Ameliorative Effect of Ethanoic Extract of *Vernonia amygdalina* (*Del*) Leaves on Reproductive Functions of Letrozole-induced Polycystic Ovarian Syndrome in Wistar Rats

By

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A DISSERTATION SUBMITTED TO THE DEPARTMENT OF BIOLOGICAL SCIENCES, COLLEGE OF BASIC AND APPLIED SCIENCES, MOUNTAIN TOP UNIVERSITY, PRAYER CITY, OGUN STATE, NIGERIA, IN PARTIAL FUFILLMENT OF THE REQUIREMENTS FOR THE AWARD OF BACHELOR OF SCIENCE (B.Sc.) DEGREE IN BIOCHEMISTRY

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CERTIFICATION

It is hereby certified that this work was carried out by Ibeawuchi, Innocent Chidiadi, matriculation number 17010102014, of the Department of Biological Sciences, Biochemistry, Mountain Top University, Ogun State, Nigeria, under the supervision of Dr. F.J. Femi-Olabisi.

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APPROVAL PAGE

This project has been read and approved as having met the requirements of the Department of Biological Sciences, Mountain Top University, Ogun state, Nigeria, for the award of Bachelor of Science (B.Sc.) degree in Biochemistry.

…… ……

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(Head of Department)

DECLARATION

I, Ibeawuchi, Innocent Chidiadi, hereby declare that this project entitled **"Ameliorative Effect of Ethanoic Extract of *Vernonia amygdalina* (*Del*) Leaves on Reproductive Functions of Letrozole-induced Polycystic Ovarian Syndrome in Wistar Rats"** is a record of my research work. All sources of information have been specifically acknowledged.

……. ……..

Ibeawuchi, Innocent Chidiadi **Date**

DEDICATION

This report is dedicated to God Almighty, To my parents Mr/Mrs Ibeawuchi, My siblings- Jerryson, Davidson, Miracle and Harrison.

ACKNOWLEDGEMENTS

Am forever indebted to God Almighty who has been my helper and strength in this research project and the journey of badging an honorable degree.

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ABSTRACT

Polycystic Ovary Syndrome (PCOS) is an endocrine disorder known by symptoms such as hyperandrogenism, amenorrhea, and polycystic ovaries. Being an endocrine disorder, its associated with metabolic dysfunction of the endocrine gland which results to an imbalance of the sex hormones which include reduced level of follicle stimulating hormone and progesterone increased level of luteinizing hormone, Insulin and testosterone which causes cyst to form in the antral ovarian follicle. Among the various reference drugs used in the treatment of PCOS combination of metformin with clomiphene citrate used in this study, has shown to exhibit a good therapeutic effect in the management of PCOS. Ethnomedicine such as *Parquetina nigrescens* leaves, *Aspargus Racemosus, Tinospora Cordifolia* etc. have been reported to contain phytocompounds which showed therapeutic effect in annulling the comorbidities associated with PCOS. Therefore, this study was aimed at evaluating the ameliorative effect of ethanoic extracts of *V. amygdalina* leaves on reproductive and metabolic dysfunctions associated with letrozole-induced PCOS in female Wistar rats.

The effects of ethanoic extract of *V. amygdalina* leaves (EEVAL) on reproductive hormones, at the doses of 50 and 100 mg/kg body weight on PCOS-induced by letrozole was evaluated in female Wistar rats as well as their lipid profile. Twenty female Wistar rats (170.81 $\pm$5.00g) were completely randomized into 5 groups (A - E) of 4 each. The animals in group A received 0.5 ml of distilled water orally on daily basis for 21 days while the letrozole rats in groups B, C, D and E also received orally 0.5 ml of distilled water, 7.14mg/kg of metformin and 2mg/kg clomiphene citrate (reference drugs) and different volume of the extract conforming to 50 and 100 mg/kg body weight of EEVAL respectively. The administration of EEVAL at 100 mg/kg B.W. to PCOS significantly ($p<0.05$) increased serum FSH and progesterone concentration, as well as HDL-C concentration when compared to the control group. It ameliorated PCOS-induced elevated serum LH, Insulin and Testosterone concentration and as well as TG, TC and LDL-C in the female rats. In conclusion, the ethanoic extract of *V. amygdalina* leaves has ameliorative effects on letrozole-induced reproductive and metabolic dysfunctions associated with PCOS rats, therefore it can be explored in the management of PCOS.

Keywords: *Vernonia amygdalina*, Letrozole, Polycystic ovarian syndrome, reproductive hormones

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LIST OF ABBREVIATIONS

PCOS: Polycystic Ovarian Syndrome

FSH: Follicle Stimulating Hormone

LH: Luteinizing Hormone

INSR: Insulin Receptor

LHCGR: Luteinizing Hormone Choriogonadotropin Receptor

FSHR: Follicle Stimulating Hormone Receptor

YAP1: Yes-Associated Protein 1

C9orf3: Chromosome 9 Open Reading Frame 3

PCOM: Polycystic Ovarian Morphology

NIH: National Institute of Health

AMH: Anti-Mullerian Hormone

T2DM: Type 2 Diabetes Mellitus

IGF-1: Insulin-like Growth Factor

GnRH: Gonadotropin Releasing Hormone

SHBG: Sex Hormone Binding Globin

BMI: Body Mass Index

HDL: High Density Lipoprotein

TG: Triacylglycerol

TC: Total Cholesterol

LDL: low Density Lipoprotein

MMPS: Matrix Metalloproteinases

ECM: Extracellular Matrix

HMG; Human Menopausal Gonadotropin

ESHRE: European Society of Human Reproduction and Embryology

ASRM: American Society for Reproductive Medicine

AES: Androgen Excess Society

PCO: Polycystic Ovaries

IR: Insulin Resistance

TNFα: Tumor Necrosis Factor α

IL6: Interleukin 6

TZDs: Thiazolidinediones

HSD: Hydroxysteroid Dehydrogenase

OCPs: Oral Contraceptive

CC: Clomiphene Citrate

IVF: Invitro Fertilization

GC-MS: Gas Chromatography Mass Spectroscopy

FAI: Free Androgen Index

VAT: Visceral Adipose Tissue

ACTH: Adrenal Corticotropic Hormones

B.W: Body Weight

EEVAL: Ethanoic Extract of *V. amygdalina* Leaves

CHAPTER ONE

1.0 Introduction

Medicinal plants contain chemicals with therapeutic characteristics, they are employed in both traditional and modern medicine (Ademosun *et al.,* 2017). *Vernonia amygdalina* is another name for bitter leaf, it is a common medicinal plant in the south-eastern part of Nigeria, which has a strong bitter flavour that is predominant in Africa (Ademosun *et al.,* 2017). Polyphenols, saponins and alkaloids, amongst other secondary metabolites, has been described to be in abundance in its leaf (Ademosun *et al.,* 2017).

V. amygdalina is broadly used in the management and control of a variety of diseases, the leaves are useful in the preparation of herbal medicine (Ifeoluwa *et al.,* 2018). *V. amygdalina* is one of Africa's (West Africa) and Asia's most well-known plants. *V. amygdalina* is a plant that lives for a long time among the *Asteraceae* family. In Africa, *V. amygdalina* has extensively been used to cure and/or control a widespread of ailments in both humans and animals (Ifeoluwa *et al*., 2018). In several African countries, particularly Nigeria, the leaves have anti-fever qualities and are used as a quinine supplement (Ifeoluwa *et al*., 2018). The plant is used to treat malaria in several parts of Africa (Tugume *et al.,* 2016).

Different phytocompounds obtained from various parts of the plant are accountable for the plant's activities (Ifeoluwa *et al.,* 2018), and several pharmacological studies have reported the anthelminthic, anticancer, antioxidant, antimicrobial, and antidiabetic potential of the plant (Anh *et al.,* 2019). According to estimates from the World Health Organization (WHO), PCOS affects over 116 million women worldwide (Bharathi *et al.,* 2017). Polycystic ovary syndrome (PCOS) affects almost 27% of females during their reproductive age (Stephanie 2021).

Cysts in the ovaries, increased levels of male steroid hormones (androgens), and irregular menstrual periods are the symptomatic degrees of the syndrome. Thus, PCOS may be defined as a heterogeneous endocrine disorder characterized by the existence of ovarian cysts, anovulation, and endocrine heterogeneity, all of which have a significant effect on a woman's life (Escobar-Morreale, 2018). Other varying symptoms amongst female individuals may include hyperandrogenism, menstrual disturbances, and differing sizes of cysts in the ovaries, though there are significant variations between people (Escobar-Morreale, 2018). Young adults with this condition may experience a few comorbidities which may include obesity, type 2 diabetes,

infertility, endometrial dysplasia, cardiovascular conditions, and psychotic disorders (El Hayek *et al.,* 2016). A decrease in Sex Hormone Binding Globin (SHBG) levels is one of the signs of PCOS, and it is linked to insulin resistance and Hyperandrogenism (Pinola *et al.,* 2015). Selective reproductive hormones to study include; Estradiol, Progesterone, Testosterone, Follicle Stimulating Hormone (FSH), Luteinizing Hormone (LH) and Insulin.

Therefore, this study was aimed at evaluating the ameliorative effect of ethanoic extract of *Vernonia amygdalina* (*Del*) leaves on reproductive functions of letrozole-induced polycystic ovarian syndrome in Wistar rats

1.1 Statement of Problem

Hyperandrogenism**,** ovulatory disorder and polycystic ovaries are the diagnostic features of PCOS. Being an endocrine disorder amongst female individuals, selective hormones are studied such as progesterone, testosterone, FSH and LH. Obesity and type 2 diabetes mellitus are few metabolic dysfunctions associated with PCOS.

Using ethanoic extract of *V. amygdalina* leaves phytoconstituents such as flavonoids which have been reported to contain several pharmacologic properties which also include antidiabetic properties (Anh *et al.,* 2019), which is an effective measure in tackling PCOS. Various in-vitro, and in-vivo experiment are carried out using the proposed plant extract, to compare the therapeutic effects of clomiphene citrate and metformin used in PCOS treatment, but none has been reported on PCOS-induced by letrozole.

1.2 Justification of Study

Traditional medicine has laid a claim on the reproductive and antihyperlipidemic effect in *V. amygdalina* leaves. It is therefore important to provide a scientific validation for this claim by employing various analytical methods such as phytochemical screening, UV-Visible spectroscopy and Gas chromatography-Mass spectroscopy analytical method to determine the phytoconstituent of *V. amygdalina* leaves and the ameliorative effects on reproductive and metabolic dysfunctions associated with letrozole-induced PCOS rats.

1.3 Aim of Study

The aim of this study is to determine the ameliorative effect of ethanoic extract of *Vernonia amygdalina* (*Del*) Leaves on selected reproductive hormones and insulin associated with letrozole-induced Polycystic Ovarian Syndrome in Wistar Rats

1.4 Specific Objectives of Study

The specific objectives of this study are as follows:

i. To evaluate the phytochemical components in the ethanoic extract of *Vernonia amygdalina* leaves through phytochemical screening, UV-Visible spectroscopy and GCMS,
ii. To induce PCOS using letrozole on female Wistar rats and monitor the oestrous cycle,
iii. To assay selected hormones such as testosterone, progesterone, follicle stimulating hormone, luteinizing hormone and insulin,
iv. To determine the serum lipid profile of PCOS rats,
v. To evaluate the associated metabolic disturbance associated with PCOS such hyperglycemia and increased body weight.

CHAPTER TWO

2.0. LITERATURE REVIEW

2.1. Historical Perspective of Polycystic Ovarian Syndrome

Stein and Leventhal, who pioneered the study of PCOS as a syndrome characterized by oligo-amenorrhea and polycystic ovaries, as well as hirsutism, acne, and obesity, initially discovered the condition in 1935 (Azziz *et al.,* 2016). Stein and Leventhal published a study in 1935 on a group of seven women who shared common traits such menstrual problems, hirsutism, and ovarian hypertrophy (Stein and Leventhal, 1935). He also addressed the absence of menstruation in women with enlarged ovarian volume and recommended ovarian wedge resection, after which two of the seven patients who regained a regular menstrual cycle following surgery became pregnant (Stein *et al.,* 1948).

Surgical treatment became less common when medicinal treatments like as clomiphene citrate, follicle stimulating hormone (FSH), and urinary source became accessible (Wang and Gemzell, 1980). In the early 1990s, during a National Institute of Health (NIH)-sponsored meeting on the illness, were formal diagnostic criteria for PCOS published and extensively utilized (Dorota *et al.,* 2017). Despite the fact that it is now commonly accepted that it is multifactorial and partly hereditary, a number of candidate genes have been proposed (Dorota *et al.,* 2017).

Insulin Receptor (INSR), Luteinizing Hormone/Choriogonadotropin Receptor (LHCGR), Follicle Stimulating Hormone Receptor (FSHR), Yes-Associated Protein 1 (YAP1), and Chromosome 9 open reading frame 3 (C9orf3), also known as aminopeptidase, are some examples of genes that may be linked to the syndrome (Liu *et al*., 2016; Zhao *et al.,* 2016). Finally, in November 2006, the Androgen Excess Society (now known as the Androgen Excess and PCOS Society) issued diagnosis guidelines, which were primarily focused on tying PCOS criteria to metabolic and other long-term comorbidities (Azziz et al., 2009).

The discovery that PCOS can be separated into four phenotypes based on three characteristics: hyperandrogenism, oligoanovulation, and polycystic ovaries, and that the Rotterdam 2003 and Androgen Excess Society 2006 criteria were simply deferrals of the NIH 1990 criteria, was a watershed moment in our understanding of PCOS (Azziz, 2021). As a result, diagnostic

classifications based on clinical symptoms such as the 1990 NIH Criteria, 2003 Rotterdam Criteria, and 2006 Androgen Excess-PCOS Criteria were developed (Azziz et al., 2009).

2.2. Polycystic Ovarian Syndrome

Polycystic ovarian syndrome (PCOS) causes little pearl-sized cyst clusters to emerge from the ovary on a regular basis (Yildiz *et al*., 2012). A cyst is an immature egg packed with fluid (Yildiz *et al*., 2012). PCOS, commonly known as "Stein-Leventhal Syndrome," is a complex, heterogeneous condition that affects 8-20% of females of reproductive age around the world (De Leo *et al.*, 2016). It affects women between the ages of 18 and 44 and is caused by genetic or environmental factors (De Leo *et al.,* 2016). Hyperandrogenism, amenorrhea, and polycystic ovaries are all symptoms of PCOS (Unluturk *et al.,* 2016).

This endocrinopathy has been associated to a variety of metabolic problems, such as dyslipidemia and insulinemia, as well as an elevated risk of type 2 diabetes, metabolic syndrome, and cardiovascular consequences (Unluturk *et al*., 2016). Despite its widespread occurrence, the exact cause of this disease is unknown (Crespo *et al.,* 2018). PCOS is a set of symptoms that women of childbearing age experience (Patel, 2018). It is caused by an imbalance in female sex hormone, which causes cysts to form in the antral ovarian follicle (Patel, 2018). The reproductive system is controlled by the hypothalamic-pituitary gonadal axis (HPG).

The release of gonadotropin releasing hormone (GnRH) from the hypothalamus stimulates the anterior pituitary gonadotropes to release LH and FSH, which leads to the production of ovaries (Chauldhari *et al*., 2018). The mechanism of GnRH production is altered because of insufficient progesterone peaks during the luteal stage of the menstrual cycle, resulting in an increase in LH output (Unluturk *et al.,* 2016). The adrenal gland contributes to hyperandrogenism in PCOS by stimulating the secretion of androgens by the ovaries as well as the synthesis of androgens when LH levels rise (Unluturk *et al.,* 2016).

Lifestyle adjustments, such as nutrition and physical activity, oral contraceptives, and metformin, which regulates the menstrual cycle, minimize insulin resistance, and help restore normal body weight (Kim, 2020).

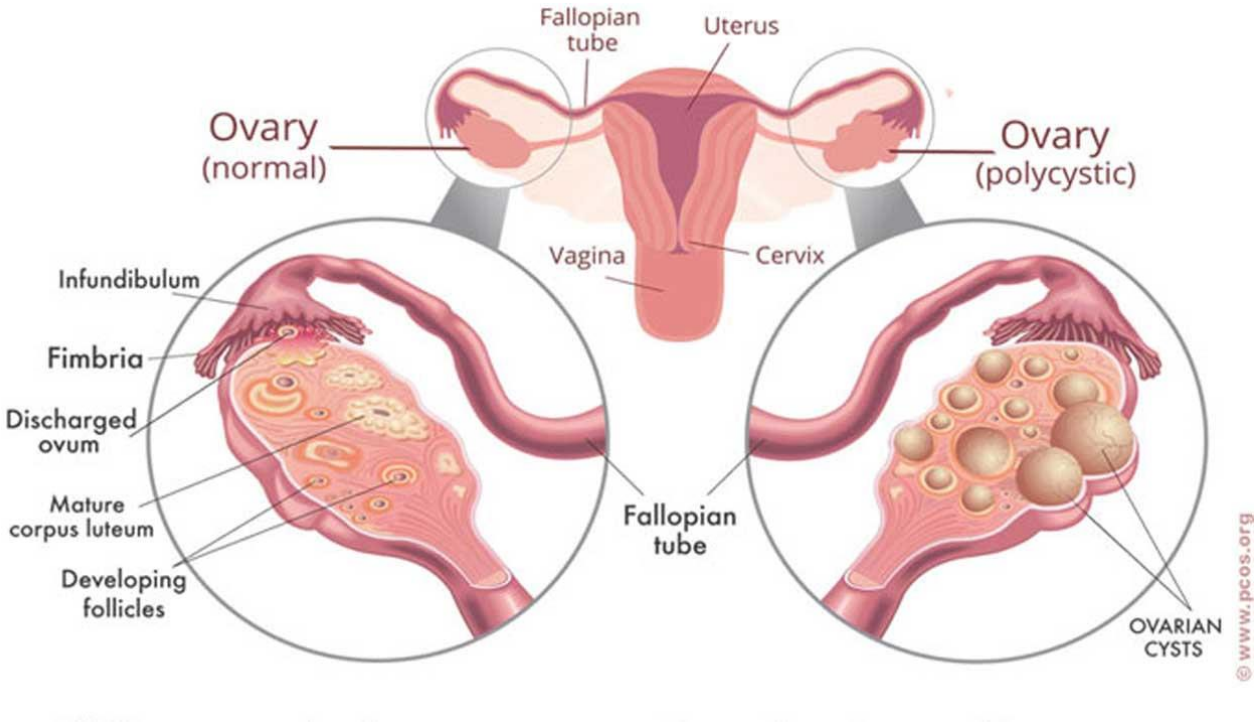


Figure 1: The normal ovary and polycystic ovary.

Source: Adunwoke, 2019.

2.2.1. Ovarian Dysfunction of PCOS

Primary ovarian failure, also known as primary ovarian insufficiency, is a dysfunction that affects women before they reach menopause (Tracy *et al.,* 2016**).**

It is defined as the ovary's failure to act adequately in its role as a reproductive organ in women under the age of 40 (Pellegrini, 2016). Primary ovarian dysfunction might occur when the ovary fails to function normally due to the natural gonadotropin stimulation delivered by the brain and pituitary gland (Pellegrini, 2016), and when gonadotropin stimulation in the hypothalamus and pituitary is insufficient results to secondary ovarian dysfunction.

The term "premature ovarian insufficiency" (POI) was chosen because it covers a wide range of disorders, and the term "insufficiency," rather than "failure," indicates to the possibility of intermittent ovarian development, which can lead to ovulation and pregnancy (Panay *et al.,* 2020). When a female child is born, her primordial follicles contain 700,000 to 1 million oocytes (Panay *et al.,* 2020). The reproductive lifetime is determined by the pool's survival duration, which is generally 400 ovulated cycles. The loss of these follicles causes POI, which results in infertility and a reduction in estrogen hormone output.

New-onset menstrual irregularities, which can range from infrequent to excessively frequent menses preceding amenorrhea, can be the first sign of ovarian insufficiency in some women (Torrealday, 2017). Anovulation refers to the absence or lack of ovulation, whereas oligo-ovulation happens when ovulation is unbalanced but not fully absent (Bharathi *et al.,* 2017). When ovulation is unbalanced but not completely missing, it’s known as oligo-ovulation (Rachel, 2020).

The most prevalent causes of secondary ovarian failure are diseases of the pituitary gland or hypothalamus, but there is a problem receiving hormone signals from the brain to them (Pellegrini, 2016). Pituitary growths, such as prolactinomas, can produce hyperprolactinemia, which can lead to secondary ovarian insufficiency (Pellegrini, 2016). The reason of secondary ovarian insufficiency is still unknown (Pellegrini, 2016). A multitude of reasons might cause secondary ovarian failure, including some medicines, intense exercise, and eating disorders. Some women with secondary ovarian failure may be able to carry children if they have menstrual periods (Pellegrini, 2016).

The stoppage of antral follicles is a characteristic of PCOS that can be reversed by increasing circulating FSH levels (Stephen and Kate, 2020). Anomalies in gonadotropin regulation, secretion, and action, as well as intraovarian factors, have all been related to the etiology of anovulation (Stephen and Kate, 2020). A higher amount of anti-müllerian hormone (AMH) is connected to anovulation and hyperandrogenism in women with polycystic ovarian morphology (PCOM) (Alebic *et al.*, 2015).

2.2.2. Metabolic Disturbances in PCOS

Females of reproductive age are susceptible to PCOS (Bozdag *et al*., 2016), and has severe metabolic, reproductive, and psychological effects, manifesting as a rise in type 2 diabetes mellitus (T2DM), anxiety, depression, and cardiovascular risk factors, all of which pose a significant health burden (Kakoly *et al*., 2018). Several theories have been proposed to understand the underlying pathophysiology of the distinct components of metabolic syndrome, which is central due to the complex systemic interactions between them (Swaramya *et al.,* 2018).

Hyperinsulinemia and type 2 diabetes, as well as cardiovascular risk factors such as visceral obesity, atherogenic dyslipidemia, and hypertension, are all part of the metabolic syndrome.

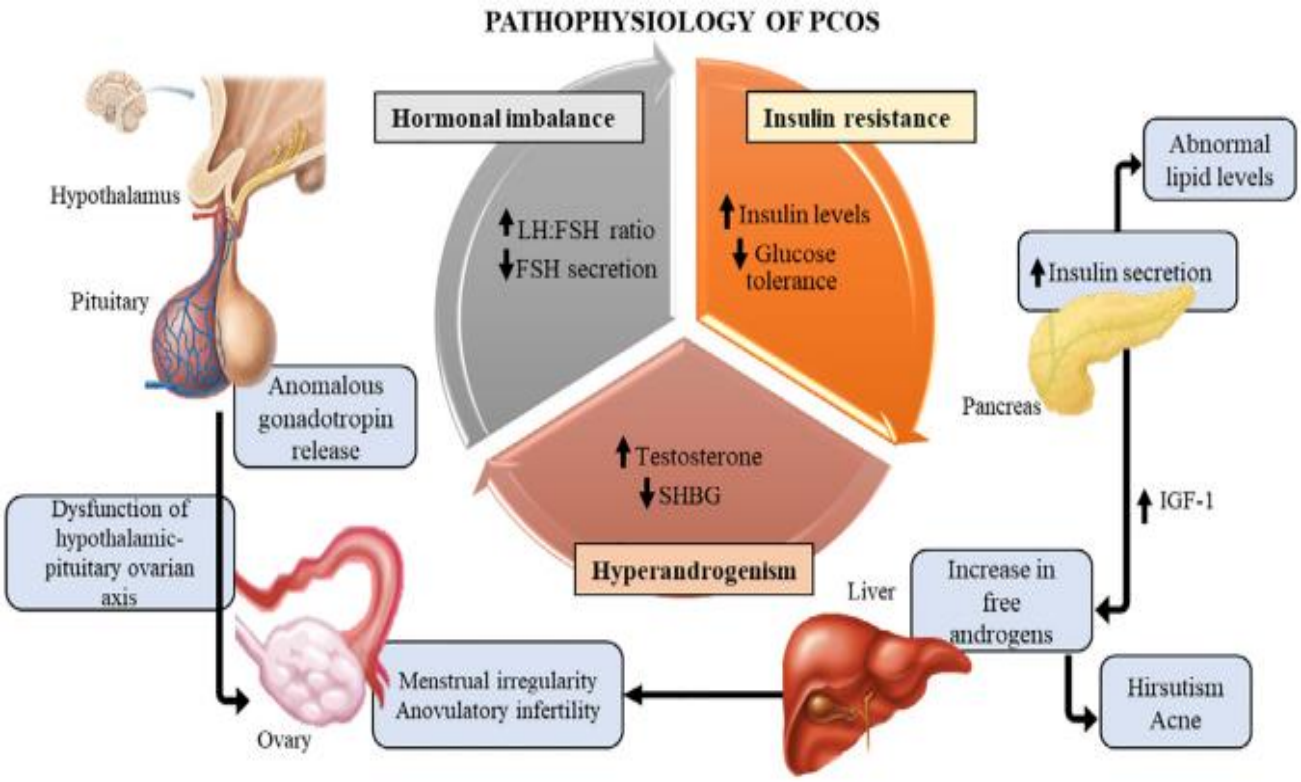


Figure 2. Pathophysiology of PCOS

Source: Walters *et al.*, 2018.

2.2.2.1. Insulin Resistance (Type 2 Diabetes Mellitus)

Insulin resistance occurs when the physiologic response to insulin stimulation in target tissues such as the liver, muscle, and adipose tissue is reduced (Seong *et al.*, 2019).

Insulin resistance affects both fat and lean patients with PCOS (Stepto *et al*., 2019). Obese PCOS individuals are affected 70-95 percent of the time, while lean PCOS patients are affected 30-75% of the time (Randeva *et al.,* 2012). High insulin levels are a sign and a main source of symptoms in PCOS (Diamanti-Kandarakis and Papavassiliou, 2006). When insulin levels are high, excess testosterone production in the ovaries can prevent ovulation (Corbould *et al*., 2005). Insulin binds to cell surface receptors that resemble the insulin-like growth factor-1 (IGF-1) receptor in structure (Macut *et al.,* 2017).

