CHAPTER ONE

1.0 Introduction

A variety of illnesses are unique to each gender. The dysfunction of female reproductive organs is referred to as gynaecological problems (Patel, 2018). Although some of these female conditions are treatable, others are lethal, chronic, and have a negative impact on fertility (Patel, 2018). Consequential to the increase in the invasion of and exposure to chemicals that are an endocrine disruptor, hormonal imbalances are becoming more common (Patel, 2018). A few reproductive hormonal abnormalities are amenorrhoea, anovulation, fibroid, endometriosis, polycystic ovarian syndrome, and infertility (Patel, 2018).

Polycystic ovarian syndrome is a heterogeneous disorder that affects women of reproductive age (Mikhael *et al*., 2019). This is a disorder that is principally characterized by signs and symptoms of hyperandrogenism and ovulatory dysfunction which disrupts the function of the hypothalamicpituitary - ovarian (HPO) axis (Baskind and Balen, 2016; Mikhael *et al*., 2019; Witchel *et al.*, 2019). Some clinical features include hirsutism, oligomenorrhea, chronic anovulation, and infertility (Witchel *et al.*, 2019). The polycystic ovarian syndrome is a clinical disorder with various reproductive metabolic features involving hormonal imbalances, insulin resistance, cardiovascular diseases (CVD), and type 2 diabetes (Emamalipour *et al.,* 2019). The most common cause of anovulatory infertility is PCOS (Mohammad and Seghinsara, 2017). Studies have shown that approximately 90% to 95% of anovulatory women seeking infertility remedy have this disorder, this is usually discovered after treatment for infertility (Jahan and Wing, 2020). Women with this disorder have been studied to have elevated levels of luteinizing hormone (LH) and reduced level of follicle-stimulating hormone (FSH), linked with high levels of androgens and insulin (Kumar *et al*., 2016). Also, insulin resistance, a consequence of hyperinsulinemia affects women with PCOS (Kumar *et al.,* 2016). Patients with PCOS have shown to have impaired glucose tolerance with type 2 diabetes (Ollila *et al.,* 2017; Kakoly *et al.,* 2018). Women with a family history of type 2 diabetes or those at higher risks of obesity are susceptible to PCOS (Ollila *et al.,* 2017). There is currently a scarcity of information about the prevalence of PCOS in Nigerian women (Apata *et al*., 2018). In a research conducted on Nigerian women, it was reported that most infertile Nigeria women have PCOS (Apata *et al*., 2018).

According to the National Institute of Health (NIH) diagnostic criteria, an estimated 5-15% of women of reproductive age suffer polycystic ovarian syndrome making it a common endocrine system disorder in women (Azziz, 2016). PCOS has a wide-ranging health effects including severe metabolic, reproductive and psychological risks (Doherty *et al.,* 2015; Arentz *et al.,* 2017). Insulin resistance which has been underpinned to be the pathogenesis of PCOS has been discovered to affect up to 75% of lean women and up to 95% of obese women (compared to 62% in BMI matched non-PCOS) (Cassar *et al*.,

2016; Arentz *et al.,* 2017). Since PCOS is a complex metabolic-endocrine condition (Kwon *et al.,* 2020), lifestyle and diet play a significant role in addition to pharmacotherapeutics (Barrea *et al.,* 2019). Recent research suggests that complementary and alternative treatments, such as herbal medicines and acupuncture, may help with PCOS symptoms, although there is inadequate proof of their efficacy and safety (Kwon *et al.,* 2020).

Traditional medicine has been in existence long ago and its use by women has increased in recent years (Li *et al.,* 2020); this comprises the use of herbal medicines which contain active pharmacological components which have therapeutic effects on female reproduction (Yang *et al*., 2019). Through drug discovery and drug design, modern medications are gotten from these herbal plants (Adeyomoye and Adewoye, 2018). This has engendered more research towards the identification of medicinal plants. *Parquetina nigrescens* is an example of a medicinal plant with various therapeutic effects (Adeyomoye and Adewoye, 2018). Mainly on traditional remedies, *Parquetina nigrescens,* has been used as popular folk medicines (Adeyomoye and Adewoye, 2018). *Parquetina nigrescens* occur in a large part of Africa, from Senegal east to Sudan and South through Central and East Africa to Zambia, Angola, and eastern Zimbabwe (Kola-Mustapha *et al*., 2019). Its leaves in boiling or infusion with other plant species are widely used in the treatment of menstrual disorders (Aborisade *et al*., 2017). Aqueous leaf extraction administered orally on diabetic rats showed a significant reduction in the level of blood glucose which was comparable to that of non-diabetic rats and those given standard drugs (Cruz, 2012) diabetes which is a metabolic complication of PCOS.

Several scientific investigations on the identification of active components in medicinal plants for efficient therapeutic interventions have reported using a variety of instruments. *In silico* drug development is a useful approach because very large numbers (up to millions) of drug compounds can be screened (Carpenter *et al*., 2018), which is likely to be more difficult, time-consuming, and costly using experimental approaches (Taguchi and Turki, 2020). Also, gas chromatography is an advanced technique, widely used for the analysis of almost every type of organic compound, including those that are not volatile in their original state but can be converted to volatile derivatives (Pagliano *et al.,* 2018; Libretexts, 2021). Separation of the different components is achieved based on their relative vapour pressure and affinities for the stationary phase (Raja and
Barron, 2021). Mass spectrometry is an analytical technique that identifies the molecules and elements present in a sample via the operation of gaseous ions both in electric and magnetic fields on the basis of their mass-to-charge ratios (Brown and Beynon, 2020). Gas chromatography - Mass Spectroscopy (GC-MS), is one of the most efficient techniques used in metabolomics. It possesses excellent separation capability, sensitivity, and reproducibility; which tends to achieve greater chromatographic resolution power (David and Rostkowski, 2020).

The cure of this common endocrine disorder, polycystic ovarian syndrome, is not known (Smith and Sanyal, 2018; Corte *et al*., 2020). However, various management options have been studied to alleviate the progression of this disorder (Rocha *et al*., 2019), some synthesised drugs such as metformin, clomiphene citrate and letrozole have been studied to have therapeutic effects on the clinical features associated with PCOS (Yu *et al*., 2017; Costello *et al*., 2019). Although these drugs help in the management of PCOS, however, they have limitations such as adverse side effects, costs and the delivery time. Hence, using this ethanoic extract of *P. nigrescens* leaves for the treatment of PCOS settles the limitations associated with these synthetic drugs. *P. nigrescens* leaves are cost effective and has less side effects being an herb. It is with these backgrounds that the aim of this study is based on, hence the aim of investigating the effects of *Parquetina nigrescens* on the polycystic ovarian syndrome.

1.1 Statement of the Problem

The prevalence of PCOS globally is estimated to be in the range of 6% and 26% (Wolf *et al.,* 2020), Nigeria reports the prevalence of PCOS at 18.1% and 12.2% based on the Rotterdam criteria (Omokanye *et al.,* 2015). Drugs such as Clomiphene citrate and metformin have been used in the management of PCOS (Davar *et al*., 2011; Hajishafiha *et al.,* 2013; Rezk *et al*., 2018), but it has been reported to have various side effects. As a result of this, the therapeutic effects of ethanoic extracts of *Parquetina nigrescens* leaves on letrozole-induced PCOS was investigated in this study.

1.2 Justification of the Study

Several studies have been carried out to investigate the leaves of *Parquetina nigrescens* on its control of diabetes and menstrual disorders (Olatubosun *et al.,* 2018) which are the key symptoms of polycystic ovarian syndrome. Traditional medicine has also laid claim to the therapeutic effects of *Parquetina nigrescens* on these two metabolic disorders associated with PCOS. It is, therefore, imperative to provide scientific validation on this claim. This study, therefore, seeks to investigate the effect(s) of ethanoic leaf extract of *Parquetina nigrescens* on letrozole-induced polycystic ovarian syndrome in rats.

1.3 Aim of Study

This study aims to evaluate the bioactive components in ethanoic extract of *Parquetina nigrescens* leaves and its reproductive effect such as oestrous cycle and hormones associated with letrozoleinduced PCOS rats.

1.4 Specific Objectives of Study

The specific objectives of this study were:

- To evaluate the phytochemical constituents in ethanoic extract of *Parquetina nigrescens* leaves via phytochemical screening, UV-Visible Spectroscopy and GC-MS.
- To induce PCOS in rats using letrozole
- To monitor the oestrous cycle
- To assay for reproductive hormones such as testosterone, progesterone, luteinizing hormone and follicle stimulating hormone
- To determine the serum lipid profile of PCOS rats
- To evaluate the associated metabolic disturbances associated with PCOS such as hyperglycaemia, increased weight and hyperinsulinemia.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Historical Perspective of Polycystic Ovarian Syndrome

Polycystic ovarian syndrome is a disorder first diagnosed and defined in the year 1935 by Stein and Leventhal (1935) having originally described the condition with the three main diagnostic features: amenorrhoea, polycystic ovaries and hyperandrogenism (Azziz, 2021). Although, Stein and Leventhal are recognised as being the first to investigate polycystic ovarian syndrome, Vallisneri, an Italian scientist, also then identified a young, infertile woman with clear, white ovaries the size of pigeon eggs in 1721 (Azziz, 2021). Despite the fact that much progress was made in characterizing the condition from the late 1950s to the late 1980s, there was still a lot of uncertainty in the field since no diagnosis criteria had been settled upon (Weatherall *et al.,* 2006; Ali, 2019).

In April 1990, a conference held at the National Institutes of Health (NIH) identified what is now known as "classic" PCOS based on a survey of meeting participants (Zawadzki and Dunaif, 1992; Azziz, 2021). Eventually, in November 2006, the Androgen Excess Society which is presently called the Androgen Excess and PCOS Society published its diagnosis guidelines, which were primarily based on linking PCOS criteria to metabolic and other long-term morbidities (Azziz *et al.,* 2009). The discovery that PCOS could be divided into four phenotypes (A–D) based on these three characteristics: clinical and/or biochemical hyperandrogenism, oligoanovulation and polycystic ovaries (Azziz, 2021; Balen *et al.*, 2003; Rosenfielf and Erhmann, 2016).

2.2 Polycystic ovarian syndrome

Polycystic ovarian syndrome (PCOS) is a common endocrine and heterogeneous disorder which affects females of reproductive age (Patel, 2018; Leo *et al.*, 2016; Kakoly *et al.*, 2018). It is the major cause of chorionic anovulation and anouvulatory infertility (Leo *et al.*, 2016; Bani and Majdi, 2017). It is associated with a variety of reproductive (hyperandrogenism, oligo or anovulation, infertility); metabolic (gestational diabetes mellitus (GDM), impaired glucose tolerance (IGT), type 2 diabetes (T2DM), cardiovascular disease (CVD), cardiovascular risk factors); and psychological (depression, anxiety, low self-esteem, disordered eating, psychosexual dysfunction) characteristics (Kakoly *et al.*, 2018). It is a constellation of symptoms which affects women of child bearing age (Patel, 2018). Polycystic ovarian syndrome (PCOS) is one of the most common endocrine disorders in reproductive-age women in the United States, with a prevalence of 4-12% (Richard, 2019). During routine gynaecologic appointments, up to 10% of women are diagnosed with PCOS (UCLA, 2015). According to some European reports, the prevalence of PCOS ranges between 6.5-8% (Richard, 2019). Despite its

prevalence, the precise cause of this disease has remained unknown. (Crespo *et al*., 2018). According to the PCOS consensus workshop group, the diagnostic criteria for PCOS is defined as the presence of at least two of the following criteria:

i. Oligo-anovulation
ii. Hyperandrogenism with clinical or biochemical signs
iii. Appearance of polycystic ovary on ultrasound examination (Bassim, 2018; Wawrzkiewicz *et al.,* 2020).

2.2.1 Classification of PCOS

PCOS occurs as a resultant of imbalances female sex hormone which leads to the development of cysts in the antral ovarian follicle (Patel, 2018). Four phenotypes of PCOS has been discovered with unique pathophysiology in each group: full-blown, non-polycystic ovaries, non-hyper androgenic and ovulatory phenotypes (Rodrigues *et al*., 2019). The phenotypes categorised are as a result of the presentation of the PCOS. The full blown PCOS (phenotype A) is the most common phenotype with prevalence of 67.7% (Sachdeva *et al*., 2019) having hyperandrogenism (HA), ovulatory dysfunction (OD), and polycystic ovarian morphology (PCOM). The non-polycystic ovaries PCOS (phenotype B) is presented with hyperandrogenism and ovarian dysfunction. The non-hyperandrogenic PCOS (phenotype D) which is presented with ovarian dysfunction and polysystic ovarian morphology, the last phenotype which is phenotype C is called the ovulatory phenotype is presented with hyperandrogenism and polycystic ovarian morphology (Lizneva *et al*., 2016).

Table 1: Classification of polycystic ovarian syndrome phenotypes

Parameter	Phenotype A	Phenotype B	Phenotype C	Phenotype D
PCOS features	HA/OD/PCOM	HA/OD	HA/PCOM	OD/PCOM
HA	+	+	+	−
OD	+	+	−	+
PCOM	+	−	+	+
NIH 1990 criteria	X	X		
Rotterdam 2003 criteria	X	X	X	X
AE-PCOS 2006 criteria	X	X	X	

Note: AE-PCOS = Androgen Excess & PCOS Society; HA = hyperandrogenism; NIH = National Institutes of Health; OD = ovulatory dysfunction; PCOM = polycystic ovarian morphology.

Source: Adapted from Lizneva et al. (2016)

2.2.2 Ovarian Dysfunction

Ovarian dysfunction, also known as ovarian insufficiency, is described as the failure of the ovary to function properly in its position as an endocrine organ or as a reproductive organ in women of reproductive age (i.e. younger than 40 years of age) (Pellegrini, 2016). Ovarian dysfunction, as defined by the World Health Organization, can be caused by a primary ovarian disorder or it can be caused by secondary factors.

Ovarian dysfunction can either be primary or secondary. It is considered primary if the ovary fails to act normally as a result of natural gonadotropin stimulation provided by the hypothalamus and pituitary gland. (Pellegrini, 2016) and secondary if the hypothalamus and pituitary do not have adequate stimulation of gonadotropin (Pellegrini, 2016).

The hypothalamic-pituitary gonadal axis (HPG), controls the reproductive system (AcevedoRodriguez *et al*., 2018) The anterior pituitary gonadotrophs are stimulated to release luteinising hormone (LH) and follicle stimulating hormone (FSH) by the release of gonadotropin releasing hormone (GnRH) from hypothalamus which leads to the production of steroids from the ovaries (Chauldhari *et al*., 2018; Rodrigues *et al*., 2019). The mechanism of secretion of the GnRH are changed as a result of lack of adequate progesterone peaks via the luteal phase of the menstrual cycle leading to the increase in secretion of LH (Unluturk *et al.,* 2016). An increase in LH level stimulates the secretion of androgens by the ovaries and also stimulates the synthesis of androgens, this adrenals contribute to hyperandrogenism in PCOS (Unluturk *et al.,* 2016). Hyperandrogenism can lead to: hirsutism, androgenic alopecia and acne (Jałowiecka, 2020).

2.2.2.1 Primary Ovarian Dysfunction

Primary ovarian dysfunction is commonly referred to as, primary ovarian insufficiency (POI) (premature menopause or early menopause) (Pellegrini, 2016). Fuller Albright, a Harvard endocrinologist, came up with the term "primary ovarian insufficiency" to emphasize that the "primary" defect was located within the ovary (Panay *et al.,* 2020).

The word ‘premature ovarian insufficiency' was preferred because it includes both spontaneous and iatrogenic disorders, and ‘insufficiency', rather than failure; it; represents the likelihood of intermittent ovarian development, which may result in ovulation and even pregnancy (Torrealday, 2017; Panay *et al.,* 2020). A recent global prevalence study of POI was concluded to be as high as 3.7% (95% confidence interval) i.e. within the range of 3.1 to 4.3 (Golezar *et al.,* 2019). This study also claimed that the prevalence was higher in countries with a low or medium human development index (Golezar, 2019).

Every female child is born with 700,000 to 1 million oocytes in their primordial follicles (Panay *et al.,* 2020). The reproductive lifespan is determined by the pool's survival time, which is usually 400 ovulated cycles (Garcia *et al*., 2019). POI occurs as a consequence of the loss of these follicles with subsequent infertility and the loss of production of estrogen hormone (Torrealday, 2017). POI could be cause by the reduction in the primordial follicle pool through high destruction of the follicles or can also be caused by problems in the maturation of primordial or developing follicles (Panay *et al.,* 2020). Genetics, recreational drug usage, autoimmune disorders, pelvic surgery, or chemical exposures all play a role in the development of the disorder (Panay *et al.,* 2020). It is also likely that spontaneous POI may occur in some women as part of an aging syndrome (Panay *et al.,* 2020). The symptoms of POI has a wide range of manifestations (Torrealday, 2017).

New-onset menstrual irregularities, which can range from infrequent to excessively frequent menses before amenorrhea, are the first sign of ovarian insufficiency in some women. Other women can seek an initial medical examination due to distressing menopausal symptoms indicating an estrogen-deficient condition, such as hot flashes, dyspareunia, sleep disruptions, reduced libido, or vaginal dryness (Torrealday, 2017). While for some women, the diagnosis of POI may be discovered only during an infertility examination (Torrealday, 2017).

2.2.2.2 Secondary Ovarian Dysfunction

The ovaries are normal in secondary ovarian failure, but there is a problem getting hormone signals from the brain to them. Diseases of the pituitary gland or hypothalamus are the most common causes (Mikhael *et al*., 2019).

Pituitary tumours, such as prolactinomas, can cause hyperprolactinemia, which can also lead to secondary ovarian insufficiency (Pellegrini, 2016). A pituitary adenoma that secretes ACTH and causes Cushing syndrome is a significant but an unusual cause of secondary ovarian insufficiency (Pellegrini, 2016). Cushing syndrome may manifest as symptoms of androgen excess, and it is easy to confuse it with polycystic ovary syndrome, delayed congenital adrenal hyperplasia, or an androgen-producing tumour of the adrenals or ovary (Pellegrini, 2016). The GnRH pulse generator is the physiologic origin of the stimulus from the CNS to release gonadotropins for ovarian stimulation (Pellegrini, 2016). Certain medications, too much exercise, and certain eating disorders may all cause secondary ovarian failure. Some women with secondary ovarian failure may be able to have children if they have menstrual cycles (Pellegrini, 2016).

2.2.3 Metabolic Disturbances Associated With PCOS

Visceral obesity, insulin resistance, compensatory hyperinsulinemia, impaired glucose metabolism, dyslipidemia, inflammation, endothelial dysfunction, and hypertension are all examples of metabolic disturbances associated with PCOS (Dantas *et al*., 2013; Macut *et* al., 2020). Furthermore, metabolic disturbances has been linked to an increased risk of type 2 diabetes, as well as preclinical and clinical cardiovascular illnesses in various studies (Petrie *et al.,* 2018). Metabolic disturbance is comparable to PCOS in many ways, including the prevalence of abdominal obesity and insulin resistance (Alves *et al*., 2017). Insulin resistance and androgen excess, as well as environmental factors, appear to play a role in dyslipidemia in PCOS (SanchezGarrido and Tena-Sempere, 2020; Unluhizarci *et al*., 2021). A number of lipid abnormalities have been discovered in PCOS patients (Wild *et al*., 2011; Liu *et al*., 2019). The most common lipid pattern is a drop in High density lipoprotein (HDL-C) and an increase in triglycerides (DiNicolantonio and O'Keefe, 2018), which is linked to insulin resistance. The most atherogenic lipid profiles are found in obese women with PCOS (Spałkowska *et al*., 2018).

2.2.3.1 Insulin Resistance and hyperinsulinemia

Majority of women with PCOS have insulin resistance which leads to type two diabetes mellitus (Jałowiecka, 2020). Insulin resistance is a major cause of metabolic disturbance and a factor in the development of PCOS (Phinney and Volek, 2019). This physiological function is manifested in obese patients as a result of their body's excess adipocytes. Hyperinsulinemia is a complication of insulin resistance, which arises when the beta cells of the islet of Langerhans in the pancreas release an excessive amount of insulin (Jałowiecka, 2020). Some women with PCOS exhibit normal sensitivity to insulin or low-level insulin resistance, which is also a feature for Rotterdam criteria (Macut, 2017).

Insulin works by binding to the cell surface receptors which has complementary structure to insulin-like growth factor-1 (IGF-1) receptor (Macut. 2017). This increases the transfer of insulinresponsive glucose transporter 4 (GLUT4) from intracellular vesicles to the cell surface, which promotes glucose uptake (Fischer *et al*., 2019). The activation of phosphatidylinositol 3-kinase is responsible for this operation (PI3-K) (Macut, 2017).

Insulin and insulin-like growth factor-1 regulate sex hormone biosynthesis; these two molecules are known as the most important extra-ovarian factors that stimulate androgen development (Jałowiecka, 2020). Insulin also suppresses hepatic sex hormone binding globulin (SHBG) activity while modulating LH pulse amplitude. According to this perspective, insulin resistance and hyperinsulinemia (Jałowiecka, 2020; Rothenberg, 2017), appear to be the most significant extraovarian factors causing PCOS symptoms. (Barber, 2016). Insulin also enhances steroidogenesis in ovarian theca and granulosa cells in response to LH stimulation. (Rothenberg, 2017). Insulin signalling has a direct relationship

with inflammation and is known to affect fertility and weight gain. Inflammation impairs insulin action and glucose tolerance, and there is a possibility that inflammation has an effect on hyperandrogenism, insulin resistance, and abdominal obesity in PCOS patients (Phinney and Volek, 2019). Insulin resistance and hyperinsulinemia can cause both endocrine and reproductive symptoms in people with PCOS. Excess body fat, which is another common feature among these women (i.e. PCOS women), can aggravate the situation. Insulin resistance can, however, be seen in many people of average weight (Moghetti and Tosi, 2021). In a mouse model of diet-induced obesity and hyperinsulinemia, which showed reduced fertility and increased serum testosterone, similar to human PCOS, the researchers discovered that: in lean normoinsulinemic animals, selective repression of the insulin receptor in theca cells had no effect on the animal. However, in obese hyperinsulinemic animals, it strengthened reproductive and endocrine characteristics (Moghetti and Tosi, 2021). Implying the direct insulin's effects on the ovary are crucial in assessing fertility (Moghetti and Tosi, 2021).

There is also evidence to suggest that insulin resistance could be the result of PCOS rather than the cause (Moghetti and Tosi, 2021). In this regard, a few studies using multi-step hyperinsulinemic glucose clamps have shown that insulin resistance develops quickly in women after supraphysiological androgen administration (Moghetti and Tosi, 2021). Insulin-induced glucose uptake was impaired in hyper androgenic women relative to healthy controls in a two-step glucose clamp study, as predicted (Moghetti and Tosi, 2021).

Hyperandrogenemia is caused by IR and hyperinsulinemia, which stimulates ovarian theca cells to secrete androgens and increases the effect of LH on ovarian androgen production (Wang *et al.*, 2019). SHBG secretion is inhibited by both androgens and insulin, resulting in an increase in free and bioactive androgen levels, as well as a worsening of clinical androgen excess (Polak *et al.,* 2016). Furthermore, since IR plays a key role in the development of metabolic syndrome and cardiovascular disease in PCOS women, its treatment (dietary changes, insulin-sensitizing medications, and bariatric surgery) is thought to be crucial for both reducing IR and alleviating its effects (Faghfoori *et al.,* 2017).

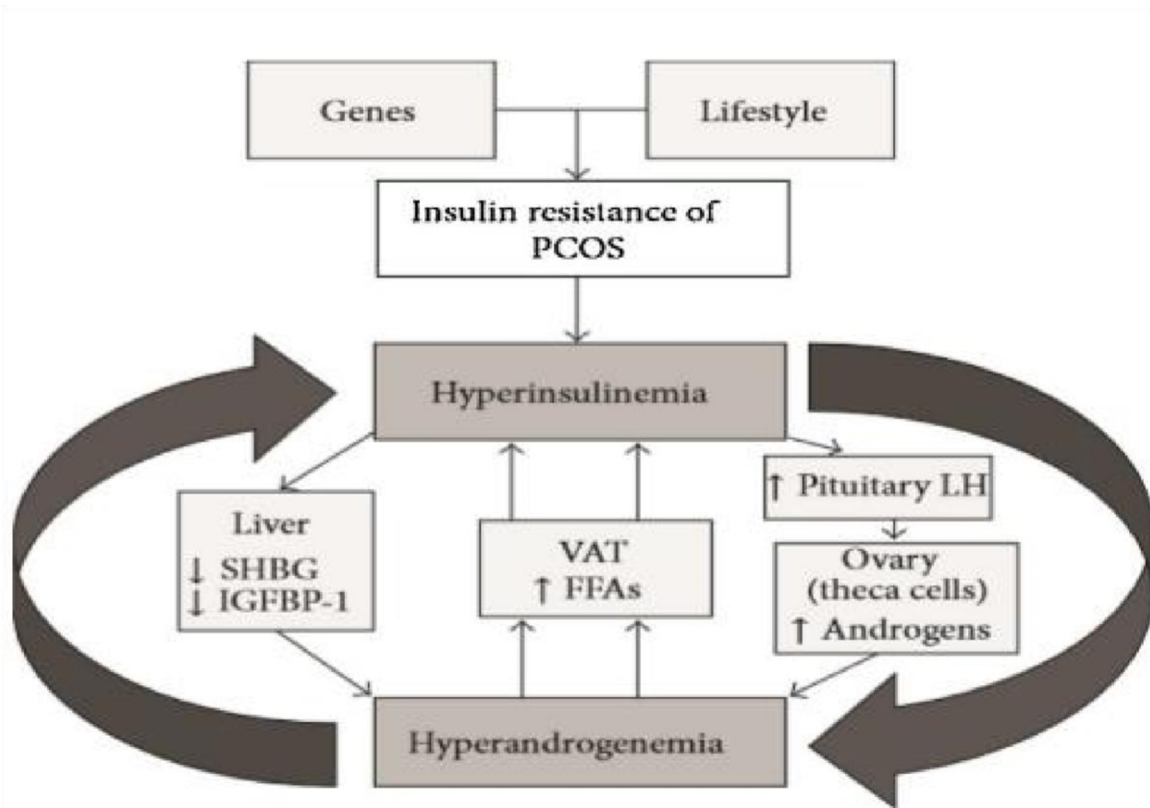


Figure 1: Pathway showing how insulin resistance affects the theca cells hence affecting its function

Source: Ajmal *et al.* (2019)

2.2.3.1 Visceral Obesity

Obesity is believed to play a role in the pathophysiology of PCOS, resulting in a more extreme phenotype of the condition. Menstrual dysfunction and increased androgen concentrations have been linked to rising adiposity (Escobar-Morreale, 2018; Golub *et al.,* 2020). Obesity worsens hyperandrogenism in PCOS (Zeng *et al*., 2020). A rise in androgen levels is linked to a reduction in sex hormone binding globulin (Rothenberg, 2018). Furthermore, since adipose tissue contains many steroidogenic enzymes that transform androstenedione into testosterone and testosterone into dihydrotestosterone (DHT), a more potent androgen, increased adiposity can contribute to hyperandrogenism (Yanes *et al.,* 2017; Rothenberg *et al.,* 2018). In several studies of obese teenage girls, there is a proportional increase in free testosterone concentration as BMI rises (Rothenberg, 2018). Insulin resistance can be present in normal-weight PCOS patients; however, insulin resistance is exaggerated when obesity is present (Rothenberg *et al.,* 2018). Furthermore, obese teenage girls with PCOS have higher insulin resistance and hyperinsulinemia than obese patients without PCOS (Rothenberg, 2018; Phinney and Volek, 2019). PCOS patients have visceral adiposity (Durmus, 2017). Obesity increases metabolic and reproductive outcomes of PCOS (Glueck, 2018). Obese people with PCOS have a more extreme phenotype than non-obese women with PCOS, with more severe menstrual irregularity, infertility, miscarriage, pregnancyinduced hypertension, gestational diabetes, prematurity, biochemical and clinical abnormalities (Gluek, 2018).

