory, analgesic and antispasmodic effects (Shams *et al*., 2005). The essential oils of this product inhibit contractions of the uterus induced by prostaglandins; it also causes blood discharge in shorter time minimizing pain of dysmenorrhea (Shams *et al*., 2005). Its fruit

contains anethole (dopamine like) that binds to dopamine receptor and suppress contractions induced by prostaglandins (Modaressnejad *et al*., 2006).

Zingiber officinale is reported to inhibit production of prostaglandins thus its traditional use in treatment of dysmenorrhea (Ozgoli *et al*., 2009). Menastil a combination of calendula oil and mint oil is reported to hinder transmission of messages from the uterine nerve cells to brain by shortening the nerve cells axons. This helps delayed transfer of pain messages from brain to the uterus (Mirabi *et al*., 2014). *Matricaria chamomilla* has anti-inflammatory and antispasmodic effects hence its use in treatment of dysmenorrhea (Barene *et al*., 2003). *Valeriana officinalis* is used traditionally as a menstruating herb to reduce pain where it works by blocking the calcium channels (Gilani *et al*., 2005). *Cinnamomum zeylanicum* essential oil and tannin components are responsible for its antispasmodic effect. Eugenol contained in the essential oil of its bark block/inhibit biosynthesis of prostaglandins and reduces inflammation (Akhavan *et al*., 2009). *Zataria multiflora* flavonoids are reported to inhibit contractions induced by cell polarization and block the calcium channels causing antispasmodic effect on the smooth muscle (Irvani, 2009). The peppermint oil provides an antispasmodic activity on the uterine muscle by blocking the calcium channels (Bahmani *et al*., 2015).

The alkaloids in several plants have been shown to block the influx of extracellular calcium through the receptor operated calcium channels inhibiting spasmodic activity in the rat uterus. During uterine contraction, there is opening of the smooth muscle calcium channels and L type channels, extracellular calcium enter and phospholipase C is activated increasing inositol 1,4,5-triphosphate (IP3) production. Sarcoplasmic reticulum releases calcium leading to myometrial contraction (Sukwan *et al*., 2014). In this way the plant phytochemicals are thought

to block the influx of extracellular calcium into the uterine muscle reducing the hyper contractions of dysmenorrhea.

2.4.3 Effect of *Asparagus racemosus* on various ailments

Asparagus racemosus of family Asparagacea is a widely used medicinal plant in treatment of a wide range of illnesses and also as a female reproductive tonic (Kinage and Chaudhari, 2016). It is commonly known as *Shatavari* in Hindi which means ‘a woman possessing 100 husbands’ since it has various rejuvenating effects in female reproductive organs (Mishra *et al*., 2013; Kumar *et al*., 2008). In Kenya its local name is ‘Kitaplelit’in Kalenjin.

Figure 1: *Asparagus racemosus* root

Literature on *Asparagus racemosus* shows it has been used in treatment of various illnesses including: gastrointestinal ailments, diabetes, coronary heart diseases, diarrhea, dyspepsia, upper respiratory tract infections, ulcers, cancer, as an antioxidant and to enhance libido in males

(Shaha and Bellan, 2017; Singh and Sinha, 2015; Chitme *et al*., 2009). *Asparagus racemosus* '*queen of herbs'* has the highest capacity to solve the many female reproductive system problems. No wonder its placed in the 6 plants of *Ayurveda*- the traditional Hindu system of medicine that is known to promote people's health (Kinage and Chaudhari, 2016; Mishra *et al*., 2013; Kumar *et al*., 2008). The major bioactive components present in the root of *Asparagus racemosus* are the steroidal saponins, flavonoids, isoflavonoids, glycosides, minerals like Calcium, Magnesium, Iron, Phosphorus and vitamins A; B1; B2, C, E (Shaha and Bellankimath., 2017; Joshi, 2016). *Asparagus racemosus* is among the phytoestrogen rich plants (Joshi, 2016). Phytoestrogens are able to induce biological responses in animals and mimic or modulate the actions of endogenous estrogens by binding to their receptors (Whitten and Patisaul, 2001). Majorly the phytoestrogenic activity of *Asparagus racemosus* is due to the steroidal saponins which can enable estrogen hormone balance (Joshi, 2016).

2.4.3.1 *Asparagus racemosus* use in management of disorders associated with menstruation

Asparagus racemosus has been used to alleviate menstrual disorders including: pre-menstrual syndrome, amenorrhea, menorrhagia (excess), irregular bleeding, disorders associated with menopause and dysmenorrhea (Kinage and Chaudhari, 2016). Nomina and Paramkush, (2012) reported that *Asparagus* together with another herbal compound Yashtimadhu was effective in reducing the amount of bleeding, duration of bleeding and inter-menstrual bleeding. Due to its known source of phytoestrogens that mimic estrogenic hormones, *Asparagus racemosus* has also been used to minimize adverse symptoms of menopause (Khulbe, 2015). The cause of primary dysmenorrhea is not clearly defined but theories suggest that it could be due to elevated secretion of prostaglandins especially PGF2α that stimulate the uterine myometrium to cause contractions; estrogen/progesterone imbalance; acute anteflexion of the uterus and hypersensitivity of

individual to pain. Prostaglandins over production due to estrogen progesterone imbalance is thought to be the major cause of primary dysmenorrhea (Iacovides *et al*., 2015; Dawood, 2006). Decrease in levels of progesterone hormone acts as a precursor for increased prostaglandin production ((Iacovides *et al*., 2015). Regression of the corpus luteum during the luteal phase removes the stabilizing effect on the endometrial lysosomes (Iacovides *et al*., 2015). There is production of Phospholipase A2 that promotes production of arachidonic acid from the cell membrane phospholipids. The arachidonic acid is converted to cyclic endoperoxidases leading to release of prostaglandin E2 (PGE2) and prostaglandin F2alpha (PGF2α) that mediate myometrial contractions, blood vessel vasoconstriction and hypersensitivity of the nerve fibers to pain (Iacovides *et al*., 2015).

PGF2α levels in the uterine muscle correlate positively with the estradiol levels and negatively with the progesterone levels. Increased levels of estradiol increase PGF2α synthesis and secretion causing uterine muscle spasms hence dysmenorrhea (Xue *et al*., 2014)). High levels of progesterone causes an antagonist reaction. Progesterone has the ability to convert estradiol into low active estrone, causing less production of prostaglandin which helps alleviate dysmenorrhea (Fang *et al*., 2017).

Alleviating the above discomforts by *Asparagus racemosus* is not clear and the literature is scanty. An isolated review by Shaha and Bellankimath (2017) for instance suggests that it is the saponins in asparagus that counteracts the effect of prostaglandins thereby stopping the contractions and subsequent pain. Conventional treatments are mainly analgesics like ibuprofen which are prostaglandin inhibitors but have adverse effects on the user.

2.5. Other physiological supportive effects of *Asparagus racemosus*

2.5.1 *Asparagus racemosus* used to enhance fertility

Asparagus racemosus and asparagus containing preparations are reported to increase the weight of accessory glands in female (Siriwardene *et al*., 2010). Histological study on ovaries of immature female rats showed that *Asparagus racemosus* may increase ovary weight in young females and enhance folliculogenesis and ovulation (Arali and Prasad, 2016). It is also reported to support deeper tissue and buildup of blood; prepare the womb for conception; as a postpartum tonic to normalize and repair prolapsed uterus (Kinage and Chaudhari, 2016; Sahrawat *et al*., 2014). Presence of various phytochemical compounds in *Asparagus racemosus* root extract is said to be the reason for its use as a potent female tonic. The active compounds majorly in the root are the steroidal saponins (Mishra *et al*., 2013).

2.5.2 *Asparagus racemosus* as an anti-arbotifacient

Asparagus racemosus has been used as an anti-arbotifacient (Khulbe 2015) since it contains saponin glycoside A4 that produces specific and competitive blockage to pitocin induced contraction thereby reducing spontaneous uterine contraction (Kinage and Chaudhari., 2016).

2.5.3 *Asparagus racemosus* as a galactogogue

Asparagus racemosus extract increases milk production in women by enhancing the development of the mammary gland alveolar tissue (Shaha and Bellan, 2017). Singh and Sinha, (2015) evaluated the effect of *Asparagus racemosus* in lactating mothers and found that levels of prolactin were three times higher than the controls.

2.6 Raman Spectroscopy

Spectroscopy is the use of absorption, emission or scattering of electromagnetic radiation by matter to qualitatively or quantitavely study matter (atoms, molecules, atomic or molecular ions

or solids) (Kalantri *et al*., 2010). Raman spectroscopy for years has been established as a spectroscopic technique for analysis of molecular materials of all types quantitatively. The technique is a non-contact characterization that does not require sample preparation. Raman measurement entails directing a focused laser onto the sample and recording the energy profile of the light scattered. The various compounds produce a unique spectrum due to excitation of the vibrational modes of the molecule. The conventional methods used in hormonal analysis though highly sensitive have limitations (Geisler *et al*., 2000). Some of these methods like enzyme-linked immunosorbent assay, high pressure liquid chromatography and radioimmunoassay all require sample pre-treatments like preparation of complex antibodies and blurring in specific and non-specific recognition boundaries which maybe complicated and costly (De meulenaer *et al*., 2002; Geisler *et al*., 2000). Thus uncomplicated, rapid, highly sensitive, less costly and selective detection method, the Raman spectroscopy, has been employed and has become a top priority for researchers (Liu *et al*., 2018).

Raman spectroscopy is a very sensitive and molecular specific technique, often used in combination with Fourier transform Infrared (FT-IR) spectroscopic technique for a complete sample spectral profiling. Spectral profiles of the sample depicting peaks and valleys of different rotational and vibrational transitions are obtained with Raman spectroscopy. The available Raman spectrometer can be fed with one of the two monochromatic lasers either 532nm or 785nm. A beam of red or green laser light is delivered to Raman through an optical fiber that gives the beam total internal reflection. Depending on the laser being used, the shutter delivers the beam to the bandpass filter. The bandpass filter then directs the light beam to the beam splitter which splits the beam into two equal portions. Fifty percent of the beam is reflected and the other 50% goes to the sample to enable scattering which is measured by use of imaging

spectrometer and CCD camera. The Spectrometer operates with 1800, 1200 and 600 BLZ grating. It is equipped with a backscattered illuminated CCD camera for acquiring the spectral profiles of the samples being analyzed. The measurements are taken in the darkroom hence minimal fluorescence from the background light. Sample focusing is done using a motorized stage, computer with STR software, and a microscope of various objective lens x10, x20, x50 and x100 for visualization. The motor-driven stage scans through the sample to collect data at different spots of the sample.

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Study area

The study was carried out in Olenguruone area in Nakuru County, Kenya. The County stretches an area of 7462 km^2, with a population of about 1, 603, 325 with 9 sub counties as per the Kenya Population and Housing Census (2009). Olenguruone is a division in Kuresoi Sub County with a population of about 32,030 according to the Kenya National Bureau of Statistics (2010). It is bordered to the east by Mau forest and to the west by Mau national reserve. It lies in Latitude of -0.5833^0 and longitude of 35.6833^0. The study area was chosen because *Asparagus racemosus* is widely used traditionally to treat dysmenorrhea. This study is part of an on-going project at the School of Pharmacy, University of Nairobi.

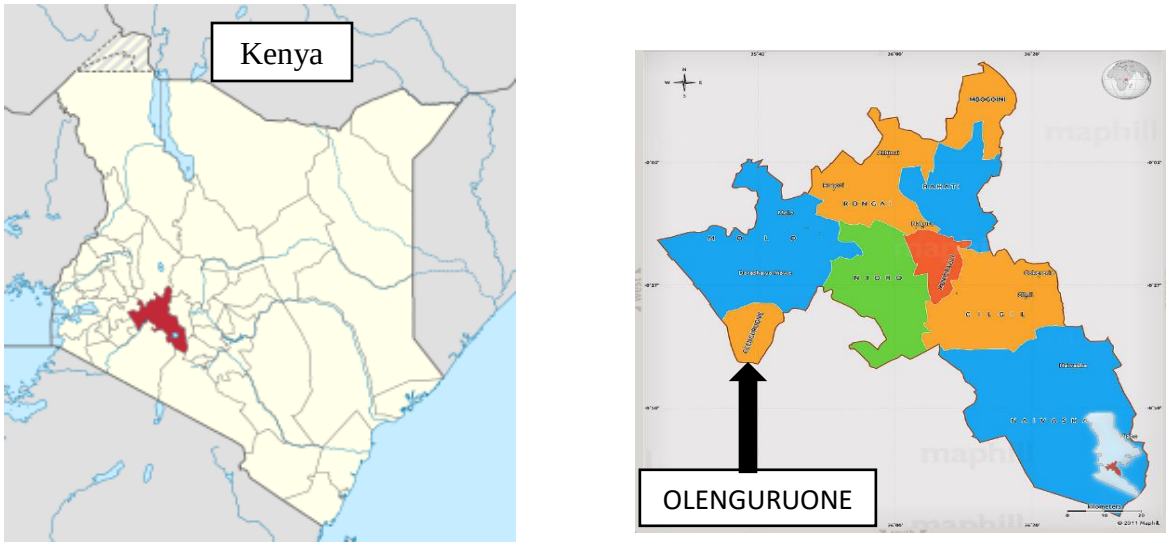


Figure 2: Map of Olenguruone division in Nakuru County on map of Kenya (http://www.maphill.com/kenya/rift-valley/nakuru/olenguruone/location © 2011)

3.2 Plant collection, preparation and extraction

3.2.1 Plant collection and preparation

The plant roots were harvested and transported to the laboratory in the Department of Veterinary Anatomy and Physiology (DVAP), University of Nairobi. The roots were washed and allowed to dry under the shade for two weeks. They were chopped into smaller pieces using a knife, ground into fine powder using Cunningham Grinder and packaged in 200g sachets and stored in clean airtight containers. To minimize degradation and loss of moisture, the powder was stored in a dark area until needed. The plant was also brought to School of Biological Sciences (University of Nairobi) for botanical identification. Voucher specimen number (RI2019/08) was obtained for future reference.

