CHAPTER ONE

1.0 Introduction

Polycystic ovary syndrome (PCOS) is an endocrine condition that affects women during their reproductive years (Smith, 2018). It is a disorder that affects a woman's hormone levels and causes women to generate more male hormones than they should (Sullivan, 2019). This hormonal imbalance causes them to miss menstrual cycle making it difficult for them to conceive (Sullivan, 2019).

Certain medicinal plants such as Aloe Vera, flax seed, and hazel nut may play a crucial role in treating PCOS (Ashkar *et al.,* 2020). Because of the contribution of various useful phytoconstituents present in different plant sections, most medicinal plants are unique in their ability to treat and cure various human ailments such as PCOS, Diabetes and other ailments (Konappa *et al.,* 2020). Aloe vera, chamomile, and cinnamon have compounds that can affect lipid profiles (Aloe vera, chamomile, Aloe vera, and Camellia sinensis), insulin resistance (cinnamon, chamomile, Aloe vera, and Camellia sinensis), blood glucose (Aloe vera, cinnamon, and Camellia sinensis), and hormones (Aloe vera, silymarin, Chamomile, Fenugreek, Camellia sinensis, Heracleum persicum, Potentilla, Menta spicata, Foeniculum vulgar, licorice and Marrubium) and ovarian tissues (Aloe vera, Chamomile, Camellia sinensis, Mentha spicata and silymarin) (Ashkar *et al.*, 2020).

Plants play an important role in disease prevention and recovery such as PCOS, and they can also help to avoid and mitigate the side effects of traditional therapies. They can be a source of biologically and pharmacologically important chemical compounds (Casuga *et al.,* 2016). Herbal medicine are used in the prophylactic treatment and alleviation of diseases and their effects as complementary or alternative medicines (Danladi *et al.*, 2018).

Vernonia amygdalina also known as bitter leaf which has a bitter taste is a plant that is widely used in Nigeria. Alkaloids, saponins, glycosides and tannins are the antinutritional constituents of *Vernonia amygdalina* which gives its bitter taste (Kadir, 2020). The *Vernonia amygdalina* has been shown to boost sperm parameters, however no studies have been done on its impact on immunosuppression-related infertility (Kadir, 2020). The determination of analyte contained in medicinal plants has been reported using a variety of instruments and techniques, including GC-MS, FT-IR, and NMR (Ruthiran *et*

al., 2017). GC-MS is used to identify the abundant analyte found in *Vernonia amygdalina* in the therapy and management of PCOS in this study.

PCOS has been reported as a prevalent disease amongst women in Africa and in Nigeria specifically. Whilst so many synthetic therapeutic options are used in the management of the disease, studies have shown that they have toxic side-effect and that they sometimes seem not to perform their physiological function. Hence, studies exploring the treatment and management of PCOS using natural phyto-constituent should be encouraged and may be effective in the amelioration of the disease through an effective drug-delivery system. Due to the exhaustive search of the author, no study on this had been conducted using *Vernonia amygdalina.* Thereby, this study aims to evaluate the toxicology effect of *Vernonia amygdalina* in the effective treatment of the PCOS disease.

1.1 Statement of the problem

The study was carried out to evaluate the safe dose of the ethanolic extract of *Vernonia amygdalina* leaves that will be used for the treatment of PCOS

1.2 Justification of the study

Ethnobotanical survey has had claim to the therapeutic potential of *Veronina amygdalina* leaves in the treatment of PCOS, therefore, it is imperative to scientifically evaluate its safety.

1.3 Aim of the study

The aim of this study is to evaluate the bioactive components of *Vernonia amygdalina* leaves and discover a dosage of the solvent extract that can revert letrozole induced polycystic ovarian syndrome female rats.

1.4 Objectives of the study

1. To induce polycystic ovarian syndrome in female wistar rats.
2. To determine the phytochemical constituents present in *Vernonia amygdalina* leaves using phytochemical screening, UV spectroscopy and GC-MS analysis
3. To evaluate the toxicological effect of *Vernonia amygdalina* as it relates to enzyme assay, kidney function indices, liver function indices and organ body weight ratio

4. To determine other biochemical effects on the ethanolic extract of Vernonia amygdalina leaves on the extract on the selected rat tissues as it relates to kidney function indices, enzyme assay, liver function indices and organ body weight ratio

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Historical perspective of polycystic ovary syndrome

Stein and Leventhal first identified PCOS as a condition characterized by oligo-amenorrhea and polycystic ovaries, as well as hirsutism, acne, and obesity, among other symptoms (Rosenfield and Ehrmann, 2016). Beginning in 1958, seminal contributions to our understanding of PCOS pathogenesis were made indicating that urinary leuteninzing hormone was enhanced by bioassay in 4 cases studied (Rosenfield and Ehrmann, 2016).

Vallisneri, an Italian medical scientist, physician, and naturalist, described a married, infertile woman with gleaming ovaries with a white surface and ovaries the size of pigeon eggs in 1721 (Szydlarska *et al.,* 2017).

In the 1980s, it was discovered that giving testosterone to female-to-male transsexuals caused polycystic ovaries, prompting the creation of ultrasonographic criteria for identifying polycystic ovarian morphology (PCOM) (Rosenfield and Ehrmann, 2016).

In 2012, the NIH's (USA) Expert Panel recommended a series of activities aimed at advancing understanding of polycystic ovary syndrome (PCOS) diagnosis and management, including the creation of basic biological and clinical markers based on a phenotype-biological approach, as well as the urgent need to extend the use of precise and reliable techniques to quantify circulating androgens (Pasquali *et al.,* 2016).

Many researchers have attempted to understand the pathophysiology of PCOS, and numerous studies have been conducted (Rocha *et al.,* 2019). Although it is now well acknowledged that it is complex and partially hereditary, a number of potential genes have been proposed (Rocha *et al.,* 2019). Insulin resistance has been observed in many women with PCOS, particularly those with Hyperandrogenism (Rocha *et al.,* 2019).

2.2 Polycystic ovarian syndrome

Polycystic ovary syndrome is an endocrine system disorder which is also known as stein-leventhal syndrome (Smith, 2018). PCOS is a female endocrine condition marked by high androgen levels, ovulatory dysfunction, and polycystic ovarian morphology, and also a set of classic clinical features such as obesity, hirsutism, alopecia, acne, irregular menses, infertility, and high blood pressure (Schneider *et al.,* 2019). Patients with PCOS are twice than likely as the general population to develop metabolic syndrome, and they are four

times more likely than the general population to develop type 2 diabetes mellitus (Williams, 2016).

The signs and symptoms appears about the time of first puberty's first menstrual cycle but can be evolve later in life as a result of weight gain (Longdom, 2020). Insulin resistance and hyperinsulinemia are the key physiopathological causes of this disorder, and inositol-mediated signaling failure can play a role in them (Laganal *et al.,* 2016).

Experiments have shown that PCOS is linked to low-grade chronic inflammation and that women with it are more likely to develop non-alcoholic fatty liver disease (Rocha and Reis, 2018). The hypothalamic pituitary ovarian axis is disrupted in PCOS, a condition characterized by signs and symptoms of excess androgen and ovulatory dysfunction (Witcher, 2019).

PCOS exact cause is uncertain and common in all ethnic group with different prevalence which depends on either body weight, lifestyle, food intake or family background. Obesity is a significant concern to women with PCOS especially in adults (Moghadam *et al.,* 2018). Women with this disorder usually have ovaries that contains very small cysts which is caused by hormonal imbalance leading to its symptoms (Kashani and Akhondzadeh, 2016). Hyperandrogenism may also be caused by a local inflammatory response of ovarian theca cells to reactive oxygen species or cytokines and chemokines produced by dysfunctional adipose tissue, according to recent research (Lagana *et al.*, 2016).

It can induce anovulatory infertility during the fertile period and be linked to an increased risk of gestational complications including miscarriage, gestational diabetes, and preeclampsia (Leo *et al.,* 2016).

Metformin, when combined with dietary and exercise improvements, increases weight loss, reduces blood sugar, and restores a regular menstrual cycle better than diet and exercise alone (Watson, 2021).

Metformin is a drug used in treating polycystic ovary syndrome as well as treating insulin resistant linked with PCOS (Kashani, 2016).Metformin can also aid in the regulation of the menstrual cycle in some women (Waldman, 2021). Metformin (Glucophage, Fortamet) is a type 2 diabetes medication which also aids in the treatment of PCOS by lowering insulin levels (Watson, 2021).

Clomiphene citrate (CC) is an estrogen receptor modulator that is non-steroidal (SERMs). SERMs' pharmacological target is to achieve beneficial estrogenic action while not having antagonistic activity in other tissues, such as the endometrium, where estrogenic activities (e.g., carcinogenesis) may be harmful. Clomiphene citrate (CC) is recommended for the treatment of anovulatory women's infertility (Al-shaikh et al., 2016). Clomiphene (Clomid) is a fertility medication that can help PCOS-affected women conceive (Watson, 2021).

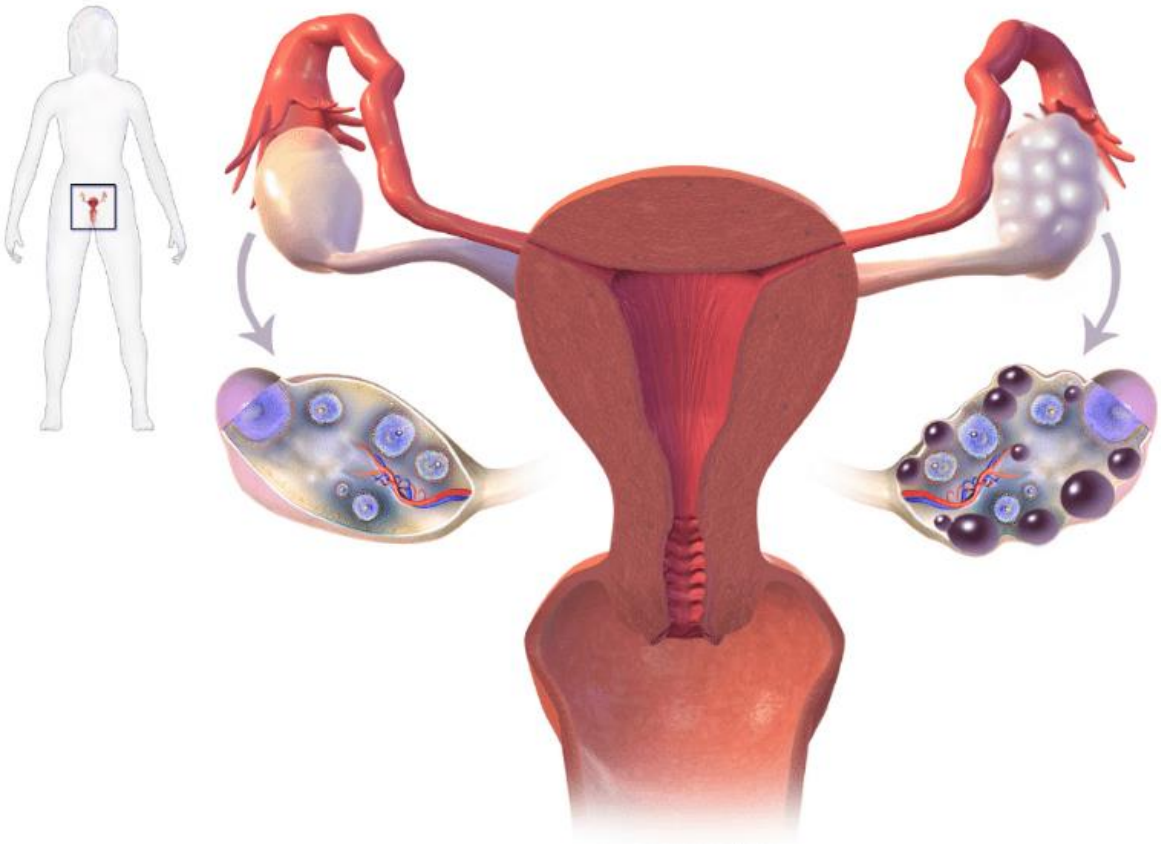

Figure 1: Schematic representation of polycystic ovary syndrome

Source: Wang (2018).

2.2.1 Ovarian Dysfunction

Ovarian Dysfunction occurs before the age of 40, the ovaries stop working and menstrual cycles stop which leads to fertility issues as well as menopause symptoms. Primary and secondary ovarian failure are the two forms of ovarian failure (Johnson, 2020). Chromosome mutations or damage from chemotherapy or radiation therapy are common causes. It is also linked to a fragile X premutation in the FMR1 gene (Mayo, 2016).

1) **Primary ovarian failure**: In women under the age of 40, primary ovarian failure (POF) is characterized by amenorrhea, hypoestrogenism, and elevated gonadotropin levels, resulting in infertility. Primary ovarian failure is a complex disorder with a variety of causes, and many genes have been linked to the phenotype (Franca *et al.*, 2017).

The following are some of the causes of primary ovarian insufficiency:

I) Insufficient ovarian follicles present at birth.

II) When the ovaries are damaged during surgery, chemotherapy, or radiation therapy, the rate of follicular atresia increases.

III) The follicles aren't working properly (as occurs in autoimmune ovarian dysfunction).

IV) There are several genetic abnormalities present (Pinkerton, 2020).

2) **Secondary ovarian failure:** This is a condition in which the ovaries quit functioning and menstrual cycles halt before age 40 (Vincent, 2016). If the hypothalamus and pituitary fail to deliver adequate gonadotropin stimulation, ovarian insufficiency is termed secondary (Vincent, 2016). Some medicines, excess exercises, wrong eating habits can also cause secondary ovarian failure which will be unable for the woman to conceive (Gleicher *et al.,* 2020). This can induce reproductive issues and symptoms of menopause. The ovaries are normal in secondary ovarian failure, but there is a problem receiving hormone messages from the brain to them (Vincent, 2016). The cause of secondary ovarian failure is the physiological lifestyle and variables such as excersise, low weight, affective and eating disorders.

2.2.2 Metabolic disturbance of PCOS

The metabolic syndrome is made up of two concepts: Cardiovascular and endocrinological causes with a focus on insulin resistance and its consequences (Chandrasekaran, 2018). PCOS phenotype appears to vary widely and is most frequently seen in the post-pubertal era. Women with PCOS have polycystic ovaries, chronic anovulation, hyperandrogenism and gonadotropin defects despite a wide range of phenotypes. In addition to the inherent characteristics of PCOS, metabolic and hormonal disorders associated with obesity, type 2 diabetes mellitus, and dyslipidemia are usual. Metabolic syndrome is a combination of these factors (Visser, 2021). Central obesity, hypertension, insulin resistance, and atherogenic dyslipidemia are all symptoms of the metabolic syndrome (Chandrasekaran, 2018).