Women with PCOS are much more likely to have visceral adiposity and elevated visceral adiposity levels, which are linked to insulin resistance (Gluek, 2018). Visceral obesity is a condition that is associated with hyperandrogenism (Zeng, 2019). Androgens induce accumulation of adipocytes in the abdominal region of the body (Milutinović *et al*., 2017), this accumulation may result to dysfunction of adipose tissue, insulin resistance and the accumulation of lipid (Zeng, 2019).

While obesity is not a diagnostic criterion for PCOS, both obese and non-obese PCOS patients have more visceral adipose tissue (VAT) than women without PCOS, VAT has been shown to be positively associated with total androgen levels, implying that obesity plays an important role in PCOS (Jena *et al.,* 2018). Obesity demonstrates as increased level of triacylglycerides (TAG), cholesterol, free fatty acids and several apo-lipoprotein abnormalities (Torre-Villalvazo *et al.,* 2018). Increase in free fatty acid causes a decrease in the sensitivity of insulin and also reduction in glucose uptake (Chow *et al.,* 2017). Free fatty acid can stimulate serine/threonine kinases thereby decreasing tyrosine phosphorylation of IRS-1, hence, encouraging insulin resistance

(Zeng, 2019). Visceral obesity and insulin resistance work in synergy; they stimulate the production of androgens in the ovaries and adrenal glands (Delitala *et al.,* 2017) and subsequently increase abdominal obesity and inflammation, hence, creating a cycle. Weight gain and obesity in women with PCOS are induced by insulin resistance, which leads to increased steroidogenesis and hyperandrogenism, this explains the close relationship between body weight and the magnitude of PCOS's hyper-androgenic characteristics (Barber *et al.,* 2019).

In the largest analysis of urinary steroid profiles in women with PCOS (n = 178) versus BMImatched control women (n = 100), Barber *et al.* (2019) found a connection between PCOS and increased 5-alpha reductase activity. Jena *et al.,* (2019) analysed 58 women with newly diagnosed PCOS and BMI-matched controls, it was discovered that cases had greater visceral abdominal fat thickness (p=0.003) than controls. Furthermore, despite having a normal BMI, non-obese women with PCOS had increased visceral adiposity and higher inflammatory markers compared to matched non-obese controls (Jena *et al*., 2019).

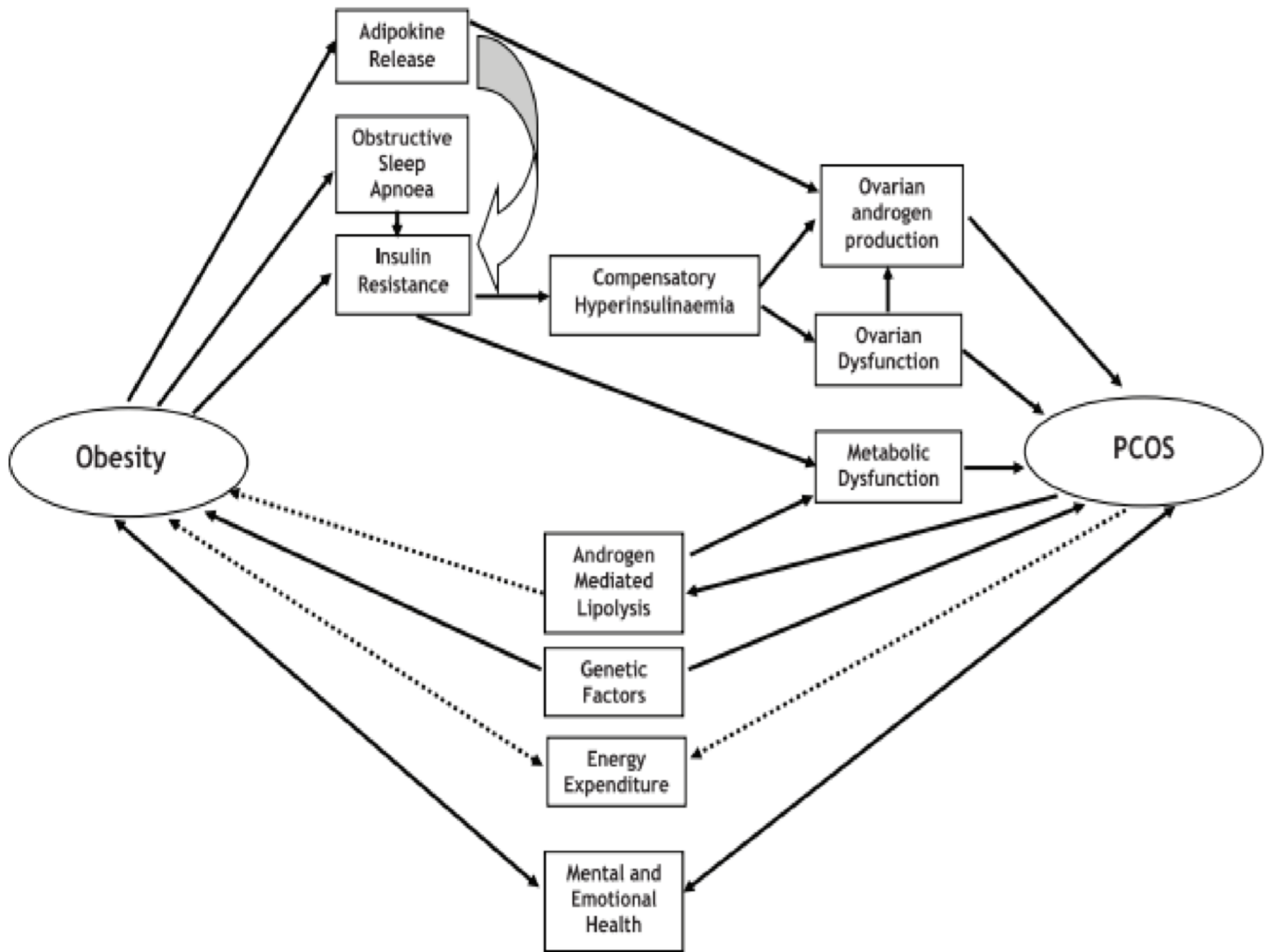


Figure 2: Summary of the mechanisms that relate obesity to PCOS. PCOS stands for polycystic ovary syndrome

Source: Barber *et al.* (2019)

2.2.3.2 Cardiovascular Risk factors

The etiology of PCOS frequently involves insulin resistance, and this places women at a higher risk for cardiovascular disease (Osibogun *et al.,* 2019). Women with PCOS are more susceptible to have CVD risk factors, as a result of the unusual hormonal pattern which are primarily mediated by insulin resistance, dyslipidemia, and hyperandrogenism ((Scicchitano *et al*., 2012; Osibogun *et al.,* 2019). Several previous research have found that various measures of subclinical cardiovascular disease, such as coronary artery calcium scores, C-reactive protein, carotid intimamedia thickness, and endothelial dysfunction, are more common in women with PCOS (Hughan *et al.,* 2016; Osibogun *et al.,* 2019).

2.2.3.3 Hypertension

Women with PCOS are more susceptible to cardiovascular risk factors such as hypertension (Gandevani *et al.,* 2018; Özkan *et al*., 2020) and this has contributed to the fact that women with PCOS are more likely to experience cardiovascular disease (CVD) later in life (Özkan *et al*., 2020). Over 30% of women with the syndrome had a blood pressure of 130/85 mmHg, and high baseline BP was found to be an effective predictor of CVD (Djuro *et al.,* 2019). Women with PCOS are more likely to experience pregnancy-related complications such as hypertension, pre-eclampsia, gestational diabetes, and increased neonatal morbidity (Djuro *et al.,* 2019). Metformin has been shown to improve metabolic, cardiovascular, and thrombotic events in diabetic patients. As a result, it is likely that some of the cardiovascular events in PCOS pregnancies may be improved with metformin therapy (Balen *et al.,* 2016).

The association between high levels of androgens and an increased prevalence of cardiovascular disease is clearly established in PCOS patients. According to a study by Shroff *et al*., 2007, the prevalence of subclinical coronary atherosclerosis (an increase in the build-up of plaque on the arteries that supplies the heart with blood) in obese young women with PCOS was found to be 5 times higher than in a reference group of healthy women with normal ranges of serum hormones and cholesterol (39.0 percent vs. 9.9 percent) (Amiri *et al.,* 2020).

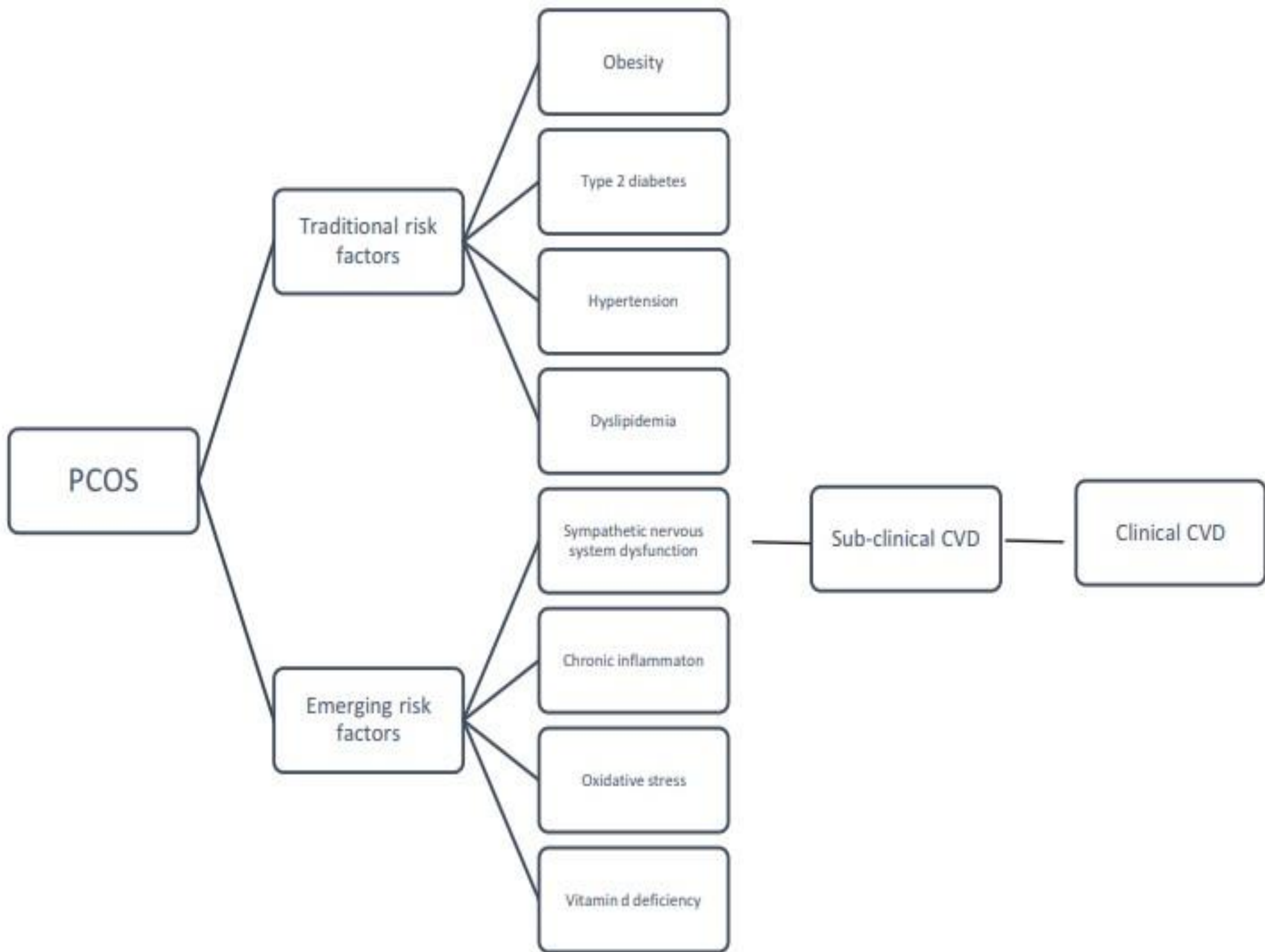


Figure 3: Cardiovascular Risk Factors in PCOS

Source: Kakoly *et al.* (2018)

2.2.3.4 Dyslipidemia

Considering the fact that PCOS has a lot of heterogeneity and individual differences, dyslipidaemia is one of the most common symptoms. (Wang *et al*., 2019). Nearly 70% of PCOS patients in the United States and Brazil, and 36% in the Mediterranean, are affected, 48.3% in China exhibiting increased levels of low-density lipoprotein (LDL), triglycerides (TG), and reduced levels of high-density lipoprotein (HDL) (Wang *et al*., 2019). Many PCOS patients are obese and have atherogenic dyslipidaemia, both of which are linked to an increased risk of CVD (Vine *et al*., 2017). Women suffering from PCOS have lipid abnormalities. According to a recent study, women with PCOS frequently have mild hypercholesterolemia (Pergialiotis *et al*., 2018). There are several lipid profiles present in patients with PCOS, these are: low levels of high-density lipoprotein cholesterol (HDL-C), high triglyceride (TG), total cholesterol (TC), and low-density lipoprotein cholesterol (LDL-C), as well as slightly higher lipoprotein concentrations (Ghaffarzad *et al*., 2016).

Studies have shown that hyperandrogenism is a major cause of lipid abnormalities; however, variations in lipid-related genes facilitate the production of hyperandrogenism (Liu *et al*., 2019). Women with PCOS and mild hypercholesterolemia levels have a higher body mass index (BMI) and higher fasting insulin and IR levels than women with normal cholesterol levels (Pergialiotis *et al*., 2018). Since centrally located adipocytes tend to have a negative effect on blood lipids, the ratio of waist-to-hip is higher in PCOS women making it more vulnerable to dyslipidaemia; centrally distributed adipose tissue may also secrete a variety of adipokines into circulation to increase inflammation, hence, indicating that adipokines are associated with inflammation in PCOS (Liu *et al*., 2019). PCOS patients have high level of LDL and high levels of triglycerides (Seyam *et al.,* 2018), HDLs are dependent on the development of the metabolic syndromes (Shaman *et al.,* 2017). A significant increase in LDL has been discovered in PCOS and these levels of LDL decrease after treatment with statins (Seymam *et al.,* 2017). Women with PCOS have more dyslipidaemia, and smaller LDL particles, which increases their risk of cardiovascular diseases (Kumar *et al*., 2017). As a result, an irregular lipid profile influences the pathological progression of PCOS (Liu *et al.,* 2019).

2.3 Diagnostic Criteria of PCOS

The European Society of Human Reproduction and Embryology/ American Society for Reproductive Medicine Rotterdam consensus (ESHRE/ASRM) came up with the diagnostic criteria for PCOS in 2006, stating the requirement of two or three features: anovulation or oligoanovulation, Clinical and/or biochemical hyperandrogenism, and polycystic ovarian morphology (PCOM) seen on an ultrasound (Mohammad and Seghinsara. 2017).

The Androgen Excess Society (AES) considered that androgen excess is an important factor in the development and pathogenesis of PCOS and it was then established that androgen excess must be present and followed by either oligo-amenorrhoea or PCOM or both (Mohammad and Seghinsara.

2017).

2.4 Prevalence of PCOS

The global prevalence of PCOS is estimated to be in the range of 6% and 26% (Manisha *et al.,* 2020). A systematic and meta-analysis was conducted by Bozdag *et al.* (2016) on other studies that concluded the re prevalence of PCOS according to at least one of the approved diagnostic criteria. It was discovered that the global prevalence ranged from 6% to 10% and this estimate was dependent on the diagnostic criteria used. The range of prevalence on Rotterdam criteria was 8 to 13%, this criteria has four phenotypes (Neven *et al.,* 2018). Several factors contribute to the variability in PCOS prevalence estimates. First, depending on the data collection area, the sample population's racial, cultural, and age distribution will be heterogeneous (Manisha *et al.,* 2020). These factors can have an impact on how hyperandrogenism manifests clinically and how ovarian follicles appear on ultrasonography over time (Manisha *et al.,* 2020). Another factor is due to expense, time, and personnel constraints, performing biochemical and ultrasound tests for the diagnosis and exclusion of related disorders may be economically challenging (Manisha *et al.,* 2020). Also, sampling technique for study may not accurately represent population prevalence (Manisha *et al.,* 2020).

2.5 Symptoms of PCOS

PCOS manifests itself in a variety of ways, with signs and symptoms appearing on a spectrum and varying in intensity. PCOS symptoms include irregular menstrual cycles and hyperandrogenism (hirsutism, acne, and alopecia), as well as fertility issues, obesity, and physiological issues. (Balen, 2017). PCOS signs are discovered prior to the onset of puberty in its clinical phenotype (Victorin, *et al.,* 2020). PCOS is five times more likely to grow in female children born to PCOS mothers. (Crisosto *et al.,* 2020; Risal *et al.,* 2020; Victorin *et al.*, 2020), as well as the fact that neonates have a longer

anogenital distance (AGD) (Barrett *et al.,* 2018), with elevated levels of ovarian AMH (Detti *et al.,* 2019) indicative of antral follicle numbers (Victorin, *et al.,* 2020).

Anovulation, also known as oligo-ovulation, is a popular PCOS symptom. PCOS causes hyperandrogenism because certain cysts release androgens, which cause virilisation or the expression of male-like characteristics in females, these characteristics include: weight gain, abdominal and subcutaneous fat, hirsutism (facial and body hair), male-pattern alopecia (hair loss), clitoromegaly (enlargement of the clitoris), deep voice, seborrhea (oily skin), acne, and other visible symptoms of hyperandrogenism (Patel, 2018). Aside from these morphological characteristics, there is a change in metabolic profile. Insulin resistance (IR), is one of the most common symptoms of PCOS (Wang *et al.,* 2019). Hyperinsulinemia occurs as a result of IR which can lead to diabetes mellitus. (Patel, 2018). Fat accumulation across the belly, also known as central adiposity, is caused by high insulin levels (Meldrum, *et al.,* 2017). Body mass index (BMI) of 30 or higher is seen in the majority of females with PCOS (Gupta *et al.,* 2019). Hypertension, cardiovascular issues, dyslipidemia, etc. are co-morbidities of PCOS (Ding *et* al., 2018; Patel, 2018; Wu *et al*., 2020). Sugar cravings, excessive urination, delayed recovery, nausea, blurred vision, tingling sensations, mood swings, anxiety, and depression episodes are common in PCOS patients (Patel, 2018). This is appropriate, given that both of these conditions are linked to diabetes. Pelvic pain, fever, nausea, vomiting, urinary problems, constipation, and other symptoms are common in patients (Patel, 2018). The abnormal urinary and bowl movement is caused by massive cysts pressing against the bladder or rectum (Patel, 2018). Another symptom of PCOS is sleep apnea (a sleep disorder in which breathing regularly stops and starts). This is caused by a change in sex steroid levels (Patel, 2018). A characteristic of PCOS is a mucus-deficient endocervix and smooth vagina, which can be seen during a pelvic exam (Patel, 2018). Skin produces light brown or black patches as a result of the hormonal imbalance in PCOS, a disorder known as acanthosis nigricans (González-Saldivar *et al* 2017). This skin pigmentation is more common on the abdomen, armpits, legs, and breasts. PCOS symptoms, on the other hand, can be thought of as a spectrum, as the signs differ between races and individuals (Balen, 2017). Hirsutism is moderate or absent in PCOS females of South Asian and Scandinavian descent because the androgen sensitivity of the pilo-sebaceous glands differs, but hirsutism is more prevalent in PCOS patients of Middle Eastern and Mediterranean origin (Patel, 2018).

2.6 Etiologies of PCOS

The etiology of PCOS is unknown (Abbott *et al*., 2005). The causes of PCOS are uncertain, and as a result, the treatments currently unavailable (Watson, 2021). PCOS has a central role in which insulin resistance (IR) and hyperandrogenism (HA) are key etiologies and primary endocrine features (Wang et al. 2019). HA and IR may both play a role in the pathology of PCOS (Wang et al., 2019). PCOS is the result of environmental and genetic influences. All of these conditions are related to high androgen exposure during the earliest stage, a state of reaction oxidation state (ROS), the immune system, and endocrine abnormalities. (Wang *et al.,* 2019).

Hyper-secretion of luteinising hormone (LH), and hyperinsulinemia may all play a role in the pathophysiology of PCOS, but another hypothesized pathophysiological mechanism includes ovarian hyperandrogenism, because greater ovarian follicular growth is implicated in follicular atresia (Fenichel et al., 2017; Shaaban et al., 2019). A metabolic, hormonal, nutritional, or toxic shift happening during embryonic development and early phases of female gonad differentiation could be a cause of any of these defects (Shaaban et al., 2019). But though the specific origin remains unknown, each irregularity can be attributed to this common factor. Numerous studies have revealed that the development of PCOS requires the combination of several genetic and environmental variables. (Shaaban *et al.,* 2019).

Lifestyle, nutrition, or other infectious factors can aggravate PCOS (Ajmal et al., 2019). Insulin resistance and hyperinsulinemia have a deleterious effect on the ovaries, contributing to anovulation (Ajmal et al., 2019). PCOS has the additional effect of elevating gonadotropinreleasing hormone (GnRH), follicle-stimulating hormone (FSH), luteinizing hormone (LH), and prolactin levels (Ajmal et al., 2019). Furthermore, research shows that genetics are important in the development of PCOS (Ajmal *et al.,* 2019). PCOS is reported to be caused by 241 gene variants, according to databases (khan *et al.,* 2019; Ajmal *et al.,* 2019). PCOS is caused by a deficiency in the transcriptional activity of a gene caused by polymorphism (Ajmal et al., 2019). Mostly, these genes include the androgen receptor, luteinizing hormone receptor, folliclestimulating hormone receptor, and leptin receptor genes (Ajmal et al., 2019). Ovarian dysfunction is caused by a gene abnormality that disrupts the biological mechanism (khan *et al.,* 2019).

2.7 Management Options of PCOS

There are many management options in the treatment of PCOS, most of these management mechanism basically helps in combating the symptoms/manifestations of this disorder (Barber *et al.,* 2019; Jałowiecka, 2020). It is recommended that aggressive lifestyle changes, especially in the area of diet,

be used to start managing PCOS changing to a healthy diet lifestyle helps in combating the insulin resistance and controlling the weight which are associated with the symptoms of the disorder. This lifestyle modification mechanism can lead to weight loss, increase anovulation indirectly, and lower systolic arterial pressure (Hugo *et al.,* 2016). Weight loss mechanism is the most effective management strategy for PCOS, however, the implementation of this strategy is challenging and it has a very high rate of failure (Barber *et al.,* 2019). Although bariatric surgery is a great substitute to this lifestyle implementation to achieve an effective longterm weight-loss, this strategy cannot be implemented by a great amount of the population (Barber *et al.,* 2019).

Enhanced appetite causes weight-gain after loss of weight and therefore it is hence logical to develop therapy that supress appetite enhancement to maintain weight loss (Barber *et al.,* 2019). Treatments to improve hyperlipidaemia, in addition to appetite suppression, may also have a positive effect on hyperandrogenemia in PCOS (Barber *et al.,* 2019). The application of mindfulness to lifestyle strategies is an alternative innovative weight-loss approach that is completely scalable on a population level and can be implemented by everyone (Barber *et al.,* 2019).

Metformin, oral contraceptives, anti-androgens, clomiphene citrate, and thiazolidinediones are also used to manage various presentations of PCOS (Rath, 2018). Metformin is widely used to treat most of the clinical symptoms of PCOS, either alone or in conjunction with other medications (Rath, 2018). It was concluded by a group of researchers that metformin therapy decreases hyperinsulinaemia and hyperandrogenaemia, independent of changes in the body weight (Rath, 2018; Yang *et al.,* 2018; Sharma *et al.,* 2019). These changes were linked to significant, long-term improvements in menstrual irregularities and the reestablishment of ovulation in a large number of patients (Rath, 2018; Kravos *et al.,* 2021). When lifestyle intervention (LSI) is inadequate, metformin is well-established for patients with PCOS and compromised glucose homeostasis or type 2 diabetes mellitus (T2DM), as well as for the treatment of menstrual irregularity in women who are unable to use oral contraceptives (Teede, *et al.,* 2018; Kravos *et al.,* 2021). Oestrogen suppresses androgen and adrenal activity while oral contraceptive pills (OCPs) minimize hyperandrogenism (Rath, 2018). At bedtime, glucocorticoids such as dexamethasone 0.5 mg or prednisolone 5 mg decrease androgen production. Cyproterone acetate has been shown to be effective in the treatment of hirsutism (prevents hirsutism recurrence) (Rath, 2018). There is still no definitive response as to how long PCOS can be treated with metformin, but it appears that when PCOS patients are overweight or obese, continued care is needed for long-term clinical benefit (Kravos *et al.,* 2021). Other treatment options for weight loss and menstrual cycle restoration include a multi-component lifestyle plan that includes food, exercise, and behavioural interventions (Kim *et al.,* 2020) should be carried out implemented. Clomiphene citrate prevents negative feedback loop by binding to estradiol receptors in the hypothalamus and as a result leads to the increased secretion

of FSH (Mitwally *et* al., 2016; Rose and Brown, 2020). Clomiphene possesses estrogenic and anti-estrogenic effects, although the exact mechanism of action is unknown (Wallach *et al.,* 1984). Clomiphene stimulates the secretion of gonadotropins, folliclestimulating hormone (FSH), and luteinizing hormone (LH), which leads to ovarian follicle development and maturation, ovulation, and subsequent development and function of the coprus luteum, resulting in pregnancy (Holesh *et al*., 2017). Gonadotropin production can be triggered by direct stimulation of the hypothalamic-pituitary axis or by estrogens competing with endogenous estrogens in the uterine, pituitary, or hypothalamus. Clomiphene does not appear to have progestational, androgenic, or antrandrogenic effects, nor does it appear to interact with pituitaryadrenal or pituitary-thyroid function.

2.8 Oestrous Cycle

The sexual cycle in rodents is referred to as the oestrous cycle (Ajayi and Akhigbe, 2020). Although, cycles usually differ between species, with animals following the cycle best suited to a specific environment or reproductive strategy (Janko *et al.,* 2016). The oestrous cycle of rodents is analogous to the human sexual cycle, also known as the menstrual cycle (ovarian and uterine cycles) (Ajayi and Akhigbe, 2020). Oestrous cycles are distinguished by morphological changes in the ovaries, uterus, and vagina that occur during the four different phases known as proestrus, estrus, metestrus, and diestrus (Figure 4) (Ajayi and Akhigbe, 2020; Auta and Hassan, 2016) and this cycle lasts for 4 to 5 days. Cell types seen in vaginal smears are commonly used to identify these stages (Ajayi and Akhigbe, 2020). The reproductive process and estrous cycle of mice begin on the 26th day after birth, with the opening of the vaginal canal, which occurs about 10 days before vaginal cornification (Ajayi and Akhigbe, 2020). Apoptosis-mediated vaginal opening is a significant secondary character in mice that is used to predict puberty (Ajayi and Akhigbe, 2020).

