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The Impact of Hunteria umbellata Aqueous Extract on the Morphology of Reproductive and Metabolic Organs in Rats with Polycystic Ovary Syndrome

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The Impact of *Hunteria umbellata* Aqueous Extract on the Morphology of Reproductive and Metabolic Organs in Rats with Polycystic Ovary Syndrome

by

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Abstract

Polycystic Ovary Syndrome (PCOS) affects 20% of women globally, characterized by inflammation, systemic issues, and hormonal imbalances, notably hyperandrogenism and insulin resistance, which impact fertility. Hunteria umbellata Extract (HUE) contains compounds like flavonoids, tannins, and terpenoids, known for their antioxidative, anti-inflammatory, and hypoglycemic effects (Ajiboye et al., 2017). Considering HUE's diverse phytochemical profile, we explored its potential for alleviating PCOS symptoms. Using testosterone propionate (TP) and a high-fat diet, we induced and maintained PCOS features in a rat model for 56 days, replicating PCOS pathophysiology. TP-induced insulin and leptin resistance contributed to obesity and PCOS hallmarks, effectively mimicking the condition.

HUE demonstrated dose-dependent effects on weight gain, reducing it at lower doses but showing complex interactions at higher doses. Ovarian histology showed PCOS-induced irregularities, while HUE-treated groups exhibited normal follicular development, possibly due to flavonoids. Uterine histology indicated endometrial hyperplasia in PCOS-induced rats, but HUE treatment increased uterine gland numbers, suggesting hormonal modulation. Kidney weight increase in PCOS-induced groups may be attributed to androgen administration, with potential benefits from HUE, especially at moderate doses. Gene analysis hinted at potential hormonal modulation by HUE, though statistical significance was not reached. Flavonoids may lower estrogen levels, possibly through AMP-activated protein kinase (AMPK) pathways (Chen et al., 2017).

In summary, HUE displayed various effects on weight gain, potential modulation of ovarian morphology, and impacts on uterine histology and renal fibrosis in a PCOS rat model. These findings underscore the need for further investigation into HUE's active constituents, mechanisms, and clinical applications for PCOS.

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Chapter I: Introduction

Reproductive success is dependent on the development and maintenance of reproductive systems, endocrine activity, and communication between these different systems. In reproduction, the hypothalamic-pituitary-gonadal axis is crucial. Successful reproduction in female animals hinges on the cyclical production of sex and sex-related hormones across the hypothalamus-pituitary-ovarian (HPO) axis (Estienne et al., 2021). At each level of the axis, specific hormones are produced to engage in either positive or negative regulatory mechanisms. Within this axis, hypothalamic neurons produce and release Gonadotropin-Releasing Hormone (GnRH), a crucial hormone in regulating gonadotropin secretion, comprising Follicle-Stimulating Hormone (FSH) and Luteinizing Hormone (LH). These hormones in turn regulate gametogenesis and steroidogenesis in the gonads. The steroid hormones are transported through a circulatory system in a feedback loop onto the pituitary gland and hypothalamus, inhibiting the release of GnRH, FSH and LH, thereby completing a negative feedback circuit. Additionally, in females just prior to ovulation, there is a change in the estrogen-induced negative feedback to positive feedback via a mechanism which causes stimulation of GnRH and LH, resulting in the GnRH/LH preovulatory surge that triggers ovulation. Understanding how the reproductive system works and the signals that affect it can serve as a useful tool in treating reproductive disorders.

Polycystic ovarian syndrome (PCOS) is one of the most common endocrinological disorders affecting women of reproductive age globally with a prevalence of 5 to 20% based on the type of criteria employed and population studied (Ehrmann, 2005). PCOS dramatically affects reproductive capabilities and often results in infertility. In the USA, the total cost of diagnosing and providing medical treatment to PCOS is up to 4.4 billion dollars with 12.2% (533 million dollars) of which is used in providing infertility care (Ehrmann, 2005). It is more prevalent in

Mexican American women and least prevalent in Asian women (Smet et al., 2018). This syndrome is a complex heterogeneous endocrine disorder which is defined by 3 main criteria: Oligoovulation or Anovulation, Hyperandrogenism, and Polycystic Ovaries, after other possible illnesses have been ruled out. (Teede et al., 2010). However, it has other clinical and biochemical features such as obesity, diabetes mellitus, hirsutism, insulin resistance, hyperinsulinemia. PCOS dramatically affects reproductive success and often results in infertility (Azziz et al., 2001).

Due to the complexity of the pathophysiology of PCOS, an ultimate cure has not been found. Instead, what is more common is the use of pharmaceuticals to manage the symptoms, although they often come with a relatively high cost and potential side effects. PCOS is identified by a dysregulation of the hypothalamic-pituitary-ovarian axis, leading to irregular GnRH secretion (hypersecretion). This, in turn, influences FSH and LH levels, resulting in elevated LH to FSH causing ovarian dysfunction. Although the exact etiology of PCOS is not known, insulin resistance (IR) seems to play a significant role in the development of this disorder (DeClue et al., 1991). Experiments conducted both in vivo and in vitro indicate that hyperinsulinemia, a downstream effect resulting from insulin resistance (IR), may contribute to heightened ovarian androgen biosynthesis and reduced hepatic production of sex hormone-binding globulin. (Adams et al., 2004; Rosenfield, 2015).

Evidence has shown that the use of plants as a form of medicine dates as far back as 5,000 years ago (Petrovska et al., 2012). It was plants that were the primary source of treatment and prophylaxis up until the advent of iatrochemistry in the 16th century. Plant parts such as the leaves, fruits, and bark can all be used to make concoctions that can treat a variety of illnesses (Oboh et al., 2018). For example, several studies indicate that garlic boosts our immune system, ginger improves digestion, and aloe vera soothes burns and skin irritation. As synthetic drugs become less

effective and their contraindications increase, millions of people turn to herbal remedies and traditional medicines for their health needs. In various traditional healing practices, different components of Hunteria umbellata hold significance for treating numerous local diseases. In Germany, the plant is used as an aphrodisiac and in lowering heart rate (Boone, 2006). In West Africa, it is utilized as an anti-inflammatory medication, for managing obesity, and in fertility treatments (Adeneye et al., 2010). Across various regions in Asia, this medication is frequently used for its antidiabetic and anti-helminthic effects (Hung et al., 2012).

Hunteria umbellata belongs to Apocynaceae family which are commonly found in west and central African countries (Falodun et al., 2006). It is a small deciduous tree, and various parts of the plant have therapeutic effects. The seeds are locally used to manage diabetes, the leaves and pulp help prevent dystocia and miscarriages, while the bark is effective against fever and stomachache (Igbe et al., 2009). *Hunteria umbellata* (HU) has been discovered to contain rich amounts of tannins, flavonoids, alkaloids, anthraquinone, cardiac glycosides, phlobatannins, and various indole alkaloids, including eburnamonine, eburnamine, and hunterine. These compounds have been observed to exhibit long-lasting hypotensive and hypoglycemic properties (Adeneye et al., 2012; Ajiboye et al., 2017; Ibeh et al., 2007). Several studies have also highlighted in great detail these hypoglycemic properties, elucidating its mechanism of action on animals (Adeneye et al., 2010; Adejuwon et al., 2013: Falodun et al., 2006). Yoruba herbalists in Nigeria are known for using a cold infusion of the plant's seeds to treat hyperlipidemia and obesity - two other clinical features usually associated with PCOS (Adeneye et al., 2011).

Like numerous herbs used in folkloric medicine, there is no agreement on the ideal level of HU concentration that is appropriate for physiologic effect or on the safety and efficacy of HU seed usage. Nonetheless, various studies, including the work of Adeneye and Adeyemi in 2009,

propose a suitable therapeutic dosage ranging from 50 mg/kg to 500 mg/kg. The specific dosage depends on the part of the plant utilized and the extraction method employed (Adeneye & Adeyemi, 2009; Ajiboye et al., 2017). With limited treatments available for PCOS, exploring HU seed, known for managing its associated metabolic issues, offers a promising avenue for therapy. This study represents a novel approach, as no previous research has investigated HUE's effects on PCOS. By examining how HU seed may impact the underlying mechanisms of PCOS, we aim to uncover new treatment possibilities for this multifaceted disorder.

Therefore, the aim of this study was to evaluate the effects of the *Hunteria umbellata'* s aqueous extract (HUE) on the induction and maintenance of PCOS. Our specific goals were to use an in-vivo model to investigate the impact of HUE on the onset of PCOS and to determine if HUE effects on the maintenance of PCOS are both dose- and time- dependent in Sprague Dawley (SD) rats. Further, we evaluated the effects of administering HUE in different doses on reproductive hormones, histological architecture of different organs, and other hematological parameters in female SD rats. The results of this experiment will provide insights into how a more cost-effective and readily available plant could alleviate the symptoms associated with PCOS, a syndrome currently lacking a definitive cure. Our hypothesis suggests that oral administration of HUE will demonstrate a dose- and time-dependent decrease in the persistence of PCOS symptoms in female Sprague-Dawley rats. This reduction is expected to be reflected in changes observed in organ morphology through histopathology.

Chapter II: Literature Review

Hypothalamic-Pituitary-Ovarian Axis (HPO Axis)

Understanding the pathophysiology of PCOS requires a comprehension of the intricate interplay among systems that regulate and modulate hormones essential for the normal functioning of the reproductive system. The Hypothalamic-Pituitary-Ovarian (HPO) axis plays a central role in the regulation of the menstrual cycle and reproductive functions (Mikhael et al., 2019). This intricate system involves the hypothalamus, pituitary gland, and ovaries, orchestrating hormonal interactions crucial for female fertility. One fundamental concept within this axis is the "2 cell 2 gonadotropin" theory of estrogen production. The process begins with Gonadotropin-Releasing Hormone (GnRH) synthesized in the hypothalamus in a pulsatile manner. GnRH is released from the median eminence into fenestrated capillaries, then transported to the anterior pituitary gland via portal circulation (Marques et al., 2022). This activates pituitary release of gonadotropin hormones (LH and FSH), which then stimulate the synthesis and secretion of gonadal sex steroids like estradiol and testosterone (Kauffman, 2022). Two separate ways of GnRH secretion exist: pulsatile and surge modes. Pulsatile mode involves episodic bursts of GnRH released into portal circulation, with undetectable GnRH concentrations between pulses. Surge mode, observed in females during the pre-ovulatory phase, shows persistent presence of GnRH in portal circulation (Maeda et al., 2010). The significance of GnRH pulsatility in LH and FSH secretion was initially shown in rhesus monkeys by eliminating endogenous GnRH secretion through hypothalamic radiofrequency. Pulsatile GnRH restored gonadotropin secretion, while continuous GnRH only produced a temporary response. Transitioning from continuous to pulsatile GnRH administration facilitated the recovery of gonadotropin secretion (Belchetz et al., 1978).

GnRH then acts on the anterior pituitary gland, stimulating the release of gonadotropins: Follicle-Stimulating Hormone (FSH) and Luteinizing Hormone (LH). The stimulatory effects of GnRH on LH and FSH secretion differ due to pulsatility, varying stimulatory effects of GnRH, and other influencing factors (Singh et al., 2023). FSH and luteinizing hormone (LH) collaboratively regulate animal reproduction via specific G protein-coupled receptors (GPCRs) under physiological conditions. They also influence steroid hormone production, cellular metabolism, growth, and various physiological activities, thereby eliciting specific biological effects on the hypothalamus, pituitary, ovary, testis, and other target tissues (Wang et al., 2021) FSH primarily targets the ovaries, initiating the follicular phase of the menstrual cycle. Within the ovaries, the "2 cell" aspect of the 2 cell 2 gonadotropin estrogen biosynthesis theory, involves the collaboration of two distinct types of cells: theca cells and granulosa cells in the production of androgen and ultimately estrogen (Liu & Hsueh, 1986). Theca cells, under LH stimulation, produce androgens like androstenedione. Subsequently, these androgens travel to granulosa cells, where they undergo aromatization—a process facilitated by FSH. This conversion ultimately leads to the production of estrogens, primarily estradiol (Singh et al., 2023).

The produced estrogen plays a crucial role in the menstrual cycle, influencing the proliferation of the uterine endometrium during the follicular phase and preparing the uterus for potential embryo implantation (Xu et al., 2022). Furthermore, estrogen levels exert feedback effects on the HPO axis; in a negative-feedback loop, high estrogen levels inhibit the release of GnRH, FSH, and LH, regulating the overall hormonal balance. (Beshay & Carr, 2017). Estradiol (E2) positive feedback results in a significant rise in GnRH secretion, leading to the 'GnRH surge', which in turn prompts a substantial 'LH surge' from the pituitary, initiating ovulation (Kauffman, 2022). The reason behind LH initially responding negatively to estrogen and later establishing a

positive feedback relationship remains unclear. Numerous studies have explored this phenomenon, with some researchers attributing it to synaptic transmission speed (Christian & Moenter, 2008), while others propose that the sustained presence of estrogen induces an upsurge in Glutamate and Gamma Amino Butyric Acid (GABA) transmission within GnRH neurons. (Robinson et al., 1991). Crucially, GnRH neurons do not possess sex steroid receptors (such as estrogen receptor α, androgen receptor, or progesterone receptor) responsible for both positive and negative feedback. Therefore, the regulation of GnRH secretion by sex steroids is indirect, happening through other steroid-sensitive brain cells upstream that interact with GnRH cells (Cheong et al., 2015; Kauffman, 2022).

It is generally believed that the adrenal glands and the ovaries produce testosterone roughly equally in response to their respective tropic hormones, LH and Adrenocorticotropic hormone (ACTH) (Horton et al., 1966). Androgen secretion, triggered by tropic hormones, is regulated through intraglandular paracrine and autocrine mechanisms. In the different regions of the body, testosterone is produced in equal amounts by the ovaries and adrenal glands, while the other half is produced by peripheral conversion of circulating androstenedione (Xu & Qiao, 2022). Recent research indicates that slight excesses of androgens disrupt the negative feedback of female sex hormones, a prevalent issue linked to polycystic ovarian syndrome (PCOS) (Rosenfield & Ehrmann, 2016).

Estrogen Receptors

Estrogen mainly influences biological functions through estrogen receptors (ER) at the gene level. It has been noted to impact ovarian functions through autocrine or paracrine actions, notably enhancing FSH effects on granulosa cells (GC). ERs are expressed in both granulosa cells (GC) and theca cells (TC) within developing follicles (Gustafsson et al., 2003) Additionally,

estrogen is crucial for maintaining reproductive function and plays vital roles in multiple bodily systems, including musculoskeletal, cardiovascular, immune, and central nervous systems (Tenti, et al., 2020)

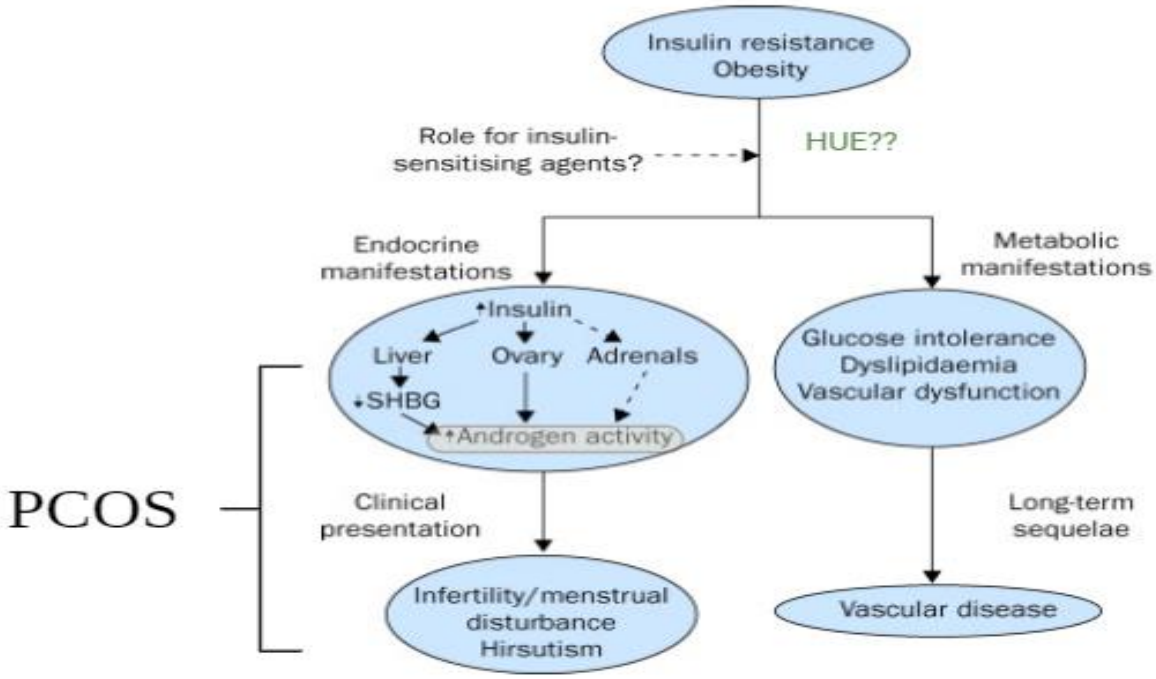
Estrogen exerts its effects through genomic pathways involving estrogen receptors ERα and ERβ. When estradiol binds to either receptor in the cytoplasm, it triggers a conformational change, leading to receptor dimerization. This complex moves to the nucleus, where it binds to chromatin at ERE sequences and modulates the transcription of target genes by recruiting transcription factors. In addition, rapid non-genomic pathways are recognized, where estrogen elicits swift responses. These responses involve the activation of various second messengers like cyclic adenosine monophosphate (cAMP) and calcium ions (Ca2+), or the initiation of intracellular kinase pathways (Walters, 2020; Xu et al., 2021).

Recent research has identified the G-protein-coupled estrogen receptor (GPER) as a mediator of estrogen's non-genomic signaling. Studies also demonstrate that GPER can rapidly activate multiple signal transduction cascades, including mitogen-activated protein kinase (MAPK), protein kinase C, and phosphatidylinositol 3-kinase (PI3K). Additionally, estradiol (E2) may bind to ERα and ERβ located in the plasma membrane, influencing cellular signaling through rapid membrane-initiated events, which are part of non-genomic signaling pathways (Xu et al., 2021). Considering these factors, altered expression of ovarian estrogen receptors (ERs) could play a significant role in conditions like PCOS, which are characterized by ovulation dysfunction. Studies have shown that disrupting the ERα gene in mice leads to a phenotype resembling PCOS, marked by elevated LH levels and ovaries containing multiple hemorrhagic and cystic follicles. Similarly, abnormal expression of ERβ in mouse ovaries results in inconsistent development of dominant follicles (Zhou et al., 2021)

Polycystic Ovarian Syndrome (PCOS)

Figure A

Illustration portraying the morbidities associated with PCOS and a potential targeted approach to alleviate the metabolic disturbances Figure adapted from (Harborne et al., 2003).