This increases the transfer of insulin-responsive glucose transporter 4 (GLUT4) from intracellular vesicles to the cell surface, which enhances glucose absorption. The activation of phosphatidylinositol 3-kinase (PI3-K) causes this process (Macut *et al.,* 2017). Hyperinsulinemia, which occurs when insulin mimics the effect of LH and elevates Gonadotropin-releasing Hormone (GnRH) indirectly, causes excess androgens (Puttabyatappa and Padmanabhan, 2018). Insulin lowers the quantity of SHBG, a critical circulatory protein that controls testosterone levels (Rojas *et al.,* 2014). Insulin signaling has been connected to inflammatory disorders such as infertility and weight gain.

Inflammation reduces the action of insulin and glucose tolerance, and it may have a role in hyperandrogenism, insulin resistance, and belly obesity in PCOS patients (Phinney and Volek, 2019). Hyperandrogenism and its clinical manifestations are exacerbated by insulin resistance (Cassar *et al.*, 2016). PCOS elevates the risk of type 2 diabetes regardless of BMI, accounting for 23% of type 2 diabetes in young women, however the underlying mechanisms of insulin resistance in PCOS remain unexplained (Kakoly *et al*., 2018).

According to some evidence, insulin resistance may be the result of PCOS rather than the cause (Moghetti and Tosi, 2021). Insulin resistance develops quickly in women after supraphysiological testosterone administration, according to a few studies using multi-step hyperinsulinemic glucose clamps (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).

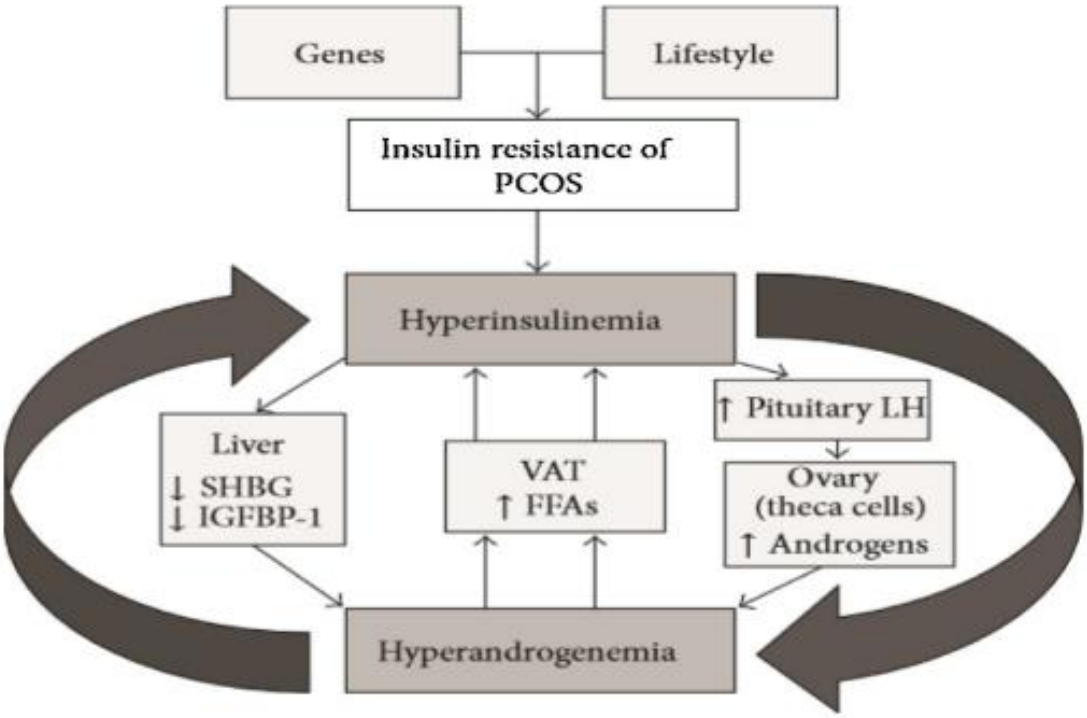


Figure 3: Pathway showing the effects of Insulin resistance

Source: `Ajmal *et al.*, 2019.

2.2.2.2. Visceral Obesity

Obesity, especially visceral obesity, which is common in both overweight and underweight PCOS women, aggravates and worsens both metabolic and reproductive outcomes (Azziz *et al.,* 2016). Obesity produces insulin resistance and compensatory hyperinsulinemia by increasing adipogenesis while decreasing lipolysis (Legro, 2012).

By upregulating ovarian androgen activity, obesity alerts the thecal cells to LH activation and increases functional ovarian hyperandrogenism (Pavaleanu *et al.,* 2016). Obesity raises levels of inflammatory adipokines, leading to increased insulin resistance and adipogenesis (Glueck and Goldenberg, 2019). Obesity has been linked to a disruption in the hypothalamic-pituitary-ovarian axis, which could contribute to PCOS (Legro, 2012).

Reduced visceral fat would control hunger, glucose levels, lipolysis, and SHBG, all of which would control androgen action in the ovary (Ouchi *et al*., 2011). Obesity is not a diagnostic criterion for PCOS, however both obese and non-obese PCOS individuals have more visceral adipose tissue (VAT) than women who do not have the disorder. VAT has also been shown to be linked to total androgen levels, implying that obesity may play a role in PCOS (Jena *et al.,* 2018).

Visceral obesity has been associated to insulin resistance, dyslipidemia, and cardiovascular disease, and women diagnosed with PCOS are more sensitive to these problems (Pavaleanu *et al*., 2016; Papadakis *et al*., 2017). (Zeng, 2020). Adipocyte accumulation in the abdominal region of the body is caused by androgens (Milutinovi *et al.,* 2017), which can lead to adipose tissue dysfunction, insulin resistance, and fat accumulation (Zeng, 2019). Moreover, non-obese females with PCOS exhibited improved visceral adiposity and higher inflammatory cytokines than matched non-obese controls, while having a regular BMI (Jena *et al.,* 2018).

Increase levels of triglyceride (TAG), cholesterol, free fatty acids, and various apolipoprotein abnormalities are all signs of obesity (Torre-Villalvazo *et al.,* 2018). A rise in free fatty acid induces a drop in insulin sensitivity as well as a drop in glucose absorption (Chow *et al.,* 2017).

2.2.2.3. Dyslipidemia

Dylipidemia, which is one a cardiovascular risk factors associated with PCOS, is described as a high level of lipids (cholesterol, triglycerides, or both) or a low level of high-density lipoprotein (HDL) cholesterol (Michael *et al*., 2019).

It has been suggested that dyslipidemia has a role in the progression of PCOS (Michael *et al*., 2019). PCOS is characterized by decrease levels of HDL-cholesterol, high triglycerides (TG), total cholesterol (TC), and low-density lipoprotein (LDL) cholesterol (Ghaffarzad *et al.,* 2016). Women diagnosed with PCOS have increased levels of dyslipidemia, such as smaller LDL particles, increasing their risk of heart disease (Kumar *et al*., 2017).

As a result, the pathological course of PCOS is influenced by an irregular lipid profile (Diamanti-Kandarakis *et al.,* 2007). Dyslipidemia, also known as atherogenic dyslipidemia, is the most common metabolic disruption in PCOS, as are conditions of Insulin resistance (Macut *et al.,* 2011). In the early stages of PCOS development, it was discovered that androgen excess generates more atherogenic LDL cholesterol particles, resulting in long-term atherogenic potential with a longer duration for oxidative transformation of LDL cholesterol. (Macut *et al*., 2011).

PCOS patients have low level of LDL and high levels of triglycerides (Zhang *et al.,* 2016), 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 reduces after treatment with statins (Seymam *et al.,* 2017). Women diagnosed with PCOS have more dyslipidemia, and smaller LDL particles, which increases their risk of cardiovascular diseases (Kumar *et al*., 2017). Lipid abnormalities are common in women with PCOS, and moderate hypercholesterolemia is also common (Pergialiotis *et al.,* 2018).

2.2.2.4. Hypertension

This is also one of the cardiovascular risk factors related to PCOS (Gandevani *et al.,* 2018). Over 30% of women diagnosed with PCOS had a blood pressure of less than 130/85 mmHg, and high baseline blood pressure has been identified as a significant predictor of cardiovascular risk (Azziz *et al.,* 2016). Even after adjusting for BMI (Body Mass Index), hypertensive women had higher levels of glucose, insulin, and lipids (Glintborg *et al.,* 2016).

Furthermore, hypertension was discovered at a young age, particularly when blood pressure was tested in pregnant PCOS women (Glintborg *et al.,* 2016). According to Shroff *et al*., young obese women diagnosed with PCOS have a 5-fold greater rate of preclinical coronary atherosclerosis than the overall population, and coronary artery calcification is four times more common in the study group than in the control group (39.0% vs. 9.9%). (Amiri *et al.,* 2020).

Oral contraceptives could formerly trigger the coagulation cascade, interleukins, and other inflammatory cytokines, raising the risk of thromboembolic events and overall cardiovascular disease in women with the syndrome (Glintborg *et al*., 2016).

2.3. Diagnostic Criteria of PCOS

Only the incidence of clinical and biochemical hyperandrogenism, as well as oligo/amenorrhea anovulation, are included in the National Institutes of Health (NIH) Criteria for PCOS, which were developed in 1990. (Zawadski and Dunaif, 1992). In addition to the two NIH criteria, the Rotterdam Criteria added polycystic ovarian morphology on ultrasound as a new criterion in 2003. (Zawadski and Dunaif, 1992).

The European Society of Human Reproduction and Embryology/ American Society for Reproductive Medicine Rotterdam consensus (ESHRE/ASRM) established and extended the diagnosis of PCOS (Table 1), which requires two of the three features: anovulation or oligo-ovulation, clinical or biochemical hyperandrogenism, and ultrasound-detected polycystic ovarian morphology (PCOM) (Zawadski and Dunaif, 1992). Finally, the Androgen Excess Society defines PCOS as hyperandrogenism with ovarian malfunction or polycystic ovaries (Azziz *et al.,* 2006).

As a result, the Androgen Excess Society (AES) found that excess androgen is a critical event in the development and pathogenesis of polycystic ovarian syndrome, and that androgen excess must occur before either oligomenorrhea or PCOM, or both (Azziz *et al.*, 2006). In recent years, PCOS has been seen as a difficult illness defined by the presence of two of the three criteria: hyperandrogenism, ovarian dysfunction, and polycystic ovaries (Tracy *et al.,* 2016).

Table 1: Diagnostic Criteria for PCOS

National Institute of Health (1990)	Rotterdam (2003)	Androgen Excess-PCOS Society (2006)
Clinical and/or biochemical indications of hyperandrogenism, chronic anovulation (Both criteria needed)	Anovulation, oligo-, and/or anovulation Hyperandrogenism manifests itself clinically and/or biochemically. Ovaries with polycystic cysts (Two of three criteria needed)	Ovarian dysfunction (oligo-anovulation and/or polycystic ovarian morphology) Clinical and/or biochemical indications of hyperandrogenism (Both criteria needed)

Source: Adapted from Tracy *et al*, (2016).

2.3.1. Hyperandrogenism

The most frequent clinical manifestation of PCOS is hyperandrogenism (Xin *et al.,* 2020). The majority of PCOS patients with hyperandrogenism have issues with steroid secretion, which results in aberrant folliculogenesis and failure of dominant follicle selection (Azziz *et al.,* 2016).

Hyperandrogenism manifests itself in form of obesity, hairiness, acne, and androgenetic alopecia (Xin *et al.,* 2020). Excess androgens hinder folliculogenesis by disrupting endogenous androgen production (Deswal *et al.,* 2018). It also promotes the formation of primordial follicles and an increase in antral follicles at the early gonadotropin level (Rosenfield and Ehrmann, 2016). GnRH production from the brain activates the release of gonadotropin hormone from the pituitary (Walters *et al.,* 2018). FSH acts on the FSH receptor in ovarian granulosa cells, converting androgens to estrogens, which promote follicle formation.

LH activates the LH receptor in ovarian theca cells, promoting androgen development, whereas FSH acts on the FSH receptor in ovarian granulosa cells, converting androgens to estrogens, which promote follicle formation (Ashraf *et al.,* 2019).

SHBG levels in the blood drop in PCOS women, contributing to an increase in free testosterone levels (Deswal *et al.,* 2018). Insulin suppresses hepatocytes' production of SHBG, a process that may be aggravated in women with PCOS as a result of hyperinsulinemia (Azziz *et al.,* 2016). Dysregulation of the neuroendocrine system has been linked to an imbalance in the hypothalamic-pituitary-ovarian axis, leading in gonadotropin excess (Ashraf *et al.,* 2019). In PCOS, GnRH stimulation stimulates the synthesis of LH rather than FSH, which result in a significant increase in the LH:FSH ratio (Walters *et al.,* 2018).

2.3.2. Ovulatory Dysfunction

The stoppage of antral follicles is a characteristic of PCOS that can be reversed by increasing circulating FSH levels (Stephen *et al.,* 2020).

Ovulatory dysfunction is diagnosed after a year post-menarche, and it is characterized by irregular menstrual cycles, which are defined as below 21 days or above 45 days in women between 1-3 years post-menarche, and below 21 days or above 35 days in women over 3 years post-menarche to perimenopause (Teede *et al.,* 2018). Ovulation can be determined using luteal phase biochemistry serum progesterone levels (Teede *et al.,* 2018).

2.3.3. Polycystic Ovaries (PCO)

An ultrasound scan image of the polycystic ovaries is used to classify PCO (Teede *et al.*, 2018). It is more common than PCOS because the ovaries contain a large density of partially developed follicles (Teede *et al*., 2018).

The ovaries' folicles, which are where the ova develop, are normally packed with fluid (Teede *et al.,* 2018). In normal circumstances, only a few follicles mature at a time and are dispersed throughout the ovary; however, if the follicles behave abnormally, PCO is diagnosed (Teede *et al.,* 2018). The formation of a larger number of follicles at the same moment, as well as their placement, distinguishes this (Teede *et al.,* 2018).

PCO does not give a distinct appearance or pain in the pelvic region; instead, it is most likely caused by a cyst, which usually goes away on its own (Teede *et al.,* 2018). Cysts can only be treated surgically if they are present, therefore PCO does not require surgery (Teede *et al.,* 2018).

2.3.4. Phenotypes of PCOS

PCOS is divided into four phenotypes: phenotype A, phenotype B, phenotype C, and phenotype D.

Table 2: PCOS phenotypes and its associated Diagnostic Criteria

Phenotypes	A	B	C	D
	Classic	NIH	Ovulatory	Normal androgenic
Hyperandrogenism	Yes	Yes	Yes	No
Chronic anovulation	Yes	Yes	No	Yes
Polycystic ovaries	Yes	No	Yes	Yes
NIH 1990	X	X	X	X
Rotterdam 2003	X	X	X	X
AE-PCOS Society 2006	X	X	X	X

Source: Adapted from Tracy *et al.*, (2016).

2.3.4.1. PCOS Phenotype A and B

Classic and NIH PCOS refers to PCOS phenotypes A and B, which are often known as the "full" PCOS phenotype (Azziz, 2018). Compared to ovulatory or non-hyperandrogenic phenotypes (C and D), women with typical PCOS have more hairs, are obese, have more irregular menstrual patterns, and are likely to develop insulin-resistant, dyslipidemic fatty liver, with an increased risk of metabolic syndrome (Lizneva *et al*., 2016).

2.3.4.2. PCOS Phenotype C

Patients with phenotype C (ovulatory PCOS) exhibit modestly elevated serum insulin, atherogenic lipids, and androgen levels compared to those with classic and non-hyperandrogenic PCOS (Guastella *et al.,* 2010). Phenotype C, which includes hyperandrogenism, polycystic ovaries, and no ovarian dysfunction, is included in the 2006 AE-PCOS Society criteria and the 2003 Rotterdam criteria (Lizneva *et al.,* 2016). Metabolic syndromes are more common in ovulatory PCOS than in other types of PCOS (Guastella *et al.,* 2010).

2.3.4.3. PCOS Phenotype D

It has normal androgen levels with slightly elevated hormone levels and the least metabolic impairment of the PCOS phenotypes (Zhang *et al.,* 2009). Ovarian dysfunction, polycystic ovaries, and no hyperandrogenism constitute phenotype D, which was introduced by Rotterdem criteria in 2003. (Lizneva *et al.,* 2016). These patients have normal testosterone levels and minor endocrine dysfunction, such as insulin resistance and a decreased metabolic syndrome prevalence (Lizneva *et al.,* 2016).

Phenotype D had the least metabolic inefficiency, a higher amount of sex hormone-binding globulin, and a lower LH/FSH ratio than healthy controls (Jamil *et al.,* 2016).

2.4. Prevalence of PCOS

PCOS prevalence is estimated between 3% and 10% depending on geographic area and race/ethnicity, however it is unknown for specific subpopulations (Wendy *et al.,* 2018). PCOS prevalence influences the diagnostic criteria, manifestations, and population examined (Wendy *et al.,* 2018).

According to the National Institutes of Health, Rotterdam, and the AE-PCOS Society's diagnostic criteria, PCOS prevalence rates were 6 percent and 10%, respectively.

The prevalence of Rotterdam characteristics varied between 8% and 13%. (Tripathy *et al.*, 2018). Obese women were likely to exhibit phenotypes A and B, which were linked to hyperandrogenism, insulin resistance, and a poor cardiometabolic profile (Tripathy *et al.*, 2018). Metabolic syndrome was the most prevalent trait in the study (Tripathy *et al.*, 2018). The prevalence rates, clinical symptoms, and pathogenesis of PCOS in women are indicated in (figure 4).

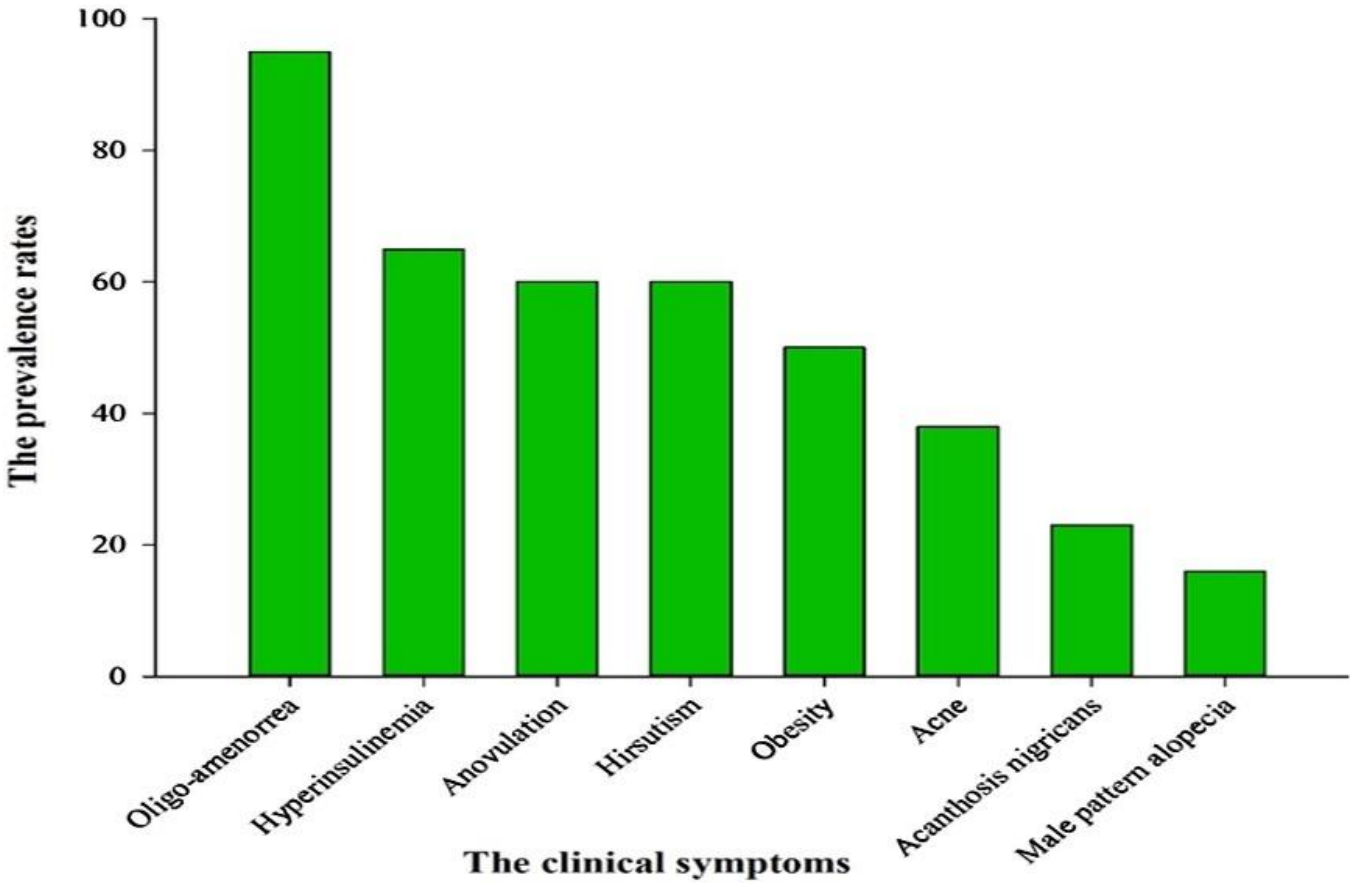


Figure 4. The prevalence rates of clinical symptoms PCOS women

Source: Adapted from Zahra *et al.*, (2018).

The oligo-amenorrhea has the highest prevalence rate of about 92%, hyperinsulinemia 65%, anovulation and hirsutism 60%, obesity 50% and acne 40% (Zahra *et al.,* 2018).

2.5. Symptoms of PCOS

Individuals diagnosed with PCOS have a problem with the hypothalamic-pituitary-gonadal axis (figure 5), which affects steroidogenesis and leads to hyperandrogenism (Laura *et al.,* 2018). Steroidogenic dysregulation in theca cells of the ovaries boosts the level of circulating androgens. Women diagnosed with PCOS have reduce amounts of SHBG, which elevates free testosterone levels (Laura *et al.,* 2018).

Hormonal imbalances encourage early follicular growth, resulting in anovulation, amenorrhea, polycystic ovaries, and infertility (Laura *et al.,* 2018). As a result of insulin resistance (IR), hyperandrogenism is connected to abdominal fat storage and hyperinsulinemia (Laura *et al.,* 2018). Inflammation is associated to the metabolic repercussions of PCOS, such as IR, dyslipidemia, and T2DM (Laura *et al.,* 2018). As adipocytes create fatty acids through lipolysis, visceral obesity boosts inflammatory mediator levels in the blood, resulting in dyslipidemia (Laura *et al.,* 2018).

Increased levels of oxidative stress markers, as well as an imbalance of pro- and anti-coagulant mediators, are found in women with PCOS (Laura *et al.,* 2018). Hemostatic and oxidative disorders, as well as inflammation, insulin resistance (IR), and dyslipidemia, all raise the cardiovascular risk of these people (Laura *et al.,* 2018**).** One of the most common PCOS symptoms is IR (Wang *et al.,* 2019). Hyperinsulinemia, which can develop to diabetes mellitus, is a side effect of IR (Patel, 2018). High insulin levels produce central adiposity, or fat deposition across the belly button (Meldrum, *et al*., 2017).

In the majority of women diagnosed with PCOS, a BMI of 30 or more is observed (Gupta *et al.,* 2019). Co-morbidities of PCOS include hypertension, cardiovascular problems, dyslipidemia, and so on (Patel, 2018). Sugar cravings, frequent urination, fatigue, nausea, impaired vision, tingling feelings, mood swings, anxiety, and depression bouts are all typical in PCOS patients (Patel, 2018). Anovulation or oligovulation, in which some male-like characteristics are

manifested in females as a result of cyst-producing androgens, is the most prevalent symptom of PCOS (Madnani *et al.*, 2013).

PCOS, commonly known as "hyperandrogenism," causes a wide range of male symptoms. (Madnani *et al.*, 2013). Weight increase, belly and subcutaneous fat, hirsutism, male-pattern alopecia, clitoromegaly, deep voice, seborrhea, and acne are all visible indications of hyperandrogenism (Madnani *et al*., 2013). Obesity, type 2 diabetes, obstructive sleep apnea, heart disease, depression, acanthosis nigricans, autoimmune thyroiditis, endometrial cancer, and breast cancer are all connected to problems (Nicandri *et al*., 2012).

Women with PCOS are more prone to miscarriage and experience pregnancy problems (Rees *et al.*, 2016). A recent meta-analysis found that the risk of gestational diabetes, pregnancy-induced hypertension, preeclampsia, premature delivery, and caesarean section is much higher (Rees *et al.*, 2016). Endometrial cancer is connected to PCOS, although it's unclear if this risk is independent of other PCOS-related risk factors such as obesity, diabetes, metabolic syndrome, and anovulation (Barry *et al.*, 2014).

