# ANTI-INFLAMMATORY EFFECT OF ALPHA LIPOIC ACID AND OLIVE OIL ON LETROZOLE-INDUCED POLYCYSTIC OVARIAN SYNDROME IN RAT MODEL: A COMPARATIVE STUDY

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**A PROJECT SUBMITTED TO THE DEPARTMENT OF BIOCHEMISTRY, COLLEGE OF BASIC AND APPLIED SCIENCES, MOUNTAIN TOP UNIVERSITY, IBAFO, OGUN STATE, NIGERIA.**

**IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE AWARD OF BACHELOR OF SCIENCE (B.Sc.) IN BIOCHEMISTRY**

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**SEPTEMBER, 2022**

## CERTIFICATION

This is to certify that this project titled **"ANTI-INFLAMMATORY EFFECT OF ALPHA-LIPOIC ACID AND OLIVE OIL ON LETROZOLE-INDUCED POLYCYSTIC OVARIAN SYNDROME IN RAT MODEL: A COMPARATIVE STUDY"** was carried out by me, IGENE, Precious Evy, with matriculation number 18010102009. This project meets the requirements governing the award of Bachelor of Science (B.Sc.) Degree in Biochemistry, Department of Biochemistry of Mountain Top University, Ogun State, Nigeria and is approved for its contribution to knowledge and literary presentation.

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## DECLARATION

I, IGENE, Precious Evy, hereby declare that this research project titled **"ANTI-INFLAMMATORY EFFECT OF ALPHA-LIPOIC ACID AND OLIVE OIL ON LETROZOLE-INDUCED POLYCYSTIC OVARIAN SYNDROME IN RAT MODEL: A COMPARATIVE STUDY"** was written under the supervision of Dr F.J Femi-Olabisi is a product of my research work. Information derived from various sources has been duly acknowledged in the text and a list of references is provided. This project has not been previously presented anywhere for the award of any degree or certificate.

_____ _____

IGENE, Precious Evy Date

## APPROVAL PAGE

This project has been approved as having met the requirements of the Department of Biochemistry, College of Basic and Applied Sciences, Mountain Top University, Ibafo, Ogun State, Nigeria for the award of Bachelor of Sciences (B.Sc) degree in Biochemistry.

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## DEDICATION

This research is dedicated to God Almighty for giving me knowledge and understanding towards the completion of this report. I also dedicate this project to my parents, Mr S. G. & Mrs G. O. Igene for their timely support.

## ACKNOWLEDGEMENT

My deepest appreciation goes to God for the inspiration for this project work and also made it possible for it to be successful by His immeasurable mercies. I also want to thank the Igene family; Samuel Igene, Glory Igene, Marvellous Igene, Testimony Igene, Dominion Igene and my grandma, also my uncles; Victory Owedeh, Oke Owedeh, Obaro Owedeh, and Oreva Owedeh, and my aunty, Faith Eyube for their physical, financial, moral and spiritual support.

I also appreciate my project supervisor, Dr F.J. Femi-Olabisi, for encouraging me to do my best throughout this project work.

I acknowledge the HOD Biochemistry, Dr O. T. Kayode and other staff of the department; Dr Ayodele, Mrs I. O. Kolawole and Prof. G.O. Ajayi for their immeasurable support towards imparting knowledge to us and also preparing us for the outside world.

A big thank you to Mr Taiwo, the animal housekeeper and to all the laboratory technologists who lent my project colleagues and I support many times. I also want to acknowledge Mr Idoko Godwin and Mr Yemi Ogundele for their enormous support and encouragement.

A million thanks to my project colleagues in the same project group as mine; Oparandu Jeffrey, Bankole Isaac and Asaluwala Olamide. Thank you for working together with me as a team to make this project a success. It was fun working with you guys and I cannot wait to meet you guys at the top.

A billion thanks to my school daddy, Dr A. O. Young, and my Chaplain, Pastor Olumide Adesina for providing me with moral, spiritual, physical, and financial support.

To all members of the Student Representative Council (SRC) executives 2021/2022 set, Eunice Olawale, Olaiya Opemipo, Adeleke Wisdom, Oroja Sinmioluwa, Oyeyele Oyedamola, Thompson David, and many others that should have been mentioned, I am very grateful for being there for me. God bless you all.

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## LIST OF ABBREVIATIONS

**PCOS-** Polycystic Ovarian Syndrome

**FSH-** Follicle Stimulating Hormone

**LH-** Luteinizing Hormone

**PCOM-** Polycystic Morphology

**NIH-** National Institute of Health

**T2DM-** Type 2 Diabetes mellitus

**GnRH-** Gonadotropin Releasing Hormone

**SHBG-** Sex hormone binding protein

**LDL-** Low-density lipoprotein

**BMI-** Body mass index

**ASRM-** American Society for Reproductive Medicine

**ESHRE-** European Society for Human Reproduction and Embryology

**AES-** Androgen Excess Society

**PCO-** Polycystic Ovaries

**IR-** Insulin Resistance

**TNF-α-** Tumor Necrosis Factor-alpha

**IL-6-** Interleukin- 6

**CC-** Clomiphene Citrate

**OCP-** Oral contraceptive pills

**IVF-** *In-vitro* fertilization

**GnRHag-** Gonadotropin Releasing Hormone agonist

**RIA-** Radioimmunoassay

**SERM**- Selective Estrogen Receptor Modulator

**hCG**- human chorionic Gonadotropin

**DHLA**- Di-hydro-lipoic acid

**AMPK**- AMP-activated protein kinase

**ROS**- reactive oxygen species

**NF-Kb**- nuclear factor-kappa B (NF-kB)

**TACE-** TNF-converting enzyme

**HPO-** Hypothalamic-pituitary-ovarian

## ABSTRACT

Polycystic ovarian syndrome (PCOS) is a multi-symptom endocrine disorder that results from androgen excess, particularly testosterone, and ovarian dysfunction that disrupts HPO axis function. Women with PCOS possess an altered immune response system which presents a low-grade chronic inflammatory state in them. Serum cytokine levels in the letrozole-induced PCOS rats will be investigated after treatment with Alpha lipoic acid and Olive oil and the results compared with a healthy control group. Thirty-five rats were used for this experiment, each group consisting of seven rats. Group 1 was the control group, while Groups 2 – 5 were induced with 1ml/1kg of letrozole for 22 days. Drug administration for the rats distributed into five groups are as follows: the non-PCOS control group was administered 0.5ml of saline water, the PCOS control group was administered 0.5ml of distilled water, and three PCOS-induced groups were administered 2mg/kg of clomiphene citrate and 7.14mg/kg of metformin, 1mg/kg of alpha lipoic acid and 4ml/kg of olive oil respectively for 14 days. At the end of the experimental period, the rats were anaesthetized with diethyl ether and sacrificed by jugular puncture 25 hours after the last treatment. The serum Tumor Necrosis Factor (TNF-$\alpha$) and Interleukin (IL-6) levels of letrozole-induced polycystic ovarian syndrome rats were assayed for and the results were analyzed. It was reported in this study, that letrozole-induced PCOS Wistar rats possess high serum levels of TNF- $\alpha$ and IL-6. Alpha lipoic acid and Olive oil exert anti-inflammatory potentials against IL-6 released into the bloodstream compared favourably with metformin and clomiphene citrate. Olive oil showed a great anti-inflammatory potential against TNF- $\alpha$ release into the bloodstream. Therefore, it can be exploited as a management option in the treatment of PCOS.



**Keywords**: Alpha lipoic acid, Chemokines, Cytokines, Inflammation, Interferons, Interleukins, Interleukin-6, TNF Olive oil, PCOS, TNF- $\alpha$

# CHAPTER ONE

## 1.0 Introduction

Polycystic ovarian syndrome (PCOS), a multi-symptom endocrinopathy results from androgen excess, particularly testosterone, and ovarian dysfunction (Rosenfield *et al.*, 2016) disrupting the HPO axis function (Hochberg *et al.,* 2011). PCOS increases serious complications among females. One in five or six females experiences severe difficulties with infertility and unpredictable menstrual cycles (Torie, 2016). PCOS causes an imbalance in hormones such as prolactin, luteinizing hormone, follicle stimulating hormone, and gonadotrophin-releasing hormone (Marx *et al.*, 2003). Although PCOS has been known for more than 70 years, the diagnosis is still controversial and lacks a clear description (Yildiz *et al.*, 2012).

PCOS is among the most common metabolic/endocrine disorders in reproductive-aged women (Ding *et al.,* 2017). Women with hyperandrogenic phenotypes are more likely than healthy women of the same age to have type 2 diabetes mellitus, hypertension, and dyslipidemia at a younger age due to the syndrome's varied clinical presentations (Popovic *et al.,* 2019). Deficiencies in hypothalamic-pituitary feedback, elevated secretion of the luteinizing hormone (LH), early granulosa cell luteinization, abnormal oocyte maturation, and early arrest of activated primary follicles are all factors in persistent hyperandrogenism with typical clinical signs of hirsutism, irregular menstruation, persistent anovulation, and infertility (Palomba *et al.*, 2017). The metabolic features of this syndrome include insulin resistance, impaired glucose tolerance, type 2 diabetes mellitus, dyslipidemia, and cardiovascular risk factors.

Approximately 90% of non-ovulatory infertility cases are triggered by PCOS (Balen & Rutherford, 2007). For this reason, some scholars doubt PCOS is an evolutionary contradiction (Casarini *et al.,* 2016). Due to the heterogeneity and unpredictability of this disease, many academics have explored its pathogenesis and treatment in recent years, but is not fully understood and may be influenced by inheritance, environment, and internal embryonic factors (Ben & Younis, 2014).

The symptoms determine how PCOS in women should be treated. These could include menstrual disorders, androgen-related symptoms, or infertility caused by ovulatory disruption. These include Weight induction, Ovulation Induction, Metformin, Alpha Lipoic acid, Olive oil, etc.

Metformin improves ovarian androgen production, endometrial growth and the proliferation of the theca cells through several mechanisms, including lowering insulin levels and changing how insulin affects these processes (Sam *et al.,* 2003).

Alpha lipoic acid (ALA) is an organosulfur molecule produced naturally by humans, animals, and plants. Research has proven that alpha lipoic acid contributes to the insulin signalling cascade's activation. A handful of PCOS-afflicted women had appreciable changes in insulin, glucose, and BMI (400 mg daily) after administering ALA supplementation for 12 weeks. ALA enhances insulin synthesis, lowers testosterone, and controls menstrual cycles (Angela, 2020).

Olive, an ancient tree cultivar of the Mediterranean Area (Olea europaea; family Oleaceae), is the source of olive oil, a liquid fat. The fruit of the olive tree is pressed to get it. It naturally contains a lot of healthy monounsaturated fats and a little quantity of saturated fat (Olive Herb Co, 2022).

Extra Virgin Olive Oil is a kind of virgin olive oil with a perfect aroma and flavour and free acidity of ≤8% (Olive Herb Co, 2022). Higher production standards for this oil preserve a number of the oil's beneficial properties, including its anti-cancer polyphenols, antioxidants, and other bioactive compounds that also reduce inflammation and support healthy cholesterol levels (Eckelkamp, 2021).

The active organ, adipose tissue, secretes adipokines, hormones, and cytokines. It is linked to endocrine functions that control immune system function, inflammatory response, lipid and glucose metabolism, and reproductive ability (Lecke *et al.,* 2013). Excess central fat may cause second-rate chronic inflammation in PCOS-afflicted women, which is mediated by proinflammatory cytokines (Sathyapalan & Aktin, 2010).

Olive oil and ALA will be used in this study to treat letrozole-induced PCOS in rats. Also, the serum cytokine levels in letrozole-induced PCOS rats will be investigated after treatment and compared with a healthy control group.

## 1.1 Statement of the Problem

PCOS-afflicted women are afflicted with low-grade chronic inflammation caused by proinflammatory cytokines and insulin resistance (Tosatti *et al.,* 2020). The aim of this study is to observe the cytokine levels in letrozole-induced PCOS rats after treatment with Olive oil and Alpha lipoic acid.

## 1.2 Justification of the Study

In PCOS, an altered immunological response is present. Amato *et al.,* (2003) and Xiong *et al.,* (2011) demonstrate that serum fluid levels of TNF- $\alpha$ and IL-6 levels in PCOS patients are higher. Therefore, there is a need to study the anti-inflammatory potential of Olive oil and Alpha lipoic acid in letrozole-induced PCOS rats.

## 1.3 Aim of the Study

The aim of this study is to assess the anti-inflammatory potential of Alpha lipoic acid and Olive oil on inflammatory proteins, in this case, cytokines (IL-6 and TNF- $\alpha$) on letrozole-induced rats. Plasma cytokine levels will be evaluated using ELISA.

## 1.4 Specific Objectives

The purpose of this study is to:

- To induce PCOS in rats using letrozole
- To evaluate cytokine levels (Interleukin-6 and TNF- $\alpha$) of letrozole-induced PCOS rats

# CHAPTER TWO

## 2.0 Literature Review

### 2.1 Historical perspective of the polycystic ovarian syndrome

The history of PCOS is not properly documented in the Egyptian papyri, although there are hints in later ancient medical documents. However, "women whose menstruation is shorter than three days or is scant are strong, with a healthy complexion and a manly aspect; but they are not concerned with delivering children or becoming pregnant," writes Hippocrates (460 BC-377 BC) (Hanson *et al.*,1975).

Approximately 98–138 AD philosopher Soranus of Ephesus wrote, “sometimes it is typical to not menstruate at all... It is also normal in persons who possess a manly body type... we observe that majority of non-menstruating women are robust, like manly and sterile women” (Eastman *et al.*, 1991).

Ambroise Pare, a renowned Renaissance surgeon, and obstetrician (1510–1590 AD) made the most direct observation when he said, "Many women when their flowers are stopped, come to have a certain manly nature, whence they are called Viragines, meaning, stout or manly women; consequently, their voice is loud and bigger, like unto a man’s, and they become bear” (Par, 1634).

These claims, dated more than two millennia, describe a collection of PCOS-suggestive symptoms, such as irregular menstruation, male habits, sub-infertility, and obesity. They also use words to describe the disorder that nowadays would be equivalent to "occasionally" or "many," showing that it was widespread enough to warrant mention (Azziz *et al.*, 2011).

Another description of PCOS from 1721 states “Young married peasant women, moderately overweight and infertile, with two abnormally sized ovaries, rough, glittery and whitish, just like pigeon eggs” (Insler & Lunenfeld, 1990). Sclerocystic abnormalities in the ovary were first described in the nineteenth century, which resulted in significant awareness of the syndrome (Chereau, 1844). At the Association of Obstetricians and Gynecologists of 1935, Stein and

Leventhal presented their research work, discussing seven women with amenorrhea, hirsutism, obesity, enlarged ovaries, and multiple small cysts (Stein & Leventhal., 1935). This information provided a more detailed explanation of the syndrome (Szydlarska *et al.,* 2017).

A requirement for PCOS diagnosis that calls for gynecologic competence is the presence of polycystic ovaries, which are inconsistently associated with the symptoms that can be used to identify the illness (Goldzieher *et al.,* 1962). The inclusion of an abnormal secretion of gonadotropin as a secondary diagnostic tool for the syndrome was prompted by the 1970 documentation by the RIA that levels of LH in the blood and the ratio of LH to FSH were high (Yen *et al.,* 1970).