3.2.2 Aqueous extraction

Extraction was done following a method used by Chitme *et al*., (2009) by placing 400g of the root powder in a volumetric flask and two litres of distilled water added. The mixture was stirred at room temperature and left to soak for 48 hours. Subsequent filtration using cotton wool was done until fine filtrate was obtained. The filtrate was freeze dried for 48hours to obtain the fine powder. The powder was then weighed to determine the percentage dry yield. The yield was recorded (20 grams) and stored in a refrigerator.

3.3 *Asparagus racemosus* aqueous root extract phytochemical screening

Kenyan *Asparagus racemosus* phytochemical screening was carried out following the method used by Periyasamy and Mahalingam (2010) to determine whether saponins, alkaloids, flavonoids, tannins, terpenoids and glycosides were present.

3.3.1 Test for saponins (foam test)

Fifty milligrams of *Asparagus racemosus* root extract powder was added to 20 ml of distilled water and thoroughly shaken in a graduated cylinder for 15 minutes. The mixture was then observed for foam formation of 1cm that persisted for 15 minutes (Tiwari *et al*., 2011).

3.3.2 Test for alkaloids

1ml of Mayer's reagent was added to one gram *Asparagus racemosus* root extract powder placed in a test tube. The set up was observed for the appearance of white creamy precipitate (Banu and Cathrine, 2015).

3.3.3 Test for flavonoids

One gram of *Asparagus racemosus* aqueous root extract was added to a solution of ammonium hydroxide (10%). Appearance of yellow colour would indicate presence of flavonoids (Banu and Cathrine, 2015).

3.3.4 Test for tannins (ferric chloride test)

Fifty milligrams of *Asparagus racemosus* root powder was dissolved in 5ml distilled water. Three drops of neutral 5% ferric chloride solution were then added. A dark green colour would indicate presence of tannins (Banu and Cathrine, 2015).

3.3.5 Test for terpenoids -Liebermann-Burchard Test

5 ml of distilled water was added to 50 milligrams of *Asparagus racemosus* root powder. One ml of the resulting solution was then mixed with 2ml of chloroform and filtered. To the filtrate, two ml acetic anhydride was added boiled and then cooled. Upon cooling, 2ml of concentrated Sulphuric acid was added. Formation of a brown ring at the junction would indicate presence of terpenoids (Tiwari *et al*., 2011).

3.3.6 Test for cardiac glycosides-Keller-Killian test

One ml of ferric chloride (3.5%) in acetic acid was added to one ml of *Asparagus racemosus* prepared by adding 50 milligrams of the powder in 5ml of distilled water. This was followed by drop wise addition of 1.5 ml concentrated sulphuric acid. A brown ring forming at the interface due to presence of de-oxy sugar was an indication of cardenolides. A pale green color in upper layer as a result of steroid nucleus indicated cardiac glycosides present (Wangia *et al*., 2017).

3.4 Experimental animals and welfare

Female Wistar rats were obtained from Biochemistry Department, University of Nairobi. The rats were transported to the Department of VAP animal house where they were housed. The beddings were wood shavings that were replaced every other day. The animals were maintained under standard environmental conditions of 12 hour light and 12 hour dark cycles, temperatures of 24-25° C. They were provided with water *ad-libitum* and adequate accessible rat-pellets from Bellmill feeds limited (Kenya). The animal house was kept clean, dry and well ventilated. Before study commencement, Animal welfare and Ethics clearance was sought from the Faculty of Veterinary Medicine Biosafety and Animal Ethics committee.

3.5 Experimental design

3.5.1 Acute oral toxicity study

A total of 12 nulliparous non pregnant female rats weighing between 180-220 g were used for this study. Three rats were assigned to each dose level according to the OECD guideline 423. (The number of rats that died at each dose level determined if further testing was required by either repeating with 3 additional animals at the same dose level or three at lower dose level). The rats were weighed and fasted overnight but had *ad-libitum* access to water. Just before administration of the extract the rats were weighed and values recorded. Through abdominal

gavage, three animals were administered with 300mg/kg body weight dose level of *Asparagus racemosus* aqueous extract (start dose level according to OECD). The rats were not allowed access to rat pellets for the next 4 hours of intense observation. The rats were observed for clinical symptoms of toxicity such as tremors, lacrimation, diarrhea, convulsions or death. The rats were then examined daily for 14 days according to OECD guidelines. Their weights were recorded on days 1, 7 and 14. Three rats (control) were fasted overnight but only received 0.5ml physiological saline. The control rats were also weighed on days 1, 7 and 14 just before humane sacrifice of all rats was undertaken. If none of the rats died upon receiving 300 mg/kg of the plant extract, same procedure was repeated at 2000 mg/ kg. If all 3 rats survived at 2000 mg/kg exposure a limit test was carried out at 5000 mg/ kg using 3 additional rats.

3.5.2 Effect of the *Asparagus racemosus* aqueous extract on estrus cyclicity, reproductive hormones, mating success, gestation length and litter size

3.5.2.1 Effect of extract on estrus cyclicity

Twenty nulliparous non pregnant normal cyclic female Wistar rats weighing between 240-280 grams were used for the study. Vaginal flushing was done daily using physiological saline between 9-10 am for 20 days. Approximately 0.5ml of vaginal fluid from each rat vaginal wash was placed on a clean microscope slide and observed under light microscope at x100 magnification. The estrus cycle stages were differentiated according to their epithelial cytological features as either proestrus, estrus, metestrus or diestrus. Only rats with regular estrus cycles were used. On day 20, twenty normal cyclic rats were randomly picked and caged in 4 groups each with five animals. Group 1 received oral gavage of high dose (600 mg/kg) of *Asparagus racemosus* root extract, group II received low dose (300 mg/kg) of the plant extract, group III received 20 mg/kg body weight of ibuprofen (positive control) and group IV received

0.5ml of physiological saline (negative control). The animals received the treatments every other day from 9-10am for 14 days. Vaginal flush was done daily between 8 to 9 am for all the rats and estrus stages recorded.

3.5.2.2 Effects of *Asparagus racemosus* extract on reproductive hormones

A drop of blood (approximately 50 µl) from the tail tip was collected daily from all rats for 14 days and placed on a microscope slide. The microscope glass slide was initially prepared by making 5 patches of the silver paste (SPI supplies, USA) using a brush and allowing it to dry for 20 minutes. The conductive silver paste was composed of silver nanoparticles: 35-60% per weight; 1-methoxy-2 propanol acetate: 10-30% weight; batyl acetate: 10-30% weight and acrylic resin: 5-10% weight. Blood (≈50µL) was obtained from the tail tip of the rat by first cleaning with cotton wool soaked with warm ethanol and a small cut made over the tail vein using a sterilized surgical blade then squeezed gently.

Figure 3: Figure showing blood smear on the silver paste patch

Blood was then smeared on the silver paste patch (Figure 3) and allowed to dry for about 30 min, then taken for analysis using the Raman spectroscopy. To obtain the hormonal profiles, confocal

laser Raman microscope system (STR, Seki Technotron Corp) equipped with a 785 nm laser (produces less fluorescence in the spectrum) together with a spectrometer (Princeton Instruments) was used. The experimental specifications used included: excitation wavelength of 785 nm, beam spot size 68.47μm, the excitation power of 18.55mW, the number of accumulations was 5, exposure time of 10 seconds, x 10 microscope objective lens with 0.3 numerical aperture and 26.5 f-numbers. A grating of 600BLZ-750nm and the center wavelength of 855.20nm was used so as to cover a wider spectral region. Spectrometer was calibrated using the strong Raman peak of silicon wafer and the peak value was 520.86nm before each sample analysis. From each of the five spots of the sample, five spectra were collected. The obtained Raman spectra for all samples were then smothered and any auto-fluorescence background removed using Vancouver Raman Algorithm. Data was then normalized and plotted using ORIGIN 2015 software. The same procedure was repeated for the standard hormones (progesterone, 17β-estradiol, follicle stimulating hormone and luteinizing hormone) from NovaTech Immundiagnostica GmbH-Technologie to obtain their spectra.

Figure 4: The Raman spectrometer set up (Seki Technotron Corp).

3.5.2.3 Effects of *Asparagus racemosus* extract on mating success, gestation length and litter size.

After 14 days of extract administration as described in section 3.5.2.1, three males were introduced into each cage. Vaginal flushing and microscopy was done daily between 8-9 am to determine presence or absence of spermatozoa. Mating success was ascertained by confirming the presence of spermatozoa. The day spermatozoa was seen was recorded as day 1 of pregnancy and the female was put in a separate cage. The females were monitored and weighed daily until they littered. The litter size (number of pups) and gestational length (days) for all rats was recorded.

3.6 Effect of *Asparagus racemosus* extract on uterine contractility

Ten nulliparous non pregnant female rats 4 months old were used for the study. All rats received 2 mg/kg b/w intra peritoneal stilboestral injection 24 hours before the onset of the experiment to enhance sensitivity of the uterus. The rats were humanely sacrificed using di-ethyl ether 24 hours after the injection. Immediately thereafter, the uterine horns were carefully harvested and excised of connective tissues. Uterine horns were placed in a dish of de Jalon's solution. De Jalon solution was composed of NaCl (9 g/litre), $NaHCO_3$ (2.1 g/l), Glucose (0.5 g/l), KCl (0.402 g/l), $CaCl_2$ (0.24 g/l), Sucrose (4.5g/l), NaH_2PO4 (0.142g/l). The de Jalon solution was maintained at 35± 0.5^0C and incessantly bubbled with 95% oxygen and 5% carbon dioxide mixture. A uterine strip of 2cm in length was cut and mounted vertically within the organ bath with one end fixed and the other one attached to an isotonic force transducer (ML500/A, AD Instrument) coupled with Power Lab data acquisition system (Power Lab 8/30). The force of contraction (grams) and frequency of contraction (beats per minute) were obtained and analyzed using Chart5 software for windows.