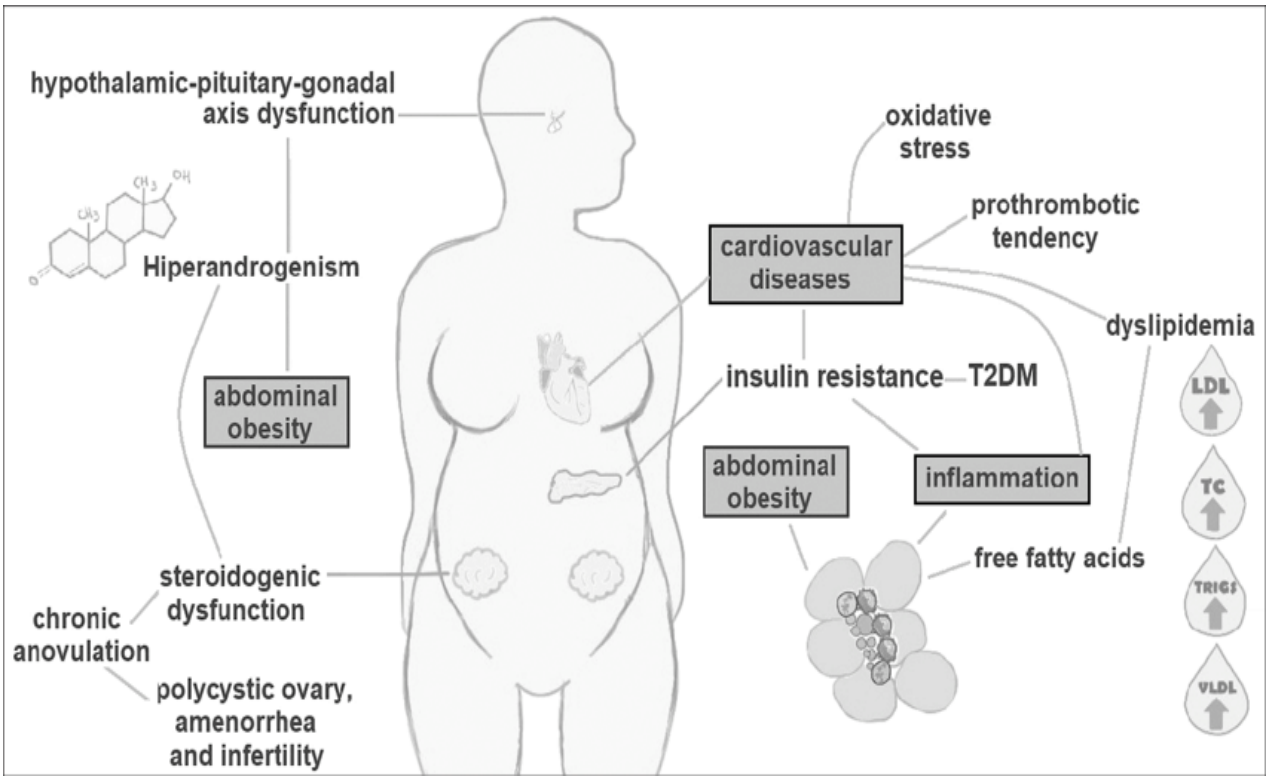


Figure 5. Associated symptoms of PCOS

Source: Adapted from Laura *et al.*, (2018).

2.6. Etiologies of PCOS

The cause of PCOS is unknown (Dunaif, 2016), although it is obvious that it is a multifaceted disorder (Aytan *et al.*, 2016). Although, in 2019, Wang *et al.*, ascertained that hyperandrogenemia (HA) and IR are central etiology and primary hormonal features of PCOS (Wang, *et al.,* 2019). They proposed that the key causes of PCOS are HA and IR, which may interact in the pathophysiology of the condition (Wang, *et al.,* 2019)

Despite the limitations in our understanding of the etiology of PCOS, certain related biochemical abnormalities have been well recognized, and inflammation is likely to play a significant role in developing these metabolic imbalances (Carvalho *et al.,* 2017a). A poor lifestyle, food, or other infectious mediators intensify PCOS (Ajmal *et al.,* 2019). IR and HA affect the function of the ovaries, which result to an increase in androgen levels, which contributes to anovulation (Ajmal *et al.,* 2019).

The levels of GnRH, FSH, LH, and prolactin are all affected by PCOS (Ajmal *et al.,* 2019). Genetic factors, in addition to environmental factors, have a role in the etiology of PCOS. PCOS is thought to be caused by 241 gene variants, according to databases (Ajmal *et al.,* 2019). PCOS is caused by a deficiency in the transcriptional activity of a gene caused by polymorphism (Ajmal *et al.,* 2019).

The genes that code for the androgen receptor, LH receptors, FSH, and leptin receptors are largely responsible for this (Ajmal *et al.,* 2019). Physical activity also benefits patients' health by lowering insulin resistance and enhancing reproductive biomarkers such as antral follicle count and blood levels of sex hormones, gonadotropins, and AMH (Al-Eisa *et al.,* 2017).

2.7. Management Options of PCOS

The treatment technique varies depending on the clinical symptoms and underlying cause, which can be separated into four categories: ovulatory dysfunction, hyperandrogenism, improving insulin resistance, and infertility treatment (Zimmerman *et al.,* 2019). It's critical to treat and control all of the symptoms of PCOS in order to avoid health problems. PCOS is a continuing condition that requires ongoing attention (Zimmerman *et al.,* 2019).

Depending on the symptoms, PCOS can be treated with lifestyle changes, medical treatments such as oral contraceptives, hormonal drugs, and infertility medications such as insulin sensitizers and ovulation induction agents.

2.7.1. Lifestyle Modification

Lifestyle measures, including as good dietary integration, increased physical movement, and the deployment of behavioral techniques, are the initial treatments for PCOS, given the implications of obesity for reproductive, metabolic, and psychosocial health (Teede *et al.,* 2011, Lim *et al.,* 2013). Treatment of overweight women diagnosed with PCOS who lose a small amount of weight improves biochemical reproduction, insulin resistance, and surrogate markers of fat distribution (Moran *et al.,* 2011).

Studies have demonstrated that changes in lifestyle, such as food, exercise, and attitude, have a positive impact on body weight, insulin resistance, and testosterone levels (Moran *et al.,* 2011). There are no precise, evidence-based guidelines for preparing a PCOS-friendly food (Orio *et al.,* 2016). According to the Structured Activity Training Program, physical exercise improves anthropometric parameters, insulin sensitivity, lipid profile, cardiovascular inflammatory markers, and the menstrual cycle in women diagnosed with PCOS (Orio *et al.,* 2016).

150 minutes of moderate level exercise per week or 75 minutes of strenuous intensity exercise per week are the general recommendations for exercise (Teede *et al.,* 2018). It is recommended that at least 250 minutes of moderate-intensity activity or 150 minutes of vigorous-intensity activity per week for modest weight loss, weight recovery prevention, and increased health benefits (Teede *et al*., 2018). Goal planning, self-monitoring, stimulus control, problem-solving, self-assertion training, slow eating, strengthening change, and preventing recurrence are some behavioral techniques for weight management (Brennan *et al.,* 2017).

Because PCOS is a continuing disorder with an elevated risk of complications, such as type 2 diabetes, the most critical and straightforward step for women with PCOS is to improve their lifestyle (Carmina, 2012).

2.7.2. Medical Treatments

2.7.2.1 Oral Contraceptive Pills (OCPs)

When used by PCOS women, OCPs can help manage excessive hair growth and acne by lowering male hormone levels (Leo *et al.,* 2009). OCPs which contain estrogen and progestin can help in the control of the symptoms of PCOS by bringing the menstrual cycle back to normal (Leo *et al.,* 2009).

The optimal contraceptive for women with PCOS should restrict antagonist follicle growth and lower androgen levels, counteract androgen effects on peripheral sebaceous units, restore estrogen and progesterone balance in the endometrium, and give adequate menstrual cycle control (Wiegratz *et al.*, 2003).

2.7.2.2 Hormonal medications

In addition to or instead of OCPs, antiandrogens can be given to women diagnosed with PCOS who seek to reduce hyperandrogenic clinical symptoms (e.g., hirsutism, acne, alopecia) (Katsambas and Dessinioti., 2010). When used in conjunction with an OCPs, spironolactone, an aldosterone antagonist, has been revealed to aid with hirsutism and acne (Saha *et al*., 2011).
Spironolactone, an aldosterone antagonist, has an antiandrogenic action at high doses (Badawy and Elnashar, 2011). Antiandrogens, such as spironolactone, flutamide, and cyproterone acetate, are favored as first-line medicines for hirsutism and acne because they inhibit androgen release by blocking androgen receptors (Badawy and Elnashar, 2011). Flutamide is a selective nonsteroidal antiandrogen that is as effective as spironolactone in the treatment of hirsutism and is more effective than OCPs in improving fatty, inflammatory, and lipid structural parameters when used in combination with metformin, but its use is inadequate due to hepatotoxicity (Ibanez and Zegher., 2006).

When compared to spironolactone and flutamide, finasteride, which suppresses the formation of dihydrotestosterone, is beneficial in lowering hirsutism but less effective in improving hirsutism (Koulouri and Conway., 2008). Finasteride does not appear to be as effective as Flutamide in treating acne (Archer and Chang., 2004). Every antiandrogen should be avoided during pregnancy due to the risk of feminizing a male child (Archer and Chang, 2004). To avoid hepatotoxicity, flutamide is used with metformin (Ibanez and Zegher, 2006).

Glucocorticoids (e.g., prednisone, dexamethasone) reduce adrenal androgen secretion and are used in combination with Clomiphene Citrate (CC) to recover ovulation and pregnancy rates and suppress the endometrium's reverse estrogen action in women with CC-resistant PCOS (Elnashar *et al.,* 2006). In women diagnosed with PCOS, glucocorticoids are also used to treat the clinical symptoms of androgen hyperplasia (Parsanezhad *et al.,* 2002). When adrenal androgen levels are high due to deteriorating glucocorticoid-related insulin resistance and connection with

osteoporosis, glucocorticoids should be explored only after other medicines have been tested (Badawy and Elnashar., 2011).

Gonadotropin-releasing hormone agonist (GnRHa) is another option in treating acne and hirsutism in people who don't respond to OCPs (Acien *et al.,* 1997). When combined with OCPs, hirsutism has shown to be more active in lowering the score than OCPs alone (Ciotta *et al.,* 1996). In another study, hirsutism women with PCOS who were administered GnRHa alone or in combination with OCPs or flutamide saw significant reductions in their hirsutisms (De Leo et al., 2000).

Although, GnRHa has shown to help with acne, few research has compared it to other acne treatments (Couzinet *et al.,* 1986). GnRHa therapy, on the other hand, is expensive and can lead to menopausal disorder, headaches, and bone loss (Lupoli *et al.,* 1997).

2.7.2.3 Insulin sensitizers

Drugs that are commonly used to treat metabolic issues associated with PCOS by reducing insulin resistance and restoring normal insulin levels are included in this category (Lauretta *et al*., 2016).

As insulin resistance is lowered, the accompanying testosterone level decreases, which results in an increase menstrual cycle (Geller *et al*., 2011). Metformin is a mass-produced biguanide that is used to treat insulin resistance and menstrual abnormalities in PCOS patients as a first-line treatment (Lauretta *et al.,* 2016). Metformin helps persons with PCOS improve their insulin tolerance by boosting glucose absorption and consumption (Geller *et al.,* 2011).

Unlike other insulin-regulating medicines that cause hypoglycemia or hyperglycemia as a side effect, it regulates glucose levels (Sivalingam *et al*., 2014). Metformin lowers insulin levels via inhibiting the CYP17 cytochrome, which is involved in the generation of androgens, as well as boosting SHBG and lowering free testosterone levels (Lashen, 2010). Metformin has also been demonstrated to improve PCOS patients' lipid profiles (Wulffelé *et al.,* 2004). When metformin is taken with clomiphene citrate, ovulation and pregnancy rates in infertile PCOS patients are increased (Dasari and Pranahita, 2009).

Despite flutamide's lack of safety in experimental animals, metformin combine with antiandrogens like flutamide has a synergetic effect in obese PCOS women (Pasquali and

Gambineri, 2006). Thiazolidinediones (TZDs), often called glitazones, are medicines that block the enzyme 11-ß-HSD (Hydroxysteroid Dehydrogenase), which is responsible for cortisol conversion. They include rosiglitazone and pioglitazone (Lauretta *et al.,* 2016). They are the second-line therapeutic option for insulin-resistant PCOS women (Stout and Fugate, 2005). TZDs have been proven to recover ovulation and conception rates in clomiphene-resistant PCOS women (Froment and Touraine, 2006).

TZDs lower excess androgens by boosting SHBG levels and redistributing them in adipose tissue (Brettenthaler *et al.,* 2004).

2.7.2.4 Ovulation Inducing Agent

The medicine of choice for treating anovulatory infertile women is clomiphene citrate (CC) (Dhaliwal *et al*., 2020). CC boosts FSH levels by blocking the estrogen receptor in a negative feedback loop (Dhaliwal *et al.,* 2020). It is specified for the treatment of anovulatory PCOS patients, although pregnancy rates vary dramatically depending on BMI.

For BMI less than 30, the of pregnancy increases, while for BMI greater than 30, the chances of pregnancy decreases (Legro *et al.,* 2007). Clomiphene citrate (Serophene or Clomid) is a selective estrogen receptor modulator that binds to estrogen receptors thereby inducing ovulation by increasing the output of pituitary gonadotropins. CC acts directly by synthesizing a surge of LH which could cause ovulation within a couple of days (Lucidi, 2019).

In a case of ovulation induction when fertility is longed-for, clomiphene citrate is suggested to be the first line of treatment (Vause *et al.,* 2010). Some women with patients after being administered with clomiphene citrate turns out to be resistant. Therefore, second-line of treatment can be considered. (Spritzer *et al.,* 2015). Laparoscopic ovarian drilling, exogenous gonadotropins and *in vitro* fertilization have been considered to be the second-line of treatment of PCOS when CC with or without metformin fails to achieve fertility (Spritzer *et al.,* 2015).

Tamoxifen is similar to CC, which is used to treat anovulation in people who do not respond to CC therapy (Dhaliwal *et al*., 2020). Combination trials of clomiphene and tamoxifen demonstrated a considerable increase in pregnancy rate due to tamoxifen's potential effect on uterine lining (Dhaliwal *et al*., 2020). Gonadotropins such as human menopausal gonadotropin (HMG) and recombinant FSH are second-line therapy for anovulatory infertile PCOS patients (Melo *et al.,* 2015).

In PCOS patients, low-dose FSH treatment is successful at inducing ovulation and increasing conception rates (Melo *et al.,* 2015). In-vitro fertilization (IVF) is indicated as a third-line treatment option for PCOS women with infertility who do not have any problems (Melo *et al.,* 2015).

2.8. Medicinal Plants For PCOS

Clinical evidence on medicinal plants with an ameliorative effect on PCOS in humans, according to Zahra *et al.,* (2018), includes *Mentha spicata* (Spearmint), *Cinnamomum zeylancum* (Cinnamon), *Grifola frondose* (Maitake), *Origanum majorana* (Sweet Marjoram), *Trigonella foenum-graceum L* (Fenugreek), *Phoenix dactylifer* (Soybean). Plants such as *Asparagus Racemosus, Tinospora Cordifolia, Grifola frondose and Lepidium meyenii,* have been commended for their ability to reduce PCOS symptoms while also having hypoglycemic and anti-obesity characteristics (animal research) (Sudhakar *et al*., 2017).

2.8.1. *Aspargus Racemosus* (Shatavari)

In Indian medicine, *Asparagus racemosus* (Asparagaceae) has long been employed (Ayurvedic, 2014).

It stimulates regular ovarian follicle development, regulates the menstrual cycle, and revitalizes the female reproductive system thanks to its phytoestrogen (Ayurvedic, 2014). It also helps with hyperinsulinemia therapy (Ayurvedic, 2014). Aside from the aforementioned effect, *A. racemosus* has pharmacological effects such as neurological disorders, dyspepsia, tumors, inflammation, neuropathy, hepatopathy, antiulcer, antioxidant, antidiarrheal, immunological modulatory activity, prevents ageing, extends life, and improves mental performance (Kumar *et al.,* 2008).

2.8.2. Tinospora Cordifolia (Guduchi)

The hypoglycemic characteristics of Tinospora cordifolia (Menispermaceae) are widely documented (Chandrasekaran *et al.,* 2012). Tinospora Cordifolia is an anti-inflammatory herb with a lot of power (Chandrasekaran *et al.,* 2012). Chronic inflammation in the tissues causes insulin imbalance and ovarian cysts. It improves insulin sensitivity, revitalizes all bodily tissues, and speeds up metabolism (Ayurvedic, 2014).

2.8.3. *Foeniculum vulgare* (Shatapushpa)

Seeds of *Foeniculum vulgare* (Apiaceae) are a useful supplement for managing PCOS. Phytoestrogens are abundant in them (Jungbauer *et al*., 2014). The phytoestrogen concentration of *Foeniculum vulgare* helps to reduce insulin resistance and inflammation in PCOS patients (Jungbauer *et al*., 2014). It's also thought to aid with the cellular imbalance that causes PCOS' metabolic problems (Jungbauer *et al*., 2014).

2.8.4. *Vernonia Amygdalina* (Bitter Leaf)

2.8.4.1. Origin

V. amygdalina, a medicinal plant, and its bioactive chemicals were researched in this study to see if they could proffer a curative effect on the reproductive hormones associated with PCOS. *V. amygdalina* is a promising medicinal plant native to Asia and Africa (Alara *et al.,* 2017). It is commonly referred to as "bitter leaf" in English due to its bitter flavor. It was named after English botanist William Vernon (Alara *et al*., 2017).

V. amygdalina (Figure 5) is one of Africa's (West Africa) and Asia's most well-known plants (Oluwaseun *et al.,* 2017). *V. amygdalina* is a perennial shrub in the *Asteraceae* family that is also called *Shuwaka* in Hausa, *Ewuro* in Yoruba, *Oriwo* in Edo, and *Olubu* in Igbo (Gashe & Zeleke, 2017).

2.8.4.2. Morphology

V. amygdalina is a plant that can reach a height of 10 m and bears elliptic-shaped petiole leaves with a diameter of 6 mm (Habtamu & Melaku, 2018). It is a tropical African vegetable that has been domesticated in many parts of West Africa, notably Nigeria, where it is used in soups (Habtamu & Melaku, 2018). Fats, proteins, fibers, minerals, amino acids, carbohydrates, and vitamins are abundant in the leaves of *V. amygdalina* (Alara *et al.*, 2017).

This plant is said to generate flavonoids, alkaloids, saponins, tannins, phenolics, terpenes, steroidal glycosides, triterpenoids, and many types of sesquiterpene lactones, among other bioactive components (Quasie *et al*., 2016; Luo *et al.,* 2017). Antimicrobial, antimalarial, antithrombotic, antioxidant, anti-diabetic, laxative, hypoglycemic, anthelmintic, anti-inflammatory, cathartic, anticancer, antifertility, antifungi, antibacterial characteristics are all provided by these bioactive chemicals (Alara *et al*.,2017c).

Figure 6: Leaves of *Vernonia amygdalina*

Source: Adapted from Udochukwu *et al.,* (2015).

2.8.4.3. Taxonomy

Table 3: Scientific Classification of *V. amygdalina*

Kingdom	Plantae
Phylum	Spermatophyta
Sub-Phylum	Angiosperms
Class	Dicotyledon
Order	Asterales
Family	Asteraceae
Genus	*Vernonia*
Species	*Amygdalina*

Scientific Name: *Vernonia amygdalina*

Source: Adapted from Sani *et al.,* (2012).

2.8.4.4. Pharmacological usage

V. amygdalina is a widespread African shrub plant with significant medicinal effect, and it's frequently employed in the treatment and control of a variety of ailments (Ifeoluwa *et al*., 2018).

Ganiyu *et al*. (2020) concluded that bitter leaves aqueous extract had neuroprotective qualities through regulation of key biomolecules in relation to neurological deterioration in Alzheimer's disease. Thi Xuan *et al*. (2020) stated that vernonioside V, a novel stigmastane, and a known flavonoid, cynaroside, were extracted from the leaves of V. amygdalina and could be used to treat inflammatory illnesses. Vernonioside V suppresses lipopolysaccharide-induced pro-inflammatory cytokine production and reactive oxygen species formation in all isolates.

Oluwaseun *et al*. (2019) claimed that using microwave-assisted extraction procedures, they were able to extract higher yields of bioactive chemicals from *V. amygdalina* leaves, which might potentially be used as a medicinal source for a variety of ailments.

2.8.5. Gas Chromatography-Mass Spectrometry (GC-MS) Analysis

The analytical technique of GC-MS is used to separate mixtures of volatile organic chemicals and identify each constituent compound (Eleanora *et al.,* 2018). It combines gas chromatography and mass spectrometry, two well-known analytical techniques (Eleanora *et al.,* 2018). Gas chromatography uses a well-designed column with varying component retention time to separate complicated mixtures (Eleanora *et al.,* 2018).

Various detectors connected to the GC column's end can then be used to evaluate the isolated chemicals (Eleanora *et al*., 2018). In the case of GC-MS, the detector is a mass spectrometer, which allows each compound form to be identified (Eleanora *et al.,* 2018).

2.8.5.1. Principle of GC-MS

Robert Finnigan devised the GC-MS technique in the 1960s, and it is used to study liquid, gaseous, or solid samples (Igwe *et al*., 2016). A capillary column coated with a stationary (liquid or solid) phase is used in a gas chromatograph (GC) to efficiently vaporize the sample into the mobile phase and separate it into its many components (Igwe *et al.,* 2016). The compounds are propelled by an inert carrier gas like as helium, hydrogen, or nitrogen (Igwe *et al*., 2016). Each compound elutes from the column at a different time based on its boiling point and polarity as the mixture's components are segregated (Igwe *et al*., 2016). The retention time of a chemical is the time it takes for it to elute (Igwe *et al.,* 2016).

The GC-MS, on the other hand, offers a broader method for characterization of organic chemicals found in plant extracts MS since it combines a fast separation technique with a time-dependent identification module (Igwe *et al*., 2016).

2.8.5.2. Application of GC-MS

In a variety of scientific areas, GC-MS offers a wide range of applications (Grant., 2021). GC-MS is commonly used in environmental science to assess the quantities of organic contaminants (Grant., 2021). It's been used to distinguish between different types of hydrocarbons in a sample for testing and bioremediation (Grant., 2021). GC-MS has been used in forensic toxicology and criminal cases, as well as drug detection in law enforcement (Grant., 2021).

Explosive's research, anti-doping and drug detection, and astrochemistry for the study and evaluation of samples acquired from distant moons in our solar system have all employed the approach (Grant., 2021).

Improved sample detection, better sensitivity, a wider range of analyzable samples, and faster performance are all advantages of GC-MS, allowing for a wide range of new applications in a variety of sectors (Susha, 2019). Medicine, environmental monitoring, food and fragrance analysis, pharmaceutical application, forensic application, biological analysis, chemical warfare, geological study, and industrial use are just some of the fields where this technology might be used (Susha, 2019).

2.8.6. UV-Visible spectroscopy

2.8.6.1. Principle of UV-Visible spectroscopy

Ultraviolet and visible absorption spectroscopy (short for UV-Vis spectroscopy) is a spectroscopy approach that involves estimating the attenuation (strength/intensity weakening) of a light beam after it passes through a sample or reflects from a sample surface. The principle of UV-Visible Spectroscopy is based on the absorption of ultraviolet or visible light by chemical molecules, which results in the production of unique spectra. Spectroscopy is based on the interaction of light and matter. When matter absorbs light, it undergoes excitation and de-excitation, resulting in the production of a spectrum (Niamh, 2019).

UV-Visible spectroscopy is extensively employed in analytical chemistry, particularly during the quantitative examination of a specific analyte (Niamh, 2019).

2.8.6.2. Application of UV-Visible spectroscopy

A UV-Vis spectrophotometer measures the intensity of light passing through a sample solution in a cuvette and compares it to the intensity of light before it passes through the sample. A UV-Vis spectrophotometer must have a light source, a sample holder, a dispersive device to separate the different wavelengths of light (e.g., a monochromator), and a suitable detector. UV-Visible spectroscopy is a simple and inexpensive technology that enables for sample recovery and good differentiation between transparent substances without the need of derivatives (Niamh, 2019). Food and agriculture, pharmaceutical research, life science, quality control, petrochemistry, traditional chemistry, and the cosmetic sector are some of the applications of UV-Visible spectroscopy (Niamh, 2019).

2.9. Phytochemical Constituents and PCOS

Alternative treatments, particularly phytotherapy, may be employed in place of commercially available drugs because there is presently no definite and ideal remedy for hormonal problems and their clinical features, and chemical medications have a variety of negative effects (Abasian *et al.*, 2018). Medicinal plants have gotten a lot of interest in recent years since they contain therapeutic chemicals and have few adverse effects (Abasian *et al*., 2018).

Because of its antioxidant and anti-inflammatory qualities, raspberry fruit extract reduced testosterone levels by inhibiting the NF-kB pathway and improved ovarian tissue symptoms such as the number of developed follicles, granulosa layer thickness, and corpus luteum number, according to a study (Bardei, 2015). By aromatizing testosterone and converting it to estradiol, *V. agnus-castus* fruit extract stimulates aromatase activity and reduces testosterone levels (Feizollahi *et al.*, 2021). This extract contains phytoestrogens (Abasian *et al*., 2018). This plant includes flavonoids that can bind to estrogen receptors, including as apigenin, vitexin, and penduletin (Niroumand *et al*., 2018).

Isoflavonoids with a phenolic ring have been demonstrated to inhibit the activities of 3-HSD and 17-HSD (Abasian *et al*., 2018). Women with PCOS have a lower total antioxidant status, and oxidative stress is considered a pathogenic aspect of the disorder (Abasian *et al*., 2018). According to evidence, reactive oxygen species (ROS) levels in ovarian tissue grow in PCOS, generating an imbalance in the oxidant and antioxidant systems (Zhang *et al.*, 2019). Soybean isoflavonoids have an antioxidant effect due to the aromatic ring and genistein (Rajan and Balaji, 2017).

2.10. Oestrous Cycle of Female Rats

The term used to describe the reproductive cycle of rodents such as rats is known as the oestrous cycle (Auta and Hassan, 2016). Analogous to the human sexual cycle (also known as the menstrual cycle, ovarian and uterine cycles), it is also similar to the lunar cycle (Auta and Hassan, 2016).

Unlike the human menstrual cycle, the oestrous cycle of rodents such as rats is mainly comprised of proestrous, oestrous, and diestrous categorized by the existence of nucleated epithelial cells (fig. 7A), cornified cells (fig. 7B), and polymorphonuclear cells (fig. 7D) respectively (Mahadevan and Balamuthu, 2016). Metaestrous is considered as the transition period between

oestrous and diestrous phases. Hence, this phase has all the three cell types in the vaginal smear (fig. 7C) (Mahadevan and Balamuthu, 2016).