Shortly after, another research suggested that the hyperandrogenemia in hirsute amenorrheic women was of ovarian origin. Plasma-free testosterone was known to be a sign of hyperandrogenism in these women (Rosenfield *et al.,* 1972). In the 1980s, polycystic ovarian morphology (PCOM) was discovered to be caused by testosterone treatment in female-to-male transsexuals, thus the ultrasonographic criteria for the identification of PCOM were developed. (Futterweit *et al.,* 1986) (Franks *et al.,* 1989).

While this was going on, the syndrome's considerable insulin resistance was discovered to occur irrespective of obesity and was linked to hyperandrogenism (Barbieri *et al.,* 1983; Chang *et al.,* 1983; Dunaif *et al.,* 1989) The production of ovarian androgen is afterwards stimulated by insulin, (Barbieri *et al.,* 1986) particularly in conjunction with LH, according to in vitro investigations (Hernandez *et al.,* 1988; Cara *et al.,* 1988). These investigations suggested that an excess of ovarian androgen may be caused by hyperinsulinemia. A GnRH agonist (GnRHag) test, which promotes the coordinated activity of the ovarian follicle in response to endogenous LH and FSH production, revealed a previously undetected form of hyperandrogenism in phenotypes A and B of PCOS women in 1989, according to findings presented at the time (Robert & David, 2016).

Over time, the syndrome's metabolic, cardiovascular, and reproductive hazards have become more prominent, as it is of the most predominant endocrinopathies in reproductive-aged women and causes severe menstruation and fertility complications (Ehrmann, 2005).

## 2.2 Prevalence Criteria of Polycystic Ovarian Syndrome

The precise causes of PCOS are yet to be fully understood, but it is supposed that the genetic background (Jones & Goodarzi, 2016) is aggravated by lifestyle and environmental factors (Merkin *et al.,* 2016; Kulhan *et al.,* 2017) may specify an individual's tendency to this disorder. PCOS predominantly affects overweight/obese women and is frequently associated with metabolic syndrome and increased risk for infertility, cardiovascular disease, and endometrial cancer (Hardiman *et al.,* 2003).

The clinical diversity of the disorder, the conceivable age and ethnicity effects, the absence of a fitting and universal definition for PCOS, made it difficult to assess the worldwide incidence of PCOS.

Three groups have suggested the PCOS diagnostic criteria (Zawadski *et al.,* 1992; ESHRE, T. R., & ASRM-Sponsored PCOS Consensus Workshop Group, 2004; Azziz *et al.,* 2006) While there were uniformities present among these criteria, some significant differences exist causing the assessment of epidemiological studies on PCOS difficult (Sirmans & Pate, 2013). However, several studies have testified to a universal PCOS prevalence of 2% to 26% (Jalilian *et al.,* 2015) which is dependent on the diagnostic criteria, population, BMI, and sample size.

## 2.3 Polycystic ovarian syndrome

Polycystic ovary syndrome is a common endocrine disorder (Goodman *et al.,* 2015) named after the distinctive "cysts" that can develop after the aggregation of ovarian follicles on the ovaries, but it's vital to understand that this is only a symptom and not the actual cause of the disorder (Dunaif *et al.,* 2013). The aggregation of ovarian follicles into a cyst-like form was brought on by an imbalance in hormones. The frequently affected hormones are androgens, Estrogen, Progesterone, Testosterone, and Luteinizing Hormone (Kavitha & Thomas, 2018).

Polycystic ovarian syndrome (PCOS) is common in women and adolescents of reproductive age, brought upon by hormonal imbalance (Azziz *et al.,* 2004). It is identified and determined by a ring of androgen excess symptoms, ovarian dysfunction, and ultrasound-confirmed polycystic ovarian morphology. PCOS-afflicted women are presented with signs of ovarian dysfunction, metabolic abnormalities and hyperandrogenism. (Trikudanathan, 2015). High androgen levels

result in increased hair growth, acne, and disruption of the brain's ovulation-inducing signals, which makes ovulation irregular. Additionally, it makes the follicles accumulate (American College of Obstetricians and Gynecologists, 2015; American Society for Reproductive Medicine, 2003).

Insulin resistance and obesity are common among these women, which raises their risk of T2DM and glucose intolerance. High insulin levels make people hungrier and make them gain weight. Additionally, the body produces more androgens in the presence of excess insulin.

The ovaries may form tiny sacs (follicles) packed with fluid, nevertheless, they might not always release eggs. This problem is known as ovarian dysfunction, a disturbance of the ovaries' hormone-producing capacity that results in irregular ovulation and menstrual cycle issues (Mayo Clinic, 2020).

The "polycystic" form of the ovaries found in PCOS patients is triggered by the aggregation of ovarian follicles at different stages of maturity (Dewailly *et al.,* 2014). Ovarian follicles are not cysts, since they contain a single oocyte. Sadly, the confusion over the definition of the syndrome distracts from the underlying pathology of the condition

Accordingly, the 2012 National Institutes of Health Office for Disease Prevention-Sponsored Evidence-Based Methodology Workshop on Polycystic Ovary Syndrome, (Johnson, 2012) advised that a new name was needed for PCOS. This proposal was braced by professional associations. There is disagreement about what the different names for the disorder mean, and this leads to conflict with others (Escobar-Morreale, 2018). The issue with the suggested names is that existing definitions of PCOS comprise women who might not have elevated androgen levels and that PCOS women, particularly those of average weight and those with normo-androgenetic phenotypes, (Moghetti *et al.,* 2013) have no evidence at all of the insulin resistance, increased cardiovascular risk, or metabolic dysfunction. The most elegant answer would be to acknowledge Stein and Leventhal's initial observations (Stein & Leventhal., 1935) and continue to call PCOS after them (Idiculla, 2014). For decades, the phrase 'Stein-Leventhal syndrome' has been used to refer to PCOS, (Azziz, 2014) and it is recognized among health officials, patients, and other organizations.

## 2.4 Classification of the polycystic ovarian syndrome

The strict National Institutes of Health (NIH) criteria that were published in 1990 underlined the fact that PCOS is a metabolic/reproductive disorder by mandating the simultaneous presence of anovulation and hyperandrogenemia on either a biochemical or clinical level (hirsutism/acne) for the diagnosis. Due to a general certainty that these women's polycystic morphology (PCOM) was a distinctive finding, the exclusion of ovarian PCOM resulted in a relative number of clinical inconsistencies (Macut et *al.*, 2012).

Although recent studies have now amply shown that PCOM on ultrasound may be detected in roughly 20–30% of women who are normally ovulating but not hyper-androgenic, for a substantial number of doctors (particularly Europeans), this distinction was essential for the diagnosis. This misconception incited the conception of new diagnostic criteria in 2004 (Rotterdam), which analyzed the presence of all three characteristics (ovarian dysfunction, polycystic ovaries, and hyperandrogenism on ultrasound) and identified PCOS with two of the three criteria present (Macut et *al.*, 2012) (Table 1).

There are three alternative sets of diagnostic criteria and four possible sub-phenotypes for the syndrome. There are four different PCOS phenotypes currently recognized: phenotypes A, B, C, and D. To address a patient's presenting symptoms, identify tailored treatment goals, and prevent the syndrome's long-term health effects, a timely diagnosis and the identification of particular sub-phenotypes are crucial (Mumusoglu & Yildiz, 2020) (Table 1).

**Table 1 Diagnostic criteria for PCOS**

| NIH 1990 | Rotterdam 2003 | AE-PCOS society 2006 |
|---|---|---|
| Long-lasting anovulation | Oligo or anovulation | Biochemical and clinical evidence of hyperandrogenism |
| Hyperandrogenism | Hyperandrogenism<br>Polycystic ovaries | Dysfunction ovaries<br>Polycystic ovary morphology |

NIH-National Institute of Health, AE-PCOS-Androgen Excess & PCOS Society

Source: Diamanti & Legro (2006)

In 2012, the National Institute of Health (NIH) consensus panel (Lizneva et *al.,* 2016) proposed the phenotypic technique of classifying PCOS using the possible combinations of these criteria:

• Phenotype A: Hyperandrogenism, Ovarian dysfunction, and Polycystic ovaries (full-blown syndrome)

• Phenotype B: Hyperandrogenism and Ovarian dysfunction (non-polycystic ovary PCOS)

• Phenotype C: Hyperandrogenism and Polycystic ovaries (ovulatory PCOS)

• Phenotype D: Ovarian dysfunction and Polycystic ovaries (non-hyperandrogenic PCOS) (Macut et *al.,* 2012) (Table 2).

Compared to phenotypes C and D, hirsutism, obesity with irregular cycles, insulin resistance, dyslipidemia, and hepatic steatosis are more prominent in phenotypes A and B. They also have a greater potential for developing metabolic syndrome (Dr Radha, 2021).

Menstrual abnormalities, clinical and biochemical hyperandrogenism and a considerably higher BMI are all characteristics of PCOS women with phenotype A. Additionally, these women run the risk of having negative metabolic and cardiovascular outcomes and they run the danger of developing several follicles (Dr Radha, 2021).

Androgen levels are normal in the mildest form of metabolic syndrome and endocrine dysfunction in phenotypic D, which is a less severe kind (Dr Radha, 2021).

Phenotype-A is common in clinically examined populations, whereas phenotype-C is prevalent in unselected populations. PCOS patients presented to the clinics are more obese and hyper-androgenic with a more severe phenotype and are of advanced metabolic risks compared with patients with PCOS in unselected populations.

**Table 2 PCOS phenotypes, their definitions, and characteristics.**

| PCOS phenotypes | Definition | Characteristics |
|---|---|---|
| A | Full-brown PCOS | HA + OD + PCO |
| B | Non-polycystic ovary PCOS | HA + OD |
| C | Ovulatory PCOS | HA + PCO |
| D | Non-hyperandrogenic PCOS | OD + PCO |

HA-Hyperandrogenism, OD-Ovarian dysfunction, PCO-Polycystic ovaries

Source: Sachdeva et *al.*, (2019).

## 2.5 Etiology of polycystic ovarian syndrome

The actual cause of polycystic ovarian syndrome (PCOS) is unknown. However, aberrant hormone levels are suspected to be responsible for this (NHS, 2019).

Insulin resistance is a typical characteristic of PCOS caused partly by adipose tissue dysfunction (Goodarzi et *al.*, 2011). The body's tissues are resistant to insulin's effects in this situation. As a result, the body must create more insulin to compensate (NHS, 2019) This causes compensatory hyperinsulinemia, which keeps glucose levels normal but affects ovarian androgen production negatively (Goodarzi et *al.*, 2011).

Hyperinsulinemia appears to have done so primarily by causing a partial "outflow" from ovarian desensitization to luteinizing hormone (LH), with the result that ovarian steroids are hyperresponsive to LH (Ehrmann et *al.*, 1995; Nahum et *al.*, 1995; Willis et *al.*, 1996). The disorder is characterized by an over secretion of LH. LH is required for the production of gonadal steroidogenic enzymes and the secretion of sex hormones. As a result, PCOS is LH-dependent, and any medication or disease that lowers LH levels lowers ovarian steroidogenesis (Robert & David, 2016). Hyperinsulinemia also reduces the liver's ability to synthesize SHBG. As a result, the concentration of free testosterone in the serum rises, resulting in peripheral androgen activity (Leroith et *al.*, 1995).

Several PCOS-afflicted women possess an imbalance in certain hormones, including raised levels of testosterone, prolactin (only in some women with PCOS), and low levels of sex hormone-binding globulin (SHBG) (NHS, 2019).

Several research works also indicated familial hyper-androgenetic phenotype in PCOS, signifying maternal inheritance and hence the contribution of genetic factors, particularly genes governing steroid hormone biosynthesis (Prapas *et al.*, 2009). Furthermore, the reformed transcription of genes in PCOS mothers, that partake in androgens synthesis alters the androgen exposure level in the uterus (Xita & Tsatsoulis, 2006). The distribution of male-type fat in female offspring has been linked to increased LH secretion, altered thecal cell differentiation, and exposure of the fetus to an excess of androgen in the uterus, based on a hypothesis (Xita et *al.*, 2010). In addition, epigenetic changes and maternal nutrition influences fetal programming

(Xita & Tsatsoulis, 2006; Dumesic et *al.*, 2014). On the contrary, research has proven a proper aromatization of the placenta, when exposed to high levels of androgens from the mother, does not cause PCOS in the female fetus. (Dumesic et *al.*, 2014.) A suggestion was made on the genomic basis of hyperandrogenism in PCOS stating that genes involved in steroid synthesis especially cytochrome P450 are considered candidate genes in the pathophysiology of PCOS. These candidate genes have been studied to delineate their association with PCOS (Legro et *al.*, 1998).

### 2.6 Symptoms of Polycystic Ovarian Syndrome

There is a significant array of symptoms among PCOS-afflicted women and, for an individual., these might change with time (Balen *et al.*, 1995). The imbalance in the hormonal secretion from the HPO axis causes a variety of symptoms in them (Figure 1). There are three major characteristic symptoms of PCOS: Ovarian dysfunction, Hyperandrogenism, and Polycystic morphology.

#### 2.6.1 Ovarian Dysfunction

Normal cyclic menstruation precedes a normal ovulatory function (Fritz & Speroff, 2011). The duration of a menstrual cycle is between the first day of menstrual bleeding in one cycle and the first day of menses in the next cycle. The average menstrual cycle is 28 days long, with the majority of cycles lasting 25 to 30 days (Presser, 1974). Menstruation at regular intervals strongly suggests but is not proof of, ovulation (Obgyn Key, 2016).

The vast majority of PCOS-afflicted women (60-85%) have severe menstrual dysfunction (Obgyn Key, 2016). Oligomenorrhea and amenorrhea are the most common abnormalities. Polymenorrhea refers to menstrual cycles that occur at intervals of less than 25 days, whereas oligomenorrhea refers to menstrual cycles that last more than 35 days (Hallberg et *al.*, 1966).

It is observed in less than 2% of untreated PCOS women. Menstrual dysfunction in women with PCOS typically begins before menarche, but many reports regular cycles for varying intervals before the onset of oligo/amenorrhea (Fritz & Speroff, 2011).

Anovulatory women rarely experience regular menstruation in general. However, anovulatory hyperandrogenic women tend to have somewhat more frequent regular cycles (Jennifer & Jack,

2019). In studies of menstrual function in women with hyperandrogenism, approximately 15-40% are eumenorrheic, despite evidence of oligo-anovulation. The incidence of eumenorrhea among PCOS-afflicted women is significantly increased with the application of the Rotterdam diagnostic criteria due to the inclusion of hirsute eumenorrheic women with polycystic ovaries (Obgyn, 2016).

### 2.6.2 Hyperandrogenism

In women with PCOS, hyperandrogenism is clinically manifested as acne, hirsutism, and androgenic alopecia. Other indicators of increased androgen levels include menstrual irregularities, weight gain, and insulin resistance (Ashraf et *al.*, 2010).

Hirsutism, one of the primary symptoms of PCOS hyperandrogenism, is defined as the presence of terminal hair in a masculine pattern on the face and/or body. In PCOS women, the prevalence of hirsutism ranges between 60% and 80% (Azziz et *al.,* 2009; Pasquali & Gambineri, 2014; Mara *et al.,* 2016; Keen et *al.,* 2017). The grading of hirsutism varies according to the ethnicity of the population. Hirsutism in PCOS women is caused by increased free testosterone levels in the blood and a more active form of testosterone. Hirsutism is the most consistent and dependable symptom used to assess clinical hyperandrogenism (Ashraf et *al.,* 2010). Ferriman and Gallwey (Ferriman & Gallwey, 1961) described the Ferriman-Gallwey (FG) score, as a visual scoring method for clinically assessing the grade of hirsutism.