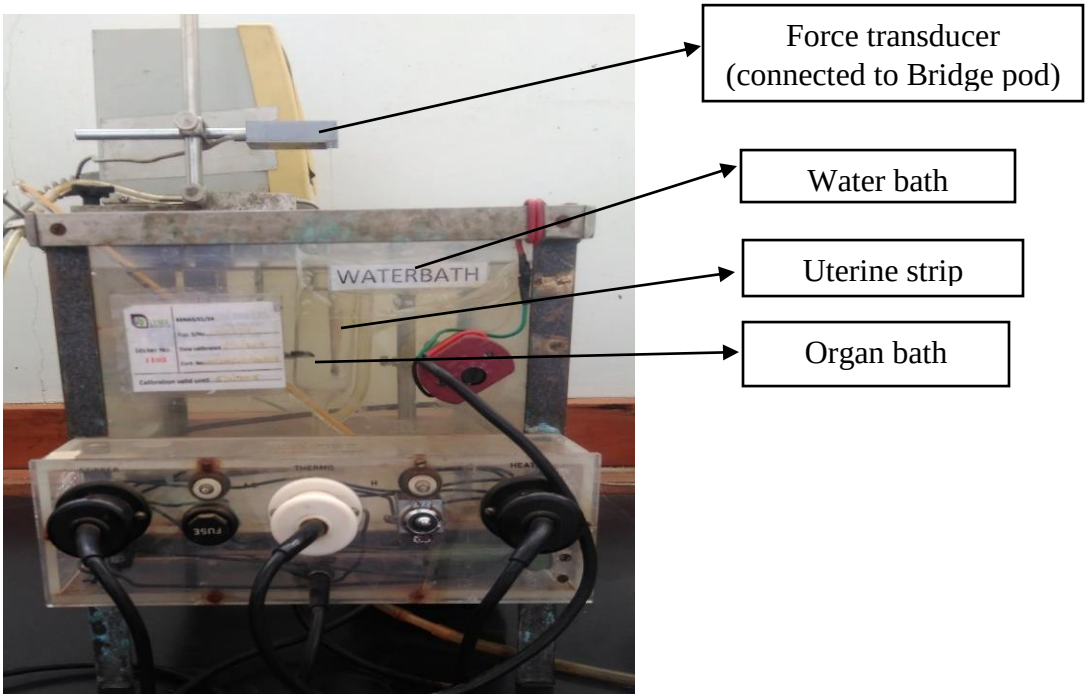


Figure 5: The organ bath set up and its associated components

One uterine strip at a time was mounted in the organ bath and after stabilizing for 20minutes, negative control readings were recorded for 10 minutes. The extract concentrations were prepared: 20 mg/ml by adding 0.02g *Asparagus racemosus* root extract powder to 1ml distilled water, 40mg/ml by adding 0.04g , 80 mg/ml by adding 0.08g and 160 mg/ml by adding 0.16g of the *Asparagus racemosus* root extract powder to 1ml distilled water. The strip was exposed to various concentrations of the plant extract beginning with the lowest concentration (20 mg/ml). The uterine strip was washed 3 times with de Jalon solution and allowed 20min rest before exposure to the next concentration (40 mg/ml) of plant extract and contractions recorded for 10 min. This was repeated until the strip was exposed to all the extract organ bath concentrations (20, 40, 80, 160 mg/ml). The procedure was repeated six times using fresh uterine strips. The effect of extract concentrations on the frequency of uterine contraction as well as contraction strength were noted. The frequency was given by the event counts determined by the number of peaks in the selected region. The amplitude was given by the means of the data points in the

selected region over a period of 10 minutes. The treatment readings were compared with the control readings for any significant difference.

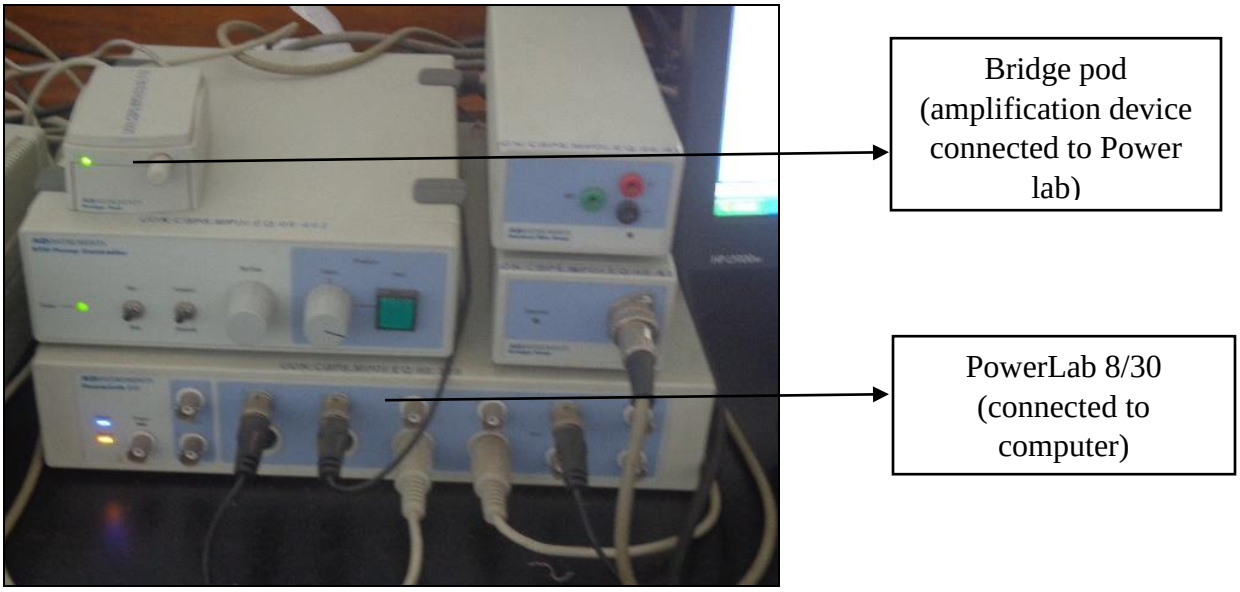


Figure 6: The PowerLab data acquisition system (AD Instrument)

3.7 Data analysis

Data collected was entered into MS- excel data sheet and cleaned to remove any incorrect and incomplete data set. The results were recorded as Mean ± SEM. SPSS was used to analyze the data and graphs were produced using Graphpad Prism version 8. Descriptive statistics was carried out on the outcome variables. One-way ANOVA followed by Bonferroni post hoc test were used to compare the outcomes between groups. P-values ($P < 0.05$) were considered significant.

CHAPTER FOUR

4.0 RESULTS

4.1 Phytochemical compounds of Kenyan *Asparagus racemosus* root extract

Phytochemical screening of Kenyan *Asparagus racemosus* root extract displayed presence of saponins, flavonoids, terpenoids and glycosides. Alkaloids and tannins were absent (Table 1).

Table 1: Phytochemical compounds of Kenyan *Asparagus racemosus* aqueous root extract

+ shows presence of the phytochemical compound, - shows absence of the phytochemical compound

	Saponins	Alkaloids	Flavonoids	Tannins	Terpenoids	Glycosides
Asparagus racemosus	+	-	+	-	+	+

4.2 Acute oral toxicity study

Asparagus racemosus aqueous root extract intra-abdominal gavage did not cause toxic effect on the rats. The animals had minimal activity during the first 1 hour but resumed normalcy within four hours of observation. No signs of toxic effect like restlessness, diarrhea, reduced behavioral activity, increased respiratory rate and labored breathing or death was recorded for all the dose levels, through the 14 days of observation. Even at the highest dose level of 5000 mg/kg body weight, the animals did not show any effects of toxicity of the plant extract. The rats resumed normal activity after dose extract administration and the extract was ruled nontoxic. There was no significant difference in the weights of the animals in the treated groups compared to the control group ($P > 0.05$). All test animals together with the control animals gained weight showing no adverse effect. Therefore, from these results working dose of 600 mg/kg body

weight was taken as the high dose and 300 mg/kg body weight as the low dose level for the study.

Table 2: Animal body weights for the control group and *Asparagus racemosus* treatment groups.

Asparagus racemosus (mg/kg)	Fasting day	Day 0	Day 1	Day 7	Day 14
Control	227.33 ± 4.92	213.63 ± 2.66	225.07 ± 3.88	235.77 ± 4.57	241.93 ± 5.32
300	231.30 ± 7.67	221.33 ± 6.97	223.63 ± 5.79	232.57 ± 7.56	238.33 ± 10.3
2000	241.07 ± 14.51	226.53 ± 11.9	238.97 ± 16.18	251.76 ± 13.3	257.50 ± 13.3
5000	244.43 ± 3.34	230.2 ± 4.07	247.03 ± 5.90	257.27 ± 6.5	263.4 ± 5.67
P-value	0.505	0.458	0.293	0.200	0.245

Table 2 shows the Mean ± SEM of animal body weights for the treatment groups and the negative control. P value ($P > 0.05$) showing no significant difference in weights due to *Asparagus racemosus* at the various dose levels; 300, 2000, 5000 mg/kg body weight for the 14 days of observation compared to the control group. *Asparagus racemosus* did not cause any mortality even at the limit dose of 5000 mg/kg b/w.

4.3 Effect of *Asparagus racemosus* root extract on cyclicity, mating success, gestation length and litter size

4.3.1 Effect on estrus cyclicity

Irregular pattern of estrus cycle was observed in animals treated with the high dose (600 mg/kg) and the low dose (300 mg/kg) body weight of *Asparagus racemosus* root extract compared to the

negative control. The proestrus phase was more frequent than metestrus and diestrus in the treatment groups compared to the control group (Table 3, 5 and 6). There was a significant difference ($P < 0.05$) in the frequency of proestrus phase in the groups treated with the plant extract compared to control groups. Metestrus and diestrus phases also had a significant difference in the treatment groups compared to control groups ($P < 0.05$). There was no significant difference in the estrus phase in the treatment groups compared to the control group. The animals treated with ibuprofen had prolonged proestrus and shorter metestrus and diestrus phase (Table 4). The negative control that received 0.5 ml physiological saline showed regular estrus cyclicity (Table 3).

Table 3: Frequency of appearance of each estrus cycle phase over a 14 day period for the negative control group.

	Estrus cyclicity pattern for negative control group					
	Rat 1	Rat 2	Rat 3	Rat 4	Rat 5	**Mean no of days**
Proestrus	4	3	3	3	5	3.6
Estrus	3	4	3	3	4	3.4
Metestrus	4	3	4	4	2	3.4
Diestrus	3	4	4	4	3	3.6

Table 3 shows estrus cycles pattern for the negative control rats over the 14 day treatment period using physiological saline.

Table 4: Frequency of appearance of each estrus cycle phase over a 14 day period after administration of ibuprofen 20 mg/kg body weight.

	Estrus cyclicity pattern for ibuprofen treated rats					
	Rat 1	Rat 2	Rat 3	Rat 4	Rat 5	**Mean no. of days**
Proestrus	6	6	6	6	7	**6.2****
Estrus	4	3	3	4	3	**3.6**
Metestrus	2	3	2	2	2	**2.0**
Diestrus	2	3	3	2	2	**2.4***

Table 4 shows frequency of appearance of estrus cycle phase over a 14 day period after rats were administered with 20 mg/kg b/w of Ibuprofen as positive control. The appearance frequency of proestrus phase was significantly increased compared to the control. *P < 0.05, **P < 0.01.

Table 5: Frequency of appearance of each estrus cycle phase over a 14 day period after administration of *Asparagus racemosus* (300 mg/kg) body weight.

	***Asparagus racemosus* 300 mg/kg estrus cyclicity pattern**					
	Rat 1	Rat 2	Rat 3	Rat 4	Rat 5	**Mean no of days**
Proestrus	6	3	6	6	6	**5.4***
Estrus	4	4	4	4	3	**3.8**
Metestrus	2	2	1	1	3	**1.8***
Diestrus	2	4	3	3	2	**2.8**

Table 5 shows frequency of appearance of each stage of estrus cycle over 14 day treatment with *Asparagus racemosus* at 300 mg/kg body weight. The appearance frequency of proestrus and estrus phases were significantly more compared to the control group. There was subsequent

reduced appearance frequency of metestrus and diestrus phases compared to the negative control group. *P < 0.05

Table 6: Frequency of appearance of each estrus cycle phase over a 14 day period after administration of *Asparagus racemosus* (600 mg/kg) body weight.

	Estrus cyclicity pattern for 600 mg/kg *Asparagus racemosus*					
	Rat 1	Rat 2	Rat 3	Rat 4	Rat 5	**Mean no. of days**
Proestrus	6	5	6	6	7	**6****
Estrus	4	5	4	4	3	**4**
Metestrus	1	3	1	1	1	**1.4****
Diestrus	3	2	3	3	3	**2.8**

Table 6 shows frequency of appearance of each estrus cycle phase over a period of 14 day treatment with 600 mg/kg body weight *Asparagus racemosus*. The appearance frequency of proestrus and estrus phases were significantly increased compared to the control group. There was subsequent reduced appearance frequency of the metestrus and diestrus phase compared to the control group. **P < 0.01

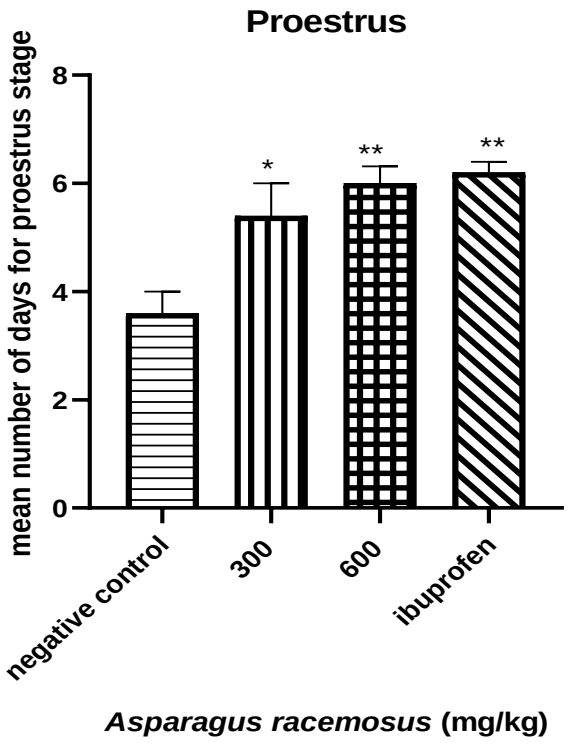


Figure 7: *Asparagus racemosus* effect on proestrus phase of the estrus cycle

The data is presented as mean ± SEM. *P < 0.05, **P < 0.01, ***P < 0.001. There was increased appearance frequency of proestrus in the treatment groups compared to negative control within the 14 day observation period (P < 0.001).