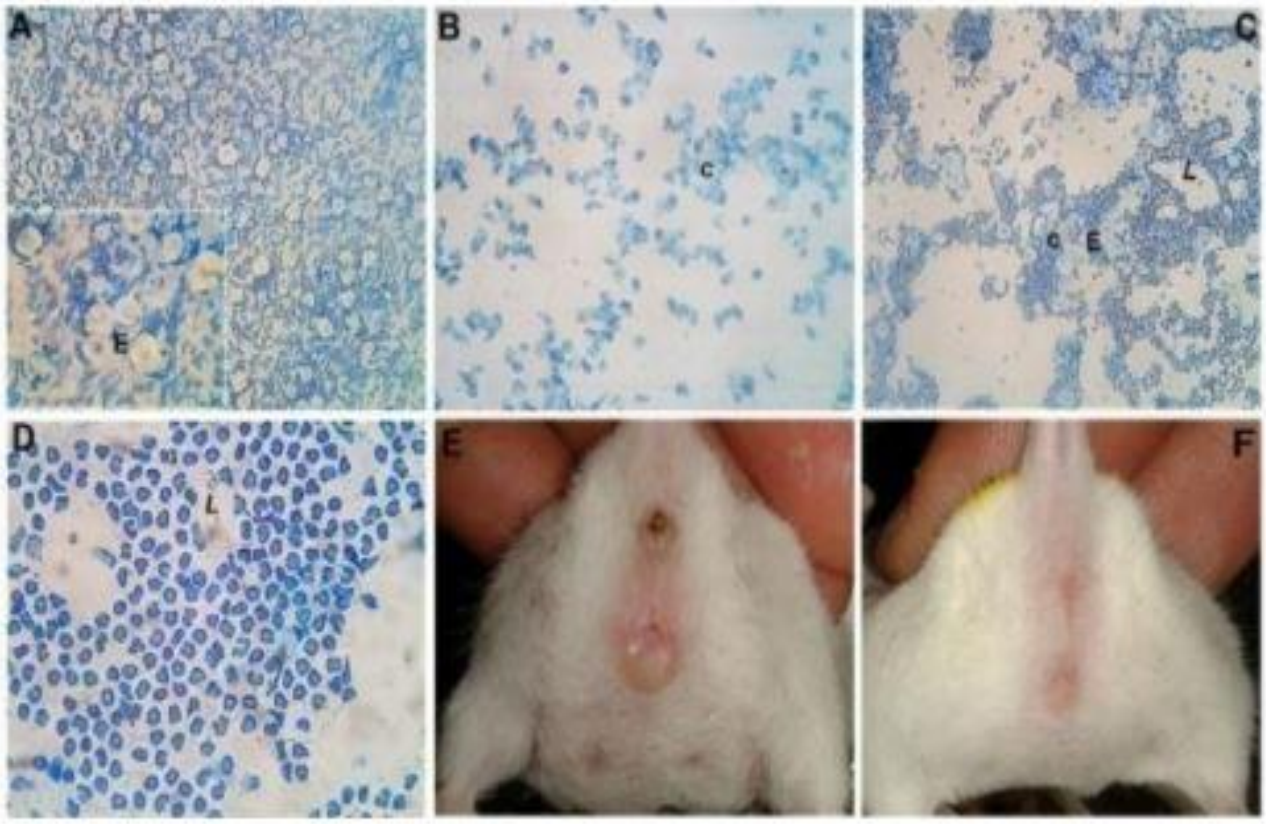


Figure 7. Physiological and cytological characterization of different phases of rat oestrous cycle

Source: Adapted from Mahadevan and Balamuthu, (2016).

2.10.1. Proestrous

The first stage of the oestrous cycle, marked by the growth of both the endometrium and ovaries, as well as the presence of round nucleated cells when viewed under a microscope, hence, it lasts within 14 hours (Auta and Hassan, 2016).

2.10.2. Oestrous

The second stage of the oestrous cycle, which is categorized by a receptivity to a male and a desire to reproduce (Auta and Hassan, 2016). It is also referred to as "heat" or "in heat" stage, pheromones may also be secreted only at this stage, and the presence of squamous cells under a microscope may also indicate the presence of these cells, hence, it lasts between 24-48 hours (Auta and Hassan, 2016).

2.10.3. Metaestrous

In the oestrous cycle, the third stage is characterized by sexual inactivity and the formation of the corpus luteum, hence, it lasts between 6-8 hours and is categorized by the presence of the proestrous, oestrous, and diestrous (all of which can be seen under a microscope) (Auta and Hassan, 2016).

2.10.4. Diestrous

The final stage of the oestrous cycle, occurring immediately before the beginning of the next proestrous cycle, and characterized by a functional corpus luteum and an increase in the blood concentration of progesterone. It lasts between 48–72 hours and is categorized by the presence of irregular non-nucleated cells and large numbers of leucocytes, as well as the presence of irregular non-nucleated cells (Auta and Hassan, 2016). Rat exhibiting prolonged polymorphonuclear cells or cornification of cells in vaginal smears was classified as anestrous (Mahadevan and Balamuthu, 2016).

Any such changes observed in oestrous cycle reflect the alteration in the functional integrity of the hypothalamic–pituitary–gonadal axis (Mahadevan and Balamuthu, 2016). Despite the fact that diverse species of rats may have slightly different reproductive cycles, the female rat's oestrous cycle is one of the most well-characterized polyestrous cycles, meaning that it is estrous cycle throughout the year, and reproductive cycles (Ekambaram *et al.*, 2017). Prolactin, LH, and FSH levels are low during the oestrous cycle and rise in the afternoon of the proestrous phase.

Estradiol levels begin to rise during metaestrous, peaking during proestrous, and then reverting to baseline during estrous. Progesterone secretion rises during metaestrous and diestrous, then falls subsequently. Then, near the conclusion of proestrous, the progesterone level climbs to its second peak. In most cases, puberty begins at 6-8 weeks when the estrous cycle begins; each cycle lasts 4-5 days on average (Auta and Hassan, 2016). The oestrous cycle is polyestrous, meaning that there is more than one oestrous cycle during a specific yearly time period, with an estrous period of approximately 12 hours during each cycle (Ekambaram and Joseph, 2017).

An increase in estradiol concentration is associated with vaginal unfolding, and a vaginal opening occurs at the first ovulation in rats during the first ovulation (Auta and Hassan, 2016). About 30 days after birth, female rats reach puberty, which is followed by the pulsatile release of LH (Ekambaram and Joseph, 2017). Until the initial proestrous, the anestrous lasts about 8 to 9 days. The first proestrous, oestrous, metaestrous, and diestrous are then followed by the second proestrous, estrous, metaestrous, and diestrous. Metaestrous occurs only when there is no prospect of conceiving naturally (Ajayi and Akhigbe, 2020).

In proestrous, follicles mature quickly before ovulation at the end of the month. In a unilateral ovarian segment, two to four big follicles can be detected at this period (Sato *et al.,* 2016). Follicles become corpora lutea at ovulation, which feature more basophilic and smaller luteal cells than later phases of the cycle. These corpora luteal begin to regress during metaestrous, and their regress continues into diestrous. The pattern of development and regression repeats itself in the next estrous stage (Sato *et al.,* 2016). Rodent ovaries contain a significant number of corpora luteal because regressive corpora luteal can survive up to 14 days (Sato *et al.,* 2016).

Several studies have offered several ways for monitoring the estrous cycle based on changes in the animal's physiology and morphology (Ajayi and Akhigbe, 2020) Visual inspection, vaginal smear/cytology, urine biochemistry, vaginal wall impedance, and histological investigation of the reproductive organs are only a few of these methods (Ajayi and Akhigbe, 2020). Although animal studies have shown that vaginal cytology is the best means of assessing the estrous cycle (Ajayi and Akhigbe, 2020).

2.11. Letrozole

The medication letrozole, also known as, “Femara” is an oral medication (2.5 mg) that is used to stimulate ovulation in women who have PCOS and unexplained infertility (Rachel., 2020).

It is an aromatase inhibitor with less anti-estrogenic properties than other estrogenic agents (Rachel., 2020). Aromatase inhibitors are drugs that block the synthesis of estradiol rather than the interaction of estradiol with receptors (Pritts *et al.*, 2011). These inhibitors function by stopping the aromatase enzyme from converting androgens to estrogens in a process known as "aromatization" (Soni *et al.*, 2020).

Letrozole, instead, is frequently used in the treatment of CC resistance, which occurs when CC fails to stimulate ovulation after at least three treatment cycles and despite increasing dosages of the medication (Rachel., 2020). In addition, letrozole is typically administered once daily for five days, and it inhibits the conversion of androgens in the body to estrogen, which occurs when estrogen is inhibited (Rachel., 2020).

When estrogen is inhibited, the pituitary gland receives a signal to produce FSH, which stimulates the ovary to produce an egg, as described above (Ataollah *et al*., 2016). Some women taking letrozole produce more FSH than a woman would naturally produce when ovulating, causing them to release more than one egg at once (Alex Mlynek, 2018). Letrozole is an organic compound that is a member of the diphenylmethane class of organic compounds (Wishart *et al.*, 2018).

Diphenylmethanes are compounds that contain a diphenylmethane moiety, which is a methane with two hydrogen atoms replaced by two phenyl groups. Diphenylmethanes are methanes that contain two phenyl groups (Wishart *et al.*, 2018). It is used to treat breast cancer in postmenopausal women at its early phase, who have already received 5 years of adjuvant tamoxifen therapy. Letrozole is a drug that has been approved by the Food and Drug Administration (FDA) (Wishart *et al.,* 2018). Women after menopause with locally advanced or metastatic breast cancer, whether or not it is hormone receptor positive, should consider this medication as a first-line treatment option (Wishart *et al.*, 2018).

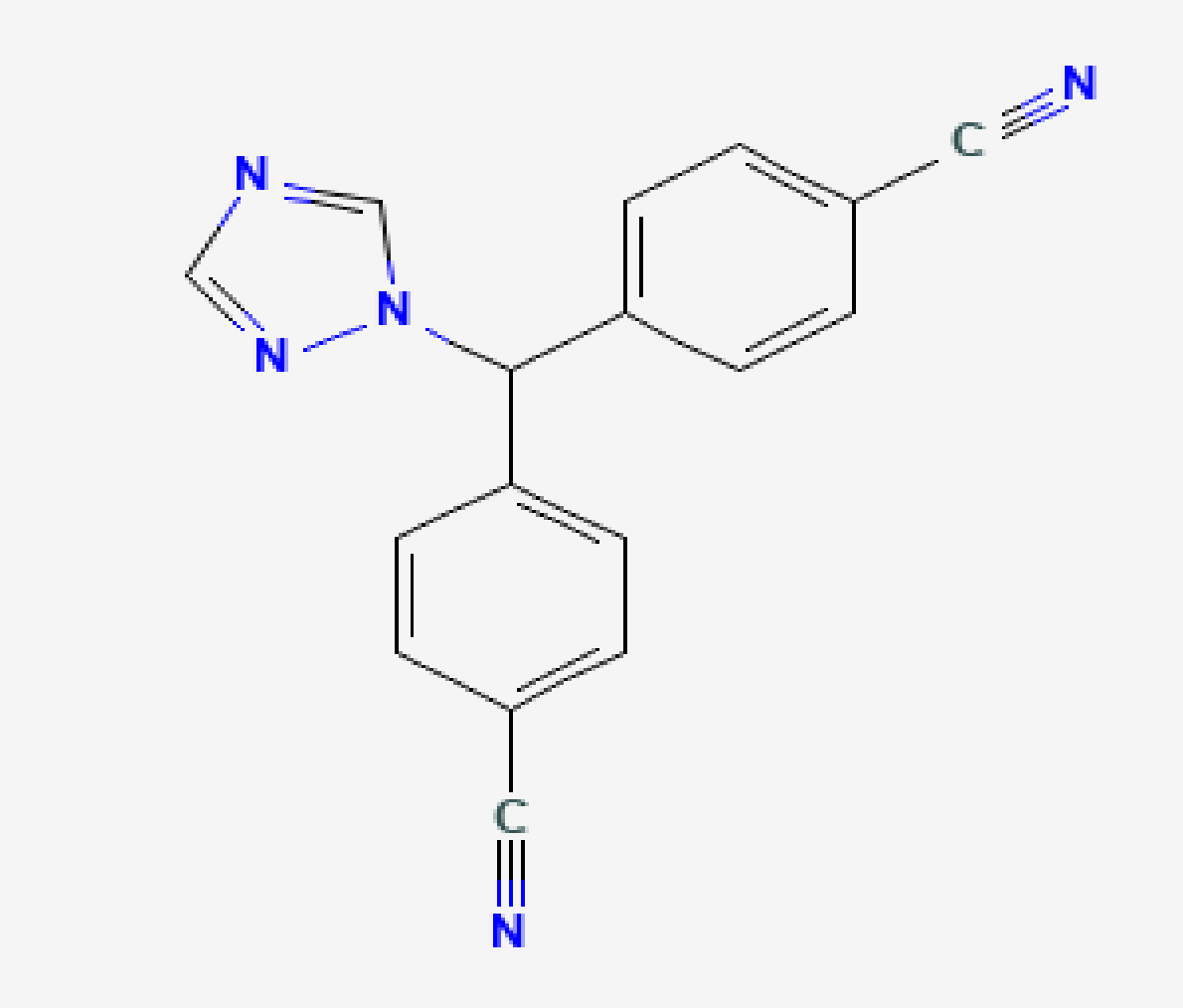


Figure 8. Chemical structure of Letrozole

Source: Adapted from NCBI, (2021).

FSH is produced by the pituitary gland as a form of negative feedback, and it is this production that causes ovulation or oligovulation (Casper., 2004). Letrozole, when administered early in the menstrual cycle, has a similar effect to cyclosporine without reducing estrogen receptors (Casper., 2004). At the University of Toronto, letrozole has been studied as a reproductive medicine in recent years, with promising results (Mitwally and Casper., 2006).

According to the findings, the medication appears to be completely eliminated from the body within a few days of the last tablet being taken (Mitwally and Casper., 2006). Due to the lack of evidence that this medication is harmful to the developing fetus at this time, it appears to be a safe fertility medication (Mitwally and Casper., 2006). In a study conducted at a tertiary referral infertility clinic in Dhaka, 35 anovulatory infertile patients who were nonresponsive to CC were included (Begum *et al*., 2009). Begum *et al*. (2009) reported a high success rate of 77.77% for follicular formation when using letrozole at a dose ranging from 2.5 to 5 mg. Another study conducted by Badawy *et al.* (2007) found that taking 5 mg letrozole in PCOS patients who had previously been treated with CC resulted in an effective induction rate of 62 percent in the majority of cases.

2.11.1. Mechanism of Action of Letrozole

Letrozole is a non-steroidal aromatase inhibitor of type II (Drugbank, 2020). By competitively preventing the aromatase enzyme (cytochrome P-450 19), it blocks an important stage in the manufacture of estrogen in the ovary and other necessary organs.

This phase involves the change of C-19 androgens to C-18 estrogens (Rose and Brown, 2020). Estrogen and estradiol are primarily produced by the action of the aromatase enzyme in postmenopausal women, which converts adrenal androgens, which include primarily androstenedione and testosterone, into estrogens and progesterone, respectively (Novartis, 2018). It is possible to suppress estrogen biosynthesis in peripheral tissues as well as cancer tissue by targeting and inhibiting the aromatase enzyme specifically (Novartis, 2018).

As a result of competitively binding to the heme of aromatase's cytochrome P450 subunit, letrozole inhibits estrogen biosynthesis throughout the body, resulting in a decrease in estrogen production in all tissues (Begum and Siddiqui, 2009). While letrozole treatment has been shown to significantly reduce serum estrogen, estradiol, and estrone sulfate levels in women, it has not

been shown to have any effect on aldosterone synthesis, adrenal corticosteroid synthesis and thyroid hormone synthesis (Novartis, 2018).

2.12. Hormonal Studies

A decrease in SHBG levels is one of the markers of PCOS, and it is linked to insulin resistance and Hyperandrogenism (Pinola *et al.,* 2015). The hormones studied include; Estradiol, Progesterone, Testosterone, FSH, LH and Insulin.

2.12.1. Estradiol

Estradiol (E2), also known as oestradiol, is the primary female sex hormone, a steroid and an estrogen. It is named after and plays a significant role in the regulation of the oestrous and menstrual cycles in females (Oyebanji *et al.,* 2018).

Estrogen secretion in PCOS women is characterized by chronic secretion rather than the cyclic pattern associated with an ovulatory cycle (Oyebanji *et al.,* 2018). Serum estradiol (E2) levels in PCOS vary, but are typically in the 60–90g/mL mid-follicular phase range. In contrast, serum levels of estrone (E1) are usually higher than those of E2, reversing the E1:E2 ratio seen in normal women. In the presence of androgen excess, this abnormality is caused by increased peripheral aromatization of androgens to estrogens (androstenedione to E1 and testosterone to E2) in extra-glandular tissues (Oyebanji *et al.,* 2018).

In the presence of an excess of androgen, chronic estrogen discharge is clinically significant because, it increases the risk of endometrial hyperplasia and, probably, the development of endometrial carcinoma (Jeffrey and Ralph, 2014).

2.12.2. Progesterone

Progesterone is an endogenous steroid and progestogen sex hormone that plays a role in the human menstrual cycle, pregnancy, and embryogenesis, among other things (Oyebanji *et al.,* 2018). Progesterone absence in PCOS has been linked to hyperinsulinemia and/or insulin resistance (Oyebanji *et al.,* 2018).

A high progesterone level indicates that ovulation occurred and the egg was released from the ovary, whereas a low progesterone level indicates that ovulation did not occur, resulting in anovulation in PCOS (Oyebanji *et al.,* 2018). Women with PCOS are generally anovulatory, and as a result, their serum progesterone levels are low and non-ovulatory (Oyebanji *et al.,* 2018).

Ovulation occurs infrequently in this group of women, resulting in luteal progesterone concentrations comparable to those found in normal women (Oyebanji *et al.,* 2018).

Among the progestins, 17-hydroxyprogesterone levels have been shown to be significantly elevated in women diagnosed with PCOS (Oyebanji *et al.,* 2018). This is as a result of theca cell production of this androgen precursor hormone (Jeffrey and Ralph, 2014). In inflammatory studies of androgen production, 17-hydroxyprogesterone responses to gonadotropin stimulation were found to be the most effective, in distinguishing PCOS women from normal ovulating women (Jeffrey and Ralph, 2014).

2.12.3. Testosterone

The primary male sex hormone and anabolic steroid is testosterone (Oyebanji *et al.,* 2018).

In premenopausal women, high androgen levels, also known as hyperandrogenemia, are linked with menstrual cycle irregularities and antagonistic metabolic features such as insulin resistance, central obesity, dyslipidemia, and chronic inflammation, which may lead to an increased cardiovascular risk (Oyebanji *et al.,* 2018). Only about 1% of testosterone exists in an unbound, or free, state and is thus bioactive. SHBG levels are abnormally low in women with PCOS, resulting in an increase in the bioactive fraction of testosterone that stimulates oily skin, acne, and hirsutism (Oyebanji *et al.,* 2018).

Serum free testosterone elevations may also explain hirsutism in women with normal serum total testosterone levels (Jeffrey and Ralph, 2014).

2.12.4. Follicle Stimulating Hormone (FSH)

FSH is a glycoprotein polypeptide hormone and a gonadotropin (Sampurna *et al*., 2017). FSH is produced and released by the anterior pituitary gland's gonadotropic cells and regulates the body's development, growth, puberty maturation, and reproductive processes (Sampurna *et al*., 2017).

The reduction in serum FSH level result to atresia in the current cohort's smaller follicles, which lack adequate sensitivity to FSH to survive. Although an elevation in LH stimulates ovarian theca cells to synthesize more androgens, a lack of FSH may be the more immediate cause of anovulation in PCOS patients (Oyebanji *et al.,* 2018). FSH levels in the blood are usually normal or slightly low in PCOS (Oyebanji *et al.,* 2018).

Attempts to characterize abnormal pulsatile FSH release in PCOS women have been fruitless due to the relatively long serum half-life of FSH, which makes identifying individual pulses impractical (Jeffrey and Ralph, 2014). As a result, a high level of LH and FSH absence may result in menstrual cycle disruption, infertility, decreased sex drive and vaginal dryness, and osteoporosis, which can lead to bone fractures and, ultimately, polycystic ovarian syndrome (Oyebanji *et al.,* 2018).

2.12.5. Luteinizing Hormone (LH)

LH, also known as lutropin, is a hormone secreted by gonadotropic cells in the anterior pituitary gland that plays a significant role in regulating ovulation function in women (Oyebanji *et al.,* 2018).

LH also aids in the regulation of the menstrual cycle and egg production (ovulation) (Oyebanji *et al.,* 2018). LH levels in the blood are elevated in the majority, but not all, of the women with PCOS (Oyebanji *et al.,* 2018). Increased LH levels are more common in PCOS women who are lean and non-obese than in those who are overweight or obese (Oyebanji *et al.,* 2018). Because LH levels can vary dramatically over short periods of time, it is probably unwise to base a diagnosis on LH levels in individual patients (Oyebanji *et al.,* 2018).

LH secretion in PCOS women is distinguished by episodic bursts every 60 minutes, whereas in normal women the pulse frequency varies from every 90 minutes in the early follicular phase to every 2–4 hours in the mid-luteal phase (Oyebanji *et al.,* 2018). The pattern of LH secretion in PCOS likely reflects the unrestricted activity of the hypothalamic GnRH pulse generator, which appears to be insensitive to progesterone-induced negative feedback (Jeffrey and Ralph, 2014).

2.12.6. Insulin

A peptide hormone synthesize by beta cells in the pancreatic islets is insulin (Oyebanji *et al.,* 2018).

It regulates carbohydrate, fat, and protein metabolism by increasing the absorption of glucose from the blood into fat, liver, and skeletal muscle cells (Oyebanji *et al.,* 2018). When glucose levels in the blood are high, pancreatic beta cells secrete insulin into the blood; when glucose levels are low, insulin secretion is inhibited (Oyebanji *et al.,* 2018). When compared to weight-matched eumenorrheic women, both obese and non-obese women diagnosed with PCOS have

insulin resistance and release more insulin in response to a standard glucose challenge (Oyebanji *et al.,* 2018).

The level of insulin resistance is subtle, and fasting insulin levels are frequently normal (Oyebanji *et al.,* 2018). A normal fasting insulin level, on the other hand, does not rule out the possibility of insulin resistance (Oyebanji *et al.,* 2018). While women with PCOS are generally euglycemic, there is compelling evidence that they are at risk of developing type 2 diabetes mellitus in the future (Jeffrey and Ralph, 2014). Insulin is considered to enhance blood free testosterone levels in two ways: first, by promoting ovarian testosterone production and secretion, and second, by reducing hepatic SHBG output directly (Mayer *et al*., 2015).

Insulin was found to boost the production of testosterone by theca cells from women with PCOS, according to a study (Zeng *et al.,* 2020). Insulin Growth Factor Receptors (IGFRs) have been discovered in ovarian cells, which also produce IGF-binding proteins (Amutha and Rajkumar, 2017). In follicle production, IGF-1 and IGF-II, which are structurally related to insulin, perform a comparable role. (Ipsa *et al.,* 2019).

CHAPTER THREE

3.0 Materials and Methodology

3.1 Materials

3.1.1 Drugs, Assay Kits, Test strips and other Chemicals and Apparatus

Letrozole (2.5mg), Metformin (500mg), Clomiphene Citrate (50mg), Absolute Ethanol, Bitter leaves (*V. amygdalina)*, Warring blender, Rotatory evaporator, Beakers, Funnel, Whatman's No.1 filter paper, measuring cylinder, Jute bag, Hot air oven and Plastic container, UV-Visible Spectrophotometer, cuvette, Distilled water, conical flask, measuring cylinder, test tubes, test tube racks, 2N sodium hydroxide, chloroform, 10% ammonia, 10% ferric chloride, 0.2% ninhydrin reagent, 10% sodium hydroxide, 2% hydrochloric acid, glacial acetic acid and ferric chloride.

High-Density Lipoprotein (HDL), Low Density Lipoprotein (LDL), Cholesterol, Triglyceride assay kits were products of Randox Laboratory, Co-Atrim, United Kingdom. Progesterone, Insulin, Estradiol, Follicle Stimulating Hormone, Testosterone and Luteinizing Hormone assay kits were manufactured by Accu-bind Diagnostics Laboratories, Freiburg, Germany. Accu-check Active strip Compact Plus Glucometer and Test Strips were used.

3.1.2 Experimental plant

Fresh leaves of *Vernonia amygdalina* were obtained from the premises of the male hostel area of Mountain Top University Ogun State Nigeria. The plant was authenticated by Nodza George at the department of Plant Biology, University of Lagos, Lagos, Nigeria which a voucher specimen number 8751 was deposited at the Herbarium of the Plant Biology department.

3.1.3 Experimental Animal Specimen

Twenty, healthy female Wistar rats (170.81 ± 5.35g) were obtained from the animal holding unit of the Mountain Top University, Ogun State, Nigeria. The animals were kept in a well-ventilated house condition and fed with rat pellets (Vital Feeds, Grand Cereals, Lagos, Nigeria) and water.

3.2 Methodology

3.2.1 Preparation of ethanoic extract of *V. Amygdalina leaves*

A known weight (4kg) *V. amygdalina* leaves was washed under running water to remove contaminants, which was sun dried to expel some water content and oven dried at 50ºC, to attain a constant weight of 1kg. When completely dry, was pulverized with a warring electric blender. The powdered material was weighed (1kg) and soaked in 3L of absolute (concentrated) ethanol for 48hrs. The extract was sieved with a jute bag and poured in a plastic container and filtered with Whatman's No. 1 filter paper. The filtrate was subjected to the rotatory evaporator to recycle back the absolute ethanol (solvent), as well as obtaining the concentrate of the plant sample. The concentrate was kept in a hot air oven at 50ºC until completely dry. Ethanoic extract of *V. amygdalina* yielded 6.705% with a deep green colour (Tietz 1995).