2.2.2.1. Insulin resistance and PCOS

Insulin resistance is characterized as a decreased glucose response to a given amount of insulin, and it is most commonly caused by problems with the insulin receptor and post-receptor signaling (Morley and Tang, 2017). Women with PCOS are more insulin resistant than women without the condition who are of similar weight. Insulin resistance is seen in 10-15% of slim PCOS women and 20-40% of obese PCOS women, and women with PCOS have a higher risk of developing type 2 diabetes (Morley and Tang, 2017).

The pathogenesis of PCOS is complicated by insulin resistance and the hyperinsulinemia that results from it. Insulin controls metabolic and mitogenic pathways that are separate from one another (Chandrasekaran, 2018). In all ethnic groups, there is a clear connection between increased insulin resistance and PCOS (Vignesh and Mohan, 2018).

Insulin resistant has been related to PCOS and hypernandrogenism, and has been confirmed to be higher in obese PCOS women than non-obese PCOS women, along with other hormonal abnormalities (Tehrani *et al.,* 2016). However, the cause and effect relationship has not been established (Tehrani *et al.,* 2016). Both slim and obese women with PCOS are found to be more insulin resistant than non-affected weight-matched controls, despite the fact that it can be found in up to 50% of women with PCOS (Tehrani *et al.*, 2016).

The enzyme cytochrome P450c 17-α in both the ovaries and the adrenal gland is stimulated by high levels of insulin in the blood to produce more male hormones (Al-

shaikh *et al.,* 2016). Insulin resistance is commonly caused by obesity. Obesity and insulin resistance both increase your chances of developing type 2 diabetes (Watson, 2021).

Insulin has two essential functions that contribute to hyperandrogenism in PCOS (Chelakkadan *et al.*, 2018).

1) Serum sex hormone binding globulin production is inhibited in the liver (SHBG).

2) IGFBP-1 production in the liver is inhibited, allowing for higher levels of IGF-1 and more local activity (Chelakkadan *et al.*, 2018).

2.2.2.2. Obesity and PCOS

Obesity is frequent in women with PCOS, with 40–80% of women suffering from the illness being overweight or obese. PCOS aggregation in families strongly suggests a genetic predisposition to the condition (Barber *et al.,* 2019).

Obesity exacerbates PCOS, which is a genetic condition. Based on epidemiological data and more recently corroborated by genetic studies, there is a strong link between obesity and PCOS (Barber *et al.,* 2019). The effects of weight gain and obesity on the development of PCOS are mediated by a number of mechanisms (Barber *et al.,* 2019). Obese PCOS women have greater total and free T levels than non-obese PCOS women, according to previous research (Gabineri *et al.,* 2002).

Obesity is linked to PCOS (Barber *et al.,* 2019). Excess adiposity has received a lot of attention because obesity, particularly in the abdominal region, is present in 50% of women with PCOS, and because obesity develops in mid-childhood and increases during puberty (Vilmann *et al.,* 2012). Obesity is also thought to exacerbate the clinical phenomenology and the development of PCOS particularly among people who are genetically predisposed to it (Vilmann *et al.,* 2012).

2.2.2.3 Type 2 diabetes and PCOS

The metabolic condition type 2 diabetes mellitus (T2DM) is characterized by chronic hyperglycemia. Due to a higher risk of heart disease, stroke, peripheral neuropathy, renal failure, blindness, and amputation, it is linked to a shorter life expectancy (Shaha and Vella, 2014).

Insulin deficiency is a symptom of type 2 diabetes, which is characterized by pancreatic β-cell failure and insulin resistance in target organs (Chatterjee *et al.*, 2017). Insulin resistance is common in women with PCOS; their bodies can produce insulin but not efficiently utilize it, increasing their risk of type 2 diabetes (Forslund *et al.*, 2020). Several investigations, however, have suggested that insulin resistance plays a key role in the syndrome's etiology (Pelusi and Pasquali, 2004). Women with PCOS frequently have aberrant glucose metabolism and lipid profiles as a result of insulin resistance, and have a higher risk of type 2 diabetes and cardiovascular disease over time (Pelusi and Pasquali, 2004). Type 2 diabetes is usually prevented or controlled with exercise and a healthy diet, research has shown that PCOS is a major independent risk factor for diabetes (Maria, 2019).

2.2.2.4 Dyslipidemia and PCOS

Dyslipidemia is defined as a condition in which the levels of low-density lipoprotein or triglycerides are abnormally high and also a conition in which your blood contains too much of one or more types of lipid (FAT) (Yuan et al., 2021). Lipids are found in three different forms in the blood which are high density lipoprotein (HDL), Low density lipoprotein and triglycerides. Dyslipidemia is defined as a condition in which the levels of low-density lipoprotein or triglycerides are abnormally high (Yuan *et al.*, 2021). In women with polycystic ovary syndrome, dyslipidemia is a prevalent metabolic condition in PCOS (Kim and Choi, 2013). Because insulin resistance is a fundamental pathophysiology of PCOS, dyslipidemia in women with PCOS may resemble that of insulin-resistant women (Kim and Choi, 2013). Patients with severe dyslipidemia, lipid-lowering medications, notably nicotinic acid and fibrates, should be utilized as first-line therapy for ALP in PCOS (Rizzo *et al.*, 2008).

In PCOS individuals, annovulation has been linked to dyslipidemia, women with anovulatory PCOS have higher TC, TGs, and LDL-C levels and lower HDL-C levels than women with ovulatory PCOS, according to a research and also in women of reproductive age, obesity can cause dyslipidemia, irregular ovarian cycles, and anovulation (Liu *et al.*, 2019).

2.2.2.5 Hypertension and PCOS

Hypertension is referred to as high blood pressure (Salman *et al.,* 2015). Hypertension is a major factor in the development of cardiovascular disease. Women with PCOS have a higher prevalence of hypertension, which may contribute to their increased risk of cardiovascular disease (Bentley-lewis, 2011). Other background variables of the individual, such as race and ethnicity, may influence hypertension in women with PCOS (Antipolis, 2020).

PCOS women are more likely to be overweight or obese, diabetic, or have high blood pressure, all of which are risk factors for heart disease and stroke (Reckelhoff, 2007).

Despite the long list of symptoms that commonly accompany PCOS, the actual mechanism(s) that cause hypertension in PCOS women are unknown. Many of the symptoms associated with PCOS, such as increased body mass index and the prevalence of metabolic syndrome, which includes insulin resistance and type 2 diabetes, have been linked to elevated blood pressure (Reckelhoff, 2007). Living a healthy lifestyle is the first step in preventing high blood pressure (Grassi, 2019). Maintaining your weight, eating a nutritious diet, and exercising regularly are all things you can do right now to lower your risk of developing high blood pressure (Grassi, 2019).

2.3 Diagnostic criteria of PCOS

The Rotterdam criteria have been used to diagnose PCOS since 2004, and they include the existence of two of the three criteria: oligo-/anovulation, hyperandrogenism, or polycystic ovaries (Lauristen and Laeger, 2019).

2.3.1 Anovulation/Oligoovulation

Anovulation is when an egg does not develop and a woman does not ovulate in some menstrual cycles which may result in irregular periods or no periods at all. When a woman experiences anovulation, she is unable to conceive (Berry, 2017). It is one of the most common causes of infertility. An imbalance of the hormones that cause a woman to ovulate can cause anovulation, which can be a symptom of PCOS (Hernandez-rey, 2018).

Oligoovulation occurs when ovulation occurs infrequently or irregularly, and is typically described as eight or fewer periods per year (Waldman, 2021). Oligoovulation is a condition in which women have infrequent ovulation and their eggs are released infrequently. Women with oligoovulation have a hard time determining their fertile

cycles. Compared to anovulatory women, oligoovulatory women may have milder symptoms and a less severe phenotype (Martin, 2016).

2.3.2. Hyperandrogenism

Hyperandrogenism is a medical disorder in which females have elevated levels of androgens (Chappell and Schutt, 2018). Women with PCOS are characterized by hyperandrogenism. The development of excess androgens is induced by the disturbance of normal ovarian or adrenal function. Impaired folliculogenesis is the first effect of androgen excess in PCOS (Ashraf *et al.,* 2019).

Hyperandrogenism may also be caused by a local inflammatory response of ovarian theca cells to reactive oxygen species or cytokines and chemokines produced by dysfunctional adipose tissue (Lagana *et al.,* 2016).

2.3.3. Polycystic ovaries

An ovary with 12 or more follicles measuring 2 to 9 mm in diameter (or 25 or more follicles using new ultrasound technology) or a volume greater than 10 mL on ultrasonography is considered polycystic. A single ovary that meets one or both of these criteria is enough to diagnose polycystic ovaries (Williams *et al.,* 2016). Ultrasound is rarely used to confirm a diagnosis of PCOS when polycystic-appearing ovaries are present (Havelock, 2018).

2.4. Prevalence of polycystic ovary syndrome

The prevalence of polycystic ovarian syndrome is estimated to be between 3% and 10%, but it varies depending on geography, race, and ethnicity (Wolf *et al.,* 2018). Because of the inconsistencies and variations in PCOS diagnostic criteria, determining the prevalence of PCOS is difficult. Polycystic ovarian syndrome affects about 10 million people worldwide, according to the PCOS Knowledge association (Wolf *et al.,* 2018). PCOS is a disease characterized by internal ovarian abnormalities. 8-15 percent of women of reproductive age are severely afflicted (Nair and balakrishnan, 2018). According to a study by Futterweit (2017), 50–75% of women with PCOS are unaware that they have the condition (Wolf *et al.,* 2018). PCOS is one of the most common disorders in women of childbearing age, with an estimated prevalence of 8–13% and significant metabolic and psychological health consequences (Mani *et al.,* 2019).

2.5. Symptoms of Polycystic ovary syndrome

About the time of their first period, some women experience symptoms. Others don't realize they have PCOS until they've gained a lot of weight or struggled to conceive (Watson, 2021).

Symptoms includes:

I) **Irregular periods:** PCOS occurs when the ovaries or adrenal glands overproduce "male" hormones (androgens) and the body has insulin resistance (Levine, 2020).The uterine lining does not shed every month due to a lack of ovulation. Some women with PCOS have less than eight or no cycles a year (Watson, 2021). According to studies, PCOS affects 87% of women who have irregular menstrual cycles (Levine, 2020).

II) **Heavy bleeding**: Since the uterine lining develops over a longer period of time, the cycles can be heavier than normal (Watson, 2021). PCOS can also induce heavy, fast-flowing periods, and sometimes, they come with huge blood clots. As the uterus sheds its lining, the anticoagulants that the body generates to protect blood from clotting cannot keep up with the high pace of bleeding, therefore blood clots are passed during menstruation (Makati, 2020).

III) **Excess hair growth:** This is caused by high levels of insulin which increases the production of male hormones called androgens and high levels of androgens can lead to excess hair growth (Pagano, 2020). Hair thinning on the scalp and top of the head in the "male-pattern" affects some women. High androgen levels, which stimulate hair follicles, are thought to be the cause of these hair changes (Harrar, 2018).

IV) **Obesity:** It is caused by excess Insulin or inability of the Insulin to function well (Pagano, 2020). PCOS and obesity have a delicate relationship. For some women, signs and symptoms of polycystic ovarian syndrome appear soon after they start having periods (William, 2011). Women with PCOS either create too much insulin or their insulin does not act properly. One of the reasons why women with PCOS gain weight or have a hard time reducing weight is the inability of insulin to operate correctly (William, 2011).

V) **Dyslipidemia:** Lipoprotein metabolism disorders, such as lipoprotein overproduction and deficiency, are known as dyslipidemia (Soni *et al.,* 2018). Changes in the PCOS oocyte environment are influenced by lipid metabolism. In PCOS follicular fluids, increased levels of glycerol, lipid region, cholesterol, and a slightly higher level of low-

density lipoprotein were found (Soni *et al.,* 2017). In women with PCOS, dyslipidemia is a prevalent metabolic condition. Because insulin resistance is a fundamental pathophysiology of PCOS, dyslipidemia in women with PCOS may resemble that of insulin-resistant women (Kim and Choi, 2013).

2.6 Etiology of polycystic ovary syndrome

The etiology of PCOS is unknown, and it is considered a complex genetic trait with a high degree of heterogeneity (Cong *et al.,* 2020). In adolescence, the first clinical signs of PCOS appear. However, there is evidence that the disease began in the uterus, implying that genetic factors are involved (Andrade *et al.,* 2016).

The precise cause of PCOS is unknown to doctors. They found that high levels of male hormones inhibit the ovaries' ability to produce hormones and produce eggs normally. Excess androgen development has been related to genes, insulin resistance, and inflammation in the past (Watson, 2021).

The combination of genetic and epigenetic modifications, primary ovarian defects, neuroendocrine alterations, and endocrine and metabolic modifiers such as anti-müllerian hormone, hyperinsulinemia, insulin resistance, adiposity, and adiponectin level contributes to the dynamic pathophysiology of PCOS (Oberfield *et al.,* 2017). Many women with PCOS have female relatives who also have the condition, even though they have never been diagnosed. As with type 2 diabetes, multiple genes are likely to play a minor role in PCOS etiology, and recent genome-wide association studies have identified candidate genes (Dennett and Simon, 2016).

2.7 Management options of polycystic ovary syndrome

Due to varying aims based on symptom presentation and changes at various life phases, PCOS management differs (Manisha *et al.,* 2020). Diet and exercise are critical factors in the treatment of PCOS and obesity. Lifestyle changes such as diet and exercise are the first-line treatment for PCOS fertility and metabolic syndrome, followed by pharmacological therapy (Lua and King, 2018).

To help decrease the effects of PCOS, try to:

1) **Manage a healthy body weight:** Weight loss can help restore ovulation by lowering insulin and testosterone levels. Consult your doctor about a weight-loss program, and visit with a dietician on a regular basis for assistance in achieving your weight-loss objectives (Mayo, 2020). Eating healthily and exercising consistently are two of the most effective strategies to deal with PCOS. PCOS affects a large number of women who are overweight or obese. Losing just 5% to 10% of your body weight will help to alleviate some symptoms and make your periods more regular. It may also aid in the management of blood sugar levels and ovulation issues (Nazario, 2020).

II) **Carbohydrate intake should be limited:** Insulin levels may be raised by low-fat, high-carbohydrate diets. If you have PCOS, talk to your doctor about a low-carbohydrate diet. Choose complex carbohydrates, which take longer to elevate your blood sugar levels (Watson, 2019).

III) **Do not take coffee:** Changes in estrogen levels and hormone behavior may be connected to caffeine use. A decaf alternative, such as herbal tea, can help you feel more energized. If you can't live without your caffeine fix, switch to green tea. Green tea has been demonstrated to help those with insulin resistance (Watson, 2019).