According to the FG score, hair is scored in nine areas of the body, including the upper lip, chin, chest, upper and lower back, upper and lower abdomen, and upper and lower limbs. To determine the level of hirsutism, these nine body parts are scored from 0 to 4, with 0 representing a total absence of terminal hair and 4 representing extensive hair growth. The summation of the scores from all nine areas yields the final score used for diagnosis. Women with an FG of 8 or higher are considered hirsute (Asuncion et *al.,* 2000).

Acne is the second most common symptom of hyperandrogenism. Acne prevalence varies by ethnicity. Other studies have estimated the incidence of acne in PCOS patients to be 9.8-34 per cent (Jones et *al.,* 2004; Azziz et *al.,* 2005). Acne is caused by inflammation of the pilosebaceous glands. Increased testosterone promotes the production of a more potent form of dihydrotestosterone, causing abnormal desquamation in the follicular epithelial cells and an

increase in sebum production in sebaceous glands. The bacterium Propionibacterium acnes colonizes this accrual of epithelial and sebum cell debris, resulting in acne. Acne is classified as mild, moderate, or severe by the World Health Organization. Comedones and papules are examples of mild acne, pustules are examples of moderate acne, and nodules, cysts, and scars are examples of severe acne Tutakne et *al.*, 2003). Acne is commonly seen on the face, upper back, neck, and pectoral regions, and its severity varies from person to person.

Another symptom of a hyperandrogenic condition affecting PCOS women is androgenic alopecia, also known as male pattern baldness. Alopecia seems to be common in PCOS, ranging from 3.2 to 34.8 per cent in various populations (Azziz et al., 2004; Carmina et *al.*, 2006; Ozdemir et *al.*, 2010). It is distinguished by miniaturization, in which the growth phase of anagen is shortened by mature terminal hair on the scalp area and gradually transforms into fewer, finer vellus hair (Lee & Zane, 2007). On the one hand, females with PCOS struggle with excessive facial hair growth, while on the other hand, they struggle with thinning scalp hair. This is due to the high levels of testosterone in PCOS women, which causes hair loss in males.

The hair follicle in PCOS-afflicted women remains active with androgenic alopecia, increasing the likelihood that hair will regrow through hair therapy in them. Their anterior hairline is also usually intact with hair loss in the anterior mid-vertex area, with postero-lateral extension to the crown as a "triangular" patch (Ashraf et *al.*, 2009).

### 2.6.3 Polycystic Morphology

Gynaecologists suggest that PCOM is a very strong factor for PCOS diagnosis, whereas according to endocrinologists it constitutes a supportive but not a definitive tool for diagnosis (Macut et *al.*, 2013). The confirmation of a polycystic ovary is defined as an ovary with 12 or more follicles measuring 2–9 mm in diameter and/or increased ovarian volume (>10 $cm^3$) (Balen et al., 1995). Furthermore, several reproductive endocrinologists advocate for different criteria in clinical practice, such as >20follicles per ovary or a higher stroma/total area ratio, whereas medical imaging specialists use outdated criteria (stroma/follicles pattern) due to their extensive knowledge of them (Macut et *al.*, 2013). The majority of PCOS adolescents with PCOM have either a mother with asymptomatic polycystic ovary syndrome or a father with metabolic syndrome (Leibel et *al.*, 2006).

About 20% of reproductive-aged women have PCOM without any other signs of PCOS (hyperandrogenism and anovulation), and this percentage is even higher in adolescents, where 40-50% of girls have PCOM, which resolves over time (Macut et *al.*, 2013). On the one hand, approximately half of asymptomatic young women with PCOM do not have biochemical evidence of dysfunction in ovarian androgens and thus represent a variation of normal. On the other hand, half of the PCOM asymptomatic women have biochemical evidence of ovarian androgenic dysfunction, and nearly half of these have biochemical hyperandrogenemia, implying ovulatory PCOS despite being eumenorrheic and otherwise normal (Rosenfield, 2015).

Ovarian morphology, while required for PCOS diagnosis and reinforced by the Rotterdam criteria, necessitates caution. An experienced and meticulous ultra-sonographer who is well-versed in current criteria is required. A detailed report detailing the number of follicles and ovarian volume should always be requested for optimal results (Macut et *al.*, 2013).

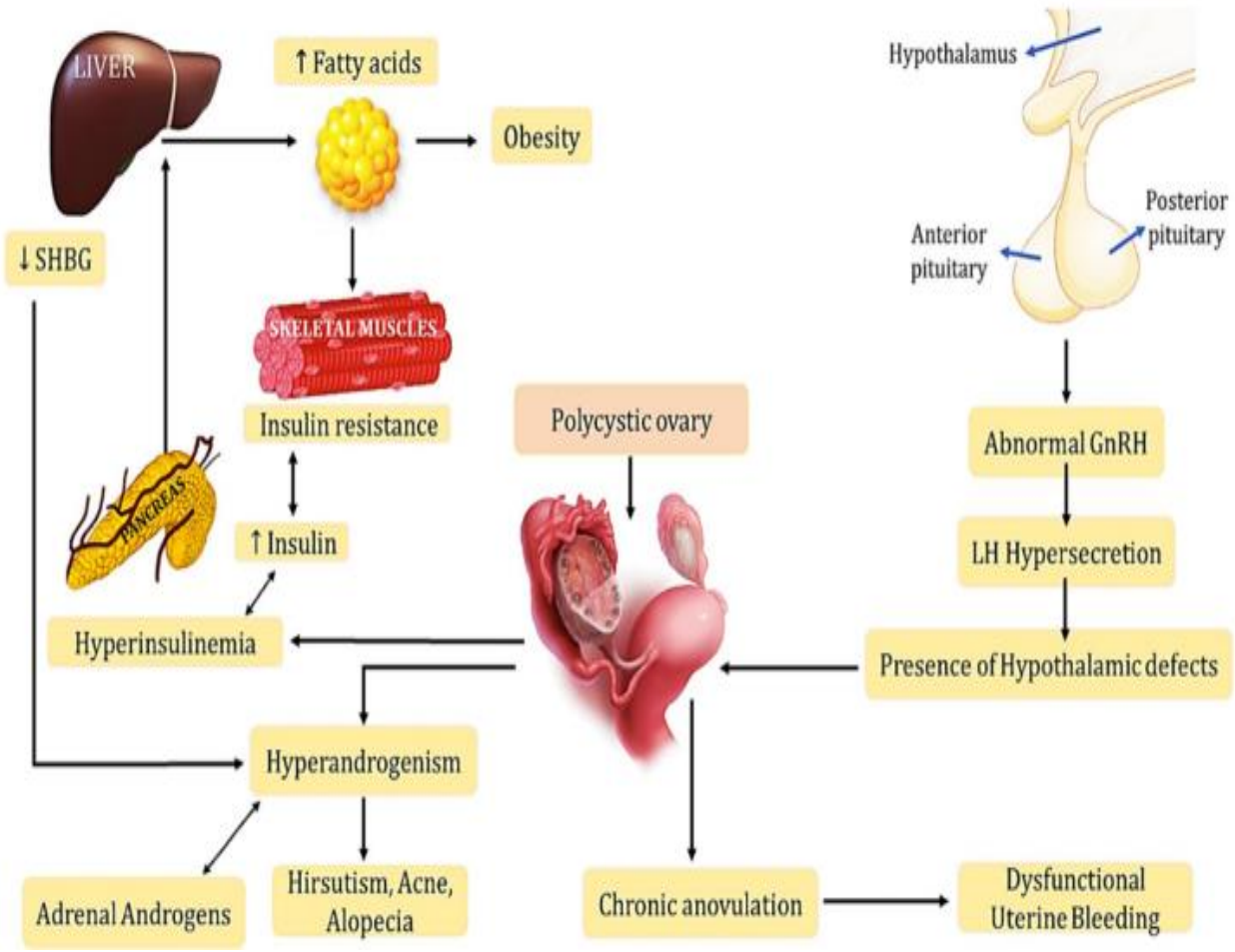


Figure 1: Implications of Polycystic Ovarian Syndrome in women's life

Source: Chaudary *et al.*, (2021)

## 2.7 Diagnosis of Polycystic Ovarian Syndrome

The criteria used in all current classifications of PCOS are the same. It is only the combination of criteria that is disputed (Zawadski & Dunaif, 1992). Diagnosis is based on the varying presence of 3 specific elements; oligo-anovulation, androgen excess, either clinical or biochemical., and the ultrasound assessment of ovarian morphology (Hoeger et *al.*, 2021). However, the accuracy of the PCOS diagnosis is proportional to the accuracy of the evaluation techniques for assessing the individual criteria, so every effort should be made to use the most appropriate methodology available (Escobar-Morreale, 2018).

In 2003, the Rotterdam European Society for Human Reproduction/American Society of Reproductive Medicine (ASRM)-sponsored PCOS consensus workshop group revised the syndrome's diagnostic criteria, establishing the following criteria: oligo/amenorrhea, clinical and biochemical signs of hyperandrogenism, and sonographically confirmed PCOS. Two of the three criteria are required for diagnosis; Sonographic features of PCOS include the presence of 12 or more follicles in each ovary measuring 2–9 mm in diameter and/or increased ovarian volume (>10 mL). An ovary in line with this definition is enough to define PCOS (Azziz, 2005; Azziz et *al.*, 2006). Some women with PCOS sonographic findings may have regular cycles without clinical or biochemical signs of hyperandrogenism. Though this is an applied functional definition of PCOS, others believe hyperandrogenism should be a fundamental part of the definition (Badawy & Elnashar, 2011).

In 2006, the Androgen Excess Society (AES) indicated that PCOS is a hyper-androgenic disorder and that the presence of hirsutism and/or hyperandrogenism constitutes a sine qua non for the diagnosis of PCOS (Azziz et *al.*, 2009). The second criterion necessary for diagnosis according to AES is either anovulation or Polycystic ovary morphology. These criteria were further consolidated in 2009 by the Androgen Excess and PCOS Society Task Force statement. Despite the presence of these several definitions, PCOS is still an excluded diagnosis of other androgenic entities. Furthermore, none of these definitions has been verified in adolescents in whom anovulation, PCOM, and hyperandrogenism usually occur for a temporary period (Arnold, 2017).

Ovulatory PCOS women appear to be less resistant to insulin than anovulatory PCOS women (Adams et *al.,* 2004). These observations limit the Rotterdam criteria's usefulness, so the Androgen Excess Society (AES) suggested that PCOS be considered an androgen excess disorder and that the NIH diagnostic criteria be used (Azziz et *al.,* 2006). The AES also suggested that women with PCOS, hyperandrogenism and ovulatory cycles be considered to have a phenotype of PCOS. Consequently, hyperandrogenism and ovulatory dysfunction, as well as hyperandrogenism, regular ovulation, and PCOS meet AES criteria for PCOS (Badawy & Elnashar, 2011).

Unless and until a broad consensus is attained on the labelling of the various PCOS phenotypes, a reasonable substitute might be to include a comprehensive description of the specific PCOS criteria matched by the individual patient as well as the comorbidities present in her specific case. For example, the consequences of a PCOS diagnosis (hirsutism, hyperandrogenemia, and oligo-ovulation) with obesity, insulin resistance, impaired glucose tolerance, and hypercholesterolemia, are vastly different from those of a PCOS diagnosis (mild oligomenorrhoea and PCOM with no evidence of androgen excess) without obesity or metabolic complications, and patients with either diagnosis meet the PCOS diagnostic. This approach may assist patients in developing realistic expectations about their disorders and may assist practitioners in guiding patients' long-term management.

### 2.8 Management options for the polycystic ovarian syndrome

Several guidelines have been offered for the therapy of PCOS-related complications such as obesity, type 2 diabetes mellitus, and all related disorders associated with metabolic syndrome, as well as psychological distress.

#### 2.8.1 Lifestyle interventions

Changes in diet, exercise, and psychological interventions aimed at improving weight can help minimize metabolic disorders associated with PCOS. This is the first line of defence in PCOS control. Increased physical activity, a healthier diet, and weight loss can all assist to minimize the metabolic irregularities associated with PCOS. Weight loss of 5-10% has been reported to treat oligo-anovulation and enhance pregnancy outcomes in PCOS-afflicted women (Bakir *et al.,*

2021). Exercise has been studied as a therapeutic in PCOS (Dos Santos *et al.*, 2020). Most exercise intervention studies are small and include a variety of exercise interventions such as aerobic, resistance, and combination exercise (Hoeger *et al.,* 2021).

The existing data suggests that a weight-loss lifestyle intervention has a good effect on hyperandrogenism and metabolic characteristics of PCOS, and also on the quality of life; however, there is less support for improved reproductive and fertility outcomes (Hoeger *et al.*, 2021).

### 2.8.2 Metformin

Metformin has the greatest data on improving menstrual cycles, glucose levels, and obesity in PCOS, mild-to-moderate reduction of insulin resistance, and minimal-to-moderate effect on altering lipid profile, hence triggering ovulation (Naderpoor *et al.*, 2015). It is inexpensive, widely available, and has a low risk of unwanted effects. The most prevalent metformin side effects are gastrointestinal symptoms, which should be stressed during patient consultation to ensure drug adherence (Hoeger *et al.,* 2021). The new recommendation recommends combining clomiphene citrate with metformin as a novel treatment, particularly in overweight or obese women with PCOS. This advice is also applicable to teens (Hoeger *et al.,* 2021). A combination of metformin and clomiphene citrate (CC) therapy has been studied and proven to be more effective than inducing ovulation alone (Bakir *et al.,* 2021).

### 2.8.3 Clomiphene citrate (CC)

As a selective estrogen receptor modulator (SERM), Clomiphene citrate (CC) prevents the brain from receiving estrogen signals, causing it to release more follicle-stimulating hormone, which triggers ovulation. Due to the numerous antral follicles found in polycystic ovaries, CC therapy is linked to an increased chance of multi-gestational pregnancy. Even though most women will ovulate while taking CC, only 50% of them will become pregnant because of the anti-estrogenic effects of clomiphene, which cause the endometrium to shrink. Furthermore, CC therapy should only last 12 cycles because longer-term therapy increases the risk of ovarian cancer owing to ovarian hyperstimulation (Bakir *et al.,* 2021).

### 2.8.4 Estrogen and progestin oral contraceptive pills (OCPs) therapy

It is applied in the treatment of acne, hirsutism and irregular menstrual cycles. Oral contraceptive pills medication can be used to regulate menstrual cycles, normalize androgen levels, and lessen the symptoms of hyperandrogenism. Additionally, it aids in lowering the likelihood of heavy and irregular menstruation brought on by a drop in hormone levels of progesterone and estrogen (Bakir *et al.,* 2021).

### 2.8.5 Anti-androgens

Spironolactone, finasteride, and flutamide are examples of anti-androgens that have been used to treat acne and hirsutism. By interacting with their receptors in peripheral cells, spironolactone and flutamide competitively suppress testosterone and dihydrotestosterone (e.g., hair follicles). Finasteride is a 5a-reductase inhibitor that prevents peripheral cells from turning testosterone into the more powerful dihydrotestosterone. Oral contraceptive pills, which centrally decrease androgen secretion, can be used in conjunction with anti-androgens. Since anti-androgens are teratogens, they should not be used during pregnancy (Bakir *et al.,* 2021).