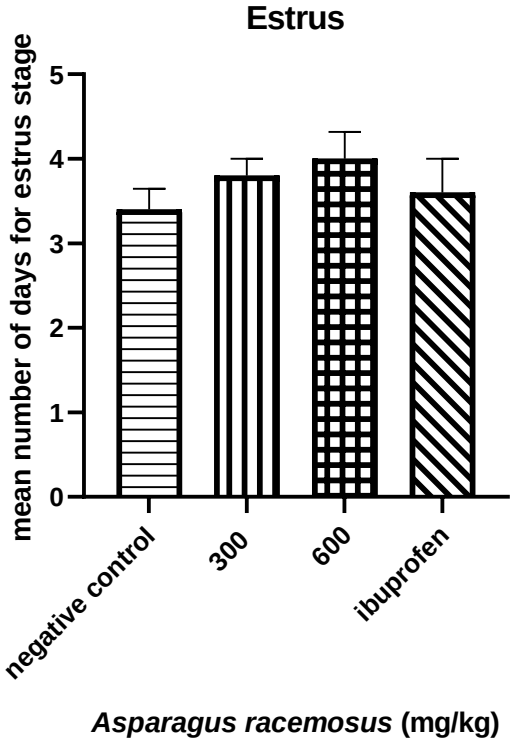


Figure 8: *Asparagus racemosus* effect on estrus phase of the estrus cycle.

The data is presented as mean ± SEM. There was non-significant disruption ($P > 0.05$) in appearance frequency of estrus phase of the estrus cycle at 300 and 600 mg/kg *Asparagus racemosus* compared to both the negative and positive control within the 14 day observation period.

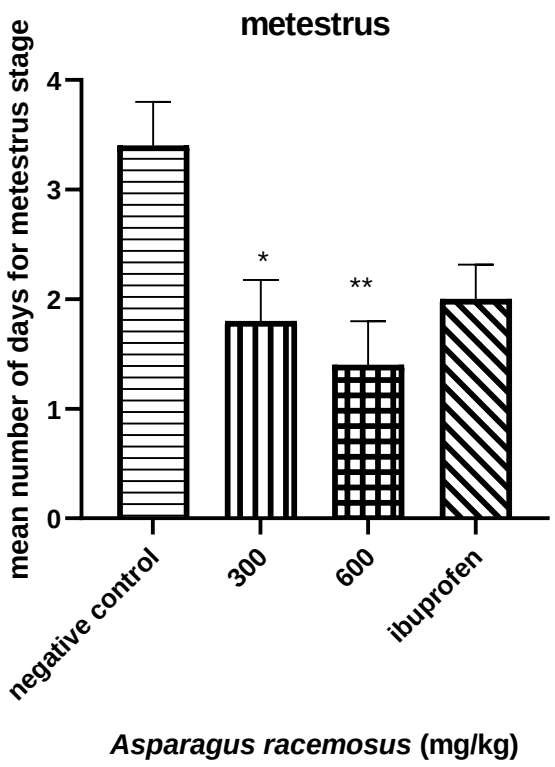


Figure 9: *Asparagus racemosus* effect on metestrus phase of the estrus cycle.

The data is presented as mean ± SEM. The frequency of appearance of metestrus decreased significantly at 300 (*P < 0.05) and 600 (**P < 0.01) *Asparagus racemosus* compared to the negative control group within the 14 day observation period.

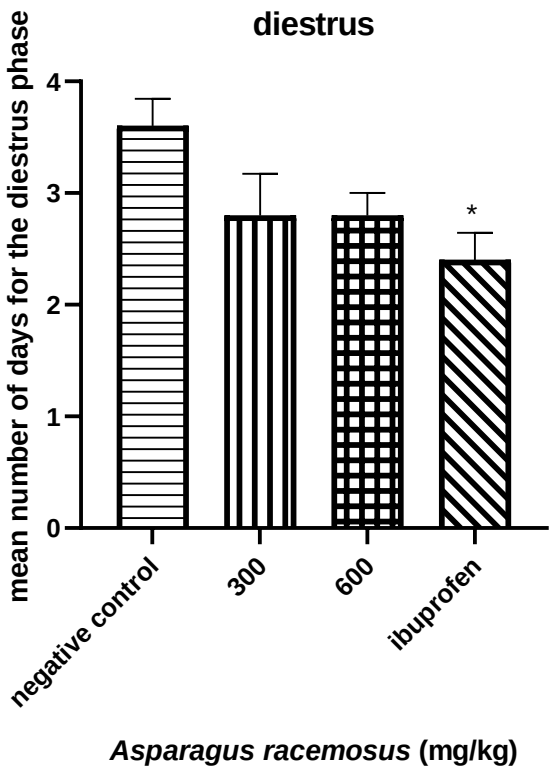


Figure 10: *Asparagus racemosus* effect on diestrus phase of the estrus cycle.

The data is presented as mean ± SEM. There was non-significant decreased appearance frequency of diestrus in the extract treated group compared to negative control group within the 14 day observation period. Ibuprofen caused a significant decrease in diestrus phase (*$P < 0.05$).

Table 7: Mean ± SEM for various phases of estrus cycle over a period of 14 days.

Treatment	Proestrus	Estrus	Metestrus	Diestrus
Negative control	3.60 ± 0.4	3.4 ± 0.24	3.4 ± 0.4	3.6 ± 0.24
600 mg/kg ASP	6.00 ± 0.32	4.00 ± 0.32	1.4 ± 0.4	2.8 ± 0.2
300 mg/kg ASP	5.40 ± 0.6	3.8 ± 0.2	1.8 ± 0.37	2.8 ± 0.37
Positive control	6.20 ± 0.2	3.60 ± 0.4	2.00 ± 0.32	2.40 ± 0.24
P-value	0.001***	0.543	0.009*	0.044*

Table 7 shows significant difference in appearance frequency of proestrus phase in the treatment groups compared to the negative control ($P < 0.001^{***}$). The estrus phase had no significant difference in the treatment group compared to negative control ($P > 0.05$). There was however a significant difference in the appearance frequency of metestrus and diestrus phase in the treatment compared to the negative control groups ($P < 0.01^{**}$ and $P < 0.05$*) respectively.

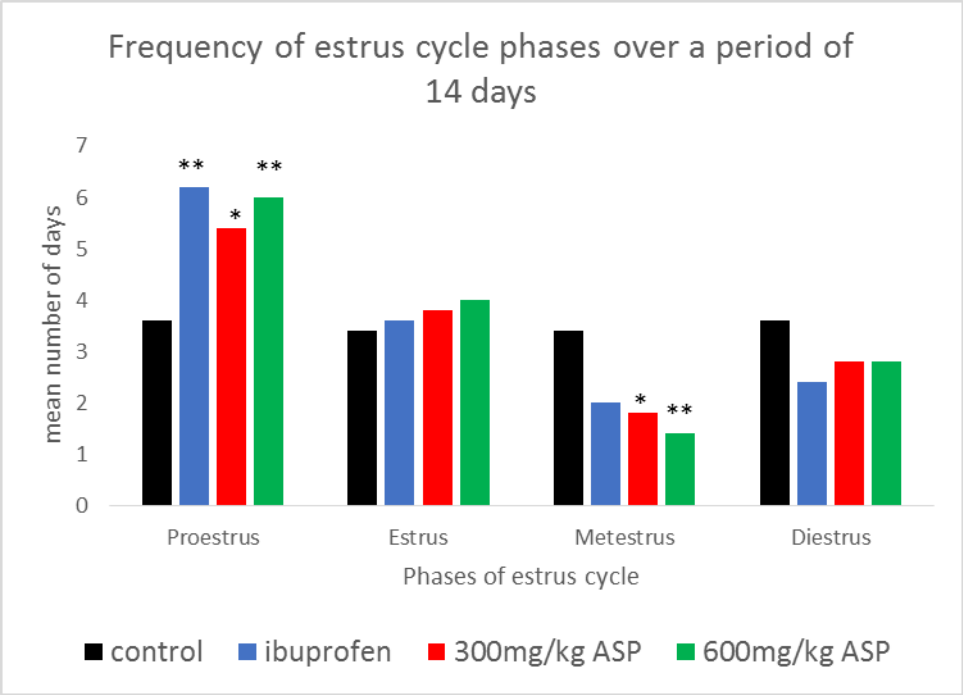


Figure 11: Effect of *Asparagus racemosus* extract on appearance frequency of estrus cycle phases compared to ibuprofen and negative control treatment groups.

There was increased appearance frequency of proestrus in the treatment groups compared to negative control ($P < 0.001$). *Asparagus racemosus* at 300 and 600 mg/kg b/w caused a non-significant increase in appearance frequency of estrus phase compared to the negative control. There was a subsequent significant reduction in the appearance frequency of metestrus ($P < 0.01$) and diestrus ($P < 0.05$) phase in the *Asparagus racemosus* treated group compared to the negative control group. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

4.3.2 Effect on mating success, gestation length and litter size

Asparagus racemosus aqueous root extract at 600 mg/kg and 300 mg/kg body weight had non-significant effect on mating success (Table 9) compared to both controls. All treated rats mated successfully. *Asparagus racemosus* extract had no significant adverse effect on conception rate as all treated rats conceived (100%) compared to negative control at 80% and positive control

40% (Table 9). All treatment group rats littered. Eighty percent of the negative control group and 40 % of the positive control group littered. All *Asparagus racemosus* extract treated and the negative control group had regular gestation length (20 ± 1 days) compared to the positive control (23 days).

Table 8: Effect of *Asparagus racemosus* on mating success, gestation length and litter size

Treatment	Mating success (%)	Conception rate (%)	Gestation length (number) Mean ± SEM	Litter size (number) Mean ± SEM
Negative control	100%	80%	21.75 ± 0.25	9.75 ± 0.5
600 mg/kg b/w	100%	100%	21.80 ± 0.2	10.60 ± 0.4
300 mg/kg b/w	100%	100%	21.80 ± 0.2	9.80 ± 0.9
Positive control	100%	40%	23.00 ± 0.0	7.50 ± 1.5
P-value			0.026*	0.144

Table 8 shows effect of *Asparagus racemosus* extract on mating success, conception rate, gestation length and litter size. There was a non-significant difference of the gestation length in the treatment groups compared to the negative control. There was a non-significant difference in litter size between treatment group compared to the negative and positive control groups.

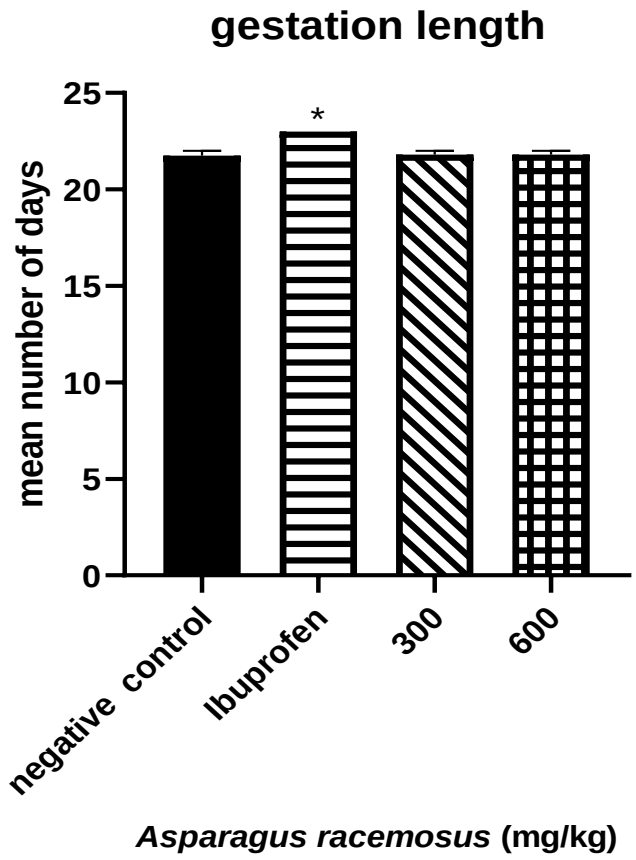


Figure 12: The effect of *Asparagus racemosus* extract on gestation length.

There was non-significant difference in gestation length in the extract treated groups compared to the negative control.