3.2.2 UV-Visible Spectroscopy

Procedure

0.48g of *V. amygdalina* 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 at the opaque portion of the cuvette and placed in the UV-Visible spectroscopy and read at different wavelengths between 200-600nm to get the different absorbance (Tietz 1995).

3.2.3 Qualitative Phytochemical Screening Analysis

The ethanoic extract of *V. amygdalina* leaves was tested for the presence of bioactive compounds using standard procedures as described by Rohini *et al.,* (2021) with slight modification.

Test for Carbohydrate (Molisch's test)

In a test tube, 2ml of plant extract was mixed with 1ml Molisch's reagent and 5 drops of strong sulphuric acid. The presence of carbohydrate is indicated by the formation of a purple or reddish colored solution (Rohini et al., 2021).

Test for Tannins

In a test tube, 1 mL of plant extract was mixed with 2 mL of 5% ferric chloride. The presence of tannins is indicated by the formation of a dark blue or greenish black solution (Rohini *et al.*, 2021).

Test for Saponins

In a test tube, 2ml of plant extract was mixed with 2ml of distilled water and shaken for 15 minutes lengthwise. The presence of saponins is indicated by the formation of foam (Rohini *et al.,* 2021).

Test for Alkaloids:

In a test tube, 2ml of plant extract was combined with 2ml of strong hydrochloric acid and 5 drops of Mayer's reagent. The presence of alkaloids is indicated by the formation of a green-colored solution or a white precipitate (Rohini *et al.,* 2021).

Test for Flavonoids

In a test tube, 2ml of plant extract was mixed with 1ml of 2N sodium hydroxide. The presence of flavonoids is indicated by the formation of a yellow-colored solution (Rohini *et al.,* 2021).

Test for Glycosides

In a test tube, 2ml of plant extract was mixed with 3ml of chloroform and 10% ammonia solution. The presence of glycosides is indicated by the formation of a pink-colored solution (Rohini *et al.,* 2021).

Test for Quinones

In a test tube, 1ml of plant extract was mixed with 1ml of strong sulphuric acid. The presence of quinones is indicated by the formation of a red-colored solution (Rohini *et al.,* 2021).

Test for Phenols

In a test tube, 1 mL of plant extract was mixed with 2 mL of distilled water and 5 drops of 10% ferric chloride. The presence of phenols is indicated by the formation of a blue or green colored solution (Rohini *et al.,* 2021).

Test for Terpenoids

In a test tube, 0.5ml of plant extract was mixed with 2ml chloroform and 2ml concentrated sulphuric acid. The presence of terpenoids is indicated by the formation of a reddish-brown colored solution (Rohini et al., 2021).

Test for Cardiac Glycosides

In a test tube, 0.5 mL of plant extract was mixed with 2 mL glacial acetic acid, 5 drops ferric chloride, and 1 mL concentrated sulphuric acid. The presence of cardiac glycosides is shown by the formation of a brown ring at the contact (Rohini *et al.,* 2021).

Test for Ninhydrin

In a test tube, 2ml of plant extract was combined with 5 drops of 0.2% ninhydrin reagent and heated for 5 minutes. The presence of ninhydrin is indicated by the formation of a blue-colored solution (Rohini *et al.,* 2021).

Test for Coumarins

In a test tube, 1ml of plant extract was mixed with 1ml of 10% sodium hydroxide. The presence of coumarins is indicated by the formation of a yellow-colored solution (Rohini *et al.,* 2021).

Test for Anthraquinones

In a test tube, 1ml of plant extract was mixed with 5 drops of 10% ammonia solution. The presence of anthraquinones is shown by the formation of pink-colored precipitate (Rohini *et al.,* 2021).

Test for Steroids

In a test tube, 1ml of plant extract was mixed with 1ml of chloroform and 5 drops of strong sulphuric acid. The presence of steroids is indicated by the formation of a brown ring (Rohini *et al.,* 2021).

Test for Phlobatannins

In a test tube, 1 mL of plant extract was mixed with 5 drops of 2% hydrochloric acid. The presence of Phlobatannins is indicated by the formation of a red-colored solution (Rohini *et al.,* 2021).

Test for Anthracyanin

In a test tube, 1ml of plant extract was mixed with 1ml of 2N sodium hydroxide and heated for 5 minutes at 100°C. The presence of Anthracyanin is shown by the formation of a bluish green color solution (Rohini *et al*., 2021).

3.2.4 GC-MS Analysis

Procedure

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 temperature of the oven 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 (Aufrere, 1976; Abraham, 1981).

3.2.5 Induction of PCOS

Twenty female Wistar rats of average weight 170.81 ± 5.23g were acclimatised for seven days under standard room condition (temperature of 22±3^{o} 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 administration 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)- were administered 0.5 ml of vehicle only (carboxy-methyl cellulose)

Group B – were administered 1mg/kg body weight of letrozole.

Group C- were administered 1mg/kg body weight of letrozole.

Group D- were administered 1mg/kg body weight of letrozole.

Group E- were administered 1mg/kg body weight of letrozole.

3.2.5.1 Confirmation of PCOS

Using light microscope, the vaginal cytology of the stages of oestrous cycle in the female rats were monitored to observe the predominant cell type in the vaginal smears which was obtained daily for 21 days during the induction period. Twenty-four hours after the last dose (1ml) of letrozole, and after an overnight 12 hours fast, the fasting blood glucose was determined. The rats were sacrificed and blood samples collected using the procedures earlier described by (Yakubu *et al.,* 2008). The serum was used for the assay of serum lipids and reproductive hormones.

3.2.5.2 Animal grouping and extract administration for pharmacological study

PCOS was induced in twenty female Wistar rats with 1ml of letrozole as described previously. Female rats which experienced irregularity in their oestrous cycle were completely randomised into 5 groups (designated A – E) of four (4) animals in each after which were treated via oral administration of different doses of the standard drugs and plant extracts for a period of 14 days.

The administration of the treatment doses according to the groups are as follows:

Group A (non-PCOS-induced control) were administered 1ml of distilled water.

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

Group C (PCOS-induced) were administered 7.14 mg/kg body weight of metformin and 2mg/kg body weight clomiphene citrate (Reference drugs).

Group D (PCOS-induced) were administered 50mg/kg body weight of ethanoic extract of *V. amygdalina* leaves.

Group E (PCOS-induced) were administered 100mg/kg body weight of ethanoic extract of *V. amygdalina* leaves.

Using light microscope, the vaginal cytology of the stages of estrous cycle in the female rats were monitored to observe the predominant cell type in the vaginal smears which was obtained daily for 14 days during the treatment period. Twenty-four hours after the last dose of plant extract and after an overnight 12 hours fast, the fasting blood glucose was determined. At the end of the experimental period, the rats were anesthetized using diethyl ether and sacrificed by jugular puncture. The rats were sacrificed and blood samples collected using the procedures earlier described by (Yakubu *et al.,* 2008). Thereafter, the ovaries, liver and kidney were isolated and separately fixed in 10% formalin for histological studies. Whereas the serum and tissue supernatants were used to carry out biochemical hormonal assays.

3.2.5.3 Vaginal Cytology

At 8-9am vaginal smears were obtained on daily basis throughout the period of study. Holding the rats at the thorax, ventral surface uppermost to provide lumbar support, vaginal secretions were collected using a cotton-tipped swabs softened with a drop of saline. Inserting into the vagina about 1-2 inches of the swab and rotated through 2-3 revolutions, to pick an adequate load of cells, the swab was gently withdrawn, rolled on the glass slide and examined under the

light microscope (x40 magnification). A digital camera (SONY corporation Digital camera, China) was used to capture the photomicrographs.

3.2.5.4 Determination of Fasting Blood Glucose Level

Using the glucometer kit (Accu-check, product of Roche Diagnostic GmbH, Sandhofer Strasse Germany), the level of fasting blood glucose was determined, after an overnight fast for 12 hours. In the morning (8:00 – 9:00 am), the tip of the tail of the rats were pricked with lancet and blood from the tail was allowed to drop on the strip which had been inserted into the glucometer. The blood glucose concentration was obtained in mg/dl for each rat in all the groups (Saidu *et al.*, 2014).

3.2.5.5 Preparation of Serum and Tissue Supernatants

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 (-4ºC) overnight. The animals were quickly dissected, the liver, kidney and ovary were excised, cleaned of fatty layers, weighed and transferred into ice cold 2M sucrose solution. Thereafter, each organ was blotted with blotting paper, cut thinly with a sterile blade and homogenized separately in ice cold 2M 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, stored frozen (-4ºC) overnight before being used for the various biochemical assays (Aufrere, 1976; Abraham, 1981).

Table 4: Dilution factor for the various assays.

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

3.2.6 Determination of Serum Concentration of Hormones

3.2.6.1 Progesterone

The serum progesterone concentration was determined using competitive Accu-Bind kits (Chen *et al*., 1991; Granoff and Abraham, 1979).

Principle

The principle is based on a one-step immunoassay that uses a competitive microplate enzyme immunoassay to assess the presence of progesterone. In order to produce an antigen-antibody complex, progesterone in the sample competes with enzyme progesterone conjugates for binding with anti-progesterone coated microplate. In the antibody-bound fraction, enzyme activity is inversely proportional to natural progesterone concentration (Chen *et al.,* 1991; Granoff and Abraham, 1979).

Procedure

25µl of standard specimen and controls was dispensed into appropriate well, 50µl of Rabbit-Anti test reagent was dispensed carefully into each well and mixed for 30 seconds, 100µl of Testosterone HRP reagent was carefully dispensed into each well and at 25ºC for 60 minutes the microplate was incubated. Using the washing buffer, the microwells was rinsed and flicked 5 times. 100µl of TMB substrate was dispensed into each well and gently mix for 10 seconds, then was incubated at 25ºC for 20 minutes, 100µl of stop solution was added to each well to stop the reaction, for 30 seconds it was mixed gently and read at 450nm absorbance for 15 minutes (Aufrere, 1976; Abraham, 1981).

Calculation

The serum progesterone concentration of the test samples was extrapolated from the calibration curve, plotting the absorbance of the standard sample at 450nm against its corresponding concentration (Chen *et al*., 1991; Granoff and Abraham, 1979).

3.2.6.2 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 idea of competitive binding between a constant quantity of rabbit anti-testosterone, goat anti-rabbit IgG-coated wells, and rabbit anti-testosterone reacted with the test sample for a constant amount of testosterone HRP conjugate. Endogenous testosterone peroxidase conjugate immunologically attached to the well competes with HRP-labeled testosterone and decreases as the concentration of testosterone in the material rises. The amount of enzyme present determines the intensity of the color produced, which is inversely proportional to the amount of unlabeled 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^{o}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 seconds and 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 standard calibration curve. Plotting the absorbance of the standard sample at 450nm against its corresponding concentration (Chen *et al*., 1991; Granoff and Abraham, 1979).

3.2.6.3 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 sandwich method's idea. The enzyme assay system employs a monoclonal antibody with high affinity and specificity directed against a particular antigenic determinant on the complete FSH molecule (enzyme conjugate and immobilized) (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 calibrator, 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 was 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 test samples (Winnink *et al*., 1990).

Calibration

The serum FSH concentration was extrapolated from the standard calibration curve. Plotting the absorbance of the standard sample at 450nm against its corresponding concentration (Winnink *et al*., 1990).

3.2.6.4 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 sandwich method's idea. The enzyme assay system employs a monoclonal antibody with high affinity and specificity directed against a particular antigenic determinant on the intact LH molecule (enzyme conjugate and immobilized) (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 was 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 (Kosasa, 1981).

Calculation

The serum LH concentration was extrapolated from the standard calibration curve. Plotting the absorbance of the standard sample at 450nm against its corresponding concentration (Kosasa, 1981).

3.2.6.5 Insulin

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

Principle

The principle is based on the interaction of streptavidin coated wells with exogenously injected biotinylated monoclonal insulin antibody to immobilize the surface of a microplate well. The enzyme-labeled antibody and a serum containing the natural antigen create a soluble sandwich complex with no competition or steric hindrance between the native antigens and antibodies. The enzyme's activity in the antibody-bound fraction is proportional to the concentration of natural antigen (Eastham, 1985).

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 (Eastham, 1985).

Calculation

The serum insulin concentration was extrapolated from the calibration curve. Plotting the absorbance of the standard sample at 450nm against its corresponding concentration (Eastham, 1985).

3.2.6.6 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).

3.2.7 Lipid Profile Determination

3.2.7.1 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:

$$\text{Cholesterol} + H_2O \longrightarrow \text{Cholesterol} + \text{Fatty acids}$$

$$+ \quad H_2O + O_2 \longrightarrow \text{Cholesterol} + H_2O_2$$

$$H_2O_2 + \text{4-Amino antipyrine} + \text{P-Hydrobenzoic acid} \longrightarrow \text{Coloured Quinonic derivatives} + 4\,H_2O$$

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 was thoroughly mixed and incubated at 37^{o} 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

3.2.7.2 Serum Triglyceride Concentration

The concentration of serum triglyceride, (TG) was determined calorimetrically (Tietz 1995).

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.

$$\text{Triglyceride} + H_2O \xrightarrow{\text{Lipases}} \text{Glycerol} + \text{Fatty acid}$$

$$H_2O_2 + \text{4-aminophenazone} \xrightarrow{\text{Peroxidase}} \text{Quinonimine} + HCl + 4\,H_2O + \text{Chlorophenol}$$

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

3.2.7.3. Serum High Density Lipoprotein-Cholesterol Concentration

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

Principle

The addition of phosphotungstic acid in the presence of magnesium quantitatively precipitates the low-density lipoprotein (LDL and VLDL) and chylomicron fractions. The cholesterol concentration in the HDL-Cholesterol fraction that remains in the supernatant is evaluated after centrifugation (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 temperature. 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

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

3.2.7.4 Serum Low Density Lipoprotein -Cholesterol Concentration

The serum Low Density Lipoprotein (LDL) cholesterol concentration can be expressed as:

LDL Cholesterol = Total Cholesterol – (HDL + TG) (Tietz 1995).

3.2.8 Data Analysis

All data were expressed as the mean of four replicates ± standard error of mean (S.E.M) except otherwise stated. Statistical analysis of the data was performed by SPSS version 26 using one way analysis of variance (ANOVA), followed by Duncan multiple range test for multiple comparison. Values were considered statistically significant at ($p < 0.05$) (Yakubu *et al.*, 2015).

CHAPTER FOUR

4.0 RESULTS

4.1 Phytochemical analysis of EEVAL.

4.1.1 Qualitative phytochemical components of EEVAL.

The result of the qualitative phytochemical screening carried out on the EEVAL (Table 7) indicated the presence of certain essential phytocompounds such saponins, alkaloids, flavonoids, quinones, phenols, terpenoids and coumarins etc.

Table 5. Qualitative phytochemical screening of EEVAL.

SECONDARY METABOLITES	LEAVES
Carbohydrate	–
Tannins	–
Saponins	+
Alkaloids	++
Flavonoids	+++
Glycosides	–
Quinones	+
Phenols	+++
Terpenoids	+++
Cardiac glycosides	–
Anthracyanin	–
Ninhydrin	–
Steroids	–
Coumarins	++
Anthraquinone	–
Phlobatannins	–

4.1.2 UV-Visible spectroscopy analysis of EEVAL.

The Ultra violet -Visible spectroscopy of EEVAL, revealed the varying absorbance of the extract at different wavelengths as shown in (Figure 9). From the UV-Vis spectroscopy curve (Figure 9), there was a spontaneous increase in absorbance value at 300nm wavelength, which lags between 320-410nm wavelengths, afterwards decreased. It can be deduced that at wavelength 320nm shows the highest absorbance peak of the most abundant phytocompound of *V. amygdalina* leaves.

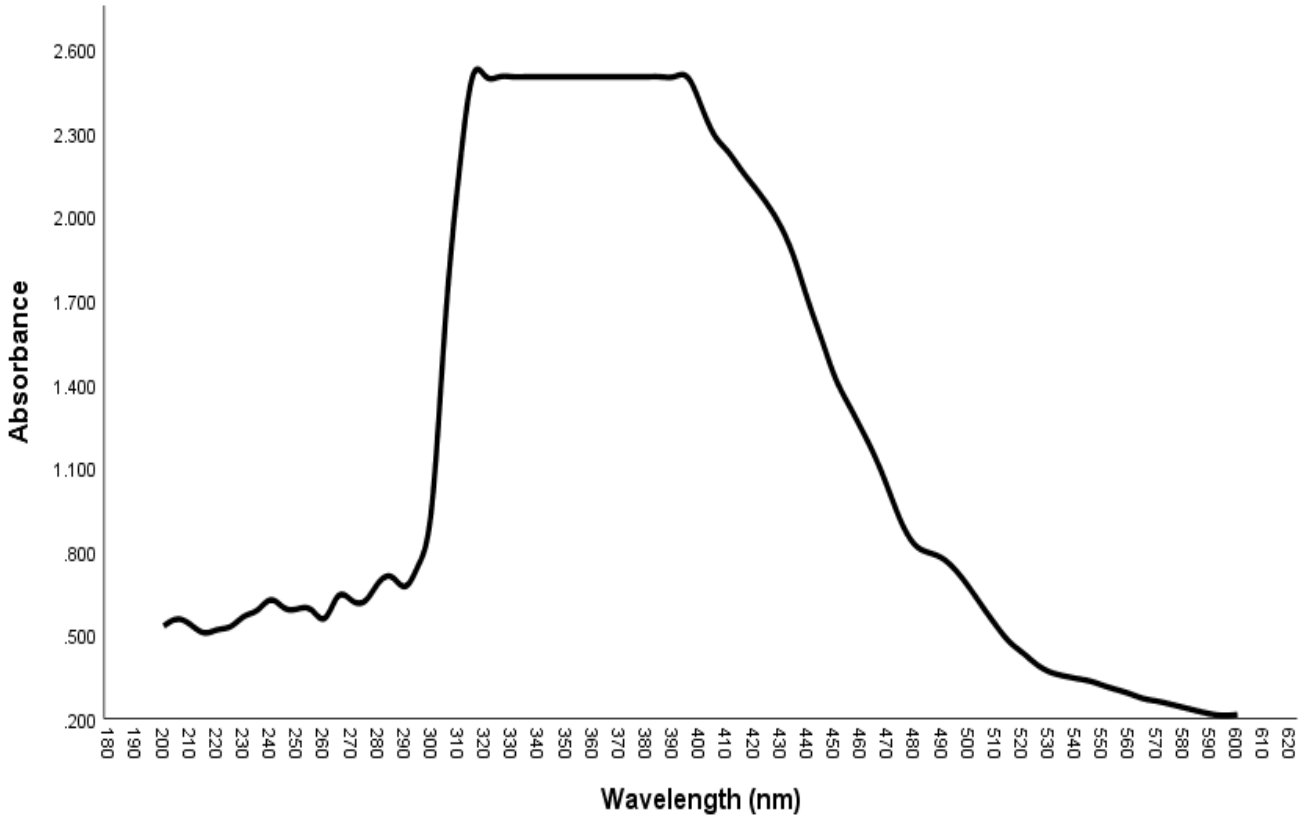


Figure 9. UV-Vis Spectroscopy of ethanoic extract of *V. amygdalina* leaves.

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

4.2.1 Chromatogram of EEVAL.

Figure 10 shows the GC-MS chromatogram of EEVAL. Peak 7 with the retention time of 4.606 was identified as **1-Fluorooctane** ($CH_3(CH_2)_7F$), as the major phytoconstituent of *V. amygdalina* leaves while other peaks were of other phytoconstituents present in the plant.

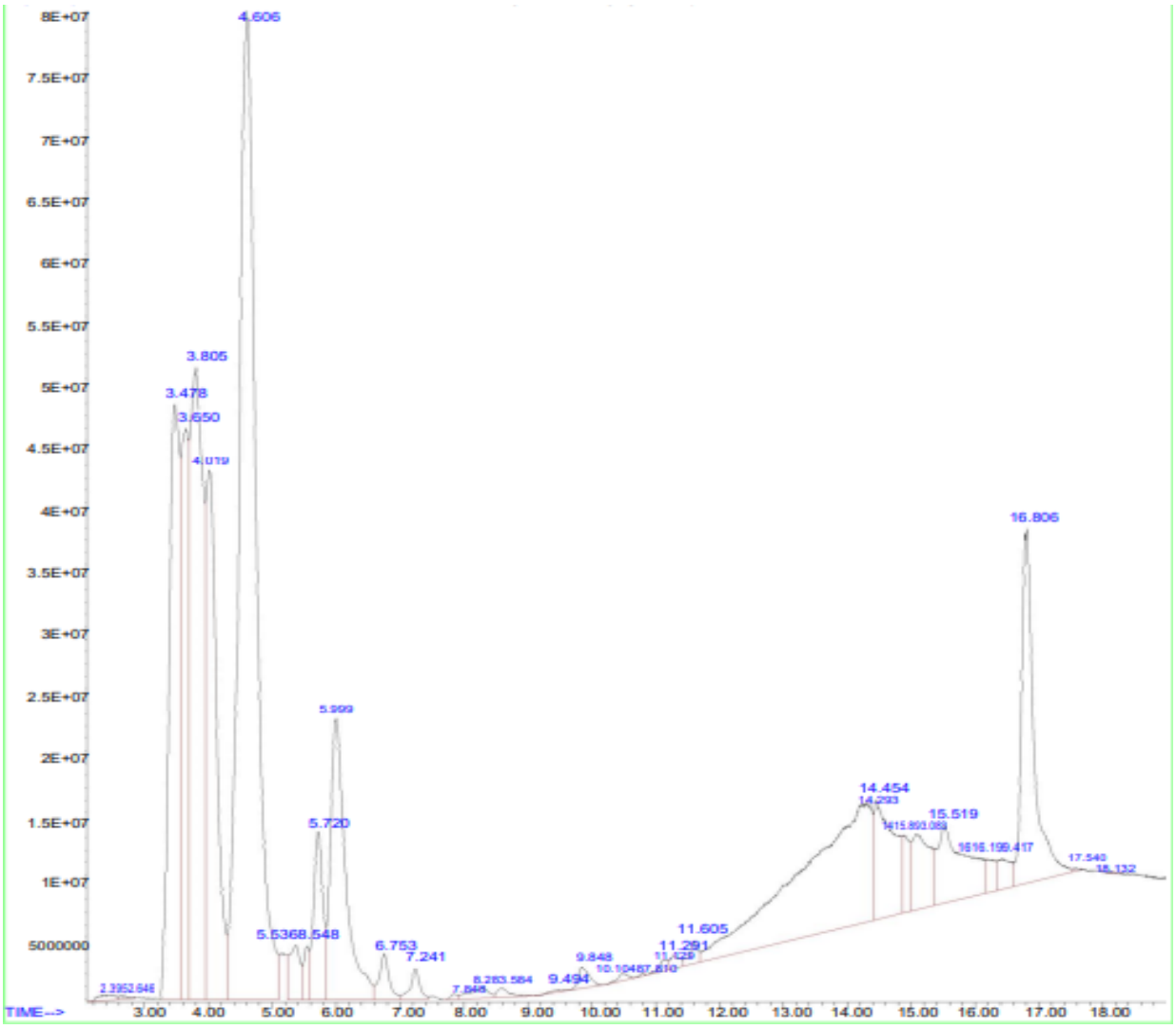


Figure 10. GC-MS chromatogram of ethanoic extract of *V. amygdalina* leaves.

Table 6. Phytocompounds of EEVAL.