Puberty in female rats is followed by the pulsatile release of luteinizing hormone (LH) about 30 days after birth (Ekambaram and Joseph, 2017). The anestrus lasts around 8 to 9 days until the first proestrus. Then comes the first proestrus, estrus, metestrus, and diestrus. Metestrus only happens when there is no chance of natural conception (Ajayi and Akhigbe, 2020). Follicles mature rapidly in proestrus before ovulation at the end of proestrus. Two to four large follicles can be seen in a unilateral ovarian segment at this time (Sato *et al*., 2016). At ovulation, follicles turn into corpora lutea, which have more basophilic and smaller luteal cells compared to later stages of the cycle (Sato *et al*., 2016). At the stage of metestrus, these corpora luteal begin to regress, and their regress continues into diestrus stage. In the next estrous stage, the pattern of growth and regression repeats itself (Sato *et al*., 2016). Regressive corpora luteal can last for up to 14 days, as a result of this, rodent ovaries contain a large number of corpora luteal (Sato *et al*., 2016). The menstrual cycle in humans, on the other hand, has three phases: menstrual, proliferative (follicular), and secretory (luteal) (Ajayi and Akhigbe, 2020). This cycle starts when a

child reaches puberty. From the start of one menstrual cycle to the start of the next, it usually takes about 28 days (Ajayi and Akhigbe, 2020). The ovulatory process occurs in the middle of the cycle, between the proliferative and secretory stages (Mukhopadhyay *et al.,* 2019) and here, ovulation occurs during the night of estrus 10-12hours after an LH surge (Paccola *et al*., 2018). High estrogen levels are associated with the proliferative process, while high progesterone levels are associated with the secretory phase (Ajayi and Akhigbe, 2020). The estrous cycle is always disrupted in rats with PCOS, mainly due to the irregularity of steroid hormones responsible for the regulation of ovarian function (Ndeingang *et al*., 2019).

Several studies have proposed different methods for measuring the estrous cycle based on physiology and anatomy changes in the animal (Ajayi and Akhigbe, 2020). For example, some of these approaches are visual evaluation, vaginal smear/cytology, Urine biochemistry, vaginal wall impedance, and histological analysis of the reproductive organs (Auta and Hassan, 2016; Ajayi and Akhigbe, 2020). Although vaginal cytology has been reported to be the best method of assessment of estrous cycle in animal studies (Ajayi and Akhigbe, 2020).

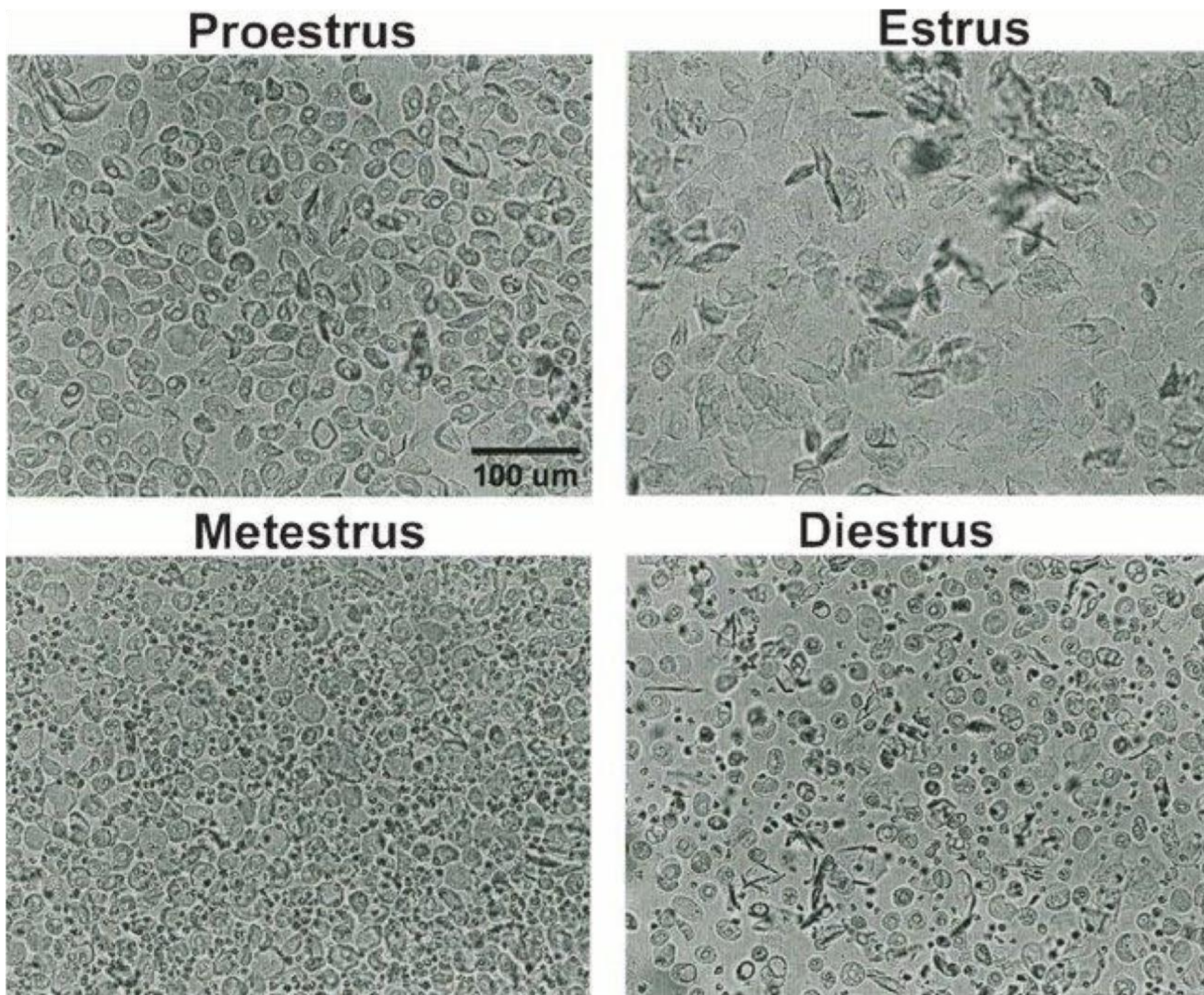


Figure 4: Four phases of oestrous cycle of Wistar rat

Source: Hubscher *et al.,* (2005)

2.9 Medicinal Plants for PCOS Management

Medicinal plants are said to play an important role in the prevention and treatment of microbial diseases in traditional folklore (Shakya, 2016; Elansary *et al.,* 2018). In economically depressed regions of the world, such as Africa, medicinal plants are an important part of disease treatment (Elansary *et al.,* 2018). The South Mediterranean region, which includes Egypt, is rich in medicinal plants that have been used in alternative medicine for thousands of years (El-Ghani, 2016; Elansary *et al.,* 2018). Despite the twentieth century's remarkable progress in synthetic organic medicinal products, over 25% of prescribed medicines in industrialized countries are still made from natural sources derived directly or indirectly from plants (El-Ghani, 2016). Traditional medicine is defined by the World Health Organization (WHO) as the sum total of all knowledge and practical skills, whether explicable or not, used in the diagnosis, prevention, and treatment of physical, mental, and social imbalances, relying solely on practical experience and observation passed down from generation to generation, whether verbal or in writing (Che *et al.,* 2017; ElGhani, 2016). Traditional medicine has been described by the World Health Organization (WHO) as one of the most reliable ways to achieve universal health coverage for the world's population (El-Ghani, 2016).

2.9.1 *Parquetina nigrescens*

Parquetina nigrescens is an herbaceous, perennial twine belonging to the family *Asclepiadaceae* (Femi-Olabisi, *et al.,* 2020; Adeyomoye and Adewoye, 2018). This plant is a monotypic genus of *Parquetina* and having only *Parquetina nigrescens* (Afzel.) Bullock, as its only species (Sopeyin and Ajayi, 2016). *Parquetina nigrescens* is commonly known as, African parquetina (English), Kwankwanin (Hausa), Mgbidim gbe (Igbo), Otonta (Asaba), Ewe Ogbo (Yoruba), and Inuwu elepe (Ife) (Kayode and Yakubu, 2017; Femi-Olabisi *et al.,* 2020). It is commonly found in the villages and forests of Senegal and Nigeria, this plant can also be found in parts of Ghana (FemiOlabisi *et al.,* 2020). It is mostly herbaceous, but as it ages, it become woody (Sopeyin and Ajayi, 2016). It has a woody base of 7-8m long (Femi-Olabisi *et al.,* 2020). It has broad coriaceous leaves that are 10-15cm long and 6- 8cm wide, as well as a fleshy coriaceous corolla that is pink, maroon, or deep crimson to black-violet on the inside and pubescent or hirsute stamens with pollen in tetrads (Sopeyin and Ajayi, 2016).

All the parts of this plant has different therapeutic effects. *P. nigrescens* is mostly used by traditional healers in the treatment of anaemia in humans, helminthiasis, gonorrhoea, menstrual disorders and it is also used as a cardiac-tonic and in the treatment of wounds. It is also commonly used as an aphrodisiac (Sopeyin and Ajayi, 2016). Different researches have unravelled many properties of the plants including

its catecholamine-like effects, antioxidant, antibacterial, antifungal properties, hypoglycaemic activities, anti-inflammatory and antipyretic properties (Parham *et al.,* 2020).

Scientists have recently reported phytochemical constituents of *Parquetina nigrescens* fruit as containing reducing sugars, alkaloids, saponins, cardiac glycosides, flavonoids, cardiac glycosides, steroids, tannins, phlobatannins, cardenolides, phenolics, anthraquinones, triterpenes with alkaloids and proximate analysis (Uwaya *et al.,* 2017; Kayode and Yakubu, 2017). The leaves of this plant is mostly used for traditional remedies and as a result, most scientific investigations are carried out using the leaves (Kola-Mustapha, 2019).

Parquetina nigrescens has been investigated scientifically by several researchers for a number of pharmacological activities (Airaodion *et al.,* 2019). A group of researchers reported that *P. nigrescens* exhibited antidiabetic and antihyperlipidemic activity (Femi-Olabisi *et al.,* 2020). Kayode and Yakubu (2017) reported that the aqueous extracts of *P. nigrescens* leaves restored both the physical and biochemical indices of male sexual activity via changes in the reproductive hormones in paroxethin-induced sexually dysfunction rats. Adeyomoye and Adewoye (2018) reported that the methanoic extracts of *P. nigrescens* leaves possess active phytochemicals which are useful in improving the oxidative stress and renal dysfunction in diabetes mellitus. Also, Okunrobo *et al.* (2014) reported that the methanoic extracts of the fruit bark of *P. nigrescens* exhibits potential dose-dependent analgesic effect both centrally and peripherally in animal models (mice). In the same vein, Oloyede *et al.* (2017) concluded that extracts (both aqueous and ethanol) of *P. nigrescens* leaves was found to exhibit antimicrobial effect is the gastrointestinal region of wistar rats. Femi-Olabisi *et al.* (2020) also reported that aqueous extract of *P. nigrescens* leaves administered 100mg/kg to female wistar rats reduced the effect of mifepristone on the liver and the kidney and this had no morphological change to the kidney, liver and uterus of the animal. Lastly, Banwo *et al.,* (2020) reported that the mixture of methanol extracts of both *Parkia biglobosa* and leaves of *P. nigrescens* exerted in equal proportion was most effective in castor oilinduced diarrhoea study with high antioxidant activities documented.

Figure 5: Leaves of *Parquetina nigrescens*

Source: Ademola (2019)

2.8.2 *Cinnamomum verum* (Cinnamon)

Cinnamomum verum commonly called Cinnamon, a common spice and flavour has been used traditionally as a medicine in the treatment of various diseases (Sachan *et al*., 2018). This herb was identified as an insulin potentiating agent about 20 years ago (Dou *et al*., 2018). Cinnamon extracts work as an amplifier of insulin-receptor function and an inhibitor of the enzyme that prevents insulin-receptor attachment (Dou *et al*., 2018). Cinnamon contains procyanidin polyphenol type-A polymers, which can increase insulin receptor autophosphorylation and inhibit protein tyrosine phosphatase I (Anderson *et al*., 2004). In insulin-responsive tissues of people with extreme obesity or non-insulin-dependent diabetes mellitus (NIDDM), insulin receptor kinase autophosphorylation and subsequent phosphorylation of its major substrate are dramatically reduced (Meyts, 2016; Dou *et al*., 2018). PCOS is presented with insulin resistance and obesity. Cinnamon has anti-obesity effects (Soliman *et al*., 2012). Cinnamon extract has been proven to help glucose consumption and alleviate insulin resistance caused by high fructose diets by enhancing the insulin signaling pathway (Qin *et al*., 2010). The impact of cinnamon in the treatment of PCOS was confirmed by its effect in lowering the levels of insulin growth factor 1 (IGF-1) and increasing the levels of insulin growth factor binding protein 1 (IGFBP-1) in the plasma and ovarian tissue (Dou *et al*., 2018).

2.8.3 *Vitex agnus-castus* (Pepper tree)

Vitex agnus-castus commonly known as pepper tree. The fruit and seed of this plant is used to regulate the menstrual cycle of an individual, it is majorly used in the regulation of hormonal imbalances (Feyzollahi *et al*., 2021). A rat model of letrozole-induced PCOS was used to study the effect of Vitex agnus-castus fruit on KISS-1 gene expression (Feyzollahi *et al*., 2021). Treatment of PCOS mice with Vitagnus extract resulted in a considerable return of the LH/FSH ratio to normal, a significant decrease in testosterone, and a significant increase in estrogen. In PCOS mice, Vitagnus therapy resulted in fewer follicular cysts, a higher number of antral and Graafian follicles, and a thinner theca layer. These findings revealed that Vitagnus extract alleviated the symptoms of ovarian syndrome and restored ovulation in the ovaries (Feyzollahi *et al*., 2021).

2.8.4 *Camellia sinensis* (Green Tea)

Camellia sinensis also called green tea. The leaves of this plant is rich in flavonoids and is used as a medicinal plant in many countries (Tehrani *et al.,* 2017). This plant has been discovered to reduce the risk of cardiovascular diseases, fasting blood glucose and checks diabetes in diabetic patients (Tehrani *et al.,* 2017). Green tea drinking has been shown to help overweight and obese women with PCOS lose weight, reduce insulin resistance, and lower free testosterone levels. It was discovered and reported that drinking of green tea has a substantial effect on the weight loss of women with PCOS (Tehrani *et al.,*

2017). Green tea consumption can aid in weight loss, lower BMI, reduce insulin resistance, and lower free testosterone in overweight and obese PCOS women (Tehrani *et al.,* 2017).

2.9 Gas Chromatography Mass Spectrometry (GC-MS)

Gas chromatography-mass spectrometry is an analytical technique (Medeiros, 2018). It is the combination of two methods (Gas chromatography and mass spectrometry) to form one technique which is used for the analysis of organic mixtures (Medeiros, 2018). Gas chromatography is used to isolate the components of a mixture, while mass spectrometry is used to characterize each component separately. A sample containing a number of organic compounds can be evaluated qualitatively and quantitatively using a combination of the two techniques (Medeiros, 2018). The volatile and thermally stable substitutes in a sample are separated by GC, while the analyte is fragmented by GC-MS and classified based on its mass. It becomes GC-MS/MS after adding a mass spectrometer.

In the 1950s, Roland Gohlke and Fred Mclafferty developed the use of mass spectrometer as the detector in gas chromatography (Hites, 2016; Silva *et al.,* 2016). This instrument has been simplifeied as a result of the development of affordable and small sized computers, and this has also influenced in the time taken for a sample to be analysed (Gruber, 2020). The GC-MS is built on two major techniques: the gas chromatography and the mass spectrometer. The gas chromatograph makes use of capillary column which is determined by the column's dimensions (length, diameter, and film thickness) as well as the phase properties (Harvey, 2020). As the sample moves the length of the column, the difference in chemical properties between various molecules in a mixture will distinguish the molecules (Ozlem, 2016). The molecules elute from the gas chromatograph in various periods of time (called retention time), allowing the mass spectrometer downstream to trap, ionize, accelerate, deflect, and detect the ionized molecules separately (Falaki, 2018). The mass spectrometer achieves this by ionizing each molecule and detecting these fragments based on their mass to charge ratio (Rajawat and Jhingan, 2019). Combining the two processes decreases the risk of error since two separate molecules are unlikely to behave similarly in a gas chromatograph and a mass spectrometer (Charde *et al.,* 2013). As a result, when an identifying mass spectrum occurs at a specific retention period in a GCMS study, it usually means that the analyte of interest is present in the sample (Luedemann *et al.,* 2008).

2.9.1 Principle of GC-MS

The primary aim of instrument analysis is to determine how much of a substance is present (Ratiu *et al.,* 2017).

The GC/MS instrument separates chemical mixtures (the GC component) and distinguishes the compounds at a molecular level (the MS component) (Charde *et al.,* 2013). This is accomplished by comparing the relative concentrations of the various atomic masses in the resulting spectrum (Nicolescu, 2017). There are two types of analysis: comparative and original. Comparative analysis compares a given spectrum to a spectrum library to see if its characteristics are present in any of the library's samples (Charde *et al.,* 2013).

The carrier gas (helium) vaporizes the sample and sweeps it into a chromatographic column after it is pumped into the GC inlet (Abdelrahman, *et al.,* 2017). The sample passes through the column, and the compounds that make up the mixture of interest are distinguished by their interactions with the column's coating (stationary phase) and the carrier gas (mobile phase) (Apeh *et al.,* 2020). The column's final section passes through a heated transfer line before terminating at the ion source's entrance, where compounds eluting from the column are transformed to ions (Falaki, 2018). A pulse of electrons ionizes the sample molecules, forming molecular ions and smaller ions with distinct relative abundances that serve as a "fingerprint" for the molecular structure (Libretexts, 2020). The ions are separated by the mass analyzer and then detected (Meier*et al.*, 2018).

2.9.2 Applications of GC-MS

GC-MS offers improved sample detection, higher sensitivity, a wider range of analyzable samples, and quicker performance, allowing GC-MS to be used in a variety of new applications (Cheriyedath, 2019). The various applications of GC-MS are found in: Environmental monitoring, Food and Fragrance Analysis, Pharmaceutical Applications, Forensic Application, Biological Analysis, Industrial Application and so on (Cheriyedath, 2019).

2.9.2.1 Application of GC-MS in Biological Compound Analysis.

Experiment design provides an effective suite of statistical methods when attempting to discover the essential factors and then optimize through these factors (Sudha *et al.,* 2017).

Plants produce essential oils for a variety of purposes, including defence against bacterial, fungal, and viral infections (Tariq *et al.,* 2019). As a result, they are a good source of biologically active substances, molecules as well as fractions (García-Salinas *et al.,* 2018). GC-MS is widely accepted that a crude phytomedicine must be analyzed critically, and the best search tool for this approach is metabolomics (Duan *et al.,* 2018; Salem *et al.,* 2020).

2.10 Principle of UV-Visible Spectrophotometer

Ultraviolet and visible absorption spectroscopy (often abbreviated to UV-Vis) is a method of spectroscopy that involves calculating the attenuation (strength/intensity weakening) of a light beam after it passes through a sample or reflects from a sample surface. The UV-Visible Spectroscopy principle is based on chemical compound’s absorption of ultraviolet or visible light, which results in the formation of distinct spectra. The interaction between light and matter is the basis of spectroscopy. Excitation and de-excitation occur as matter is absorbed by light, resulting in the formation of a spectrum. In analytical chemistry, UV-Visible spectroscopy is commonly used, especially during the quantitative analysis of a specific analyte.

Table 2: Summary of the defining histological features of the rat female reproductive tract during diestrus, proestrus, estrus, and metestrus

Stages	Vagina	Uterus	Ovaries
Diestrus	The epithelium is established at the lowest level, with variable leukocyte infiltration. Proliferation and thickening of epithelial cells (no visible stratum granulosum) occurs, along with a decrease in leukocyte infiltration.	A slit-like lumen that is small and avascular. Low columnar epithelium forms the inside. Initially, there are few mitoses, but as the process advances, the number of mitoses increases. There are just a few degenerate cells in between. At the end of the stage, there is stromal edema.	The corpora lutea is large. It may be finely vacuolated. The development of fibrous tissue occurs in the central cavity.
Proestrus	Polymorphs appear occasionally. There is very little degeneration or desquamation. Gradually, the stratum granulosum (which marks the beginning), the superficial mucoid layer, and the stratum corneum are formed. At the end of the cycle, the mucoid cells are completely cornified and display a superficial mucoid layer with some desquamation.	Mitoses can be found in epithelial cells with little to no degeneration and no invasion of inflammatory cells. Dilatation occurs, particularly near the end of the stage	Corpora lutea is prone to degeneration. There are typically cytoplasmic vacuoles present. Proliferation of fibrous tissue occurs in the central cavity.
Estrus	The superficial mucoid and cornified layers are gradually shed. Epithelium height is reduced. There are cell debris present. Mitotic figures are lost. Infiltration of leukocytes occurs over time	Estrus begins with the appearance of noticeable degeneration/necrosis of epithelial cells, usually in the glands. Mitotic function is lost. Infiltration of leukocytes. Dilatation will last until the end of estrus.	Corpora lutea degeneration is present. A few small corpora lutea with basophilic cell cytoplasm, a central fluid-filled cavity, and no fibrous tissue.
Metestrus	The beginning is characterized by a nearly complete detachment of the cornified layer (generally residual squames in lumen). Desquamation continues, with depletion of the stratum granulosum and upper germinativum. Infiltration of leukocytes.	Endometrial epithelial cells continue to degenerate. Mitotic activity is restored; both (mitotic activity and degeneration) are visible together.	The fluid cavity in the corpora lutea can still exist. It's a lot smaller than it was at diestrus. Cells are slightly basophilic. Fibrous tissue is generally absent.

Source: Adapted from Westwood (2008).

2.11 Aromatase inhibitors

Aromatase inhibitors prevent the ovaries from producing estrogen. Aromatase inhibitors operate by inhibiting the enzyme aromatase, which converts testosterone into estrogen in minute concentrations in the body (Ratre *et al.,* 2020). Examples of aromatase inhibitors are: Anastrozole, Letrozole, and Exemestane. Letrozole belongs to this group of drugs known as non-steroidal aromatase inhibitors (Franik *et al.,* 2018; NCBI, 2021).

2.11.1 Letrozole

Letrozole is a third-generation aromatase inhibitor that was first identified in 1990 as an oral nonsteroidal type II aromatase inhibitor (Drugbank, 2021). This means it has no effect on cortisol, aldosterone, or thyroxine levels (Wishart *et al*., 2018; MedlinePlus, 2018; Franik *et al.,* 2018).

Letrozole is sold under the brand name "Femara" (Mlynek, 2018).
Aromatase inhibitors, a form of drug that inhibits estradiol synthesis rather than inhibiting the estradiol-receptor interaction (Pritts *et al.,* 2011). These inhibitors work by inhibiting the action of aromatase enzyme which converts androgens to estrogens in a process called "aromatization" (Drugbank, 2020; Soni *et al.,* 2020; Shi *et al*., 2020; Pritts *et al.,* 2011). Letrozole, which is one of these inhibitors, was approved for use in the treatment of breast cancer in 1997 (Ratre *et al*., 2020), and by 2001, it had been used successfully in anovulatory women, the medication is now widely used by physicians and patients for the treatment of ovulation dysfunction and regulated ovarian hyper stimulation (Pritts *et al.,* 2011). The drug is used as a first-line treatment for women with PCOS (Mlynek, 2018), and infertility (Gurevich, 2020). Letrozole is a nitrile that belongs to the triazole family. It functions as an anticancer agent and an inhibitor of the enzyme (aromatase) EC 1.14.14.14 (NCBI, 2021). Aromatase inhibitors such as Letrozole or anastrazole have been administered as a treatment of PCOS women with clomiphene citrate (CC)-resistant anovulation (Shi *et al*., 2020).

2.11.1.1 Mechanism of Action of Letrozole

Letrozole is a non-steroidal type II aromatase inhibitor (Drugbank, 2020). It prevents an essential step in the biosynthesis of estrogen in the ovary and other required tissues, this step involves the conversion of C-19 androgens to C-18 estrogens by competitively inhibiting aromatase enzyme (cytochrome P-450 19) (Rose and Brown, 2020). The use of letrozole results in the increase in androgen level and decrease in estrogen levels in the tissues distributed round the body (Rose and Brown, 2020). Letrozole is used to treat infertility in the same way as clomiphene citrate is used to treat infertility for a long period of time. Letrozole for infertility uses a similar negative feedback loop to increase FSH secretion, but it does so by decreasing estradiol development in the ovary and other tissues (Rose and Brown, 2020).

The application of letrozole for the treatment of breast cancer is quite different from its application in the infertility therapy (Rose and Brown, 2020).

2.11.1.2 Pharmacology of Letrozole

Letrozole is a third-generation aromatase inhibitor, third generation in because it was developed using new molecular modelling techniques which were designed to modify better binding to the catalytic site in aromatase (Andrianov *et al.,* 2019; Rose and Brown, 2020). Before the advent of third generation aromatase inhibitor, there was first and second aromatase inhibitor. The first generation inhibitor, although blocked estrogen biosynthesis but inhibited the biosynthesis of cortisol, aldosterone and thyroid hormone (Ghosh and Egbuta, 2016; Rose and Brown, 2020), while the second generation aromatase inhibitors although were more safe to use but they were not selective in inhibiting biosynthesis of estrogen. The third generation was then designed to meet the aim of selectivity and potency (Rose and Brown, 2020).

Following oral administration, letrozole has a bioavailability of 99.9%. It has a 42-hour terminal half-life after a single dose, but it takes 2 to 6 weeks to reach steady-state concentration (steadystate half-life is 118 h) (Rose and Brown, 2020), this is as a result of letrozole being metabolise into inactive metabolites by cytochrome P-450 enzymes: 3A4 and 2A6 (Rose and Brown, 2020). Cytochrome P-450 2A6 has a more significant affinity to letrozole (and more effective metabolism) than 3A4, but is saturated with a letrozole dose of approximated 2.5 mg. Hence, letrozole is extremely effective in suppressing the synthesis of estrogen (Rose and Brown, 2020). Aromatase expression is regulated by cAMP and gonadotropins in the ovary (Zhou *et al.,* 2016). Aromatase has a high affinity for androgen and letrozole (Koss and Frick, 2019; Rose and Brown, 2020). As testosterone binds to an aromatase molecule in ovarian tissue, it is easily converted to estradiol, which has a low affinity for it, and the estradiol molecule is released into the cytoplasm. This release then makes the active site for the aromatase readily available to bind competitively with an androgen or letrozole. Letrozole circulates with 55 percent of its weight bound to albumen, and its pharmacology reduces aromatase activity by more than 99 percent (for a dose of 2.5mg) (Rose and Brown, 2020). Estradiol can still be released during the follicular process of the menstrual cycle in premenopausal women treated with letrozole for infertility, either due to a reduction in letrozole availability due to letrozole's half-life, the treatment regimen, or enhanced aromatase expression mainly due to increased FSH and FSH receptor availability (Casper and Mitwally, 2011; Rose and Brown, 2020).