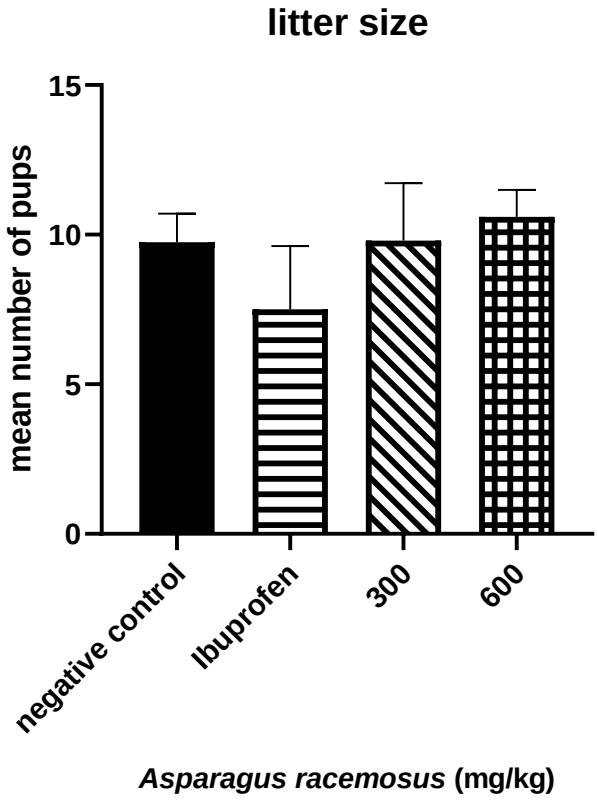


Figure 13: The effect of *Asparagus racemosus* extract on mean number of pups born.

600 mg/kg and 300 mg/kg of *Asparagus racemosus* extract resulted in a non-significant higher mean number of pups compared to the negative and the positive control groups.

4.4 Effect of *Asparagus racemosus* root extract on reproductive hormones

The bands from the animals treated with 600 mg/kg (high dose) of *Asparagus racemosus* aqueous root extract showed high intensity compared to those of the negative and positive controls.

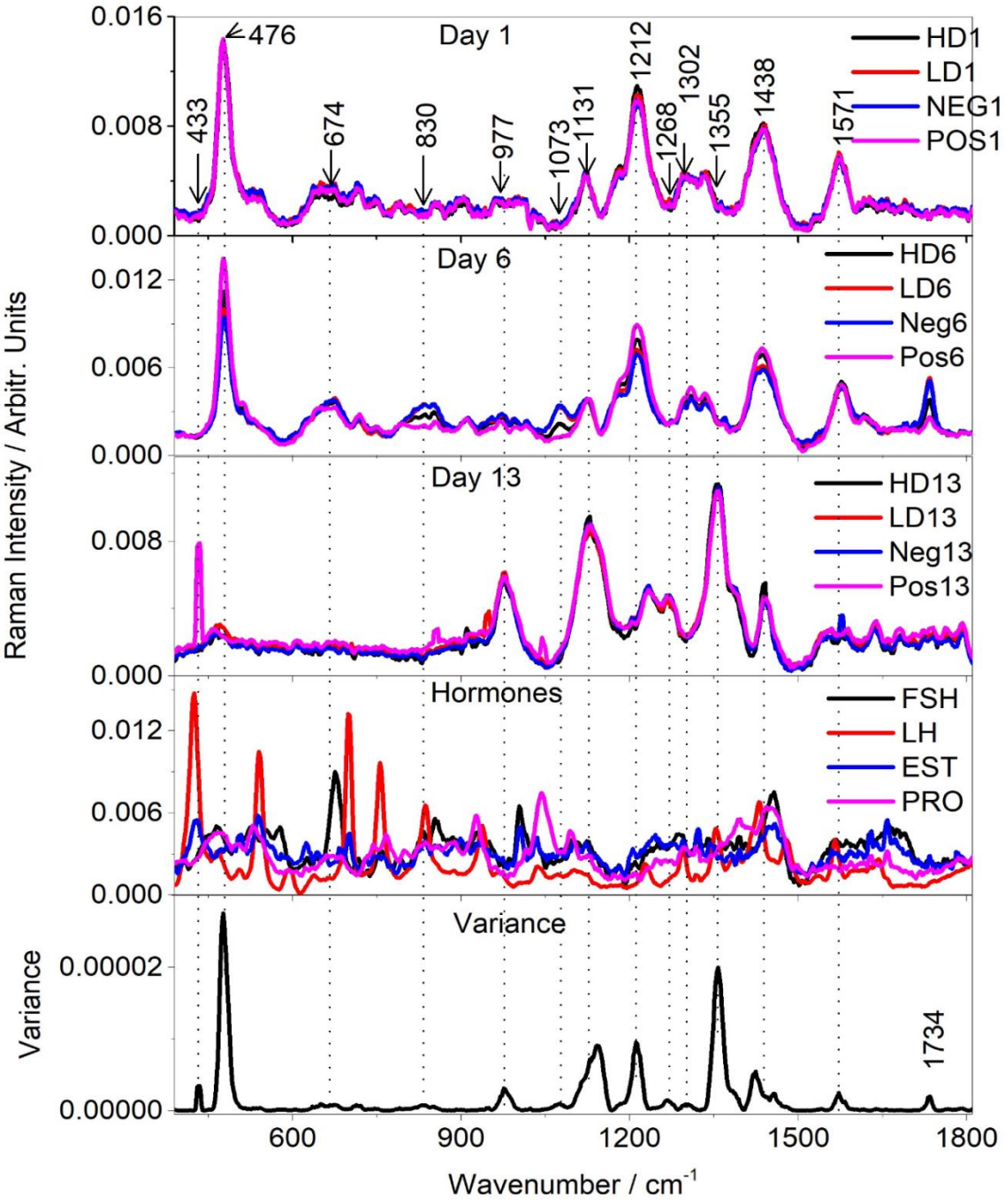


Figure 14: Raman spectra for day 1, 6 and 13; the spectrum for standard hormones and the variance of the mean across the treatment groups.

KEY: HD: high dose (600 mg/kg) *Asparagus racemosus* group; LD; low dose (300 mg/kg) *Asparagus racemosus* group; NEG: negative control (0.5 ml physiological saline) group; POS: positive control (20 mg/ml ibuprofen) group.

The spectra were analyzed for different days. The peaks show spectra for day 1, 6, 13, standard hormones, and spectrum for the variance of the mean across the treatment groups. Only the prominent peaks are shown (Figure 14).

From the spectra (Figure 14), day 1 shows no variations in the hormone intensity peaks in the treatment groups compared to the negative control and the ibuprofen treated group (positive control).

On day 6 there are significant variations in the level of hormones in the blood.

The 476 cm^{-1} peak was influenced by FSH and progesterone. There was high intensity in positive control group, followed by high dose, low dose and least effect in negative control group.

Peak 674 cm^{-1} was influenced by FSH and progesterone. The intensity was high in negative control, followed by low and high dose. With least effect seen in the positive control.

Peak 830 cm^{-1} was mainly influenced by LH and FSH. High hormone intensity was seen in the negative control, low and high dose. Least effect seen in the positive control.

Peak 1212 cm^{-1} (Figure 14) was influenced by estradiol, FSH and0 LH in the positive control, high, low dose and least in negative control.

Peak 1438 cm^{-1} (Figure 14) was influenced by LH and progesterone with a high hormone intensity in positive control, followed by high, low dose and least effect in the negative control. Peak 1734 cm^{-1} (Figure 14) was influenced by estradiol, FSH and progesterone by the low dose followed by negative control, high and least in positive control.

On day 13, the peak 433 cm^{-1} was influenced by LH in the positive control group.

Peak 476 cm^{-1} was influenced by hormone FSH and progesterone. The significant difference was caused by high and low dose of the extract.

Peak 1131 cm^{-1} (Figure 14) was influenced by hormone progesterone with high intensity caused by high and low dose of the extract compared to the controls.

Peak 1268 cm^{-1} (Figure 14) was influenced by hormone FSH and estradiol with high intensity caused by the high dose, low dose, negative and positive control.

Peak 1438 cm^{-1} (Figure 14) was influenced by hormone FSH, LH and progesterone. The significant difference in the intensity was due to high dose of the extract compared to the controls.

Peak 1355 cm^{-1} (Figure 14) was influenced by LH. The significant difference in the intensity was due to high and low dose extract compared to the control.

Table 9: Effect of *Asparagus racemosus* on intensity of hormone in the blood

Treatment	Intensity of hormone (FSH, LH, estradiol, progesterone)
Negative control 0.5ml physiological saline	31.04 ± 1.27
Positive control 20 mg/kg ibuprofen	38.45 ± 1.53
300 mg/kg *Asparagus racemosus*	31.31 ± 1.31
600 mg/kg *Asparagus racemosus*	39.49 ± 1.81
P value	0.0001***

Table 9 shows the intensity of *Asparagus racemosus* at 600 and 300 mg/kg on reproductive hormone levels compared to ibuprofen (positive control) and the negative control. *Asparagus racemosus* caused an increase in intensity dose dependently. There was a significant difference in the intensity (concentration) of hormone ($P < 0.001$***).

4.5 In vitro studies on isolated rat uterine contractility

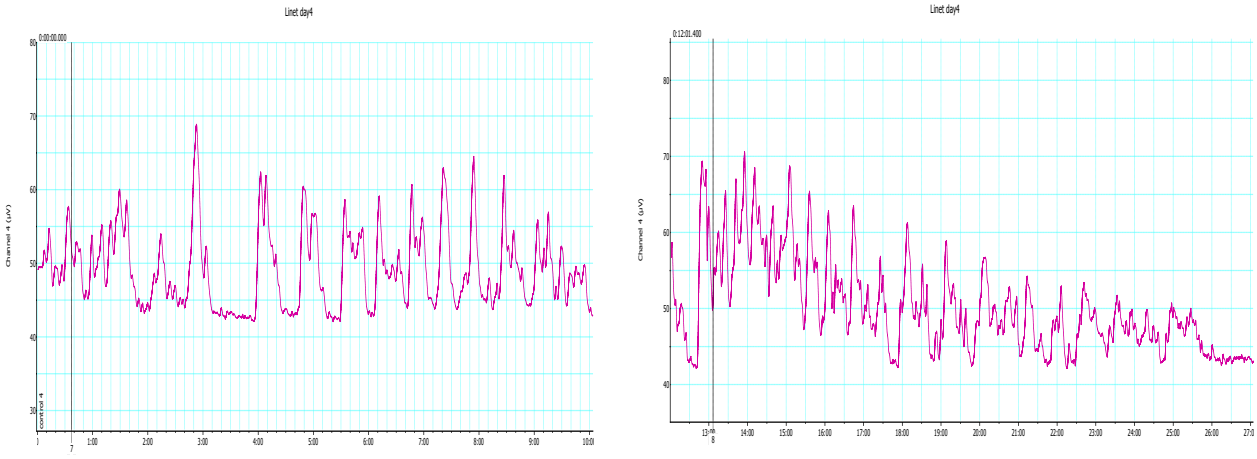
4.5.1 Force/ amplitude of contraction

Percentage contraction was obtained by the formula (% contraction = force of contraction after treatment - control contraction, divided by control contraction ×100).