2.8. Medicinal plants in the treatment of polycystic ovary syndrome

2.8.1 *Aloe vera*: *Aloe vera,* commonly known as the "Natural healer", has a number of health benefits when consumed. *Aloe vera (L.). A. barbadensis Mill*. is one of nature's oldest recognized medicinal plants, and is often referred to as a miracle plant or natural healer. It is a member of the liliaceae family, which contains around 250 species, but only two of them, *A. barbadensis Mill.* and *A. arborescens Mill*. are considered the most significant (Katte *et al.*, 2018). The use of Aloe gel enhanced reproductive efficiency by modulating the luteinizing hormone receptor, androgen receptor, aromatase, and steroidogenic acute regulatory which can change the ovarian placental steroid status (Nair and Balakrishnan, 2018). Drinking aloe juice aids in the removal of toxins which are contained in our bowels and the cleansing of the digestive tract, which is crucial if one have PCOS (Bennett, 2019). A study was conducted by Marhanjan *et al.* (2010) on the effect of *Aloe barbadensis Mill.* Formulation on letrozole induced PCOS rat model which

exhibited the efficacy of Aloe vera gel formulation as a possible therapeutic agent in the prevention and management of PCOS (Marhanjan *et al.* 2010).

Figure 2: ***Aloe Vera***

Source: Almanac (2021).

2.8.2 *Linum usitatissimum*: The botanical name of flax seed is *Linum usitatissimum* which belong to the family linaceae. *Linum usitatissimum* supplementation has the ability to reduce the amount of follicles and ovarian volume in polycystic ovaries, enhanced menstrual cycles, and has no effect on body weight, blood sugar, or hirsutism (Nair and Balakrishnan, 2018). As a result, it should be investigated further as a potential new drug source for PCOS (Nair and Balakrishnan, 2018).

A study was conducted by Jelodar *et al.* (2018) on the effects of hydro alcoholic of flaxseed which shows that flaxseed increases the level of progesterone and decreases testosterone (Jelodar *et al.*, 2018). *Linum usitatissimum* is an herbal medicine which reduces the body weight, insulin concentration in patients with polycystic ovary syndrome (Haidari *et al.*, 2020). The most significant lignan present is secoisolariciresinol diglucoside (SDG), which is found in *Linum usitatissimum*. Excess testosterone appears to be reduced by lignans, which is thought to have a role in PCOS pathogenesis (Farzana *et al.*, 2015).

Figure 3: ***Linum usitatissimum*** (Mahesh *et al.*, 2020)

2.8.3 *Corylus avellana:* *Corylus avellana* conists of sitosterols, squalene, campesterol, and stigmasterol and tocopherol. Due its ability to control gonadotropins, hormones, serum lipid parameters, and antioxidant properties, it has been found to be effective in PCOS (Nair and Balakrishnan, 2018). *Corylus avellana* oil has been found to help treat PCOS by controlling gonadotropins, steroids, and serum lipid parameters, as well as having antioxidant properties (Demirel *et al.*, 2016).

Figure 4: ***Corylus avellana*** (Burgress, 2019).

2.8.4 *Mentha piperita L*: *Mentha piperita L* also known as peppermint is a medicinal herb that has gotten a lot of attention from the food and pharmaceutical industries due to its health benefits (Rasouli *et al.*, 2017). Pepper Mint has anti-androgen properties; it helps to lower testosterone levels and maintain hormonal balance. In both animals and women, *Mentha piperita* has antiandrogenic properties. In Iran, Turkey, India, the Middle East, Europe, and Canada, *mentha piperita* is widely used in folk medicine for flatulent colic, appetite, stomach pain, fever, nausea, vomiting, and digestion (Amoura *et al.*, 2015). A study was conducted by Abouelnaga *et al*. (2015) indicating the potential effects of *Mentha piperita* on letrozole-induced PCOS in female albino. In the study, females with PCOS had significant changes in blood testosterone, estrogen, LH, and FSH activity. Ovarian cysts with a reduced granulosa layer, atretic follicles, and a small number of corpora lutea were found in the PCOS group. Furthermore, the PCOS group caused significant changes in uterine tissue, including necrosis in stromal mesenchymal cells and hyperplasia of luminal epithelial cells which showed promising potential as an alternative medicine for the treatment of PCOS. (Abouelnaga *et al.,* 2015).

Figure 5: ***Mentha piperita L*** (Lim, 2018).

2.8.5 *Vernonia amygdalina*

V. amygdalina, is one of Africa's and Asia's most well-known plants. It is the most widely cultivated plant of the Vernonia family, which contains over 1,000 shrub species (Alara *et al.,* 2017). *Vernonia amygdalina Del* (Asteraceae) is a perennial shrub found in Africa's tropical regions. It is used as a vegetable as well as for medicinal purposes. Because of its diverse pharmacological uses, the plant has been used in folkloric medicine to treat a variety of diseases (Danladi *et al.,* 2018).

Many research on this plant have indicated that it contains flavonoids, saponins, alkaloids, tannins, phenolics, terpenes, steroidal glycosides, triterpenoids, and several forms of sesquiterpene lactones, among other bioactive compounds (Alara *et al.,* 2017). They possess different pharmacological properties such as antimicrobial, animalarial, anti-diabetic, antioxidant, laxative, antithrombotic, antifertility, anticancer, anthelmintic, anti-fungi, hypoglycemic due to their bioactive compounds (Alara *et al.,* 2017). It was named after William Vernon, an English botanist (Oyeyemi *et al.,* 2018). Vernonia amygdalina Del (Asteraceae) is a small shrub with dark green leaves and rough bark native to tropical Africa, but it has been domesticated in many parts of West Africa (Oyeyemi *et al.,* 2018). It is a perennial plant that grows to a height of 1 to 6 meters and it is a soft-wooded shrub that can regenerate quickly Fats, proteins, fibers, minerals, amino acids, carbohydrate, and vitamins abound in *vernonia amygdalina* leaves (Alara *et al.*, 2019).

Vernonia amygdalina Del is a multipurpose, fast-regenerating shrub with soft wood (Oyeyemi *et al.,* 2018). Its bitter flavor has earned it the nickname "bitter leaf," and it is also known by a variety of regional names in various languages. The bitter taste is caused by anti-nutritional phytochemicals found in the plants. The leaves are eaten as a leafy green vegetable. The high mineral and vitamin content has made it an essential part of the human diet (Oyeyemi *et al* 2018). In Nigeria, The Hausas calls it Ewuro, The Igbos calls it Onugbu, and The Yoruba calls it Shiwaka (Oyeyemi *et al.,* 2018). In Uganda, it is called Umubirizi or Mululuza. In Ethiopia, it is called ebichaa. In Tanzania, it is call Omubilizi. In Ghana, it is called Awonwono. In togo, it is called aluma or gbondutsi. In sierra leone,it is called Nje nyani In rwanda, it is called Umubilizi (Oyeyemi *et al.,* 2018).

Figure 6: *Vernonia amygdalina* (Udochukwu *et al.,* 2015)

2.8.5.1 Traditional uses of *Vernonia amygdalina*

Vernonia amygdalina is commonly used as a cooking herb and a food vegetable. It has a plethora of folkloric applications. The leaves are eaten as an appetizer, and the leaves' extract is used to facilitate digestion in Nigeria. Hausa women in Northern Nigeria eat it in the belief that it improves physical attractiveness (Oyeyemi *et al.,* 2018). The Leaf extract has been shown to be a viable alternative to hops in beer brewing (Oyeyemi *et al.,* 2018). In Africa, *Vernonia amygdalina* has long been used for the conventional treatment and/or control of a variety of diseases in both humans and animals. The leaves have anti-fever properties and are used as a quinine substitute in many African countries, including Nigeria. In various parts of Africa, the plant is used as a malaria treatment (Oyeyemi *et al.,* 2018).

Table 1. Folkoric uses of Vernonia amygdalina

Use in ehnomedicine	**Parts of plants used**	**Country or region**	**Method of extraction**
Malaria	Leaves	Southern Uganda	Infusion
Convulsion, Stomache	Leaves, root	Uganda	Infusion
Diabetes	Leaves	Nigeria	Boiling
Liver diseases	Leaves	Rwanda	Crush a handful and boil in 3L of water and 1 glass of banana wine
Febrile convulsion, fever, malaria	Leaves, root	Tanzania	Squeezing

Source: Oyeyemi *et al.*, (2018)

2.9 Gas Chromatography Mass Spectrometry

Gas chromatography–mass spectrometry (GC–MS) is widely acknowledged in the metabolomics community as one of the most reliable, repeatable, and widely used analytical platforms for metabolomics research (Beale *et al.,* 2018). The separation and analysis of multi-component mixtures such as essential oils, hydrocarbons, and solvents is accomplished using gas chromatography. To make the readings more meaningful, different temperature programs can be used, for example, to distinguish between substances that behave similarly during the GC phase (Fauzi *et al.*, 2017).

The hyphenated analytical technique Gas Chromatography–Mass Spectrometry (GC-MS) combines the separation capabilities of gas-liquid chromatography with the detection characteristic of mass spectrometry to identify distinct compounds within a test sample. The volatile and thermally stable substitutes in a sample are separated by GC, whereas the analyte is fragmented by GC-MS and identified by its mass. The inclusion of a mass spectrometer transforms it into GC-MS/MS (Chauhan *et al.,* 2014). Alcohols, alkaloids, nitro compounds, long chain hydrocarbons, organic acids, hormones, esters, and aminoacids 15 can all be detected using GC–MS, which only includes a small amount of plant extracts (Konappa *et al.,* 2020). Because of its high sensitivity in resolving, characterizing, and quantifying complex mixtures of organic compounds, GC/MS has become an indispensable analytical method for addressing specific questions regarding the chemical constituents of works of art (Sutherland, 2018). Gas chromatography-Mass spectrometry (GC-MS) is an analytical approach that is hybrid which combines the separation capabilities of GC with the detection capabilities of MS to improve sample analysis performance. Although GC can isolate volatile components in a sample, MS can help fragment them and classify them by mass (Susha, 2019). In the field of volatile analysis, GC/MS is the most commonly mentioned technique (Peieria *et al.,* 2020).

2.9.1 Principle of GC-MS

GC/MS combines two separate technologies, gas chromatography and mass spectrometry, to examine complex organic and biological combinations (Hussain and Maqbool, 2014). The GC-MS instrument is made up of two major components. By passing the sample through a stationary phase (Mobile phase) placed in the inert gas column, converting the sample into pure chemical pulses based on their volatility

(Hussain and Maqbool, 2014). When substances depart a chromatography column, a mass spectrophotometer is used to obtain their spectra, electrical and/or magnetic fields are used to isolate ions inside the mass spectrophotometer to achieve phase ion separation (Hussain and Maqbool, 2014). The mass spectrophotometer defines and quantifies compounds based on their mass-to-charge ratio (m/z). These spectra can be saved and studied on the system (Hussain and Maqbool, 2014). A chromatogram of this signal as a function of time yields a series of symmetrical peaks that reveal some sample composition information (Hussain and Maqbool, 2014). The retention time of the peaks or the region under the peaks provide a quantitative measure of the sum of each component when compared to the retention time of some norm (Hussain and Maqbool, 2014).

2.9.2. Application of Gas Chromatography-Mass Spectrometry

GC-MS offers improved sample detection, higher sensitivity, a wider range of analyzable samples, and quicker performance, allowing GC-MS to be used in a variety of new ways.

I) Medicine: GC-MS is used in screening tests to identify a variety of metabolic disorders that are present at birth. It detects small amounts of compounds in the urine of people who have inherited metabolic disorders. Oils in ointments, creams, and lotions can also be detected by this device (Susha, 2019).

II) Environmental Monitoring: Gas chromatography (GC) plays an important role in the detection and quantification of common contaminants in the environment today (Sltos and Galceran, 2018). The major application of GC-MS is monitoring environmental pollutants. It is commonly used in the detection of dibenzofurans, dioxins, herbicides, sulfur, pesticides, phenols, and chlorophenols in air, soil, and water (Susha, 2019).

III) Biological analysis: Narcotics, barbiturates, alcohols, and medications such as anticonvulsants, anesthetics, antihistamines, sedative hypnotics, and anti-epileptic medicines can all be detected using GC-MS. It may also be used to detect contaminants and metabolites in blood, as well as to profile fatty acids in microorganisms (Susha, 2019).

IV) Forensic application: GC-MS can help any forensic laboratory by allowing for the deconvolution of coeluted components while also increasing the sensitivity of minor components. This is why GC–MS is the gold standard in the forensic investigation of

trace evidence like ignitable liquids and medicines (Bridge *et al.,* 2018). In forensic toxicology, GC-MS is commonly used to identify poisons and steroids in biological materials, as well as in anti-doping labs to detect performance-enhancing chemicals like anabolic steroids (Susha, 2019).

V) Pharmaceuticals: GC-MS is utilized in research and development, production, and quality control in the pharmaceutical business. It's a technique for detecting contaminants in active pharmaceutical components. GC-MS is utilized in medicinal chemistry for chemical synthesis and characterization, as well as pharmaceutical biotechnology (Susha, 2019).

2.10. UV-visible spectrophotometry

Ultra-violet spectrophotometer is a type of physical optical spectrophotometer that uses light in the visible, ultraviolet, and near-infrared ranges (Skoog *et al.,* 2004). It is based on the Beer-Lambert law, which states that the absorption of a solution is proportional to the concentration of the absorbing species in the solution and the length of the direction (Skoog *et al.,* 2004). As a result, it can be used to determine the absorber concentration in a solution over time. UV-VIS spectrophotometry has been the most effective analytical tool in the modern laboratory for the last 37 years, and for the last century. it is vital to understand how fast absorption changes with concentration (Skoog *et al.,* 2004).

2.10.1. Principles of UV-visible spectrophotometry

Absorption occurs when radiation produces an electronic transition inside the structure of a molecule or ion in the visible or ultraviolet field (Sharma et *al.,* 2004). The absorption of light by a sample in an ultraviolet or visible fluid (Sharma et *al.,* 2004). Light energy promotes electrons from their ground state orbital to a higher energy excited state orbital, also known as an anti-bonding orbital.

Three forms of orbitals of the ground state could be theoretically involved (Sharma et *al.,* 2004).