Pathophysiology

Polycystic ovarian syndrome may be correlated with neuroendocrine, metabolic, and ovarian abnormalities. As a heterogeneous disorder, PCOS has an unknown etiology. A primary disorder of gonadotropin secretion, ovarian hyperandrogenism and insulin resistance are the major etiologies of PCOS (Azziz et al., 2001). PCOS may involve heightened gonadotropin-releasing hormone (GnRH) pulse frequency, leading to an elevation in both the frequency and pulse amplitude of LH over FSH production. Consequently, this abnormality contributes to an increased circulating LH/FSH ratio resulting in immature follicles (Saadia, 2020). As a result, ovarian

follicles may not respond to FSH, anti-mullerian hormone (AMH) may be elevated, follicular arrest may occur, and testosterone, estradiol, and dehydroepiandrosterone (DHEA) may be secreted in greater quantities (Lauritsen et al., 2014). AMH is intricately linked to PCOS, where it is frequently elevated. This hormone, produced by small ovarian follicles reflects ovarian reserve, indicating the quantity of remaining eggs. In PCOS, elevated AMH levels are associated with an abundance of small follicles, a defining characteristic (Rudnicka et al., 2022). This abundance leads to irregular menstrual cycles and infertility in PCOS. These conditions can lead to elevated circulating androgens as a result of disrupted ovarian steroid hormone synthesis (Gargus et al., 2022). Apart from issues related to ovulatory dysfunction, including polycystic ovaries observed through ultrasound and histopathological examination, PCOS is associated with hyperandrogenemia, abnormal gonadotropin levels, hirsutism, hyperinsulinemia, and insulin resistance (Abraham Gnanadass et al., 2021; Azziz et al., 2001; Azziz et al., 2004; Hoeger et al., 2021). The hypothalamic–pituitary–ovarian axis is abnormally affected by high insulin levels (Chang et al., 1986). These insulin-induced changes play a role in suppressing post-receptor effects, elevating free fatty acids, releasing androgens and cytokines into the bloodstream. This cascade leads to increased deposition of leptin by adipocytes in the abdomen and notable rises in blood plasma levels of these substances. (Zeng et al., 2020). Furthermore, an increase in androgen levels in the blood may inhibit adiponectin, reducing insulin sensitivity and increasing insulin levels in the body (Moghetti et al., 2021). Hyperinsulinemia and increased cellular androgen production contribute to elevated circulating testosterone levels by reducing sex hormone-binding globulin (SHBG) (Pugeat et al., 2010; Simó et al., 2012). The exacerbation of these factors may further worsen the progression of the disease.

Criteria for diagnosing PCOS.

The authors of a 1935 report on their findings of seven cases in which they described patients suffering from oligo- or amenorrhea in addition to polycystic ovaries on both sides (Stein and Leventhal, 1935). Among these patients, only three were obese, and five had hirsutism. In one case, the patients with acne were thin and in another, they were obese and hirsute. Polycystic ovaries are different from PCOS, even though many women with PCOS have them. In many patients with PCOS, abnormal gonadotropin concentrations, such as elevated LH/FSH ratios, have been observed. (Azziz et al., 2004) Considering the heterogeneity of the disorder and the conflicting findings from published studies, an expert meeting was held in 1990 at the National Institute of Child Development and Health (NICHD). A consensus derived from these data highlights that PCOS is primarily characterized by (in order of importance): (1) clinical manifestations such as hirsutism and/or biochemical indications of hyperandrogenism, (2) persistent anovulation, and (3) the exclusion of related disorders like congenital adrenal hyperplasia, androgen-secreting tumors, hyperprolactinemia, and thyroid disease (Zawadzki, 1992). A consistent hallmark of PCOS is the presence of elevated androgen levels, either manifested clinically or biochemically. Consequently, the initial diagnostic step involves confirming the existence of symptoms indicative of excess androgen, such as hirsutism. Additionally, androgenic acne, alopecia, and dysfunction of the ovaries may indicate androgen excess, although their association is less strong than that with hirsutism. (Azziz et al., 2001; 2004).

Classification of PCOS Based on Phenotypes: Unraveling the Complexity (Khan et al., 2019).

Phenotype A and Phenotype B - Classic PCOS:

Classic PCOS, represented by Phenotype A and Phenotype B, is defined by a combination of oligoovulation and hyperandrogenism, along with either the presence or absence of polycystic ovaries as determined by ultrasound. Women falling into this category commonly experience heightened menstrual dysfunction, increased insulin secretion, and elevated insulin resistance. Additionally, they face an increased risk of metabolic syndrome, obesity, and atherogenic dyslipidemia. The noteworthy aspect of significantly elevated levels of anti-mullerian hormone (AMH) further emphasizes the ovarian component inherent in this classic presentation.

Phenotype C - Ovulatory PCOS:

Phenotype C is characterized by individuals who show polycystic ovaries on ultrasound, maintain regular menstrual cycles, and exhibit hyperandrogenism. While these individuals maintain ovulatory cycles, they often have mildly elevated serum insulin, atherogenic lipids, and androgen levels. Notably, high hirsutism scores and an increased prevalence of metabolic syndromes set ovulatory PCOS apart from classic and non-hyperandrogenic PCOS. It is worth noting that higher socioeconomic status could play a role in influencing ovulation patterns within this phenotype.

Phenotype D – Non-Hyperandrogenic PCOS :

Unlike the classic PCOS phenotypes, Phenotype D distinguishes itself by having normal androgen levels, slightly elevated endocrine levels, and the least degree of metabolic dysfunction. Individuals in this group commonly have regular menstrual cycles with occasional irregularities. Phenotype D challenges the conventional understanding of PCOS, highlighting the syndrome's

multifaceted nature and emphasizing the need for personalized diagnostic and management approaches.

Heterogeneity of PCOS and Possible Pathways

The majority of women with polycystic ovary syndrome (PCOS) develop this endocrine disorder during their reproductive years, with a prevalence of 5% to 20%, depending on the diagnostic criteria (Ehrmann, 2005) Gonadotropin secretion was adopted as an alternative diagnostic tool and research was focused on the possible neuroendocrine origins of the syndrome (Yildiz et al., 2003). Dysregulation of androgen secretion is thought to cause PCOS due to functional ovarian hyperandrogenism (FOH) (Hernández-Jiménez et al., 2022). This research, which led to the conclusion that PCOS is a type of FOH caused by a dysregulation of androgen release, changed our thinking of this syndrome. In place of deficient enzyme activity, a steroidogenic disorder is believed to be caused by enzyme dysregulation. (Wawrzkiewicz-Jałowiecka et al., 2020).

Significant insulin resistance has also been demonstrated to occur independently of obesity in the disorder of hyperandrogenism (Chang et al., 1983). The mechanisms underlying insulin resistance have been recently delineated; insulin resistance induces lipolysis at the liver and skeletal muscle levels, leading to an accumulation of non-esterified fatty acids. This lipid buildup in the liver triggers activation of the diacylglycerol/protein kinase C axis, which inhibits the insulin receptor, thereby disrupting insulin signaling and gluconeogenesis (Petersen & Shulman, 2018) Additionally, in skeletal muscle, inhibition of phosphoinositide-3 kinase and phosphorylation of insulin receptor substrate 1 result in impaired insulin signaling, altering the expression of GLUT-4 and glucose uptake (Armanini, et al., 2022). The results of in vitro studies have shown insulin stimulates the production of ovarian androgen, particularly in conjunction with LH (Ding et al.,

2021). In addition to elevated serum LH levels and the LH to FSH ratio (Littlejohn et al., 2007), FSH levels are normal to slightly suppressed and are not sufficiently high to stimulate normal follicle development (Rosenfield et al., 2015). PCOS has never included gonadotropin levels in any of the diagnostic criteria due to the pulsatile nature of LH production, which makes it difficult to detect characteristic deviations in blood samples (Winters et al., 2000).

Insulin resistance and type 2 diabetes are associated with PCOS; the connection between insulin and androgen levels introduces a confounding factor. Insulin potentially influences androgen synthesis, metabolism, and/or clearance at various levels, including the pituitary, ovarian, and hepatic levels (Zhu et al., 2021). Studies suggest that hyperinsulinemia might impact excessive androgen production in the ovaries. In a study by Burghen et al., (1980), it was observed that women with PCOS exhibited higher insulin responses during oral glucose tolerance tests, unrelated to obesity. The presence of acanthosis nigricans in women with typical PCOS raised the possibility of insulin resistance, akin to individuals with rare insulin resistance syndromes displaying acanthosis nigricans. The findings of this study led to a new area of study on the mechanisms that link insulin resistance to PCOS. Adipocytes, skeletal muscle, and cardiac muscle are insulin-responsive target tissues that regulate glucose homeostasis by up taking glucose and inhibiting liver glucose synthesis (Roberts et al., 2007). The action of insulin on hepatic glucose production may be mediated by insulin's suppression of lipolysis, which results in a decrease in circulating free fatty acid levels (Nakamura et al., 2009).

A metabolic action such as glucose uptake, glucose production, or lipolysis requires increased amounts of insulin due to insulin resistance, which results in insulin being less effective at mediating these metabolic actions. (Arner 2005; Ek et al., 2002; Kobaly et al., 2014). Women can also develop visceral fat mass due to androgens (Tsilchorozidou et al., 2003). An increased

production of insulin, the abnormal actions of insulin, the decrease of hepatic insulin clearance, or the combination of these metabolic changes may result in hyperinsulinemia (Ezeh et al., 2022). As a result of excessive serine phosphorylation of insulin receptors, many PCOS patients are insulin resistant, impairing glucose oxidation in cells (Oróstica et al., 2020). According to Dunaif et al. (1995) suggested this abnormality may be attributed to a serine/threonine kinase external to the insulin receptors. A study conducted in vitro found that insulin stimulated the synthesis of androgen from stromal and thecal ovarian tissue (Ehrman et al., 1992).

Increased ovarian P450c17α activity, a key enzyme in the biosynthesis of androgens, is reported in obese and non-obese hyperinsulinemic females. The potential exacerbation of ovarian androgen biosynthesis in PCOS could be attributed to reduced serum adiponectin levels. Adiponectin is known to exert inhibitory effects on ovarian androgen production; thus, its suppression may contribute to increased androgen biosynthesis in the ovaries of individuals with PCOS (Barber et al., 2021) The levels of sex hormone-binding globulin (SHBG) at the hepatic level are lower in women with PCOS due to the inhibitory action of insulin, boosting free testosterone levels in the bloodstream (Nestler et al., 1991). SHBG, a liver-produced glycoprotein, exhibits high affinity and specificity for binding sex steroids. Clinical observations and literature reports have indicated a negative correlation between circulating SHBG levels and markers of non-alcoholic fatty liver disease (NAFLD) and insulin resistance, both common comorbidities associated with PCOS (Qu & Donnelly, 2020). According to these studies, insulin may be a pathogenetic factor in the onset of hyperandrogenism in some women with PCOS. Pharmacological agents such as glucosidase inhibitors can decrease insulin secretion or increase insulin sensitivity. (Tiwari & Rao, 2002). As a result of both insulin resistance and decreased insulin clearance, PCOS is known to produce hyperinsulinemia (Chang et al., 1989). Numerous

studies using conventional methods have investigated the mechanisms underlying insulin resistance in PCOS. (Borzan, et al., 2021; Zeng et al., 2020). The development of abdominal obesity may be attributed to hyperandrogenism by increasing the number of skeletal muscle fibers that are less insulin sensitive (Holmang and Bjorntorp, 1992). Alongside traditional regulators like FSH and LH, insulin-like growth factor (IGF) plays a role in regulating both insulin and androgens (Li et al., 2023).

*Hunteria umbellata (*HU) and Possible Mechanism of Action

Some of HU's active phytochemicals are responsible for its hypoglycemic and hyperlipidemic effects. There have previously been reports that HU contains alkaloids, flavonoids, saponins, and tannins (Adeneye and Adeyemi, 2009; Ibeh et al., 2007). It is also well documented in literature that the presence of glycosides, flavonoids, alkaloids, saponins, phytosterol, terpenoids, and tannins account for the hypoglycemic and hypolipidemic activities of medicinal plants. (Adeneye et al., 2010; Udinyiwe & Aghedo, 2022).

In a recent research study, Erinidine was obtained after in vitro derivatization, which may enhance insulin sensitivity (Adejuwon et al., 2013). The antihyperglycemic effect of erinidine was found to be weakly mediated through glucosidase inhibition mechanisms in vitro. (Adeneye et al., 2012). For erinidine to exert its glucose uptake inhibitory effect, it may need to be bio-transformed into its active metabolite(s) (Adejuwon et al., 2013). In vitro assays show poor biological activity for some extracts, but their active metabolites may become pharmacologically active when transformed into their active metabolites in vivo (Farnsworth, 1993).

The action of several alkaloids on beta-glucosidase inhibits intestinal glucose uptake and leads to hypoglycemia. In response to the treatment of PCOS, present studies suggest that HU may

contribute to concomitant steroidogenic reversal, from downstream effect on glucose metabolism as one of the possible mechanisms for its therapeutic advantages (Adeneye and Adeyemi, 2009).

There is evidence that flavonoids inhibit prolactin levels and increase ovulation by interfering with dopamine-2 (D-2) receptors (Seidlova-Wuttke & Wuttke, 2017). Additionally, flavonoids may increase ovulatory cycles due to their effects on estrogen receptors (ERs) (Falodun et al., 2006).

There is a link between abnormal steroid biosynthesis in PCOS women, which is associated with cytochrome P450 and 17-hydroxylase (CYP17A1) and enhanced CYP17A1 activity enhances androgen production and secretion (Ding et al., 2018; Hajiaghayi et al., 2016). In the synthesis of steroid hormones, the enzyme CYP17A1 plays a key role. (Moka & Sumithra, 2022) There are increased androgen levels in women with polycystic ovarian syndrome (PCOS) because of increased Cyp17A1 expression and changes in gene expression in granulosa cells (Kakuta et al., 2018). The CYP17A1 gene might be suppressed by HU is another potential pathway. Cyst formation in the ovary can be further suppressed by all the mechanisms above, which could be beneficial for treating PCOS.

Furthermore, HU may increase the utilization of glucose in peripheral tissues, as well as reduce the intake of food and the absorption of glucose in the intestinal tract. (Adeneye et al., 2011; Ibeh et al., 2007). Studies have also demonstrated that HU contains a high amount of alkaloids, which may have accounted for the antihyperglycemic action of HU that was mediated via intestinal glucose uptake inhibition. (Adejuwon et al., 2013; Ajiboye et al., 2017). HU may also lower glucose absorption by inhibiting sucrase and maltase activity in CaCo-2 intestinal cells in a similar manner to glycoside inhibitors (Tiwari & Rao, 2002). The lack of stimulation of endogenous insulin secretion in HU prevents hyperglycemia and hyperinsulinemia, commonly associated with

antidiabetic drugs such as metformin, usually prescribed for the maintenance of PCOS symptoms (Adeneye et al., 2012). Additionally, it may treat PCOS since insulin resistance is more prevalent in women with PCOS. As HU improves insulin sensitivity, it may also reduce insulin levels and, consequently, androgen levels (Okolafor & Ekhaise, 2021). Thus, HU might become a key alternative drug in the treatment of PCOS. HU, however, may phosphorylate and activate AMP-activated protein kinase (AMPK) in the liver, which in turn may lead to a variety of pharmacologic effects, including inhibition of glucose and lipid synthesis, due to the nature of the phytochemicals present (Chen, et al., 2017; de Freitas Junior et al., 2017).

Conclusively, the molecular mechanisms underlying the HU action appear to be complex and remain a topic of considerable debate. In order to improve therapeutic innovation, it is imperative that we evaluate all possible mechanisms of action.

Chapter III: Hypotheses

We hypothesized**:**

- H_0: HUE doses administered orally will not have an ameliorative effect on the persistence of PCOS in female Sprague-Dawley rats based on changes to their organ morphology.

- H_1: HUE doses administered orally will have an ameliorative effect on the persistence of PCOS in female Sprague-Dawley rats based on changes to their organ morphology.

- H_0: Oral administration of HUE will not have a dose- and time-dependent attenuating effect on PCOS symptoms in female Sprague-Dawley rats based on histopathological evidence.

- H_2: Oral administration of HUE will have a dose- and time-dependent attenuating effect on PCOS symptoms in female Sprague-Dawley rats based on histopathological evidence.

Chapter IV: Materials and Methods

Approval

The research protocols employed in this study were approved by the Institutional Animal Care and Usage Committee (IACUC) of St. Cloud State University, St. Cloud, Minnesota, USA. The research was conducted in compliance with the National Institute of Health's Guide for the Care and Use of Laboratory Animals (National Library of Medicine, 2011). All experiments were carried out in the vivarium at the integrated science and engineering laboratory facility (ISELF) building at St. Cloud State University, St. Cloud, Minnesota.

Seed Acquisition and Preparation

Hunteria Umbellata seeds were obtained from Falana Farms in Ibadan, Oyo State Nigeria. The identity of the seed was validated by Dr. Taofikat Adesalu of the Department of Botany, University of Lagos, Nigeria. The seeds were cleaned, rendered free of dust, and incubated at 40°C till well dried; before being blended to a fine powder using Waring commercial blender (Waring Products Division, New Hartford, Conn. USA). It was then stored in a polythene bag in a dry area at room temperature until used.

Aqueous extraction of HU seeds

Hunteria umbellata extract (HUE) were performed using an updated Adeneye et al., (2010) protocol. 25 g of dried seeds were ground into a fine powder using a Waring commercial blender. Following this, 500 ml of distilled water is added to the powder to make solution, allowing it to boil continuously for 24 hours before being filtered through a clean muslin cloth. After filtering, petri dishes with the liquid filtrate were placed in an incubator at 37°C for 10+ hours until completely dried. Residues were then scraped, ground, and stored in a refrigerator in a sealed container at 4°C. Different doses of the HU solution used for administration were made

from this powdered residue. The doses used were determined based on results from previously conducted research study (Adeneye et al., 2010; 2011)

Experiment 1: Pilot Study

Effect of aqueous extract of Hunteria umbellata on body weight, water intake, and feed consumption in Sprague Dawley rats.

The main objective of this preliminary experiment was to determine the effect aqueous extract of *Hunteria umbellata* would have on body weight, water intake and feed consumption in Sprague Dawley rats. Based on the results, we were able to ascertain the safety and acceptability of the extract as well as provide valuable insight for the main study's success. Twenty (20) juvenile female Sprague Dawley rats weighing 41 ± 5.17g around 3 weeks old were used in this experiment. The animals were housed at the ISELF Vivarium of the Department of Biological Sciences, St. Cloud State University, Minnesota throughout the study. They were maintained under a lighting regimen of 12 hours under light and 12 hours in the dark at 25℃. These rats were obtained as pups from adult rats that were housed and bred in the same facility. The rats were weighed at the beginning of the experiment and randomly assigned to one of four groups: **control group** (regular water; n=5), **low-dose group** (100 mg/kg body weight - *Hunteria umbellata*; n=5), **medium-dose group** (200 mg/kg body weight - *Hunteria umbellata*; n=5) and **high-dose group** (400 mg/kg body weight - *Hunteria umbellata*; n=5). Throughout the 7-day research period, daily weight measurements were taken for all rats, and the quantity of consumed HUE was documented. Rats had ad libitum access to their feeds, which consisted of a standard diet comprising 4% fat, 16% protein, and 49% carbohydrates. Additionally, various doses of HUE were administered alongside the regular rat feed.

Experiment 2: Main Study

Effect of Hunteria Umbellata Aqueous Extract on the Morphology of Reproductive and Metabolic Organs in Sprague Dawley Rats with Polycystic Ovary Syndrome (PCOS)

The objective of this study was to determine the effect of the oral administration of HUE on the induction and maintenance of PCOS based mainly on the changes to the histoarchitecture of different organ-systems. Organs of emphasis included the ovaries, kidney, liver, uterus. We also determined the effects on body weights and specific organ weights.