4.5.1.1 Effect of *Asparagus racemosus* on force of uterine contraction

The tracings recorded before and after exposing the uterine strip to various *Asparagus racemosus* aqueous root extract concentration in the organ bath shows a decline in force of contraction (Figures 15-18). There was significant difference in force of contraction compared to the control ($P < 0.0001$) (Table 12). Figure 20 and Table 10 shows percentage decrease in force of

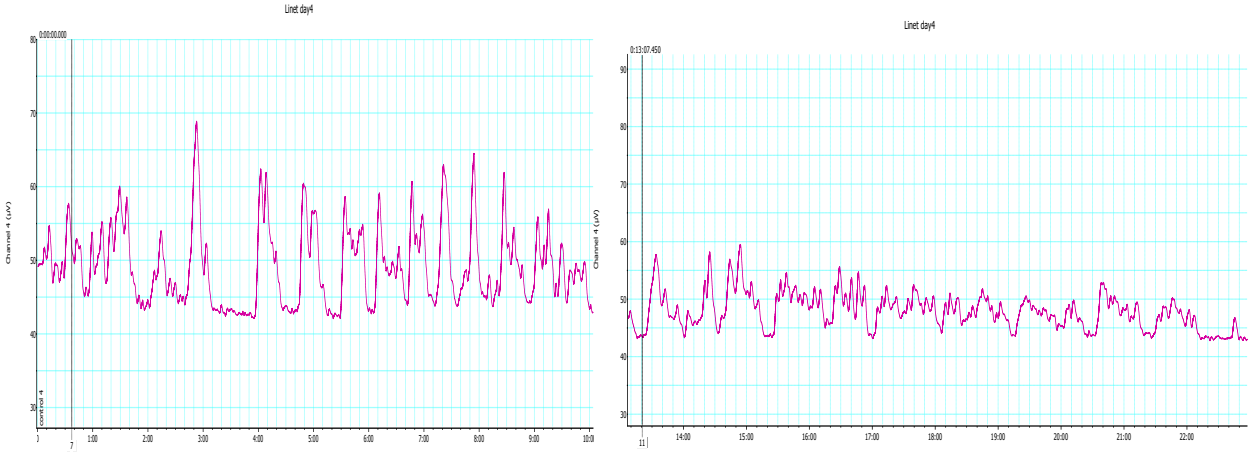
contraction. 20 mg/ml *Asparagus racemosus* root extract decreased the force of contraction by -0.146% (Table 10, Figure 20). 40 mg/ml of *Asparagus racemosus* decreased the force of contraction by -4.87 % (Table 10, Figure 20). 80 mg/ml *Asparagus racemosus* root extract concentration, decreased the percentage force of contraction by -7.97% (Figure 20). 160 mg/ml of *Asparagus racemosus* decreased the force of contraction by -19.55% (Figure 20). The results showed that *Asparagus racemosus* aqueous root extract caused a decrease in the force of contraction in the isolated rat uterine muscle in a dose dependent manner. There was a significant difference in the reduced force of contraction due to *Asparagus racemosus* at 160 mg/ml compared to the negative control ($P < 0.001$***) (Table 12).



a) Control pattern (de Jalon alone) b) Effect of 20 mg/ml on uterine contraction

Figure 15: Tracings of force and frequency of uterine contraction before and after exposure to 20 mg/ml *Asparagus racemosus* extract

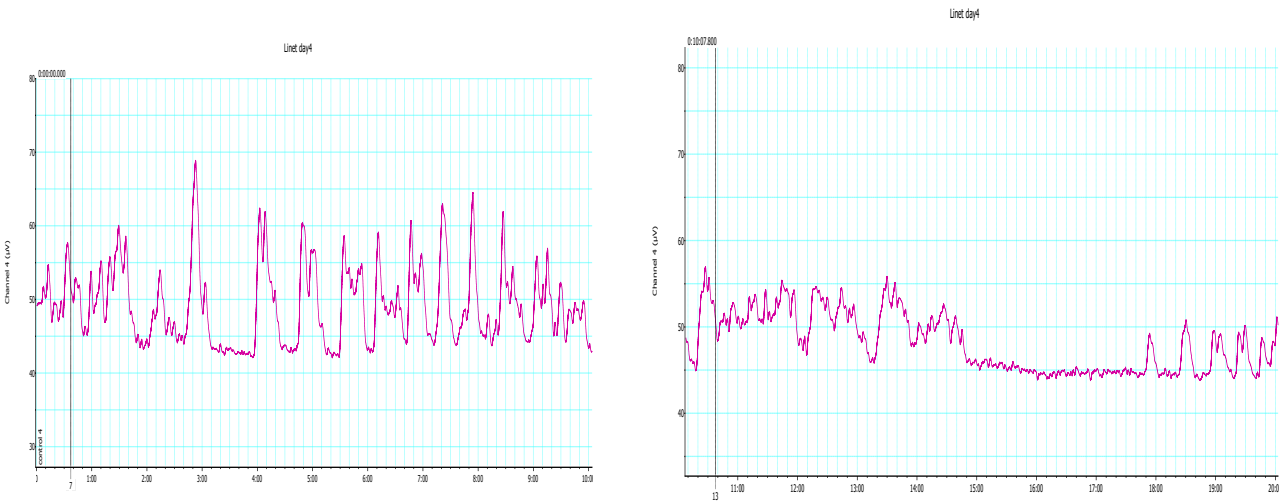
a) Shows control readings (force and frequency) of contraction before exposure of the isolated non-pregnant uterine strip to the treatment, and (b) shows the uterine contractions after exposure to 20 mg/ml *Asparagus racemosus* root extract. The tracings demonstrates a decline in the force and frequency of contraction.



a) Control pattern (de Jalon alone) b) Effect of 40 mg/ml on uterine contraction

Figure 16: Tracings of force and frequency of uterine contraction before and after exposure to 40 mg/ml *Asparagus racemosus* extract

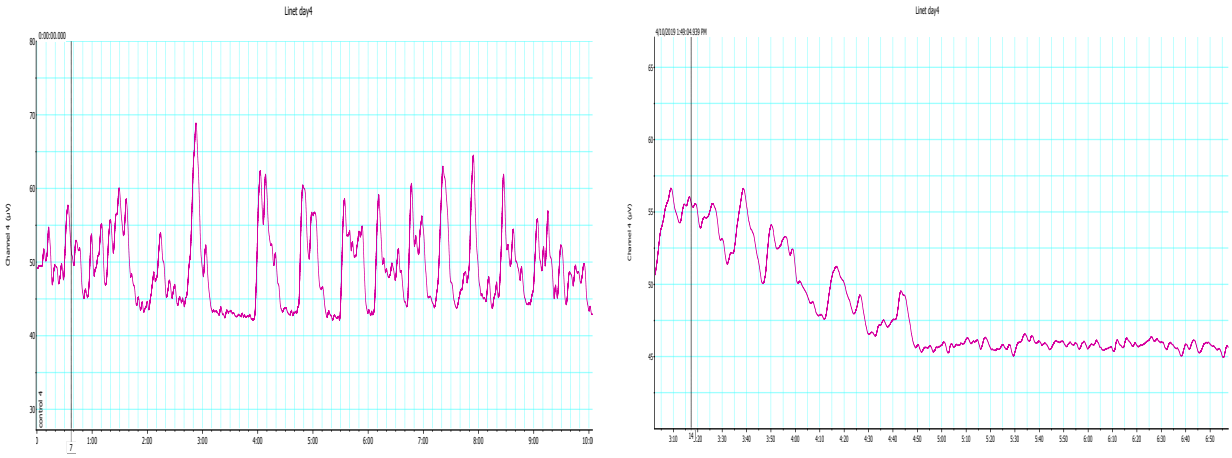
a) Control pattern for force and frequency of contraction of the isolated non-pregnant rat uterine tissue when exposed to de Jalon solution, b) force and frequency of isolated uterine contraction upon exposure to 40 mg/ml *Asparagus racemosus* extract. The tracing demonstrates a decline in both frequency and force of contraction.



a) Control pattern (de Jalon alone) b) Effect of 80mg/ml on uterine contractility

Figure 17: Tracings of force and frequency of uterine contraction before and after exposure to 80 mg/ml *Asparagus racemosus* extract

a) Control pattern for the force and frequency of isolated non pregnant uterine tissue contraction when exposed to de Jalon solution, b) force and frequency of isolated non pregnant uterine tissue contraction upon exposure to 80 mg/ml *Asparagus racemosus* extract. The tracings show a decline in the frequency and force of contraction.



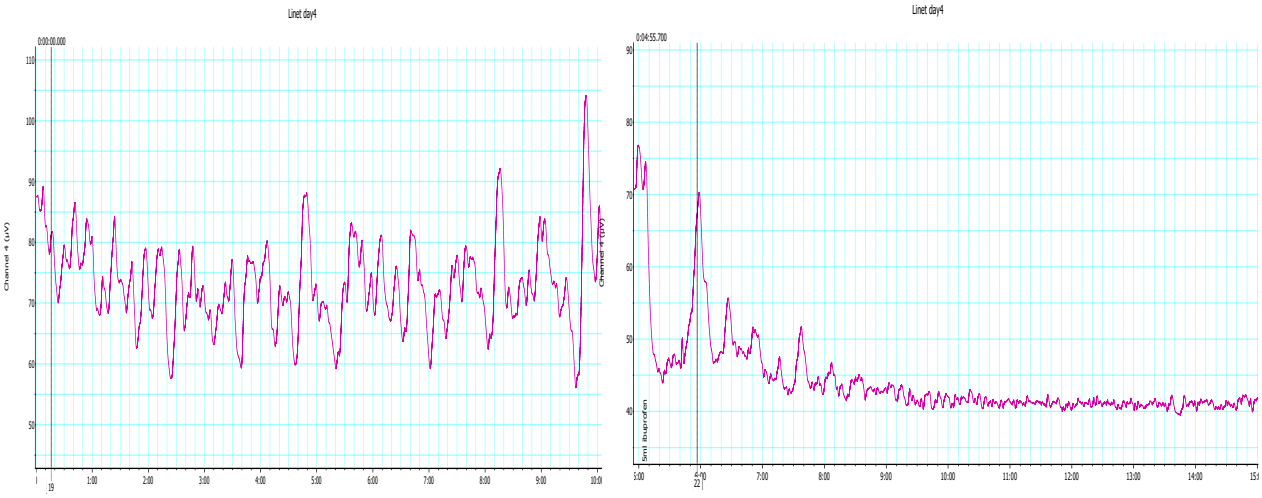
a) Control pattern (de Jalon alone) b) Effect of 160 mg/ml on uterine contractions

Figure 18: Tracings of force and frequency of uterine contraction before and after exposure to 160 mg/ml *Asparagus racemosus* extract.

a) Control pattern for the force and frequency of isolated non pregnant uterine tissue contraction when exposed to de Jalon solution, b) force and frequency of isolated non pregnant uterine tissue contraction upon exposure to 160 mg/ml *Asparagus racemosus* extract. The tracings show a decline in the frequency and force of contraction.

4.5.1.2 Effect of ibuprofen

The mean ± SEM of uterine force of contraction before and after adding ibuprofen in the organ bath shows a reduction in force of contraction (Table 10). The percentage decrease in force of contraction was -13.38% compared to the control contractions (Figure 20). Figure 19 are the tracings for positive control before and after exposure to ibuprofen.



a) Control readings (de Jalon alone) b) Effects of 5ml ibuprofen on uterine contraction

Figure 19: Tracings of force and frequency of uterine contraction before and after exposure to Ibuprofen

a) Control pattern for force and frequency of non-pregnant isolated uterine tissue contraction when exposed to de Jalon solution, b) force and frequency of non-pregnant isolated uterine tissue contraction after exposure to 5ml of ibuprofen in the organ bath. The tracings show a decline in the frequency and force of contraction.

Table 10: Effect of *Asparagus racemosus* root extract and ibuprofen on isolated uterine amplitude (force of contraction).

Dose (mg/ml)	Study	Rat 1	Rat 2	Rat 3	Rat 4	Rat 5	Rat 6	**MEAN**	**SEM**
20	Control	53.49	47.42	74.99	43.98	39.59	65.62	**54.18**	**5.56**
	Treatment	53.43	46.97	72.08	43.23	38.95	65.29	**53.32**	**5.3**
	% contraction	-0.1	-0.95	-3.89	-1.72	-1.6	-0.5	-0.15	0.55
	Control	53.49	47.42	74.99	43.98	39.59	65.62	54.18	5.56
	Treatment	52.36	46.67	64.14	43.37	36.06	64.08	**51.12**	**4.64**
40	% contraction	-2.1	-1.57	-14.47	-1.39	-8.91	-2.35	-5.13	2.20
	Control	53.49	47.42	74.99	43.98	39.59	65.62	54.18	5.56
	Treatment	50.36	46.10	60.45	42.75	34.41	63.05	**49.52**	**4.43**
80	% contraction	-5.85	-2.78	-19.39	-2.81	-13.07	-3.92	-7.97	2.77
	Control	53.49	47.42	74.99	43.98	39.59	65.62	54.18	5.56
	Treatment	48.90	31.37	65.33	30.41	30.28	60.62	**44.48**	**6.55**
160	% contraction	-8.59	-33.84	-12.89	-30.87	-23.52	-7.62	-19.55	4.67
Ibuprofen	Control	71.79	55.13	55.12	56.96	49.65	67.99	**59.44**	**3.49**
	Treatment	59.64	51.64	51.54	40.54	42.14	63.53	**51.51**	**3.74**
	% contraction	-16.91	-6.34	-6.5	-28.83	-15.13	-6.55	-13.38	3.64

Table 10 shows the mean percentage decrease in isolated uterine force of contraction. The decrease in the force/ amplitude of uterine contraction was dose dependent, with highest decline at 160 mg/ml (-19.55) compared to positive control (-13.38).