1. Alpha molecular (Bonding)
2. Pie (Bonding) orbital molecular
3. Atomic orbital n (non-bonding)

Furthermore, the transistion may involve two types of anti bonding orbitals

1. Orbital of o* (Sigma star)

2. Pie* orbital (Pi atar)

2.11 Letrozole

Letrozole decreases estrogen levels in postmenopausal women by preventing the conversion of adrenally-generated androstenedione to estrone by aromatase in peripheral tissues, similar to other aromatase inhibitors (Scholar, 2007). Letrozole is an aromatase inhibitor that is use to induce ovulation in anovulatory infertile women with an endometrial thickness of more than 56 mm. It works by suppressing the enzyme aromatase, which decreases estrogen production (Guang *et al.,* 2018). Letrozole stimulates ovulation by inhibiting estrogen synthesis and increasing the release of follicle-stimulating hormone (FSH). Letrozole has been shown to reduce estrogen levels by at least 97% to 99% (Guang *et al.,* 2018). Letrozole was also found to be successful in clomiphene-resistant individuals, resulting in ovulation in 62% of instances and pregnancy in 14.7% (Guang *et al.,* 2018). Sweating, hot flashes, joint discomfort, and weariness are the most prevalent adverse effects of letrozole (Scholar, 2007). Letrozole has become increasingly widely used for ovulation induction in the PCOS patient group, and it is now regarded to be first-line therapy (Barakat *et al.,* 2018).

2.11.1 Mechanism of action letrozole

Letrozole, often known as Femara, is a generic oral medication that inhibits estrogen production (anti-estrogen drug). Because it includes the enzyme aromatase, which creates estrogen, estradiol, and estrone, the adrenal glands are the primary source of circulating estrogens (Ogburu, 2020). Letrozole inhibits aromatase's active site as well ascytochrome P450 19A1's electron transfer chain (Nabholtz, 2008). High levels of luteinizing hormone, FSH, and uterine weight loss result as a result of this (Nabholtz, 2008). The induction of Letrozole reduces the availability of estrogens, causing estrogen-dependent tumors to regress (Nabholtz, 2008). Letrozole is a very strong and specific AI that inhibits intracellular aromatase enzyme activity in the key places where it is located, thereby suppressing whole-body aromatization. Letrozole suppresses the growth or induces the regression of hormone-responsive breast cancers in vivo by efficiently suppressing estrogen production (Bhatnagar, 2007).

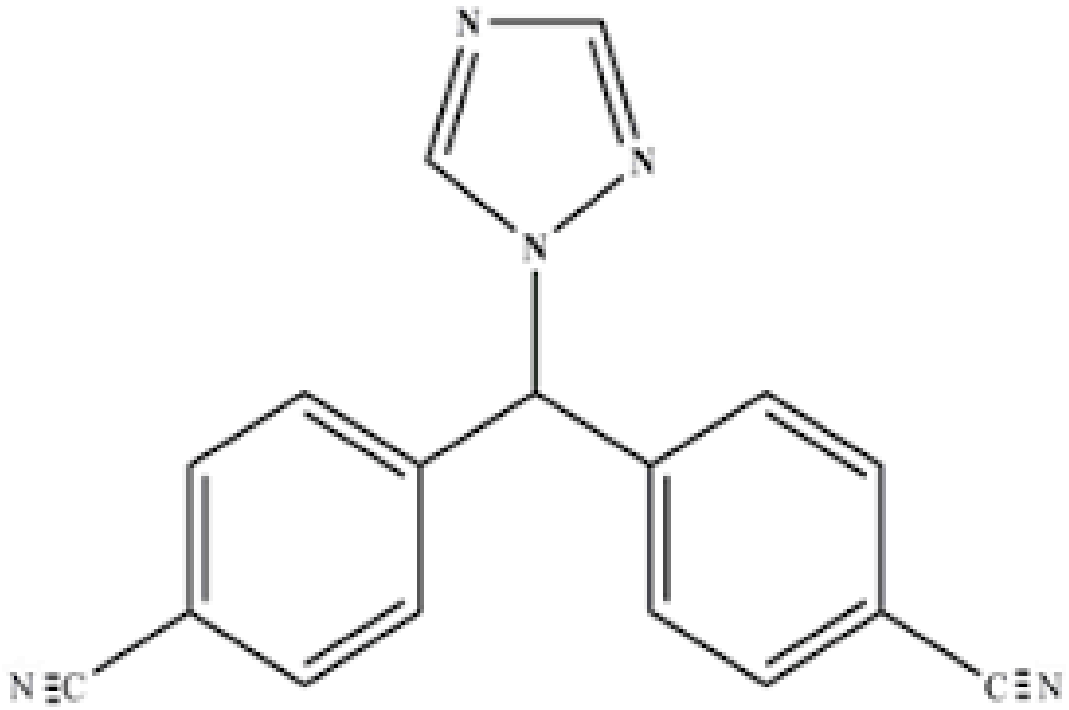

Figure 7: Chemical structure of letrozole

Source: Bijari *et al.,* (2018).

2.12. PCOS associated organs

The ovary, uterus, liver and kidney and vaginal canal constitute up the female reproductive system. The ovary, liver and kidney all have a similar anatomy that is tailored to their respective tasks (Picut and Remick, 2016). The mammary glands (breast glands) are also taken into account because they are crucial during pregnancy (Picut and Remick, 2016).

2.12.1. Ovary and PCOS: Ovaries are small, oval-shaped paired gonads linked to the back of the uterus' wide ligament and placed on either side of the uterus in relation to the lateral wall of the pelvic (Soloyan *et al.,* 2019). The ovary's surface is covered with a layer of waldeyer's germinal epithelium, which is made up of columnar epithelial cells (Soloyan *et al.,* 2019). The rapid maturation of ovarian primordial follicles into primary follicles, as well as an increase in the number of granulosa cells per primary follicle, is a sign of PCOS. Follicles accumulate in the ovarian cortex after that, but do not progress properly toward ovulation or atresia (Makris, 2019). As eggs partially mature but are not released, numerous bubble-like cysts may grow on the surface of one or both ovaries in a patient with PCOS. These eggs remain in follicles that enlarge but do not open. On a single ovary, a woman with PCOS may develop 25 or more cysts (Harrar, 2021).

2.12.3 Liver and PCOS: The liver is a vital organ that performs a variety of functions in the body, including protein and blood clotting factor creation, triglyceride and cholesterol formation, glycogen synthesis, and bile generation (Benjamin, 2020). The liver is a large organ that can be found on the right side of the stomach (Benjamin, 2020). Nonalcoholic fatty liver disease is twice as common in women with polycystic ovarian syndrome, with androgen excess leading to disease progression (Vassilatou, 2014). Although the natural history of NAFLD is unknown, and hepatic steatosis appears to be a relatively benign condition in the majority of patients, limited evidence suggests that advanced liver disease is more common in obese PCOS patients with NAFLD (Vassilatou, 2014). Patients with polycystic ovary syndrome are more likely to develop nonalcoholic fatty liver disease. Obesity and insulin resistance are thought to be the primary causes of NAFLD in PCOS (Vassilatou, 2014).

2.12.4 Kidney and PCOS

All vertebrates have a pair of kidneys, which are bean-shaped organs. They remove waste from the body, keep electrolyte levels regulated, and keep blood pressure in check (Kaygusuz et al 2013). The kidneys are located toward the rear of the abdomen, one on each side of the spine. To make room for the liver, the right kidney is slightly smaller and lower than the left. Male kidneys weigh 125–170 g, while female kidneys weigh 115–155 g (Nath et al 2013).

The kidneys' primary function is to maintain homeostasis. This means they keep track of fluid levels, electrolyte balance, and other factors that keep the body's internal environment stable and comfortable. Other functions of the kidney include (Knoedler et al 2015)

2.13. Percentage organ body weight ratio

Changes in organ weight acts as an indicator to identify organ damage that has been chemically induced, although it can be difficult to analyze because a change in organ weight can reflect chemically induced changes in overall body weight (Lazic *et al.*, 2020). Interpreting the organ weight data can be difficult because you have to identify whether a chemically induced compound affects the organ directly through changes in the body weight (Lazic *et al.,* 2020). To calculate the ratio (relative organ weight), the organ of the animal is dividing by the body weight of the animal the multiplied by hundred. Unfortunately, with this method, the ratio fails to adjust the differences in the body weight between groups properly, but this is mostly ignored (Michael et al., 2007). The percentage organ- body weight ratio was obtained using the following expression:

$$\text{Percentage organ-body weight ratio} = \frac{\text{Organ weight}}{\text{Total body weight of the animals}} \times 100$$

2.14. Liver function indices

Serum total protein, albumin, globulin, direct bilirubin, aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase total bilirubin and albumin: globulin ratio, are all biochemical markers of liver function that are measured (Imafidon and Okunrobo, 2012). The total serum protein test determines how much protein is present in the blood. It also assesses the levels of albumin and globulin, two key protein groups found in the blood (Butler, 2019). Albumin is primarily produced in the liver. It aids in the prevention of blood spilling from blood vessels. Albumin is also necessary for tissue growth and repair as it aids in the transport of various drugs and other substances through the bloodstream. Globulin is made up of three types of proteins: alpha, beta and gamma (Busher, 1990). The liver produces some globulins whereas the immune system produces others. Hemoglobin binds to some globulins. Other globulins fight infection by transporting metals like iron into the bloodstream. Serum protein electrophoresis can split serum globulin into numerous groupings (Busher, 1990). Alanine aminotransferase is an enzyme that is mostly found in the liver. Enzymes are proteins that help the body perform clinical processes (Liu *et al.,* 2014). ALT activity is assessed not just to diagnose liver illness but also to keep track of one's overall health (Liu *et al.,* 2014). Various factors such as viral hepatitis, alcohol intake and medicine, influence ALT activity. Because of the worldwide obesity epidemic, the influence of metabolic disorders on ALT variation has recently sparked attention. (Liu *et al.,* 2014). The enzyme AST is primarily located in the liver, although it can also be found in the muscles. AST is released into the bloodstream when your liver is damaged (Xu and Higgins, 2015). The level of AST in your blood is measured by an AST blood test (Xu and Higgins, 2015). The test can assist your doctor in determining whether you have liver damage or disease (Xu and Higgins, 2015). ALP is a membrane–bound glycoprotein that catalyzes the breakdown of phosphate monoestersat basicv pH levels. Intestinal ALP, Placental ALP, germ cell ALP and tissue nonspecific alkaline phosphatase ALP are the four isoenzyme of alkaline isoenzymes of alkaline phosphatase based on the place of tissue expression (Sharma *et al.,* 2013). Low ALP activity is caused by micronutrients such as zinc and magnesium (Sunda *et al.,* 2017).

2.15 Kidney function indices

Creatinine is a standard metric for assessing kidney function. Creatinine is a subunit of creatinine phosphate in muscle that the body produces at a fairly steadypace dependent on muscle mass (Gowda *et al.,* 2019). Males have normal creatinine clearance test valve of 110-150ml/min whereas females have a normal creatinine clearance test valve of 100-130ml/min. Calculating glomerular filtration rate from serum creatinine concentration is recommended by the national kidney disease education program. (Gowda *et al.,* 2019). Urea is a nitrogenous byproduct of protein and amino acid catabolism that is created by the liver and transported throughout the intracellular and extracellular fluid (Gowda et al., 2019). Urea is filtered out of the blood by the glomeruli of the kidney and partially reabsorbed with water (Gowda *et al.,* 2019). The most often used clinical indices for determining renal function are based on the concentration of urea in the blood (Gowda *et al.,* 2019). Increased blood urea nitrogen (BUN) is linked to kidney disease, urinary tract blockage due to a kidney stone, cognitive heart failure, dehydration, fever, shock, and gastrointestinal bleeding. High BUN levels can occur late in pregnancy or as a result of consuming excessive amounts of protein-rich foods (Gowda *et al.,* 2019). BUN levels more than 100mg/dL indicate significant kidney injury whereas lower BUN levels indicate fluid overload (Gowda *et al.,* 2019).

CHAPTER THREE

3.0 MATERIALS AND METHOD

3.1 Materials

3.1.1 Plant Material and Authentication

Fresh leaves of bitter leaf were collected at Mountain Top University, Ogun State. The plant was authenticated at the Department of plant biology, University of lagos, Nigeria. A voucher specimen (UIH 8751) was deposited at the herbarium of the department.

3.1.2 Assay kits and Drugs

Albumin, bilirubin, total protein, globulin, creatinine, uric, urea, aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP). Letrozole. Whatmann's filter paper #1, rotary evaporator, conical flask, beaker, hot air oven, weighing balance, jute bag.

3.1.3 Chemicals and reagents

NaOH, Diethyl ether, ethanol, sulphuric acid, chloroform, 0.02% ninhydrin reagent

3.2 METHODOLOGY

3.2.1 Preparation of Solvent Extracts

The identified sample were thoroughly rinsed under running water to remove contaminants, it was dried under the sun and thereafter, it was weighed on a weighing balance until it attained a constant weight. It was blended with the aid of an electric blender oven dried at 50 degree Celsius when it attained a constant weight. It was pulverized with the aid of an electric blender and was soaked with ethanol in ratio 1:4 for 48hours and sieved with a jute bag. The supernatant was filtered with a whatmann's filter paper #1. The filtrate was then concentrated in a rotary evaporator machine at 78 degree Celsius. At the end of the concentration, the rotary evaporator had successfully separated the solvent from the solvent extract. The ethanolic extract was then collected in a beaker and evaporated to dryness in a hot air oven at 50 degree Celsius, after which it was weighed to get the percentage yield.

3.2.2 Phytochemical screening of the extract

3.2.2.1 Secondary metabolites

Vernonia amygdalina leaves was screened for secondary metabolite present as described according to Arvindganth *et al.*, (2015).

3.2.2.1.1 Qualitative Phytochemical Analysis

The aqueous solvent plant extract was tested for the presence of bioactive compounds using standard methods. 2g of solvent extract of *Vernonia amygdalina* and 50ml of water was filtered with the aid of filter paper. The following were the phytochemicals that were tested:

3.2.2.1.2 TEST FOR CARBOHYDRATE

Procedure: 1ml of molisch test was poured into a conical flask, 5 drops of sulphuric acid was added and 2 ml of filtered solvent extract was also added (Arvindganth *et al.,* 2015).

3.2.2.1.3 TEST FOR TANNINS

Procedure: 2ml of filtered ethanolic extract of *Vernonia amygdalina* and 2ml of ferric chloride was poured into a conical flask (Arvindganth *et al.,* 2015).

3.2.2.1.4 TEST FOR SAPONIN

Procedure: 2ml of filtered ethanolic extract of *Vernonia amygdalina* and 2ml of distilled water was poured into a conical flask and it was shaken for 15 minutes (Arvindganth *et al.,* 2015).

3.2.2.1.5 TEST FOR ALKALOID

Procedure: 2ml of filtered ethanolic extract of *Vernonia amygdalina* and 2ml of concentrated hydrochloric acid was poured into a conical flask. Then, few drops of Mayer's reagent was also added (Arvindganth *et al.,* 2015).

3.2.2.1.6 TEST FOR FLAVONOID

Procedure: 2ml of filtered ethanolic extract of *Vernonia amygdalina* and 1ml of 2N sodium hydroxide was poured into a conical flask (Arvindganth *et al.,* 2015).