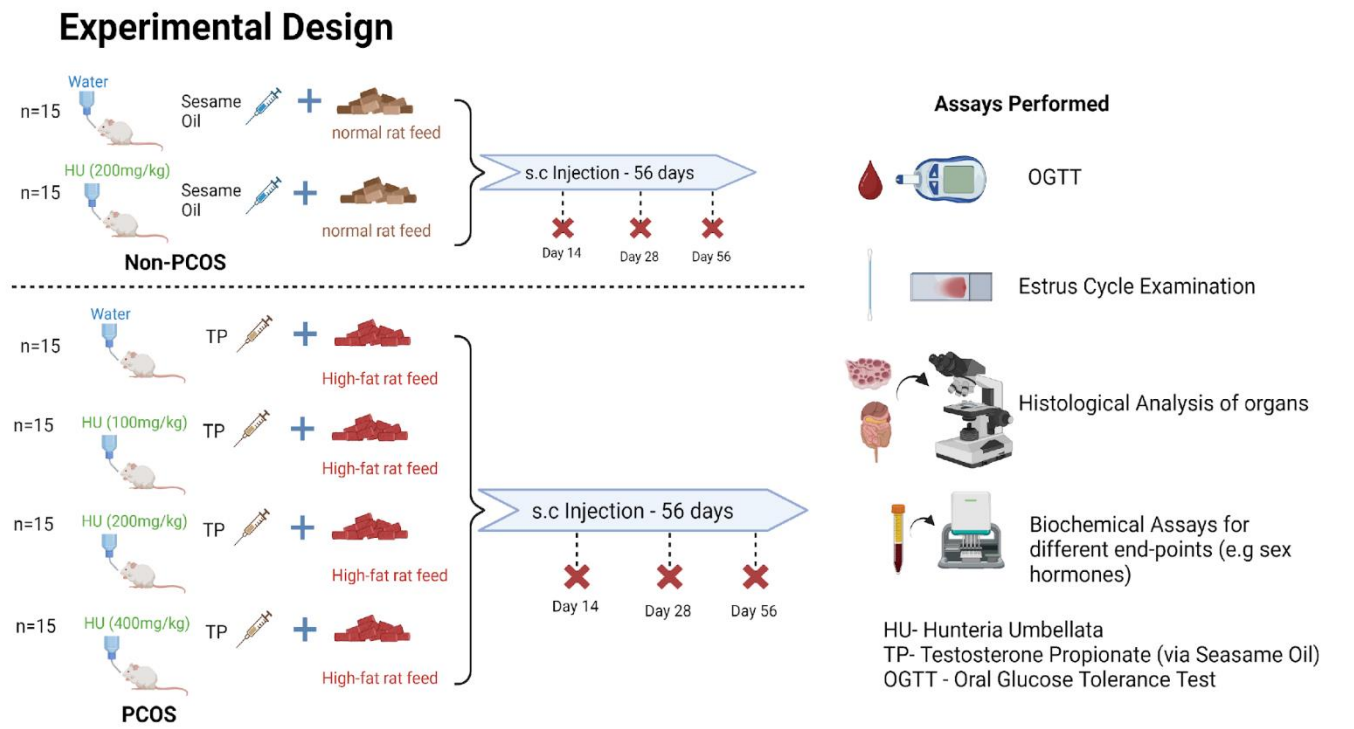
Experimental Animals

90 female Sprague Dawley rats weighing approximately 56 ± 8.7g body weight (BW), at 3 weeks of age, were placed in two major groups, either non-PCOS (nPCOS) (n=30) or Testosterone Propionate (TP)-induced PCOS (n=60) group. The nPCOS were allotted to either control (regular water- RW_nPCOS) or *H. umbellata* at 200 mg/kg of BW (mid-dose- MD_nPCOS). PCOS-induced rats were further divided into 4 groups: regular water - RW_PCOS, HUE at 100 mg/kg of BW (low-dose- LD_PCOS), HUE at 200 mg/kg of BW (mid-dose - MD_PCOS) or HUE at 400 mg/kg of BW (high-dose - HD_PCOS). Commencing from the third week, a cohort of 30 rats, comprising 15 rats in the nPCOS group and 15 rats in the PCOS group, were provided ad-libitum access to regular water. The remaining 60 rats were given water containing varying doses of HUE. Starting in the fourth week, the PCOS induction injection began and continued for 56 days. The time points at 14, 28, and 56 days are crucial for evaluating the effects of HUE over time and assessing any time-dependent factors. Beginning at the 4^{th} week of life, each rat in the PCOS group was fed with a high-fat (HF) diet for 28 days and the nPCOS rats were fed the normal rat diet for the same period. The high fat diet contained 29% fat, 26% proteins and 30% carbohydrate (Envigo Teklad Diets). This contrasts with the 4% fat. 16% protein and

49% carbohydrates present in the regular Teklad Global rodent diet. Research studies have indicated that the PCOS induction technique of TP + high fat diet can generate both metabolic and reproductive characteristics similar to human PCOS (Zhai et al., 2012). TP directly contributes to increased androgen levels, while a high-fat diet may contribute to insulin resistance and obesity, creating a synergistic effect that can disrupt normal ovarian function. Additionally, excessive testosterone and a high-fat diet have been associated with increased inflammation in the body. Chronic inflammation as well, further exacerbates the associated conditions of PCOS (Rudnicka et al., 2021). During this feeding period, each PCOS rat were subcutaneously injected with TP (1mg per 100 g BW; dissolved in sesame oil; Sigma Aldrich Co, St. Louis MO) daily. Each non-PCOS rat received the same volume of the diluent sesame seed oil, subcutaneously. Rats were maintained under standard conditions in the ISELF vivarium.

Figure B

This illustration demonstrates the experimental design utilized for the research work.



Euthanization

On the 2nd, 4th, and 8th weeks post-PCOS induction, 5 rats from each of the 6 groups were euthanized by cervical dislocation and decapitation following approved IACUC guidelines.

Plasma Collection

Whole trunk blood was collected into EDTA tubes upon decapitation. Blood samples were centrifuged at 3000 rpm for 10 minutes and plasma was harvested and stored at -80°C for subsequent glucose assay and other end points analyses.

Hematological Analyses

Whole blood samples from each rat were collected into EDTA tubes at euthanization. Hematological analyses were done at Idexx BioAnalytics Laboratory, North Grafton, Massachusetts, U.S.A

Harvest of Organs

At euthanization, ovaries, uterus, kidneys, livers, from each rat were harvested, weighed, sectioned, and placed in plastic histological cassettes and stored at -80^{o}C until further histological processing.

Histology

The updated histology protocol of Feldman et al., (2014) was used. Cut Pieces of ovaries, kidney, liver, lungs, uterus, and rats were packed into small containers and stored at -80^{o}C. Prior to analyses collected tissue samples were dehydrated through a series of ethanol and xylene baths with a Leica automated tissue processor 1050 (Leica, Wetzlar, Germany). Dehydrated samples were embedded in paraffin using a Thermo Scientific Microm EC 350–1 embedding station (Waltham, MA). Sectioning was performed at 5.0 μm thickness using the Reichert-Jung cassette microtome (Leica, Wetzlar, Germany). Staining was carried out using hematoxylin and eosin staining techniques as described by Carson (1997). Masson Tri-chrome stain was also done following Van De Vlekkert et al., (2020) protocol. Slides were checked for normalcy and/or morphological changes.

Gene Expression

In examining the mRNA expression levels of estrogen receptors (ER) and androgen receptors (AR) in rat kidneys, the study adhered to a well-defined methodology. Kidney samples were procured from three distinct groups: non-PCOS control rats, PCOS-induced rats treated with regular water, and PCOS-induced rats treated with varying doses of Hunteria umbellata extract. The total RNA extracted from these samples underwent cDNA synthesis via a reverse transcription kit. Subsequently, Quantitative Real-Time PCR (qPCR) was employed, utilizing gene-specific primers for ERα, ERβ, and AR. The reference gene, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), facilitated normalization through the comparative Ct method. Statistical analysis using GraphPad Prism enabled the identification of noteworthy differences in gene expression among experimental groups. To ensure data reliability, quality control measures, such as no-template controls and standard curves, were meticulously implemented. The gene assay and analysis followed the detailed protocol outlined by Pfaffl et al., (2001), incorporating specific primer sequences for accuracy and consistency.

Morphometric Assessments of Tissue Structures.

In this study, we utilized ImageJ for the histomorphometry analysis of kidney tissue histology slides. Following the preparation of histological slides using hematoxylin and eosin (H&E) staining, high-resolution images were captured using a microscope equipped with a digital camera. The acquired images were then processed through ImageJ, where the software's calibration feature ensured precise measurements by referencing a known scale from the microscope. Regions of Interest (ROIs) were systematically selected to represent specific areas within the tissue section, and ImageJ's segmentation tools were employed for the accurate delineation of histological structures (glomerulus) from the background. Quantitative

measurements, encompassing parameters such as area, perimeter, and density of the specific cell types (glomerulus) and tissue components, were obtained using ImageJ's built-in tools and plugins. The obtained data was exported and subjected to statistical analysis to identify significant differences among the experimental groups.

Statistical Analyses

Data was collected for body weight, ovary weights, kidney weights, liver weights, uterus weights, oral glucose tolerance test (OGTT), All gathered information was presented as the mean ± standard error of the mean (SEM). The impact of the oral administration of HUE on the data was assessed through the implementation of the Two- way analysis of variance technique (ANOVA) utilizing GraphPad Prism 10.1 software. A significance threshold of $P < 0.05$ was chosen. In cases of significant differences, Tukey’s post hoc test was employed to delineate the specific means.

Chapter V: Results

Experiment 1: Pilot Study

Effect of aqueous extract of Hunteria umbellata on body weight, water intake, and feed consumption in Sprague Dawley rats.

The outcomes align with our expectations. HUE did not exhibit any detrimental effects on the rats, nor did it impede their regular eating and drinking habits. The administration of this extract was facilitated through their water consumption. The findings from this initial experiment provided valuable insights and laid the foundation for the success of the main study.

Experiment 2: Main Study

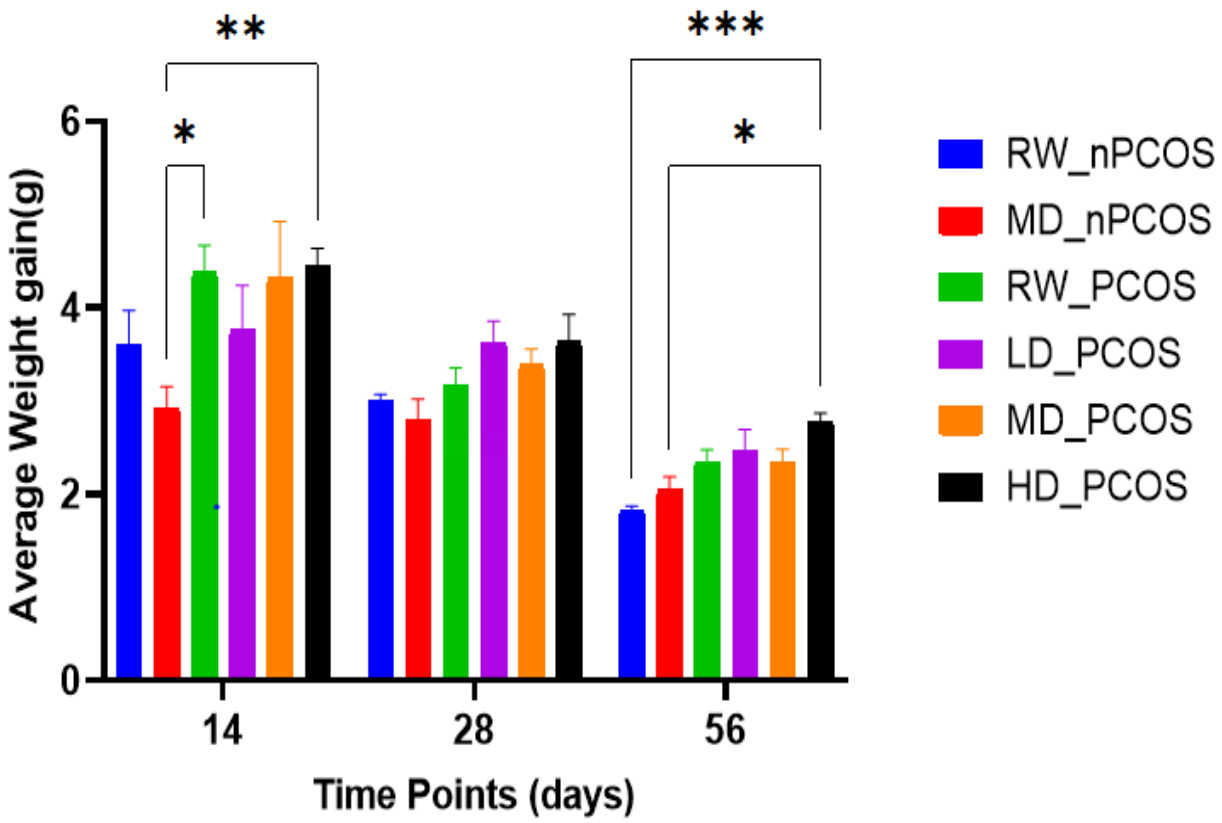
Effect of PCOS and HUE on Average Daily Body Weight Gain and Absolute Weight Gain

The influence of orally administered HUE on body weight is visually represented in Figures 1 and 2. These figures demonstrate a discernible effect on daily body weight gain, which is both time-dependent and treatment-dependent across all HUE dosage levels (Low-Dose - LD =100mg/kg, Medium-Dose - MD =200mg/kg, High-Dose =400mg/kg). Furthermore, the oral administration of HUE did not yield significant alterations in mean body weight gain across the different groups (RW_nPCOS: 2.82 ± 0.523g, MD_nPCOS: 2.59 ± 0.273g, RW_PCOS: 3.3 ± 0.590g, LD_PCOS: 3.29 ± 0.413g, MD_PCOS: 3.35 ± 0.569g, HD_PCOS: 3.63 ± 0.482g). Notably, while these differences did not reach statistical significance, it is worth noting that the MD_nPCOS treatment group exhibited the lowest body weight gain, totaling 2.594g. Figure two shows the weight gain trend over 56 days, it becomes evident that the control group consistently exhibited the lowest body weight over the 56-day period, with the MD_nPCOS group closely following. This pattern notably became prominent around the 21st day, preceding which the observable effect of HUE in mitigating weight gain in the absence of PCOS was apparent. Several key observations emerged from the results. Notably, the average daily body weight gain showed

a decrease with age. On day 14, significant differences in daily weight gain were identified between nPCOS, MD_nPCOS, and all other PCOS rats. Furthermore, the daily administration of HUE did not exert a significant impact on the daily body weight gain in PCOS rats each day. In summary, there was no significant effect observed for PCOS or HUE administration on the daily body weight gain after 28 days of PCOS induction.

Figure 1

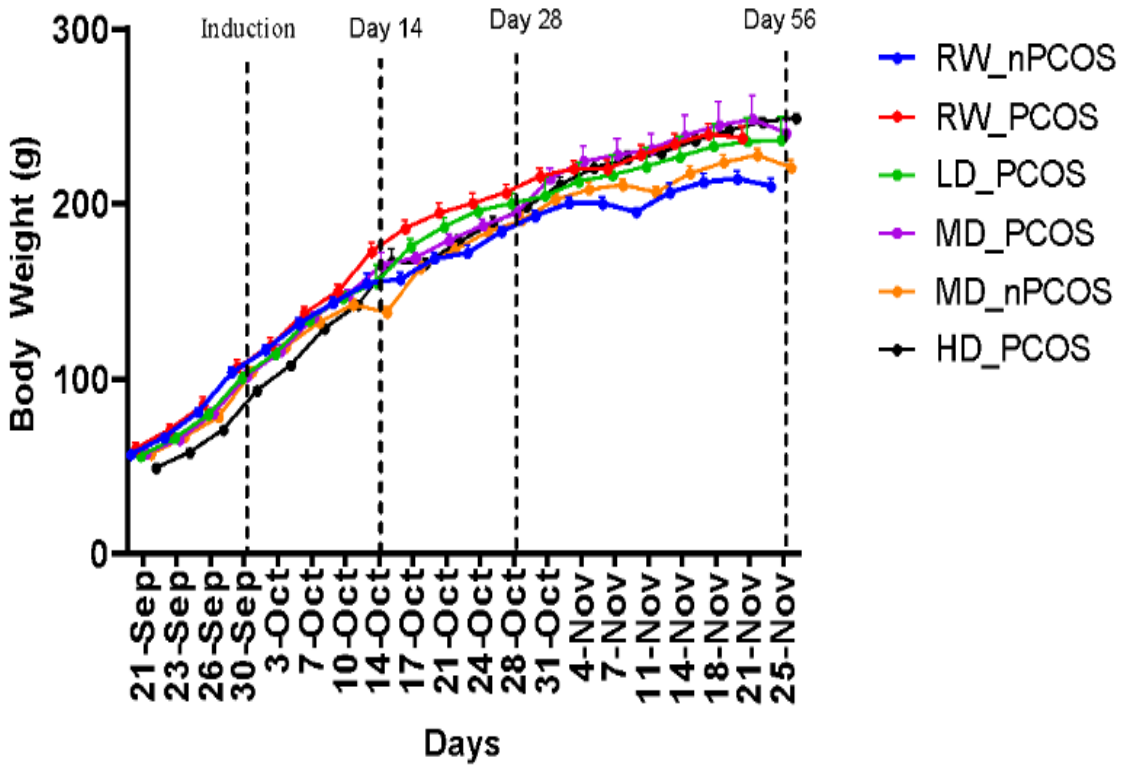
Average Daily Body Weight Gain



Note. Graph showing the Average Body Weight Gain Over 56 Days for Six Experimental Groups (RW_nPCOS, MD_nPCOS - 200mg/kg HUE, RW_PCOS, LD_PCOS – 100mg/kg HUE, MD_PCOS – 200mg/kg HUE, HD_PCOS – 400mg/kg). Values are represented as Mean ± SEM.

* showing statistically significant difference at $P<0.05$

Absolute Weight Gain Trend



Note. Graph Showing the Absolute Bodyweight Gain Trend Over 56 Days for Six Experimental Groups (RW_nPCOS, MD_nPCOS - 200mg/kg HUE, RW_PCOS, LD_PCOS – 100mg/kg HUE, MD_PCOS – 200mg/kg HUE, HD_PCOS – 400mg/kg). Values are represented as Mean ± SEM.

Visceral Organ Weight

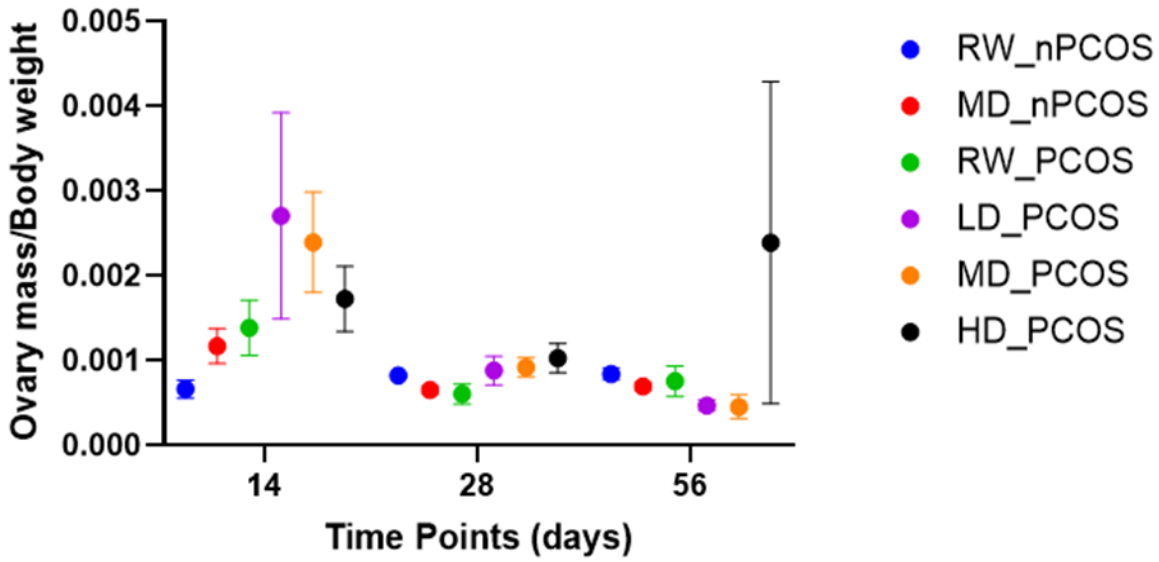
Effect of the PCOS & HUE on Ovary Weight in Female Sprague-Dawley Rats

The impact of HUE administration via the oral route on ovarian weight is shown in Figure 3. There was a tendency for an enlargement in ovarian weight following a 14-day treatment regimen of HUE on PCOS induced group, coupled with an observable declining trend with ascending doses of HUE. While no statistically significant effect was observed, there was a tendency for relative ovarian weight to decrease with age, and this decrease remained unaffected

by HUE treatment. Notably, HD_PCOS rats exhibited a wide variation in ovarian weight, indicating potential individual differences within this group.