### 2.8.6 Gonadotropin therapy

In instances where clomiphene citrate and metformin are not effective, recombinant follicle-stimulating hormone (FSH) and human chorionic gonadotropin (hCG) can be used to induce ovulation. It is possible to inject exogenous gonadotropins to simulate the physiological processes of follicle formation. A dominant follicle is administered FSH to help it expand to a specific size, and then human chorionic gonadotropin is utilized to trigger ovulation. To reduce the dangers of multiple pregnancies and ovarian hyperstimulation, this therapy must be continuously monitored with imaging and laboratory tests (Bakir *et al.,* 2021).

### 2.8.7 In-Vitro Fertilization (IVF)

This is used to treat infertility in women who have not had success with other ovulation-inducing treatments. In IVF, eggs are removed from the ovaries and combined in vitro with sperm to create embryos. Viable embryos are then put into the uterus. Compared to women without

PCOS, PCOS-afflicted women have comparable success and live birth rates. Ovarian hyperstimulation as a result of gonadotropin therapy, which is administered before oocyte retrieval to stimulate follicular development, and multi-gestational pregnancy due to the transfer of several embryos are risks of the technique (Bakir *et al.,* 2021).

### 2.8.8 Surgical interventions

Bariatric surgery is an excellent treatment for obesity and PCOS symptoms, especially after all other treatment options have failed and should be made available to individuals who are extremely obese (Oritz-Flores *et al.,* 2018). The hazards, however, include nutritional and surgical issues, and pregnancy should be avoided for a full year after the procedure (Hoeger *et al.,* 2021).

To rebalance and enhance ovarian function in PCOS, ovarian tissue is damaged with a surgical needle or a laser beam during a minimally invasive surgical procedure known as laparoscopic ovarian drilling (Hoeger *et al.,* 2021). It entails making 10 or more perforations with a laser or cautery in the ovary. It's believed that ovarian theca ablation causes ovulation by reducing androgen production. It can be applied in the treatment of anovulation that is resistant to clomiphene citrate (Bakir *et al.,* 2021). Compared to medical ovulation induction alone, this drilling may lower the live birth rate in PCOS anovulatory women and clomiphene citrate resistance (Bordewijk *et al.,* 2020). Remember that women who undergo laparoscopic ovarian drilling are still vulnerable to the dangers of surgery, including anaesthesia-related issues, infection, and adhesions (Hoeger *et al.,* 2021).

## 2.9 Letrozole

Letrozole with the chemical name 4,40-[(1H-1,2,4-triazol-1-yl) methylene] bis-benzonitrile, is an aromatase inhibitor responsible for the conversion of androgens into estrogens, lowering estrogen production in the body by 97% to 99%, thereby increasing the production of follicle-stimulating hormone (FSH) (USADA, 2021; Kar, 2013) (Figure 2). The additional levels of FSH stimulate ovarian follicle(s) to develop and mature in the ovary and help to correct hormonal imbalances which prevent women from ovulating. It is a fertility medication for patients with PCOS (CNY Fertility, 2021).

In the early 2000s, fertility specialists recognized the estrogen-lowering mechanism of letrozole and began exploring its potential in the treatment of infertility (Pavone & Bulun, 2013). Research has shown that Letrozole can help significantly increase pregnancy and live birth rates for individuals with PCOS (Roy et *al.,* 2012; Banerjee et *al.,* 2012). Those with PCOS commonly use Letrozole to induce ovulation in combination with different fertility treatments to increase their chances of getting pregnant.

Constant administration of letrozole to female rats induces PCOS-associated metabolic and endocrine/reproductive abnormalities at adult age (Maliqueo, et *al.,* 2013; Kafali et *al.,* 2004).

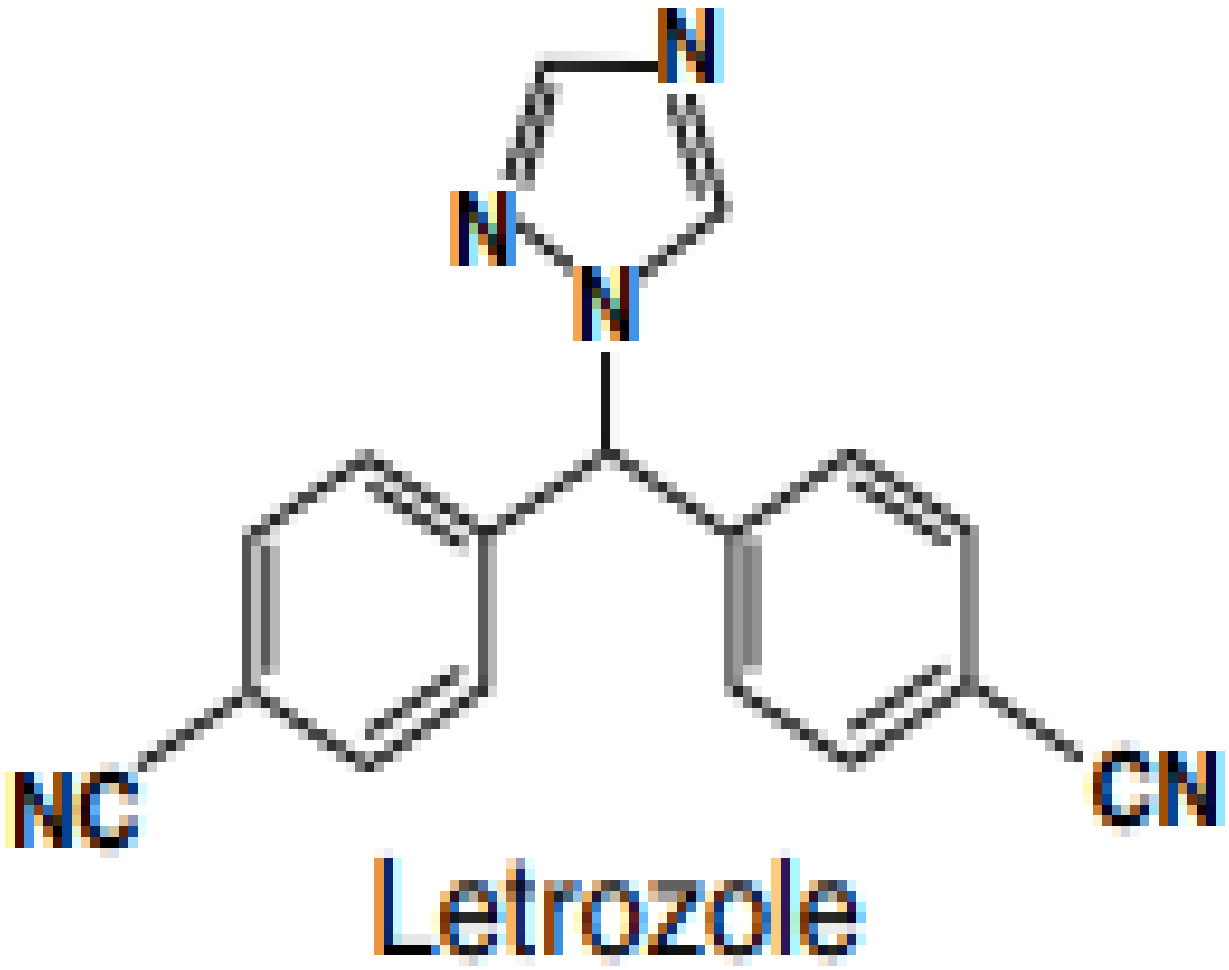


Figure 2: Molecular structure of the aromatase inhibitor, Letrozole.

Source: Ajayi (2007)

## 2.10 Alpha-Lipoic Acid (ALA)

Alpha lipoic acid (also known as Lipoicin or Thioctan) is a fatty acid synthesized by the body from cysteine, an amino acid (Anna et *al.,* 2021; Jillian, 2022). It has an impact on the eyes, brain, and nervous system as it is a fat-soluble substance that can pass across the blood-brain and hemostatic barriers (Gomes & Negrato, 2014; Ciudin et *al.,* 2017). Through aerobic metabolism, glucose is predominantly converted into energy with the help of oxygen. Its amphipathic structure, which is made up of an eight-carbon chain with two oxygen atoms in the carboxylic group and two sulfur atoms in the terminal section, allows it to serve as a biological carrier of electrons (Anna et *al.,* 2021).

ALA can be found in nature in two different states: reduced (dihydrolipoic acid, or DHLA) (Figure 3), and oxidized (cyclic disulfide) (Figure 4). Through redox processes, these forms can be changed into one another (Solmonson & DeBeradinis, 2018).

Since Burt Berkson employed ALA in diabetic animal models in the 1970s, ALA has been used in therapeutic settings. It considerably reduced the unpleasant symptoms brought on by untreated diabetes complications. After that, he concluded that it might be helpful for diabetic neuropathy (Andreea Rotaru, 2020; Agathos et *al.,* 2018).

ALA is also an antioxidant, neutralizing free radicals that damage cells at the genetic level. It is also available in certain foods and as a supplement (Jennifer et *al.,* 2022). It functions as an antioxidant by neutralizing free radicals in the cytoplasm and cell membrane when it is in its reduced form. Due to its interaction with the nuclear transcription factor (NF-kB), it reduces the release of free radicals and cytotoxic cytokines (Anna et *al.,* 2021).

Additionally, ALA promotes glucose uptake by stimulating the redistribution of glucose transporters to the plasma membrane and tyrosine phosphorylation of insulin receptor substrate increasing sugar absorption in both insulin-sensitive and insulin-resistant muscle tissues as demonstrated by Konrad and colleagues. It activates AMPK, a protein kinase that enhances the cell's ability to absorb glucose.

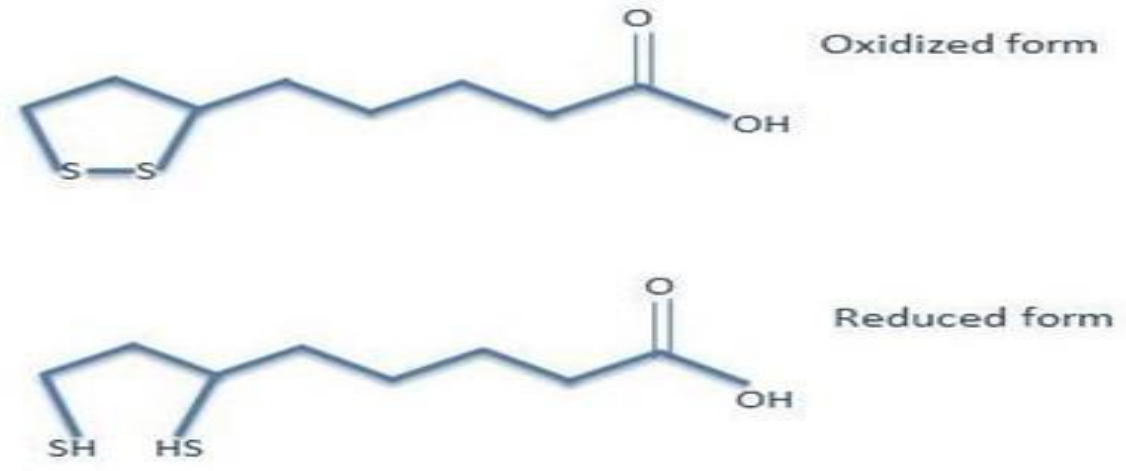


Figure 3: Oxidized (cyclic disulfide) and Reduced (dihydrolipoic acid -DHLA) forms of Alpha lipoic acid.
Source: Solmonson & DeBeradinis, 2018

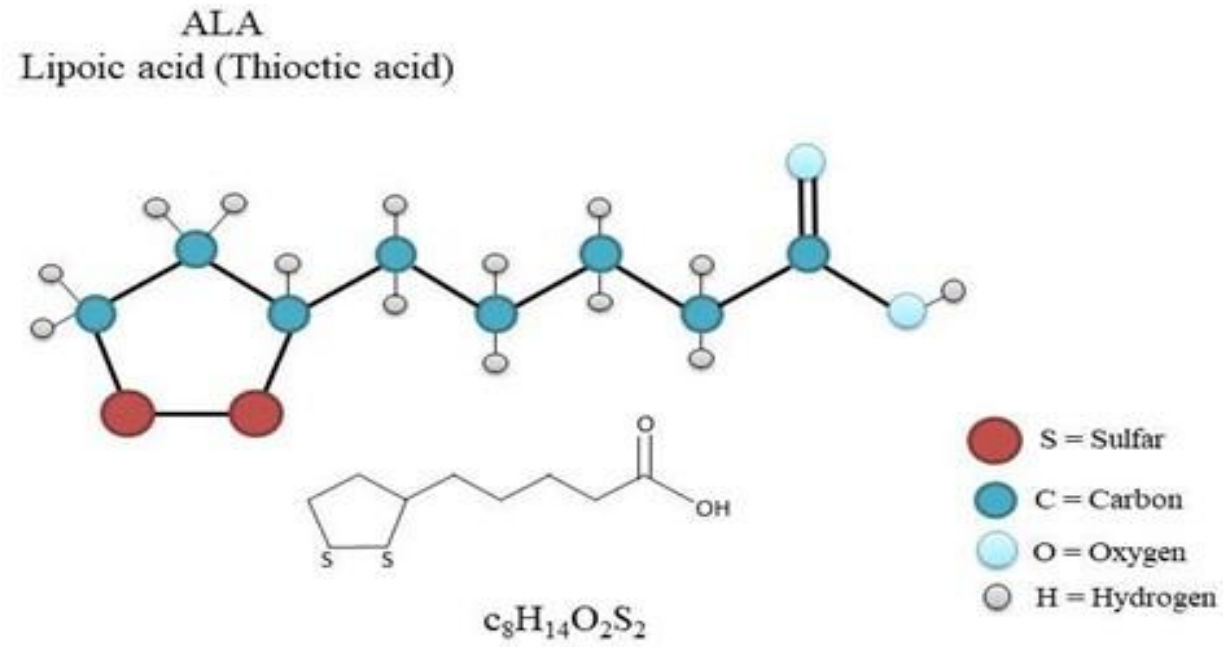


Figure 4: Alpha lipoic acid, an amphipathic molecule with a chain of eight carbon atoms, two oxygen in the carboxylic group and two sulfur located in the terminal part.
Source: Solmonson & DeBeradinis, 2018

By minimizing the damage done by free radicals, enhancing the speed of nerve communication, and restoring normal neurological sensitivity, ALA safeguards the nerves. ALA is helpful for peripheral neuropathies, especially diabetic neuropathy, where it greatly lessens uncomfortable feelings (Amara et al., 2019; Mijnhout et al., 2012; Mrakic-Sposta et al., 2018).

Additionally, ALA can repair and recycle endogenous antioxidants such as glutathione, coenzyme Q, vitamin C, and vitamin E. This amplifies its effects and defends against reactive oxygen species assaults on nerves and blood vessels (Perez-Matos et al., 2017).

ALA is the only antioxidant that can combat free radicals in every area of the neuron. The peculiarity of ALA is that it functions both inside the cell, in an environment that is both watery and oily, or rich in lipids, like the membrane of neurons (Tutelyan et al., 2019).

Although alpha lipoic acid has anti-inflammatory properties, it does not work as well as nonsteroidal anti-inflammatory drugs. It may help to reduce systemic inflammation over time, but there are no immediate effects (Li et *al.,* 2015).