3.2.2.1.7 TEST FOR GLYCOSIDE

Procedure: 2ml of filtered ethanolic extract of *Vernonia amygdalina* and 1ml of 2N of sodium hydroxide was poured into a conical flask (Arvindganth *et al.,* 2015).

3.2.2.1.8 TEST FOR QUINONES

Procedure: 1ml of ethanolic extract of *Vernonia amygdalina* and 1ml of concentrated sulphuric acid was also poured into a conical flask (Arvindganth *et al.,* 2015).

3.2.2.1.9 TEST FOR PHENOLS

Procedure: 1ml of ethanolic extract of *Vernonia amygdalina* and 2ml of distilled water was poured into a conical flask. Then, few drops of 10% ferric chloride was also added (Arvindganth *et al.,* 2015).

3.2.2.1.10 TEST FOR TERPENOIDS

Procedure: 0.5ml of solvent extract of *Vernonia amygdalina* and 2 ml of chloroform was added into conical flask. Then, 2ml of concentrated hydrosulphuric acid was also added (Arvindganth *et al.,* 2015).

3.2.2.1.11 TEST FOR CARDIAC GLYCOSIDES

Procedure: 0.5ml of solvent extract of *Vernonia amygdalina* was poured into a conical flask. 2ml of glacial acetic acid containing few drops of ferric chloride was added (Arvindganth *et al.,* 2015).

3.2.2.1.12 TEST FOR NINHYDRIN

Procedure: 2ml of the solvent extract of *Vernonia amygdalina* was poured into a conical flask. Few drops of 0.2% ninhydrin reagent was added and healed for 5 minutes (Arvindganth *et al.,* 2015).

3.2.2.1.13 TEST FOR COUMARINS

Procedure: 1ml of 10% sodium hydroxide was added to 1ml of the solvent extract of *Vernonia amygdalina* and poured into a conical flask (Arvindganth *et al.*, 2015).

3.2.2.1.14 TEST FOR ANTHRAQUINONES

Procedure: 1ml of solvent extract of *Vernonia amygdalina* was poured into a conical flask. Few drops of 10% of ammonia solution was added (Arvindganth *et al.,* 2015).

3.2.2.1.15 TEST FOR STEROIDS

Procedure: 1ml of solvent extract of *Vernonia amygdalina* was poured into a conical flask. 1ml of chloroform and few drops of concentrated sulphuric acid was added (Arvindganth *et al.,* 2015).

3.2.2.1.16 TEST FOR PHLOBATANNINS

Procedure: 1ml of solvent extract of *Vernonia amygdalina* was poured into a conical flask. Few drops of 2% hydrochloric acid was added (Arvindganth *et al.,* 2015).

3.2.2.1.17 TEST FOR ANTHRACYANINE

Procedure: 1ml of solvent extract of *Vernonia amygdalina* was poured into a conical flask. 1ml of 2N sodium hydroxide was added and heated for 5 minutes at 100 degree Celsius (Arvindganth *et al.*, 2015).

3.2.3 UV-visible spectrophotometer

The absorbance and wavelength of the peaks were determined for the solvent plant extract by a wavelength scan between 200nm and 600nm. The UV-visible spectrophotometer were recorded on a UV-Vis spectrophotometer (Arvindganth *et al.*, 2015).

3.3 GC-MS

The GC-MS study was carried out using a Hewlett Packard Gas Chromatograph (Model 6890 series) fitted with a flame ionization detector and a Hewlett Packard 7683 series 250 °C MS transfer line temperature injector. The GC was fitted with an HP-5MS (30 x 0.25 mm) fused silica capillary column, 1.0μm film thickness. The oven temperature was kept at 50 ° C for 5 min of held time and increased at a rate of 2 ° C / min from 50 to 250 ° C, using helium gas (99.999 percent) as a carrier gas at a steady flow rate of 22 cm / s. A 1.0micron extract (1 mg diluted in 1 ml of absolute alcohol) was injected at a 1:30 split ratio. Agilent Science Network Mass Spectrometer (Model 5973 series) coupled with Hewlett Packard Gas Chromatograph (Model 6890 series) with NIST08 Library software database was studied by MS. Mass spectra were taken at 70 eV/200 °C, 1 scan/s scanning rate. Compound recognition was carried out using the NIST08 Library database. The mass spectrum of each unknown compound was compared with the known compounds contained in the library software database (Lian *et al.*, 2013)

3.4 Experimental animals

Sixteen female rats were used in the study which was gotten from Mountain Top University, Ogun state, Nigeria. The animals were acclimatized for one week to standard housing condition (temperature 25^{o}C) and were kept in a well-ventilated house whereby animal feed and water were given to them during the course of this study. Every morning upon entering the animal house, rats were exposed to light for 12 hours and darkness for another 12 hours.

A total number of 16 rats were segregated or divided into 4 groups with each containing 4 rats and they were labelled head, body, tail and head and body and were all induced with letrozole and carboxyl methyl cellulose except the normal control group which was given only Carboxyl methyl cellulose.

Group A– Normal control

Group B – PCOS + H_2O

Group C – PCOS +Clomiphene citrate + Metformin

Group D – PCOS + 50mg/B.W of *Vernonia amygdalina*

Group E – PCOS + 100mg/B.W of *Vernonia amygdalina*

Administration was done orally for 21 days. After 21 days, they were treated for 14 days. The rats were anaesthetized and blood was collected via jugular puncturing. The liver, kidney and ovary were isolated and kept in the ice-cold sucrose solution.

3.4.1 Sample collection

The weight of each rat was checked every week using an analytical balance. All the experimental animals were fasted for 12 hours before the period of administration of letrozole and carboxyl methyl cellulose. 1ml of letrozole and carboxyl methyl cellulose was given to Group A, Group B, Group C and Group D and E

3.4.2 Preparation of tissue and serum supernatants

The method described by Yakubu *et al* (2008) was used to prepare the tissue and serum supernatants. All the experimental animals were fasted for 12 hours after the administration of plant extract, clomiphene citrate, distilled water and silver nitrate. The rats were weighed individually and thereafter anaesthetized using 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 centrifuge tubes and left at room temperature for 10minutes to clot. The blood samples were centrifuged at 3000rpm for 10minutes.The animals were quickly dissected the ovary, Kidney and Liver were collected from each rats. Two big sample bottles were used and labelled (Histological and sucrose solution). Ovary, kidney and liver of rats that were labelled head were put into the big sample bottles labelled Histology and formalin was added into the bottles so as to preserve the organs. Ovary, kidney and liver of all the rats labelled

head, body and tail were inserted into the big sample bottle labelled sucrose solution. Afterwards, the collected blood samples were centrifuged at 3000rpm for 10minutes, the supernatant was collected into a fresh sample bottle, stored frozen ($4^{o}C$) overnight before being used for the various biochemical assays.

Table 2: Dilution factor for the various assays

Assays	Serum	Liver	Kidney
ALT	×5	×30	
ALP	×5	×30	×60
AST	×5	×30	
BUN	×5		
Creatinine	×5		
Albumin	×5		
Globulin	×5		
Total Protein	×5		×60
Albumin-Globulin ratio	×5		
Urea	×5		
Uric acid	×5		
Direct Bilirubin	×5		
Total Bilirubin	×5		

3.5. Biochemical assessment

3.5.1 Determination of liver function indices

3.5.1.1 Total protein activity

The method was described by Knipe (1998) was used to determine serum total protein activity.

Principle:

Pyrogallol red complexes with proteins in an acid environment containing molybdate ions. The resulting blue-coloured complex absorbs maximally at 600 nm. Therefore the optical density at 600 nm is directly proportional to the protein concentration of the samples.

Sample test for kidney sample

200 µL of diluted kidney solution was poured into all the labelled test tubes, 1ml of Reagent 1 of total protein kit was added. Thereafter it was incubated for 30minutes at room temperature (25 degree Celsius). The absorbance of each test tubes containing the minutes was read at 540nm (Knipe, 1998).

Sample test for serum sample

200 µL of diluted serum solution was poured into all the labelled test tubes, 1ml of Reagent 1 of total protein kit was added. Thereafter it was incubated for 30minutes at room temperature (25 degree Celsius). The absorbance of each test tubes containing the minutes was read at 540nm (Knipe, 1998).

Sample test for liver sample

200 µL of diluted liver solution was poured into all the labelled test tubes, 1ml of Reagent 1 of total protein kit was added. Thereafter it was incubated for 30minutes at room temperature (25 degree Celsius). The absorbance of each test tubes containing the sample was read at 540nm (Knipe, 1998).

Calculation:

$$\text{Total protein (g/dl)} = \frac{A_{sample} \times \text{Concentration of standard}}{A_{standard}}$$

A_{sample} =Absorbance of the test sample

$A_{standard}$ = Absorbance of the standard

Concentration of standard = 5.95g/dl

3.5.1.2 Serum total and direct globulin concentration

The method was described by Evelyn and Malloy (1938) was used to determine serum total and direct bilirubin concentration

Principle:

Direct bilirubin in the serum reacts with diazotized sulphanilic acid in the alkaline medium to form blue colored complex. Total bilirubin was determined in the presence of caffeine benzoate and acetate as accelerators to form azobilirubin (albumin bound bilirubin)

Procedure:

For the determination of total bilirubin, 25 µL of reagent 2 was placed into 100 µL of reagent 1 after which 500 µL of reagent 3 was added subsequently. Thereafter, 1000 µL of appropriately diluted serum was added to the mixture. The blank was constituted by replacing the serum with distilled water without reagent 2. For the determination of direct bilirubin, 25 µL reagent 2 was added to 100 µL of reagent 1 followed by the addition of 1000 µL of 0.9% NaCl. A known volume (100 µL) of diluted serum was added to the mixture. The blank was constituted by replacing serum with distilled water without adding reagent 2. The mixture for both total and direct bilirubin were incubated at 37^{o}C for 30 minutes after which 500 µL of reagent 4 was dispensed into the total bilirubin preparation. The mixture were further incubated at 25^{o}C for 30 minutes and absorbance was read spectrophotometric ally at 578nm

Calculation:

Total bilirubin (µmol/L) = 10.8 x A_{TB}

Direct bilirubin (µmol/L) =14.4 x A_{DB}

A_{TB}=Absorbance of total bilirubin

A_{DB} = Absorbance of direct bilirubin

10.8 = Milligrams of total bilirubin per 100ml

14.4 = Milligrams of direct bilirubin per 100ml

3.5.1.3 Serum albumin concentration

The procedure was described by Doumas *et al*, (1971) was employed for the determination of serum albumin in the animals.

Principle:

The determination of serum albumin is based on its quantitative binding to the indicator, 3, 3', 5, 5'-tetrabromocresol sulphonaphthalein (bromocresol green, BCG). The albumin-BCG coplex absorbs maximally at 578nm. The absorbance is directly proportional to the albumin concentration in the sample.

Procedure:

5µl of enzyme source (serum) was added to the sample test tubes, 5µl of water was added to the blank test tube and 5µl of standard was added to the standard test tube. 1.5ml of Reagent 1 was added to the sample test tubes, blank test tube and standard test tube, which the solution was mixed and kept in the hot-air oven for 10 minutes at 37^0 C, which the absorbance of the samples was read against the blank using UV-Visible spectroscopy at 630nm wavelength.

Calculation:

$$\text{Albumin concentration (g/dl)} = \frac{A_{sample} \times \text{Concentration of standard}}{A_{standard}}$$

A_{sample} = Absorbance of the test sample

$A_{standard}$ = Absorbance of the standard

Concentration of standard = 4.68g/dl

3.5.1.4 Serum globulin concentration

The serum globulin level was assayed using the method described by Tietz (1995) by subtracting the concentration of serum albumin from the serum protein content. The concentration was expressed in g/dl.

Globulin (g/dl) = Total protein –Albumin

3.5.1.5 Serum albumin-globulin ratio

The determination of serum albumin-globulin ratio was done using the method described by Melnick et al. (1940) by dividing the concentration of serum albumin by serum globulin content. It is expressed mathematically as:

$$\text{Albumin-globulin ratio} = \frac{\text{Serum albumin (g/dl)}}{\text{Serum globulin (g/dl)}}$$

3.5.2 Determination of kidney function indices

3.5.2.1 Serum urea concentration

The procedure described by Veniamin and Vakirtzi (1970) was used for the determination of urea in the serum

Principle:

Urea in the serum is hydrolyzed to ammonia in the presence of urease. The ammonia is then determined photometrically by Berthellot's reaction as derived by the follows:

Urea + H_2O --------- $2NH_3 + CO_2$

NH_3 + hypochlorite + phenol ---------- indophenols (blue compound)

Procedure:

To 6 ml of reagent 2 was added to 24 ml of reagent 1 which was used as the working solution. The standard was constituted by substituting the sample with 1 µL of standard reagent. To 10 µL of standard, 1 µL of working reagent was added while for 10 µL was added to 1 µL of working reagent. The mixture for both the sample and standard were mixed and the absorbance was read at 546nm after exactly 30 seconds. Another absorbance was read A_2 exactly 60 seconds.

Calculation: Urea concentration (mg/dl) = $\frac{A_{sample} \text{ x Concentration of standard}}{A_{standard}}$

Concentration of standard = 50mg/dl

3.5.2.2 Serum creatinine concentration

The method described by Bartels and Bohmer (1972) was used for the determination of serum creatinine.

Principle:

Creatinine in alkaline solution reacts with picric acid to form a red colored complex. The amount of the complex formed is directly proportional to the creatinine concentration and spectrophotometrically at 490nm.

Procedure:

To 100 µL of diluted enzyme source, 1 ml of working reagent was added. The blank was constituted by replacing the sample with 0.1ml of distilled water. The resulting mixture was read after 30 seconds and the absorbance A_1 of the standard and sample were read

respectively. Exactly 2 minutes later, the absorbance A_2 of standard and sample were also read spectrophotometrically at 490nm.

Calculation:

$$A_2 - A_1 = A_T$$

Concentration of creatinine in the sample (mg/dl) = $\frac{A_T \text{ X Concentration of standard}}{A_{standard}}$

3.5.2.3 Serum uric acid concentration

Serum uric acid concentration was determined according to the method described by Tietz (1995)

Principle:

Uric acid is converted by uricase to allantoin and hydrogen peroxide which under the catalytic influence of peroxidase, oxidises 3, 5-Dichloro-2-hydroxybenzenesulfonic acid and 4-aminophenazone to a red violet quinoeiminie compound.

Procedure:

0.5ml or working reagent was added to 0.01ml of the diluted serum sample. The standard was constituted by replacing the serum with 0.01ml of standard reagent. The blank was constituted by replacing the test sample with 0.01ml of distilled water. The resulting mixture was incubated at 37℃ for 5minutes and the absorbance of the serum samples and the standard were read spectrophotometrically at wavelength of 520nm against reagent blank within 30 minutes.