Figure 3

Relative Ovary Weight



Note. Graph Showing the Relative Ovary Weight Over the 56 Days for Six Experimental Groups (RW_nPCOS, MD_nPCOS - 200mg/kg HUE, RW_PCOS, LD_PCOS – 100mg/kg HUE, MD_PCOS – 200mg/kg HUE, HD_PCOS – 400mg/kg). Values are represented as Mean ± SEM.

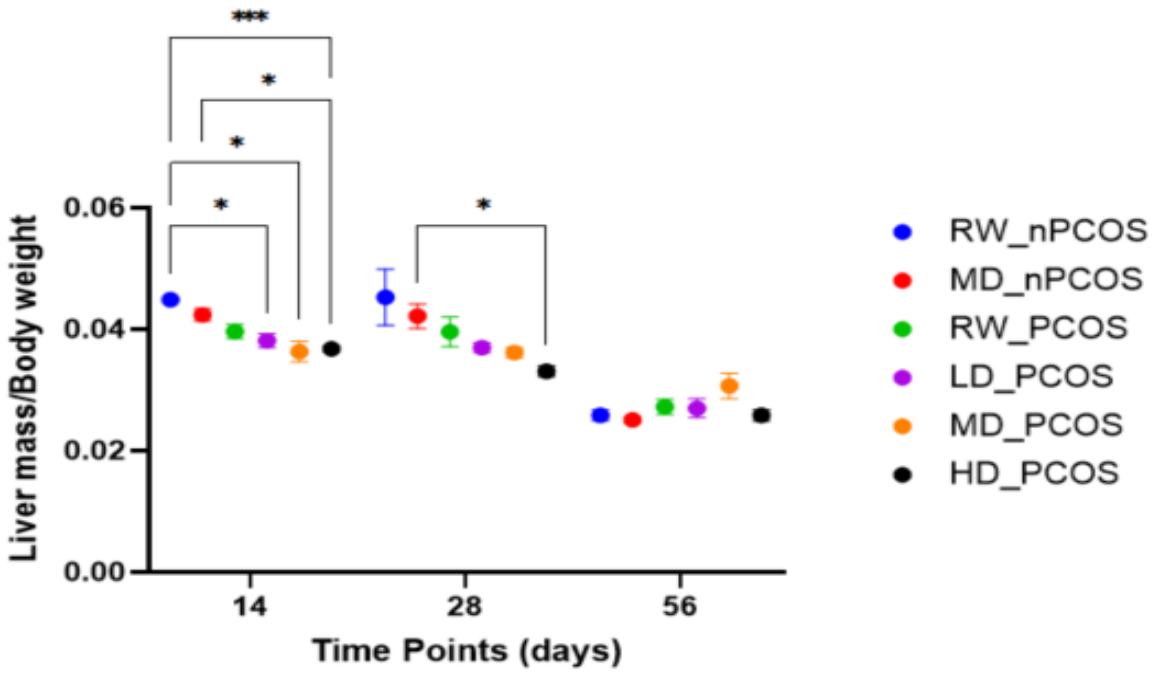
Effect of PCOS and HUE on Liver Organ Weight in Female Sprague-Dawley Rats

The impact of oral administration of HUE on liver weight is depicted in Figure 4. There was a reduction in liver dimensions observed on day 56 post PCOS induction. Subsequent statistical scrutiny divulged significant disparities encompassing time, treatment, and their interaction within the groups. Specifically, the analysis revealed a significant discrepancy on day 14 between the control RW-nPCOS group and all three PCOS-induced groups subjected to varying doses of HUE treatment. Additionally, a notable difference surfaced between the MD-nPCOS and HD-PCOS subgroups on day 14. As the study progressed to day 28, the only statistically

significant differentiation among the cohorts remained confined to the MD-nPCOS and HD_PCOS comparison. By the day 56 assessment, there were no significant differences observed among any of the treatment groups, at a significance level of $p < 0.05$. Although there was statistical difference in liver weights between MD_nPCOS and HD_PCOS rats on day 14 and 28, these differences did not persist at day 56 post-induction.

Figure 4

Relative Liver Weight



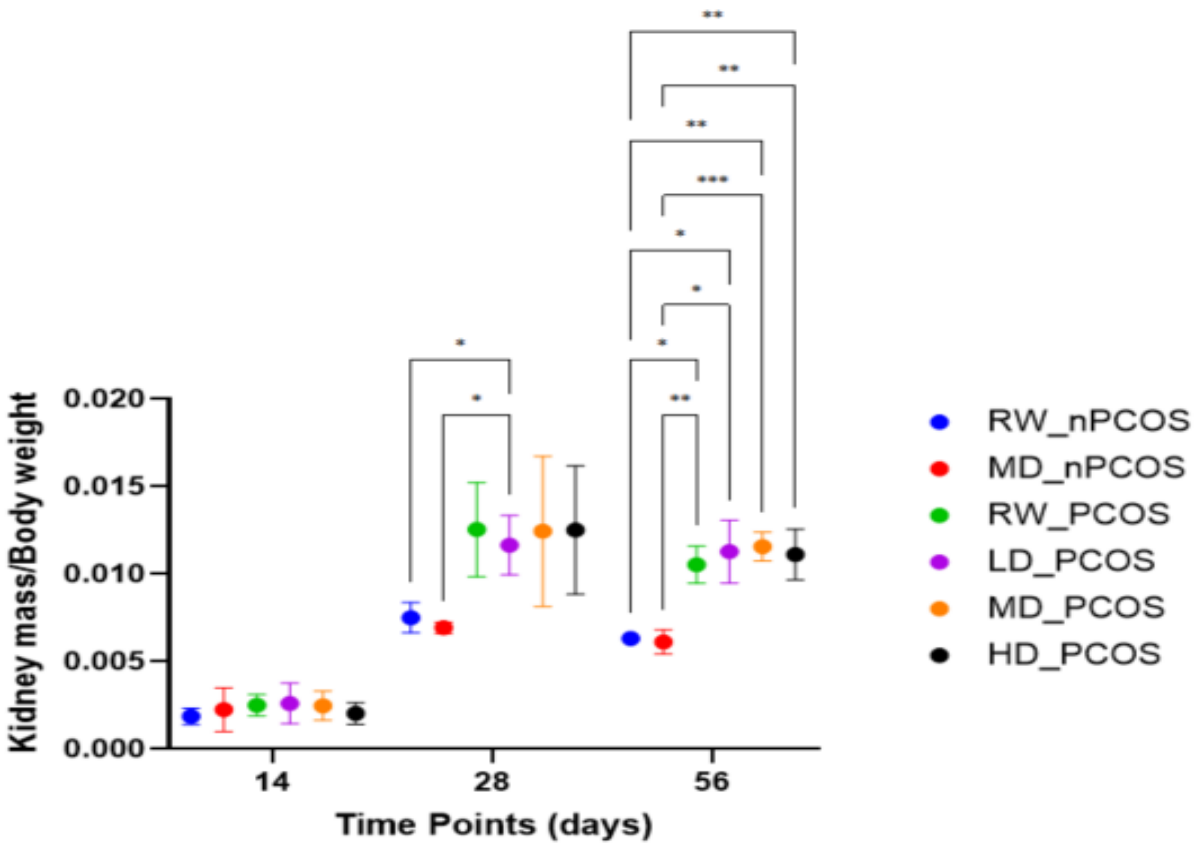
Note. Graph Showing the Relative Liver Weight Over the 56 Days for Six Experimental Groups (RW_nPCOS, MD_nPCOS - 200mg/kg HUE, RW_PCOS, LD_PCOS – 100mg/kg HUE, MD_PCOS – 200mg/kg HUE, HD_PCOS – 400mg/kg HUE). Values are represented as Mean ± SEM.
* showing statistically significant difference at P<0.05
*** showing statistically significant difference at P<0.001

Effect of PCOS or HUE on Kidney Organ Weight in Female Sprague-Dawley Rats

Figure 5 shows the effect PCOS and HUE treatment on relative kidney weight. The graphical representation clearly illustrates an increase in kidney dimensions after a 28-day treatment period across all PCOS-induced groups. This stands in stark contrast to the previous 14-day benchmark, wherein no discernible variations in size were observed across the six groups. The graphical data further suggests that the final two time points—28 days and 56 days—exhibit a comparable trend in relative kidney weight. Employing mixed-effects analysis, discernible effects emerged across time, treatment, and their interaction in relation to kidney weight. Subsequent post hoc scrutiny unveiled a statistically significant divergence at the 28-day juncture between the LD-PCOS group and both nPCOS groups. No effect of PCOS or HUE the investigation advanced to the 58-day time point, statistical significance remained evident, with intergroup analyses indicating a distinct and statistically significant contrast in the means of relative kidney weights between the non-PCOS and PCOS groups.

Comparing the means of the diverse PCOS-induced groups to either RW-nPCOS or MD-nPCOS unveiled noteworthy differences. However, no significant disparities surfaced among the PCOS-induced groups themselves, nor among the non-PCOS group. Furthermore, a temporal effect was observable across the various groups when evaluating means at different time points.

Relative Kidney Weight



Note. Graph Showing the Relative Kidney Weight Over the 56 Days for Six Experimental Groups (RW_nPCOS, MD_nPCOS - 200mg/kg HUE, RW_PCOS, LD_PCOS – 100mg/kg HUE, MD_PCOS – 200mg/kg HUE, HD_PCOS – 400mg/kg HUE). Values are represented as Mean ± SEM.

* showing statistically significant difference at P<0.05

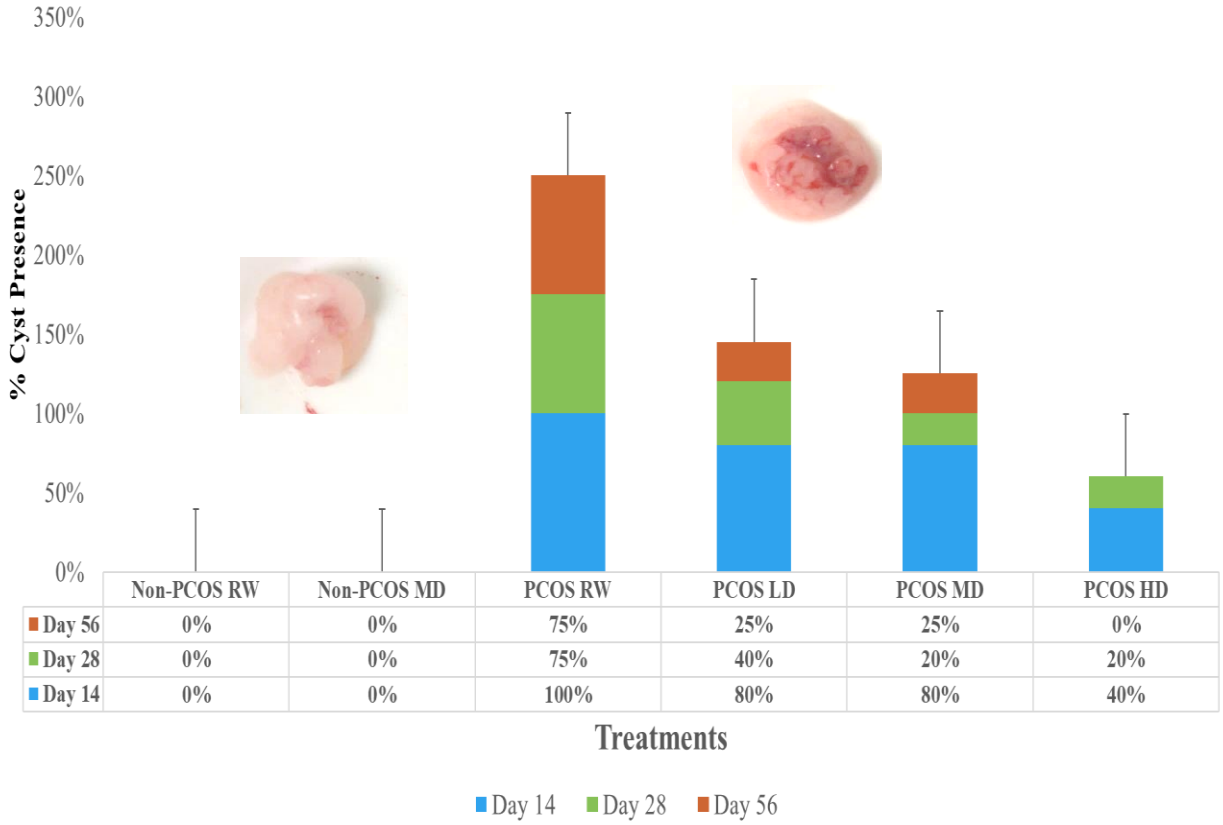
** showing statistically significant difference at P<0.01

Effect of HUE on the Incidence of PCOS (Follicular Cyst) in Female Sprague-Dawley Rats

Figure 6 shows the percentage of morphological ovarian cysts at time of euthanasia. On days 14, 28, and 56 post-PCOS induction, no follicular cysts were observed on the ovaries of the nPCOS group. In the PCOS-induced group, there was a noticeable trend towards a reduced percentage of animals presenting cysts, and this trend appeared to be both time and treatment-dependent. Progressing through the treatment groups to higher doses revealed a lower percentage of animals with cysts, with 40% of the animals presenting cysts compared to higher levels of cysts at lower doses on day 14. Besides the treatment effect, a time-dependent influence was evident, indicating a gradual decrease in PCOS incidence based on cyst observation. Overall, among the PCOS rats, the administration of HUE significantly reduced the incidence of PCOS. At the highest dose of HUE administration, no morphological ovarian cysts were observed 56 days after PCOS induction.

Figure 6

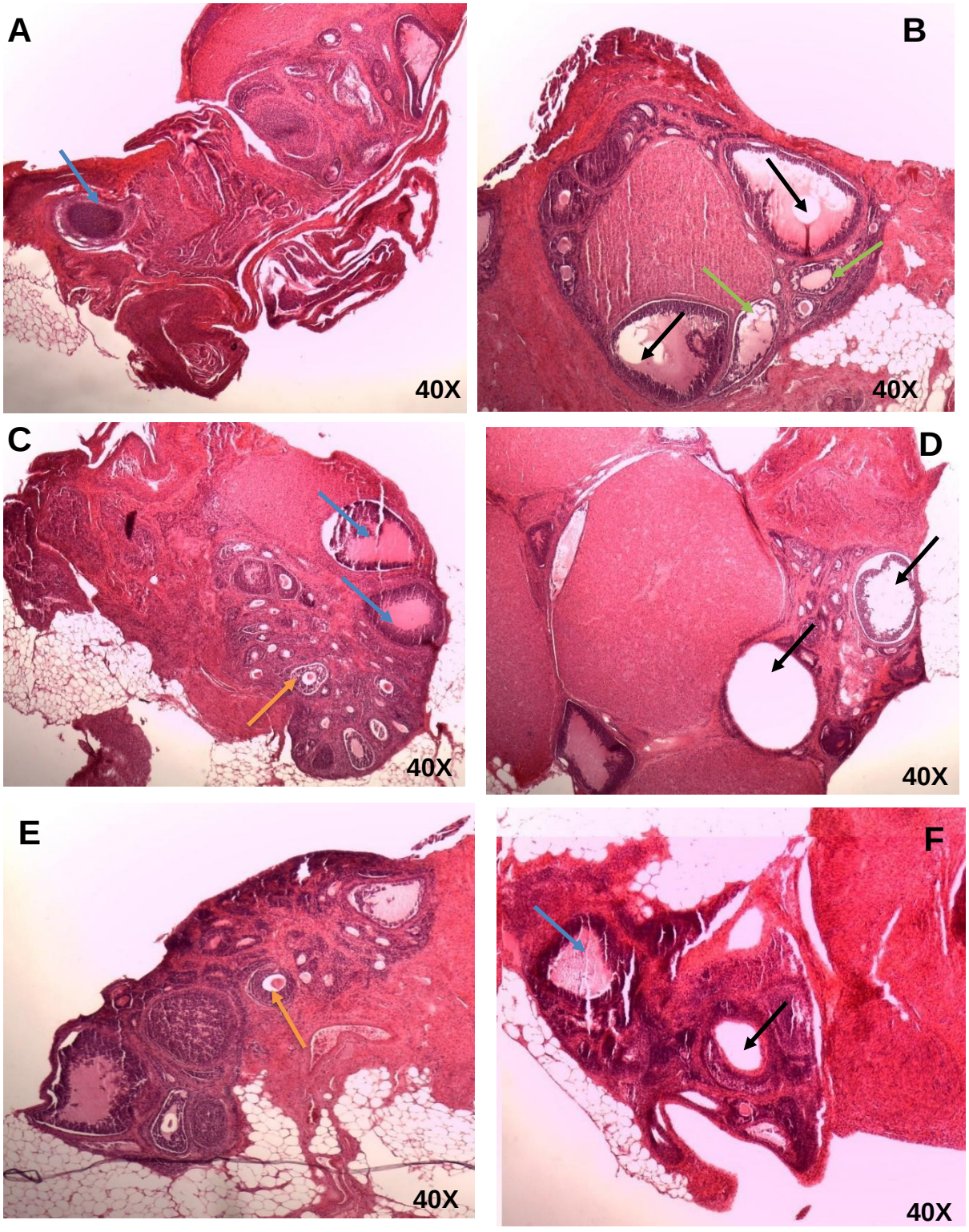
Incidence of PCOS (Based on Follicular Cyst)



Note. Graph Showing the Incidence of PCOS based on Identifiable Follicular Cyst Over the 56 Days for Six Experimental Groups (RW_nPCOS, MD_nPCOS - 200mg/kg HUE, RW_PCOS, LD_PCOS – 100mg/kg HUE, MD_PCOS – 200mg/kg HUE, HD_PCOS – 400mg/kg HUE).