**2.11 Olive Oil**

Olive oil is a fluid fat obtained from olives (the fruit of Olea europaea; family Oleaceae), a traditional tree crop of the Mediterranean Basin. It is produced by the pressing of the olive fruit. It is naturally high in beneficial Monosaturated fats and low in dreadful Saturated Fat (Olive Herb Co, 2022).

It has 14.5% of total saturated fat, 13% of which is Palmitic acid and 1.5% is stearic acid and >85% of total unsaturated fat, consisting of 70% – 73.5% monounsaturated and 15.5% polyunsaturated fat. The monosaturated fats consist of 70% Oleic acid and 0.3% - 3.5% Palmitoleic acid, while the polyunsaturated 15% Linoleic acid and 0.5% $\alpha$- Linoleic acid. Olive oil has a melting point of -6.0°C and a boiling point of 190°C - 215°C for extra virgin olive oil (Sarah, 2015).

There are two types of olive oil: virgin olive oil and refined olive oil. Extra virgin olive oil is a type of virgin olive oil (Eckelkamp, 2021).

Extra Virgin Olive Oil is a virgin olive oil of perfect aroma and flavour with a free acidity below 8% (Olive Herb Co, 2022). Higher production standards for extra virgin olive oil preserve a number of the oil's beneficial properties, including its anti-cancer polyphenols, antioxidants, and other bioactive compounds that also reduce inflammation and support healthy cholesterol levels (Eckelkamp, 2021).

Secondary plant metabolites known as phenolic compounds are produced either naturally or in response to stress (Naczk & Shahidi, 2004). When the olive fruits are crushed to produce the olive oil industrially, the synthesis of these compounds takes place in virgin olive oils (Pérez et *al.,* 2014).

The activity of hydrolytic and oxidative enzymes, and also the initial presence of glycosides in the fruit tissue, are all closely correlated with the presence of phenolic compounds (Pérez et *al.,* 2014). They have an aromatic ring with at least one hydroxyl linked to it in terms of chemical structure. Phenolic compounds are classified into lipophilic (α, β, and γ-tocopherols and tocotrienols) (Ambra et al., 2017) or hydrophilic. The most significant lipophilic phenolic component found in virgin olive oils is α -tocopherol (>90% of tocopherols) (Franco et *al.,* 2014), with a mean concentration of 150.7 mg/kg, and reaching levels of up to 400 mg/kg (Ambra et *al.,* 2017. Around 36 hydrophilic phenolic compounds have been identified in olive oil and assembled into six categories based on their chemical structure (Figure 5) (Cicerale et *al.,* 2009). The phenolic compounds are mainly accountable for the organoleptic characteristics (aroma and flavour) (Valli et *al.,* 2014; Procida et *al.,* 2016) and oxidative stability of the oil (Jiménez-Sánchez *et al.,* 2016). The mean phenolic content in extra virgin olive oil is 551.4 mg/kg (ranging from 50–800 mg/kg).

Consuming foods high in phenolic compounds regularly has been shown to lessen the risk of developing chronic diseases (Salas-Salvadó et *al.,* 2016; Tresserra et *al.,* 2014), primarily due to their ability to modulate low-grade inflammation (Tangney & Rasmussen, 2013)

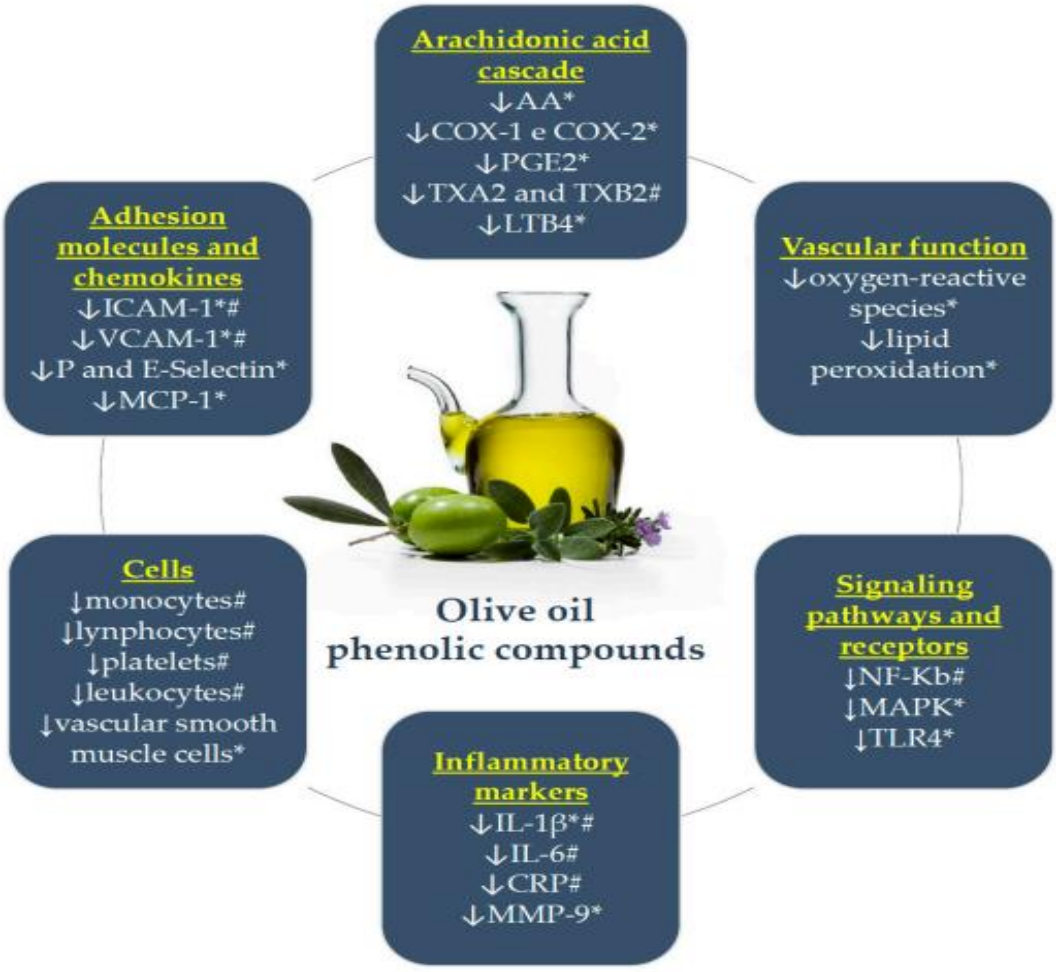


Figure 5: Main anti-inflammatory effects of phenolic compounds.

Source: de Souza *et al.*, (2017)

## 2.12 Cytokine as an Inflammatory protein

The mechanisms of capillary constriction, leukocytic aggregation, redness, heat, pain, edema, and usual loss of function in inflammatory reactions act as a mechanism for harmful chemicals and damaged tissue clearance (Merriam-Webster Dictionary, 2022). Inflammation is a phagocytic cell's innate immune mechanism. The five cardinal symptoms of inflammation are heat, discomfort, redness, tumour, and loss of function (Ferrero-Miliani et *al.*, 2007) Cells in the body and the blood initiate the inflammatory response when signalling pathways are coordinated to regulate levels of inflammatory mediators (Lawrence, 2009). The alleviation of acute inflammation and restoration of tissue homeostasis are mechanisms by which cells and molecules interact in acute inflammatory responses, thus preventing further injury or infection. Unfortunately, if this goes untreated, the inflammation can become chronic and lead to long-term health problems (Zhou et *al.*, 2016).

At inflammatory sites, a variety of immunological and parenchymal cells create inflammatory lipids, formed from plasma membrane lipids and a majority of them are Arachidonic Acid metabolites. Some lipids are pro-inflammatory, whereas others are regulatory and help to keep inflammation in check (Pathway medicine, 2017).

Cytokines are proinflammatory mediators synthesized from physical or biochemically stressed tissues (Dinarello et *al.*, 2000) Other names for cytokines include chemokine (cytokines having chemotactic activity), lymphokine (cytokines produced by lymphocytes), interleukin (cytokines made by one leukocyte and acting on other leukocytes), and monokine (cytokines produced by monocytes). Cytokines can affect the cells that release them (autocrine action), on close cells (paracrine action), or, in certain cases, on cells that are far away (endocrine action) (SinoBiological., n.d.).

Cytokines regulate cellular activities by their attributes which are synergism (which occurs when the collective effect of two cytokines on cellular activity is larger than the additive effects of individual cytokines), redundancy (many different cytokines can mediate the same or comparable tasks), and pleiotropy (the properties of one cytokine inhibits or offsets the effects of another cytokine).

They serve as a mediator for inflammatory responses in cellular organelles to fight infections (pro-inflammatory cytokine response). However, cytokines aid the development of acute inflammation into a chronic one causing diabetes, asthma, cancer, etc (Dalanon et *al.*, 2021). Drugs are produced to target the diseases associated with cytokines as a method of treating chronic inflammation.

Immunoregulatory cytokines that promote inflammation are collectively referred to as proinflammatory cytokines (IL-1, IL-6, and TNF- $\alpha$). They play a part in the stimulation of inflammatory responses and are mostly generated by activated macrophages. The ratio of proinflammatory to anti-inflammatory cytokines determines the overall outcome of an inflammatory response (Zhang & An, 2007)

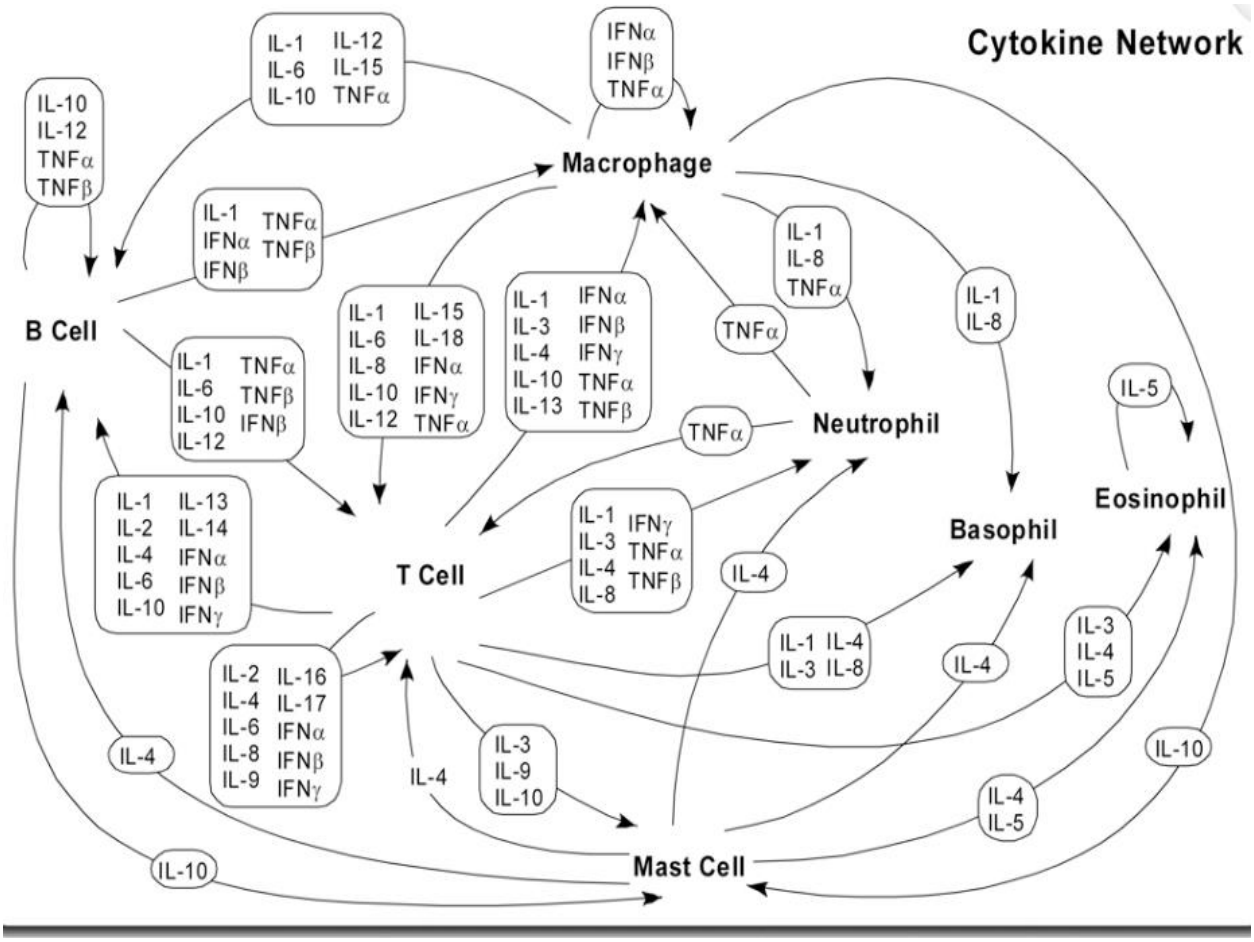


Figure.6: Cytokine network.

Source: Zhang & An (2007)

### 2.12.1 Interleukins

Like other cytokines, interleukins are also quickly and briefly secreted in response to a stimulus, such as an infectious agent, as opposed to being stored by cells. It plays a role in the control of neural, regenerative, and metabolic processes. It stimulates the production of acute phase proteins whose plasma concentrations increase or decrease in response to inflammation (Health matters, n.d.).

Interleukins play important roles in the differentiation and activation of immune cells, and also proliferation, maturation, migration, and adhesion possessing both pro- and anti-inflammatory properties. The main role of interleukins is to modulate growth, differentiation, and activation during inflammatory and immune responses. They have both paracrine and autocrine functions (Justiz & Qurie, 2021).

The gene for human interleukin-6 (Figure 7) is located on chromosome 7p21, having a total amino acid composition of 212, including a 28-amino-acid signal peptide. Interleukin- 6 is synthesized by T and B lymphocytes, fibroblasts, and macrophages (Petes *et al.*, 2018).

It has been demonstrated that IL-6 is crucial for the neuronal response to nerve damage. There is proof that IL-6 plays a role in the neuropathic pain behaviour that develops after a peripheral nerve lesion. Additionally, it can influence some endocrinological abnormalities and the balance of hormones. Patients with lipid problems and insulin resistance had higher IL-6 concentrations (Zhang & An, 2009).