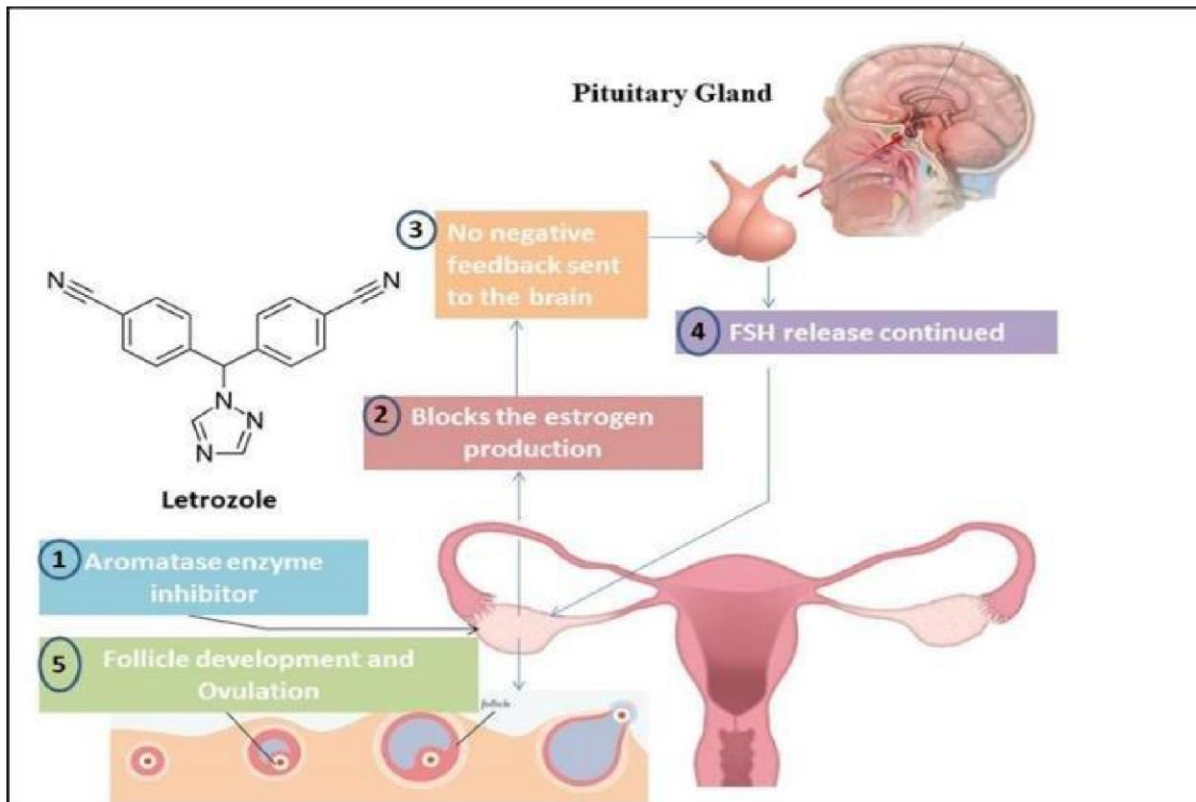


Figure 6: Mechanism of Action of Aromatase inhibitors (such as letrozole)

Source: Maladkar *et al.,* (2020)

2.12 Phytochemical Constituents and PCOS

Phytochemicals are wide variety of compounds made by plants, but is mainly used to describe those compounds that may affect human health (Grusak, 2002). Medicinal properties of plants are normally dependent on the presence of certain secondary metabolites also known as active principles (Grusak, 2002). Some of these secondary metabolites include alkaloids, phenols, anthraquinones, glycosides, saponins, tannins, flavonoids, cardenolides, chalcones, terpenoids and phlobatannins.

Alkaloids belong to a group of secondary metabolites that are synthesised from amino acids and contain one or several nitrogen atoms as constituents of heterocycles and it has been reported to decrease the blood glucose and insulin levels (Baldeon *et al.,* 2012). Alkaloids in *P. nigrescens* extract has been reported to have antihyperinsulinemic effect due to its anti-diabetic activity (Switi *et al*., 2014).

Tannins are astringent, bitter plant polyphenolic compound thst bionds to and precipitates proteins and various other organic compounds such as alkaloids (Katie and Thorington, 2006). Tannins may stimulate the transportation of glucose and inhibition of adipocytes differentiation in β-cells of insulin resistant type 2 diabetic patients (Riedl and Hagerman, 2001).insulin resistance along with its compensatory hyperinsulineia, are hallmarks of PCOS, which puts women with this condition at an increased risk of impaired glucose tolerance and T2DM (Ehrmann *et al.,* 1999; Legro *et al.,* 1999).

Seeing that there is currently no definitive and ideal cure for hormonal disorders and their clinical manifestations, because chemical medications have a variety of side effects, alternative treatments, especially phytotherapy, may be used in place of commercially available drugs (Abasian *et al.,* 2018). Since medicinal plants contain active compounds and have minimal side effects, they have received a lot of attention in recent years (Abasian *et al.,* 2018). Licorice, raspberry, and soybean, for example, are some plants that contain high levels of phytoestrogens such as: biochanin A, daidzein, genistein, and formononetin (Krizova *et al.,* 2019). These plants' anti-androgenic properties cause androgen levels to drop in PCOS patients (Rajan and Balaji, 2017; Abasian *et al.,* 2018). According to a study, it was discovered that raspberry fruit extract reduced testosterone levels by inhibiting the nuclear factor kappa light chain enhancer of activated B cells (NF-kB) pathway, and improved ovarian tissue symptoms such as the number of growing follicles, granulosa layer thickness, and corpus luteum number, as a result of its antioxidant and antiinflammatory properties (Bardei, 2015). According to Jelodar *et al*., (2015) consuming *V. agnuscastus* fruit extract increases aromatase activity and lowers testosterone levels by aromatizing testosterone and converting it to estradiol (Feizollahi *et al.,* 2021). Phytoestrogens are present in this extract (Abasian *et al.,* 2018).

This plant, *P. nigrescens*, contains flavonoids such as apigenin, vitexin, and penduletin that can bind to estrogen receptors (Niroumand *et al*., 2018). According to research carried out in both human and mouse testicular microsomes, it was discovered that genistein had the ability to inhibit 3 beta-Hydroxysteroid dehydrogenase (3-HSD) and 17 beta-hydroxysteroid dehydrogenase (17HSD) (Xu *et al.,* 2017; Abasian *et al.,* 2018). The presence of a phenolic ring in iso-flavonoids has been shown to inhibit the activities of 3-HSD and 17-HSD (Abasian *et al.,* 2018). In addition to inhibiting steroid-synthesizing enzymes, genistein may affect adenylatecyclase activity if the LH receptor does not bind to the G-protein, and block steroidogenesis-stimulated LH development if the LH receptor does not bind to the G-protein (Rajan and Balaji, 2017). Total antioxidant status declines in women with PCOS, and oxidative stress is considered a pathological feature of such condition (Abasian *et al.,* 2018). Evidence suggests that in PCOS, the levels of reactive oxygen species (ROS) in ovarian tissue rise, causing an imbalance in the oxidant and antioxidant systems (Zhang *et al.,* 2019). Because of the aromatic ring and genistein in soybean isoflavonoids, they have an antioxidant effect (Rajan and Balaji, 2017). Pollen from P. dactylifera contains antioxidant compounds such as vitamins and minerals, such as zinc and selenium, which help people with PCOS regulate their body's antioxidant balance (Abasian *et al.,* 2018). Many of the plants studied, such as Soybean, Raspberry*, A. vera, L. pumila, C. nucifera, G. max,* and *G. glabra*, have antiandrogenic properties, while others, such as *P dactylifera, G. max, C. sinensis, M. piperita,* and *T. vulgaris*, have antioxidant properties that help treat PCOS by lowering oxidative stress and increasing serum sex hormone levels (Abasian *et al.,* 2018). Various phytoestrogens and antioxidant compounds found in medicinal plants have been shown to improve PCOS symptoms and can thus be used to effectively treat this syndrome (Abasian *et al.,* 2018).

2.13 Hormone Studies

2.13.1 Progesterone

Progesterone is a hormone that aids in the preparation of the uterine lining for pregnancy (Knudtson and McLaughlin, 2019; Al-Asmahk, 2019) and is synthesized in the corpus luteum.

The hormone progesterone is essential for the normal control of female reproductive functions (Al-Asmahk, 2019). Induction of ovulation, facilitation of implantation, and preservation of early pregnancy are the key physiological activities of progesterone in the uterus and ovary (AlAsmahk, 2019). The luteal phase of the menstrual cycle is mediated by progesterone (Al-Asmahk, 2019). Granulosa cells experience changes in response to the ovulatory stimulus during the preovulatory process, resulting in terminally differentiated luteal cells. Granulosa cells secrete a lot of progesterone as they differentiate (Al-Asmahk, 2019).

Patients with PCOS who try to become pregnant via the use of fertility medications have their progesterone level examined about 7 days after their ovulation (Ray, 2018). If the level of Progesterone is high, which is a value higher than 14ng/ml, then it implies that ovulation occurred and egg was released from the ovary (Sterling, 2011). If the level of progesterone after the 7 days of presumed ovulation, is low, then it means that the egg was not released (Sterling, 2011). Sometimes, women with PCOS can have signs of ovulation occurring but when the test for progesterone is done, the result shows that no ovulation occurred, when this happens it means that the body is producing a follicle and preparing for ovulation but for some reason, the egg is not been released from the ovary (Sterling, 2011). Women with PCOS require a higher level of progesterone in other to decrease the rate of secretion of GnRH, hence resulting in insufficient plasma follicle-stimulating hormone (FSH) synthesis and excessive plasma luteinizing hormone stimulation of androgens produced by the ovaries (Marques *et al*., 2018). Women with PCOS are generally anovulatory, which means their serum progesterone levels are low and they are nonovulatory (Khmil *et al.,* 2020; Legro, 2017). In this category of women, an occasional ovulation results in luteal progesterone concentrations that are close to those of normal women (Chang and Kazer, 2014). 17-hydroxyprogesterone levels have been shown to be substantially elevated in women with PCOS (Chang and Kazer, 2014). This is due to theca cell development of this androgen precursor hormone. 17-hydroxyprogesterone responses to gonadotropin stimulation helped to better differentiate PCOS women from average in provocative studies of androgen development (Chang and Kazer, 2014).

2.13.2 Androgens

Androgens are often referred to as the "male" hormones, but these hormones are present and essential in both men and women (Antoniou-Tsigkos *et al.,* 2019). These hormones are important to the normal reproductive function, emotional well-being, cognitive function, lean muscle function and the growth, and bone strength (Gurevich, 2020). Some androgen hormone effects includes the stimulation of pubic and body hair growth, fat cell action and distribution around the body. In both male and female, androgens are the precursor to estrogens (Gurevich, 2020). Androgens are produced in the adrenal glands, the ovaries and in the fat cells (Gurevich, 2020). Androtenedione and testosterone are the main androgens and they are secreted by the adrenal gland and by the ovary. In women with PCOS, circulating levels of these androgens are elevated (Gurevich, 2020).

The levels of androgen can be measured using two methods: the total testosterone and the free testosterone. The total testosterone refers to the total amount of all testosterone including the free testosterone in the body. It has a range of 6.0 to 86ng/dl (Sterling, 2011). The free testosterone refers to the amount of testosterone that is unbound and the amount that is active in the body (Sterling, 2011). This amount ranges from 0.7 to 3.6pg/ml. an elevated free testosterone level is a sensitive indicator of

excess androgen in the body (Sterling, 2011). Other androgens such as: dehydroepiandrosterone sulphate (DHEEA-S), may be normal or just slightly above the normal range in patients with PCOS. Androstenedione levels are usually elevated in patients with PCOS (Sterling, 2011). The ovary produces 60% of the androgens while the adrenal gland secrets 40% of androgens (Chang and Kazer, 2014; Lucidi, 2019). PCOS patients often have an increased level of both total testosterone and free testosterone. A small increase in testosterone level in a woman can alter menstrual cycle and ovulation (Sterling, 2011).

2.13.3 Follicle Stimulating Hormone

Follicle-stimulating hormone (FSH) is a glycoprotein gonadotropin (Al-Matari *et al.,* 2020) secreted by the anterior pituitary in response to the hypothalamic hormone known as the gonadotropin-releasing hormone (GnRH) (Padmanabhan and Cardoso, 2020). FSH aids in the regulation of the menstrual cycle in women and promotes the development of eggs and promotes the secretion of estrogen by the ovaries (Arazi *et al.,* 2019). FSH receptors are G-protein coupled receptors which are found in the granulosa cells that surrounds developing ovarian follicles (Holesh *et al.*, 2020). FSH promotes follicular development, granulosa cell proliferation, androgen aromatization to estrogens, as well as LH receptor expression (Holesh *et al.*, 2020). Women's FSH levels fluctuate during the menstrual cycle, with the maximum levels occurring right before the ovary releases an egg (Aritonang *et al.,* 2017) in a process called Ovulation (Aritonang *et al.,* 2017). In women with PCOS, the level of FSH is usually low or within the normal reference range of 4.7 to 21.5mIU/mL (Saadia, 2020; Dewailly *et al.,* 2016; Galan, 2020).

2.13.4 Luteinizing Hormone (LH)

Luteinizing Hormone (LH) is a gonadotropin produced by the anterior pituitary gland in response to the release of high-frequency GnRH (Holesh *et al.,* 2020). Through stimulation of theca cells and luteinized granulosa cells, LH is responsible for inducing ovulation, uterine implantation of fertilized oocytes, and the ovarian production of progesterone (Holesh *et al.,* 2020). LH interacts with Theca cells in the ovary that are adjacent to granulosa cells prior to the LH surge. Androgens produced by these cells diffuse into the granulosa cells, where they are converted to estrogen for follicular development (Holesh *et al.,* 2020). The LH surge promotes follicular eruption by increasing the activity of proteolytic enzymes, which weaken the ovarian wall and enable the oocyte to move through (Laven, 2019). LH increases the ovary's development of estrogen and progesterone in women (Holesh *et al.,* 2020). During the mid-menstrual cycle, a surge of LH causes ovulation, and continued LH secretion stimulates the corpus

luteum to release progesterone (Sterling, 2011). The ovarian follicle's development is largely regulated by FSH, and the secretion of estrogen from this follicle is reliant on FSH and LH (Sterling, 2011).
LH and FSH levels usually range from 5 to 20 mlU/ml at the start of the cycle. In the early part of their cycle, most women have roughly equal levels of LH and FSH (Reed and Carr, 2018). However, there is an LH surge 24 hours before ovulation, when the amount of LH rises to around 25-40 mlU/ml (RO *et al.*, 2016; Reed and Carr, 2018). LH levels return to normal after the ovary releases the egg (Zhao *et al.,* 2016).
LH secretion is frequently elevated in PCOS women (Coutinho and Kauffman, 2019). High levels of LH lead to high amounts of androgens (i.e. male hormones like testosterone), which, in combination with low levels of FSH, leads to poor egg development and failure to ovulate (Nedresky and Singh, 2020; Jabbour, 2020). In addition, a lack of ovulation causes relative deficiencies in progesterone production by the ovary, this frequently results in the absence of monthly cycles (an/oligo ovulation) (Reed and Carr, 2018; Holesh *et al.,* 2020).

2.13.6 Insulin

Insulin is a peptide hormone that binds to receptors on the plasma membrane of target cells to coordinate an integrated anabolic response to nutrient availability (Peterson and Shulman, 2018). Insulin is a major regulator in the metabolism of cells and it is also a growth factor (Vigneri *et al.,* 2016) produced from the beta cells in the pancreatic islets of Langerhans in response to the rise of blood glucose level (Farack *et al.,* 2019). It is a hormone that plays a crucial role in the regulation of blood glucose homeostasis (Guemes *et al.,* 2016). It is involved in the accumulation of fat in addition to its function in regulating blood sugar levels (Qaid and Abdelrahman, 2016). Insulin signals fat cells to take up glucose and store it as triglycerides after the liver has used up its glycogen storage ability (Czech, 2017).
Insulin resistance occurs when cells in the muscles, body fat, and liver reject or ignore the signal that the hormone insulin is attempting to send out, in order to take glucose from the bloodstream and deposit it in our cells (Harrar, 2019). Glucose which is called blood sugar is the main source of fuel in the body (Harrar, 2019; Staels, 2017). Increased insulin resistance and hyperinsulinemia, are some of the pathophysiological basis of PCOS, along with increased androgen development and impaired folliculogenesis, all have distinct characteristics women with PCOS (Papakonstantinou *et al.,* 2016). According to some studies carried out on PCOS, it has been discovered that, Compared to age, body mass index (BMI), and insulin resistance-matched controls, women with PCOS have higher insulin resistance but also higher insulin secretion, which seems to be at least in part independent of obesity, with both slim and obese women with PCOS having reduced insulin sensitivity (Papakonstantinou *et al.,* 2016). Obesity, on the other hand, aggravates insulin resistance in PCOS (Zeng *et al.,* 2020).

Hyperinsulinemia is considered to be a significant factor in the maintenance of hyperandrogenemia, functioning both as a direct inducer of excess androgen output by theca cells and as a co-gonadotropin, enhancing the effect of the increased LH stimulus seen in the majority of PCOS women (Marshall and Dunaif, 2012). Insulin may have other effects, since it has been linked to androgen's central activities in impairing progesterone inhibition of the GnRH pulse generator (Marshall and Dunaif, 2012). Acne, facial hair development, weight gain around the waist, and dark patches of skin in the abdomen, armpits, groin, and waistline are all symptoms of PCOS (Hoeger *et al*., 2014). These symptoms are exacerbated by causing women to produce excess androgens (testosterone) as a result of the very high level of insulin (Wang *et al.,* 2019; Ashraf *et al*., 2019). This results to more androgenic features such as more hair, more acne and irregular periods (oligo/anovulation) (Azziz *et al.,* 2016).

Insulin is thought to raise free testosterone levels in the bloodstream by two distinct mechanisms: first, by stimulating ovarian testosterone biosynthesis and secretion, and second, by directly suppressing hepatic SHBG output (Mayer *et al.,* 2015; Handelsman, 2020).

According to a study, it was discovered that the production of testosterone by the theca cells from women with PCOS was stimulated by insulin (Zeng *et al*., 2020). The function of ovarian cytochrome P450C17 and circulating testosterone levels are both reduced when insulin release is reduced in women with PCOS (Rosenfield and Ehrmann, 2016). Insulin Growth Factor Receptors (IGFR) have been found in ovarian cells that also make IGF-binding proteins. (Amutha, and Rajkumar, 2017). IGF-1 and IGF-II, which are structurally related to insulin, play a similar role in follicle production. (Baptiste *et al.,* 2010; Ipsa *et al.,* 2019). Hyperinsulinemia stimulates ovarian androgen development, which leads to hyperandrogenemia in women with PCOS (Baptiste *et al.,* 2010).

2.13.7 Estradiol

Estrogen is a female hormone produced mostly by the developing follicles in the ovaries, but also in small amounts by the adrenal glands (Merchenthaler, 2018). Estradiol (E2) is the most active estrogen in the body (Telfer and Boutot, 2019). Estrogen is one of the primary female sex hormone, it is responsible for the control of functions involving the female reproductive system as well as the secondary sexual features that appear during the stages of puberty and sexual maturity (Fuentes and Silveyra, 2019). To stimulate menstruation, enough estrogen must be present to work with progesterone (Telfer and Boutot, 2019). Estradiol is the most common form of estrogen in the body, and it is produced in the ovaries (Telfer and Boutot, 2019). Estrogens are formed in other parts of the body as well, such as fat tissues, bones, skin, liver, and adrenal glands (Nwankudu, 2020). Estradiol is primarily produced in the ovaries (Telfer and Boutot, 2019). During the first part of the cycle, the follicular phase, which lasts from the start of your period to ovulation, estradiol is released from sacs that contain your eggs,

called follicles (Watson, 2019). Estradiol promotes the thickening and development of the endometrium (the lining of the uterus) (Thiyagarajan, 2020). Estradiol levels peak at the end of the follicular phase, causing the brain to release two hormones: a large burst of luteinizing hormone (LH) and a smaller burst of follicle stimulating hormone (FSH) (Holesh *et al.,* 2020). Hence ovulation is triggered causing the follicles to stop the production of estadiol (Telfer and Boutot, 2019).

The characteristic features of PCOS are, anovulation, hyperandrogenism and polycystic ovary morphology (Homer et al., 2017). Anovulation is associated with limited progesterone synthesis and moderate estradiol (E2) secretion derived primarily from peripheral extraglandular conversion (Homer et al., 2017). The increased granulosa cell response to FSH in PCOS may be due to the local output of E2 (Homer *et al.,* 2017). There's a lot of evidence that estrogen improves follicle activity, including FSH responses (Homer *et al.,* 2017).

The majority of women with PCOS are shocked to learn that their estrogen levels are normal (around 25-75 pg/ml) (Dapas *et al.,* 2020). This may be as a result of the high levels of insulin and testosterone present in PCOS women are sometimes transformed to estrogen (Rosenfield, and Ehrmann, 2016). Although aggregation of follicles will result in an increase in ovarian size as well as a slight increase in basal serum estrogen levels (Carr *et al.,* 2019). High levels of estrogen inhibit apoptosis, which is a characteristic of PCOS, in this syndrome (Carr *et al.,* 2019).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Materials

3.1.1 Collection of Plant Material

Fresh leaves of *parquetina nigrescens* plant were obtained from the premises of Mountain of Fire and Miracle Ministries, Lagos-Ibadan express way, Ibafo, Ogun State, Nigeria. The authentication number was assigned by Mr. Bolu in the University of Ilorin, Nigeria, where a voucher specimen of UIH001/0980 was deposited at the herbarium of the Botany department.

3.1.2 Experimental animals

Twenty female albino rats (*Rattus novergicus*) with an average weight of 170.81g ± 5.35g were obtained from the animal holding unit of the department of biological sciences, Mountain Top University, Ibafo, Nigeria. The animals were kept in a well-ventilated house condition (temperature of 22±3 ° C; photoperiod of 12h/12h light/dark cycle; humidity: 45-50%) and fed with rat pellet (vital feeds, Grand cereals, Jos, Nigeria) and water *ad libitum*.

3.1.3 Drugs and Assay kits

Albumin, Aspartate aminotransferase (AST), Alanine aminotransferase (ALT), Bilirubin, Cholesterol, Creatinine, High density lipoprotein Cholesterol, total protein, and triglycerides assay kits were products of Randox Laboratory, Co-Atrim, United Kingdom. Urea, Uric acid, Progesterone, Insulin, estrogen testosterone, follicle stimulating hormone and lutinising hormone assay kits were manufactured by Diagnostics Laboratories, Freiburg, Germany and Accubind. Letrozole was manufactured by Pharmadox healthcare, UK. Clomiphene citrate was manufactured by Firstsource Pharmachem Lagos, Nigeria. Metformin hydrochloride was manufactured by Sante Pharmaceuticals, France. Carboxy methyl cellulose was a product of Bakers choice, Lagos, Nigeria.

3.1.3.1 Glucometer and Test strips

Accu-chek Active strip compact plus glucometer and Accu-chek active test strip glucometer were products of Roche Diagnostic, Mannheim Sandhofer strasse, Germany.

3.1.3.2 Other chemicals and Reagents

All chemicals and reagents used were of analytical grade obtained mainly from Sigma Aldrich Ltd, Buchs, Canada.

3.1.4 Equipments and Apparatus

Stirring rod, Warring blender, Rotatory evaporator, Beakers, Funnel, Whatman's No.1 filter paper, Measuring cylinder, Muslin bag, and Hot air oven, UV-Visible Spectrophotometer (Jenway 7205), cuvette,

3.2 METHODS

3.2.1 Preparation of ethanoic extract Of *Parquetina nigrescens* leaves

The identified sample was weighed (2,141g) and rinsed under running water to remove dirt and contaminants. The rinsed sample was oven dried at 50 °C to a constant weight of 544g, after which it was then pulverised using a blender (Mikachi Blender, Model MK-1830, China). 500g of the pulverised sample was soaked in 2.5L of absolute ethanol (98.5%) inside a container covered with a lid, and kept inside a cabinet for 48hours, this mixture was stirred four times within the 48 hours. After the 48hours, the sample was then sieved using clean muslin bag, the extract was filtered using a set up containing; Whatman's number 1 filter paper, funnel and conical flasks, this set up was used in order to further separate residue from the filtrate. The filtrate was then concentrated using a rotary evaporator to get a yield of 66g (13.2% yield). The concentrate was then stored at -4 °C.

3.2.2 UV-VISIBLE SPECTROSCOPY

1.18g of *P. nigrescens* leaves was dissolved in 40 ml distilled water in a conical flask, the solution was filtered using a filter paper to give a clear solution. The plant sample solution was placed in a clean cuvette, held by the opaque sides of the cuvette and placed in the UV-Visible spectroscopy and read at wavelengths ranging from 200 to 600nm to get the wide absorbance range. Repeated readings were taken to ensure accuracy.

3.2.3 Qualitative Phytochemical Screening

The solvent extract of *P. nigrescens* leaves was tested for the presence of secondary metabolite using standard procedures as described by Roghini and Vijayalakshmi (2012) with slight modification.

3.2.3.1 Secondary Metabolites

Parquetina nigrescence leaves were screened for the presence of secondary metabolites as described:

3.2.3.2 Qualitative Screening of Secondary Metabolites

a. Carbohydrates (Molish's test)

2g of the sample was dissolved in 50ml of distilled water and then filtered using Whatman number 1 filter paper, to remove all residue. 2ml of the extract was treated with 1ml of Molish's reagent, few

drops of concentrated sulphuric acid was added to the mixture. The appearance of purple or a reddish colour suggested the presence of carbohydrates.

b. Tannins

2ml of the 5% ferric chloride was added to 1ml of the extract. The formation of a dark blue or greenish black indicated the presence of tannins.

c. Saponins

2ml of the extract was added to 2ml of distilled water and shaken lengthwise for 15 minutes. The presence of saponin was detected to cause formation of 1 cm layer of foam.

d. Alkaloids

2ml of concentrated hydrochloric acid was added to 2ml of extract. Few drops of Mayer's reagent were added to the mixture. The formation of green colour or white precipitate indicated the presence of alkaloids.

e. Flavonoids

2ml of the extract was added to 1ml of 2N sodium hydroxide. Presence of flavonoids was indicated by the formation of yellow colour solution.

f. Glycosides

3ml of chloroform and 10% ammonia solution was added to 2 ml of the extract. The formation of pink colour indicated the presence of glycosides.

g. Quinones

1ml of the extract was added to 1ml of concentrated sulphuric acid. The formation of red colour indicated the presence of quinones.

h. Phenols

1ml of the extract was added to 2ml of distilled water followed by few drops of 10% ammonia solution. Formation of blue colour or green colour indicated the presence of phenols.

i. Terpenoids

0.5ml of the extract was treated with 2ml of chloroform and concentrated sulphuric acid. The formation of red brown colour at the interface indicated the presence of terpenoids.

j. Glycosides

0.5ml of the extract was added to 2ml of glacial acetic acid and few drops of ferric chloride were added. This was under layered with 1ml of concentrated sulphuric acid. The formation of brown ring indicated the presence of cardiac glycosides.

k. Protein (Ninhydrin Test)

0.2% of ninhydrin reagent was added to 2ml of the extract and heated for 5 minutes. The formation of blue colour indicated the presence of amino acids.

l. Coumarins

1ml of 10% sodium hydroxide was added to 1ml of the extract. Formation of yellow colour indicated the presence of coumarins

m. Anthraquinones

To 1ml of the extract, few drops of 10% ammonia solution was added. Appearance of pink colour precipitate suggested the presence of anthraquinones.

n. Steroids

1ml of the extract was added to equal volume of chloroform and few drops of concentrated sulphuric acid. The presence of steroid was indicated by the formation of brown ring and the appearance of bluish brown ring indicated the presence of phytosteroids.

o. Phlobatannins

Few drops of 2% hydrochloric acid were added to 1ml of the extract. Appearance of red colour precipitate indicated the presence of phlobatannins.

p. Anthracyanine

1ml of the extract was added to 1ml, 2N sodium hydroxide and heated for 5 minutes at 100°C. The formation of bluish green colour indicated the presence of anthocyanin.

3.2.4 GC-MS Analysis

The GC-MS study was carried out using a Hewlett Packard Gas Chromatograph (Model 6890 series) fitted with a flame ionization detector and a Hewlett Packard 7683 series 250 °C MS transfer line temperature injector. The GC was fitted with an HP-5MS (30 x 0.25 mm) fused silica capillary column, 1.0µm film thickness. The oven temperature was kept at 50 ° C for 5 min of held time and increased at a rate of 2 ° C / min from 50 to 250 ° C, using helium gas (99.999 percent) as a carrier gas at a steady flow rate of 22 cm / s. A 1.0micron extract (1 mg diluted in 1 ml of absolute alcohol) was injected at a 1:30 split ratio. Agilent Science Network Mass Spectrometer (Model 5973 series) coupled with Hewlett Packard Gas Chromatograph (Model 6890 series) with NIST08 Library software database was studied by MS. Mass spectra were taken at 70 eV/200 °C,

1 scan/s scanning rate. Compound recognition was carried out using the NIST08 Library database. The mass spectrum of each unknown compound was compared with the known compounds contained in the library software database.