Figure 7

Effect of Hunteria umbellata extract on Ovary Histology (H & E)



Note. H & E-Stained Rat Ovaries Photomicrographs at 40x Magnification. Black Arrow – Fluid Filled Cystic Follicles, Blue Arrow- Corpus Luteum, Yellow Arrow: Graafian Follicle, Green Arrow: Atretic Follicle. A. RW_nPCOS, B. RW_PCOS, C. MD-PCOS (200mg/kg), D. MD_PCOS (200mg/kg), E. LD-PCOS (100mg/kg) F. HD-PCOS (400mg/kg)

Effect of PCOS & HUE on Ovary Histology (H & E)

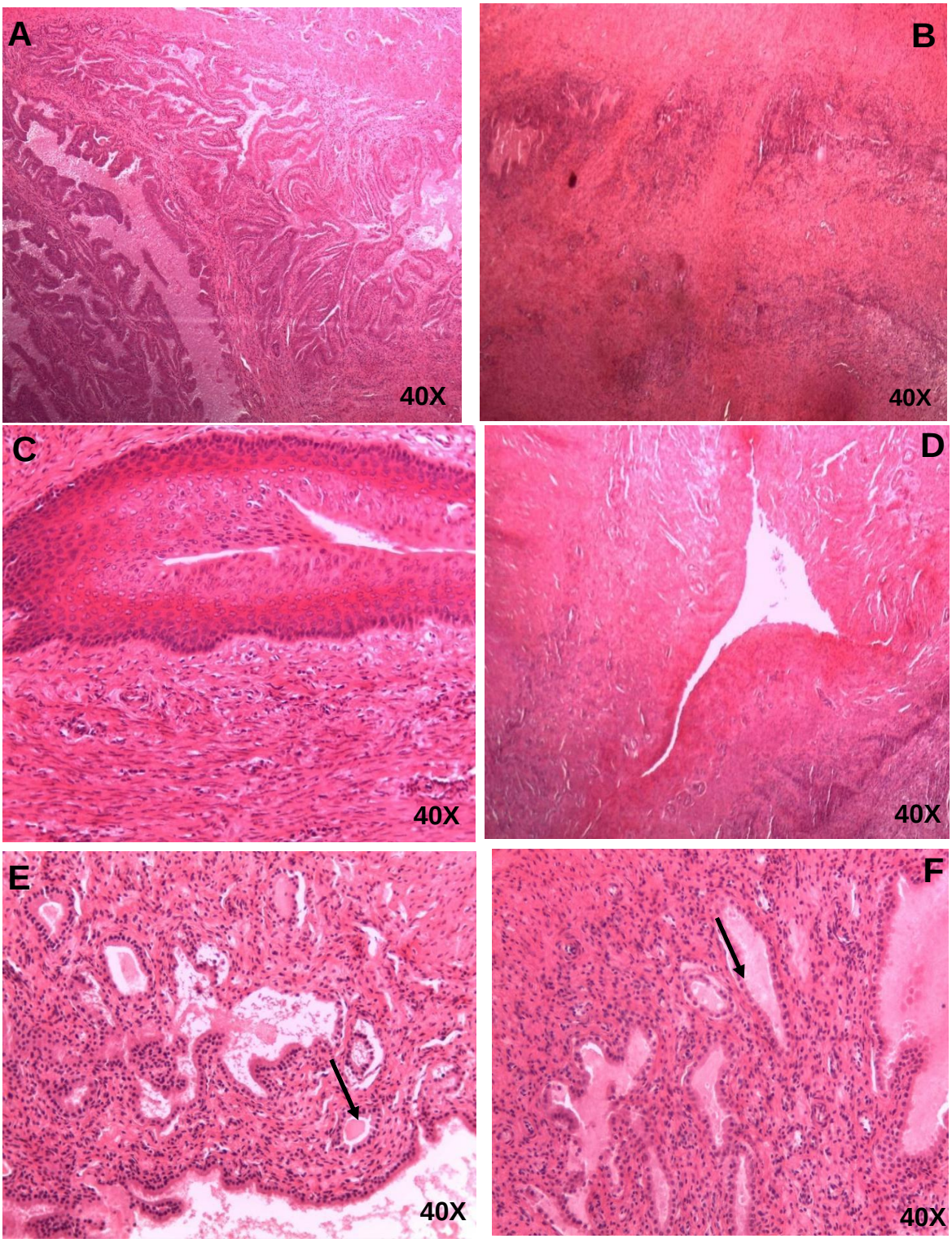
Figures 7A through 7F present cross-sectional representations of H&E-stained rat ovaries. Figures 7A and 7B depict ovaries from the nPCOS control rats, while Figures 7C to 7F illustrate ovaries from PCOS rats treated with regular water or varying doses of HUE. The histological examination of ovarian sections revealed distinctive features of PCOS in rats injected with TP. These features included irregular morphological characteristics, numerous dilated cystic follicles, atretic follicles, and the notable development of theca interna cells. Our observations indicated that both RW_nPCOS and MD_nPCOS exhibited characteristic ovarian structures, notably featuring normal follicles, graafian follicles, and corpus luteum. These findings suggest typical ovarian functionality. MD_nPCOS, in particular, displayed an elevated follicle count, potentially indicating a modulatory influence of HUE on follicular development. An intriguing observation is the increased presence of fluid-filled follicles at the highest dose, while MD_PCOS demonstrates relative normalcy with fewer such follicles.

Effect of PCOS & HUE on Uterus Histology (H & E)

Figures 8A through 8F present cross-sectional representations of H&E-stained rat uterus from both nPCOS control rats (Fig. 8A & 8B) and PCOS rats treated with regular water or different doses of HUE (Fig. 8C – 8F). The histological changes observed in the uterus underscore the intricate interplay between the ovary and uterus in the context of PCOS. RW_PCOS displayed histological characteristics consistent with endometrial hyperplasia, likely attributable to hormonal imbalances and anovulation. These alterations were consistently observed across all PCOS-induced groups, characterized by what appeared to be an increased number of uterine glands when compared to the non-PCOS groups.

Figure 8

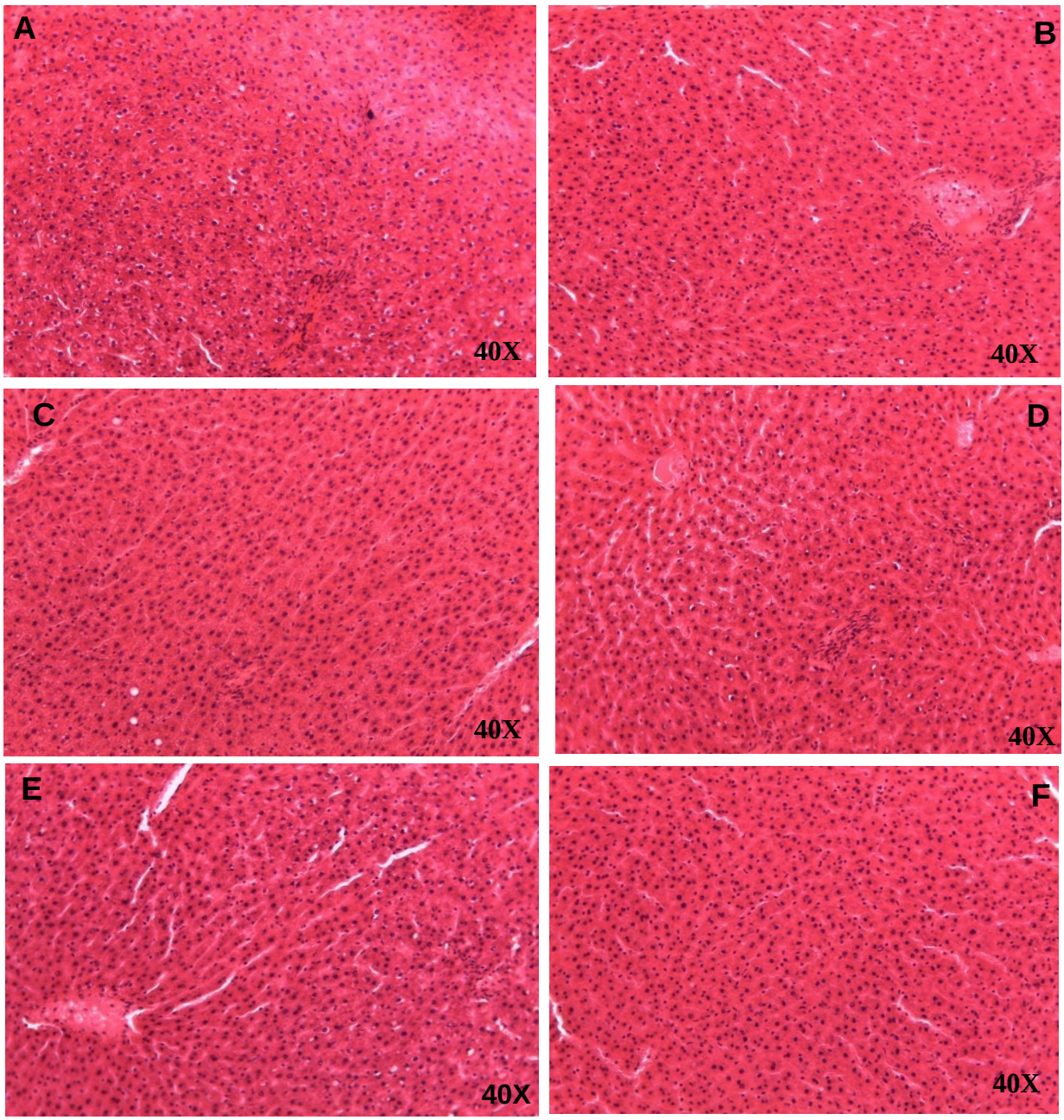
Effect of Hunteria umbellata extract on Uterus Histology (H & E)



Note. H & E-stained rat uterus photomicrographs at 40x magnification. Black arrow – Uterine Glands. A. RW_nPCOS, B. RW_PCOS, C. MD-PCOS (200mg/kg), D. MD_PCOS (200mg/kg), E. LD-PCOS (100mg/kg) F. HD-PCOS (400mg/kg)

Figure 9

Effect of *Hunteria umbellata* extract on Liver Histology (H & E)



Note. H & E-stained rat liver photomicrographs at 40x magnification. A. RW_nPCOS, B. RW_PCOS, C. MD-PCOS (200mg/kg), D. MD_PCOS (200mg/kg), E. LD-PCOS (100mg/kg) F. HD-PCOS (400mg/kg)

Effect of PCOS & HUE on Liver Histology (H & E)

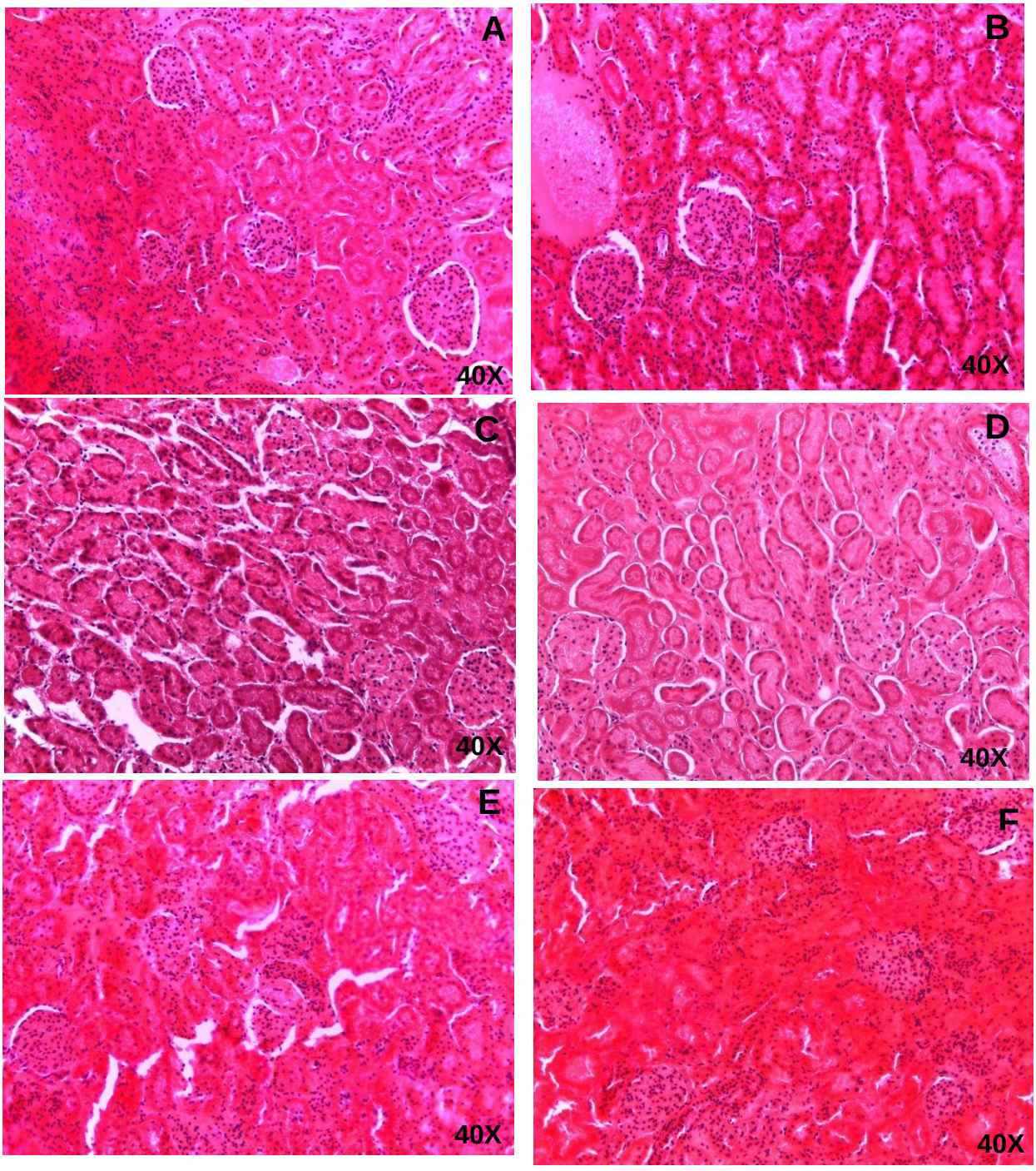
Figure 9A through 9F shows cross sectional representation of H &E-stained rat liver from both nPCOS control rats (Fig 9A & 9B) and PCOS rats treated with regular water or different doses of HUE (Fig 9C – 9F). The histological analysis of the liver yielded fascinating insights into the impact of PCOS and HUE on hepatic structure. RW_PCOS exhibited distinct characteristics associated with non-alcoholic fatty liver disease (NAFLD), specifically hepatocyte steatosis.

Effect of PCOS & HUE on Kidney Histology (H & E)

Figure 10A through 10F shows cross sectional representation of H &E-stained rat kidneys from both nPCOS control rats (Fig 10A & 10B) and PCOS rats treated with regular water or different doses of HUE (Fig10C – 10F). The observed renal architecture in the RW_nPCOS and MD_nPCOS groups can be considered as a reference for normal physiological renal structure. Nevertheless, the existence of subtle histopathological abnormalities within MD_nPCOS, despite the presence of a normal macroscopic appearance, implies that subtle renal changes may manifest even in the absence of overt pathological indications. The distinct histological anomalies observed in the RW_PCOS Group point towards significant renal perturbations.

Figure 10

Effect of Hunteria umbellata extract on Kidney Histology (H & E)



Note. H & E-stained rat kidney photomicrographs at 40x magnification. A. RW_nPCOS, B. RW_PCOS, C. MD-PCOS (200mg/kg), D. MD_PCOS (200mg/kg), E. LD-PCOS (100mg/kg) F. HD-PCOS (400mg/kg).

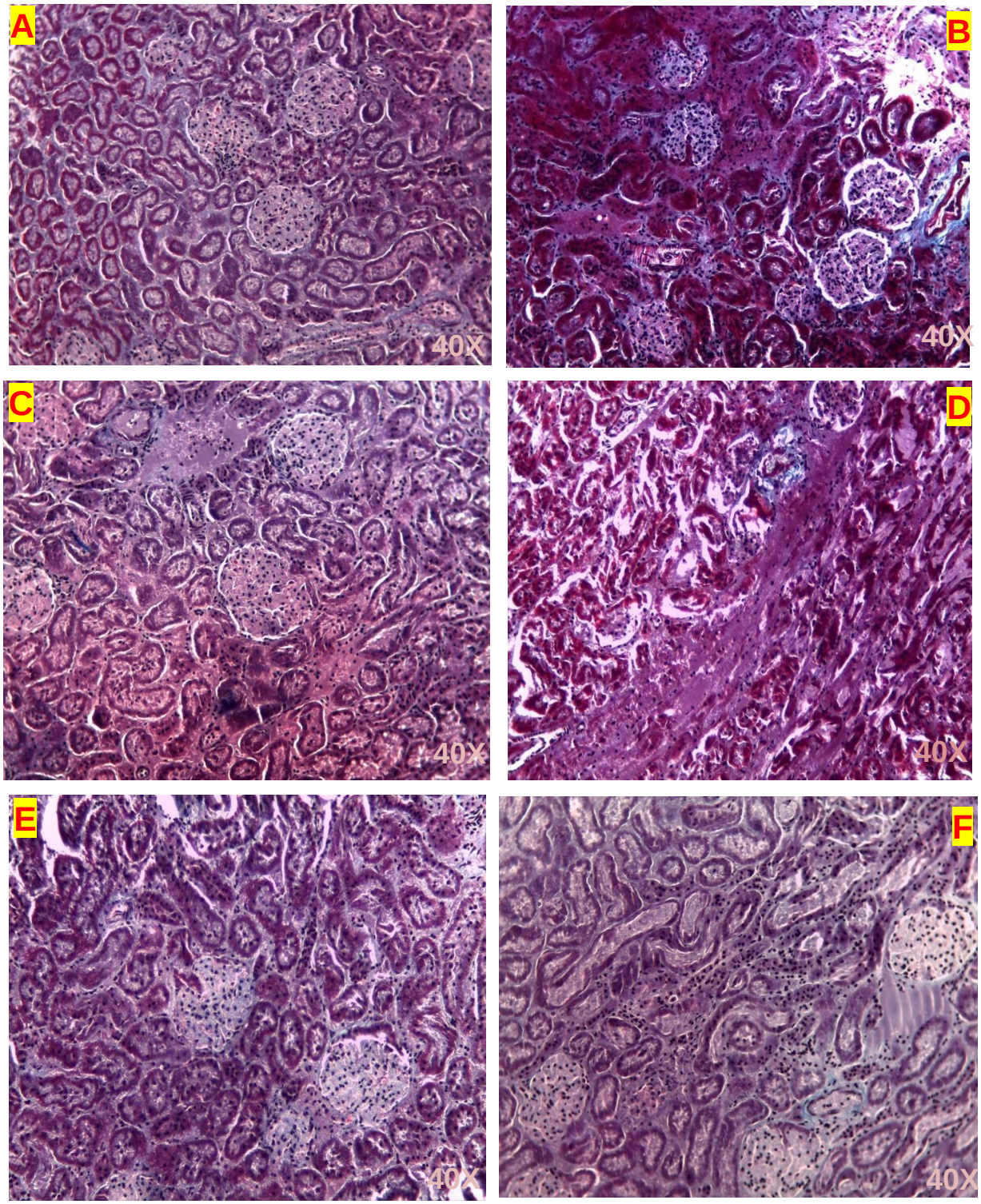
Effect of PCOS & HUE on Kidney Histology (Masson's trichrome)

Figure 11A through 11F presents a cross-sectional representation of Masson's trichrome-stained rat kidneys from both nPCOS control rats (Fig 11A & 11B) and PCOS rats treated with regular water or different doses of HUE (Fig 11C – 11F).

The notable increase in kidney size observed in the PCOS-induced groups prompted us to delve deeper into the changes occurring. Masson's trichrome stain, commonly used in histology, aids in differentiating collagen in tissue sections, providing insights into glomerular fibrosis in kidneys. This staining technique offers a potential explanation for the observed increase in kidney size, suggesting a link to glomerular fibrosis.

Figure 11

Effect of *Hunteria umbellata* extract on Kidney Histology (Masson's Trichrome)



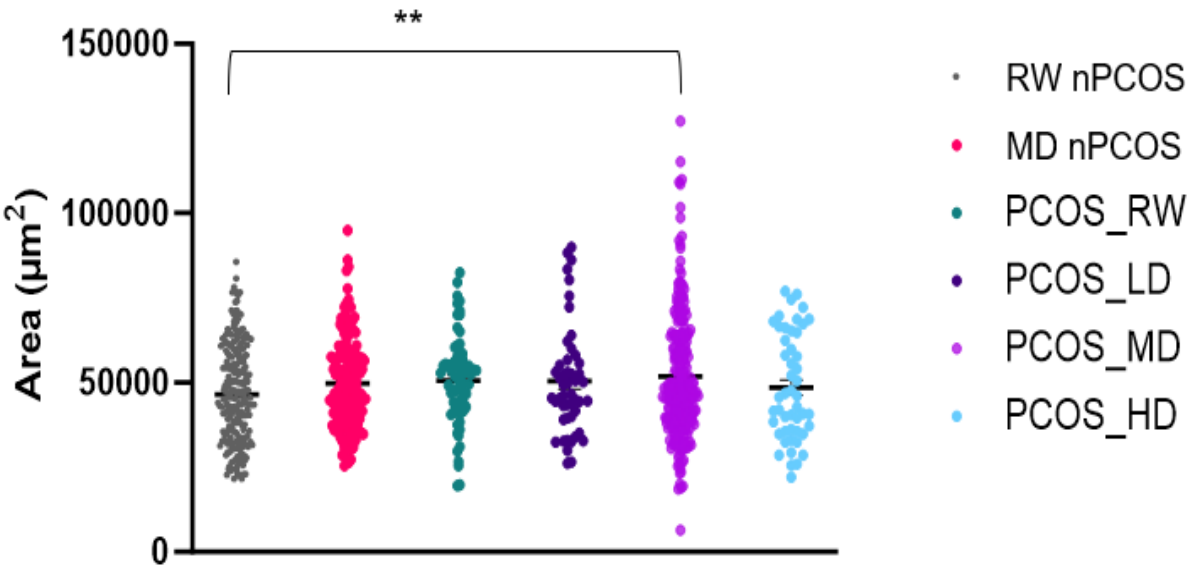
Note. Masson's Tri-chrome-stained rat kidney photomicrographs at 40x magnification. A. RW-nPCOS B. RW-PCOS C. MD-nPCOS (200mg/kg) D. MD_PCOS (200mg/kg) E. LD-PCOS (100mg/kg) F. HD-PCOS (400mg/kg)

Effect of PCOS & HUE on Glomerulus Size.