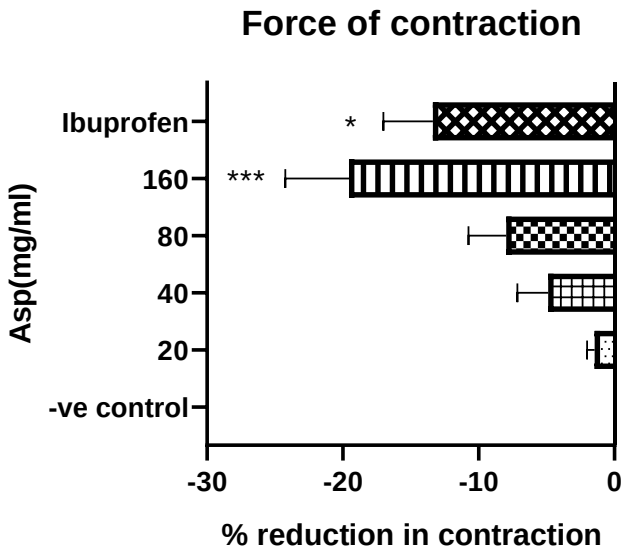


Figure 20: Effect of *Asparagus racemosus* root extract and ibuprofen on isolated uterine amplitude (force of contraction)
Key ASP: *Asparagus racemosus*

The graph shows a significant reduction in the force of isolated uterine contraction. Various *Asparagus racemosus* concentrations caused a significant reduction in force of isolated uterine contraction. At 20 mg/ml, -0.146%; at 40 mg/ml, -4.87%; at 80 mg/ml, -7.97%; at 160 mg/ml, -19.55%. Ibuprofen (positive control) also caused a reduction in force of uterine contraction.

* $P < 0.05$, ** $P < 0.01$, ***$P < 0.001$.

4.5.2 Frequency/ rate of contraction

4.5.2.1 Effect of *Asparagus racemosus* on frequency of uterine contraction

The tracings recorded before and after exposing the non-pregnant uterine strip to various *Asparagus racemosus* aqueous root extract concentration in the organ bath shows a decline in frequency of contraction (Figures 15-18). There was a significant difference in frequency of contraction ($P < 0.0001$) (Table 12). Upon exposure to 20 mg/ml of extract the frequency of

uterine contraction reduced by -5.99% (Table 11, Figure 21). At 40 mg/ml, the frequency reduced by -9.61% (Figure 21). At 80 mg/ml, the frequency of contraction decreased by -16.76%. At 160 mg/ml, there was a decrease in frequency of contraction by -25.21% (Figure 21). The reduction in frequency of contraction was dose dependent with the highest reduction in contraction seen at 160 mg/ml of *Asparagus racemosus* concentration. There was a significant difference in the frequency of contraction caused by *Asparagus racemosus* at 80 and 160 mg/ml ($P < 0.05$ and 0.0001) respectively compared to the negative control (Table12).

4.5.2.2 Effect of ibuprofen

The mean ± SEM frequency of isolated uterine contraction upon exposure to ibuprofen in the organ bath decreased (Table 11). The percentage decline in the frequency of isolated uterine contraction was -15.78% (Figure 19).

Table 11: Effect of *Asparagus racemosus* root extract and ibuprofen on isolated uterine rate (frequency) of contractions.

Dose (mg/ml)	Study	Rat 1	Rat 2	Rat 3	Rat 4	Rat 5	Rat 6	**MEAN**	**SEM**
20	Control	56	67	45	114	112	50	**74**	**12.69**
	Treatment	52	63	41	109	108	47	**70**	**12.53**
	%contraction	-7.14	-5.97	-8.89	-4.39	-3.57	-6	-5.99	0.78
40	Control	56	67	45	114	112	50	74	12.69
	Treatment	54	60	41	98	100	45	**66.33**	**10.69**
	%contraction	-3.57	-10.45	-8.89	-14.04	-10.71	-10	-9.61	1.4
80	Control	56	67	45	114	112	50	74	12.69
	Treatment	47	53	42	93	98	37	**61.67**	**10.94**
	%contraction	-16.07	-20.9	-6.67	-18.42	-12.5	-26	-16.76	2.74
160	Control	56	67	45	114	112	50	74	12.69
	Treatment	50	29	51	90	89	40	**58.17**	**10.43**
	%contraction	-10.71	-56.72	-22.22	-21.05	-20.54	-20	-25.21	6.53
Ibuprofen	Control	49	104	80	102	78	51	**77.33**	**9.7**
	Treatment	40	99	73	94	52	40	**66.33**	**10.75**
	%contraction	-18.37	-4.81	-8.75	-7.84	-33.33	-21.57	-15.78	4.4

Table 11 shows the effect of *Asparagus racemosus* and Ibuprofen on mean frequency of isolated uterine contraction. There was a dose dependent significant decrease in the frequency of uterine contraction compared to the positive control.

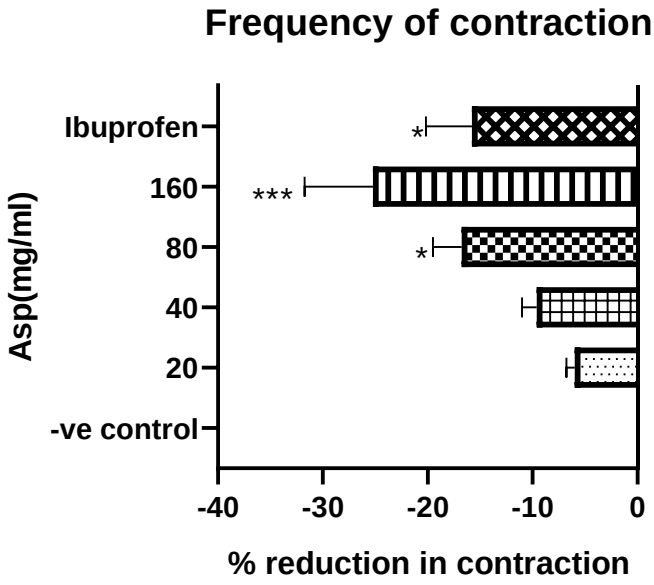


Figure 21: Effect of *Asparagus racemosus* root extract and ibuprofen on isolated uterine rate (frequency) of contraction.

Key ASP: *Asparagus racemosus*

The figure shows effect of *Asparagus racemosus* root extract and ibuprofen on frequency of isolated uterine contractions. There was a dose dependent significant decrease in frequency of isolated uterine contraction. At 20 mg/ml (-5.99%); at 40 mg/ml (-9.61%); at 80 mg/ml

(-16.76%); at 160 mg/ml (-25.21%); ibuprofen (-15.78%). * $P < 0.05$, ** $P < 0.01$, ***$P < 0.001$.

Table 12: Effect of *Asparagus racemosus* root extract on isolated uterine muscle force and frequency of contraction

Treatment (mg/ml)	Force of contraction	Frequency of contraction
Control	0.00	0.00
20 mg/ml	-1.46 ± 0.55	-5.99 ± 0.78
40 mg/ml	-5.13 ± 2.20	-9.61 ± 1.4
80 mg/ml	-7.97 ± 2.77	-16.76 ± 2.74*
160 mg/ml	-19.55 ± 4.67***	-25.21 ± 6.53***
Ibuprofen 5ml	-13.38 ± 3.64**	-15.78 ± 4.4*
P value	0.0001***	0.0001***

Table 12 shows mean ± SEM percentage decline in isolated uterine force and frequency of contraction upon exposure to *Asparagus racemosus* and ibuprofen. There was a dose dependent significant decline in the force and frequency of contraction in all treatment groups compared to the negative and positive control ($P < 0.0001$**). 160 mg/ml *Asparagus racemosus* caused the highest reduction in both force and frequency of isolated uterus contraction.

CHAPTER FIVE

5.0 DISCUSSION

5.1 *Asparagus racemosus* Phytochemistry

The phytochemical analysis of Kenyan *Asparagus racemosus* showed presence of saponins, flavonoids, glycosides and terpenoids. These results corroborate with previous reports of plant studied in the Himalayan region of India which reported presence of saponins, glycosides and absence of alkaloids (Wani *et al*., 2011). Glycosides and flavonoids promotes fertility (Telefo *et al*., 2012). The leaves of *Justicia insularis* that contained glycosides and flavonoids mixed with others from *Aloe buettneri, Hibiscus macranthus, Dicliptera verticillata* were used to alleviate painful menstruation and treat cases of infertility in women (Tefelo *et al*., 2012). *Justicia insularis* containing flavonoids and glycosides improved fertility in female rats (Telefo *et al*., 2012). The improved fertility could be due to these estrogenic compounds found in the leaf extract. It was also reported that the aqueous extracts of *Justicia insularis* cause ovarian steroidogenesis and folliculogenesis in the female rats probably due to the biochemical compounds they contain.

Rajashekar *et al*., (2012) reported on presence of flavonoids in *Pedalium murex* that improved fertility by increasing the pup body weight and the litter size. Date Palm Pollen has been used in Middle East for good health and improvement of female fertility (Koehn and Carter, 2005). It is reported to contain saponins, flavonoids, estrone, and cholesterol. In the current study, there were saponins and flavonoids in *Asparagus racemosus* which could be the reason for the improved fertility as shown by increased litter size. Study by Dande and Patil, (2012) however reported that saponins present in the *Trigonella foenum-graecum* seeds caused anti-fertility effect. *Echinophora platyloba* is reported to contain flavonoids, alkaloids and saponins and it is

effective in reducing uterine contractions. The phytochemical compounds are believed to be the reason for its use in treatment of dysmenorrhea (Bahmani *et al*., 2015). In the current study *Asparagus racemosus* showed presence of high concentration of saponins which could explain its traditional use to relieve the pain of dysmenorrhea.

Phytoestrogenic plants containing steroidal saponins and flavonoids cause increased levels of circulating estrogen in females (Mustapha *et al*., 2011). High levels of estrogen prolong the proestrus and estrus phases of the estrus cycle. Mustapha *et al*., (2011) studied aqueous and ethanolic leaf extract of *Rynchosia sublobata* containing flavonoids and saponins in the female Wistar rats and found they prolonged the proestrus phase of the estrus cycle. This study corroborates the present study as it showed that *Asparagus racemosus* root extract prolonged the proestrus phase.

5.2 Acute oral toxicity

Asparagus racemosus crude extracts are used by traditional herbalists in Nakuru County, Kenya to alleviate dysmenorrhea. Acute oral toxicity studies carried out in this study indicated that this plant did not cause any abnormal behavior or mortality even at the limiting dose of 5000 mg/kg body weight. The results are similar to those of Kumar *et al*., (2010) who reported that the extracts caused no behavioral, autonomic or central nervous system alterations nor mortality at 3200 mg/kg b/w. Prabha *et al*., (2004) also assessed safety level of *Asparagus racemosus* through acute and chronic toxicity by administration of 1000 mg/kg on both pre and postnatal rat development and found that no changes were observed in behavior or food and water intake on body weight. *Cyperus rotundus* reported to treat various illnesses including dysmenorrhea, at a dose level of 5000 mg/kg showed no toxicity, changes in behavior, difference in gross internal organs appearance and no mortalities (Al-Snafi, 2016). Toxicity study of *Amaranthus viridis*

showed no signs of toxicity or mortality of the rats when administered at a dose of 2000 mg/kg (Emmanuel *et al*., 2018). Acute toxicity studies on *Carica papaya* leaves showed no acute adverse effects or mortality at a dose of 2000 mg/kg though it was reported to cause dehydration (Afzan *et al*., 2012). All these studies corroborate this study on absence of toxicity.

5.3 Estrus cyclicity

Kenyan *Asparagus racemosus* aqueous root extract caused a significant disruption of the estrus cycle in the rats. Doses of 300 and 600 mg/kg b/w prolonged proestrus phase (Tables 5, 6 and 7; Figures 7 and 11). The proestrus and the estrus stages occurred more frequently compared to the negative control. These results corroborates those by Barhane and Singh, (2002) who also reported 100% return to estrus on postpartum cows after *Asparagus racemosus* supplement addition within 90 days of calving. There was subsequent reduction in frequency of metestrus and diestrus stages at 300 and 600 mg/kg b/w compared to the negative control in a dose dependent manner (Tables 5, 6, 7 and Figures 9, 10, 11). The reduction in the frequency of metestrus and diestrus resulted in a shift of the estrus cycle to favor proestrus and estrus phases. The reduction of metestrus and diestrus phases could be due to estrogenic nature of *Asparagus racemosus*. Indeed Daramola *et al*., (2015) reported that *Telfairia occidentalis* causes a reduction of diestrus phase favoring occurrence of estrus and proestrus due to its estrogenic property. Similar results were reported by Kage *et al*., (2009) using *Trichosanthes cucumerina.* Reduction in diestrus phase is beneficial to development and maturation of follicles due to estrogen that increases during proestrus phase and decreasing levels of FSH causing growth of the pre ovulatory follicle (Satue and Gardon, 2013). This finally propagates the next phase which is proestrus where follicles enlarge and levels of estrogen increase preparing the dominant follicle for ovulation (Daramola *et al*., 2015).