Calculation:

Concentration of uric acid (mg/dl) = $\frac{\text{Absorbance of test X Concentration of standard}}{\text{Absorbance of standard}}$

3.5.2.4 Serum blood urea nitrogen (BUN) – Creatinine ratio

The serum BUN-Creatinine ratio was determined using the method described by Tietz (2006). It is expressed mathematically by dividing the amount of serum urea by serum concentration.

3.5.3 Determination of specific enzyme activity

3.5.3.1 Alkaline phosphatase activity

The method was described by wright et al, (1972) was used for the determination of alkaline phosphatase activity**.**

Principle:

The amount of phosphate ester split within a given period of time is a measure of the phosphatase enzyme. Para-nitro phenyl phosphate was hydrolyzed to para-nitro phenyl and phosphoric acid at a pH of 10.1. The para-nitro phenol confers a yellowish color on reaction.

Procedure:

To 10 µL of diluted sample, 500 µL of reagent 1 was added. The blank was constituted by substituting the serum with 0.02 µL of distilled water. The resulting mixture was read after 1 minutes and the absorbance A_1 of the standard and sample were read respectively. Exactly 2 minutes later, the absorbance A_2 of standard and sample were also read spectrophotometrically at 490nm. Exactly 3 minutes later, the absorbance A_2 of standard and sample were also read spectrophotometrically at 405nm.

Calculation:

U/I = 2742 x Change in absorbance

3.5.3.3 Aspartate aminotransferase activity

The method described by Reitman and Frankel (1957) was used for assaying the activity of aspartate aminotransferase.

Procedure:

The enzyme catalyses the reversible reaction involving alpha-ketoglutarate and L-aspartate to form L-glutamate and oxaloacetate hydrazone formed with 2, 4-dinitrophenylhydrazine at 546nm.

Preparation of liver sample solution

50 micrometer of diluted liver sample was poured into all the labelled test tubes. 250 micrometer of reagent 1 of AST sample was added. It was incubated for 30 minutes at 37 degree Celsius. Afterwards, 2.5micrometer of 0.4N NaOH was added into solution and it was allowed to stand tor 25 degree Celsius.

Preparation of serum sample solution

50micrometer of diluted blood sample was poured into all the labelled test tubes. 250 micrometer of reagent 1 of AST sample was added. It was incubated for 30 minutes at 37 degree Celsius. Afterwards, 2.5micrometer of 0.4N NaOH was added into solution and it was allowed to stand tor 25 degree Celsius.

Preparation of blank test solution

50 micrometer of distilled water was poured into all the labelled test tubes. 250micrometer of reagent 1 of AST sample was added. . It was incubated for 30 minutes at 37 degree Celsius. Afterwards, 2.5micrometer of 0.4N NaOH was added into solution and it was allowed to stand tor 25 degree Celsius. The absorbance of all sample (Blood and liver sample) was read against the reagent blank after 5 minutes at 546nm.

3.5.3.4 Alanine aminotransferase activity

This method described by Reitmann and Frankel (1957) was used for assaying the activity of alanine aminotransferase.

Principle;

Alanine aminotransferase activity was determined by monitoring the concentration of pyruvate hydrazine formed with 2, 4-dinitrophenyl hydrazine.

Procedure:

Preparation of liver sample

50micrometer of diluted liver sample was poured in each of the labelled test tubes. 250micrometer of buffer reagent 1 of ALT was added. The solution was incubated for 30minutes at 37 degree Celsius. Afterwards 250micrometer diluted liver sample was added. It was mixed and allowed to stand for 20minutes at 25 degree Celsius. 2.5micrometer of diluted liver sample was added.

Preparation of serum sample

50micrometer of diluted blood sample was poured in each of the labelled test tubes. 250micrometer of buffer reagent 1 of ALT was added. They were incubated for 30minutes at 37 degree Celsius. Afterwards 250micrometer diluted liver sample was added. It was mixed and allowed to stand for 20minutes at 25 degree Celsius. 2.5micrometer of diluted liver sample was added.

Reagent blank test

50ml of distilled water was poured into all the labelled test tubes. 0.5ml of buffer reagent 1 of ALT was added. Afterwards, it was incubated for 30minutes at 37 degree Celsius. 250 micrometer of 2, 4-DNP was added in reagent blank. It was mixed and allowed to stand for 20minutes. 2.5 micrometer of each diluted sample (Blood, serum) was added. The absorbance was read (A_{sample}) against the reagent fblank after 5minutes.

3.5.4. Determination of the percentage organ-body weight ratio

The percentage organ-body weight ratio was obtained using the following expression:

$$\text{Percentage organ-body weight ratio} = \frac{\text{Organ weight x 100}}{\text{Total body weight of the animals}}$$

CHAPTER FOUR

4.0 Results

4.1 Phytochemical analysis

Qualitative analysis of solvent extract of *Vernonia amygdalina*

The findings of the phytochemical study carried out on the ethanolic extract of the *Vernonia amygdalina* indicated the presence of certain essential bioactive components. Qualitative analysis of solvent extract of *V.amygdalina* revealed the presence of saponins, flavonoids, alkaloids, quinone, phenols, terpenoids and coumarin while phlobatannis, antracyanine, steroids, ninhydrin, glycosides, tannins and carbohydrates were not detected. The highest were quinones and terpenoids while the lowest were saponin and coumarin (Table 2).

Table 2: Qualitative analysis of ethanolic extract of *Vernonia amygdalina*

PHYTOCHEMICAL TEST	**RESULT**
CARBOHYDRATES	-
TANNINS	-
SAPONINS	+
ALKALOIDS	++
FLAVONOIDS	++
GLYCOSIDES	-
QUINONES	+++
PHENOLS	++
TERPENOIDS	+++
CARDIAC GLYCOSIDES	-
NINHYDRIN	-
STEROIDS	-
Coumarin	+
Phlobatannins	-
Antracyanine	-

4.2 UV-spectroscopy analysis of ethanolic extract of *Vernonia amygdalina*

The ultra violet- visible spectroscopy of solvent extract of *V. amygdalina* revealed the varying absorbance of the plant extract at different wavelengths as shown in figure 8. The highest absorbance is 2.6 at 320nm while the lowest absorbance is 0.2 at 600nm

Figure 8: UV-Vis Spectroscopy analysis of ethanolic extract of *V. amygdalina* leaves.

4.3 Chromatogram of ethanoic extract of *V. amygdalina* leaves.

Figure 9 shows the GC-MS chromatogram of ethanoic extract of *V. amygdalina* leaves. Peak 7 with the retention time of 4.606 was identified as **1-Fluorooctane** (CH3(CH2)7F) (Table 3), as the major phytochemical component of *V. amygdalina* leaves while other peaks were of other phytochemical components present in the plant.

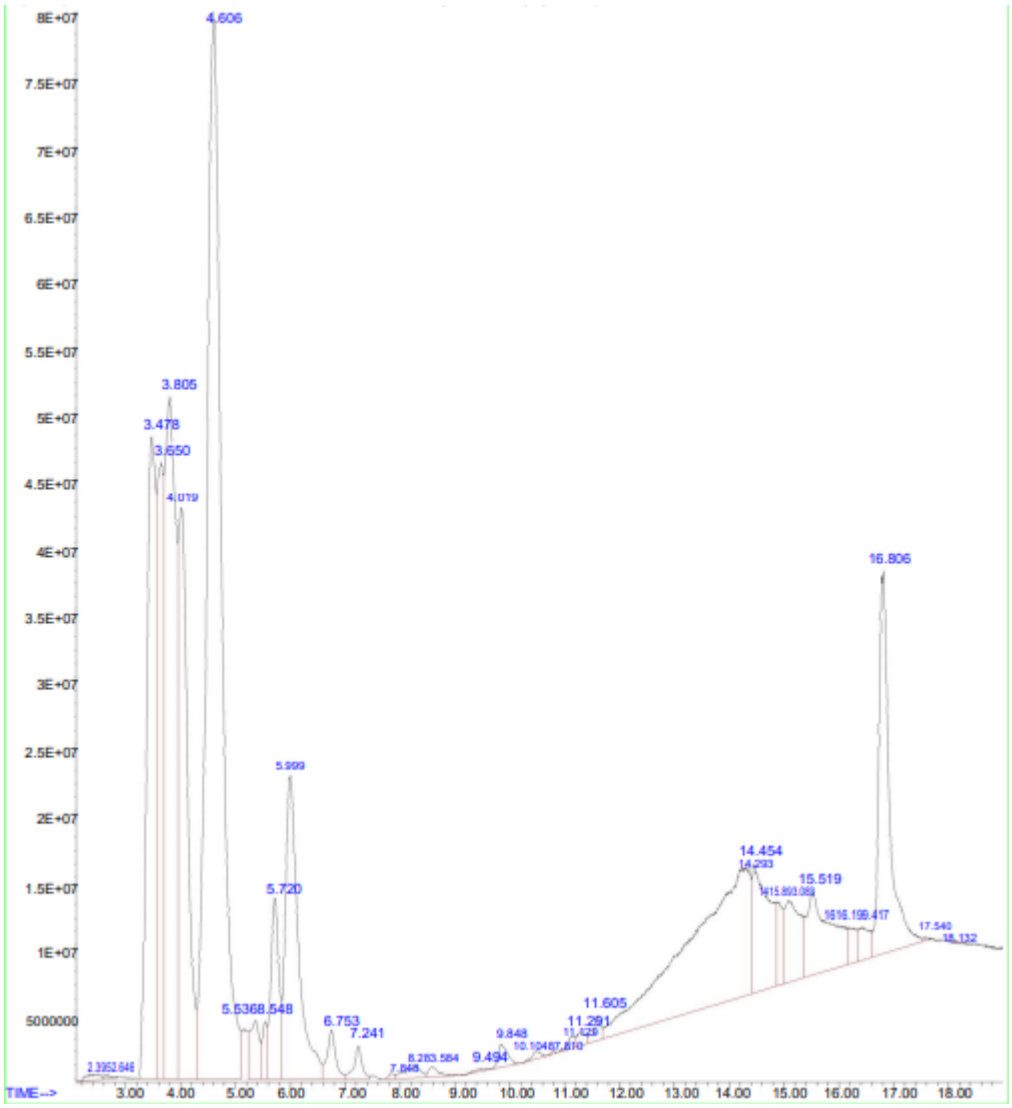

Figure 9: GC-MS chromatogram of ethanolic extract of *V. amygdalina* leaves.

Table 3: Phytochemical components of ethanolic extract of *V. amygdalina* leaves.

S/N	**Name of Compound**	**Retention Time**	**Area %**	**Chemical Formula**
1	Propanoic acid, 2-mercapto-methyl ester	2.395	0.13	C4H8O2S
2	3-Methylbenzofuran-2-carboxylic acid, 3-dimethylamino-1,2-dimethylpropyl ester	2.646	0.04	C17H23NO3
3	2-Hydroxyethyl vinyl sulfide	3.478	8.76	C4H8OS
4	Diethanolamine	3.650	6.00	C4H11NO2
5	2-Hexene, 5-methyl-, (E)-	3.805	11.55	C7H14
6	Thiirane	4.019	8.16	C2H4S
7	1-Fluorooctane	4.606	24.14	C8H17F
8	1-Butanol, 3-methyl-, acetate	5.368	0.84	C7H14O2
9	1-Butanol, 3-methyl-, acetate	5.548	0.41	C7H14O2
10	Peroxide, dimethyl	5.720	2.26	C2H6O2
11	Propanenitrile, 2-hydroxy-	5.999	6.14	C3H5NO
12	Pentane, 1-(1-	6.753	0.66	C9H20O2

	ethoxyethoxy)-			
13	Hexanoic acid, ethyl ester	7.241	0.42	C8H16O2
14	1-Pyrrolidinylacetonitrile	7.848	0.05	C6H10N2
15	Propane, 1,1,3-triethoxy-	8.283	0.28	C9H20O3
16	(.+/-.)-p-Methoxyamphetamine, N-trimethylsilyl-	8.584	0.16	C_{13}H23NOSi
17	1-Nonen-3-ol	9.494	0.02	C9H18O
18	Octanoic acid, ethyl ester	9.848	0.34	C10H20O2
19	p-Benzoquinone	10.487	0.10	C6H4O2
20	3,4-Pyridinediamine	10.810	0.02	C5H7N3
21	[1,1'-Bicyclopentyl]-2-one	11.129	0.13	C15H22O
22	-(1-Ethoxyethoxy) butyric acid, ethyl ester	11.291	0.18	C10H20O4
23	Decanoic acid, ethyl ester	11.605	0.26	C12H24O2
24	9-Octadecenoic	14.293	12.59	C18H34O2

	acid, (E)-			
25	Phthalic acid, 2-ethylhexyl tetradecyl ester	14.454	3.31	C30H50O4
26	Phthalic acid, 2-cyclohexylethyl ethyl ester	14.893	0.70	C18H24O4
27	Hexadecanoic acid, methyl ester	15.083	1.92	C17H34O2
28	Hexadecanoic acid, ethyl ester	15.519	3.16	C18H36O2
29	N-Acetyl-d-glucosamine	16.199	0.41	C8H15NO6
30	Cyclopentanecarboxylic acid, 1-methyl-3-oxo-, methyl ester	16.417	0.55	C8H12O3
31	Phytol	16.806	6.26	C20H40O
32	Urea, 1-(2,4-difluorophenyl) -3- [4- (3-methyl-5-trifluoromethyl pyrazol -1-yl) phenyl]-	17.540	0.03	C18H13F5N4O

4.4.1 Percentage relative organ-body weight ratio of letrozole-induced animals administered ethanolic extract of *Vernonia amygdalina* leaves

The relative kidney ovary weight ratio of letrozole induced animals treated with distilled water showed a significant decrease ($P>0.05$) while there was a significantly increase in the relative liver ratio of the animals. The relative liver, kidney, ovarian weight ratio of letrozole-induced animals administered 50 mg/kg body increased while there was a significant decrease in 100 mg/kg body weight of the extract (Table 4).The relative kidney weight ratio of letrozole-induced animals administered orally distilled and clomiphene citrate with metformin ($P>0.05$) whereas there was a significant increase in control animals. The relative ovarian weight ratio of letrozole-induced animals administered orally 50 and 100mg/kg body weight decreased ($P>0.05$) whereas there was a significant increase in control animals. The relative ovary weight of letrozole-induced animals administered orally 50 and 100mg/kg body weight compared favourably ($P<0.05$) with that of the control animals.