3.2.5 Experimental design

Twenty rats were completely randomised into five groups of four (4) animals and designated as A, B, C, D and E. The control, PCOS, PCOS + distilled water, PCOS + metformin + clomiphene citrate, and PCOS + ethanoic extract of *Parquetina nigrescens* leaves group. Rats in PCOS group were treated daily with letrozole (dissolved in 0.5% carboxy-methyl cellulose) at 1 mg/kg body weight concentration for 21 days to induce PCOS. Rats in PCOS+ distilled water was given only distilled water daily for 21 days. Rats in PCOS+metformin+clomiphene citrate group were treated with letrozole for 21 d followed by treatment with metformin at 7.14 mg/kg body weight and clomiphene citrate at 2mg/kg body weight concentration daily for 21 d. Rats in PCOS+solvent extract of *parquetina nigrescens* leaves group were treated with letrozole for 21 d followed by treatment with SEPN at 0.5-1ml daily for 21 d. Control rats received 0.5% carboxy methyl cellulose orally as vehicle control for 21 d. At the end of the treatment regimen, rats were fasted overnight (12-14 h) and anaesthetized with diethyl ether and sacrificed by jugular puncture. Blood samples were collected and serum was separated and used for biochemical analysis. Ovaries, were dissected out directly from the lumbar dorsal wall beneath the inferior pole of the kidneys. The kidneys and liver were also harvested from the animals.

3.2.6 Induction of PCOS

3.2.6.1 Animal grouping, induction and administration of ethanoic extract of *P. nigrescens* leaves

Twenty female wistar rats of average weight 170.81 ± 5.23g were acclimatised for two weeks under standard room condition (temperature of 22±3 °C; photoperiod of 12h/12h light/dark cycle) and fed with rat pellets and water *ad libitum*. A known amount of letrozole (1921.28mg) was dissolved in a known volume (280ml) of carboxy-methyl cellulose solution. Administration was carried out once daily for 21 days via oral route to induce PCOS and the body weight was determined every 7 days, after which were treated with the standard drugs and plant extract for a period of 14 days.

The rats were randomly grouped into five groups of four animals as follows:

Group A – (control) received 0.5 ml of vehicle only (carboxy-methyl cellulose)

Group B - received 1 ml of distilled water.

Group C received.

Group D received 1mg/kg body weight of Letrozole

Group E received 1mg/kg body weight of Letrozole

3.2.6.2 Confirmation of PCOS

The estrous cycle in the rats were observed by vaginal cytology using the light microscope to observe predominant cells present in vaginal smears, this was obtained daily for the induction period. Twenty four hours after the last dose of the letrozole, and after a 12hours overnight fast, the fasting blood glucose level of the rats were determined. The rats were sacrificed and blood samples were obtained via jugular puncturing and organ samples were harvested using procedures earlier described by (Yakubu *et al.,* 2008). The serum was used for the assay of serum lipids and reproductive hormones.

3.2.6.3 Vaginal Cytology

Vagina smears were obtained daily between the hours of 8:00am and 9:00am throughout the period of study. The rats were held at the thorax, ventral surface while providing lumbar support as far as possible. Vaginal secretion samples were obtained from the rats using cotton-tipped swabs softened with a drop of distilled water. After about 2 inches of the swab was inserted and about 3 revolutions which allowed

the cotton tip to pick a generous ampunt of vagina cells. The swab was gently withdrawn and rolled on a clean microscopic glass slide and the cells were examined under a light microscope using an objective lens of ×100. A digital camera (SONY corporation Digital camera, China) was used to capture the photomicrographs.

3.2.6.4 Determination of Fasting Blood Glucose Level

The level of fasting blood glucose was determined using the glucometer kit by Accu-Chek after an overnight fast for 12 hours. In the morning, the tip of the tail of the rats were punctured using a blood lancet , blood from the tail region was allowed to drop on the glucose test strip which was inserted into a glucometer. The fasting blood glucose concentration (mg/dL) of the rats were obtained in all the rats (Saidu *et al*., 2014).

3.2.7 Preparation of Serum and Tissue Supernatant

The method as described by Yakubu *et* al (2008) was used to prepare the serum and tissue supernatant. The rats were weighed individually and thereafter anaesthetized in a jar containing cotton wool soaked in diethyl ether. The neck area was cleared of fur and skin to expose the jugular veins. The jugular veins were displaced slightly from the neck region and thereafter cut with a sharp sterile blade. The animals were held head downwards, allowed to bleed into clean, dry sample tubes and left at room temperature for 10 minutes to clot. The blood samples were centrifuged at 4000rpm for 10 minutes to obtain the supernatant from the stock using Thermo Scientific Centrifuge (Heraeus Megafuge 8).
The sera were thereafter aspirated using Microflux pipette into clean, dry, sample bottles and were then stored frozen (-4°C) overnight. The animals were quickly dissected, the liver, kidney and ovary were excised, cleaned of fatty layers, weighed and transferred into ice cold 0.25M sucrose solution. Thereafter, each organ was blotted with clean cotton paper, cut thinly with a sterile blade and homogenized separately in ice cold 0.25M sucrose solution (1:4 w/v) based on their different dilution factors used such as kidney (x60) liver (x30) and ovaries (x80). The homogenates obtained were centrifuged at 4000rpm for 10 minutes to obtain the supernatants which were then gently collected into sample bottles, frozen (4°C) overnight before they were used for the various biochemical assays.

3.2.8 Animal grouping and extract administration for pharmacological study

PCOS was induced in twenty fenmale wistar rats with 1ml of letrozole as described previously. Female rats which experienced urregularity in their oestrous cycle were completely randomised into 5 groups (designated A – E) of four (4) animals in each as follows:

Group A – (non-PCOS-induced control) received 1ml of distilled water

Group B - (PCOS-induced) received 1 ml of distilled water.

Group C (PCOS-induced) received 0.5ml of 7.14 mg/kg body weight of metformin and 0.5ml of 2mg/kg body weight clomiphene citrate (Reference/Standard drugs).

Group D (PCOS-induced) received 50mg/kg body weight of ethanoic extract of *P. nigrescens* leaves.

Group E (PCOS-induced) received 100mg/kg body weight of ethanoic extract of *P. nigrescens* leaves.

The 0.5ml and 1ml of plant extract, metformin and distilled water which corresponded to their respective doses were administered once daily for 14 days. The female rats in each group were weighed at an interval of 7 days. At the end of the experimental period, each animal was anesthesized using diethyl ether and sacrificed by jugular puncture. Thereafter, the blood sample of each animal was collected in a blood sample bottle.

3.2.8 DETERMINATION OF SERUM CONCENTRATION OF HORMONES

3.2.8.1 PROGESTERONE

The serum progesterone concentration was determined using competitive Accu-Bind kits (Aufrere, 1976; Abraham, 1981).

PRINCIPLE

The principle is based on a one step immunoassay to determine the presence of progesterone using competitive microplate enzyme immunoassay. Progesterone present in the sample competes with enzyme progesterone conjugates for binding with anti-progesterone coated microplate to form an antigen-antibody complex. The activity of the enzyme in the antibody bound fraction is inversely proportional to the native progesterone concentration (Aufrere, 1976; Abraham, 1981).

PROCEDURE

To 0.025 ml of each calibrator, control and serum samples in the microplate wells, 0.10 ml of the conjugate was dispensed into each well. The microplate was swirled gently for 30 seconds to mix and was incubated for 60 minutes at 25 °C. The content of the microplate was decanted and 0.30 ml of the washing solution was added repeatedly four times. TMB substrate (0.10 ml) was added and incubated at room temperature for 25 minutes in the dark after which 0.15ml of stopping reagent was pipetted into each well. The absorbance was readon microplate reader at 450nm within 20 minutes after the addition of the stopping reagent.

Calculation

The serum progesterone concentration of the test samples was extrapolated from the calibration curve (figure 10) plotting the absorbance of the standard sample at 450nm aganst its corresponding concentration.

3.2.8.2 TESTOSTERONE

The serum testosterone concentration was quantitatively determined using the direct human testosterone Accu-bind kit (Chen *et al*., 1991; Granoff and Abraham, 1979).

PRINCIPLE

The testosterone EIA is based on the principle of competitive binding between testosterone HRP conjugate for a constant amount of rabbit anti-testosterone, Goat anti-rabbit IgG-coated wells and rabbit anti-testosterone reacted with the test sample. HRP-labeled testosterone competes with endogenous testosterone peroxidase conjugate immunologically bound to the well and progressively decrease as the concentration of testosterone in the specimen increases. The intensity of the color formed is proportional to the amount of enzyme present and is inversely related to the amount of un-labelled testosterone in the sample (Chen *et al*., 1991; Granoff and Abraham, 1979)..

PROCEDURE

Exactly 0.01ml of standards, control and test sample, 0.1 ml of testosterone HRP conjugate reagent and 0.05ml of rabbit anti-testosterone reagent were dispensed into each well, mixed thoroughly for 30 seconds and incubated at 37°C for 90 minutes. The microwells were rinsed and flicked for 5 minutes with distilled water. A known volume (0.1ml) of 3,3, 5,5-tetramethylbenzidine (TMB) reagent was dispensed into each well, mixed gently for 5 secondsand incubated at room temperature for 20 minutes. The blue colour completely turned yellow and the absorbance was read at 450nm with a microtitre well reader within 15 minutes of the standard solutions against its corresponding concentrations. The reaction was stopped with the addition of 1N HCl, afterwards the absorbance was read at 450nm (Chen *et al*., 1991; Granoff and Abraham, 1979)..

Calculation

The serum testosterone concentration was extrapolated from the calibration curve (figure 11).
Plotting the absorbance of the standard sample at 450nm against its corresponding concentration.

3.2.8.3 FOLLICLE STIMULATING HORMONE (FSH)

The serum FSH was quantitatively determined using the direct human serum follicle stimulating enzyme immunoassay kits (Winnink *et al*., 1990).

PRINCIPLE

This is based on the principle of sandwich method. The enzyme assay system utilizes a high affinity and specificity monoclonal antibody (enzyme conjugate and immobilized) directed against a distinct antigenic determinant on the intact FSH molecule (Winnink *et al*., 1990).

PROCEDURE

Microplate wells of each reference, control and serum samples to be assayed were in duplicate. A known weight (0.025ml) of each calibrators, control and serum samples were pipetted into appropriate wells. Exactly 0.1ml of the conjugate was dispensed into each well, except for the blank (which contained distilled water) and incubated on the thermo shaker for 30 minutes at 37 ^{o}C. Each well were washed five times with 0.3ml of working washing solution and tapped firmly against absorbing paper to ensure that it dried. Exactly 0.1ml of 3,3, 5,5-tetramethylbenzidine

(TMB) substrate was added to each well, and incubated at 25 ^{o}C in the dark for 30 minutes. A known volume of 0.15 ml of stopping reagent was placed into each well and the mixed gently for 10 seconds; the plate was read on microplate reader at 450 nm within 20 minutes after the addition of the stopping reagent. A calibration curve of absorbance standards was plotted against the concentration and this was used in the determination of concentrations for the tests samples.

Calibration

The serum FSH concentration was extrapolated from the calibration curve (figure 12). Plotting the absorbance of the standard sample at 450nm against its corresponding concentration

3.2.8.4 LUTEINIZING HORMONE (LH)

The serum LH was quantitatively determined using the direct human serum follicle stimulating enzyme immunoassay kits (Kosasa, 1981).

PRINCIPLE

This is based on the principle of sandwich method. The enzyme assay system utilizes a high affinity and specificity monoclonal antibody (enzyme conjugate and immobilized) directed against a distinct antigenic determinant on the intact LH molecule (Kosasa, 1981).

PROCEDURE

A known volume (0.025 ml) of the standard solutions, control, serum samples were placed in appropriate wells. Exactly 0.1ml of the conjugate was dispensed into each well except blank and incubated on a thermo shaker for 30 minutes at 37 °C. each well was washed five times with 0.3 ml of working washing solution and tapped firmly against absorbing paper to ensure its dryness.

Exactly 0.1ml of 3,3, 5,5-tetramethylbenzidine (TMB) reagent was dispensed into each well and incubated at 25 °C for 30 minutes in a dark place. Thereafter, 0.15 ml of stop reagent was pipetted into each well and mixed gently for 10 seconds. The absorbance of the samples were read on the microplate reader at 450nm within 20 minutes after the addition of stop reagent. Calibration curve of the standard was plotted to get the concentrations of each test sample.

Calculation

The serum LH concentration was extrapolated from the calibration curve (figure 13). Plotting the absorbance of the standard sample at 450nm against its corresponding concentration.

3.2.8.5 INSULIN

The serum insulin was quantitatively determined using microplate immunoenzymometric assay kit as described in the manufacturer's protocol version (Eastham, 1985).

PRINCIPLE

The principle is based on immobilization that takes on a surface of a microplate well through the interaction of streptavidin coated on the wells and exogenously added biotinylated monoclonal insulin antibody. The enzyme labelled antibody and a serum containing the native antigen reactive results between the native antigens and antibodies without competition or steric hindrance to form a soluble sandwich complex. The activity of the enzyme in the antibody-bound fraction is directly proportional to the native antigen concentration (Eastham, 1985).

Calculation

The absorbance of each calibrator, control and serum sample was plotted with the absorbance on the y-axis and the concentrations on the x-axis (figure 14). The insulin concentration of the test samples were extrapolated from the calibration curve obtained by plotting the absorbance of the standard solutions against its corresponding concentrations.

PROCEDURE

An aliquot (0.05ml) of the standard solution, control, serum samples were placed in appropriate wells. Exactly 0.01 ml of the insulin enzyme reagent was dispensed into each well and the microplates were swirled gently for 20 seconds. The microplates containing the reaction medium was wrapped in a plastic bag and incubated for 120 minutes at 25 °C. the wells were washed three times with 0.35ml of working substrate solution per well and aspirated using a micropipette. A known volume (0.1ml) of the working substrate was added to each well and incubated at 25 °C for 15 minutes. Exactly 0.05 ml of stopping reagent was placed into each well and mioxed gently for 20 seconds. The plate was read on microplate reader at 450nm within 30 minutes after the addition of the stopping reagent.

3.2.8.6 Estradiol

Desired number of coated wells was secured in the holder. 25µL of standards, specimens and contraols were dispensed into appropriate wells. 50µL of rabbit anti-estradiol (E2) reagent was dispensed into each well. 100µL of estradiol-HRP conjugate reagent was dispensed into each well and was thoroughly mixed for 30seconds. The samples were then incubated for 90minutes at room temperature. Thie samples in the wells were mixed and flicked 5 times using washing buffer (1X). 100µL of TMB substrate was dispensed into each well and this was gently mixed for 10 seconds, this was then incubated at room temperature for 20 minutes. The reaction was stopped by adding a stop solution to each well. This was gently mixed for 30 seconds and was ensured that all the blue colour changes turned yellow colur completely. The absorbance was read at 450nm with a microtiter well reader within 15 minutes (Tsang *et al*., 1980).

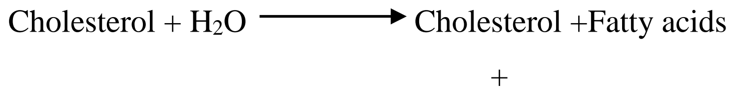
3.2.9.1 LIPID PROFILE DETERMINATION

3.2.9.1.1 Serum Total Cholesterol Concentration

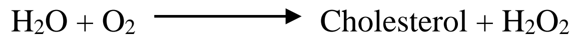
The total cholesterol concentration in the serum of the rats was carried out using the CHOD-PAP reaction (Tietz 1995).

PRINCIPLE

It is based on the following reactions:


Cholesterol + H_2O → Cholesterol +Fatty acids

+


H_2O + O_2 → Cholesterol + H_2O_2

H_2O_2 + 4-Amino antipyrine + P-Hydrobenzoic acid

→ Coloured Quinonic derivatives + 4 H_2O

PROCEDURE

A known volume (2.0ml) of the working reagent was placed into test tubes containing 0.02 ml of the appropriately diluted serum samples. The blank and standard were constituted by substituting the serum with 0.01 ml of distilled water and standard working reagent respectively. The reaction constituent were thoroughly mixed and incubated at 37°C for 5 minutes. Absorbance was spectrophotometrically read at 546nm against the blank (Tietz 1995).

CALCULATION

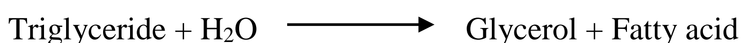
Concentration of cholesterol = $\frac{Absorbance\ of\ sample}{Absorbance\ of\ standard}$ x 5.40mM/L

3.2.9.1.2 Serum Triglyceride Concentration

Principle

The triglyceride concentration is determined after enzyme activity hydrolysis with lipases, the indicator is quinonimine formed from hydrogen peroxide, 4- aminophenazone and 4- chlorophenol under the catalytic influence of peroxidase (Tietz 1995).

Lipases


Triglyceride + H_2O → Glycerol + Fatty acid

Peroxidase

H_2O_2 + 4-aminophenazone Quinonimine + HCl + 4 H_2O + Chlorophenol

Procedure

Exactly 1000µl of the working reagent was added to well clean labelled test tubes consisting of blank, standard and sample. Thereafter 10µl of the distilled water, standard solution (200mg/dL) and serum samples were added respectively. The resulting solution was mixed and incubated for
5 minutes at 37℃. The change in absorbance of standard and sample against the reagent blank was read at 500nm (Tietz 1995).

Calculation

Concentration of Triglyceride (TG) = $\frac{Absorbance\ of\ sample}{Absorbance\ of\ standard}$ x 2.17mM/L

3.2.9.1.3 Serum High Density Lipoprotein-Cholesterol Concentration

The determination of serum High Density Lipoprotein Cholesterol (HDL) concentration (Tietz 1995).

Principle

The low-density lipoprotein (LDL and VLDL) and chylomicron fractions are precipitated quantitatively by the addition of phosphortungstic acid in the presence of magnesium. After centrifugation, the cholesterol concentration in the HDL-Cholesterol fraction, which remains in the supernatant is determined (Tietz 1995).

Procedure

Exactly 200µl of the serum samples and standard were dispensed into separate test tubes and 500µl of the reagent was added. The resulting solution was mixed and left for 10 minutes at room tenmperature. Thereafter, it was centrifuged at 4000rpm for 10 minutes and the clear supernatant was separated for determination of cholesterol content. The concentration of cholesterol was determined using enzymatic saponification procedure as described by Tietz (1995).

Calculation

HDL-C $= \frac{Absorbance\ of\ sample}{Absorbance\ of\ standard}$ x 5.25mM/L

3.2.9.1.4 Serum Low Density Lipoprotein - Cholesterol Concentration

The serum Low Density Lipoprotein (LDL) cholesterol concentration can be expressed as: LDL Cholesterol = Total Cholesterol – (HDL + TG)

Table 3: Dilution factor for the various assays

Parameters/	Serum
Total Cholesterol	×5
Triglycerides	×5
High density Lipoprotein	×5
Low density Lipoprotein	×5

3.2.10 DATA ANALYSIS

Data were analysed by Statistical Product and Service Solution (SPSS) version 26. The statistical differences between groups were determined by one-way of variance (ANOVA) followed by Duncan multiple range test for multiple correlation. Results were expressed as mean of four determinations ± standard error of mean (SEM). Values were considered statistically significant at $p<0.05$.

CHAPTER FOUR

4.0 RESULTS

4.1 Chemical constituents of ethanoic extract of *P.nigrescens* leaves

4.1.1 Secondary metabolites

Qualitative analysis of ethanoic extract of *P. nigrescens* leaves revealed the presence of alkaloids, saponins, flavonoids, Quinones, terpenoids, cardiac glycosides and coumarins. While tannins, carbohydrate, phenols and steroids were not detected (Table 3).

Table 4: Qualitative phytochemical components carried out in ethanol extract of *P.nigrescens* leaves

SECONDARY METABOLITES	**LEAVES**
Carbohydrates	--
Tannins	--
Saponins	++
Alkaloids	++
Flavonoids	++
Glycosides	--
Quinones	++
Terpenoids	++
Phenols	++
Cardiac Glycosides	++
Anthracyanine	--
Ninhydrin	--
Steroids	++
Coumarins	++
Anthraquinones	--
Phlobatannins	--

4.2 Gas chromatography-Mass spectrometry (GC-MS) analysis

Peak 9 with the retention time of 4.622 was identified as Cyclopropane, and methyl ester as the major phyto-component of *P. nigrescens* while the other peaks were representations of other phyto-components present in the plant.

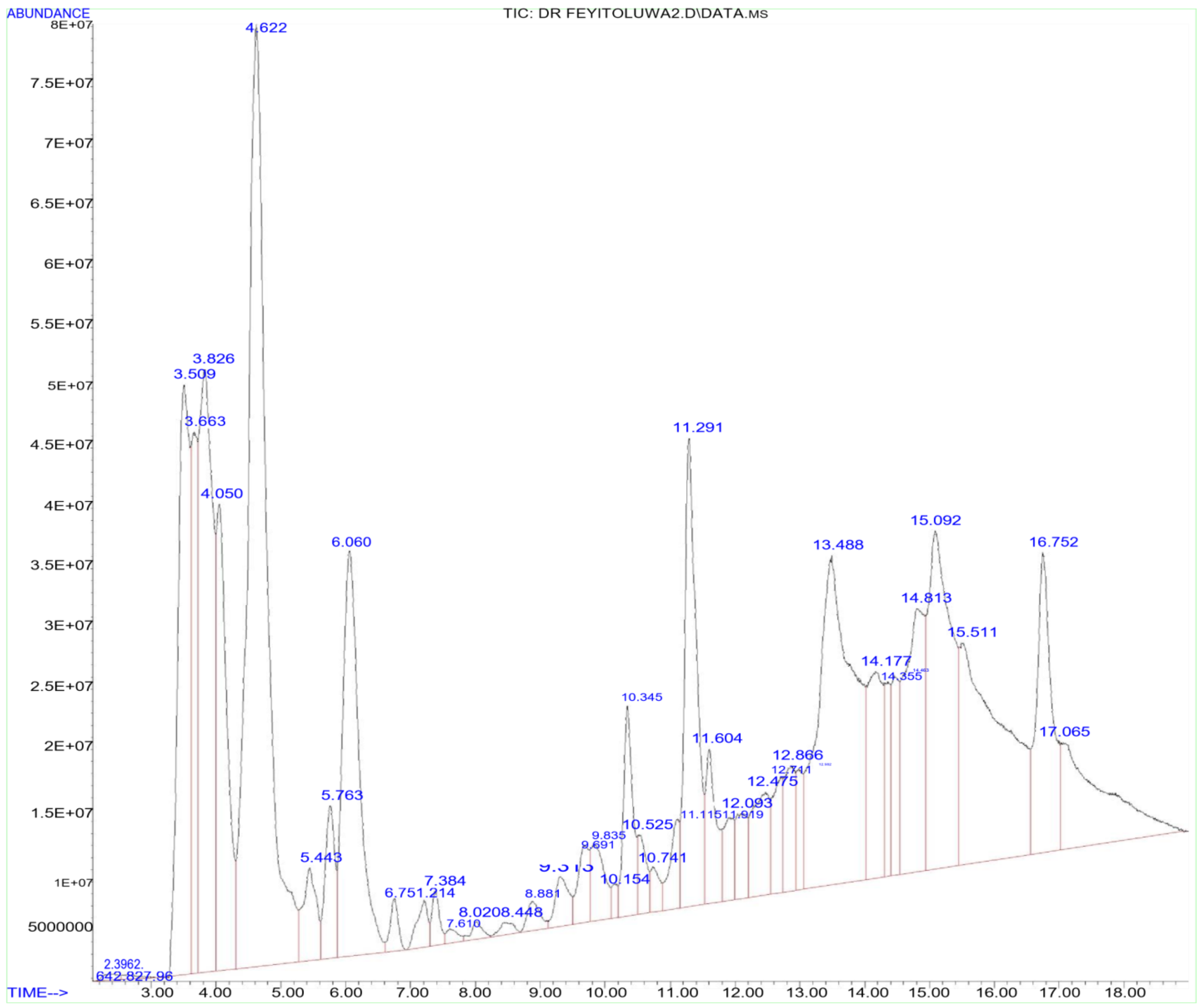


Figure 7: GC-MS chromatogram of solvent leaves extract of *P.nigrescens*

4.3 Phyto-component of ethanoic extract of *P. nigrescens* leaves

Other phyto-components of solvent extract of *P. nigrescens* leaves other than Cyclopentane, methyl ester were identified. The phytochemical components identified in the solvent extract of *P. nigrescens* leaves by GC-MS showing their peak, retention time, library ID, % of total and chemical formula is shown in Table 4.

Table 5: Phytochemical components identified by GC-MS in ethanol extract of *P. nigrescens* leaves showing the peak, name of compound, Percentage Area, retention time, chemical compound formula

Peak	Name of Compound	% Area	Retention Time	Chemical Compound formula
1	Glycerol triethyl ether	0.07	2.393	$C_{12}H_{20}O_6$
2	propriomazine	0.04	2.644	$C_{20}H_{24}N_2OS$
3	Benzo[b][1,4]diazepine-2(1H,3H)-one	0.01	2.825	$C_{15}H_{12}N_2O$
4	Fumaric acid, 2-ethoxyethyl isobutyl ester	0.01	2.975	$C_{10}H_{16}O_4$
5	D-Fructose	5.57	3.507	$C_6H_{12}O_6$
6	Hydroxylamine, O-(3-methylbutyl)-3-Buten-2-ol	2.61	4.663	$C_{10}H_{18}O_3$
7	2-Hexene, 5-methyl-,	6.74	3.826	C_7H_{14}
8	Carbonyl sulfide	4.18	4.051	COS
9	Cyclopropane, 1-methyl-2-(3-methylpentyl	14.60	4.620	$C_{10}H_{20}$
10	Furfural	1.02	5.446	$C_5H_4O_2$
11	Peroxide, dimethyl	1.19	5.765	$C_{14}H_{10}O_3$
12	Cyclopentane, 1,2-dimethyl-	5.53	6.059	C_7H_{14}
13	Pentane, 1-(1-ethoxyethoxy)-	0.35	6.753	$C_9H_{20}O_2$
14	2-Furancarboxaldehyde, 5-methyl-	0.40	7.216	$C_6H_6O_2$
15	3,4-Difluoroanisole	0.35	7.385	$C_7H_6F_2O$
16	Triethylphosphine	0.13	7.610	$C_6H_{15}OP$
17	2-[2-[2-[2-[2-[2-[2-[2-(2-	0.16	8.023	$C_{10}H_{20}O_5$

	Acetyloxyethoxy)ethoxy]ethoxy]ethoxy]ethoxy]ethox y]ethoxy]ethoxy]ethyl acetate			
18	Benzeneacetaldehyde	0.14	8.449	C_8H_8O
19	t-Butyldichlorophosphine	0.30	8.880	$C_4H_9Cl_2P$
20	5-Methylhexane-2,4-dione, enol	0.56	9.312	$C_7H_{12}O_2$
21	p-Dioxane-2,3-diol	0.69	9.693	$C_4H_8O_4$
22	Octanoic acid, 2-methyl-, methyl ester	0.87	9.837	$C_{10}H_{20}O_2$
23	2,4(3H,5H)-Furandione, 3-methyl-pyridine	0.15	10.156	$C_6H_4F_4O_3$
24	4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl	1.59	10.344	$C_6H_8O_4$
25	Ethanamine, N-ethyl-N-nitroso	0.56	10.525	$C_8H_{10}N_2O$
26	6-Methoxy-3-pyridazinethiol	0.33	10.744	C_6H_7NOS
27	Pyrrolidine, 1-methyl-	0.70	11.13	$C_5H_{11}N$
28	5-Hydroxymethylfurfural	4.55	11.288	$C_6H_6O_3$
29	2-Methoxy-4-vinylphenol	1.27	11.601	$C9H_{10}O_2$
30	1-(3,6,6-Trimethyl-1,6,7,7a-tetrahydrocyclopenta[c]pyran-1-yl)ethanone	0.70	11.920	$C_{13}H_{18}O_2$
31	Glutaric acid, heptyl 2-naphthyl ester	0.81	12.095	$C_{18}H_{34}O_4$
32	Succinic acid, 3-pentyl tridec-2-ynyl ester	1.44	12.744	$C_{22}H_{38}O_4$
33	4-Methyl-2-pentyl acetate	0.90	12.708	$C_8H_{16}O_2$
34	Benzaldehyde, 3-hydroxy-	1.12	12.865	$C_7H_6O_2$
35	Acetic acid, 8a-methyl-8-oxodecahydronaphthalen-1yl ester	0.65	12.984	$C_{16}H_{26}O_2$
36	Oxaziridine, 2-methyl-3-propyl-	9.39	13.490	$C_5H_{11}NO$
37	1-Cyclohexene-1-propanol, 2,6,6-trimethyl	2.61	14.178	$C_{12}H_{22}O$

38	Benzocycloheptene, 3-hydroxy-	0.85	14.354	$C_{15}H_{24}O_2$
39	Benzenebutanamine	1.24	14.460	$C_{10}H_{16}N_2$
40	Indan, 1-methyl-	4.13	14.810	$C_{10}H_{12}$
41	Pentanoic acid, 1,1-dimethylpropylester	6.45	15.092	$C_9H_{18}O_2$
42	Hexadecanoic acid, ethyl ester	7.56	15.511	$C_{36}H_{72}O_3$
43	Phytol	3.73	16.749	$C_{20}H_{40}O$
44	Methyl 2-hydroxy-octadeca-9,12,15-trienoate	3.73	17.062	$C_{19}H_{32}O_3$

4.4 UV-spectroscopic analysis of ethanoic extract of *Parquetina nigrescens*

The ultraviolet- visible spectroscopy of aqueous extract of *Parquetina nigrescens* leaves revealed the varying absorbance of the leaves at different wavelengths as shown in Figure 4. The highest peak was found at an absorbance value of 2.5 at a wavelength of 320nm and this absorbance value remained consistent to a wavelength of 440nm. The lowest absorbance was 0.2 at wavelength of 600nm.