Figure 12 provides a graphical representation of changes and differences occurring at the level of the glomerulus in rat kidneys from both nPCOS control rats and PCOS rats treated with regular water or different doses of HUE. No statistically significant difference was observed between all cohorts concerning glomerular size, except when comparing the RW_nPCOS to the MD_PCOS group, which exhibited a significant difference.

Figure 11

Effect of Hunteria umbellata extract on Kidney Histology (Masson's Trichrome)



Note. Scatterplot Graph Showing the Glomerulus Area Size for Six Experimental Groups (RW_nPCOS, MD_nPCOS - 200mg/kg HUE, RW_PCOS, LD_PCOS – 100mg/kg HUE, MD_PCOS – 200mg/kg HUE, HD_PCOS – 400mg/kg). Values are represented as Mean ± SEM.

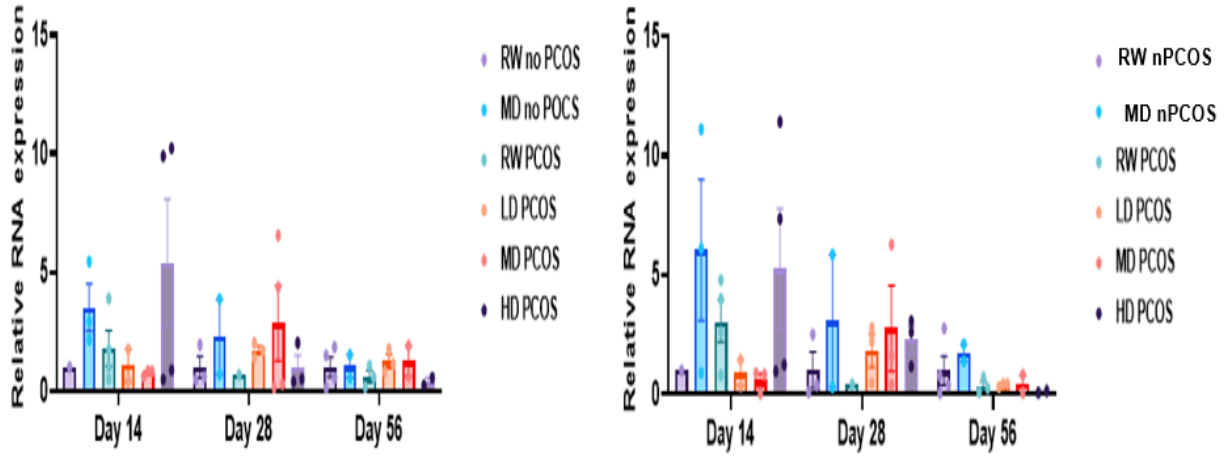
** showing statistically significant difference at $P<0.01$

Gene Expression Dynamics in Renal Tissues during PCOS Progression and HUE Administration in a Rat Model

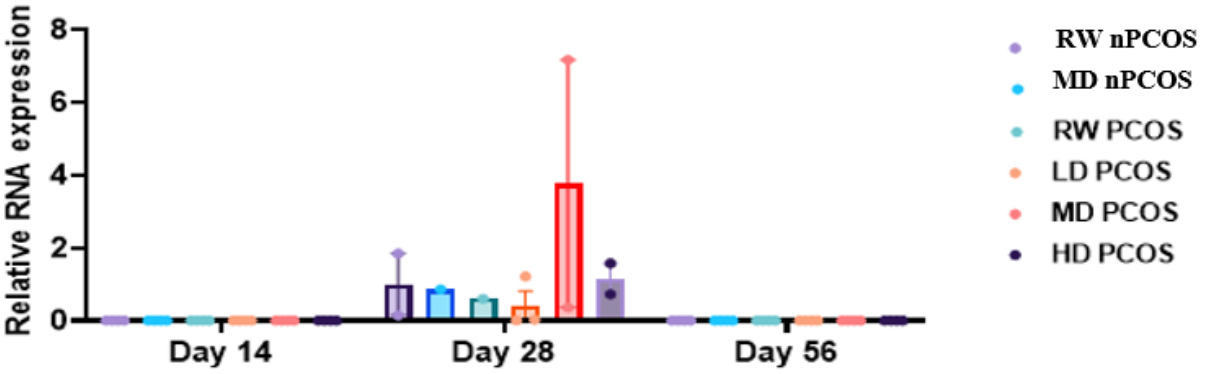
Gene analysis was performed on renal samples to explore the expression levels of estrogen alpha and beta receptors, as well as androgen receptors and GPER (G protein-coupled estrogen receptor). The measurements were normalized to the expression levels observed in the RW non-PCOS group for each respective day. Interestingly, no statistically significant differences emerged between the various groups, possibly influenced by the notable data dispersion observed. It is noteworthy to mention the unexpectedly low gene expression levels detected in this context, especially considering that receptors like ER alpha should naturally exhibit substantial presence, particularly in the kidney at baseline. Although no significant differences were observed, notable trends were discerned, with the exclusion of the no PCOS groups from the analysis. Specifically, it is apparent that the expression of estrogen receptor beta tends to decrease from the levels exhibited in the RW PCOS group by day 14 for the HUE-treated PCOS groups, and analogous trends are evident for ER alpha on the same day. By day 28, these trends reverse with a tendency toward increased expression. Strikingly, by day 56, distinctions between the various groups are less prominent. It is pertinent to mention that the androgen receptor and GPER (G protein-coupled estrogen receptor) exhibited minimal amplification at day 14 or 56, but a notable amplification was observed at day 28.

Figure 13

Kidney Estrogen Receptors (α and β)



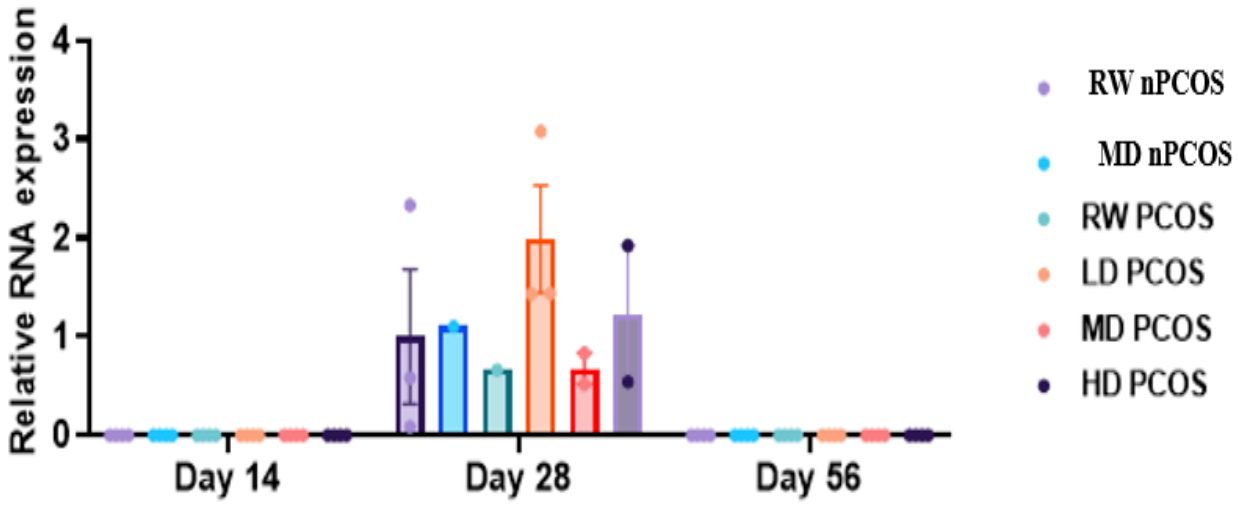
Note. Graph Showing the Kidney Estrogen α and β levels for Six Experimental Groups (RW_nPCOS, MD_nPCOS - 200mg/kg HUE, RW_PCOS, LD_PCOS – 100mg/kg HUE, MD_PCOS – 200mg/kg HUE, HD_PCOS – 400mg/kg). Values are represented as Mean ± SEM.



Note. Graph Showing the Kidney GPER levels for Six Experimental Groups (RW_nPCOS, MD_nPCOS - 200mg/kg HUE, RW_PCOS, LD_PCOS – 100mg/kg HUE, MD_PCOS – 200mg/kg HUE, HD_PCOS – 400mg/kg). Values are represented as Mean ± SEM.

Figure 15

Kidney Androgen Receptor



Note. Graph Showing the Kidney Androgen Levels for Six Experimental Groups (RW_nPCOS, MD_nPCOS - 200mg/kg HUE, RW_PCOS, LD_PCOS – 100mg/kg HUE, MD_PCOS – 200mg/kg HUE, HD_PCOS – 400mg/kg). Values are represented as Mean ± SEM.

Chapter VI: Discussion

In this study, we systematically investigated the potential benefits of *Hunteria umbellata* extract (HUE) on PCOS in the Sprague Dawley rat model. PCOS stands as the most prevalent endocrine disorder, impacting an estimated 20% of women in their reproductive years. PCOS is characterized by its inflammatory, systemic, and autoimmune endocrinopathy, as noted in the work of Patel, 2018. Among individuals with PCOS, a systemic low-grade inflammation exerts adverse effects on various facets of fertility, concurrently linked to hyperandrogenism and insulin resistance (Rudnicka, 2021). Scientific literature extensively supports the identification of compounds such as flavonoids, tannins, alkaloids, glycosides, saponins, phytosterol, and terpenoids within *Hunteria umbellata.* (Boone, 2006). These flavonoids have demonstrated a myriad of functions, encompassing the scavenging of oxygen-free radicals (Ma et al., 2021), inhibition of metal-ion chelation - rendering them potent antioxidants (Pourcel, 2007), utility in the treatment of cardiovascular diseases (Zhi et al., 2016), anti-inflammatory properties (Tang et al., 2019), and efficacy in addressing complications associated with diabetes (Varma et al., 1977). Furthermore, there is a growing body of evidence indicating that flavonoids possess the capacity to inhibit regulatory enzymes and transcription factors intricately involved in the inflammatory process (Alam et al., 2020; Maleki et al., 2019). On account of their diverse bioavailability, flavonoids have been applied to prevent degenerative diseases, such as diabetes, cardiovascular complications, cancer, and hypoglycemia (Liu et al., 2021; Wen et al., 2021). A comparable effect is substantiated by ample evidence in the literature regarding the actions of HUE (Adeneye et al., 2012; Ajiboye et al., 2017; Ibeh et al., 2007) Moreover, noteworthy applications of flavonoids have been elucidated in the context of reproductive endocrine disorders, including conditions like menopausal syndrome and endometriosis (Chen et al., 2019). Nonetheless, it remains uncertain

whether HUE exerts an influence on PCOS and the associated underlying mechanisms. Consequently, our speculation is rooted in the substantial presence of flavonoids, tannins, and indole alkaloids within HUE, suggesting its potential in mitigating PCOS symptoms.

The utilization of intraperitoneal administration of TP, in conjunction with a high-fat diet over the course of 56 days, proved effective in inducing and sustaining the characteristic condition of PCOS throughout the entire study period. TP is an exogenous anabolic steroid typically employed in the treatment of conditions such as hypogonadism and low testosterone levels. High-fat diet has the potential to induce a hypothalamic inflammatory response. This heightened expression of inflammatory factors in the hypothalamus can subsequently result in insulin and leptin resistance, ultimately contributing to disturbances in appetite regulation and the development of obesity - a comorbidity in PCOS. It is worth noting that obesity is increasingly recognized as a state of low-grade chronic inflammation, and this inflammatory state can induce peripheral insulin resistance, consequently affecting glucose and lipid metabolism. (Huang et al., 2015). Research findings have substantiated that hyperinsulinemia can stimulate the ovaries to synthesize elevated quantities of androgens, culminating in the development of hyperandrogenemia. This effect is particularly notable in the escalation of free androgens, contributing to the onset of insulin resistance (IR), as observed in studies by Huang et al., (2015). The success in establishing this disease model was experimentally confirmed in our study.

Findings obtained from the monitoring of body weight clearly demonstrated that, within the PCOS-induced model group, the rats exhibited a significant increase in body weight when compared to the control group. This observation aligns with the anticipated outcome, given that 40-80% of individuals diagnosed with PCOS are reported to be overweight or obese, contingent on the specific population under investigation. Our study's outcomes unveiled a notable disparity

at the initial time point when comparing MD_nPCOS to both RW_PCOS and HD_PCOS groups. Nevertheless, it is important to note that at higher concentrations, the phytochemical constituents within HUE may elicit a bidirectional impact on fat metabolism, implying a complex relationship between dosage and its influence on body weight regulation. (Younas et al., 2022). This assertion gains further support from the significant differences observed at the 56-day timepoint, notably between RW_PCOS and MD_nPCOS in comparison to HD_PCOS. Once more, these findings underscore the potential bidirectional influence of HUE at higher concentrations on fat metabolism. Taking into consideration the observed trend, it becomes apparent that the control group maintained the lowest body weight throughout the 56-day period, closely followed by the MD_nPCOS group. This trend notably manifested around the 21st day, prior to which HUE tended to mitigate weight gain in the absence of PCOS was shown at the highest dose was observed. Research has provided evidence indicating that flavonoids possess the capacity to decrease plasma levels of total cholesterol, triglycerides, free fatty acids (FFAs), and low-density lipoprotein cholesterol (LDL-C) in experimental animals. Simultaneously, they have been shown to elevate the levels of high-density lipoprotein cholesterol (HDL-C) and adiponectin, collectively contributing to a potential reduction in body weight.

Furthermore, the histological analysis of ovarian sections revealed distinctive hallmarks of PCOS in the rats injected with TP. In RW_PCOS, distinctive features associated with PCOS, including irregular morphological characteristics such as the presence of numerous dilated cystic follicles, atretic follicles, and the notable development of theca interna cells, were observed. Our observations indicated that both RW_nPCOS and MD_nPCOS exhibited characteristic ovarian structures, notably featuring normal follicles, Graafian follicles, and corpus luteum. These findings are indicative of typical ovarian functionality. Notably, MD_nPCOS displayed an elevated follicle

count, potentially indicating a modulatory influence of HUE on follicular development. The hormone-modulating potential of HUE may be attributed to the presence of diverse phytochemicals, such as flavonoids and alkaloids. Research conducted by Zhang et al, (2022) and Wang et al., (2019) has underscored the capacity of phytochemical-rich extracts with similar profiles to reinstate hormonal equilibrium, providing further support for our own findings. The observed influence of HUE on ovarian morphology offers insights into the potential mechanisms underpinning its effects. Notably, flavonoids, renowned for their antioxidant properties, may have the potential to counteract oxidative stress, a factor implicated in the pathogenesis of PCOS (Heshmati et al., 2019). An intriguing observation is the increased presence of fluid-filled follicles at the highest dose, while MD_PCOS demonstrates a relative normalcy with fewer such follicles. This pattern suggests the bidirectional influence of HUE at specific dosage levels. The 14-day interval marked a pivotal phase, initiating noticeable changes, and these effects became more pronounced by the 28-day mark. The sustained improvements observed until the 56-day time point prompt inquiries into adaptive responses and enduring benefits, underscoring the significance of accounting for temporal factors in botanical interventions.

The histological changes observed in the uterus serve to underscore the intricate interplay between the ovary and uterus in the context of PCOS. RW_PCOS displayed histological characteristics consistent with endometrial hyperplasia, which is attributable to hormonal imbalances and anovulation. These findings are in concordance with prior studies conducted by Wild (2002) and Virginia et al, (2020), which underscored the influence of PCOS-induced hormonal fluctuations on the endometrium. Notably, these alterations were consistently observed across all PCOS-induced groups, characterized by an increased number of uterine glands when compared to the non-PCOS groups. Uterine glands play a pivotal role in facilitating uterine

receptivity and the process of implantation. They secrete factors that are crucial for the survival and development of the conceptus. Research has shown that androgens can induce epithelial proliferation, leading to an increase in the number of endometrial glands in adult rodents. This phenomenon may explain the observed changes in our study, as the PCOS-induced groups were administered exogenous androgens. These androgens are known to directly stimulate glandular epithelial proliferation and influence the expression of regulators associated with the cell cycle and stromal-epithelial interactions These observed changes align with the results reported by Shi et al. (2019).

The histological analysis of the liver yielded insights into the impact of PCOS and HUE on hepatic structure. RW_PCOS exhibited distinct characteristics associated with non-alcoholic fatty liver disease (NAFLD), specifically hepatocyte steatosis. These observations align with the notion that insulin resistance and androgen excess play contributory roles in promoting the accumulation of lipids within the liver, as previously demonstrated by Roy et al., (2019). The statistical analysis unveiled a significant disparity in liver weight between the non-PCOS group and the PCOS group. Interestingly, HUE did not appear to exert a restorative effect on the impact induced by the PCOS disease model. Additional research endeavors are warranted to elucidate the precise influence of alkaloids, flavonoids, and other noteworthy phytochemicals found in HUE on the liver.

Notably, there was a noteworthy increase in kidney size, as indicated by kidney weight, across all groups induced with PCOS when compared to the non-PCOS group. This observation serves as an indicator of the impact of PCOS on kidney function, likely arising from the comorbidities often associated with PCOS, such as diabetes and metabolic disturbances. These comorbidities can contribute to glomerular hypertrophy and an elevated glomerular filtration rate.

Moreover, the elevated levels of androgens and insulin resistance might potentially contribute to lipid accumulation within renal cells, potentially explaining the variations in organ weight observed. Numerous studies have suggested the impact of testosterone on the kidney in the context of the PCOS induction model employed (Gagliano-Jucá, et al., 2020; Harris et al., 2020). The observed renal architecture in the RW_nPCOS and MD_nPCOS groups can be considered as a reference for normal physiological renal structure. Nevertheless, the existence of subtle histopathological abnormalities within MD_nPCOS, despite the presence of a normal macroscopic appearance, implies that subtle renal changes may manifest even in the absence of overt pathological indications. The distinct histological anomalies observed in RW_PCOS Group point towards significant renal perturbations. The presence of tubular necrosis and the presence of darker stained areas, which likely denote regions of tissue damage and fibrosis, align with the findings reported by Du Plessis et al. (2011). These observations underline the multifaceted impact of PCOS on renal health. The detection of fibrosis, notably emphasized by the darker stained areas following Masson's trichrome staining in LD_PCOS and MD_PCOS, might relate to the ongoing process of tissue remodeling. These findings are in accordance with research conducted by Prabhu et al, (2021), which underscores the significance of fibrosis in the progression of renal dysfunction. It is worth noting that MD_PCOS exhibited the most notable improvement among the PCOS-induced rats treated with HUE. The observations in HD_PCOS parallel those seen in MD_nPCOS, albeit to a lesser degree. It is noteworthy that, despite the enlarged kidneys observed in all rats in the PCOS group, there was no evidence of reduced kidney function. The relatively less pronounced histological anomalies in certain groups suggest that the administration of HUE may potentially mitigate renal aberrations commonly associated with androgen excess, particularly when administered at an appropriate dosage. These findings are in line with the research conducted by

Meihe et al., (2023), which underscores the potential of botanical interventions in alleviating renal morphological damage. Phytochemical constituents like flavonoids and alkaloids could contribute to such effects by exerting anti-inflammatory and antioxidant actions, as postulated by Hu et al., (2022) and Liao et al., *(2020)*.