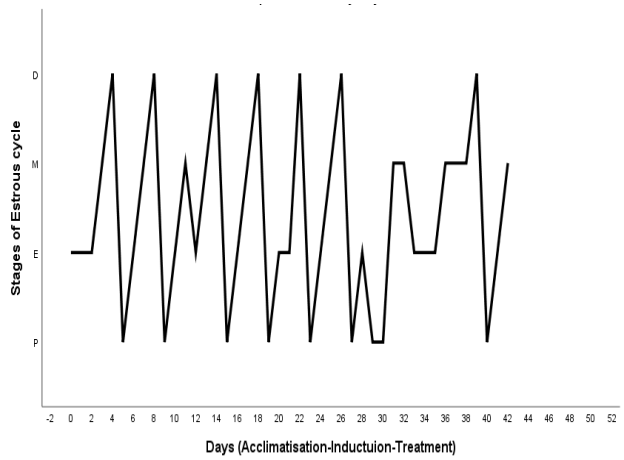
S/N	Name of Compound	Retention Time	Area %	Chemical Formular
1	Propanoic acid, 2-mercapto-methyl ester	2.395	0.13	$C_4H_8O_2S$
2	3-Methylbenzofuran-2-carboxylic acid, 3-dimethylamino-1,2-dimethylpropyl ester	2.646	0.04	$C_{17}H_{23}NO_3$
3	2-Hydroxyethyl vinyl sulfide	3.478	8.76	C_4H_8OS
4	Diethanolamine	3.650	6.00	$C_4H_{11}NO_2$
5	2-Hexene, 5-methyl-, (E)-	3.805	11.55	C_7H_{14}
6	Thiirane	4.019	8.16	C_2H_4S
7	1-Fluorooctane	4.606	24.14	$C_8H_{17}F$
8	1-Butanol, 3-methyl-, acetate	5.368	0.84	$C_7H_{14}O_2$
9	1-Butanol, 3-methyl-, acetate	5.548	0.41	$C_7H_{14}O_2$
10	Peroxide, dimethyl	5.720	2.26	$C_2H_6O_2$
11	Propanenitrile, 2-hydroxy-	5.999	6.14	C_3H_5NO
12	Pentane, 1-(1-ethoxyethoxy)-	6.753	0.66	$C_9H_{20}O_2$
13	Hexanoic acid, ethyl ester	7.241	0.42	$C_8H_{16}O_2$
14	1-Pyrrolidinylacetonitrile	7.848	0.05	$C_6H_{10}N_2$
15	Propane, 1,1,3-triethoxy-	8.283	0.28	$C_9H_{20}O_3$
16	(.+/-.)-p-Methoxyamphetamine, N-trimethylsilyl-	8.584	0.16	$C_{13}H_{23}NOSi$

17	1-Nonen-3-ol	9.494	0.02	$C_9H_{18}O$
18	Octanoic acid, ethyl ester	9.848	0.34	$C_{10}H_{20}O_2$
19	p-Benzoquinone	10.487	0.10	$C_6H_4O_2$
20	3,4-Pyridinediamine	10.810	0.02	$C_5H_7N_3$
21	[1,1'-Bicyclopentyl]-2-one	11.129	0.13	$C_{15}H_{22}O$
22	-(1-Ethoxyethoxy) butyric acid, ethyl ester	11.291	0.18	$C_{10}H_{20}O_4$
23	Decanoic acid, ethyl ester	11.605	0.26	$C_{12}H_{24}O_2$
24	9-Octadecenoic acid, (E)-	14.293	12.59	$C_{18}H_{34}O_2$
25	Phthalic acid, 2-ethylhexyl tetradecyl ester	14.454	3.31	$C_{30}H_{50}O_4$
26	Phthalic acid, 2-cyclohexylethyl ethyl ester	14.893	0.70	$C_{18}H_{24}O_4$
27	Hexadecanoic acid, methyl ester	15.083	1.92	$C_{17}H_{34}O_2$
28	Hexadecanoic acid, ethyl ester	15.519	3.16	$C_{18}H_{36}O_2$
29	N-Acetyl-d-glucosamine	16.199	0.41	$C_8H_{15}NO_6$
30	Cyclopentanecarboxylic acid, 1-methyl-3-oxo-, methyl ester	16.417	0.55	$C_8H_{12}O_3$
31	Phytol	16.806	6.26	$C_{20}H_{40}O$
32	Urea, 1-(2,4-difluorophenyl)-3- [4-(3-methyl-5-trifluoromethylpyrazol -1-yl) phenyl]-	17.540	0.03	$C_{18}H_{13}F_5N_4O$
33	Cyclopropanecarboxylic acid, 2-methyl-3-(1-propenyl)-	18.132	0.01	$C_{10}H_{16}O_2$

4.3 Oestrous cyclicity of letrozole-induced PCOS female Wistar rats after oral administration of EEVAL.

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 (Figure 11-15). 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).

1



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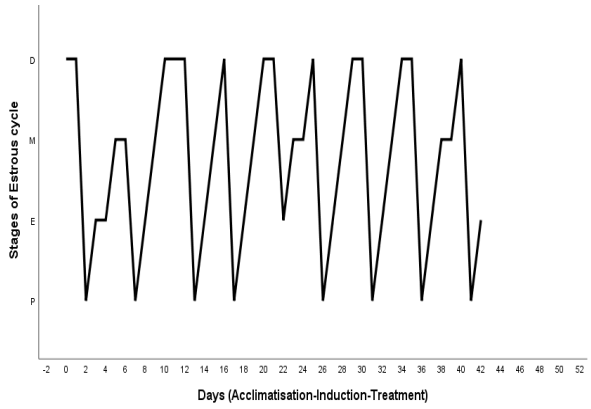

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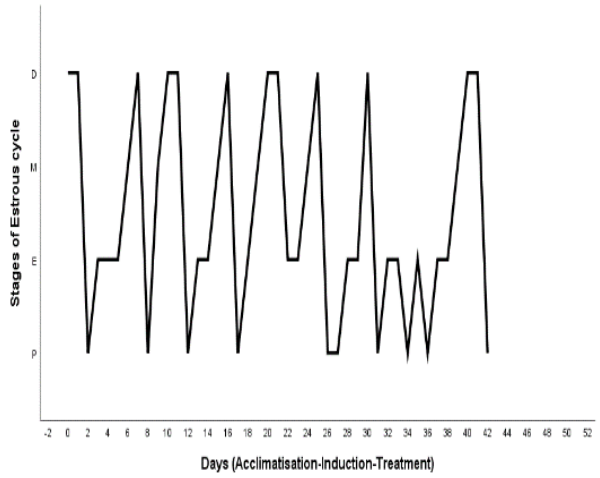

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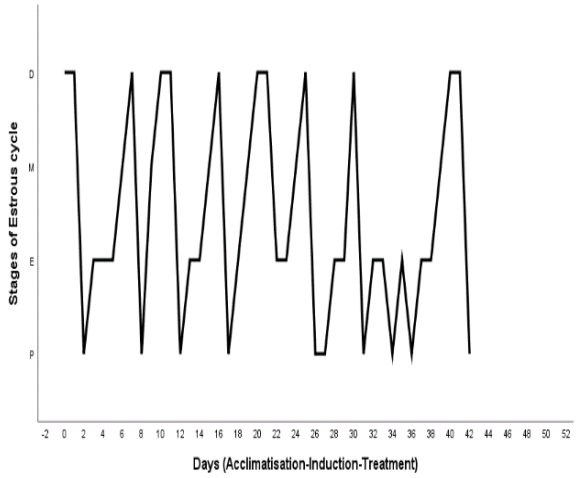
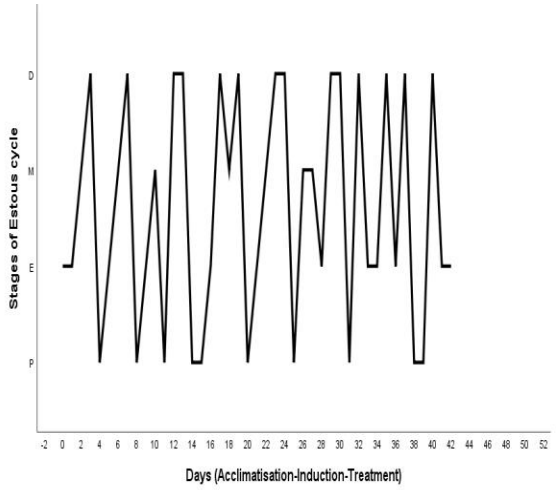


Figure 11: Oestrous cyclicity of rats in the normal control group.

P: Proestrous, E: Estrous, M: Metaestrous, D: Diestrous

1



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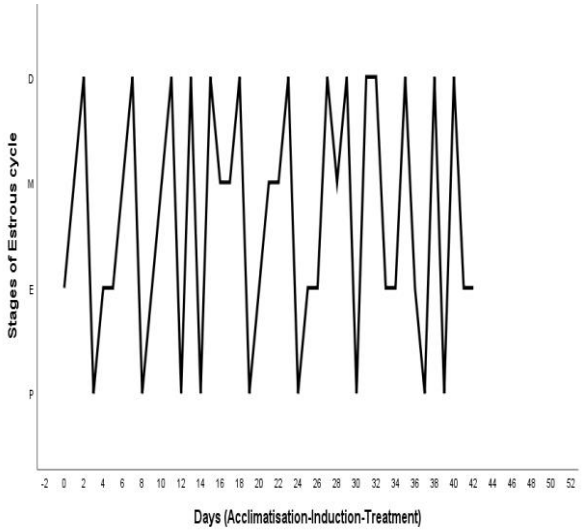

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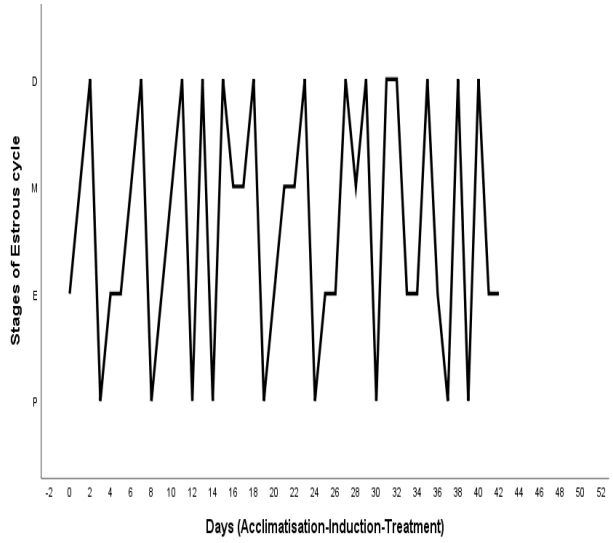

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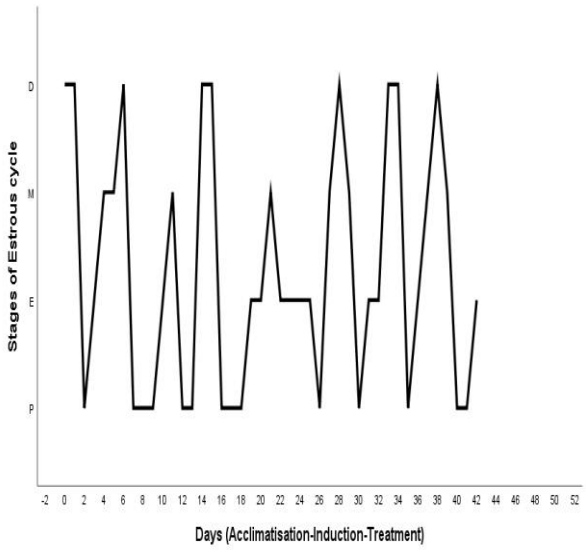
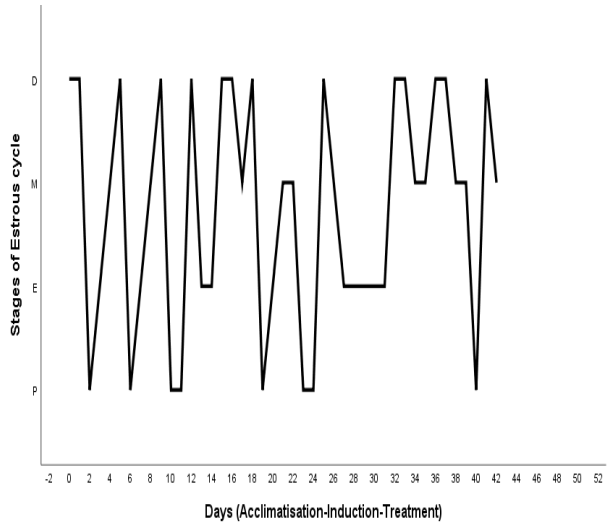


Figure 12: Oestrous cyclicity of letrozole-induced female rats untreated group

P: Proestrous, E: Estrous, M: Metaestrous, D: Diestrous

1



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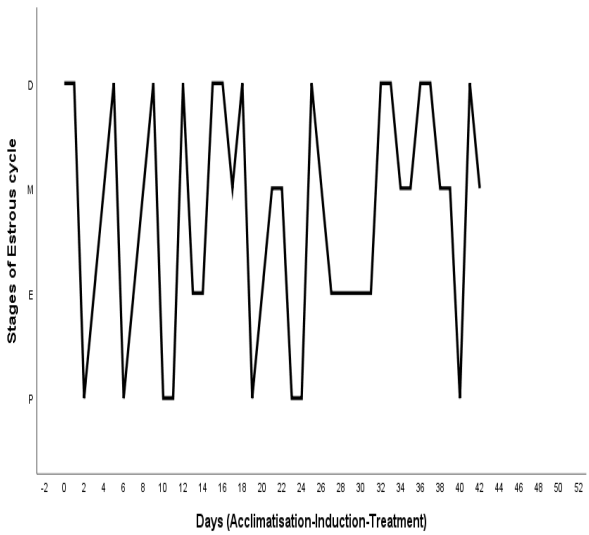

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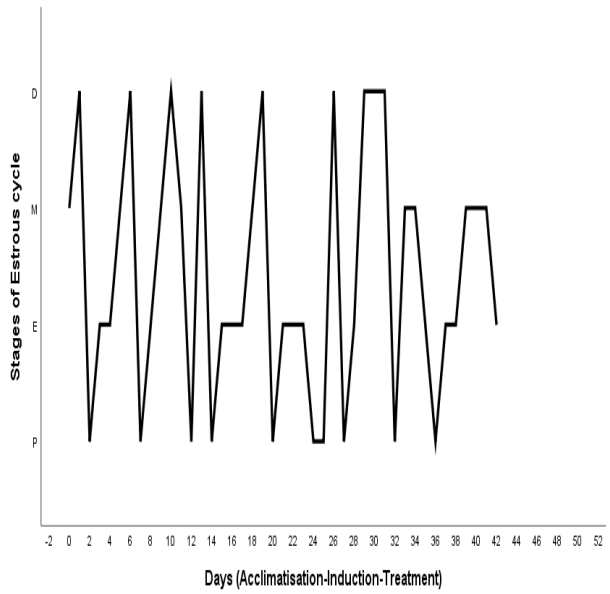

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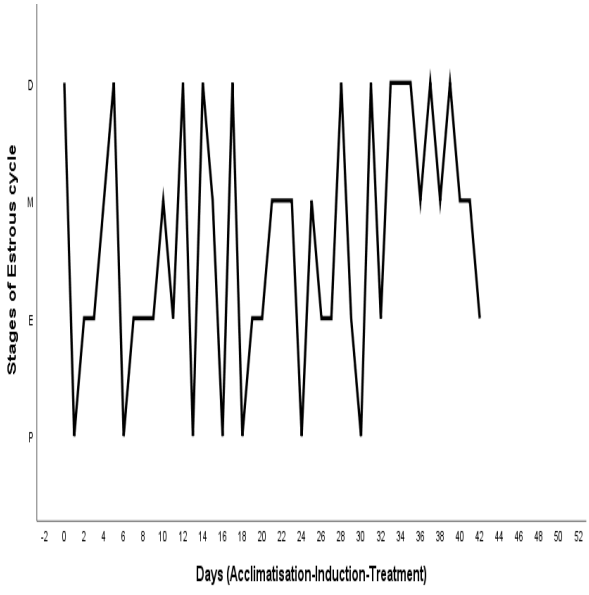

Figure 13: Oestrous cyclicity of letrozole- induced female rats treated with reference drugs (metformin and clomiphene citrate).

P: Proestrous, E: Estrous, M: Metaestrous, D: Diestrous

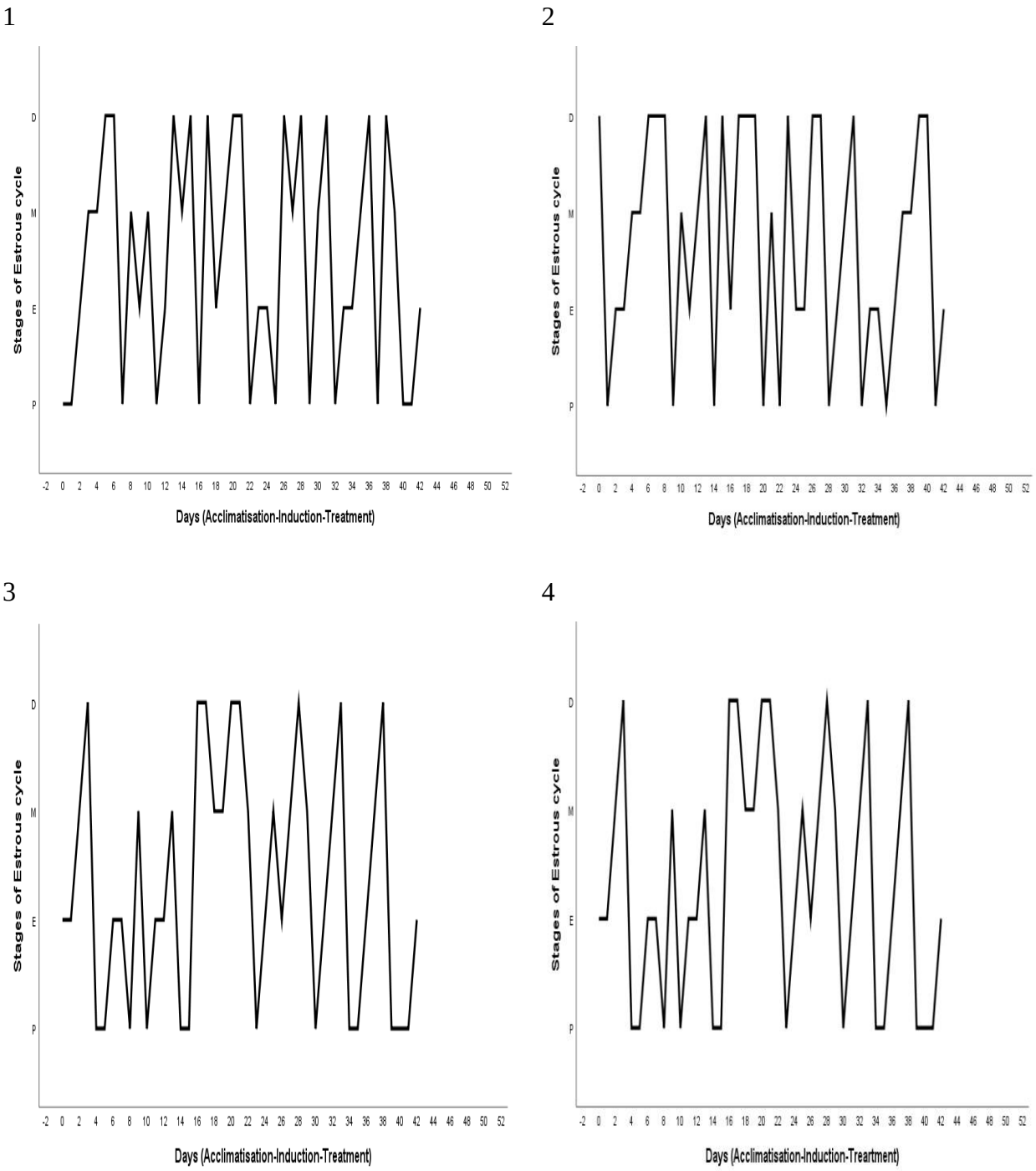

Figure 14: Oestrous cyclicity of letrozole- induced female rats treated with 50mg/kg body weight of EEVAL.

P: Proestrous, E: Estrous, M: Metaestrous, D: Diestrous

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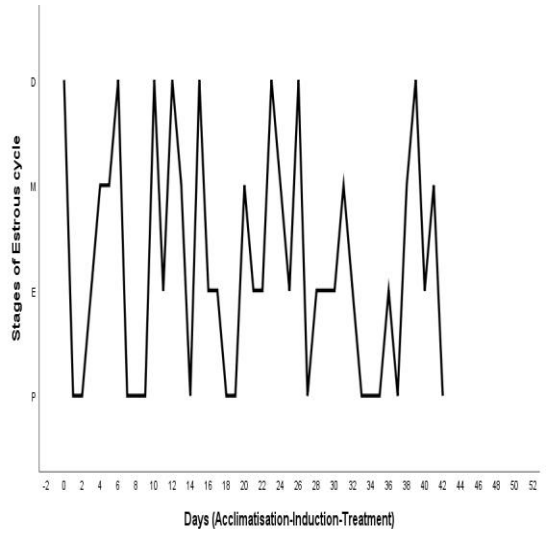

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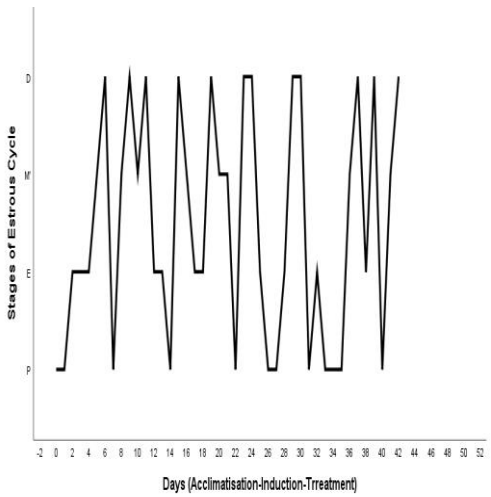

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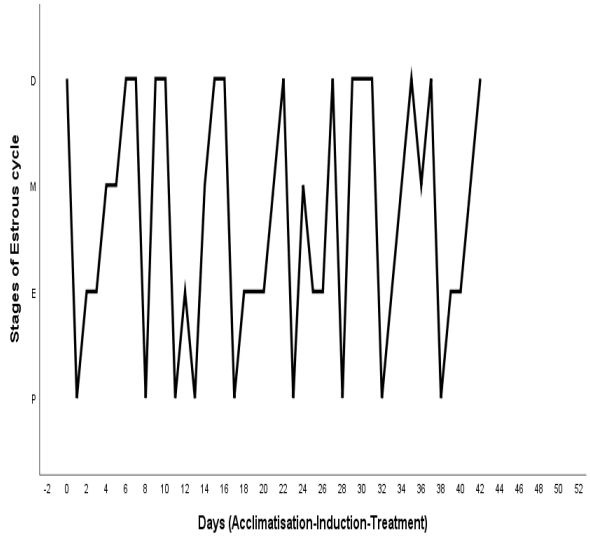

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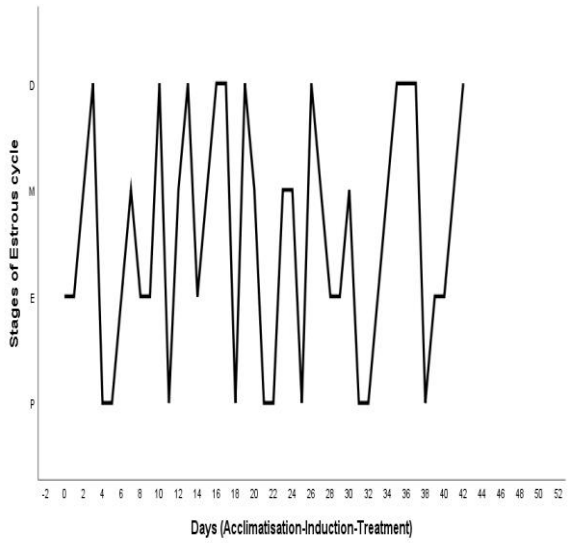

Figure 15: Oestrous cyclicity of letrozole- induced female rats treated with 100mg/kg body weight of EEVAL.

P: Proestrous, E: Estrous, M: Metaestrous, D: Diestrous

4.4 Effects of ethanoic extract of *V. amygdalina* leaves on the Fasting blood glucose levels of letrozole-treated female rats.

The fasting blood glucose levels of letrozole-treated animals administered was significantly increased ($P<0.05$) when compared with the control (Table 8). The 50 and 100 mg/kg B.W of the extract to letrozole-treated rats significantly ($p<0.05$) decreased compared with the reference drugs (Table 14).

Before induction, the administration of letrozole significantly ($p>0.05$) compared favourably with the control group. In contrast the administration of 50 and 100mg/kg B.W of the extract significantly ($p<0.05$) increased compared to the letrozole-treated rats (Table 14).

After induction and treatment, the administration of letrozole significantly ($p<0.05$) decreased compared to the control group. In contrast the administration of 50, 100mg/kg B.W of the extract and reference drugs significantly ($p<0.05$) decreased compared to the letrozole-treated rats (Table 14).

Table 7: Fasting blood glucose levels of letrozole-treated animals administered EEVAL.

	Fasting blood glucose level of rats (mg/dl)		
	Before Induction	**After Induction**	**After Treatment**
Control	74.00 ± 0.58^{a}	87.50 ± 0.96^{a}	114.50 ± 0.29^{a}
Letrozole + distilled water	75.00 ± 0.58^{a}	78.50 ± 0.96^{d}	100.00 ± 0.00^{c}
Letrozole + Metformin + Clomiphene Citrate	63.50 ± 0.29^{d}	64.00 ± 1.16^{b}	79.00 ± 0.58^{b}
Letrozole + 50mg/kg body weight of extract	80.50 ± 0.29^{c}	70.00 ± 1.16^{c}	80.50 ± 1.44^{b}
Letrozole + 100mg/kg body weight of extract	77.50 ± 0.87^{b}	68.50 ± 0.87^{c}	78.50 ± 0.29^{b}

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

4.5 Effects of ethanoic extract of *V. amygdalina* leaves on the body weight of letrozole-treated female rats.

The body weight of letrozole-treated animals was significantly increased (P>0.05) when compared with the control group. The administration of 50 and 1000 mg/kg body weight showed a significant (P>0.05) increased as well as PCOS rats administered metformin and clomiphene citrate (Table 15).

Before induction, the administration of letrozole significantly ($p<0.05$) increased compared to the control group. In contrast the administration of 50, 100mg/kg B.W of the extract and reference drugs significantly ($p<0.05$) increased compared to the letrozole-treated rats (Table 15).

After induction and treatment, the administration of letrozole significantly ($p<0.05$) increased compared to the control group. In contrast the administration of 50, 100mg/kg B.W of the extract and reference drugs significantly ($p<0.05$) increased compared to the letrozole-treated rats (Table 14).

Table 8: Body Weight of letrozole-treated animals administered EEVAL.

	Body weight of rats (g)		
	Before Induction	**After Induction**	**After Treatment**
Control	138.49 ± 1.03^{a}	150.20 ± 1.41^{a}	162.52 ± 0.66^{a}
Letrozole + distilled water	155.22 ± 0.11^{b}	189.45 ± 0.70^{c}	182.12 ± 0.81^{b}
Letrozole + Metformin (7.14mg/kg B.W) + Clomiphene Citrate (2mg/kg B.W)	192.83 ± 0.76^{e}	219.70 ± 0.01^{d}	211.44 ± 7.06^{c}
Letrozole + 50mg/kg body weight of extract	169.95 ± 0.06^{d}	179.28 ± 0.55^{b}	190.21 ± 0.80^{b}
Letrozole + 100mg/kg body weight of extract	164.10 ± 0.09^{c}	188.76 ± 1.13^{c}	186.65 ± 0.80^{b}

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

4.6 Effects of ethanoic extract of *V. amygdalina* leaves on concentration of metabolic hormones of letrozole-treated female rats.