Table 4: Effects of ethanolic extract of *Vernonia amygdalina* leaves on some organ-body weight ratio of letrozole-induced female rats

	Organ-body weight ratio (%)		
	Liver	Kidney	Ovary
Control	2.67 ± 0.02[a]	0.45 ± 0.22[a]	0.05 ± 0.01[a]
PCOS + Distilled water	2.68 ± 0.06[a]	0.51 ± 0.02[b]	0.09 ± 0.01[b]
PCOS + CC + Metformin	3.26 ± 0.07[b]	0.58 ± 0.02[c]	0.06 ± 0.01[a]
PCOS +50mg/kg body weight of extract	2.78 ± 0.13[b]	0.50 ± 0.02[a]	0.06 ±0.02[a]
PCOS + 100mg/kg body weight of extract	2.60 ± 0.10[c]	0.49 ± 0.01[a]	0.05 ± 0.01[a]

Data are means of four determination + SEM. Values with different superscript are significantly different ($P<0.05$).

4.4.2 Effects of solvent extract of *Vernonia amygdalina* leaves on serum concentration of liver function indices: albumin, globulin and albumin: globulin ratios.

The result (Table 5) showed that serum albumin and globulin of letrozole-induced PCOS female rats treated with distilled water compared favorably ($P>0.05$) with control animals. Serum albumin level of letrozole-induced PCOS animals treated with 50 and 100 mg/kg body weight of the extract significantly increased ($P<0.05$) whereas there was a significant decrease in the control animals. Serum albumin levels of letrozole-induced PCOS animals treated with 50mg/kg body weight compared favourably ($P>0.05$) control animals Serum globulin levels of letrozole-induced PCOS animals administered orally with clomiphene citrate and metformin increased significantly ($P<0.05$) while there was a decreased in the letrozole-induced PCOS animals administered orally distilled water. Serum globulin levels of letrozole-induced PCOS animals that received 100mg/kg body weight increased significantly ($P<0.05$) while there was a significant decrease in the concentration level of letrozole-induced PCOS animals that received 50mg/kg body weight of extract.

Table 5: Effects of administration of ethanolic extract *Vernonia amygdalina* leaves on serum concentration of liver function indices: albumin, globulin and albumin: globulin ratios

Groups	Albumin (g/dl)	Globulin (g/dl)	Albumin: Globulin ratio (g/dl)
Control	6.81 ± 0.69^{a}	5.60 ± 1.01^{a}	1.28 ± 0.11^{a}
PCOS + Distilled water	6.84 ± 0.08^{a}	5.07 ± 0.15^{a}	1.36 ± 0.05^{a}
PCOS + CC + Metformin	7.12 ± 0.12^{a}	6.97 ± 0.11^{a}	1.02 ± 0.00^{a}
PCOS +50mg/kg body weight of extract	7.65 ± 0.09^{a}	6.23 ± 0.14^{b}	1.08 ± 0.11^{c}
PCOS +100mg/kg body weight of extract	7.80 ± 0.52^{b}	7.00 ± 0.45^{b}	1.01 ± 0.11^{c}

4.4.3 Effects of ethanolic extract of *Vernonia amygdalina* leaves on serum concentration of some liver function indices: total protein, total bilirubin and direct bilirubin

Serum total protein levels of letrozole-induced animals administered orally distilled water compared favourably ($P>0.05$) with control animals whereas there is a significant decrease in 100mg/kg body weight ($P<0.05$) and a significant increase in letrozole induced animals administered orally 50mg/kg body weight extract.

Serum total bilirubin levels of letrozole-induced animals orally administered distilled water increased significantly ($P<0.05$) whereas there was a decrease in letrozole-induced animals administered orally clomiphene citrate and metformin. Serum total bilirubin levels of letrozole-induced animals received 100mg/kg body weight extract increased ($P<0.05$) whereas there is a significant decrease in 100mg/kg body weight extract. Serum total bilirubin letrozole-induced animals orally administered clomiphene citrate and metformin compared favourably ($P>0.05$) with control animals.

Serum direct bilirubin levels of letrozole-induced administered orally metformin and clomiphene citrate increased significantly ($P<0.05$) whereas there is a significant decrease in letrozole-induced animals received distilled water. Serum total bilirubin levels of letrozole-induced animals administered orally 50 and 100mg/kg body weight increased significantly ($P<0.05$) whereas there is a significant decrease in letrozole-induced animals administered orally distilled water and control animals.

Table 6: Effects of administration of ethanolic extract *Vernonia amygdalina* leaves on serum concentration of some liver function indices total protein, direct bilirubin and total bilirubin

Groups	Total protein	Total bilirubin	Direct bilirubin
Control	1.22 ± 0.69^{a}	1.56 ± 0.55^{a}	5.01 ± 0.82^{a}
PCOS + Distilled water	1.77 ± 0.23^{a}	2.28 ± 0.28^{a}	1.29 ± 0.68^{c}
PCOS + CC + Metformin	0.16 ± 0.20^{b}	0.31 ± 0.16^{a}	8.56 ± 0.52^{b}
PCOS +50mg/kg body weight of extract	1.38 ± 0.22^{c}	1.24 ± 0.23^{b}	6.59 ± 1.37^{c}
PCOS +100mg/kg body weight of extract	0.78 ± 0.20^{c}	1.46 ± 0.45^{c}	6.19 ± 1.68^{d}

Data are means of four determination $\pm$ SEM. Values with different superscript are significantly different ($P< 0.05$).

4.4.4 Effects of ethanolic extract of *Vernonia amygdalina* leaves on serum concentration of some kidney function indices

Serum creatinine levels of letrozole-induced animals administered orally distilled water increased significantly ($P<0.05$) whereas there was a decreased in serum creatinine levels of letrozole-induced control animals. There is a significant increase in serum albumin levels of letrozole-induced animals administered orally 50mg/kg body weight ($P<0.05$) where as there is a significant decrease in letrozole-induced animals administered orally with a mixture of clomiphene citrate and metformin with that of the 100mg/kg body weight extract. There is a significant decrease in 100mg/kg body weight ($P<0.05$) whereas there is a significant increase in control animals (Table 7).

Serum urea levels of letrozole-induced animals administered orally clomiphene citrate metformin compared favourably ($P<0.05$) with control animals. Serum urea levels of letrozole-induced animals administered orally 50 and 100mg/kg body weight extract increased significantly ($P<0.05$) whereas there was a significant decrease in control animals. Serum albumin levels of letrozole-induced animals administered orally distilled water decreased significantly ($P<0.05$) whereas there is a significant increase in letrozole-induced animals administered orally clomiphene citrate and metformin, 50 and 100mg/kg body weight extract and control animals (Table 7).

Serum uric levels of letrozole-induced animals administered orally clomiphene citrate and metformin significantly increased ($P<0.05$) whereas there was a significant decrease in control animals. Serum uric levels of letrozole-induced animals administered orally 50mg/kg body weight extract ($P<0.05$) whereas there is a significant decrease in control animals and letrozole-induced animals administered orally 100mg/kg body weight extract

Serum BUN: Creatinine ratio of letrozole induced animals administered orally clomiphene citrate and metformin and also letrozole induced animals administered orally distilled water compared favourably ($P>0.05$) with control animals Serum BUN: creatinine ratio of letrozole induced animals administered orally 100mg/kg body weight of extract increased significantly and a decrease in 50mg/kg body weight ($P<0.05$) and also a significant decrease in control animals (Table 7).

Table 7: Effects of administration of ethanolic extract *Vernonia amygdalina* leaves on serum concentration of some kidney function indices

Group	Creatinine	Urea	Uric	BUN-creatinine ratio
Control	0.34 ± 0.38^{a}	1.05 ± 0.26^{a}	0.64 ± 0.00^{a}	2.98 ± 0.44^{a}
PCOS + Distilled water	0.53 ± 0.00^{b}	0.89 ± 0.00^{a}	2.27 ± 0.00^{b}	1.68 ± 0.00^{a}
PCOS + CC + Metformin	0.50 ± 0.00^{b}	3.27 ± 0.00^{a}	3.34 ± 0.00^{c}	6.54 ± 0.00^{a}
PCOS +50mg/kg body weight of extract	0.52 ± 0.03^{c}	2.90 ± 1.15^{b}	4.87 ± 0.48^{d}	3.90 ± 0.20^{b}
PCOS + 100mg/kg body weight of extract	0.04 ± 0.02^{d}	2.15 ± 0.30^{a}	1.78 ± 0.17^{b}	4.79 ± 1.51^{c}

Data are means of four determination $\pm$ SEM. Values with different superscript are significantly different ($P < 0.05$)

4.4.5 Effects of ethanolic extract of *Vernonia amygdalina* leaves on alkaline phosphatase concentration of letrozole-induced female rats

The administration of 50 and 100mg/kg body weight extract to letrozole-induced rats significantly increased the ALP activity in the serum ($P<0.05$) whereas there is a significant decrease the ALP activity of control animals and also an increase significantly (P. 0.05) letrozole induced animals administered orally distilled water (Figure 10).

The administration of 50 and 100mg/kg body weight extract to letrozole-induced rats significantly decreased the ALP activity in the liver ($P<0.05$) whereas there is a significant increase the ALP activity in the liver of control animals. The administration of clomiphene citrate and metformin to letrozole-induced rats significantly decreased the ALP activity in the liver ($P<0.05$) whereas there is a significant increased the ALP activity in the liver ($P<0.05$) whereas there is a significant increase in control animals (Figure 10)

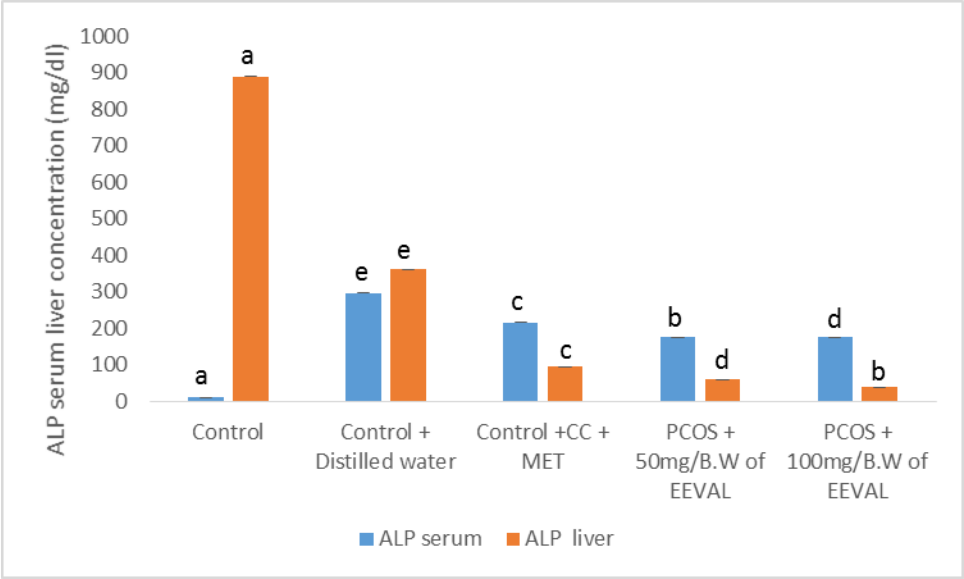

Figure 10: Effects of ethanolic extract of *Vernonia amygdalina* leaves on alkaline phosphatase concentration of letrozole-induced female rats

4.4.6 Effects of ethanolic extract of *Vernonia amygdalina* leaves on aspartate aminotransferase concentration on serum and liver of letrozole-induced female rats

The administration of 50 and 100mg/kg body weight extract to letrozole-induced rats significantly increased the AST activity in the serum ($P<0.05$) whereas there is a significant decrease in AST activity in serum of letrozole-induced animals administered orally distilled water and control animals. The administration of clomiphene citrate and metformin to letrozole-induced rats significantly decreased the AST activity in the serum ($P<0.05$) whereas there is a significant increase in AST activity in serum of letrozole-induced animals administered distilled water and control animals (Figure 11).

The administration of 100mg/kg body weight extract to letrozole-induced rats increased the AST activity in the liver ($P<0.05$) whereas there is a significant decrease in AST activity in the liver of letrozole-induced animals administered clomiphene citrate and metformin and also letrozole-induced animals administered 50mg/kg body weight of extract. The administration of distilled water to letrozole-induced rats significantly increased and also the control animals significantly increased the AST activity in the liver ($P<0.05$) whereas there is a significant decrease in AST activity in the liver of letrozole-induced rats administered orally clomiphene citrate and metformin and also letrozole-induced rats received 50mg/kg body weight extract (Figure 11).

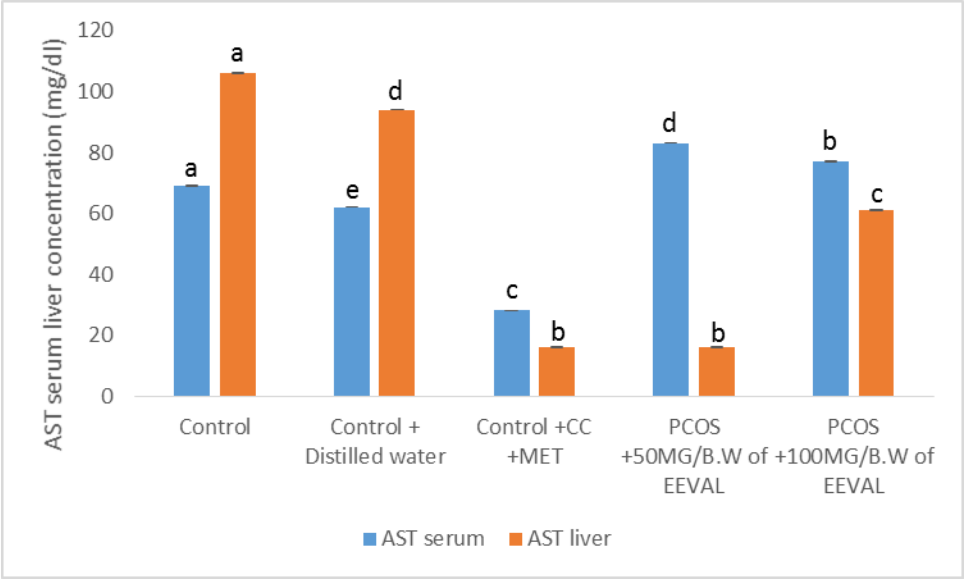

Figure 11: Effects of ethanolic extract of *Vernonia amygdalina* leaves on aspartate aminotransferase concentration of letrozole-induced female rats

4.4.7 Effects of solvent extract of *Vernonia amygdalina* leaves on alanine aminotransferase concentration on serum and liver of letrozole-induced female rats

The administration of 100mg/kg body weight of extract to letrozole-induced rats significantly increased the ALT activity in the serum ($P<0.05$) whereas there is a significant decrease in the ALT activity in the serum in letrozole-induced rats administered orally 50mg/kg body weight of extract and also control animals. The administration of clomiphene citrate and metformin to letrozole-induced rats significantly decreased the ALT activity in the serum ($P<0.05$) whereas there is a significantly increase in letrozole-induced animals administered orally distilled water (Figure 12).