Gene analysis was conducted on renal samples to investigate the expression levels of estrogen alpha and beta receptors, as well as androgen receptors and GPER (G protein-coupled estrogen receptor). Measurements have been normalized to the expression levels observed in the RW_nPCOS group for each respective day. Remarkably, no statistically significant differences between the various groups have emerged, potentially influenced by the notable data dispersion observed. It is intriguing to note the unexpectedly low gene expression levels detected in this context, particularly considering that receptors like ER beta and GPER should naturally exhibit substantial presence, particularly in the kidney at baseline (Ma et al.,2021). The expression pattern of estrogen receptor beta showed a notable decrease from the initially observed high levels in the RW_PCOS group by day 14. This decrease persisted through day 28 and remained consistently low even by day 56. It is pertinent to mention that the androgen receptor and GPER exhibited minimal amplification on day 14 or 56, but a notable amplification was observed at day 28. The underlying reasons for this selective amplification on day 28 remain to be explored, and it is notable that not all samples within each group demonstrated amplification. Studies have demonstrated the capacity of flavonoids to reduce estrogen levels. Research outcomes revealed that apigenin, a natural flavonoid, led to a reduction in estrogen levels and the LH/FSH ratio within the treated groups in comparison to the PCOS control group. (Le Bail et al., 2000) This decrease in estrogen levels may be attributed to the inhibitory action of apigenin on aromatase enzymes and its impact on 17 β-hydroxysteroid dehydrogenases (Darabi et al., 2020). To maximize the insights

on the alterations induced by flavonoids on various parameters of PCOS, it is imperative to delve into the molecular mechanisms through which flavonoids might exert their effects. Existing literature highlights that soy isoflavones have been shown to augment the antioxidant capacity of rats while also inhibiting the activation of the nuclear factor-kappa beta (NF-κB) signaling pathway. This dual action contributes to the reduction of inflammatory cytokines (Ma et al., 2021) Similar results have been reported in studies investigating the therapeutic effects of catechins. The available body of evidence, especially concerning the diverse phytochemicals found in HUE, implies that HUE may possess the potential to enhance AMPK activity, aligning with findings from previous research (Wang et al., 2017). Therefore, it is plausible to assert that one possible mechanistic pathway through which HUE exerts its effects on PCOS involves the activation of AMPK. AMPK possesses the capability to modulate glucose homeostasis and enhance insulin sensitivity by concurrently inhibiting inflammation and facilitating insulin signaling transduction. (Zhu et al., 2016)

Furthermore, it has been demonstrated that AMPK can diminish the activity of NF-κB and decrease the levels of the pro-inflammatory cytokine TNFα (Giri et al., 2004). Metformin, a medication renowned for its analogous antidiabetic effects, has been demonstrated to function as an agonist of AMPK. It possesses the capability to mitigate inflammatory responses mediated by TNFα and chemokines, as well as ameliorate granulocyte dysfunction, primarily through an AMPK-dependent mechanism [Kai et al., 2015]. The findings indicate that AMPK could potentially serve as a shared target for HUE, as similar botanical such as Baicalin appears to provide protection against PCOS through mechanisms reliant on AMPK. Moreover, studies have highlighted that the activation of AMPK by metformin can mitigate endocrine and reproductive dysfunction associated with PCOS (Fulghesu et al., 2012; Wang et al., 2019). Furthermore, an

alternative potential pathway involves the PI3K/Akt signaling pathway. This pathway is a significant downstream component of insulin signaling, serving as a mediator for insulin's influence on glucose and lipid metabolism. (Li et al., 2017). Under the condition of PCOS, PI3K/Akt signaling is usually inhibited (Zhao et al., 2017) Research has demonstrated that comparable flavonoids, such as Baicalin, inhibited the reduction in Akt phosphorylation in a manner dependent on AMPK. (Zhang et al., 2017). Findings from prior research strongly suggest that the onset and progression of PCOS are intricately linked to the phosphatidylinositol 3-kinase (PI3K)/protein kinase B (Akt) signaling pathway, as indicated by studies conducted by Qiu et al., (2020) and Xiang et al., (2021). In light of these findings, it becomes evident that a robust connection exists between the PI3K/Akt signaling pathway and dyslipidemia in the context of PCOS. Consequently, there is a pressing necessity to pinpoint novel targets that could potentially exert external regulation over this pathway, thereby enabling precise modulation of disorders related to lipid metabolism.

Flavonoids and their derivatives represent a category of naturally occurring compounds abundantly distributed within the plant kingdom. These compounds boast a diverse array of biological activities and prominently display anti-diabetic and anti-obesity properties. (Hussain et al., 2020; Li et al., 2017). Flavonoids' advantageous attributes in the realm of diabetes and obesity management are realized through the fine-tuning of specific signaling networks, notably the IRS/PI3K/Akt/GLUT4 pathway. This modulation serves to enhance glucose metabolism, facilitate glucose transport, and regulate aldose reductase activity across various cell types, including pancreatic β cells, hepatocytes, adipocytes, and skeletal muscle fibers, through the intricate pathways of glucose metabolism. (Han et al., 2017; Hardie et al., 2011; Mladenova et al., 2021).

While our study contributes valuable insights, it is imperative to recognize its inherent limitations. To delve deeper into the intricate mechanisms underpinning HUE's impact on ovarian morphology, future research could encompass further gene expression analysis, hormone profiling, and an exploration of molecular signaling pathways. Furthermore, the applicability of these findings to human populations necessitates clinical investigations to establish translatability.

Chapter VII: Conclusion

In conclusion, our study reveals promising insights into the potential of HUE in mitigating the symptoms of PCOS in a rat model. The observed effects on body weight, ovarian morphology, and renal histology suggest that HUE may hold promise as a botanical intervention for PCOS. These effects may be attributed to the presence of flavonoids and other bioactive compounds within HUE. However, further research is needed to elucidate the precise molecular mechanisms and to determine the translatability of these findings to human populations. Overall, our study contributes to the growing body of evidence supporting the potential therapeutic role of HUE in PCOS management.

References

Abraham Gnanadass, S., Divakar Prabhu, Y., & Valsala Gopalakrishnan, A. (2021). Association of metabolic and inflammatory markers with polycystic ovarian syndrome (PCOS): an update. *Archives of Gynecology and Obstetrics*, *303*, 631-643.

Adams, J. M., Taylor, A. E., Crowley Jr, W. F., & Hall, J. E. (2004). Polycystic ovarian morphology with regular ovulatory cycles: insights into the pathophysiology of polycystic ovarian syndrome. *The Journal of Clinical Endocrinology & Metabolism*, *89*(9), 4343-4350.

Adejuwon, A. A., Crooks, P. A., Fadhel-Albayati, Z., Miller, A. F., Zito, S. W., Adeyemi, O. O., & Agbaje, E. O. (2013). Antihyperglycemic profile of erinidine isolated from Hunteria umbellata seed. *African Journal of Traditional, Complementary, and Alternative Medicines*, *10*(2), 189.

Adeneye, A. A., & Adeyemi, O. O. (2009). Further evaluation of antihyperglycaemic activity of Hunteria umbellata (K. Schum) Hallier f. seed extract in experimental diabetes. Journal of Ethnopharmacology, 126(2), 238-243.

Adeneye, A. A., Sofidiya, M. O., & Adenekan, S. O. (2011). Anti-inflammatory and antioxidant activities of Hunteria umbellata seed fractions. *Pharmacologia*, *2*(6), 165-171.

Adeneye, A. A., Adeyemi, O. O., & Agbaje, E. O. (2010). Anti-obesity and antihyperlipidaemic effect of Hunteria umbellata seed extract in experimental hyperlipidaemia. *Journal of ethnopharmacology*, *130*(2), 307-314.

Adeneye, A. A., Adeyemi, O. O., Agbaje, E. O., & Sofidiya, M. O. (2012). The novel antihyperglycaemic action of Hunteria umbellate seed fractions mediated via intestinal

glucose uptake inhibition. *African Journal of Traditional, Complementary and Alternative Medicines*, *9*(1), 17-24.

Ajiboye, T. O., Hussaini, A. A., Nafiu, B. Y., & Ibitoye, O. B. (2017). Aqueous seed extract of Hunteria umbellata (K. Schum.) Hallier f. (Apocynaceae) palliates hyperglycemia, insulin resistance, dyslipidemia, inflammation and oxidative stress in high-fructose diet-induced metabolic syndrome in rats. *Journal of ethnopharmacology*, *198*, 184-193.

Alam, W., Khan, H., Shah, M. A., Cauli, O., & Saso, L. (2020). Kaempferol as a dietary anti-inflammatory agent: current therapeutic standing. *Molecules*, *25*(18), 4073.

Armanini, D., Boscaro, M., Bordin, L., & Sabbadin, C. (2022). Controversies in the pathogenesis, diagnosis and treatment of PCOS: focus on insulin resistance, inflammation, and hyperandrogenism. *International journal of molecular sciences*, *23*(8), 4110.

Arner, P. (2005). Effects of testosterone on fat cell lipolysis. Species differences and possible role in polycystic ovarian syndrome. *Biochimie*, *87*(1), 39-43.

Azziz, R., Ehrmann, D., Legro, R. S., Whitcomb, R. W., Hanley, R., Fereshetian, A. G., ... & PCOS/Troglitazone Study Group. (2001). Troglitazone improves ovulation and hirsutism in the polycystic ovary syndrome: a multicenter, double blind, placebo-controlled trial. *The Journal of Clinical Endocrinology & Metabolism*, *86*(4), 1626-1632.

Azziz, R., Woods, K. S., Reyna, R., Key, T. J., Knochenhauer, E. S., & Yildiz, B. O. (2004). The prevalence and features of the polycystic ovary syndrome in an unselected population. *The Journal of Clinical Endocrinology & Metabolism*, *89*(6), 2745-2749.

Barber, T. M., & Franks, S. (2021). Obesity and polycystic ovary syndrome. *Clinical endocrinology*, *95*(4), 531-541.

Belchetz, P. E., Plant, T. M., Nakai, Y., Keogh, E. J., & Knobil, E. (1978). Hypophysial responses to continuous and intermittent delivery of hypothalamic gonadotropin-releasing hormone. *Science*, *202*(4368), 631-633.

Beshay, V. E., & Carr, B. R. (2017). Hypothalamic–pituitary–ovarian axis and control of the menstrual cycle. *Clinical reproductive medicine and surgery: A practical guide*, 1-17.

Boone, M. J. (2006). Hunteria umbellata (K. Schum.) Hallier f. *Prota*, *11*.

Borzan, V., Lerchbaum, E., Missbrenner, C., Heijboer, A. C., Goschnik, M., Trummer, C., ... & Obermayer-Pietsch, B. (2021). Risk of insulin resistance and metabolic syndrome in women with hyperandrogenemia: a comparison between PCOS phenotypes and beyond. *Journal of Clinical Medicine*, *10*(4), 829.

Burghen, G. A., Givens, J. R., & Kitabchi, A. E. (1980). Correlation of hyperandrogenism with hyperinsulinism in polycystic ovarian disease. *The Journal of Clinical Endocrinology & Metabolism*, *50*(1), 113-116.

Chang, C., Whelan, C. T., Popovich, T. C., Kokontis, J., & Liao, S. (1989). Fusion proteins containing androgen receptor sequences and their use in the production of poly-and monoclonal anti-androgen receptor antibodies. *Endocrinology*, *125*(2), 1097-1099.

Chang, R. J., Nakamura, R. M., Judd, H. L., & Kaplan, S. A. (1983). Insulin resistance in nonobese patients with polycystic ovarian disease. *The Journal of Clinical Endocrinology & Metabolism*, *57*(2), 356-359.

Chang, R. J., Steingold, K. A., Lobo, R. A., Judd, H. L., Lu, J. K., &. (1986). The effect of bromocriptine on gonadotropin and steroid secretion in polycystic ovarian disease. *The Journal of Clinical Endocrinology & Metabolism*, *62*(5), 1048-1051.

Chen, F. Y., Wang, X., Tang, R. Y., Guo, Z. X., Deng, Y. Z. J., & Yu, Q. (2019). New therapeutic approaches for endometriosis besides hormonal therapy. *Chinese medical journal*, *132*(24), 2984-2993.

Chen, Q., Wang, T., Li, J., Wang, S., Qiu, F., Yu, H., ... & Wang, T. (2017). Effects of natural products on fructose-induced nonalcoholic fatty liver disease (NAFLD). *Nutrients*, *9*(2), 96.

Cheong, R. Y., Czieselsky, K., Porteous, R., & Herbison, A. E. (2015). Expression of ESR1 in glutamatergic and GABAergic neurons is essential for normal puberty onset, estrogen feedback, and fertility in female mice. *Journal of Neuroscience*, *35*(43), 14533-14543.

Christian, C. A., & Moenter, S. M. (2008). Critical roles for fast synaptic transmission in mediating estradiol negative and positive feedback in the neural control of ovulation. *Endocrinology*, *149*(11), 5500-5508.

Darabi, P., Khazali, H., & Mehrabani Natanzi, M. (2020). Therapeutic potentials of the natural plant flavonoid apigenin in polycystic ovary syndrome in rat model: via modulation of pro-inflammatory cytokines and antioxidant activity. *Gynecological Endocrinology*, *36*(7), 582-587.

de Freitas Junior, L. M., & de Almeida Jr, E. B. (2017). Medicinal plants for the treatment of obesity: ethnopharmacological approach and chemical and biological studies. *American journal of translational research*, *9*(5), 2050.

DeClue, T. J., Shah, S. C., Marchese, M., & Malone, J. I. (1991). Insulin resistance and hyperinsulinemia induce hyperandrogenism in a young type B insulin-resistant female. *The Journal of Clinical Endocrinology & Metabolism*, 72(6), 1308-1311.

Ding, D. C., Chen, W., Wang, J. H., & Lin, S. Z. (2018). Association between polycystic ovarian syndrome and endometrial, ovarian, and breast cancer: A population-based cohort study in Taiwan. *Medicine, 97*(39), e12608.

Ding, H., Zhang, J., Zhang, F., Zhang, S., Chen, X., Liang, W., & Xie, Q. (2021). Resistance to the insulin and elevated level of androgen: A major cause of polycystic ovary syndrome. *Frontiers in endocrinology,* 12, 741764.

Du Plessis, E. C., Prozesky, L., & Botha, C. J. (2011). The pathology of acute Nolletia gariepina poisoning of cattle. *Journal of the South African Veterinary Association, 82*(3), 144-149.

Dunaif, A. (1995). Hyperandrogenic anovulation (PCOS): a unique disorder of insulin action associated with an increased risk of non-insulin-dependent diabetes mellitus. *The American journal of medicine, 98*(1), S33-S39.

Ek I, Arner P, Rydén M, (2002) A unique defect in the regulation of visceral fat cell lipolysis in the polycystic ovary syndrome as an early link to insulin resistance. *Diabetes.;*51:484 – 492.

Estienne, A., Bongrani, A., Ramé, C., Kurowska, P., Błaszczyk, K., Rak, A., ... & Dupont, J. (2021). Energy sensors and reproductive hypothalamo-pituitary ovarian axis (HPO) in female mammals: Role of mTOR (mammalian target of rapamycin), AMPK (AMP-activated protein kinase) and SIRT1 (Sirtuin 1). *Molecular and cellular endocrinology,* 521, 111113.

Ezeh, U., Pisarska, M. D., & Azziz, R. (2022). Association of severity of menstrual dysfunction with hyperinsulinemia and dysglycemia in polycystic ovary syndrome. *Human Reproduction, 37*(3), 553-564.

Falodun A, Nworgu ZAM, Ikponmwonsa M. O. (2006) Phytochemical components of Hunteria umbellata (K. Schum.) and its effect on isolated non-pregnant rat uterus in oestrus. *Pak J Pharm Sci.19(*3):256–258.

Feldman, A. T., & Wolfe, D. (2014). Tissue processing and hematoxylin and eosin staining. *Histopathology: Methods and Protocols,* 31-43.

Fulghesu, A. M., Romualdi, D., Di Florio, C., Sanna, S., Tagliaferri, V., Gambineri, A., ... & Lanzone, A. (2012). Is there a dose–response relationship of metformin treatment in patients with polycystic ovary syndrome? Results from a multicentric study. *Human reproduction, 27*(10), 3057-3066.

Gagliano-Jucá, T., Pencina, K. M., Guo, W., Li, Z., Huang, G., Basaria, S., & Bhasin, S. (2020). Differential effects of testosterone on circulating neutrophils, monocytes, and platelets in men: Findings from two trials. *Andrology*, *8*(5), 1324-1331.

Gargus, E. S., Bae, Y., Chen, J., Moss, K. J., Ingram, A. N., Zhang, J., ... & Woodruff, T. K. (2022). An ovarian steroid metabolomic pathway analysis in basal and polycystic ovary syndrome (PCOS)-like gonadotropin conditions reveals a hyperandrogenic phenotype measured by mass spectrometry. *Biomedicines, 10(7),* 1646.

Giri, S., Nath, N., Smith, B., Viollet, B., Singh, A. K., & Singh, I. (2004). 5-aminoimidazole-4-carboxamide-1-β-4-ribofuranoside inhibits proinflammatory response in glial cells: a possible role of AMP-activated protein kinase. *Journal of Neuroscience*, *24*(2), 479-487.

Gustafsson, J. Å. (2003). What pharmacologists can learn from recent advances in estrogen signalling. *Trends in pharmacological sciences*, *24*(9), 479-485.

Hajiaghayi, M., Rezayat, F., Ghasemi, N., Soleymanian, A., Mesdaghi, M., Mosaffa, N., & Ramezani Tehrani, F. (2016). The Study of cell Proliferation, TNF-α Cytokine Production

and Expression of CD8 Marker in Peripheral Blood Lymphocytes of Women with Polycystic Ovary Syndrome (PCOS) by co-culture with Ovarian Tumor Cell Lines (SKOV3, A2780). Iranian Journal of *Endocrinology and Metabolism*, *18*(3), 187-196.

Han, Z., Cai, S., Zhang, X., Qian, Q., Huang, Y., Dai, F., & Zhang, G. (2017). Development of predictive models for total phenolics and free p-coumaric acid contents in barley grain by near-infrared spectroscopy. *Food chemistry*, *227*, 342-348.

Harborne, L., Fleming, R., Lyall, H., Norman, J., & Sattar, N. (2003). Descriptive review of the evidence for the use of metformin in polycystic ovary syndrome. *The Lancet*, *361*(9372), 1894-1901.

Hardie, D. G. (2011). AMP-activated protein kinase—an energy sensor that regulates all aspects of cell function. *Genes & development*, *25*(18), 1895-1908.

Harris, A. N., Lee, H. W., Verlander, J. W., & Weiner, I. D. (2020). Testosterone modulates renal ammonia metabolism. *American Journal of Physiology-Renal Physiology*, *318*(4), F922-F935.

Hernandez, E. R., Resnick, C. E., Holtzclaw, W. D., Payne, D. W., & Adashi, E. Y. (1988). Insulin as a regulator of androgen biosynthesis by cultured rat ovarian cells: cellular mechanism (s) underlying physiological and pharmacological hormonal actions. *Endocrinology*, *122*(5), 2034-2043.

Hernández-Jiménez, J. L., Barrera, D., Espinoza-Simón, E., González, J., Ortíz-Hernández, R., Escobar, L., & Torres-Ramírez, N. (2022). Polycystic ovarian syndrome: signs and feedback effects of hyperandrogenism and insulin resistance. *Gynecologica*l *Endocrinology*, *38*(1), 2-9.

Heshmati, J., Morvaridzadeh, M., Maroufizadeh, S., Akbari, A., Yavari, M., Amirinejad, A., & Sepidarkish, M. (2019). Omega-3 fatty acids supplementation and oxidative stress parameters: A systematic review and meta-analysis of clinical trials. *Pharmacological research, 149*, 104462.