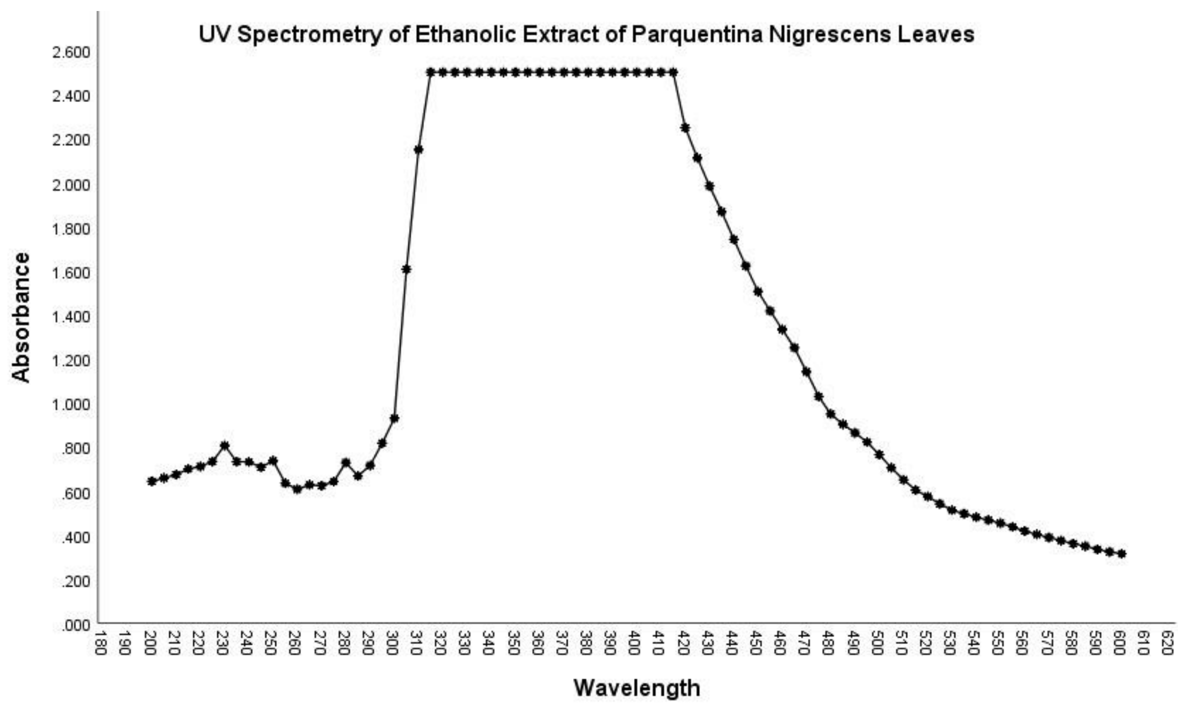


Figure 8: Ultra violet -Visible spectroscopy of *Parquetina nigrescens* Leaves

4.5 Results of pharmacological study

4.5.1 Oestrous cyclicity of letrozole-induced female wistar rats after oral administration of ethanoic extract of *Parquetina nigrescens* leaves

The daily vaginal cytology of letrozole-induced animals administered distilled water revealed inconsistent 4 – 5 days of oestrous cyclicty evident by the presence of cornified squamous anucleated cells compared to the control animals (Table 7-10). The administration of extract at 50 and 100mg/kg body weight reversed the trend of persistent presence of cornified epithelial cells in the oestrous cycle of the letrozole- induced rats in a manner that was similar to animals that received the standard drug (i.e. metformin and clomiphene citrate). On the other hand, the extract at 100mg/kg body weight did not reverse the trend of the presence of cornified epithelial cells in the oestrous cycle, but the phase was characterised by diestrous phase (i.e. large number of white blood cells).

4.6 Fasting blood glucose levels of letrozole-induced animals administered ethanoic extract of *Parquetina nigrescens* leaves

The fasting blood glucose levels of letrozole induced animals treated with distilled water was significantly ($p>0.05$) reduced compared with the control (Table 7-11). The administration of the 50mg/kg body weight of the extract to letrozole-induced animals significantly ($p>0.05$) decreased fasting blood glucose concentration of letrozole-treated animals in a manner similar to letrozole-induced animals that received the reference drugs: metformin and clomiphene citrate.

Table 6: Fasting blood glucose concentrations of Letrozole-induced PCOS rats administered with ethanoic extracts of *Parquetina nigrescens* leaves

	Fasting Blood Glucose of Rats (mg/dl)		
	Before Induction	**After Induction**	**After Treatment**
Control	74.00 ± 0.58[a]	88.00 ± 0.58[a]	114.50 ± 0.29[a]
PCOS + distilled water	75.00 ± 0.58[a]	78.00 ± 0.58[c]	100.00 ± 0.00[b]
PCOS + Clomiphene Citrate + Metformin (7.14mg/kg body weight)	63.50 ± 0.29[c]	64.00 ± 1.15[b]	79.00 ± 0.58[d]
PCOS+50mg/kg body weight of extract	63.50 ± 0.29[c]	77.00 ± 3.46[c]	76.00 ± 1.15[e]
PCOS+100mg/kg body weight of extract	83.50 ± 0.87[b]	86.50 ± 0.87[a]	86.00 ± 1.15[c]

Data are mean of four determinations ± SEM; Values with different superscripts in each column are significantly different ($P<0.05$).

4.7 Body weight of female rats administered ethanoic extract of *Parquetina nigrescens* leaves

The body weight of letrozole-induced animals administered distilled water was significantly ($p>0.05$) different from the control (Table 7). This significant difference ($p>0.05$) when compared with the control was extended to letrozole induced animals that rreceived the extracts at 50mg/kg body weight and at 100mg/kg body weight.

Table 7: Weights of Letrozole-induced animals administered with ethanoic extracts of *Parquetina nigrescens* leaves

	Weight of Rats (kg)		
	Before Induction	**After Induction**	**After Treatment**
Control	138.49 ± 1.03[a]	150.20 ± 1.41[a]	162.52 ± 0.66[a]
PCOS + distilled water	155.22 ± 0.11[b]	189.45 ± 0.71[b]	182.12 ± 0.81[b]
PCOS + Clomiphene Citrate + Metformin (7.14mg/kg body weight)	192.83 ± 0.76[d]	219.66 ± 0.01[d]	211.44 ± 7.06[c]
PCOS+50mg/kg body weight of extract	167.99 ± 0.76[c]	194.84 ± 1.22[c]	178.01 ± 1.01[b]
PCOS+100mg/kg body weight of extract	167.40 ± 0.29[c]	186.25 ± 2.47[b]	187.15 ± 0.93[b]

Data are mean of four determinations ± SEM; Values with different superscripts in each column are significantly different ($P<0.05$).

4.8 Effect of ethanoic extract of *Paquentina nigrescens* leaves on serum hormones levels of letrozole induced female rats

The administration of letrozole significantly increased the serum insulin of the female rats. The administration of the extract at 50 and 100 g/kg body weight to letrozole induced animals significantly reversed this trend in a manner similar to the letrozole treated animals.

The administration of letrozole significantly ($p>0.05$) reduced the testosterone level with respect to the normal control. Administration of 100mg/kg body of EEPNL had no significant difference compared to the standard (metformin and clomiphene citrate).

The administration of letrozole significantly decreased the serum progesterone concentration of the PCOS rats. The administration of 50 and 100mg/kg body weight of the extract to letrozole treated animals produced serum progesterone concentration that was significantly ($p>0.05$) different from those of PCOS rats. There was no significant ($p>0.05$) difference in the serum progesterone concentration of the normal control and the rats administered with 50mg/kg body weight.

The administration of letrozole significantly ($p>0.05$) decreased the serum concentration of FSH in PCOS rats. There was no significant ($p>0.05$) difference in the FSH concentration levels of the rats administered with 50 and 100 mg/kg body weight of plant extract and those administered with the standard drugs (Table 7).

The administration of letrozole significantly ($p>0.05$) increased the serum concentration of LH in PCOS rats. There was a significant ($p>0.05$) level of decrease in the rats administered with 50mg/kg body weight of plant extract and the PCOS group whereas the rats administered with 100mg/kg body weight of the plant extract showed significant increase to that of the normal control (Table 8).

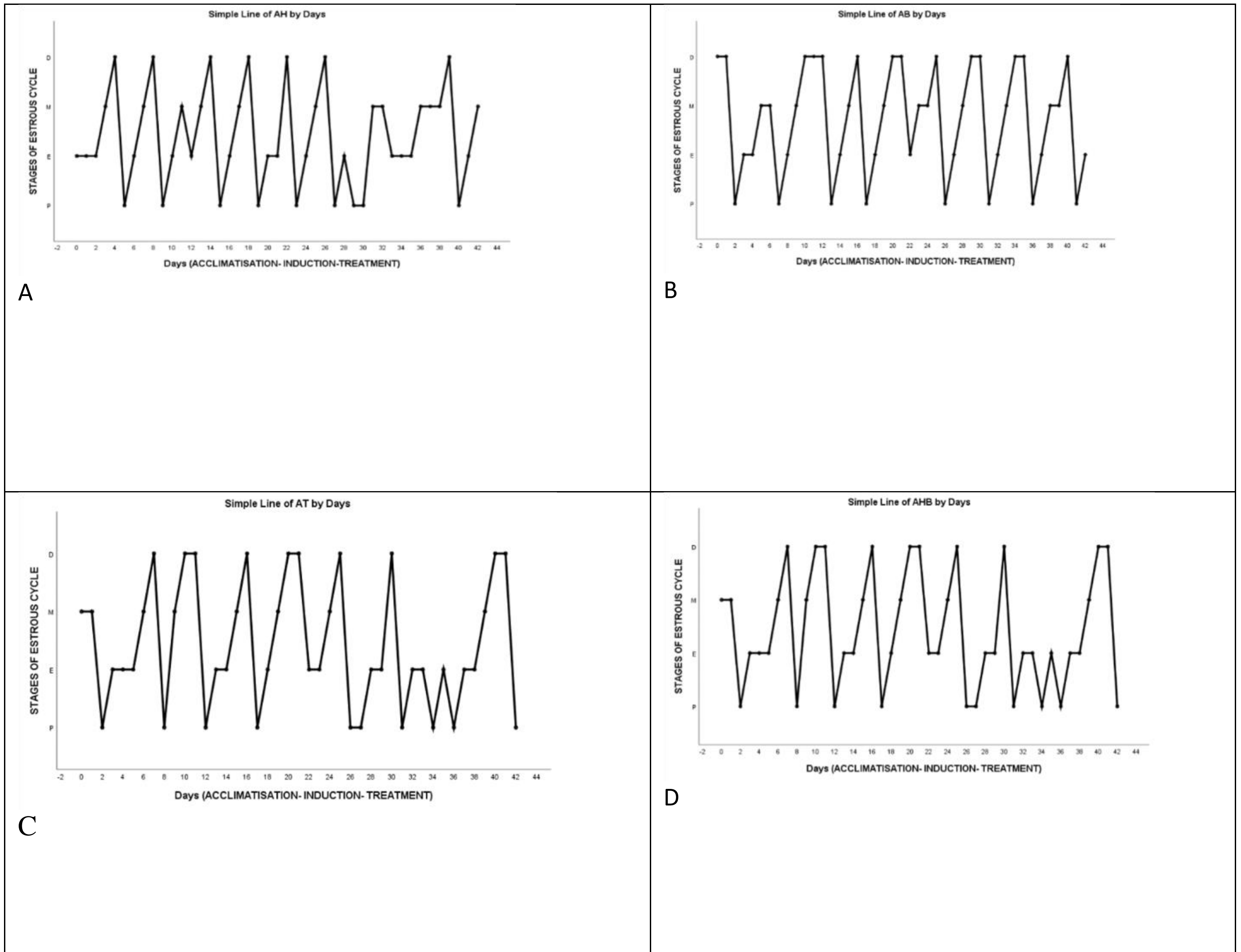


Figure 9: Estrous cyclicity of rats in the normal control group
P: Proestrus, E: Estrus, M: metaestrus, D: Diestrus

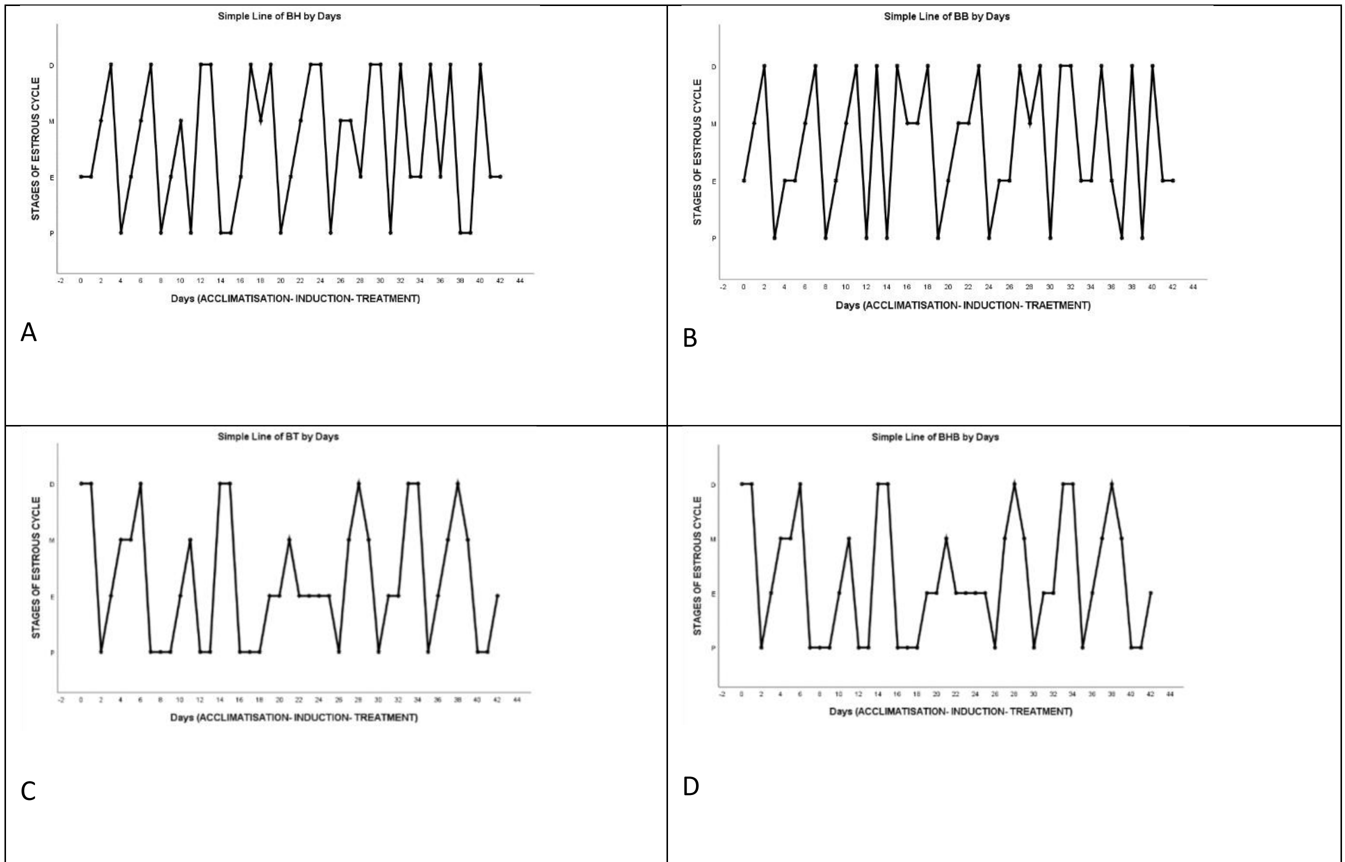


Figure 10: Estrous cyclicity of letozole- induced Female rats untreated group
P: Proestrus, E: Estrus, M: metaestrus, D: Diestrus

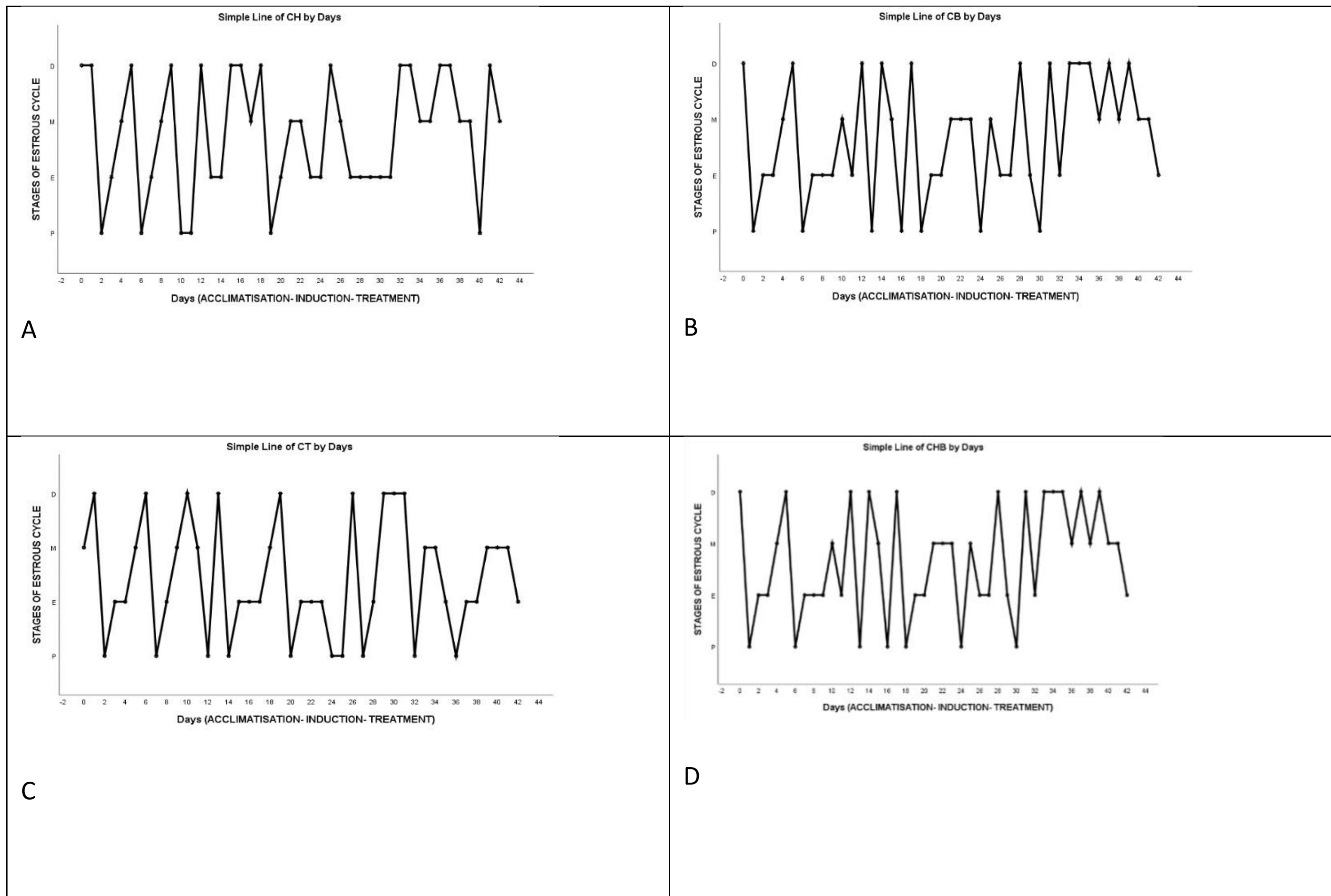


Figure 11: Estrous Cyclicity of letrozole induced female rats treated with standard drug (MET+CC)
MET: Metformin; CC: Clomiphene Citrate
P: Proestrus, E: Estrus, M: metaestrus, D: Diestrus

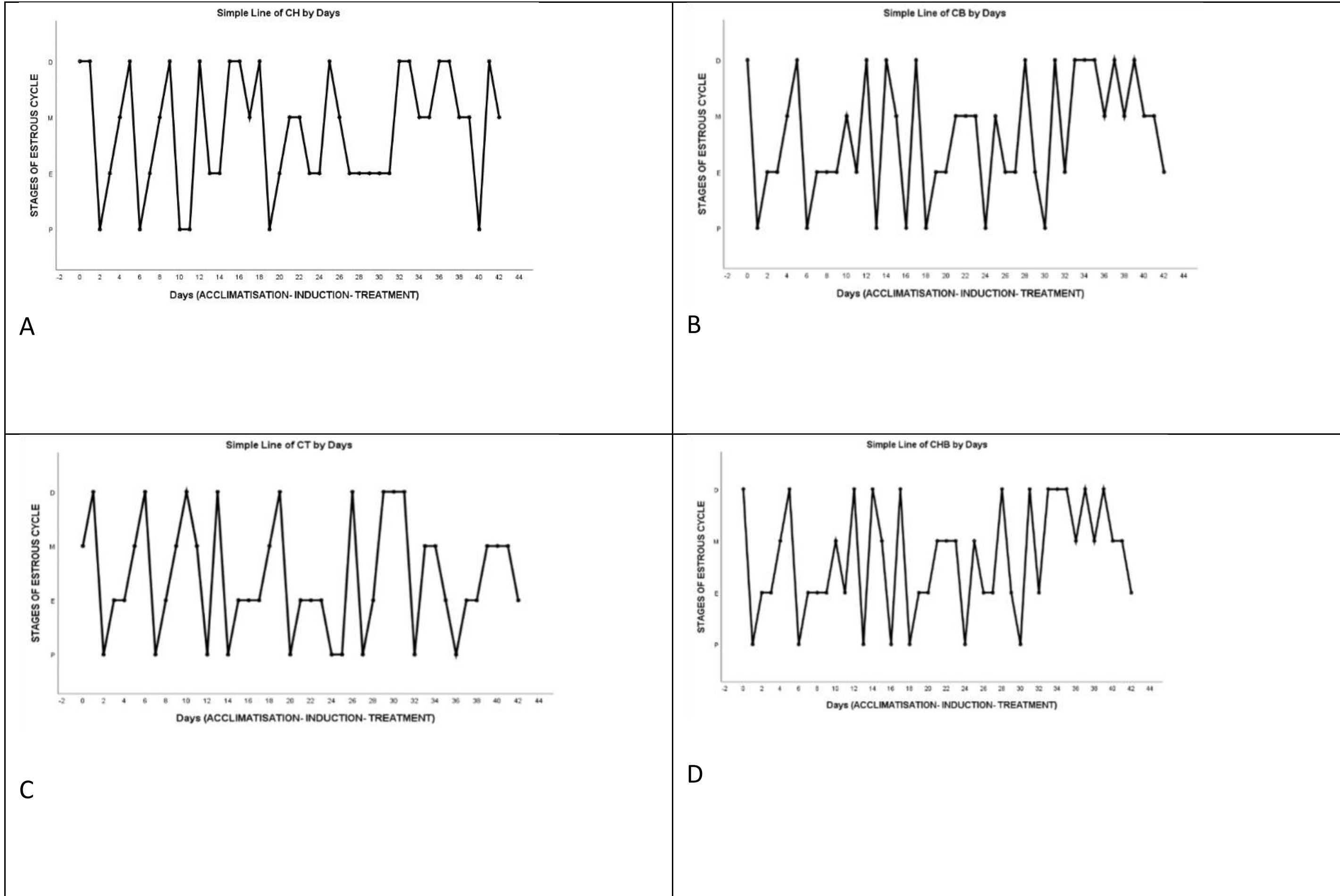


Figure 12: Estrous Cyclicity of letrozole induced female rats treated with 50mg/kg body weight of EEPNL

P: Proestrus, E: Estrus, M: metaestrus, D: Diestrus

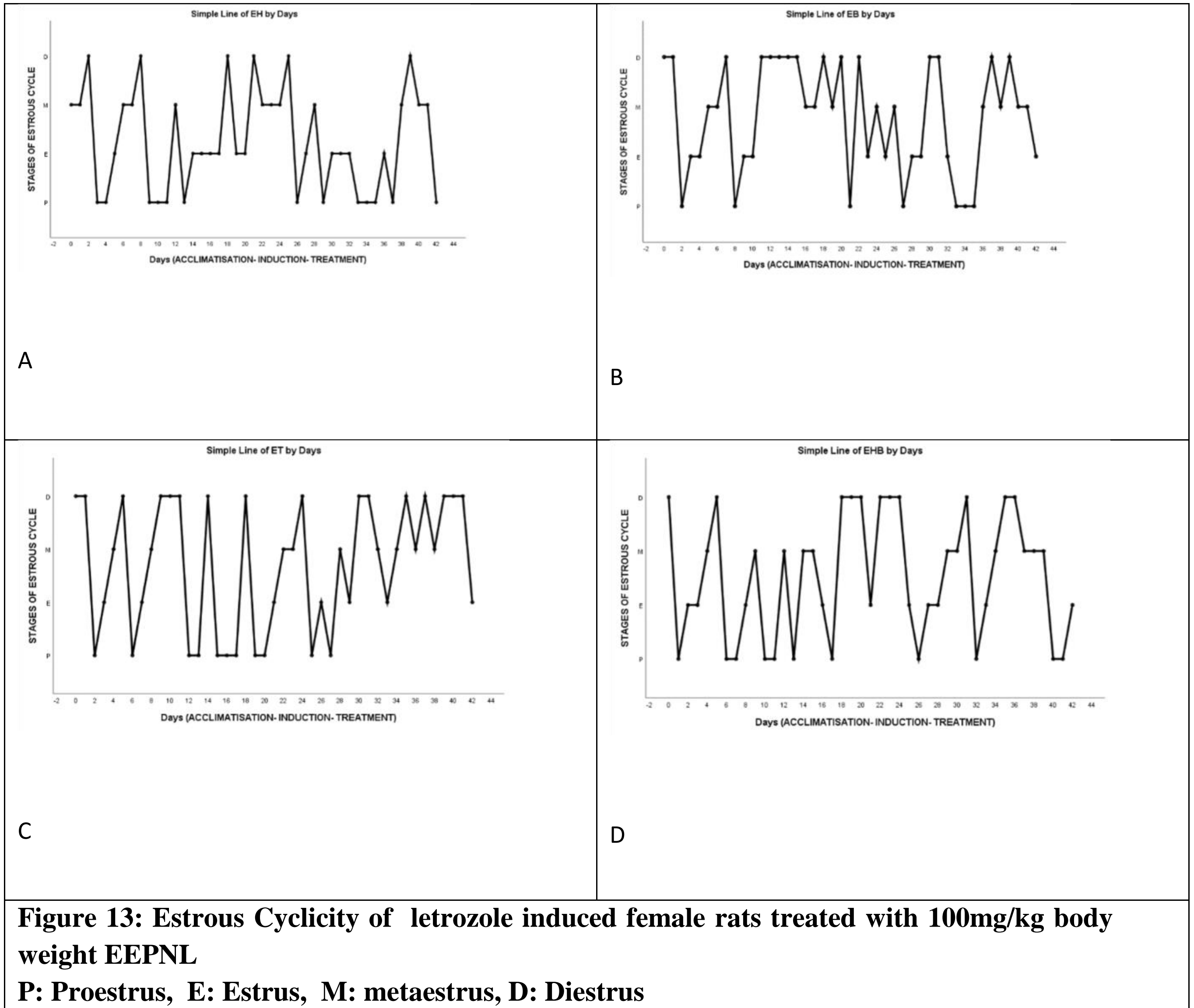


Figure 13: Estrous Cyclicity of letrozole induced female rats treated with 100mg/kg body weight EEPNL
P: Proestrus, E: Estrus, M: metaestrus, D: Diestrus

4.9 Lipid profile

4.9.1 Serum lipid profile of letrozole-induced rats after oral administration of solvent extract of *Parquetina nigrescens* leaves.