The ovarian steroids and the pituitary gonadotropins control the estrus cycle. Estradiol levels increase as levels of FSH rise. This causes growth and maturation of the ovarian follicles. Estradiol also causes endometrial wall to grow and proliferate in preparation for implantation. A disruption in the hormones results into disruption of the estrus cycle. Plants have been reported to disrupt the estrus cycle in various ways: *Cirus medica* seed extract caused prolonged proestrus and estrus stages and reduced metestrus and diestrus stages (Patil and Patil, 2009); *Crotalaria juncea* extract caused prolonged estrus and metestrus stages and reduced proestrus and diestrus stages in mice (Malashetty and Patil, 2004); *Hibiscus rosa-sinensis* flowers caused increased estrus and metestrus stages (Al-Snafi, 2018)).

In this study the estrus cycle was disrupted with prolonged proestrus and estrus decreasing metestrus and diestrus stages compared to the control. This is probably due to direct or indirect effect of *Asparagus racemosus* on pituitary gonadotropins or the ovarian steroids. In the late luteal phase of non-pregnant animals, uterine production of prostaglandin PGF2α increases. PGF2α is transported to the corpus luteum (Johnson, 2002) and initiates luteolysis. The resultant reduction in progesterone and elevated PGF2α leads to dysmenorrhea. *Asparagus racemosus* caused a decrease in the metestrus and diestrus phases and this could be the reason for its use in management of dysmenorrhea. This probably could also be due to greater influence of the extract on progesterone hormone (Figure 14) maintaining its levels and reduced uterine contraction thus managing dysmenorrhea. However for the positive control (ibuprofen), the estrus cycle was significantly disrupted; with a prolonged proestrus and the estrus phases (Figures 11 and Tables 4, 7) compared to the negative control group.

5.4 Mating success, gestation length and litter size

Mating success was determined microscopically by presence of spermatozoa in the vaginal smear. Presence of spermatozoa was evidence that mating took place and fertilization occurred. At 300 and 600 mg/kg of *Asparagus racemosus* root extract both groups had 100% mating success. This is probably due to increased concentration of estradiol that initiates estrus behavior also causing preovulatory gonadotropins surge that leads to ovulation (Smith *et al*., 2005). It therefore shows ovulation took place and the rats mated. Somina, a polyherbal product used in Pakistan caused 100 % mating and 100 % conception (Ahmed and Azmat, 2016). This study is corroborated by Yinusa *et al*., (2010), who reported that *Quassia amara* caused 100 % mating success in female Wistar rats.

For the positive and negative control 100% mating success was recorded (Table 9). Regular gestation length of the Wistar rat is 19 ± 3 days (Suckow *et al*., 2005). In this study; *Asparagus racemosus* had no significant difference in the gestation length compared to negative control. However, there was significant difference between treatment groups compared to positive control ($P < 0.05$). This study is corroborated by Datta and Bose, (2018) who reported leaf extracts of *Pterospermum acerifolium* caused no alterations in gestation length in Wistar rats. In this study, litter size was higher compared to negative and positive control (Table 8, Figure 13). However, it was a non-significant difference compared to the control group. Increased litter size due to effect of *Asparagus racemosus* extract is probably due to its reported enhancement of folliculogenesis and ovulation (Kalia *et al*., 2003). A mixture of herbs containing *Asparagus racemosus* was reported to cause uterotrophic activities in rats (Gopumadhavan *et al*., 2005). *Asparagus racemosus* is also reported to play a role in uterine receptivity and implantation by enhancing uterine blood supply thereby resulting in enhanced fertility (Prasad *et al*., 2002).

Kumar and Singh, (2001), reports an improved conception rate due to *Asparagus racemosus* on In-vitro fertilization in women compared to the placebo group. The reported increase in litter size is probably due to high saponin concentration. Somina, the polyherbal product used in Pakistan improved implantation but did not alter the litter size of Wistar rats (Ahmed and Azmat, 2016). Yinusa *et al*., (2010), reported on *Quassia amara* that caused 100% fertility on Wistar rats but a decrease in the mean litter size. *Telfairia occidentalis* caused an increase in litter size in the Wistar rat (Daramola *et al*., 2015) which also corroborates this study. Farahbod and Soureshjani, (2018) reported on various medicinal plants thought to have improved fertility in women by enhancing endometrial thickness and folliculogenesis. These include *Vitex agnus*, *Cimicifuga racemosa*, *Yucca schidigera* and *Lepidium meyenii*.

Ibuprofen (positive control) caused a decline in litter size and prolonged the gestation period compared to the negative control and treatment groups ($P < 0.05$) (Table 8). All rats had 100 % mating success; however only 2 littered. This is probably due to compromised fertilization and /or implantation. Ibuprofen is a NSAID usually taken as an analgesic. Ibuprofen blocks cyclooxygenase-2 enzyme that is responsible for the synthesis of prostaglandins and is reported to affect fertility (Sirois *et al*., 2004).

5.5 Effect of *Asparagus racemosus* on reproductive hormones

Mammalian reproductive cycle is as a result of regulated and balanced hormonal interaction which involves hypothalamic GnRH, pituitary gonadotropins and the ovarian steroid hormones. LH and FSH induce production of estradiol and progesterone (Shimada and Terada, 2002). Higher levels of progesterone leads to quiescence of the myometrium muscle hence resistance to prostaglandin stimulation. Increased progesterone levels also leads to decreased estradiol levels thus inhibiting production of prostaglandins.

In this study on the 6th day at Raman peaks 476, 1200, 1436, 1733 cm^{-1} (Figure 14) there was increased levels of progesterone probably due to *Asparagus racemosus* at 300 and 600 mg/kg. This might be due to increased levels of FSH and LH. Increased progesterone levels down regulate uterine muscle prostaglandin receptors. This probably causes an inhibition of uterine contraction leading to reduced dysmenorrhea (Iacovides *et al*., 2015). Probably this effect of the extract on progesterone levels may explain the use of *Asparagus racemosus* in dysmenorrhea management. Other plants with similar effects have previously been reported. *Vitex agnus* plant used in treatment of dysmenorrhea was found to contain essential oils which caused a decrease in the levels of FSH, LH but increased progesterone levels (Mirabi *et al*., 2014). On day 13, there was a significant influence of 300 and 600 mg/kg *Asparagus racemosus* on the hormones (Figure 14). Peaks 476, 1131 and 1438 cm^{-1} show higher influence of FSH, LH and progesterone hormone in the rats treated with 300 and 600 mg/kg of the extract. The high levels of progesterone might probably be the reason the plant is used as anti-dysmenorrhea as it causes down regulation of prostaglandin receptors on the uterine muscle. The increased levels of these hormones could also be due to its reported fertility effect.

Estradiol has various functions in the female including growth and proliferation of the endometrial layer in readiness for implantation and promoting oviduct integrity by stimulating the oviduct secretory cells to manufacture and release glycoproteins necessary for nourishment of the embryo. The increased levels of estradiol on day 13 (Figure 14) at peaks 977 and 1268 cm^{-1} compared to day 1 and 6 of *Asparagus racemosus* treatment probably explains its reported fertility enhancement properties (Kinage and Chaudhari, 2016**)**. Increased FSH and LH levels found in the present study also promotes folliculogenesis leading to enhanced estradiol thereby promoting fertility. This is also evidenced by 100 % mating success and pregnancy.

All the rats in the treatment group littered, with a higher number of pups compared to the controls (Table 8). Sharma and Bhatnagar, (2010), reported an increase in serum follicle stimulating hormone at 100 mg/kg *Asparagus racemosus* root extract thereby further corroborating the present study. Study by Gopumadhavan *et al*., (2005), however, contradicts this study as it reported that a polyherbal formulation containing *Asparagus racemosus* caused increased uterine weights and uterine glycogen levels in immature rats without altering the serum progesterone or estrogen levels. *Cimicifuga racemosus* together with clomiphene increased women fertility by increasing the levels of estradiol and progesterone alongside causing an increase in endometrial thickening (Shahin and Mohammed, 2014). *Asparagus officinalis* increased female fertility by increasing serum GnRH, FSH, LH and progesterone levels in Wistar rats (Jashni *et al*., 2016). Mice administered with *Vitex agnus* showed increased levels of FSH and LH and their fertility was enhanced (Farahbod and Soureshjani, 2018; Rafieian and Movahedi, 2017). Rats administered with ibuprofen showed significant reduction in FSH and LH levels which are necessary for successful ovulation (Agbai *et al*., 2017).

In this study the ibuprofen treated Wistar rats had estrus but probably did not ovulate and or probably had impaired implantation window. This might be the reason why most of the positive control rats did not litter. Those that littered had very minimal litter sizes. *Asparagus racemosus* influenced blood hormone levels in a dose dependent manner compared to the positive control showing better effects caused by high dose (Table 9). There was a significant difference in the intensity of hormone between the different groups compared to the control (Table 9).

5.6 Effect of *Asparagus racemosus* on uterine myometrial quiescence

Asparagus racemosus is documented as the most potent female tonic herb **(**Mishra *et al*., 2013). It is said to cleanse, provide nourishment and strength to the female reproductive organs thus used for various gynecological female problems including PMS, dysmenorrhea, amenorrhea, menopause and endometriosis (Khulbe, 2015; Mishra *et al*., 2013). The major theory for the possible cause for dysmenorrhea is increased release of prostaglandins (PG) especially PGF2 α which cause hyper contractility of the uterine muscle thus reducing blood flow hence pain (Wallace *et al*., 2010).

This study show that increasing concentration of *Asparagus racemosus* caused a significant reduction in the force and frequency of uterine contractions (Figures 20 and 21). These results corroborates other studies (Khulbe, 2015; Mishra *et al*., 2013) that reported blockage of induced contractions by *Shatavari* in the rat, pig and rabbit in a dose dependent manner hence used in management of dysmenorrhea. Study by Verma *et al*., (2005), reported anti-inflammatory activity on the uterus by *Hemidesmus indicus*, thus its use during dysmenorrhea. *Echinophora platyloba* is reported to reduce uterine contractions, thus used in treatment of dysmenorrhea (Bahmani *et al*., 2015). *Hypericum perforatum* possess an analgesic activity by inhibiting prostaglandin production that mediate pain (Bahmani *et al*., 2015; Khanavi *et al*., 2005). *Zingiber officinale* is used in management of dysmenorrhea due to its potential in inhibiting cyclooxygenase hence hindering production of prostaglandins (Ozgoli *et al*., 2009). *Asparagus racemosus* therefore could be inhibiting production of prostaglandin hence its use in management of dysmenorrhea. *Achillea willhemsii* causes anti-prostaglandin effect that produce an inhibitory effect on synthesis of arachidonic acid (Mirabi *et al*., 2014). Khatami *et al*., (2017) also reported analgesic effect on the uterine muscle in flowers of Chamomile which is used in

treatment of dysmenorrhea. From the studies, these plants have the potential to generate novel dysmenorrhea drugs and so does *Asparagus racemosus*.

This study shows that *Asparagus racemosus* significantly decreased the force and rate of uterine contraction and increased progesterone levels in the blood inhibiting prostaglandins production hence relieving uterine pain. Since *Asparagus racemosus* is not associated with any side effects on reproduction, it's thus recommended as an alternative remedy for dysmenorrhea.

CHAPTER SIX

6.0 CONCLUSIONS AND RECOMMENDATIONS

6.1 CONCLUSIONS

1. Presence of saponins, glycosides and flavonoids in the *Asparagus racemosus* aqueous root extract might be responsible for the fertility effect and its use in pain relief.

2. *Asparagus racemosus* did not show any signs of toxicity even at the highest limit dose hence non-toxic

3. *Asparagus racemosus* shows no adverse effect on mating, conception rate, gestation length and litter size. Effect of *Asparagus racemosus* on these reproductive parameters shows clearly that it is a fertility promoter. Results of this study shows increased litter size in all the extract treated groups an indication of successful fertilization and implantation. The effect might be at hypothalamic-pituitary-gonadal axis due to hormonal balance.

4. Raman spectroscopy showed great influence of LH, FSH, estradiol and progesterone hormones in the rats. LH, FSH and progesterone were influenced more especially by the high dose. *Asparagus racemosus* therefore could be working at the uterine level inhibiting prostaglandin production that results in dysmenorrhea.

5. The results of this study indicates that *Asparagus racemosus* can be employed to reduce uterine contractions induced by prostaglandins more than the commercial drug ibuprofen. All the doses produced reduction in contractions but the most effective dose was the 160 mg/ml in the organ bath.

6.2 RECOMMENDATIONS

1. Phytochemical isolation of pure compounds so as to establish if the activity can be attributed to specific compounds.
2. In this study, the plant roots were used. This might lead to a depletion and extinction of the plant. It is important to promote its propagation.
3. Further work which involve testing the plant on other smooth muscles like the trachea and ileum.
4. Evaluate effect of *Asparagus racemosus* extracts at molecular level

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