PCOS-afflicted women have increased serum IL-6 concentrations, which are correlated with androgen and insulin resistance (Peng *et al.*, 2016)

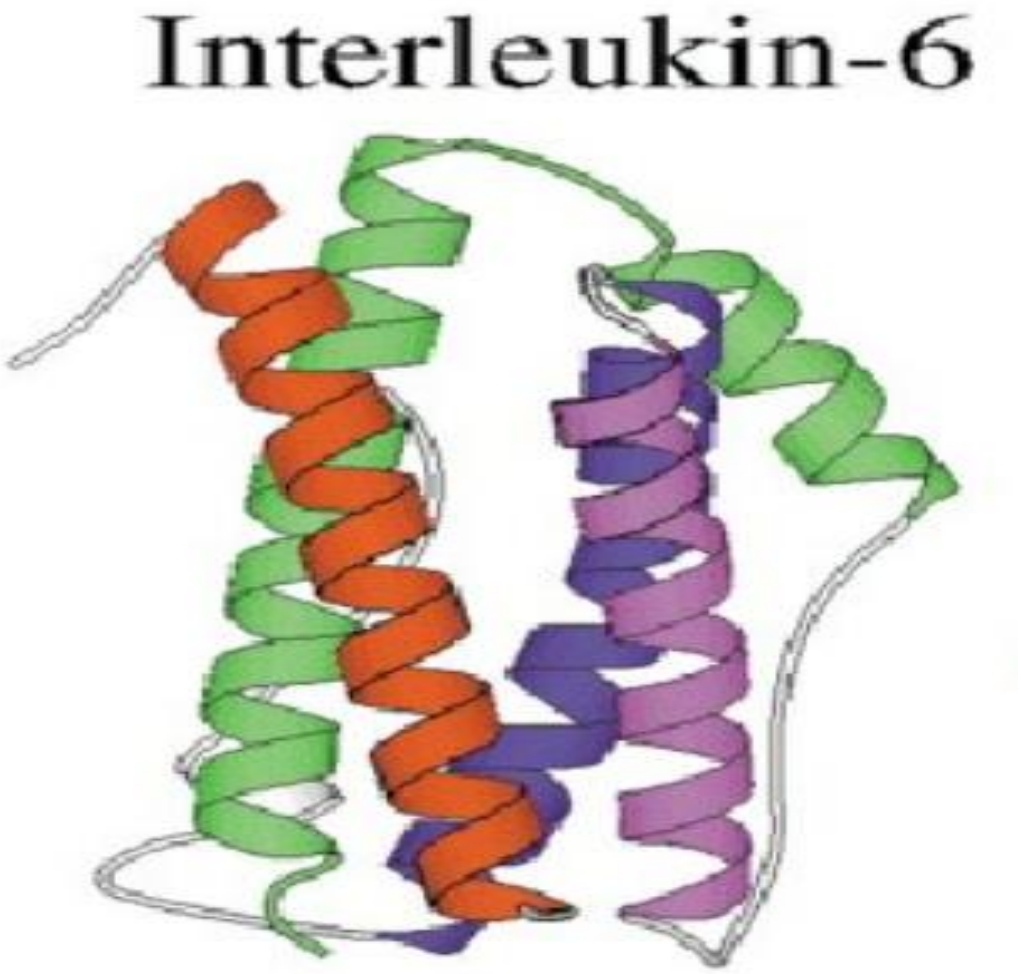


Figure 7: Crystal structure of Interleukin- 6.

Source: Bravo & Heath (2000).

### 2.12.2 Tumor necrosis factor- alpha (TNF- $\alpha$)

Tumour Necrosis Factor-alpha (TNF- $\alpha$) is a cytokine released by immune cells having the ability to inhibit tumour cell growth and cause tumour regression. It is a 157-amino acid protein produced as a membrane-bound protein (pro-TNF) and released via TNF-converting enzyme (TACE)-mediated cleavage. TNF- $\alpha$ has several roles in physiological situations such as body development and immunology, as well as pathological reactions such as inflammation, tumour growth, transplant rejection, rheumatoid arthritis, and septic shock. TNF- $\alpha$ works on the cellular level by activating certain signalling pathways that regulate cell survival, growth, and death (Figure 8) (Wang & Lin, 2008).

TNF- $\alpha$ is synthesized by a variety of cells, including macrophages, monocytes, neutrophils, T cells, and NK cells but is primarily secreted by the visceral adipocytes (Escobar Morreale *et al.,* 2011). It binds to both types of outer membrane-associated receptors on target cells, TNFR1 and TNFR2, and activates cell survival as well as pro-inflammatory nuclear factor-kappa B (NF-kB). The chemical stimulates phagocytes, causing them to engulf and eliminate pathogenic pathogens and cellular waste. It also boosts the expression of adhesion molecules on the vascular endothelium, allowing immune cells, particularly neutrophils and macrophages, to migrate to locations of tissue injury and infection (El-Tahan *et al.,* 2016).

TNF-α is a mediator of insulin resistance. Women with PCOS had increased blood TNF- $\alpha$ concentrations, as well as greater monocyte and lymphocyte circulating levels, and inflammatory infiltration in ovarian tissue. (Xiong *et al.,* 2011). Hyperglycemia can contribute to the inflammatory process of PSCO. Glucose is a primary redox substrate of mononuclear circulating cells, causing the generation of reactive oxygen species (ROS) and thereby activating nuclear factor-kappa B (NF-kB), a transcription factor involved with the release of proinflammatory mediators such as TNF- $\alpha$ or IL-6. (Rudnicka *et al.,* 2021).

Figure 8: Molecular structure of Tumor Necrosis Factor.

Source: Salmon *et al* (2018)

## 2.13 PCOS and Inflammatory proteins

Recent research suggests that the long-term metabolic effects of PCOS might be allied to a state of low-grade chronic inflammation (Repaci *et al.,* 2011).

PCOS women possess high follicular fluid and serum levels of interleukin- 6 and tumour necrosis factor-alpha (TNF- $\alpha$) (Amato *et al.,* 2003) indicating that the immune system plays a role in the etiology of PCOS. PCOS-afflicted women have greater amounts of CRP, monocytes, lymphocytes, TNF- $\alpha$, and IL-6 in their blood, as well as macrophages and lymphocytes in their ovarian tissue. Lymphocytes and macrophages secrete inflammatory cytokines such as IL-6 and TNF- $\alpha$, which activates more lymphocytes and macrophages to increase the synthesis of cytokines (Figure 9).

The presence of insulin resistance in PCOS may alter serum levels of IL-6 and TNF- $\alpha$ (Samy *et al.,* 2009). It has been shown that 50–70% of PCOS females have some element of insulin resistance (Ebejer & Calleja-Agius. 2013). Hypothalamic-pituitary-ovarian dysfunction, insulin resistance, and anovulation are thought to be caused by this peripheral and ovarian inflammation (Xiong *et al.,* 2011). Visceral obesity is linked to insulin resistance and may lead to adipocyte malfunction and low-grade inflammation in PCOS individuals (Sathyapalan & Atkin, 2010). Females with insulin-resistant PCOS produce considerably more interleukin- 6 than men. This contrasts with IL-6 levels, which are considerably greater in insulin-resistant PCOS women. Furthermore, in PCOS females with insulin resistance, the level of IL-6 generated by lymphocytes and monocytes in response to microbial stimuli is higher (Fulghesu *et al.,* 2011).

It has been proposed that PCOS has an altered immunological response (Amato *et al.,* 2003). In line with this, experimental studies suggest that in PCOS, particularly when insulin resistance is present, altered immune response to microbial and inflammatory stimuli plays an essential role in the development of low-grade inflammation (Fulghesu *et al.,* 2011).

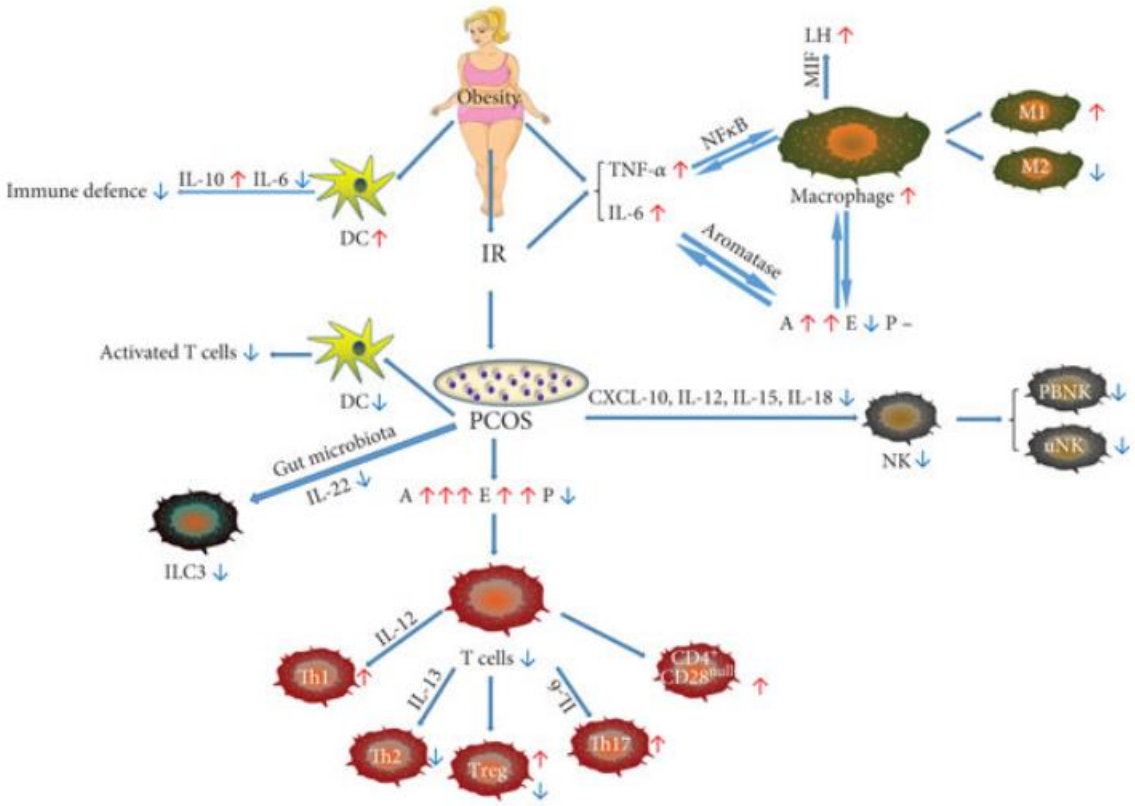


Figure.9: Immunophenotypic profiles in the polycystic ovarian syndrome. (Hu *et al.*, 2020)

# CHAPTER THREE

## 3.0 MATERIALS AND METHODS

### 3.1 Materials

Microscopic slides, cotton swabs, sterile blades, paper tape, hand & surgical gloves, foil paper, sample tubes, ice pack, tissue paper, sacrifice table, cotton wool, rat cages, feeding troughs, 0.9% normal saline, diethyl ether, volumetric flask, spatula, Eppendorf tubes, nose mask, test-tube rack, ethanol, sodium hydroxide, 10% formalin.

### 3.1.1 Experimental animals

Thirty-five female albino rats (*Rattus novergicus*) with an average weight of 162.63g ± 6g were gotten from the animal holding unit of the Department of Biochemistry, Mountain Top University, Ibafo, Nigeria. The animals were housed with adequate ventilation (temperature: 28 ± 3ºC; photoperiod of 12 hours light and 12 hours dark; humidity: 65%) and fed with rat pellets and water *ad libitum,* in compliance with animal experimentation ethics.

### 3.1.2 Drugs and Assay kits

Letrozole was manufactured by Pharmadox healthcare, Paola, Malta. Clomiphene citrate was manufactured by Firstsource Pharmachem Lagos, Nigeria. Metformin hydrochloride was manufactured by Sante Pharmaceuticals, Mougins, France. Alpha Lipoic acid was manufactured by Natrol LLC, Chatsworth, USA. Rat TNF- $\alpha$ (Tumor Necrosis Factor-alpha) ELISA Kit and Rat IL-6 (Interleukin-6) ELISA Kit were manufactured by Elabscience Biotechnology Inc, Texas, USA.

#### 3.1.2.1 Other chemicals and reagents

Analytical-graded chemicals and reagents were obtained mainly from Sigma Aldrich Ltd, Buchs, Canada were used during the course of the experiment.

### 3.1.3 Equipment and Apparatus

Beakers (500ml, 250ml, 50ml), syringe & canola (for drug administration), mortar & pestle, stirring rod, measuring cylinders (1000ml, 500ml, 100ml), dissecting kit, desiccator, syringe,

canola, analytical balance, microscope, weighing balance, centrifuge (iSG 24-place high-speed micro centrifuge CD3024), microplate reader (Thermo Scientific), Eppendorf rack, multichannel micropipette, micropipette, pipette tips, microplate well washer (Thermo Scientific), incubator (memmert), vortex (VORTEX GENIE -2 Digital).

### 3.2 Methods

#### 3.2.1 Experimental design

Thirty-five rats were uniformly distributed into five groups of seven (7) animals and designated as group 1 (non- PCOS control group), group 2 (PCOS + distilled water), group 3 (PCOS + clomiphene citrate + metformin), group 4 (PCOS + alpha lipoic acid), and group 5 (PCOS + olive oil).

Upon complete administration of treatment, the rats were anaesthetized with diethyl ether and sacrificed by jugular puncture 25 hours after the last treatment. Blood samples was drawn from each rat and the serum was separated to be used for biochemical analysis.

#### 3.2.2 Induction of PCOS

##### 3.2.2.1 Animal grouping, induction, and administration of alpha lipoic acid and olive oil

For two weeks, thirty-five female albino rats (*Rattus novergicus*) with an average weight of 162.63g ± 6g were acclimatized under standard room conditions (temperature: 28 ± 3ºC; photoperiod of 12 hours light and 12 hours dark cycle) and fed with rat pellets and water *ad libitum.* The rats were administered letrozole orally for 22 days to induce PCOS, after which were treated with the standard drugs, Alpha lipoic acid and olive oil for 14 days. The body weight was also determined every 7 days. The rats were held at the thorax, the uppermost ventral surface providing lumbar support.

The rats were randomly grouped into five of seven animals each as follows:

**Group 1** – (non-PCOS control) was administered 0.5ml of saline water.

**Group 2** – (PCOS control) and

**Groups 3** – **5** (PCOS-induced) were administered 1mg/kg of letrozole.

### 3.2.2.2 Confirmation of PCOS

The estrus cycle of the rats was observed by vagina cytology using the light microscope to observe predominant cells present in vagina smears. This was conducted daily on the rats during the period of induction.

### 3.2.2.3 Vagina cytology

Vagina cytology was carried out daily in the laboratory. The vagina smears of the rats was obtained daily between the hours of 6:00 am and 7:00 am throughout the study. The vagina of the rats was swabbed using cotton-tipped swabs softened with saline water. 2cm of the swab was inserted into their vagina and about three revolutions were made to obtain a generous amount of vagina cells. The swab was withdrawn and rolled on a clean microscopic glass slide to spread the vagina cells on it. The cells were then observed under the microscope with an objective lens of ×40.

### 3.2.3 Preparation of Serum and Tissue Supernatant

The rats were weighed and thereafter anaesthetized in a jar containing diethyl-ether-soaked cotton wool. Their jugular veins were ruptured using a sharp sterile blade and allowed to bleed into dry sterile sample tubes and left at room temperature.

The blood samples were centrifugated at 4000rpm for 10 minutes to obtain the supernatant using Thermo Scientific Centrifuge (Heraeus Megafuge 8). The sera were aspirated into dry sterile sample bottles using disposable pipettes and stored frozen at -4$^{o}$C.

### 3.2.4 Animal grouping and drug administration for pharmacological study

PCOS was induced in twenty-eight Wistar rats with 1ml/1kg of letrozole daily. Drug administration for the rats distributed into five groups are as follows:

**Group 1** – (non-PCOS control) was administered 0.5ml of saline water.

**Group 2** – (PCOS control) was administered 0.5ml of distilled water

**Group 3** – (PCOS-induced) was administered 2mg/kg of clomiphene citrate and 7.14mg/kg of metformin

**Group 4** – (PCOS-induced) was administered 1mg/kg of alpha lipoic acid

**Group 5** – (PCOS-induced) was administered 4ml/kg of olive oil

Drug administration for the female rats lasted 14 days. The rats were weighed at seven days intervals. At the end of the experimental period, the rats were anaesthetized with diethyl ether and sacrificed by jugular puncture 25 hours after the last treatment.