The administration of letrozole significantly (p>0.05) increase the serum total cholesterol, triacylglycerol, HDL-cholesterol and LDL-cholesterol concentrations. However, administration of 50 and 100mg/kg body weight of the extract significantly (p>0.05) decreased the serum total cholesterol concentration of the letrozole induced female rats. Furthermore, the 50mg/kg body weight of the extract significantly (p>0.05) elevated the serum cholesterol concentration of the animals. The letrozole treatment produced cholesterol concentration that compared favourably with the normal control group of animals (Table 8). Again, the elevation in triacylglycerol after the administration of letrozole was further increased by both doses of the extract. In contrast, the administration of letrozole to the letrozole-treated rats significantly (p>0.05) reduced the serum triacylglycerol content of the animals (Table 8). In addition, the administration of the extract to the letrozole-induced rats significantly (P>0.05) increased the serum concentration of HDL-c when compared with the normal control group of rats. The pattern of elevation of HDL-C by the extract was extended to the letrozole-treatment rats that received the standard drugs (metformin and clomiphene citrate) (Table 8). Although, letrozole treatment increased the serum concentration of LDL-C, the administration of the exact significantly (p>0.05) reduced the serum HDL-C concentration when compared with the rats in the control group and the letrozoletreated rats that received distilled water. This trend of decrease in the serum levels of LDL-C was extended to the letrozole-treated rats the received standard drugs. The level of LDL-C produced by letrozole compared favourably with those that received the 100mg/kg body weight of the extract (Table 8).

Table 8: Serum Lipid profile of letrozole-induced female rats

Groups	Total Cholesterol concentration (mmol/dL)	HDL Cholesterol concentration (mmol/dL)	Triglyceride concentration (mmol/dL)
Control	10.23 ± 0.01 [a]	4.85 ± 0.29[a]	14.50 ± 0.85[a]
PCOS + distilled water	68.76 ± 0.01[d]	0.60 ± 0.26[b]	22.28 ± 0.38[d]
PCOS + Clomiphene Citrate + Metformin (7.14mg/kg body weight)	8.03 ± 0.37[a]	0.38 ± 0.64[b]	16. 51 ± 0.18[b]
PCOS+50mg/kg body weight of extract	30.63 ± 1.76[c]	0.46 ± 0.08[b]	17.87 ± 0.47[c]
PCOS+100mg/kg body weight of extract	14.35 ± 0.41 [b]	0.80 ± 0.01[b]	15.35 ± 0.36[a]

Data are mean of four determinations ± SEM; Values with different superscripts in each column are significantly different ($P<0.05$).

4.10 Effects of administration of letrozole on serum hormone concentration of female Wistar rats

Compared to the control, there was a significant ($p<0.05$) increase in the level of serum insulin in PCOS untreated rats. The treatment with standard drugs (metformin and clomiphene), 50mg/kg body weight of EEPNL resulted in a significant regression in the level of serum insulin as compared to PCOS rats (Figure 14).

A significant increase in testosterone accompanied by a significant decrease in the levels of progesterone in rats with PCOS compared to the healthy rats in the control group. PCOS rats treated with the standard drugs (metformin and clomiphene citrate) showed a significant increase in progesterone levels compared to PCOS group (PCOS + distilled water). Consistently, a significant increase in the testosterone levels were observed in PCOS groups treated with either 50mg/kg EEPNL or 100mg/kg EEPNL compared to PCOS untreated rats (Figure 15).

A significant increase in LH accompanied by significant decrease in FSH levels was observed in rats with PCOS compared to the healthy control groups (i. e. normal control). PCOS rats treated with the standard drugs (metformin and clomiphene citrate) as well as 50mg/kg and 100mg/kg of EEPNL exhibited a significant ($p>0.05$) increase on FSH levels, while significant improvement of LH level was observed in the rats administered with 100mg/kg of EEPNL (Figure 17).

The administration of ethanolic extract of *P. nigrescens* leaves significantly increased ($p>0.05$) the serum estradiol concentration of the female rats. This significant increase in the estradiol concentration resulted in a hoger concentration of estradiol in the plant extract treated rats than the rats in the control group (Figure 19).

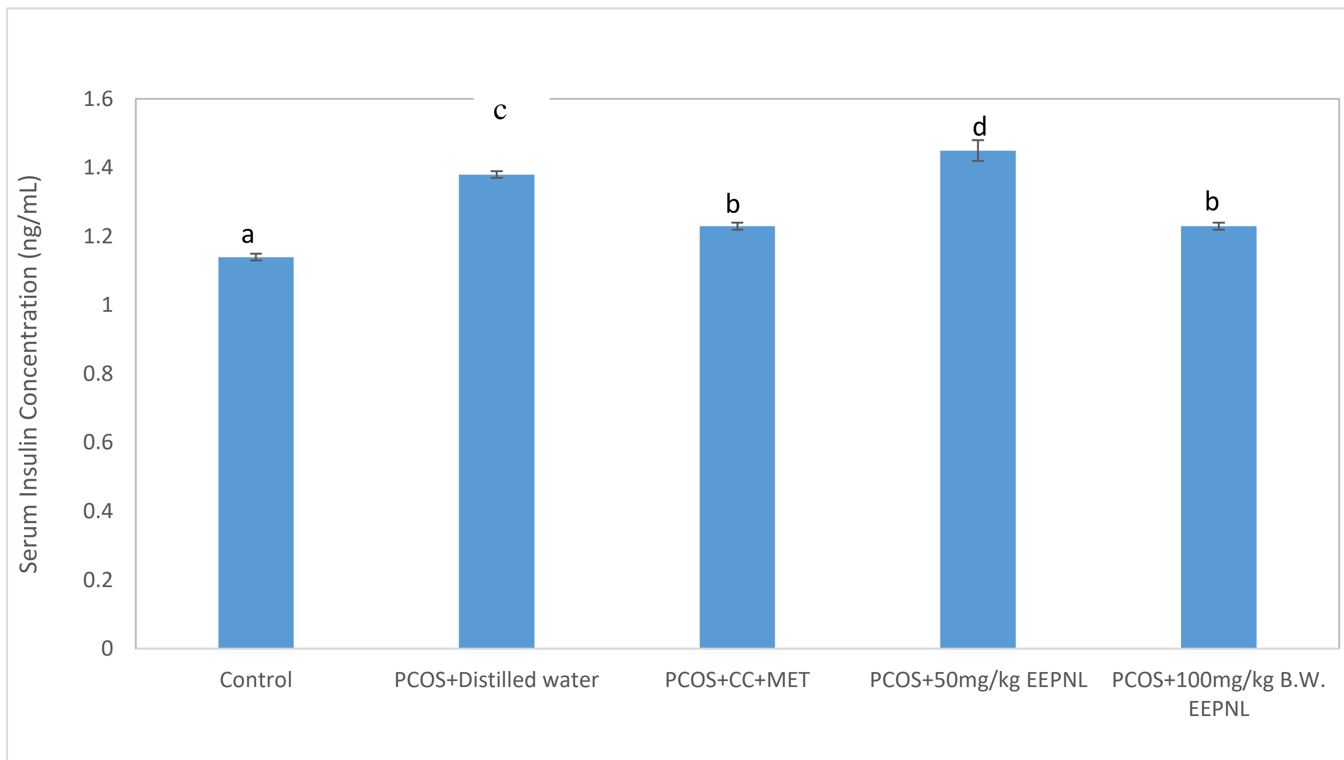


Figure 14: **The Effect letrozole-induced PCOS on serum insulin concentration of rats administered ethanolic extract of *Parquetina nigrescens* leaves**

Data are mean of four determinations ± SEM; Values with different superscripts in each column are significantly different ($P<0.05$).

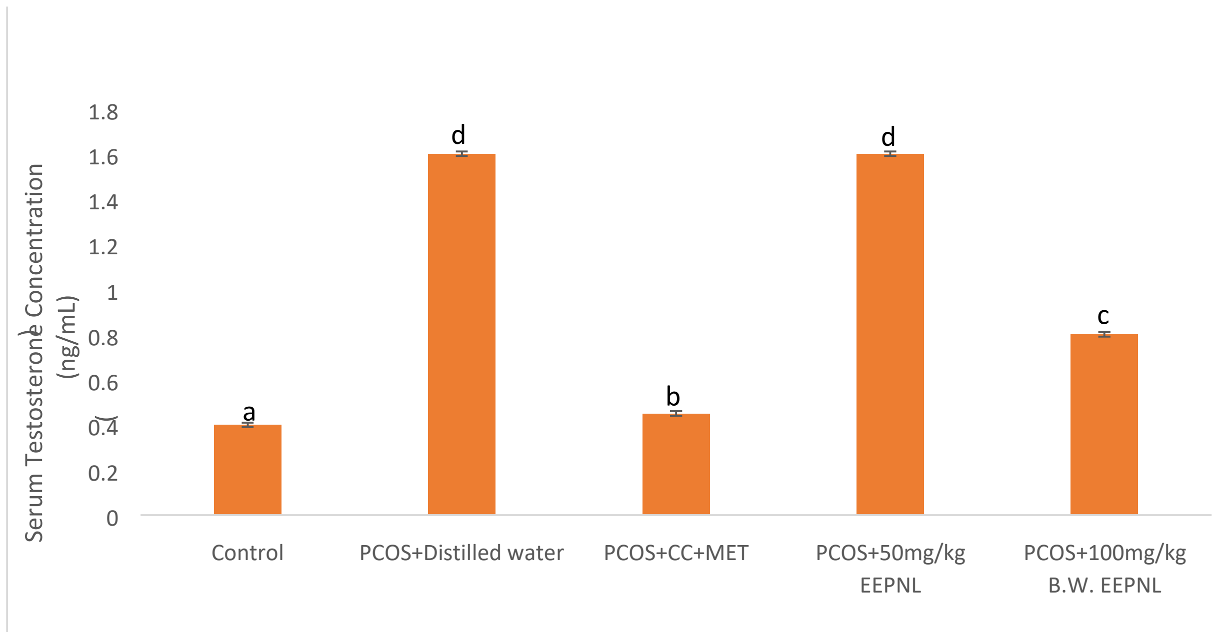


Figure 15: The Effect letrozole-induced PCOS on serum testosterone concentration of rats administered ethanolic extract of *Parquetina nigrescens* leaves

Data are mean of four determinations ± SEM; Values with different superscripts in each column are significantly different (P<0.05).

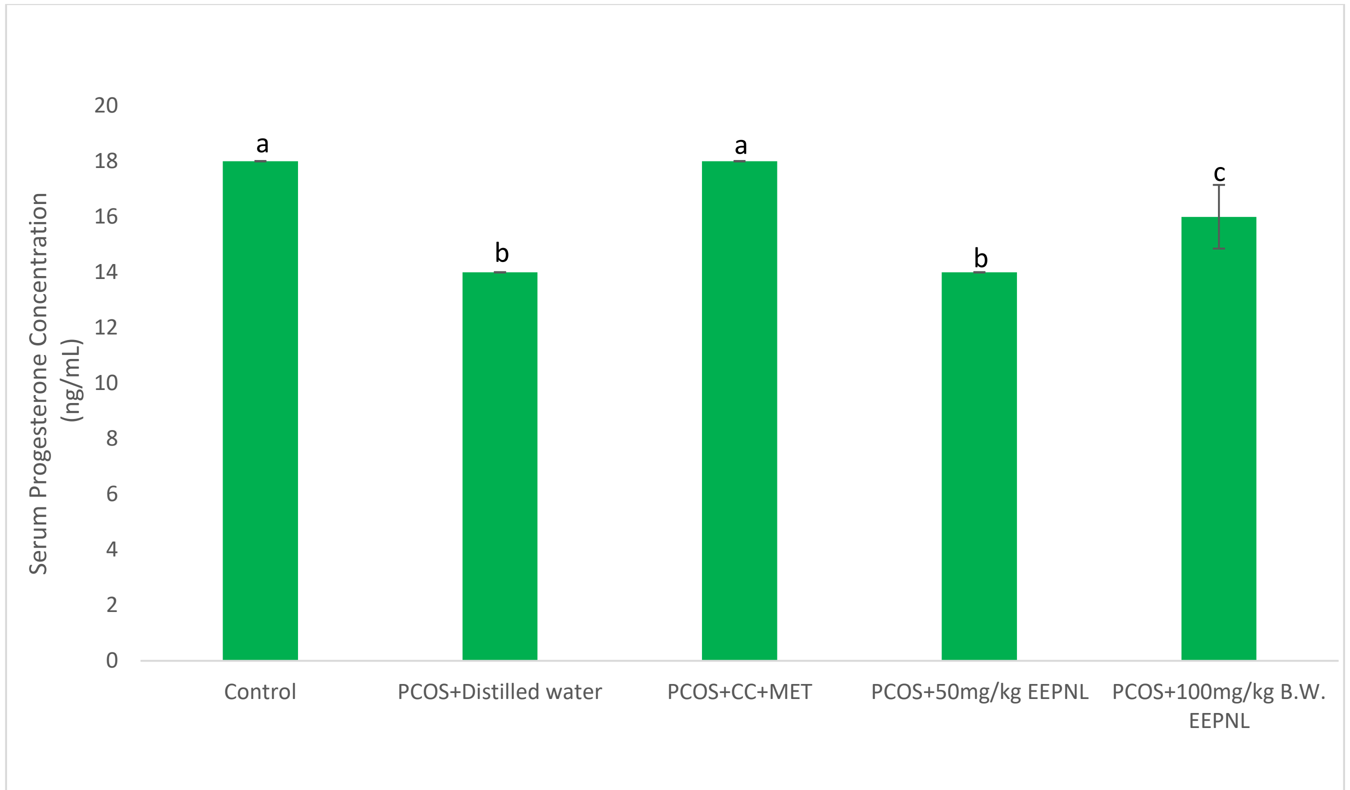


Figure 16: The Effect letrozole-induced PCOS on serum progesterone concentration of rats administered ethanolic extract of *Parquetina nigrescens* leaves

Data are mean of four determinations ± SEM; Values with different superscripts in each column are significantly different (P<0.05).

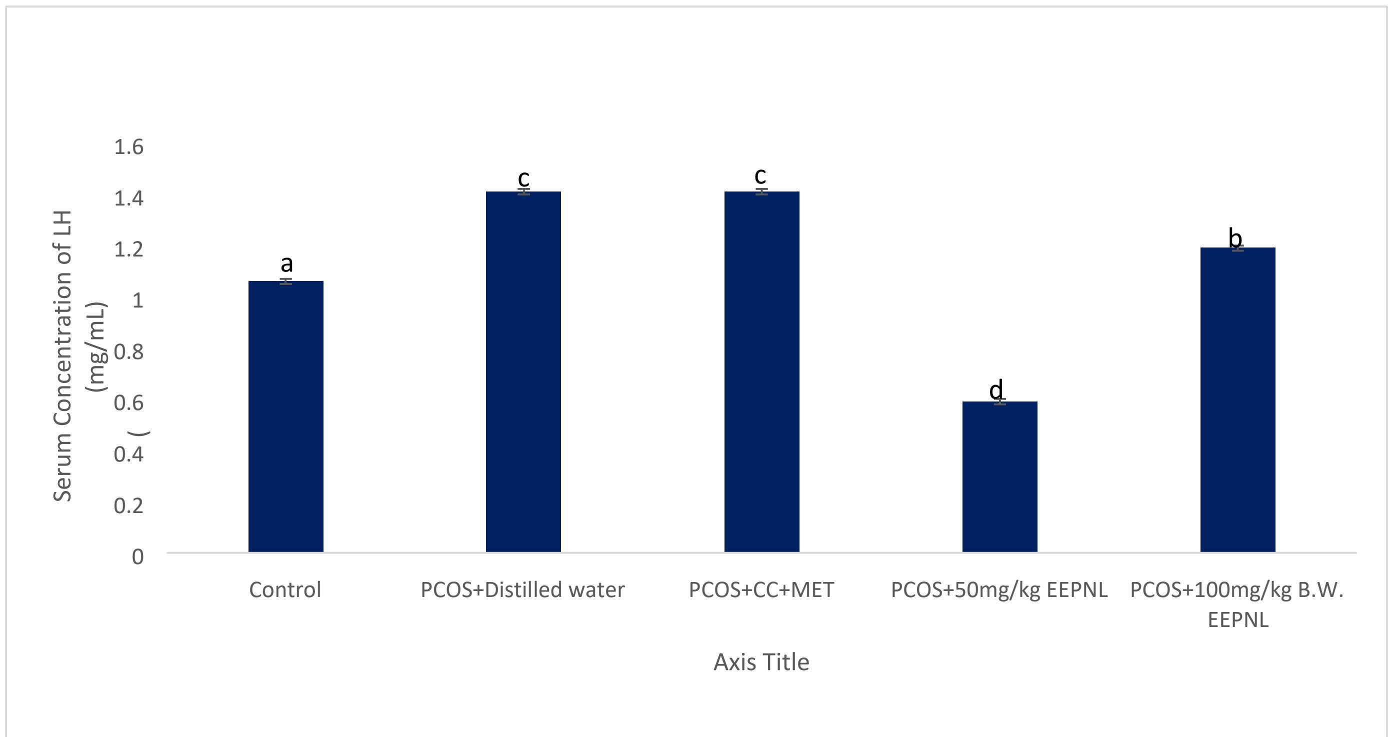


Figure 17: The Effect letrozole-induced PCOS on serum LH concentration of rats administered ethanolic extract of *Parquetina nigrescens* leaves.

Data are mean of four determinations ± SEM; Values with different superscripts in each column are significantly different ($P<0.05$).

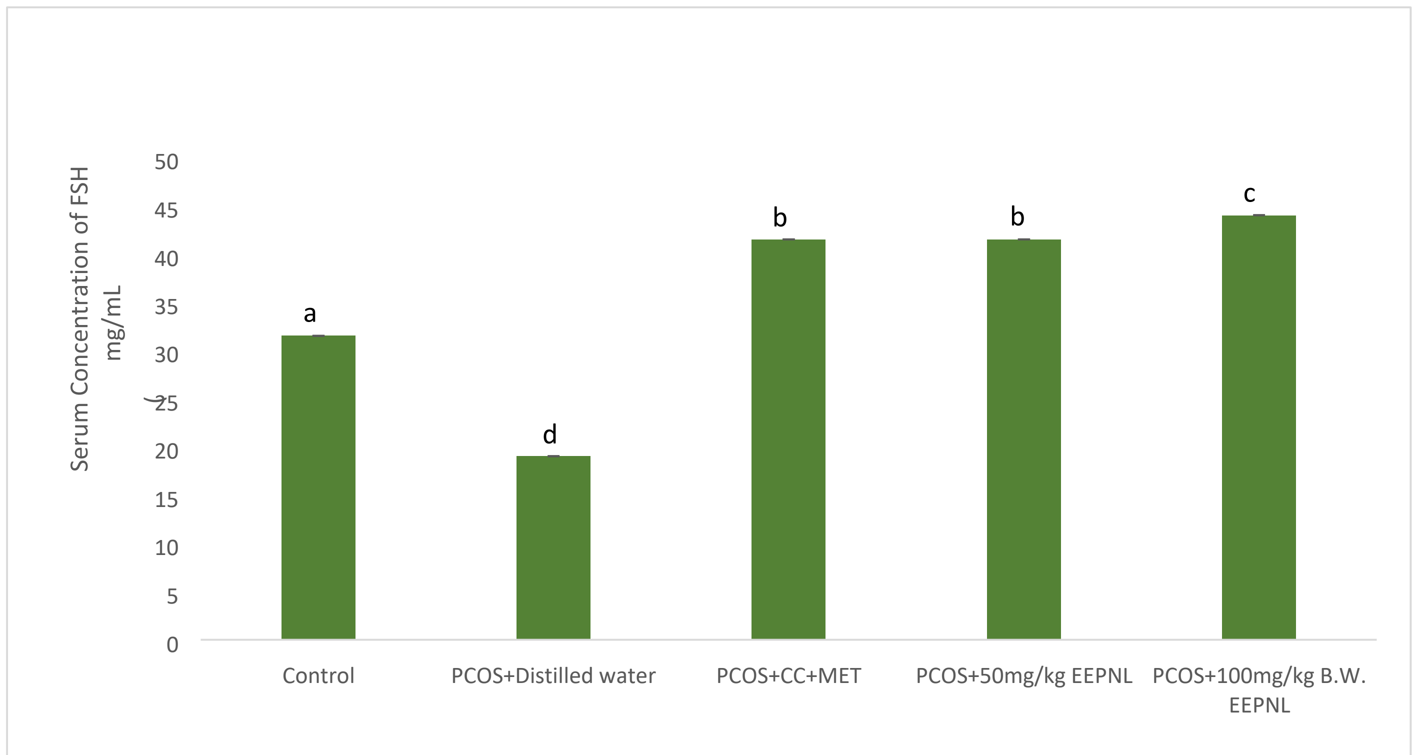


Figure 18: The Effect letrozole-induced PCOS on serum FSH concentration of rats administered ethanolic extract of *Parquetina nigrescens* leaves

Data are mean of four determinations ± SEM; Values with different superscripts in each column are significantly different (P<0.05).

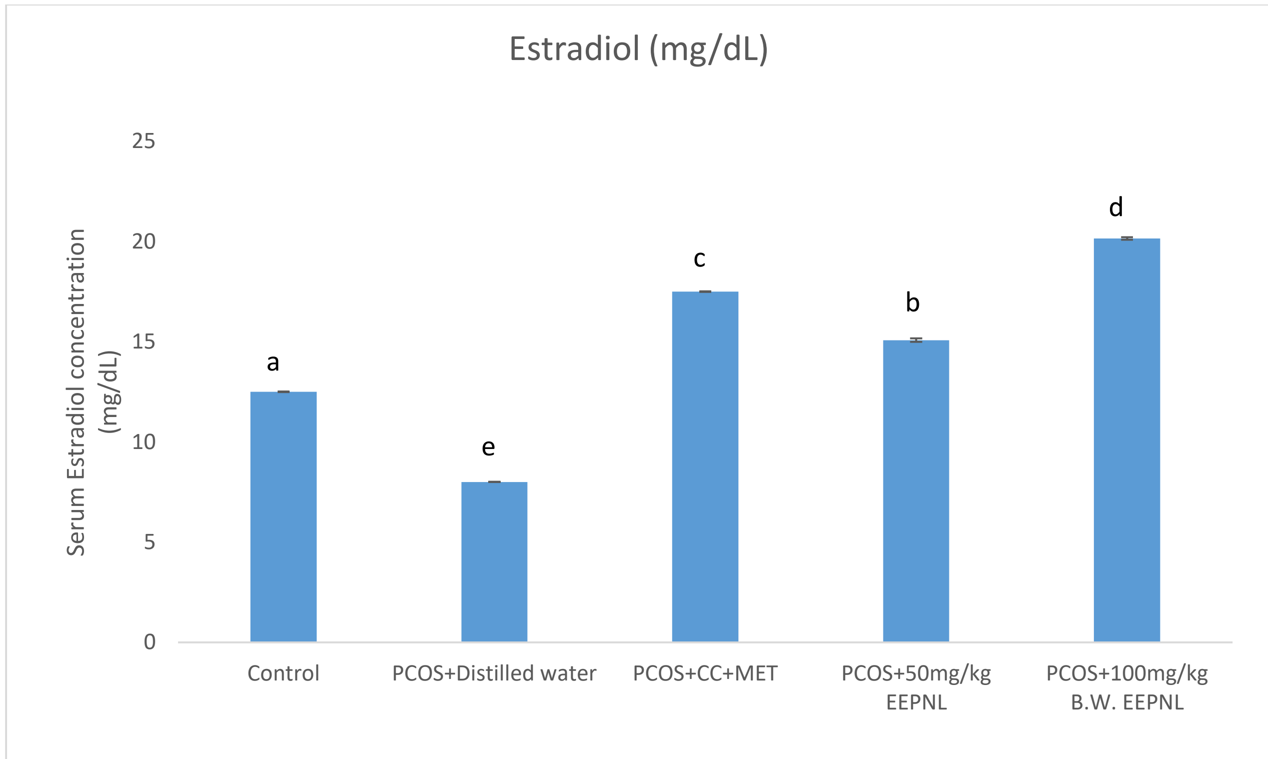


Figure 19: The Effect letrozole-induced PCOS on serum FSH concentration of rats administered ethanolic extract of *Parquetina nigrescens* leaves

Data are mean of four determinations ± SEM; Values with different superscripts in each column are significantly different (P<0.05).

CHAPTER FIVE

5.0 DISCUSSION

The most common reproductive disorder diagnosed in women of reproductive age is the polycystic ovarian syndrome (Mikhael *et al*., 2019). It is a clinical disorder that affects women having various dysfunctional reproductive and metabolic features which leads to infertility (Amal *et al.,* 2019; Emamalipour *et al.,* 2019). Insulin resistance, cardiovascular diseases (CVD), and type 2 diabetes are the main complications of PCOS (Emamalipour *et al.,* 2019). In this study, letrozole administered at 1mg/kg body weight was used to induce PCOS in female Wistar rats. It has been reported by Amal *et al.* (2019) that administration of 1mg/kg body weight of letrozole induces PCOS and its associated metabolic disorders.

This study reveals that, the oral administration of varying doses of ethanolic extracts of *P.nigrescens* leaves mitigated the induced features/symptoms of PCOS rats after 14 days of treatment. This therapeutic effect was characterised by the restoration of estrous cycle, reduction in fasting blood glucose level as well as the significant improvement of the lipid profile and sex hormones.

The oestrous cycle is always disrupted in rats with PCOS, mainly due to the irregularity of steroid hormones responsible for the regulation of ovarian function (Ndeingang *et al*., 2019). However, in this study, PCOS rats had irregular oestrous cycle while the rats in the normal control group had stable and regular oestrous cycle at the end of the induction period. Rajan *et al*. and Yang *et al* (2017) reported that letrozole-induced PCOS in rats is associated with prolonged oestrous cycle.