The administration of letrozole to female rats significantly increased ($p<0.05$) the serum insulin, LH and testosterone concentrations while the progesterone, estradiol and FSH concentrations were significantly decreased ($p<0.05$) when compared to the control group (Figure 16-20).

The administration of 50, 100 mg/kg B.W of the extract and metformin with clomiphene citrate significantly ($p<0.05$) decreased the serum testosterone, LH and Insulin concentration compared to the letrozole-treated rats. In contrast the administration of these doses significantly ($p<0.05$) increased the serum FSH, progesterone and estradiol compared to the letrozole-treated rats.

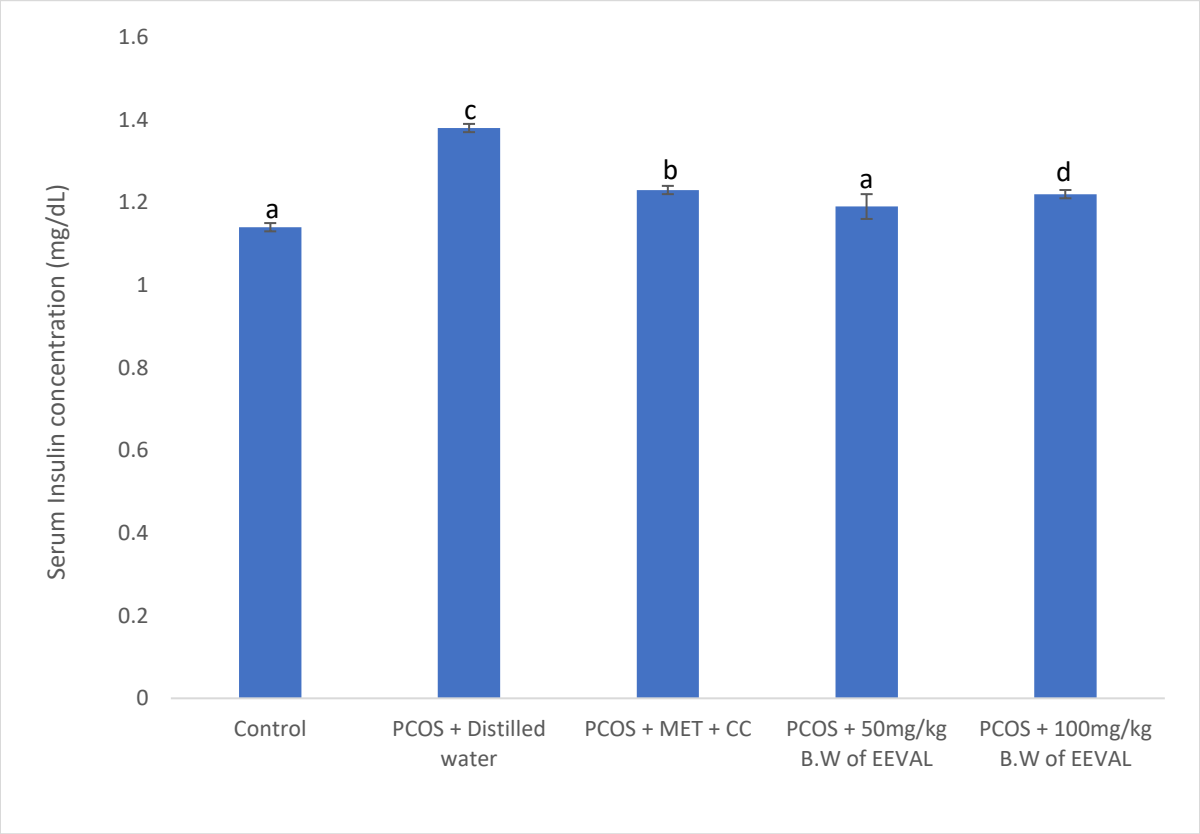

Figure 16: Serum insulin concentration of letrozole-induced PCOS rats administered EEVAL

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

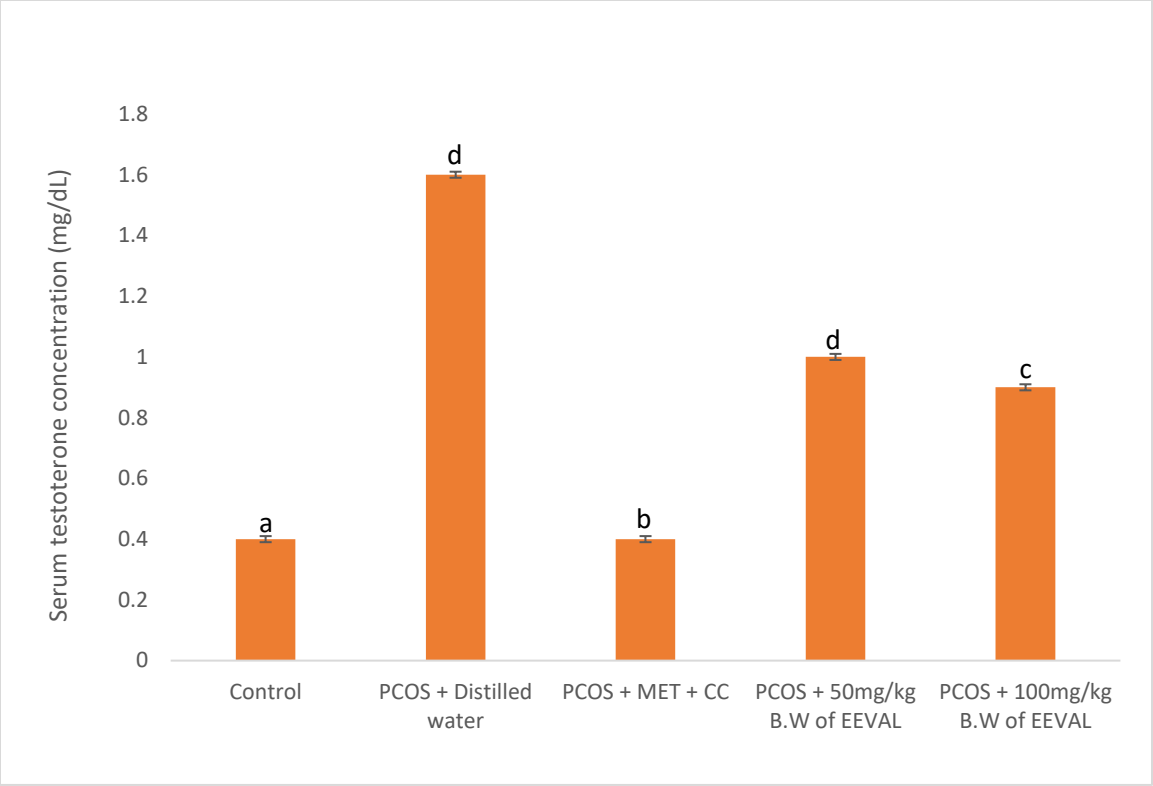

Figure 17: Serum testosterone concentration of letrozole-induced PCOS rats administered EEVAL

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

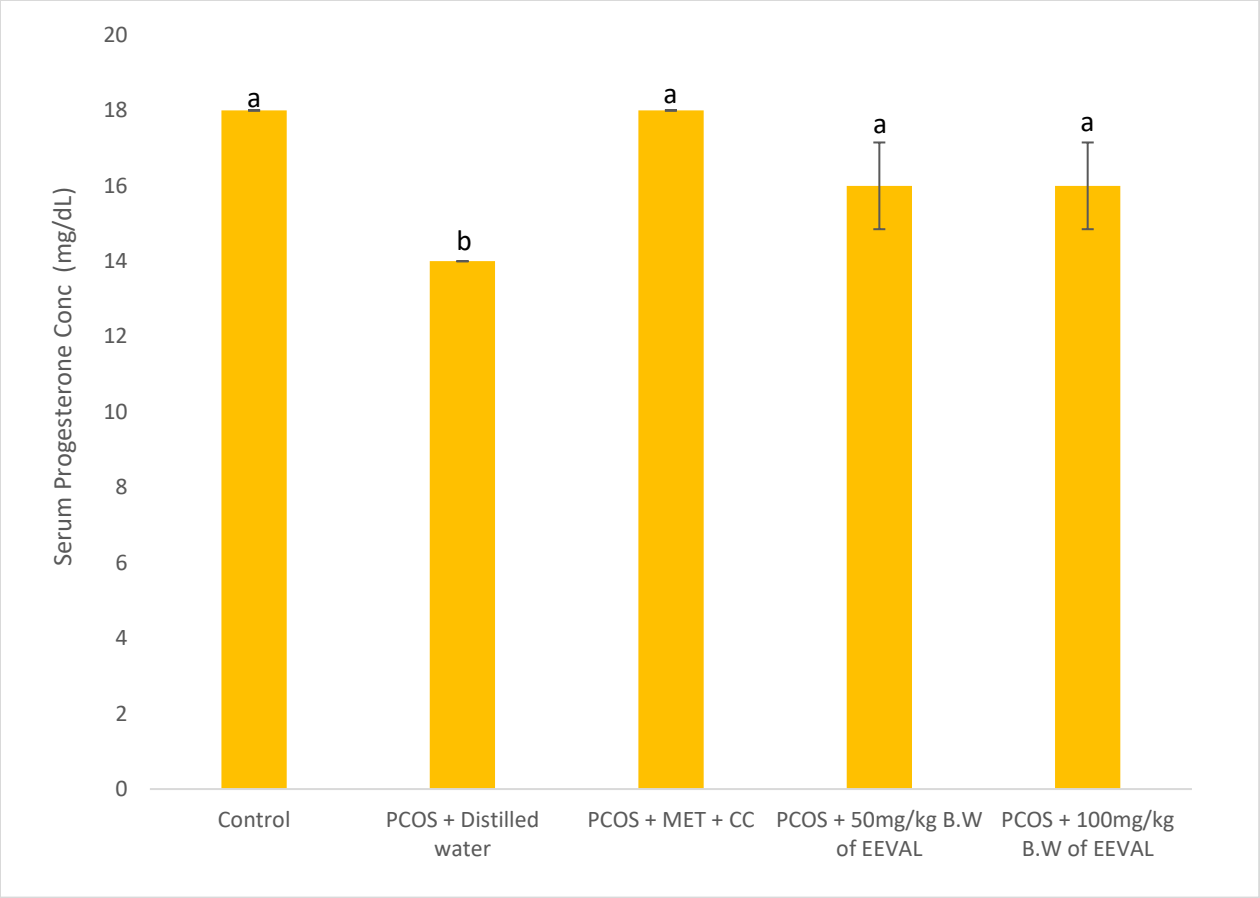

Figure 18: Serum progesterone concentration of letrozole-induced PCOS rats administered EEVAL

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

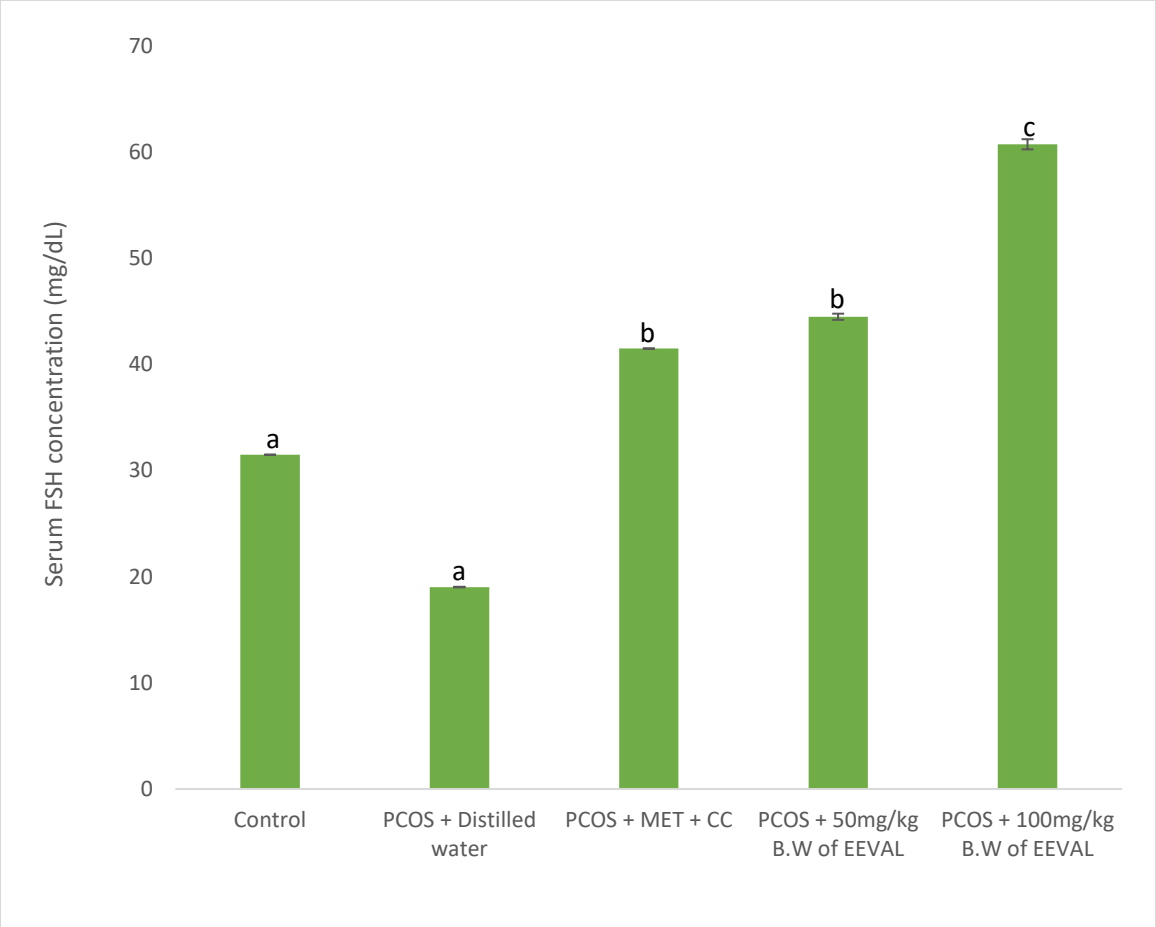

Figure 19: Serum FSH concentration of letrozole-induced PCOS rats administered EEVAL

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

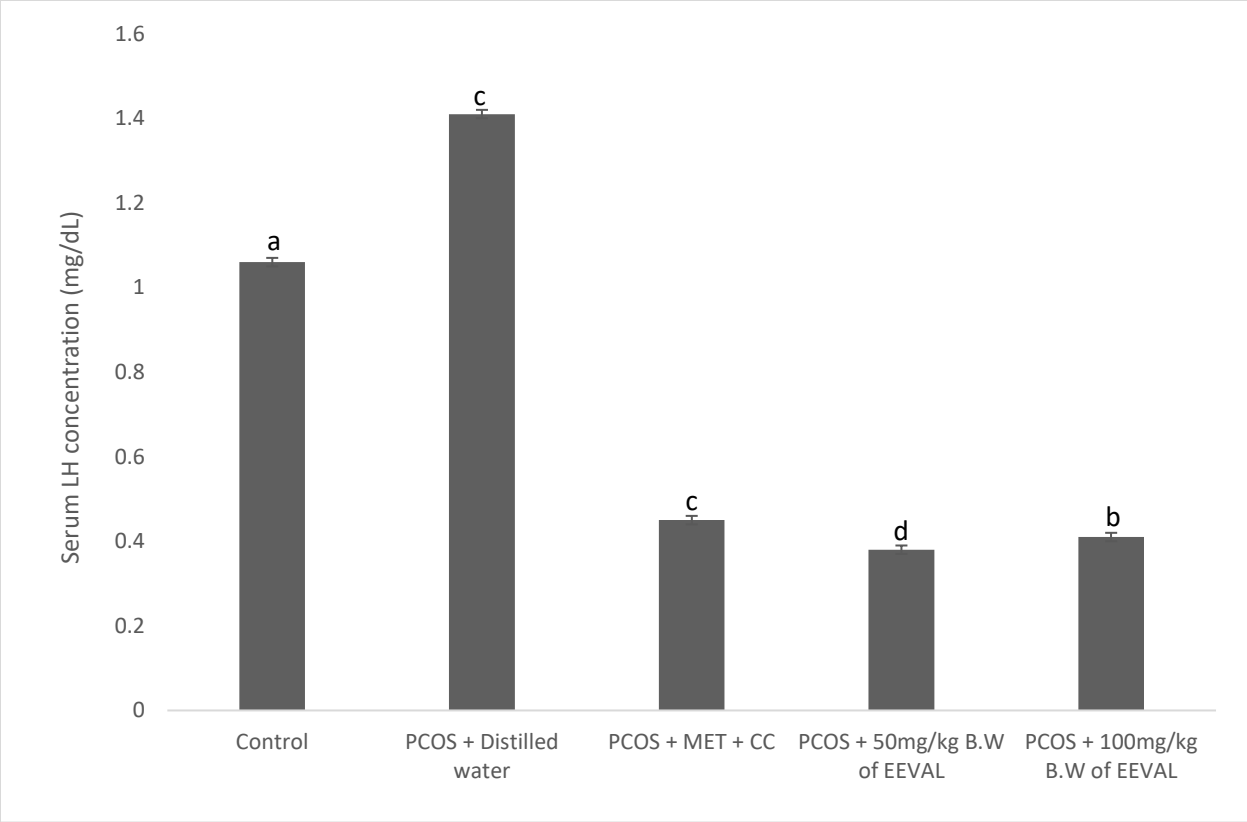

Fig. 20: Serum LH concentration of letrozole-induced PCOS rats administered EEVAL

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

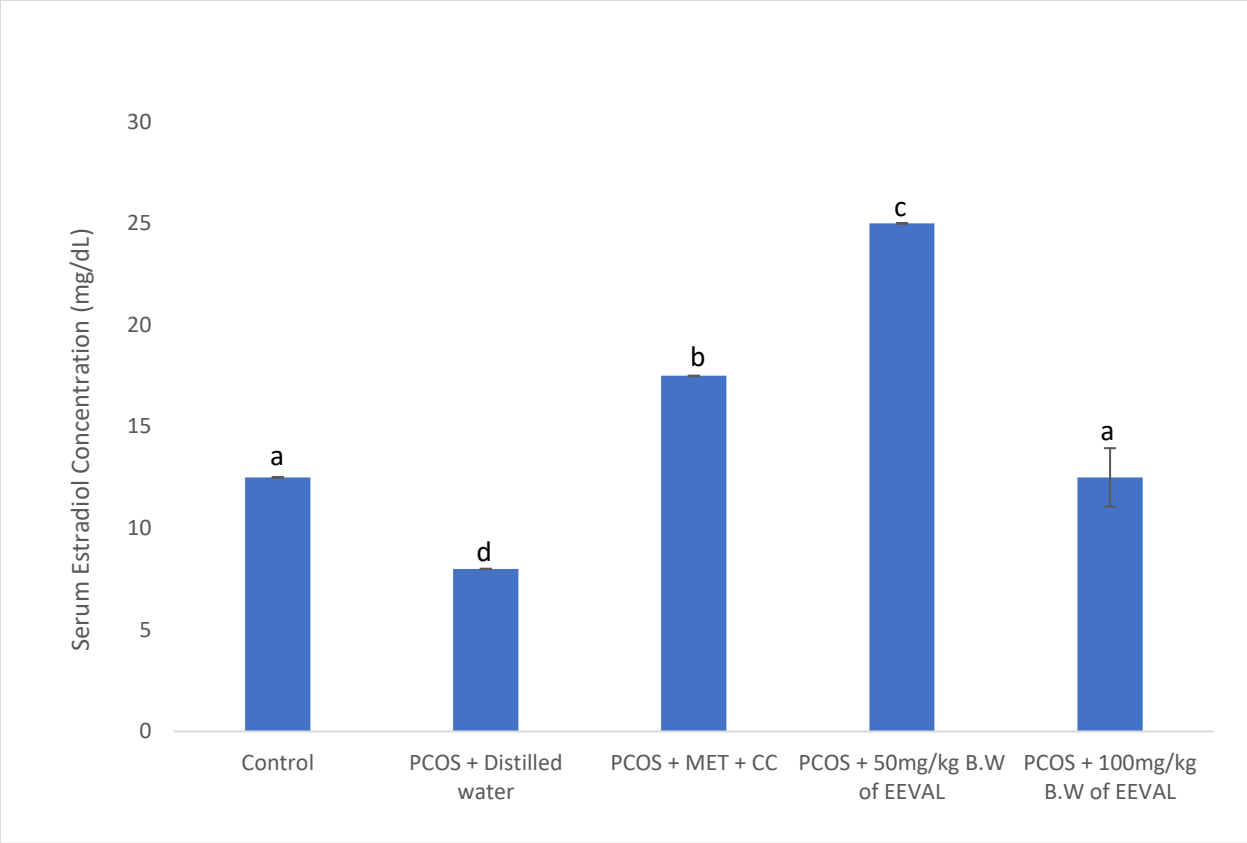

Fig. 21: Serum Estradiol concentration of letrozole-induced PCOS rats administered EEVAL

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

4.7 Lipid Profile

4.7.1 Serum lipid profile of letrozole-induced PCOS rats following oral administration of EEVAL.

The administration of letrozole significantly ($p<0.05$) increased the serum total cholesterol, triacylglycerol and LDL-C concentrations compared to the control group. However, it significantly ($p<0.05$) reduced the serum concentration of HDL-C.

The administration of 50 mg/kg body weight of the extract significantly increased the serum total cholesterol compared to the 100 mg/kg body weight of the extract (Table 9).

Also, the elevation in triacylglycerol after the administration of letrozole was significantly ($p<0.05$) increased in the letrozole-treated rats compared to the control group. The reference drugs significantly ($p<0.05$) decreased triacylglycerol concentration that significantly ($p>0.05$) decreased compared favourably with the 100mg/kg body weight of the extract (Table 9).

In addition, the effect of letrozole administration significantly ($p<0.05$) decreased the serum concentration of HDL-C compared to the control group. In contrast the 50 and 100 mg/kg body weight of the extract significantly ($p<0.05$) increased compared to the letrozole-treated rats. (Table 9).

However, letrozole administration significantly ($p<0.05$) increased the serum concentration of LDL-C compared to the control group. In contrast the 50 and 100 mg/kg body weight of the extract significantly ($p<0.05$) decreased compared to the letrozole-treated rats, also the administration of metformin and clomiphene significantly ($p>0.05$) compared favourably with the control group. (Table 9).

Table 9: Serum lipid profile of letrozole-treated rats following oral administration of EEVAL.

	TC (mmol/L)	TG (mmol/L)	HDL-C (mmol/L)	LDL-C (mmol/L)
Control	10.23 ± 0.00^{a}	14.50 ± 0.85^{a}	4.85 ± 0.29^{a}	9.06 ± 0.60^{a}
Letrozole + distilled water	68.78 ± 0.00^{c}	22.28 ± 0.38^{c}	0.60 ± 0.26^{b}	45.10 ± 0.64^{c}
Letrozole + Metformin (7.14mg/kg B.W) + Clomiphene Citrate (2mg/kg B.W)	8.03 ± 0.37^{e}	16.51 ± 0.18^{b}	0.38 ± 0.06^{b}	8.86 ± 0.48^{a}
Letrozole + 50mg/kg body weight of extract	20.25 ± 0.12^{b}	17.36 ± 0.00^{b}	3.42 ± 0.00^{c}	0.74 ± 0.00^{d}
Letrozole + 100mg/kg body weight of extract	3.20 ± 0.53^{d}	16.26 ± 0.27^{b}	4.84 ± 0.16^{a}	17.80 ± 0.17^{b}

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

TC = Total Cholesterol; TG = Triglyceride; HDL-C = High Density Lipoprotein-Cholesterol; LDL-C = Low Density Lipoprotein-Cholesterol.

CHAPTER FIVE

5.1 Discussion

Polycystic ovarian syndrome is the most frequent reproductive illness identified in women of reproductive age (Mikhael *et al.*, 2019). It is a medical condition that affects women who have a variety of defective reproductive and metabolic characteristics, resulting in infertility (Emamalipour *et al*., 2019).

In this study, female Wistar rats were given 1mg/kg body weight of letrozole to induce PCOS. Amal *et al.* (2019) found that giving 1mg/kg B.W of letrozole which causes PCOS and its accompanying metabolic problems. The oral administration of varied doses of ethanolic extracts of *V. amygdalina* leaves to PCOS rats for 14 days alleviated the produced features/symptoms, according to this study. The restoration of the oestrous cycle, a decrease in fasting blood glucose, and significant improvements in lipid profile and sex hormones were all part of this therapeutic impact.

In rats with PCOS, the oestrous phase is constantly interrupted, owing to the irregularity of steroid hormones that control ovarian function (Ndeingang *et al*., 2019). However, towards the end of the induction period, PCOS rats had an inconsistent oestrous cycle, whereas the rats in the normal control group had a stable and regular oestrous cycle. Letrozole-induced PCOS in rats is linked to a prolonged oestrous cycle, according to Yang *et al*. (2017). In animal experiments, vaginal cytology was found to be the most effective means of assessing the oestrous cycle (Ajayi and Akhigbe, 2020).

Changes in vaginal cytology are utilized to interpret alterations in hormone levels and changes in the oestrous cycle. Letrozole therapy altered the oestrous cyclicity, causing a 7-8-day cycle with a prolong or protracted oestrus phase (Nallathambi and Bhargavan, 2019). Oral metformin and clomiphene citrate administration did not entirely reverse the protracted oestrus phase, while letrozole-induced PCOS rats given 50 mg/kg B.W. had their cycle totally reversed to a normal 4-5 days cyclicity (Nallathambi and Bhargavan, 2019).

Letrozole, an aromatase inhibitor, works by blocking the enzyme aromatase, which converts androgens to estrogens, in a competitive manner (Rose and Brown, 2020). Letrozole causes an

increase in androgen levels in the ovary (Rose and Brown, 2020), with characteristics similar to human PCOS, such as hyperandrogenism and aberrant follicles (Rose and Brown, 2020).