### 3.2.5 Determination of Serum concentration of Cytokines

#### 3.2.5.1 Interleukin 6 (IL-6)

**Principle**

The serum IL-6 concentration was quantitatively determined using the Rat IL-6 (Interleukin 6) ELISA Kit produced by Elabscience Biotechnology Inc. The Sandwich-ELISA principle was applied in this experiment.

**Procedure**

The samples were diluted in a ratio of 1:8. The standards were diluted using serial dilution. The standards were centrifuge at 10,000g for 1min.

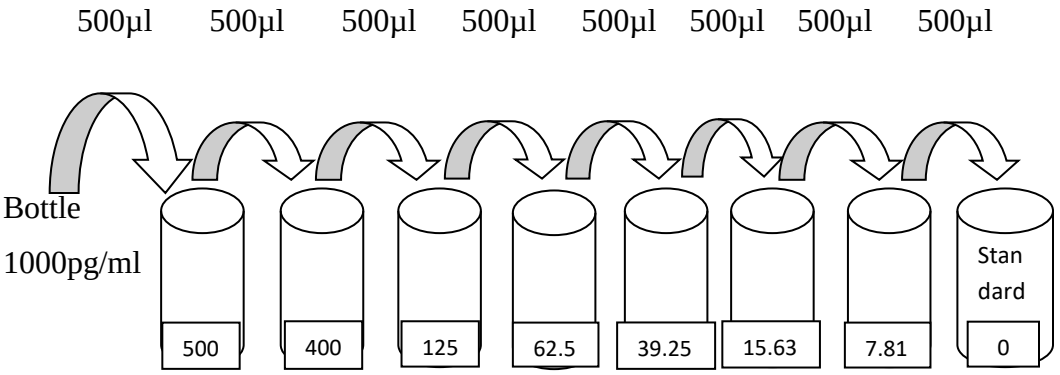


**Figure 10: Serial dilution of the standard**

Add 100µl of standard, blank, and sample to each well. The plate was enclosed with the sealer provided and incubated for 90mins at 37ºC. The liquid was decanted from each well after 90mins of incubation. 100µl of Biotinylated detection Ab was introduced into each well and covered with a new seal and incubated for 1 hour at 37ºC. The solution was decanted from each well. 350µl of wash buffer was introduced into each well and left to soak for 1min. The solution was

then decanted and patted dry with clean absorbent paper. This step was repeated 2 more times (the wash step). 100µl of HRP conjugate working solution was introduced into each well, and the plate was covered with a new seal and incubated for 30min at 37ºC. The solution was decanted from each well. 350µl of wash buffer was introduced into each well and left to soak for 1min. The solution was then decanted and patted dry with clean absorbent paper. The plate was incubated for about 15 to 30 mins at 37ºC and colour change was observed. The microplate reader was pre-heated for about 15mins before OD measurement. 500µl of stop solution was introduced into each well and the OD value of each well was determined at 450nm with a microplate reader.

### 3.2.5.2 Tumor Necrosis Factor $\alpha$ (TNF- $\alpha$)

**Principle**

The serum TNF- $\alpha$ concentration was quantitatively determined using the Rat TNF- $\alpha$ (Tumor Necrosis Factor-alpha) ELISA Kit produced by Elabscience Biotechnology Inc. The Sandwich-ELISA principle was applied in this experiment.

**Procedure**

The samples were diluted in a ratio of 1:8. The standards were diluted using serial dilution.

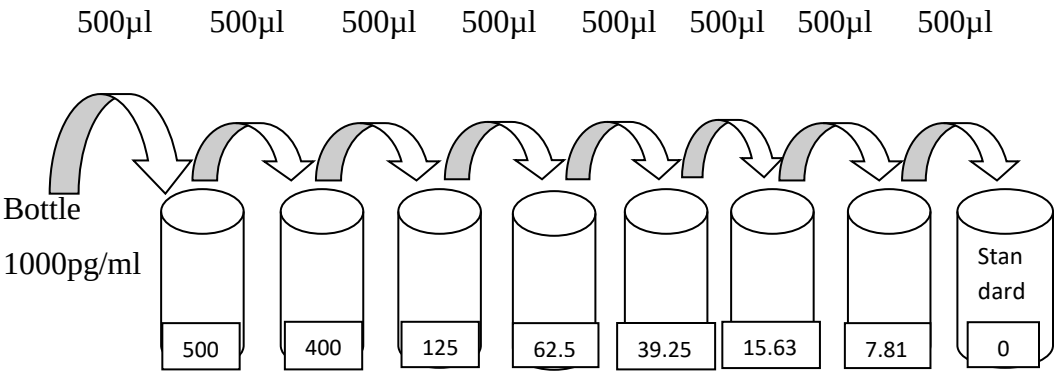


**Figure 11: Serial dilution of the standard**

100µl of standard, blank, and sample was introduced into each well and covered with the seal provided, and incubated for 90mins at 37ºC. The concentrated Biotinylated detection Ab was centrifuged at 800×g for 1min. The liquid was decanted from each well after 90mins of incubation. 100µl of Biotinylated detection Ab was introduced into each well and covered with a

new seal and incubated for 1 hour at 37ºC. The concentrated HRP conjugate was centrifuged at 800×g for 1min. The solution was decanted from each well. 350µl of wash buffer was introduced into each well and left to soak for 1min and decant the solution. The plate was then patted dry against a clean absorbent paper. This step was repeated 2 more times (the wash step). 100µl of HRP conjugate working solution was introduced into each well and covered with a new seal and incubated for 30min at 37ºC. The solution was decanted from each well. 350µl of wash buffer was then added to each well and left to soak for 1min. The solution was decanted and patted dry with clean absorbent paper (this step was repeated 5 times). 90µl of substrate reagent was introduced into each well and covered with a new seal and incubated for about 15 to 30 mins at 37ºC (the colour change was observed). The microplate reader was pre-heated for about 15mins before OD measurement. 500µl of stop solution was introduced into each well and the OD value of each well was determined at 450nm with a microplate reader.

### 3.2.6 Data Analysis

Data were analyzed using IBM SPSS version 20. The statistical differences between the groups were determined using the one-way variance (ANOVA) statistical test and Duncan's multiple range test for multiple correlations. Values were statistically significant at $p<0.05$. Results were expressed as mean ± standard error of the mean (SEM).

# CHAPTER FOUR

## 4.0 Results

### 4.1 Effects of Alpha lipoic acid and Olive oil on the Tumor Necrosis Factor- $\alpha$ (TNF- $\alpha$) levels of letrozole-induced polycystic ovarian syndrome rats

The serum TNF- $\alpha$ levels of letrozole-induced PCOS rats were significantly increased ($p<0.05$) when compared favourably to the control group. The olive oil treatment administered to the letrozole-induced PCOS rats significantly decreased the serum TNF- $\alpha$ levels to that of the control group compared to the Alpha lipoic acid and the standard drug (Figure 12).

In the untreated PCOS group, the administration of letrozole to the rats significantly increased ($p<0.05$) the serum TNF- $\alpha$ levels compared favourably to the control group.

After treatment with the administration of Alpha lipoic acid and the reference drug to the rats, the serum TNF- $\alpha$ levels of the letrozole-induced PCOS rats. significantly decreased ($p<0.05$). Even more, Olive oil significantly decreased ($p<0.05$) the serum TNF- $\alpha$ levels of the letrozole-induced PCOS compared to other treatment groups and the control group.

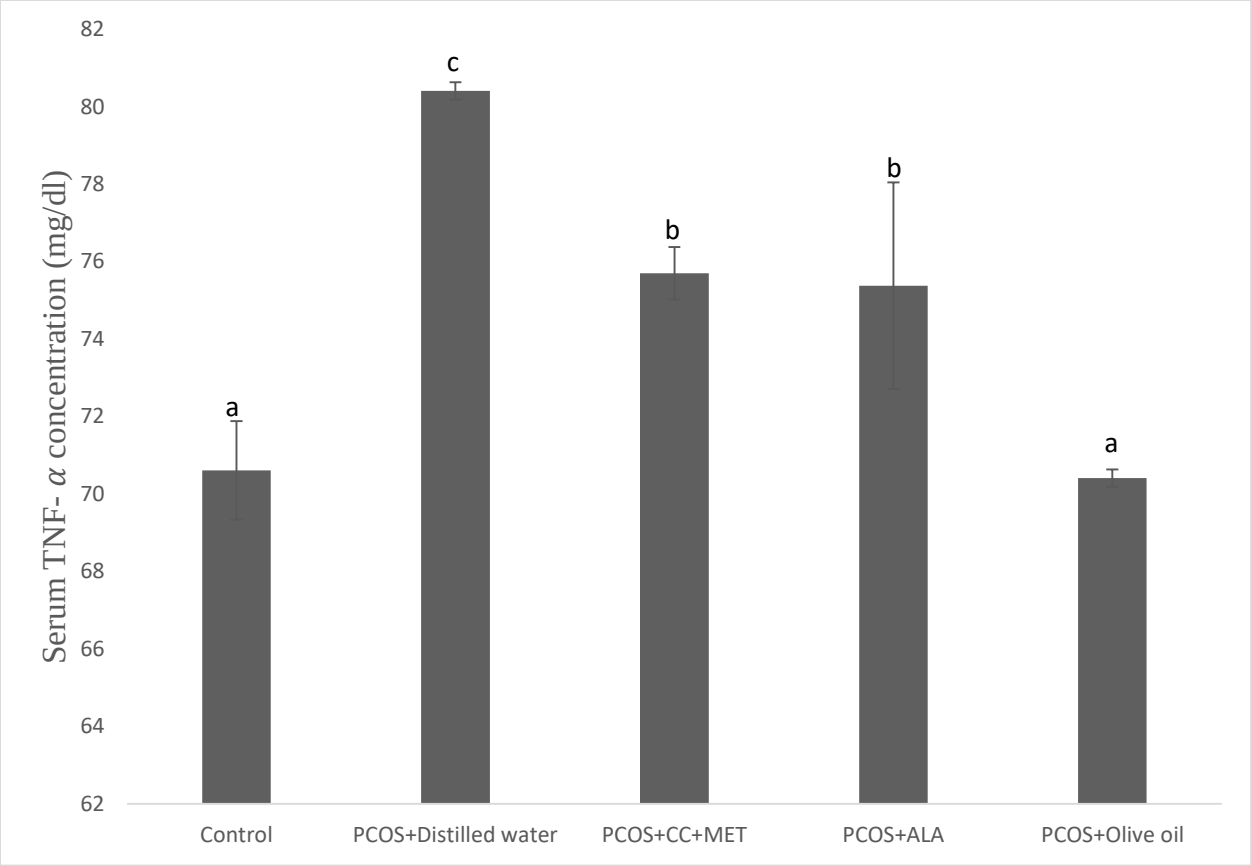


**Figure 12: Serum TNF- $\alpha$ concentration of letrozole-induced PCOS rats administered Alpha lipoic acid (ALA) and Olive oil.**

**Data are the mean of four determinations ± SEM; Each column with different values and superscripts is different significantly ($p<0.05$).**

### 4.2 Effects of Alpha lipoic acid and Olive oil on the Interleukin-6 (IL-6) levels of letrozole-induced polycystic ovarian syndrome rats

The IL-6 levels of letrozole-induced PCOS rats were significantly decreased ($p<0.05$) compared favourably to the control group (Figure 13).

In the untreated PCOS group, the administration of letrozole to the rats significantly increased ($p<0.05$) the IL-6 levels compared favourably to the control group.

After treatment with the administration of Alpha lipoic acid, Olive oil and the reference drug to the letrozole-induced PCOS rats, the serum IL-6 levels of the rats significantly decreased ($p<0.05$).

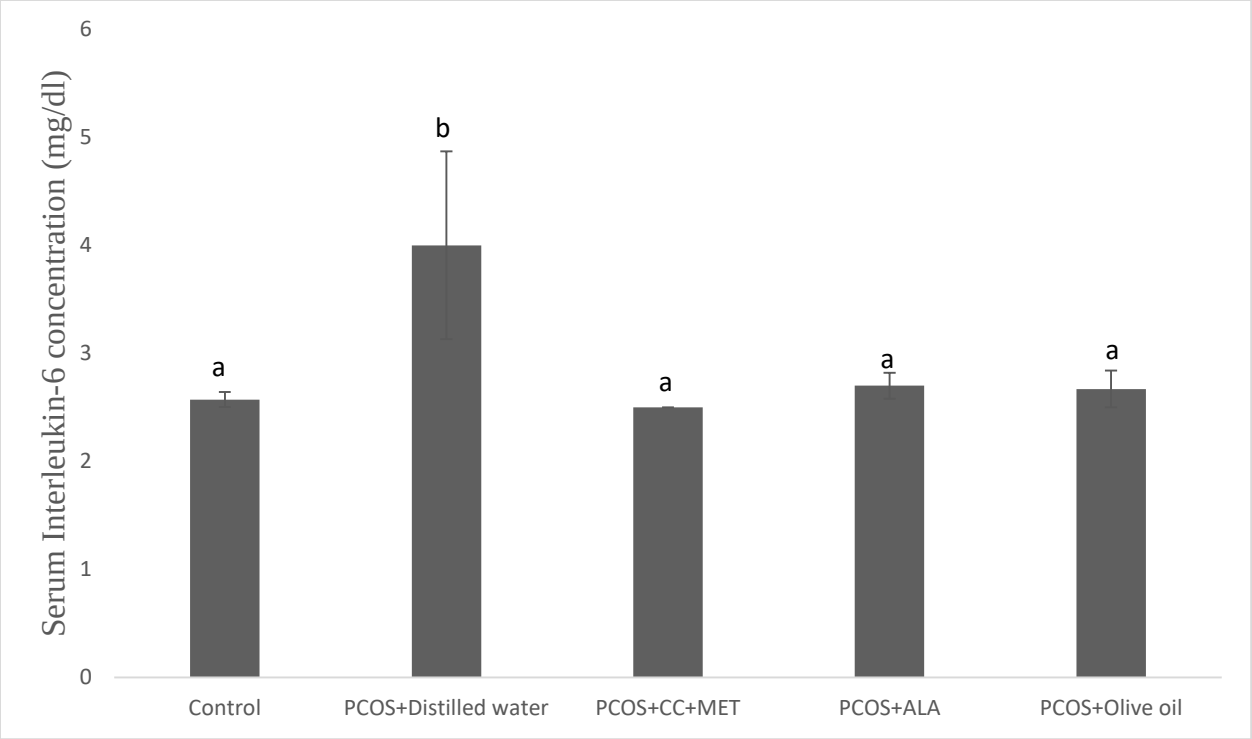


**Figure 13: Serum IL-6 concentration of letrozole-induced PCOS rats administered Alpha lipoic acid (ALA) and Olive oil.**

**Data are the mean of four determinations ± SEM; Each column with different values and superscripts is different significantly ($p<0.05$).**

# CHAPTER FIVE

## 5.1 Discussion

Polycystic ovary syndrome is an endocrine disorder (Goodman et *al.*, 2015) present in 4% – 20% (Deswal *et al.,* 2020) of reproductive-aged women. It is named after the distinctive “cysts” that can develop after the accrual of ovarian follicles on the ovaries, but it's vital to understand that this is only a symptom and not the actual cause of the disorder (Dunaif et *al.,* 2013). The "polycystic" form of the ovaries found in PCOS-afflicted patients is triggered by the accrual of ovarian follicles at different stages of maturity (Dewailly et *al.,* 2014). The accrual of ovarian follicles into a cyst-like form was brought on by an imbalance in hormones. The frequently affected hormones are androgens, Estrogen, Progesterone, Testosterone, and Luteinizing Hormone (Kavitha & Thomas, 2018).