Hoeger, K. M., Dokras, A., & Piltonen, T. (2021). Update on PCOS: consequences, challenges, and guiding treatment. *The Journal of Clinical Endocrinology & Metabolism, 106*(3), e1071-e1083.

Horton, R., Romanoff, E., & Walker, J. (1966). Androstenedione and testosterone in ovarian venous and peripheral plasma during ovariectomy for breast cancer. *The Journal of Clinical Endocrinology & Metabolism*, *26(*11), 1267-1269.

Hu, T., Yue, J., Tang, Q., Cheng, K. W., Chen, F., Peng, M., ... & Wang, M. (2022). The effect of quercetin on diabetic nephropathy (DN): A systematic review and meta-analysis of animal studies. *Food & Function, 13*(9), 4789-4803.

Huang, C. F., Yang, C. Y., Chan, D. C., Wang, C. C., Huang, K. H., Wu, C. C., ... & Liu, S. H. (2015). Arsenic exposure and glucose intolerance/insulin resistance in estrogen-deficient female mice. *Environmental health perspectives, 123*(11), 1138-1144.

Hung, H. Y., Qian, K., Morris-Natschke, S. L., Hsu, C. S., & Lee, K. H. (2012). Recent discovery of plant-derived anti-diabetic natural products. *Natural product reports, 29*(5), 580-606.

Igbe I, Omogbai EKI, Ozolua RI. (2009) Hypoglycemic activity of aqueous seed extract of Hunteria umbellata in normal and streptozotocin-induced diabetic rats. *Pharmaceutical Biology. 47*(10):1011–1016.

Kai, Y., Kawano, Y., Yamamoto, H., & Narahara, H. (2015). A possible role for AMP-activated protein kinase activated by metformin and AICAR in human granulosa cells. *Reproductive Biology and Endocrinology, 13*, 1-8.

Kakuta, H., Iguchi, T., & Sato, T. (2018). The involvement of granulosa cells in the regulation by gonadotropins of Cyp17a1 in theca cells. *in vivo, 32(*6), 1387-1401.

Kauffman, A. S. (2022). Neuroendocrine mechanisms underlying estrogen positive feedback and the LH surge. *Frontiers in neuroscience, 16*, 953252.

Khan, M. J., Ullah, A., & Basit, S. (2019). Genetic basis of polycystic ovary syndrome (PCOS): current perspectives. *The application of clinical genetics*, 249-260.

Kobaly K, Vellanki P, Sisk RK, (2014) Parent-of-origin effects on glucose homeostasis in polycystic ovary syndrome. *J Clin Endocrinol Metab.;99:*2961–2966

Lauritsen, M. P., Bentzen, J. G., Pinborg, A., Loft, A., Forman, J. L., Thuesen, L. L., ... & Nyboe Andersen, A. (2014). The prevalence of polycystic ovary syndrome in a normal population according to the Rotterdam criteria versus revised criteria including anti-Müllerian hormone. *Human reproduction, 29*(4), 791-801.

Le Bail, J. C., Champavier, Y., Chulia, A. J., & Habrioux, G. (2000). Effects of phytoestrogens on aromatase, 3β and 17β-hydroxysteroid dehydrogenase activities and human breast cancer cells. *Life Sciences, 66*(14), 1281-1291.

Li, H., Zhang, Y., Liu, C., Zhang, Y., Yang, H., Fu, S., & Lv, H. (2023). Association of insulin-like growth factor-1 with polycystic ovarian syndrome: a systematic review and meta-analysis. *Endocrine Practice, 29*(5), 388-397.

Li, T., Mo, H., Chen, W., Li, L., Xiao, Y., Zhang, J., ... & Lu, Y. (2017). Role of the PI3K-Akt signaling pathway in the pathogenesis of polycystic ovary syndrome. *Reproductive sciences, 24*(5), 646-655.

Liao, Y., Tan, R. Z., Li, J. C., Liu, T. T., Zhong, X., Yan, Y., ... & Wang, L. (2020). Isoliquiritigenin attenuates UUO-induced renal inflammation and fibrosis by inhibiting Mincle/Syk/NF-Kappa B signaling pathway. *Drug design, development and therapy,* 1455-1468.

Littlejohn EE, Weiss RE, Deplewski D, Edidin DV, Rosenfield R. (2007) Early childhood obesity as the initial sign of insulin resistant hyperinsulinism and precursor of polycystic ovary syndrome. *J Pediatr Endocrinol Metab 20*:41–51.

Liu, F., Sirisena, S., & Ng, K. (2021). Efficacy of flavonoids on biomarkers of type 2 diabetes mellitus: a systematic review and meta-analysis of randomized controlled trials. *Critical reviews in food science and nutrition, 63*(21), 4916-4941.

Liu, Y. X., & Hsueh, A. J. (1986). Synergism between granulosa and theca-interstitial cells in estrogen biosynthesis by gonadotropin-treated rat ovaries: studies on the two-cell, two-gonadotropin hypothesis using steroid antisera. *Biology of reproduction, 35*(1), 27-36.

Ma, H. Y., Chen, S., & Du, Y. (2021). Estrogen and estrogen receptors in kidney diseases. *Renal Failure, 43*(1), 619-642.

Ma, X., Li, X., Ma, L., Chen, Y., & He, S. (2021). Soy isoflavones alleviate polycystic ovary syndrome in rats by regulating NF-κB signaling pathway. *Bioengineered,* 12(1), 7204-7212.

Maeda K, Ohkura S, Uenoyama Y, Wakabayashi Y, Oka Y, Tsukamura H, Okamura H. Neurobiological mechanisms underlying GnRH pulse generation by the hypothalamus. *Brain Res.* 2010; 1364:103–115.

Maleki, S. J., Crespo, J. F., & Cabanillas, B. (2019). Anti-inflammatory effects of flavonoids. *Food chemistry*, 299, 125124.

Marques, P., Skorupskaite, K., Rozario, K. S., Anderson, R. A., & George, J. T. (2022). Physiology of GNRH and gonadotropin secretion. *Endotext [internet]*.

Meihe, L. I., Shan, G. A. O., Minchao, K. A. N. G., Zhang, X., Ping, L. A. N., Xiaoling, W. U., ... & Zheng, J. (2023). Quercitrin alleviates lipid metabolism disorder in polycystic ovary syndrome-insulin resistance by upregulating PM20D1 in the PI3K/Akt pathway. *Phytomedicine*, 154908.

Mikhael, S., Punjala-Patel, A., & Gavrilova-Jordan, L. (2019). Hypothalamic-pituitary-ovarian axis disorders impacting female fertility. *Biomedicines*, *7*(1), 5.

Mladenova, S. G., Vasileva, L. V., Savova, M. S., Marchev, A. S., Tews, D., Wabitsch, M., & Georgiev, M. I. (2021). Anti-adipogenic effect of alchemilla monticola is mediated via PI3K/AKT signaling inhibition in human adipocytes. *Frontiers in Pharmacology*, 12, 707507.

Moghetti, P., & Tosi, F. (2021). Insulin resistance and PCOS: chicken or egg?. *Journal of endocrinological investigation*, 44(2), 233-244.

Moka, M. K., & Sumithra, M. (2022). Role Of Tinospora Cordifolia and Alkaloid Rich Fraction Against Hyperandrogenisam: A In-Vitro Study on Cell Proliferation, Colony Formation and CYP17A1 Gene Expression by Qrt-PCR. *Journal of Pharmaceutical Negative Results*, 2479-2488.

Nakamura Y, Gang HX, Suzuki T, Sasano H, Rainey WE. (2009) Adrenal changes associated with adrenarche. *Rev Endocr Metab Disord.*10:19 –26

Oboh, G., Adebayo, A. A., & Ademosun, A. O. (2018). Erection-stimulating, anti-diabetic and antioxidant properties of Hunteria umbellata and Cylicodiscus gabunensis water extractable phytochemicals. *Journal of Complementary and Integrative Medicine*, *15*(1).

Okolafor, F., & Ekhaise, F. (2021). Effect of a tropical plant (Hunteria umbellata) in the management of Streptozotocin induced Diabetes Mellitus and other physiological and biochemical functions in Wistar. *Current Perspectives on Medicinal and Aromatic Plants*, 4(1), 1-12.

Oróstica, L., Poblete, C., Romero, C., & Vega, M. (2020). Pro-inflammatory markers negatively regulate IRS1 in endometrial cells and endometrium from women with obesity and PCOS. *Reproductive Sciences*, 27, 290-300.

Patel, S. (2018). Polycystic ovary syndrome (PCOS), an inflammatory, systemic, lifestyle endocrinopathy. *The Journal of steroid biochemistry and molecular biology*, 182, 27-36.

Petersen, M. C., & Shulman, G. I. (2018). Mechanisms of insulin action and insulin resistance. *Physiological reviews.*

Petrovska, B. B. (2012). Historical review of medicinal plants' usage. *Pharmacognosy reviews*, *6*(11), 1.

Pfaffl, M. W., Lange, I. G., Daxenberger, A., & Meyer, H. H. D. (2001). Tissue-specific expression pattern of estrogen receptors (ER): Quantification of ERα and ERβ mRNA with real-time RT-PCR. *Apmis*, *109*(S103), S540-S550.

Pourcel, L., Routaboul, J. M., Cheynier, V., Lepiniec, L., & Debeaujon, I. (2007). Flavonoid oxidation in plants: from biochemical properties to physiological functions. *Trends in plant science*, 12(1), 29-36.

Prabhu, Y. D., Bhati, M., Vellingiri, B., & Gopalakrishnan, A. V. (2021). The effect of γ-linolenic acid on Polycystic Ovary Syndrome associated Focal Segmental Glomerulosclerosis via TGF-β pathway. *Life Sciences,* 276, 119456.

Pugeat M, Nader N, Hogeveen K, Raverot G, Déchaud H, Grenot C. (2010) Sex hormone-binding globulin gene expression in the liver: drugs and the metabolic syndrome. *Mol Cell Endocrinol*. 316:53–59.

Qu, X., & Donnelly, R. (2020). Sex hormone-binding globulin (SHBG) as an early biomarker and therapeutic target in polycystic ovary syndrome. *International journal of molecular sciences*, 21(21), 8191

Roberts AE, Arbogast LK, Friedman CI, Cohn DE, Kaumaya PT, Danforth DR. (2007) Neutralization of endogenous vascular endothelial growth factor depletes primordial follicles in the mouse ovary. *Biol Reprod*. 76:218 –223

Robinson, J. E., Kendrick, K. M., & Lambart, C. E. (1991). Changes in the release of gamma-aminobutyric acid and catecholamines in the preoptic/septal area prior to and during the preovulatory surge of luteinizing hormone in the ewe. *Journal of neuroendocrinology*, *3*(4), 393-399.

Rosenfield RL. (2015) The polycystic ovary morphology-polycystic ovary syndrome spectrum. *J Pediatr Adolesc Gynecol;* 28:412– 419.

Rosenfield, R. L., & Ehrmann, D. A. (2016). The pathogenesis of polycystic ovary syndrome (PCOS): the hypothesis of PCOS as functional ovarian hyperandrogenism revisited. *Endocrine reviews*, 37(5), 467-520.

Rosenfield, R. L., Ehrmann, D. A., & Littlejohn, E. E. (2015). Adolescent polycystic ovary syndrome due to functional ovarian hyperandrogenism persists into adulthood. *The Journal of Clinical Endocrinology & Metabolism*, 100(4), 1537-1543.

Rudnicka, E., Kunicki, M., Calik-Ksepka, A., Suchta, K., Duszewska, A., Smolarczyk, K., & Smolarczyk, R. (2021). Anti-müllerian hormone in pathogenesis, diagnostic and treatment of PCOS. *International journal of molecular sciences*, 22(22), 12507.

Rudnicka, E., Suchta, K., Grymowicz, M., Calik-Ksepka, A., Smolarczyk, K., Duszewska, A. M., ... & Meczekalski, B. (2021). Chronic low-grade inflammation in pathogenesis of PCOS. *International journal of molecular sciences*, *22*(7), 3789.

Saadia, Z. (2020). Follicle stimulating hormone (LH: FSH) ratio in polycystic ovary syndrome (PCOS)-obese vs. non-obese women. *Medical Archives*, 74(4), 289.

Seidlova-Wuttke, D., & Wuttke, W. (2017). The premenstrual syndrome, premenstrual mastodynia, fibrocystic mastopathy and infertility have often common roots: effects of extracts of chasteberry (Vitex agnus castus) as a solution. *Clinical Phytoscience*, *3*, 1-11.

Shi, Y., Kong, X., Yin, H., Zhang, W., & Wang, W. (2019). Effect of hawthorn leaf flavonoids in dehydroepiandrosterone-induced polycystic ovary syndrome in rats. *Pathobiology*, 86(2-3), 102-110.

Simó, R., Barbosa-Desongles, A., Lecube, A., Hernandez, C., & Selva, D. M. (2012). Potential role of tumor necrosis factor-α in downregulating sex hormone–binding globulin. diabetes, 61(2), 372-382.

Singh, R., Kaur, S., Yadav, S., & Bhatia, S. (2023). Gonadotropins as pharmacological agents in assisted reproductive technology and polycystic ovary syndrome. *Trends in Endocrinology & Metabolism*, 34(4), 194-215.

Tang, J., Diao, P., Shu, X., Li, L., & Xiong, L. (2019). Quercetin and quercitrin attenuates the inflammatory response and oxidative stress in LPS-induced RAW264. 7 cells: in vitro assessment and a theoretical model. *BioMed research international*, 2019.

Tiwari, A. K., & Rao, J. M. (2002). Diabetes mellitus and multiple therapeutic approaches of phytochemicals: Present status and future prospects. *Current science*, 30-38.

Tsilchorozidou T, Honour JW, Conway GS. (2003) Altered cortisol metabolism in polycystic ovary syndrome: insulin enhances 5-reduction but not the elevated adrenal steroid production rates. *J Clin Endocrinol Metab*.;88: 5907–5913

Udinyiwe, C. O., & Aghedo, E. S. (2022). Proximate composition, phytochemical and antimicrobial activity of aqueous and ethanolic extracts of Hunteria umbellata on some clinical isolates. *Journal of Applied Sciences and Environmental Management, 26*(1), 31-36.

Van De Vlekkert, D., Machado, E., & d'Azzo, A. (2020). Analysis of generalized fibrosis in mouse tissue sections with Masson's trichrome staining. *Bio-protocol, 10*(10), e3629-e3629.

Varma, S. D., Mizuno, A., & Kinoshita, J. H. (1977). Diabetic cataracts and flavonoids. *Science, 195(*4274), 205-206.

Virginia, A. M., Virginia, T. M., & Laura, K. (2020). Androgen receptor and uterine histoarchitecture in a PCOS rat model. *Molecular and Cellular Endocrinology, 518*, 110973.

Walters, K. A. (2020). Polycystic ovary syndrome: Is it androgen or estrogen receptor?. *Current Opinion in Endocrine and Metabolic Research, 12*, 1-7.

Wang, H. Q., Zhang, W. D., Yuan, B., & Zhang, J. B. (2021). Advances in the regulation of mammalian follicle-stimulating hormone secretion. *Animals, 11*(4), 1134.

Wang, T., Jiang, H., Cao, S., Chen, Q., Cui, M., Wang, Z., & Kang, N. (2017). Baicalin and its metabolites suppresses gluconeogenesis through activation of AMPK or AKT in insulin resistant HepG-2 cells. *European Journal of Medicinal Chemistry, 141*, 92-100.

Wang, W., Zheng, J., Cui, N., Jiang, L., Zhou, H., Zhang, D., & Hao, G. (2019). Baicalin ameliorates polycystic ovary syndrome through AMP-activated protein kinase. *Journal of Ovarian Research*, 12, 1-12.

Wawrzkiewicz-Jałowiecka, A., Kowalczyk, K., Trybek, P., Jarosz, T., Radosz, P., Setlak, M., & Madej, P. (2020). In search of new therapeutics—molecular aspects of the PCOS pathophysiology: genetics, hormones, metabolism and beyond. *International Journal of Molecular Sciences*, 21(19), 7054.

Wen, K., Fang, X., Yang, J., Yao, Y., Nandakumar, K. S., Salem, M. L., & Cheng, K. (2021). Recent research on flavonoids and their biomedical applications. *Current medicinal chemistry*, 28(5), 1042-1066.

Wild, R. A. (2002). Long-term health consequences of PCOS. Human reproduction update, 8(3), 231-241.

Winters SJ, Talbott E, Guzick DS, Zborowski J, McHugh KP. (2000) Serum testosterone levels decrease in middle age in women with the polycystic ovary syndrome. *Fertil Steril*.;73:724 –729.

Xu, X. L., Deng, S. L., Lian, Z. X., & Yu, K. (2021). Estrogen receptors in polycystic ovary syndrome. *Cells*, 10(2), 459.

Xu, X. L., Huang, Z. Y., Yu, K., Li, J., & Deng, S. L. (2022). Estrogen biosynthesis and signal transduction in ovarian disease. *Frontiers in Endocrinology*, 13, 827032.

Xu, Y., & Qiao, J. (2022). Association of insulin resistance and elevated androgen levels with polycystic ovarian syndrome (PCOS): a review of literature. *Journal of healthcare engineering*, 2022.

Yildiz BO, Yarali H, Oguz H, Bayraktar M. (2003) Glucose intolerance, insulin resistance, and hyperandrogenemia in first degree relatives of women with polycystic ovary syndrome. *J Clin Endocrinol Metab.;*88:2031–2036.

Younas, A., Hussain, L., Shabbir, A., Asif, M., Hussain, M., & Manzoor, F. (2022). Effects of fagonia indica on letrozole-induced polycystic ovarian syndrome (PCOS) in young adult female rats. *Evidence-based Complementary and Alternative Medicine*, 2022.

Zawadzki, J. K. (1992). Diagnostic criteria for polycystic ovary syndrome (a rational approach). Polycystic ovary syndrome, 377-384.

Zeng, X., Xie, Y. J., Liu, Y. T., Long, S. L., & Mo, Z. C. (2020). Polycystic ovarian syndrome: correlation between hyperandrogenism, insulin resistance and obesity. *Clinica chimica acta*, 502, 214-221.

Zhai, H. L., Wu, H., Xu, H., Weng, P., Xia, F. Z., Chen, Y., & Lu, Y. L. (2012). Trace glucose and lipid metabolism in high androgen and high-fat diet induced polycystic ovary syndrome rats. *Reproductive Biology and Endocrinology*, *10*, 1-9

Zhang, J., Zhang, H., Xin, X., Zhu, Y., Ye, Y., & Li, D. (2022). Efficacy of flavonoids on animal models of polycystic ovary syndrome: a systematic review and meta-analysis. *Nutrients,* 14(19), 4128.

Zhao, H., Zhou, D., Chen, Y., Liu, D., Chu, S., & Zhang, S. (2017). Beneficial effects of Heqi san on rat model of polycystic ovary syndrome through the PI3K/AKT pathway. Daru Journal of *Pharmaceutical Sciences,* 25, 1-12.

Zhou, S., Wen, S., & Xu, L. (2021). Association of estrogen receptor genes polymorphisms with polycystic ovary syndrome: a systematic review and meta-analysis based on observational studies. *Frontiers in Endocrinology,* 12, 726184.

Zhu, R., Zheng, J., Chen, L., Gu, B., & Huang, S. (2016). Astragaloside IV facilitates glucose transport in C2C12 myotubes through the IRS1/AKT pathway and suppresses the palmitate-induced activation of the IKK/IκBα pathway. *International Journal of Molecular Medicine,* 37(6), 1697-1705

Zhu, T., Cui, J., & Goodarzi, M. O. (2021). Polycystic ovary syndrome and risk of type 2 diabetes, coronary heart disease, and stroke. *Diabetes*, 70(2), 627-637.

APPENDIX A: IACUC Approval

IACUC approval number is 4-140. Title: “The Effect of aqueous extract of Hunteria umbellata on the induction and maintenance of Polycystic Ovary Syndrome (PCOS) in the rat”.