The administration of distilled water to letrozole-induced rats significantly increased in the ALT activity in the liver ($P<0.05$) whereas there is a significant decrease in the ALT activity in the liver of letrozole-induced animals administered orally clomiphene citrate and metformin. The administration of 50 and 100mg/kg body weight extract to letrozole-induced rats significantly increased the ALT activity in the liver ($P<0.05$) whereas there is a decreased in the letrozole-induced rats administered orally clomiphene citrate and metformin (Figure 12).

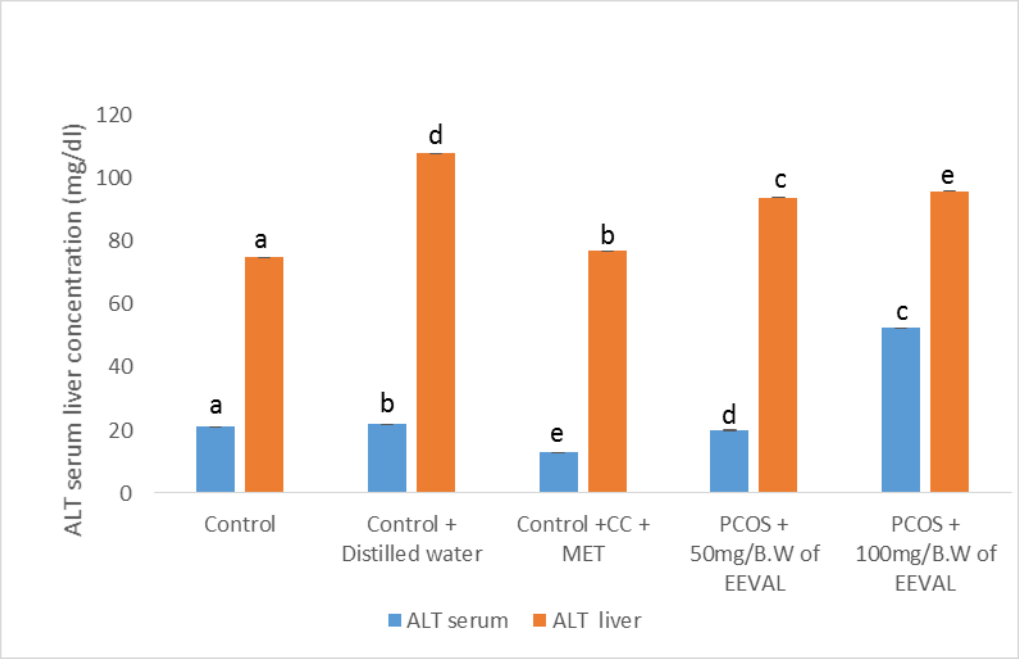

Figure 12: Effects of ethanolic extract of *Vernonia amygdalina* leaves on alanine aminotransferase concentration of letrozole-induced female rats

CHAPTER FIVE

5.1 DISCUSSION

Polycystic ovary syndrome is an endocrine system disorder which is also known as stein-leventhal syndrome (Smith, 2018). PCOS is a female endocrine condition marked by high androgen levels, ovulatory dysfunction, and polycystic ovarian morphology, and also a set of classic clinical features such as obesity, hirsutism, alopecia, acne, irregular menses, infertility, and high blood pressure (Schneider *et al.,* 2019). Medicinal plants, in general, contain bioactive chemicals that vary in kind and concentration both within and across species. Plants are potentially hazardous due to their chemical contents: as a result, certain plants employed in traditional medicine are fundamentally harmful (Merlin *et al.*, 2019).

The study investigated the toxicology effects of *Vernonia amygdalina* leaves on letrozole-induced polycystic ovarian syndrome in albino rats. Letrozole which is a non-steroidal aromatase inhibitor was used to induce PCOS orally. Clomiphene citrate belongs to the ovulatory stimulant class of drugs. It functions similarly to estrogen, a female hormone that allow eggs to grow and be released from the ovaries (Gupta and Khanna, 2018). Metformin belongs to a group of medicine known as biguanides. Metformin aids in the regulation of glucose levels in the blood. It reduces the amount of glucose you receive from food as well as the quantity of glucose your liver produces (Wang *et al.,* 2017). Clomiphene citrate is a more successful ovulation medicine, although metformin in combination with clomid can help some women who haven't ovulated after using clomid alone (Gill *et al.,* 2014). The toxicological evaluation of *Vernonia amygdalina* leaves on letrozole-induced polycystic ovarian syndrome in female wistar rats has provided additional information of its effects on the liver and kidney and organ body weight ratio of the animals. The importance of assessing the levels of albumin, globulin and bilirubin (Total and direct) in the serum of animals together with the administration of chemical compounds including this plant extract cannot be overemphasized as they are useful criteria for evaluating not only the secretory ability and or functional capacity of the liver (Femi-olabisi *et al.,* 2020).

Albumin, globulin and total bilirubin which make up the cell's protein content can be used to analyze the liver's functional capacity (Femi-olabisi *et al.,* 2020). Furthermore, changes in the concentration of albumin, globulin and bilirubin in animal serum indicate the liver's synthetic and secretory functions as well as the type of liver damage (Femi-olabisi *et al.,* 2020). The reduction in the level of computed albumin-globulin ratio may be as a result of inflammation, infection, chronic illness and liver disease which may lead to cancer or heart disease. The unaltered total protein level by letrozole was also maintained by all the doses of the extract suggest normal functioning of the live in relation to total bilirubin. This agrees with Ekam et al., (2012) there was increase in serum albumin and globulin compared to control animals. This increase in albumin and globulin levels may further corroborate the letrozole-induced related changes in the female rats. The elevated albumin level by letrozole was further aggravated by the administration of the extract suggesting a possible synergistic action between the drug and the extract in this study. This might be an indication that the extract also has the ability to further alter the biochemical changes initiated by the drug, Letrozole, in this instance. Overall, the letrozole-induced functional toxicity was further aggravated by the extract in the present study.

The kidney eliminates wastes such as uric acid, creatinine and ions ensuring that the optimal composition of body fluids is maintained (Taubert *et al.,* 2007). Blood urea and creatinine are thought to be significant contributors to renal dysfunction with higher levels of these metabolites in the blood indicating renal disease or renal damage associated with impaired kidney function (Shafee *et al.,* 2013). Renal function and physiologic reserve refer to the kidney's ability to not only filter an unwanted substance but also to reabsorb essential nutrients (Yakubu and Nurudeen, 2014). The elevated urea and uric acid of letrozole-induced animals that received 50 and 100mg/kg body weight of extract suggest that the extract at this dose might stimulate the synthesis of urea. The changes in urea and uric acid levels of letrozole-induced rays suggest that the glomerular filtration rate was affected by letrozole and the administration of 50 and 100mg/kg body weight induced glomerular filtration dysfunction. All these alterations by letrozole and extract implies an effect on the normal functioning of the kidney arising probably from the interference with the metabolic process of the metabolites, inefficient filtration by the

kidney and obstruction of the lower urinary tract, impaired glomerular and tubular reabsorption (Yakubu *et al.,* 2003). There was a significant decrease in creatinine levels of letrozole-induced ani,mals administered orally 50 and 100mg/kg body weight whereas there is a significant increase in control animals. The decrease may be due to reduced muscle, malnutrition which may lead to chronic kidney disease.

Alkaline phosphatase is an ectoenzyme of the plasma membrane and is used to check the integrity or damage to the plasma membrane (Femi-olabisi *et al.,* 2020). There is a significant decrease in the ALP activity in liver and serum upon administration of 50 and 100mg/kg body weight extract of letrozole-induced animals whereas there is a significant increase in control animals which is due to the inhibition of the enzyme activity at the cellular molecular level (Akanji *et al.,* 1993) This decrease agrees with Umezaba *et al.*, (1982) which indicate the inactivation of the enzyme molecule in situ.

AST activity levels in the serum of letrozole-induced animals administered 50 and 100mg/kg body weight extract decreased and an increase in control animals. The further mitigation of Liver AST by the administration of all the extract doses to letrozole-induced rats suggest enzyme inactivation while the increase in serum AST at 50 and 100mg/kg body weight extract suggest that these doses can elevate the diminished letrozole-induced AST activity and this could be due to contribution probably from other organ not investigated. The altered liver and serum ALT activity of animals administered letrozole suggest that the compound has no damaging effect on hepatocytes. The increase in the liver ALT in letrozole-induced rats administered all the doses of the extract shows that the extract affected ALT activity and this may be due enzyme inactivation and the reduction in the serum enzyme at 50 and 100mg/kg body weight of the extract may be due to contribution from other organ not investigated in the presence study.

5.2 Conclusion

In conclusion, *Vernonia amygdalina* aggravated letrozole-induced related increase in albumin, globulin, urea, uric direct bilirubin and serum alkaline phosphate and reduces liver alkaline phosphate, aspartate aminotransferase, total bilirubin and albumin: globulin ratio. Although they were changes in the size of the studied organs (liver), mild

alterations was evident in the Letrozole-induced female rats when compared to the control animals.

5.3 Recommendations

The result on this study reveals that further studies should be carried on the toxicological effect of *Vernonia amygdalina* leaves on Letrozole-induced female rats of which the long-term effect should be thoroughly investigated.

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APPENDIX

0.25M Sucrose Solution

171.15g sucrose was dissolved in 2 liters of distilled water.

Preparation of 7.14mg/kg of Letrozole

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. Therefore 7.1mg/kg 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 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.

Reagents for total and conjugated bilirubin

R1- 29mmol/l of sulphanilic acid and 0.17N hydrochloric acid

R2- 38.5mmol/l of sodium nitrite

R3- 0.26mmol/l of caffeine and 0.52mol of sodium benzoate

Reagent for Phosphatase

(i) Alkaline phosphatase (ALP)

Carbonate Buffer (0.1M, pH 10.1)

(a) 2.10g of sodium bicarbonate ($NaHCO_3$) was dissolved in 250ml of distilled water

(b) 7.2035g of sodium carbonate ($NaCO_3.H_20$) was dissolved in distilled water to make 250ml solution. Solution A was added to Solution B with continuous stirring until pH of the mixture comes to 10.1

Reagent for Aspartate aminotransferase (AST)

0.4N Sodium Hydroxide

16g of sodium hydroxide was dissolved in 1litre of distilled water in one litre volumetric flask.

Reagent R1 composition

Tris Buffer (pH 7.8) 110mmol/l

L-Aspartate 340mmol/l

Lactate dehydrogenase ≥ 4000 U/L

Malate dehydrogenase ≥ 750 U/L

Reagent R2 composition

CAPSO 20mmol/l

2-Oxoglutarate 85mmol/l

NADH 1.05mmol/l

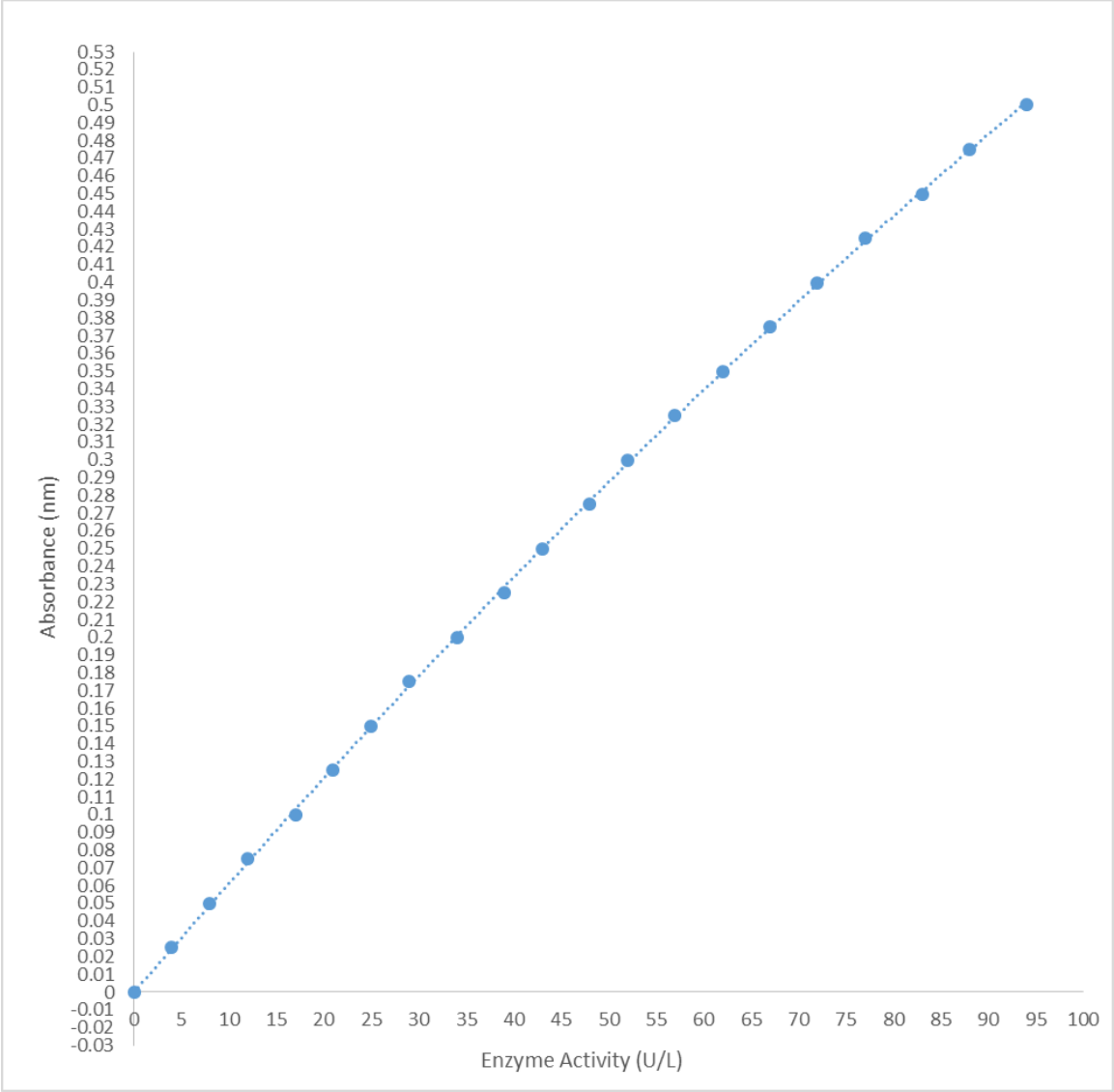

Figure 10: Calibration curve for alanine aminotransferase