Rats in PCOS groups were treated daily with letrozole at 1mg/kg each rat for 21 days inducing PCOS. In a report by Kafali et *al.,* (2004), adult rats given letrozole for three weeks developed characteristics similar to women with PCOS. Letrozole when continually administered to female rats induces PCOS-associated metabolic and endocrine abnormalities at adult age (Maliqueo, et *al.,* 2013).

Letrozole, an aromatase inhibitor, is responsible for the conversion of androgens into estrogens, lowering estrogen production in the body by 97% to 99%, thereby increasing the production of follicle-stimulating hormone (FSH) (Kar, 2013)

The histology of vaginal smears is a vital indicator of ovarian physiology (Kafali et *al.,* 2004) The vagina of the rats was swabbed using cotton-tipped swabs softened with saline water. Vaginal cytology is the most effective means of monitoring the oestrous cycle of PCOS rats.

Women with PCOS possess an altered immune response system. Several studies suggest that the presence of a low-grade chronic inflammatory state may be related to long-term metabolic effects and cardiovascular complications in PCOS (Repaci et *al.,* 2011)

In this study, the serum levels of TNF- $\alpha$ and IL-6 in the PCOS untreated group were relatively higher compared favourably to the control group. Amato et *al.,* (2003) and Xiong et *al.,* (2011) demonstrate that serum fluid levels of TNF- $\alpha$ and IL-6 levels in PCOS patients are higher.

Higher IL-6 levels have been linked to insulin resistance and testosterone levels in PCOS women compared to controls. In women with PCOS, IL-6 is a significant modulator of type 2 diabetes and cardiovascular disease. Although high blood IL-6 levels are not an inherent feature of PCOS, they should be employed as a biomarker of the syndrome (Peng *et al.*, 2016).

Serum levels of TNF- $\alpha$ were reduced by Alpha lipoic acid and Olive oil. Other studies have proven that the secretion of pro-inflammatory cytokines such as TNF- $\alpha$ & IL-6 can be decreased by the administration of Alpha lipoic acid (Cardici et *al.,* 2010).

ALA reverted the elevated serum IL- 6 levels of PCOS rats to 1% higher than the control group, 9% lesser than that of the PCOS control group, and 1% higher than that of Olive oil. ALA reverted the elevated serum TNF- α levels of PCOS rats to 1% higher than the control group, 2% lesser than that of the PCOS control group, and of the same levels as that of Olive oil.

Alpha lipoic acid causes hypermethylation at the 5′-flanking area of IL-6, which is associated with decreased mRNA expression and protein synthesis (Dinicola *et al.,* 2017).

Olive oil reverted the elevated serum IL- 6 levels of PCOS rats to that of the control group, 10% lesser than that of the PCOS control group, and 1% lesser than that of Olive oil. Olive oil reverted the elevated serum TNF- α levels of PCOS rats to 1% higher than the control group, 3% lesser than that of the PCOS control group, and 1% lesser than that of Olive oil.

Oleanolic acid present in virgin olive oils reduces IL-6 levels by AMPK which plays a modulatory role in inflammation processes. To become active, AMPK needs to be phosphorylated at its α-subunit (Thr172 residue) (Li *et al.,* 2018; Fan *et al.,* 2018). It has been reported that lipopolysaccharides (pro-inflammatory) induce the dephosphorylation of AMPK (Fan *et al.,* 2018).

Consumption of extra virgin olive oil is also linked to the proliferation of *Clostridium cocleatum,* which degrades oleuropein, causing fermentation and acting as a probiotic (Deiana *et al.,* 2018). Probiotics aid in the maintenance of the intestinal barrier through a variety of processes, including the reduction of pro-inflammatory cytokines such as TNF- $\alpha$ and IL-6, as well as the reduction of total cholesterol and LDL concentrations (Marcelino *et al.,* 2019).

Upon three weeks of administration of Alpha lipoic acid, serum TNF- $\alpha$ and IL-6 levels were significantly decreased. (Shaafi *et al.*, 2020)

Though the serum levels of Interleukin-6 were reduced by Alpha lipoic acid, it is not as significant as in Olive oil. Olive oil significantly decreased the serum levels of TNF- $\alpha$. ALA supplementation reduces IL-6 levels in older men but not in women, but has little effect on muscle mass and strength during resistance training (Cornish & Chilibeck, 2009)

Therefore, Alpha lipoic acid and Olive oil can be exploited in the study of polycystic ovarian syndrome.

## 5.2 Conclusion

It is confirmed in this study, that letrozole-induced PCOS Wistar rats possess high serum levels of Tumor Necrosis Factor (TNF- $\alpha$) and Interleukin (IL-6). Alpha lipoic acid and Olive oil exert anti-inflammatory potentials against Interleukin-6 (IL-6) release into the bloodstream compared favourably to metformin and clomiphene citrate. Olive oil showed a great anti-inflammatory potential against Tumor Necrosis Factor (TNF- $\alpha$) release into the bloodstream compared to other drugs. Therefore, Alpha lipoic acid and Olive oil can be exploited as a management option in the treatment of inflammation in women with PCOS.

## 5.3 Recommendation

Chronic and sub-acute toxicity studies should be done on virgin olive oil to study its effect on gynaecological disorders.

Further studies should be done to understand the mechanism of the anti-inflammatory potential of Alpha lipoic acid on tumour necrosis factor-alpha.

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## APPENDICE

### Preparation of 7.14mg/kg of Metformin hydrochloride

Each tablet of metformin contains an active component of 500mg administered to humans with approximately 70kg body weight. The average weight of the experimental animals was 0.21kg (209.57g). 1.496mg (active component for the average rat weight) was multiplied by 0.5607g (weight of tablet) divided by 500mg (active component per 70kg) to get 0.0017g (dosage for a rat). Therefore, 7.14mg/kg body weight metformin was used.

### Preparation of 1mg/kg of Letrozole

Each tablet of letrozole contains an active component of 2.5mg administered to humans with approximately 70kg body weight. The average weight of the experimental animals was 0.19kg (192.796g). 0.193mg (active component for the average rat weight) was multiplied by 0.1022g (weight of tablet) divided by 2.5mg (active component per 70kg) to get 0.00789g (dosage for a rat). Therefore, 1mg/kg body weight of the experimental animals was used.

### Preparation of 2mg//kg of Clomiphene citrate

Each tablet of clomiphene citrate contains an active component of 2mg administered to humans with approximately 70kg body weight. The average weight of the experimental animals was 0.21kg (209.57g). 0.00598mg (active component for the average rat weight) was multiplied by 0.3317g (weight of tablet) divided by 2mg (active component per 70kg) to get 0.0009918g (dosage for a rat). Therefore, 2mg/kg body weight of the experimental animals were used.

### Preparation of 1mg/kg of Alpha lipoic acid

Each capsule of Alpha lipoic acid contains an active component of 300mg per kg body weight. The average weight of the experimental animals was 0.21kg (209.57g). 20.957mg (active component for the average rat weight) was multiplied by 0.4404g (weight of the drug without the capsule) divided by 300mg (the active component of the drug) to get 0.03077g (dosage for a rat). Therefore, 1mg/kg body weight of the experimental animals was used.

## CALIBRATION CURVES

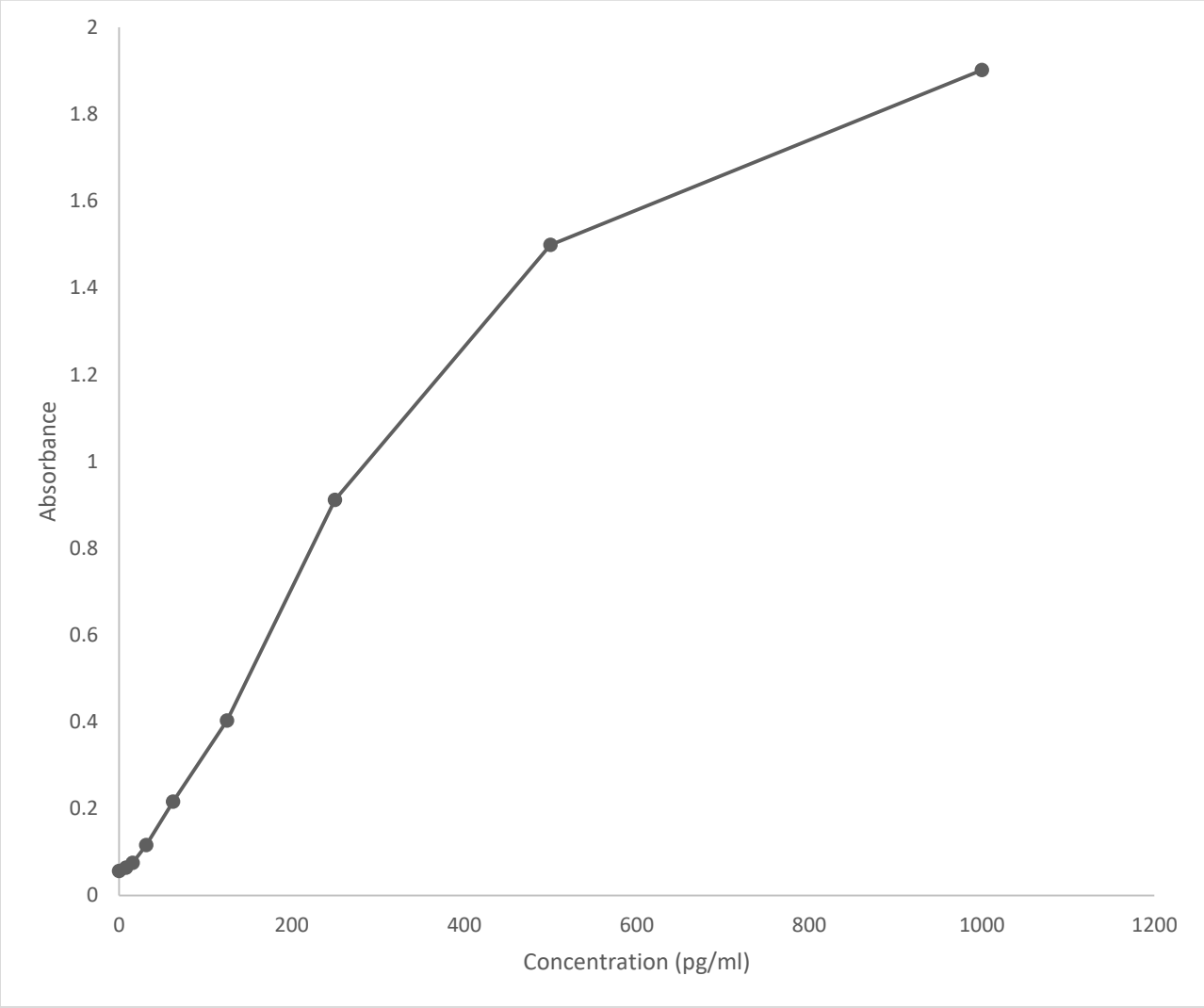


*Figure 14: Calibration curve of Interleukin-6 (IL-6) concentration (pg/ml)*

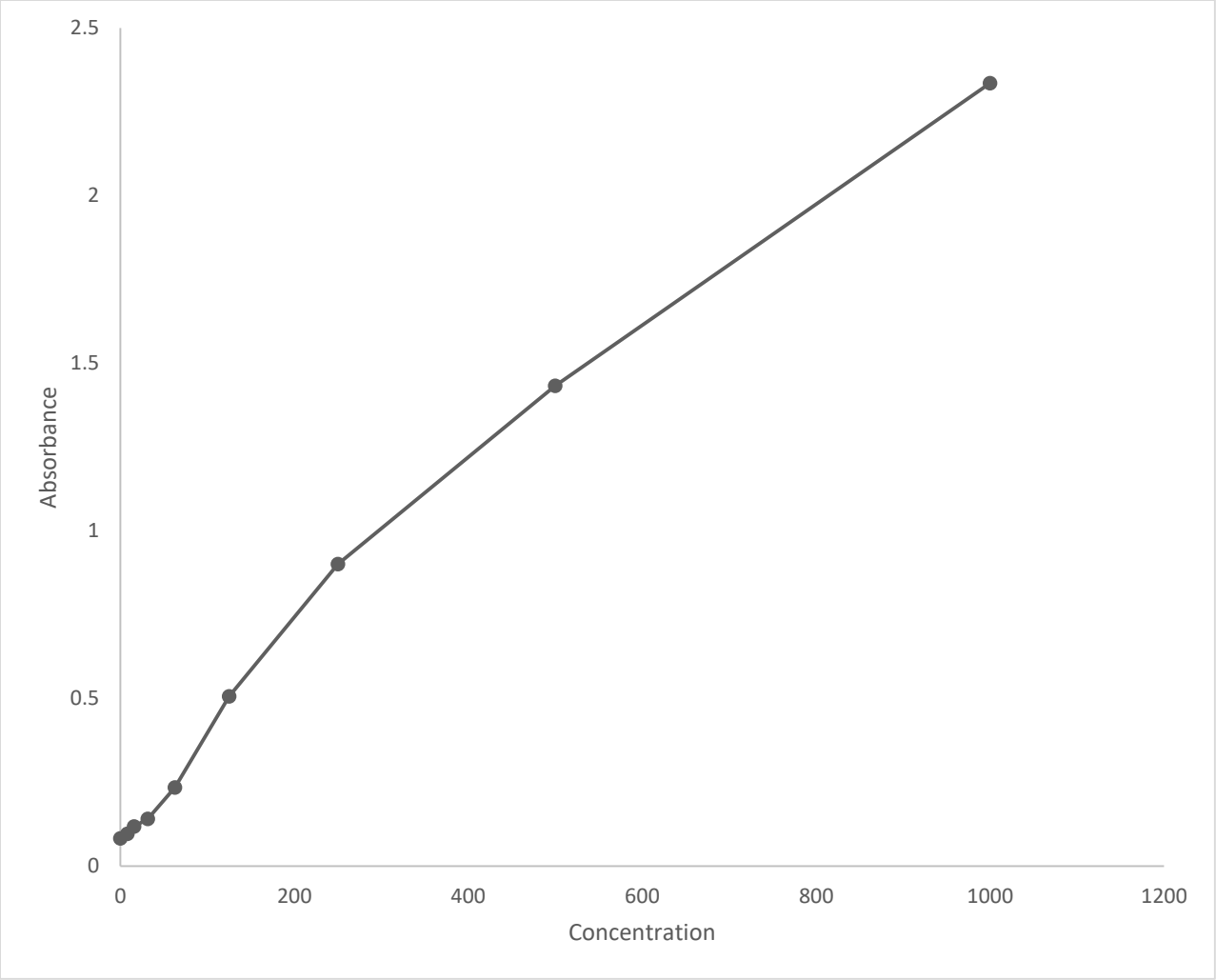


*Figure 15: Calibration curve of Tumor necrosis factor ∝ (TNF- ∝) concentration (pg/ml)*