Measurement of sex hormone levels can reveal the presence of PCOS. The consistent parameters needed to diagnosis a woman with PCOS include elevated serum testosterone, LH concentrations, and low progesterone and FSH levels (Ndeingang *et al.,* 2019). Letrozole therapy increased insulin, testosterone, and LH levels significantly ($p<0.05$) in this study, but FSH, estradiol and progesterone levels declined significantly ($p<0.05$). This conclusion supports the findings of Ndeingang *et al.*, (2019), who found that increased serum LH and low progesterone suggest the presence of PCOS. Metformin and clomiphene citrate considerably reduced high levels of testosterone and progesterone, whereas letrozole at 100mg/kg B.W. caused a significant ($p<0.05$) decrease in testosterone and LH levels while significantly (p0.05) increasing progesterone and FSH levels.

High testosterone levels indicated a buildup of androgens, presumably as a result of the inhibition of androgen substrate conversion to estrogens (Handelsman, 2020). Because letrozole presumably increased LH secretion, the elevated serum LH concentrations could be related to a reduction in estrogen synthesis in the brain and pituitary (Amal *et al.,* 2019). PCOS is characterized by the absence of progesterone's effects on follicular development in the latter stages of follicular rupture. The extract's insulin-sensitizing characteristics are supported by a significant ($p<0.05$) increase in progesterone levels in letrozole-induced PCOS rats given 100mg/kg B.W. of the extract.

This could be because the extract works in a similar way to metformin, reversing LH and causing follicular rupture and the creation of the corpus luteum, which is responsible for progesterone synthesis. Gonadotrophin-releasing hormone (GnRh) is secreted by the hypothalamus, which stimulates the pituitary gland in the brain to release FSH and LH (Slater, 2012). FSH acts directly on the receptors on the granulosa cells to increase the growth and maturation of ovarian follicles (Kumar *et al.,* 2011).

The increase in FSH concentration in letrozole-induced PCOS rats treated with 100mg/kg body weight of the extract explains the increased FSH-gonadotrophin releasing hormone in the hypothalamus, which causes the anterior pituitary gland to generate FSH to drive

folliculogenesis and ovulation. The input here is in keeping with Pasqualin and Gambineri's reports (2006). Insulin sensitizers have also been shown to help in ovulation. One of the side effects of PCOS is an imbalanced lipid profile, which can lead to dyslipidemia. This study's findings are comparable to those of Amal *et al* (2019). Total cholesterol, triglyceride, and LDL levels increased significantly ($p<0.05$) in the letrozole-induced PCOS group, while HDL levels decreased significantly ($p<0.05$). These parameters were significantly ($p<0.05$) increased after oral administration of EEVAL.

Hyperandrogenemia is to blame for the variations in hormone levels and lipid profile. Excess androgen can have negative consequences in a variety of systems (Amal *et al*., 2019). Adipocyte androgen receptors, as well as testosterone, exert anti-lipolytic actions in abdominal subcutaneous pre-adipocytes, presumably via inhibiting catecholamine-induced lipolysis selectively (Amal *et al.*, 2019). Hyperlipidemia was also connected with an upward trend in body weight in PCOS untreated rats, which could be due to letrozole's anabolic effects, which are linked to fat storage.

The weight of the rats after treatment with metformin and clomiphene citrate was significantly ($p<0.05$) lower than the weight of the rats after induction with 1mg/kg B.W of letrozole; this finding agrees with Zhang *et al*., (2017), who found that the combination of metformin and clomiphene significantly reduced body weight. Metformin's insulin-sensitizing qualities have been found in studies to enhance menstrual periods and ovulation rates in women with PCOS. Metformin may also affect hyperandrogenism, metabolic changes, and, most critically, fertility (Pasquali and Gambineri, 2006). Flavonoids have been found to have anti-diabetic properties in animals (Saba *et al*., 2010). As a result, the presence of flavonoid in the extract could be to blame for the animals' oestrus cycle being normalized, as shown with metformin administration.

Hyperglycemia is also thought to be a key indication of PCOS. In this study, however, the level of glucose in the PCOS group before induction by letrozole was significantly ($p<0.05$) higher than in the normal control group. The glucose level of the PCOS group reduced significantly ($p<0.05$) after induction and treatment with EEVAL compared to the normal control group.

As a result, insulin levels in PCOS-untreated rats were found to be considerably higher than in the control group. As a result, there was no hyperglycemia or hyperinsulinemia, which could be

due to the glucose molecules attaching to insulin receptors being good and effective. This shows that insulin resistance is not present.

5.2 Conclusion

In this study, it is confirmed that administration of letrozole induced PCOS in female Wistar rats which is associated with reproductive and metabolic dysfunctions. The 100mg/kg body weight dose of EEVAL showed an ameliorative effect on the reproductive and metabolic functions which compared favourably with the metformin and clomiphene citrate (standard drugs). Therefore, this plant is suggested to have therapeutic effect in the management of PCOS.

5.3 Recommendation

Further work should be done on the phytocompounds of *V. amygdalina* leaves via in-silico technique for pharmacological study.

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APPENDIX

2M 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 that is 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

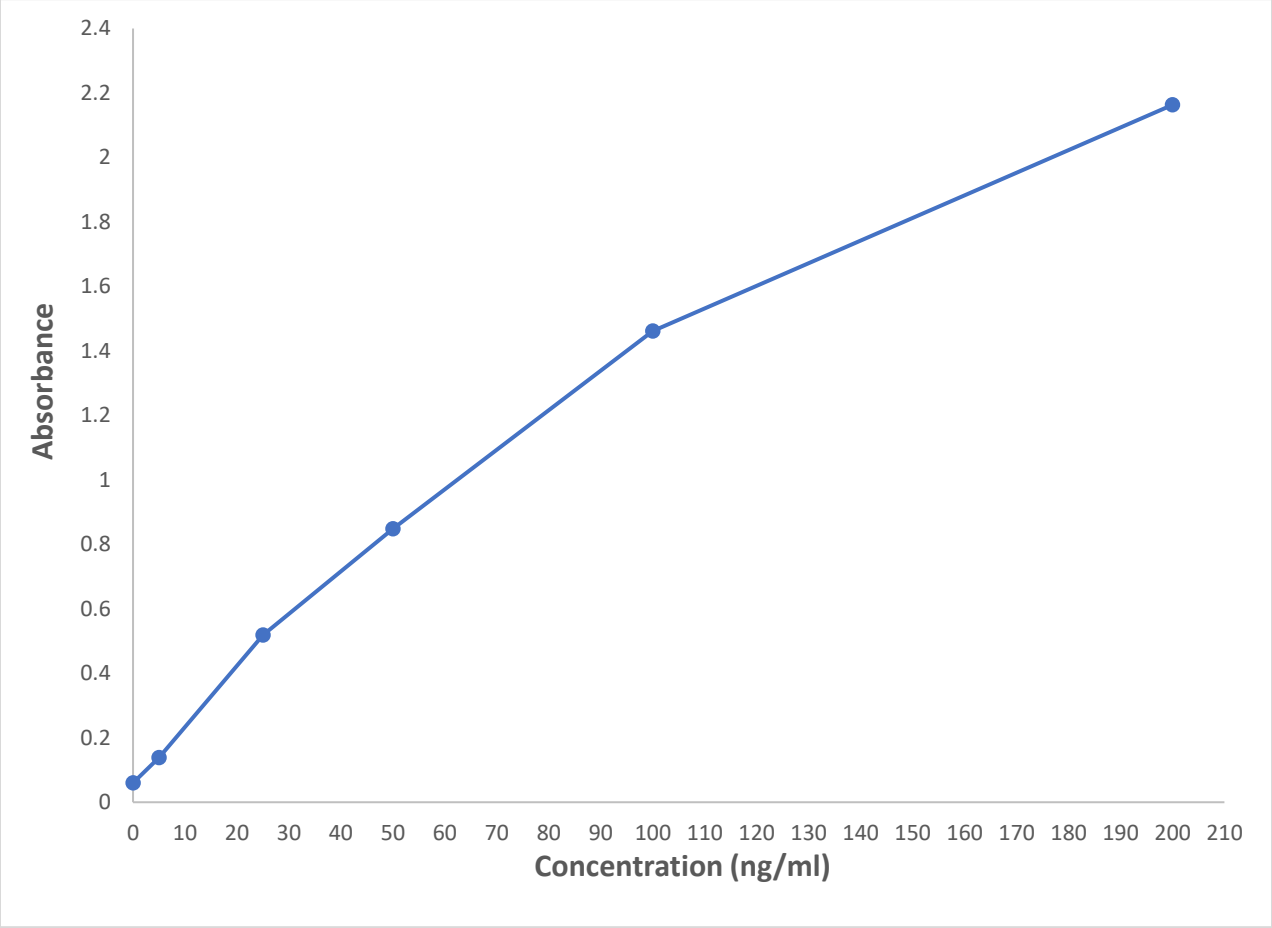

Figure 22. *Calibration curve of LH concentration (ng/ml)*

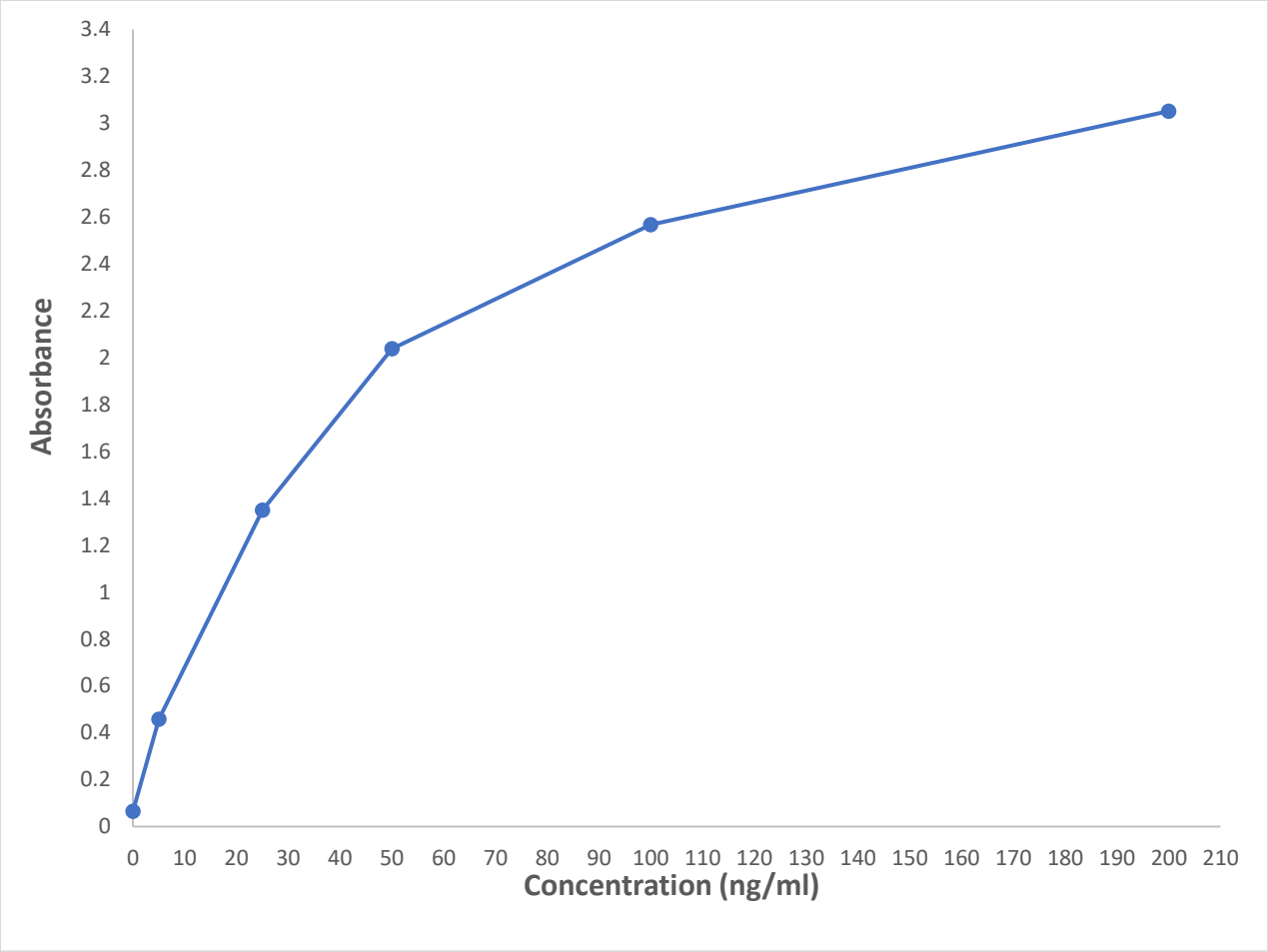

Figure 23. *Calibration curve of Insulin concentration (ng/ml)*

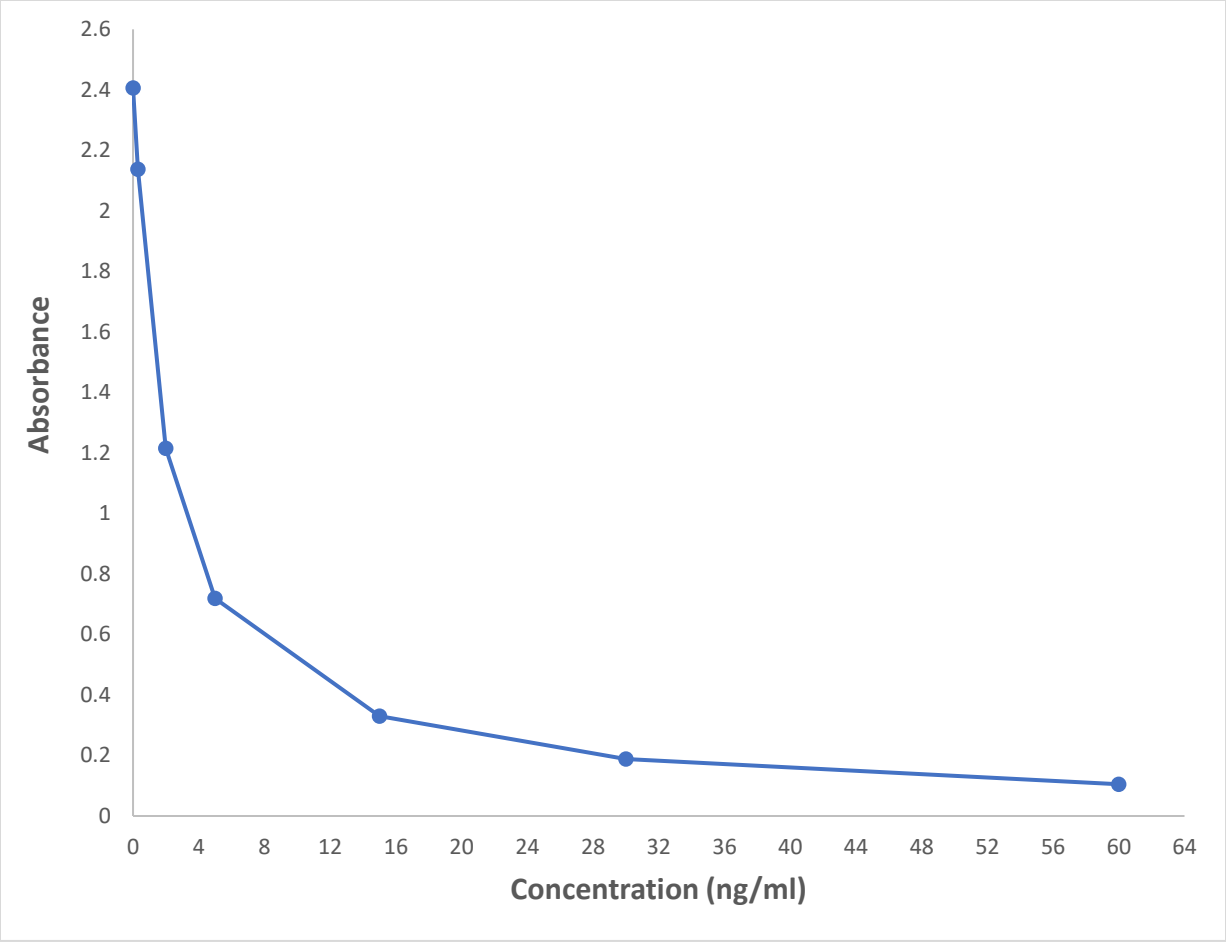

Figure 24. *Calibration curve of Progesterone concentration (ng/ml)*

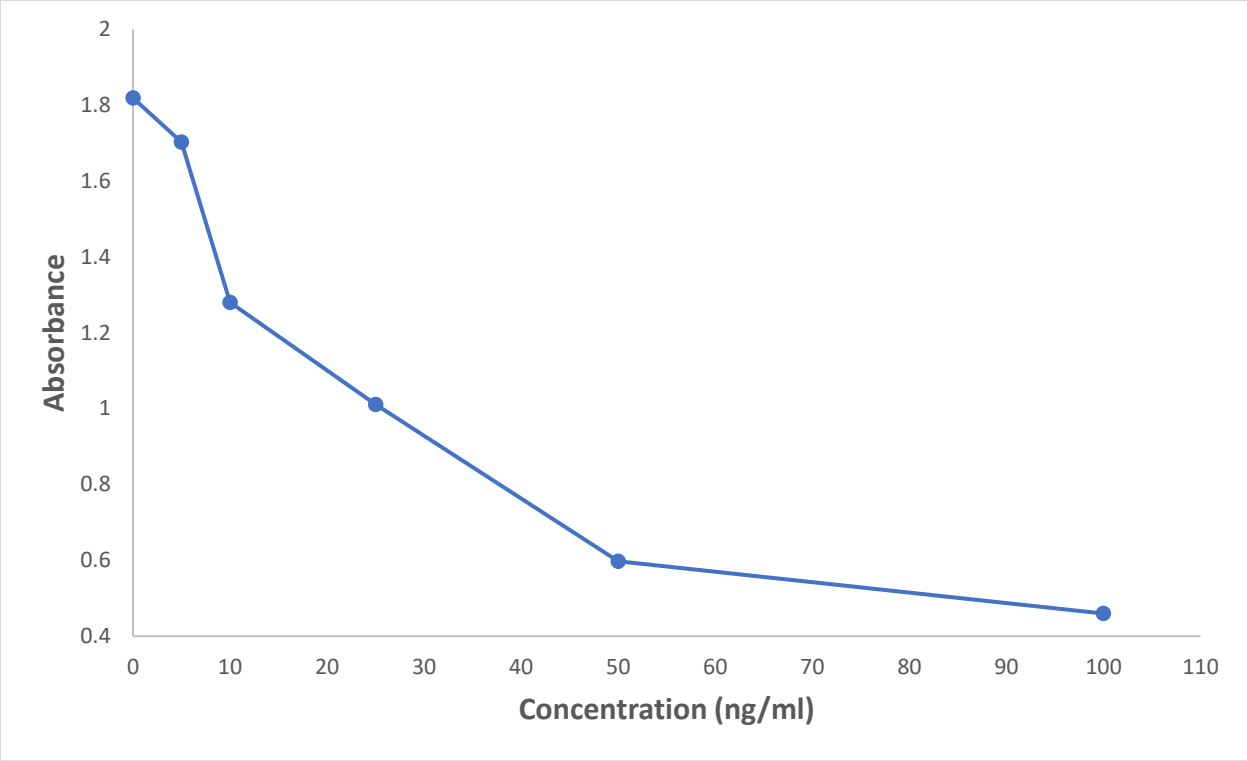

Figure 25. *Calibration curve of FSH concentration (ng/ml).*

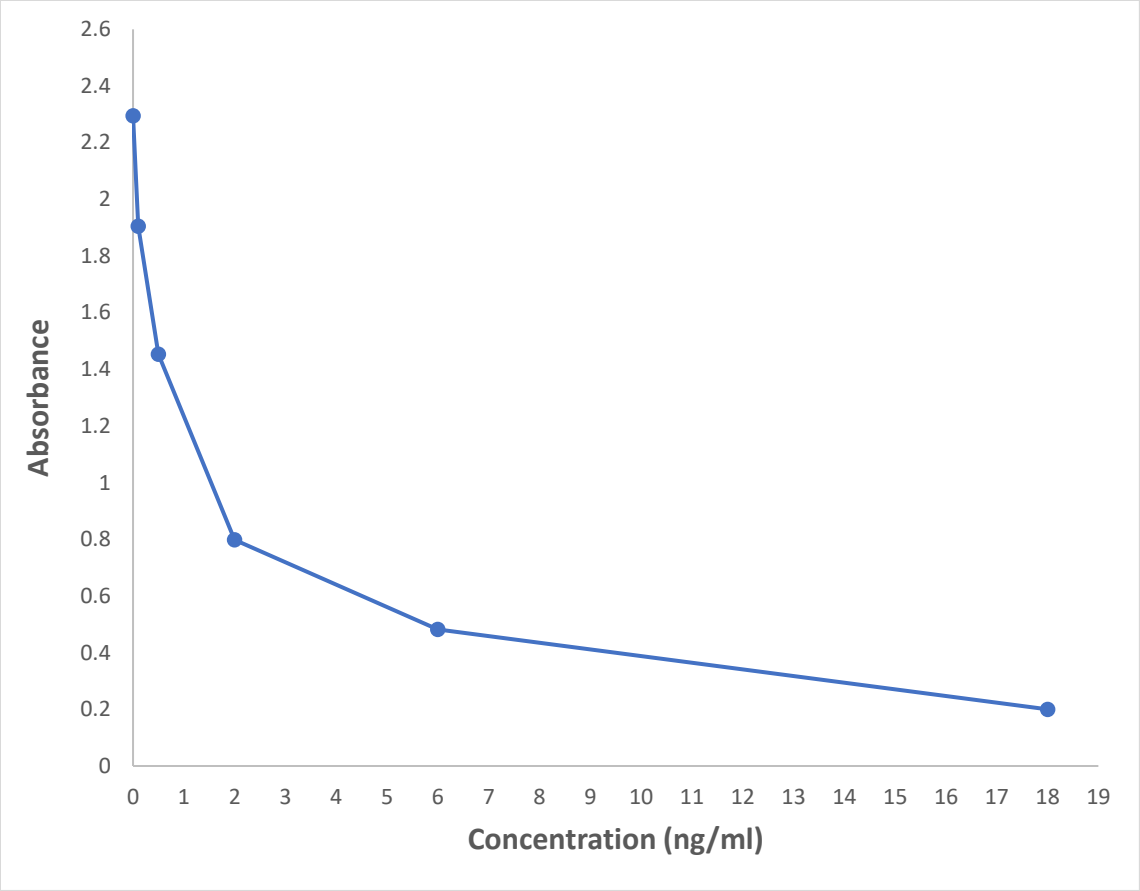

Figure 26. *Calibration of Testosterone concentration (ng/ml)*

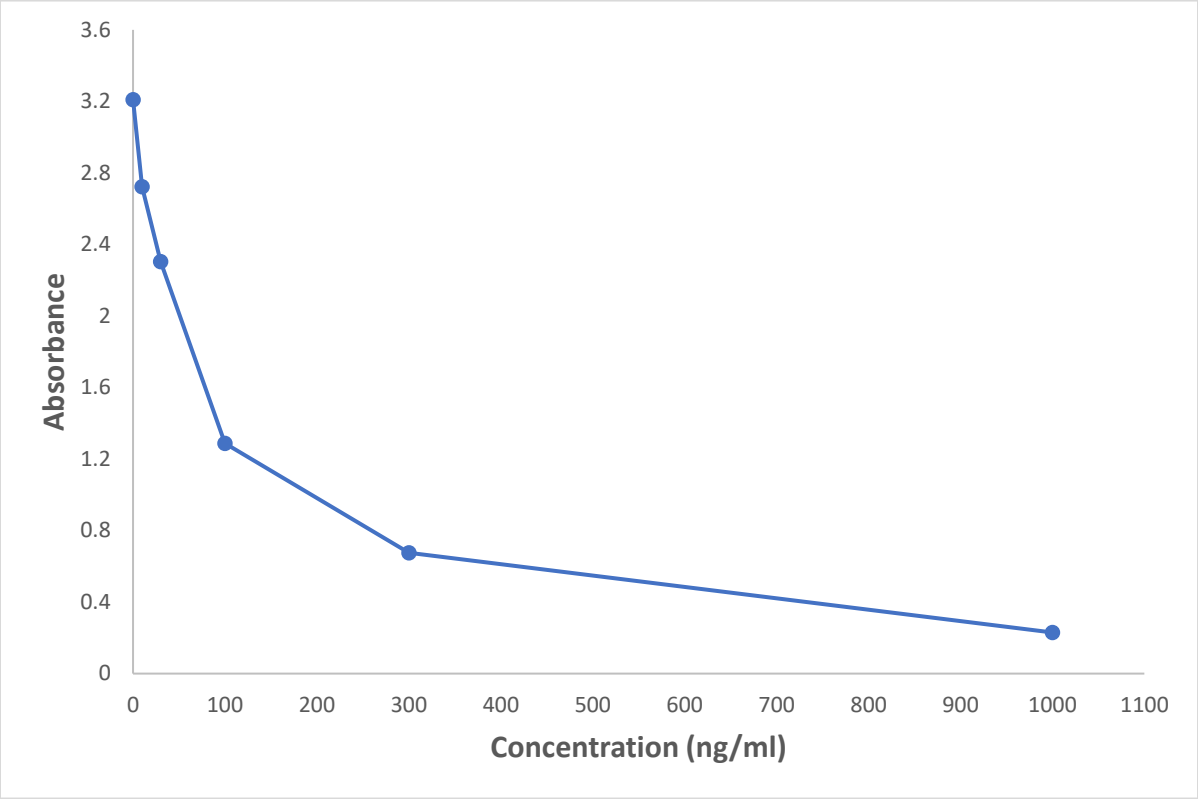

Figure 27. *Calibration curve of Estradiol concentration (ng/ml)*