Vaginal cytology has been reported to be the best method of assessment of oestrous cycle in animal studies (Ajayi and Akhigbe, 2020). Changes in vaginal cytology is used in interpreting the changes in hormonal levels and modifications in oestrous cycle, the administration of letrozole altered the oestrous cyclicity inducing prolong or persistent estrus phase with a cycle 7-8 days (Nallathambi and Bhargavan, 2019). The administration of MET and CC did not completely reverse the persistent estrus phase while the PCOS rats administered 50 mg/kg B.W. had a complete reversal of their cycle to a normal 4-5 days cyclicity (Nallathambi and Bhargavan, 2019).

Letrozole, an aromatase inhibitor acts by competitively inhibiting the action of aromatase enzyme which converts androgens to estrogens (Rose and Brown, 2020). The use of letrozole results in the increase in androgen levels in the ovary (Rose and Brown, 2020) with features analogous to that of human PCOS, including hyperandrogenism and abnormal follicles (Rose and Brown, 2020).

The presence of PCOS is indicated by the measurement of sex hormone levels. Elevated serum testosterone, LH concentrations and low progesterone and FSH are the consistent parameters levels to diagnose a woman with PCOS (Ndeingang *et al*., 2019). As evident in this study, the administration of letrozole significantly increased insulin, testosterone and LH levels while FSH and progesterone were

significantly decreased. This result agrees with the work of Ndeingang *et al*., (2019) that elevated serum LH and low progesterone as seen in this result indicates the presence of PCOS. The administration of MET and CC significantly reversed the high level of testosterone, and progesterone while the administration of 100mg/kg B.W. of letrozole caused a significant reduction in the testosterone, and LH levels although this dose (100mg/kg B.W.) increased the levels of progesterone and FSH levels. High testosterone levels reflected accumulation of androgens possibly due to the blockade of conversion of androgen substrates into estrogens (Handelsman, 2020). The high serum LH concentrations could be due to the reduction of oestrogen production in hypothalamus and pituitary due to the letrozole presumably enhanced LH secretion (Amal *et al.,* 2019).

The anti-folliculogenic action of the elevated levels of LH (hillier, 1990) and the absence of progesterone actions on follicular development in the final steps of follicular rupture are features of PCOS. The increase in progesterone concentration in letrozole induced animals administered with 100mg/kg B.W. of the extract corroborates the insulin-sensitising properties of the extract. This may be due to the extract having similar mode of action as metformin resulting in the reversal of LH leading to follicular rupture and formation of corpus luteum that is responsible for the synthesis of progesterone.

The hypothalamus secretes gonadotrophin- releasing hormone (GnRh), which stimulates the pituitary gland in the brain to release follicle stimulating hormone (FSH) and lutenising hormone (LH) (Slater, 2012). FSH stimulates the growth and maturation of ovarian follicles by acting directly on the reveptors located on the granulosa cells (Kumar *et al.,* 1997). The increase in the FSH concentration of in letrozole induced rats treated with 100mg/kg body weight of the extract explains the increased FSH-gonadotrophin releasing hormone in the hypothalamus thereby causing the anterior pituitary gland to synthesize FSH in order to stimulate folliculogenesis and ovulation. The submission here is in line with the reports of Pasqualin and Gambineri (2006). That ovulation can also be significantly improved by insulin sensitizers.

Elevated levels of estrogen has been reported to induce reproductive features found in women with PCOS such as disrupted ovulation and hormonal imbalances (Walters *et al*., 2012). Administration of letrozole causes disruption of hormones such as decreased estradiol and progesterone concentration (Carr *et al.,* 2019). When compared with PCOS group, estradiol level was significantly increased ($p < 0.05$) in rats co-administered with clomiphene citrate and metformin, and those administered with both the 50 and 100mg/kg body weight. Treatment of PCOS rats with the ethanolic extract of *P. nigrescens* leaves restored the cyclicity by modulating the aromatisation of androgens into estrogen, by lowering LH level, by improving circulating estradiol concentration and inducing ovulation. In this study, letrozole-induced PCOS rats exhibited high LH and testosterone levels but low estradiol and FSH concentrations compared with the control. The leaves of *P. nigrescens* extract exhibited an

antiandrogenic effect in PCOS rats by increasing aromatase process (i.e. the conversion of of testosterone to estradiol) leading to low level of testosterone in the body (Ndeingang *et al*., 2019).
One consequence of PCOS is the imbalance of lipid profile and the development of dyslipidaemia. The result in this study is similar to that of Amal *et al.* (2019). The administration of letrozole PCOS-induced group showed marked increase in cholesterol, triglyceride and decrease in HDL levels and LDL levels. These parameters were significantly improved after the administration of *P.nigrescens* extracts. The differences in hormone levels and lipid profile are attributed to hyperandrogenemia. The adverse effect of excess androgen may be expressed in many systems (Amal *et al.,* 2019). Androgen receptors present on adipocytes and testosterone have an anti-lipolytic effects on abdominal subcutaneous pre-adipocytes, apparently through selective inhibition of catecholamine-induced lipolysis (Amal *et al.,* 2019). The hyperlipidemia observed in the PCOS untreated rats was also correlated with an upward trend in body weight which could be as a result of anabolic properties of letrozole, associated with fat accumulation.
The weight of the rats after treatment with metformin and clomiphene citrate were significantly ($p>0.05$) lower than those after induction with 1mg/kg B.W. of letrozole, this result agrees with Zhang *et al*., (2017) that the combination of metformin and clomiphene evidently reduced the body weight. Studies have shown that metformin may significantly improve menstrual cycles and ovulation rates in women with PCOS due to its insulin-sensitizing properties. Metformin may also have important effect on hyperandrogenism, metabolic alterations and importantly on fertility (Pasquali and Gambineri, 2006). Flavonoids have been discovered to have antiglycemic effects in animal (Saba *et al.,* 2010). Hence, the presence of flavonoid in the extract may be responsible for thenormalization of the oestrus cycle in the animals as seen in administered metformin
Hyperglycemia is also considered as an important indicator of PCOS. However, in this study, the level of glucose before induction of PCOS group was significantly greater than that of the normal control. After induction and treatment, the glucose level of the PCOS group significantly decreased compared to that of the normal control group. Consequently, it was observed that the level of insulin for PCOS untreated rats significantly increased compared to the control group. Hence it can be said that there was no hyperglycaemia and hyperinsulineamia this can be as a result of good and effective glucose molecule binding to insulin receptors. This indicates absence of insulin resistance.

5.1 Conclusion

This study shows confirmed that letrozole-induced PCOS in rats is associated with reproductive and metabolic disorders. Ethanolic extracts of *P.nigrescens* leaves restored estrous cyclicity, reduced insulin level, improved lipid profile and sex hormones in PCOS rats after 14 days of treatment. This plant,

might be considered as an alternative therapeutic remedy to treat reproductive and metabolic disorders in patients associated with PCOS.

5.2 Recommendation

The bioactive components responsible for the efficacy against polycystic ovarian syndrome should be isolated and investigated for its mechanism of action.

Further studies should be carried out on the effects of ethanolic extracts of *P.nigrescens* leaves on the litters produced by letrozole-induced animals.

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APPENDIX I

0.25M Sucrose Solution

171.15g of sucrose was dissolved in small quantity of water and made up to 2 litres with distilled water.

Preparation of 7.14mg/kg of Metformin

Each tablet of metformin drug contains 500mg of active ingredient is used by humans with approximately 70kg body weight. The average weight of the experimental animals was 170.81g i.e. 0.17kg. Therefore 7.14mg/kg body weight metformin was used.

Preparation of 1mg/kg of Letrozole

Each tablet of letrozole contains 2.5mg of active ingredient letrozole is used by humans with approximately 70kg body weight. The average weight of the experimental animals was 170.81g. Therefore, 1mg/kg body weight of the experimental animals was used.

Preparation of 2mg/kg body weight of Clomiphene Citrate

Each tablet of clomiphene citrate contains 2mg of active clomiphene citrate is used by humans with approximately 70kg body weight. The average weight of the experimental animals was 170.81g. Therefore, 2mg/kg body weight of the experimental animals were used.

CALIBRATION CURVES

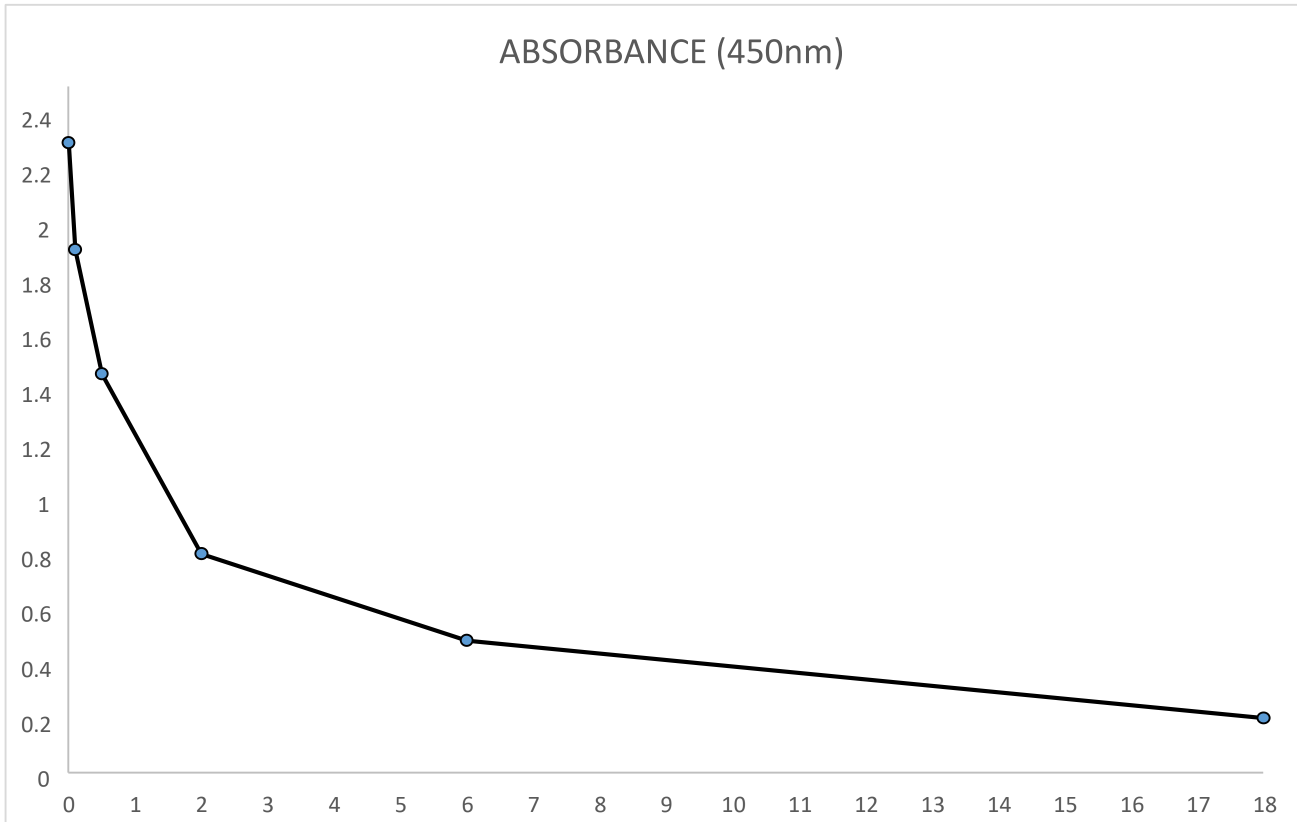

Figure 20: Calibration curve for testosterone

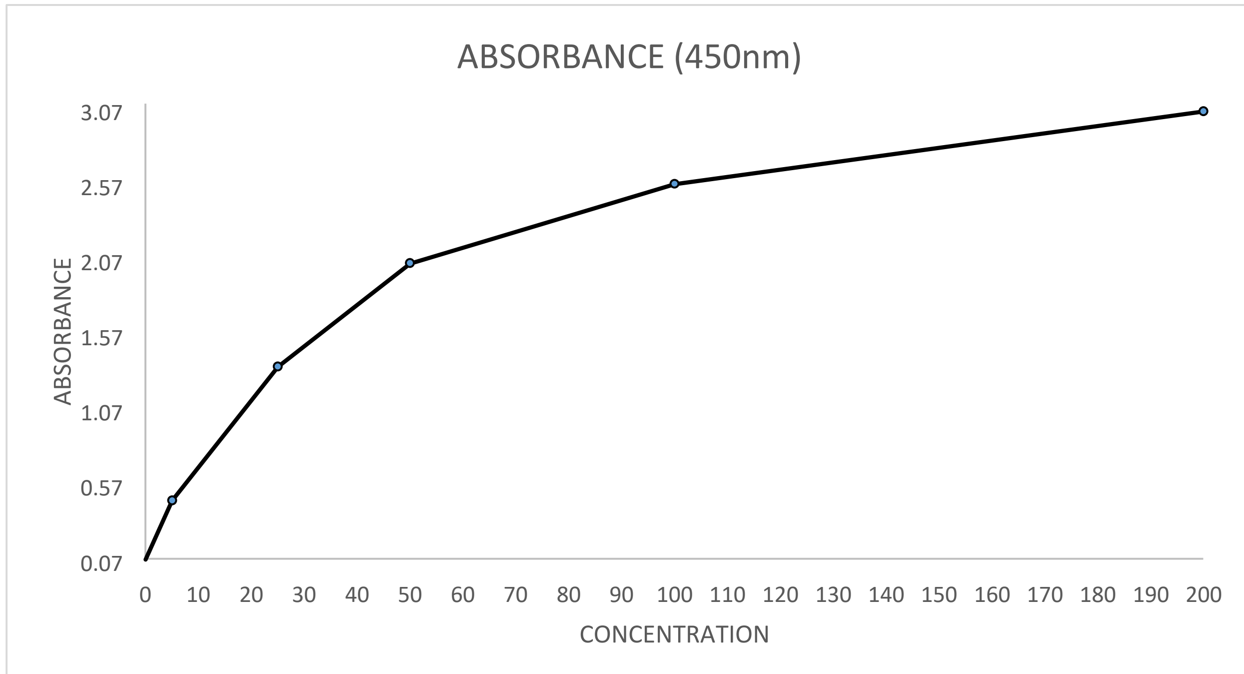

Figure 21: Calibration curve for Insulin

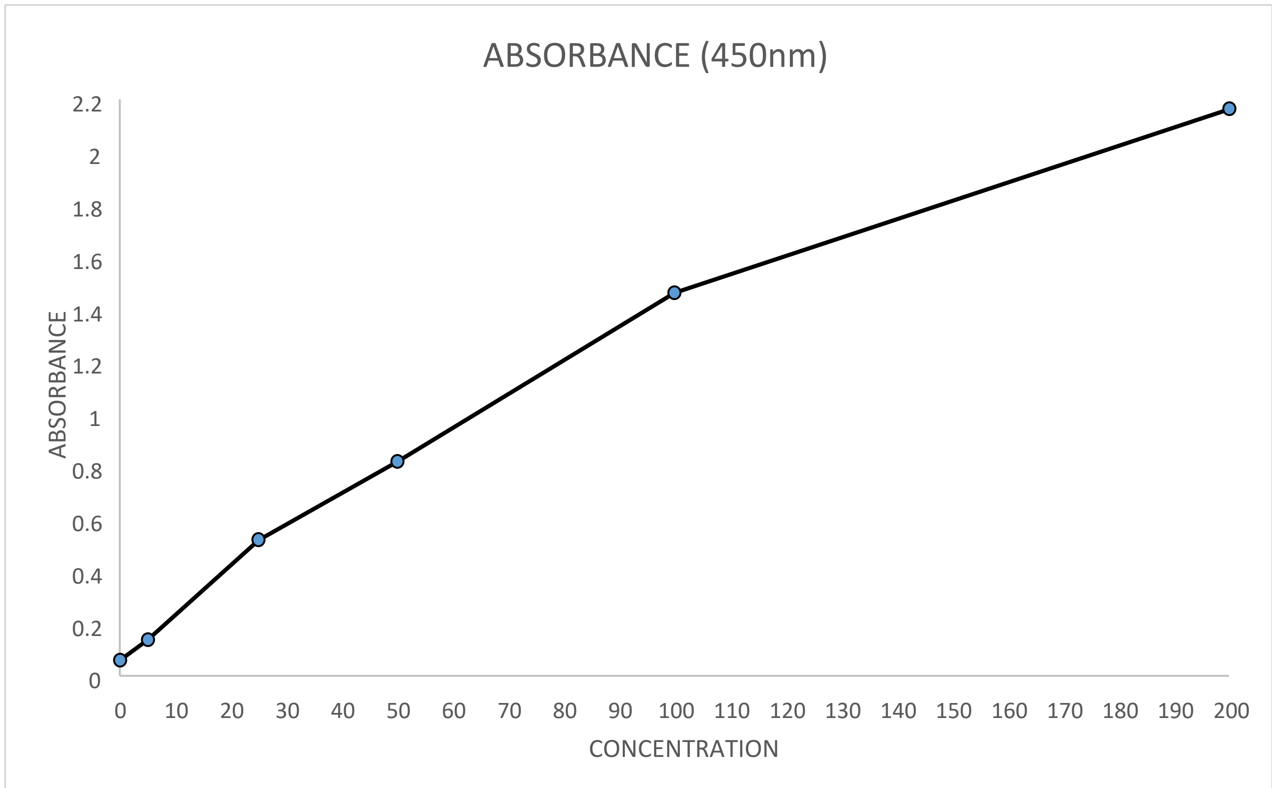

Figure 22: Calibration curve for Luteinising hormone

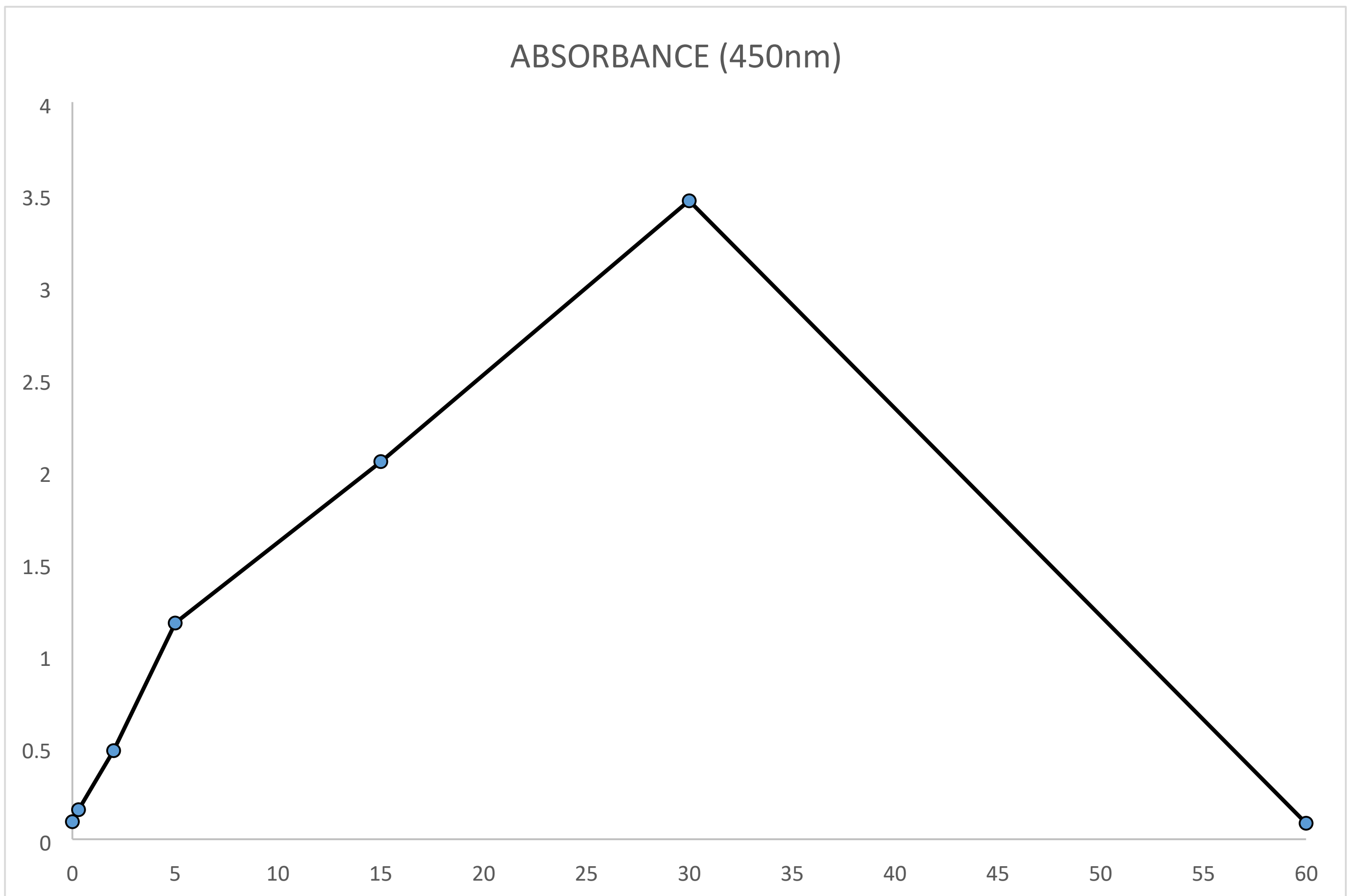

Figure 23: Calibration curve for Progesterone

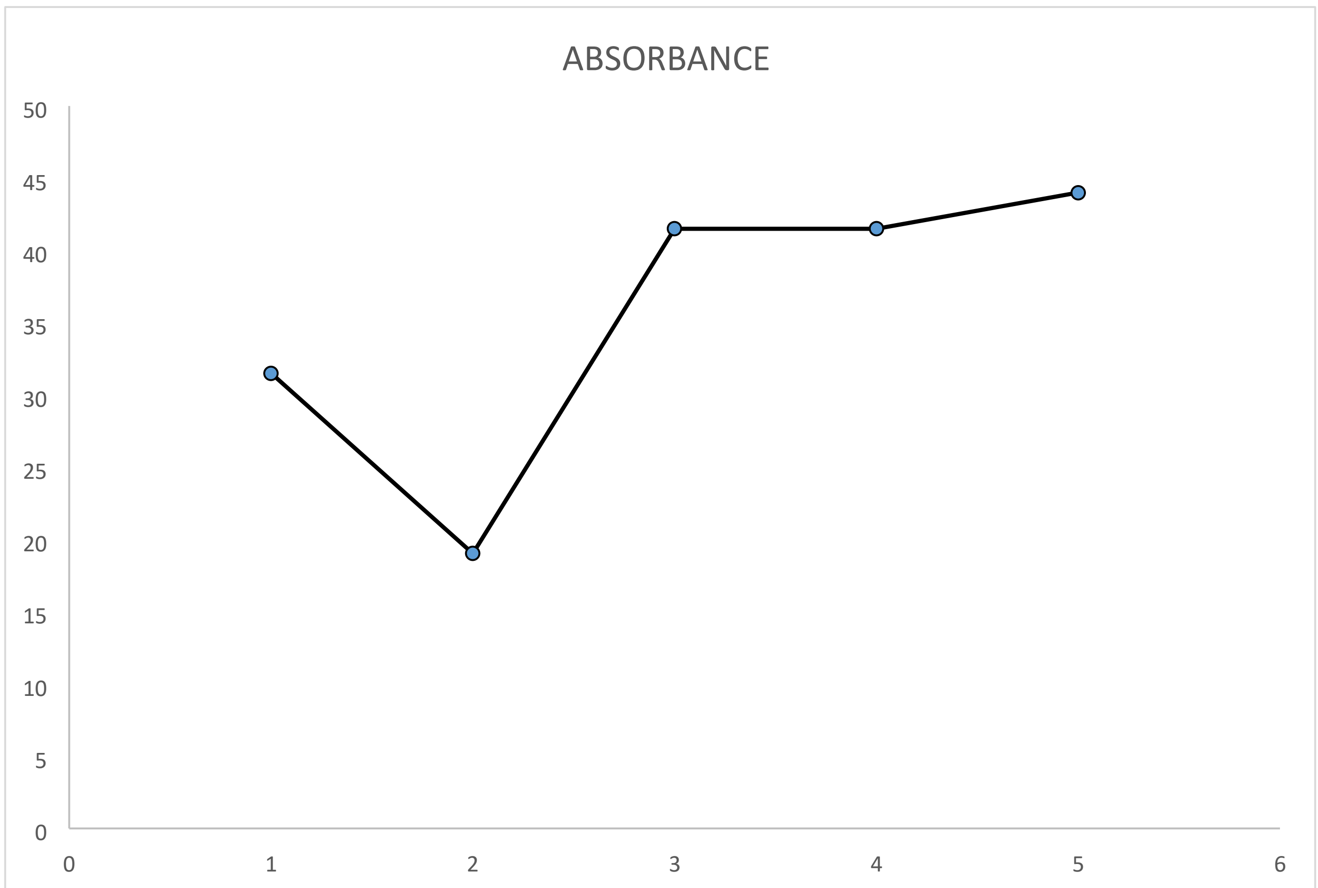

Figure 24: Calibration cure for Follicle stimulating Hormone

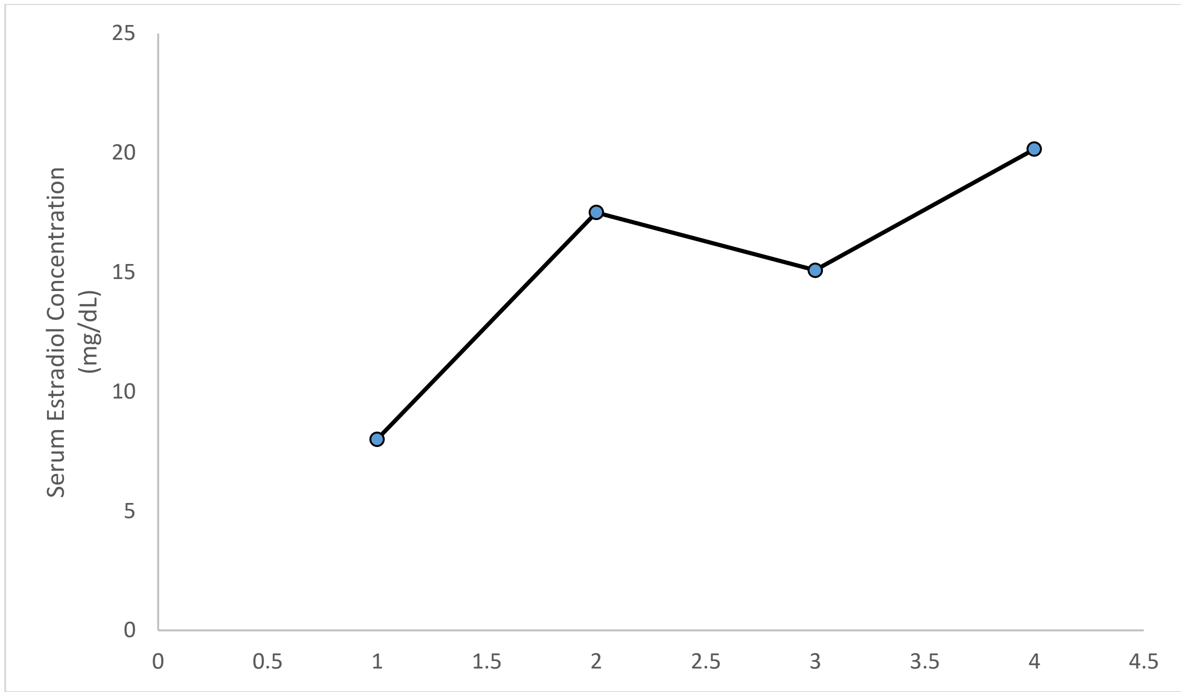

Figure 25: Calibration cure for Serum Estradiol